Intracellular infections in the pathogenesis of vascular diseases; *in vitro* studies

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Bijna alles wat je doet is onbelangrijk,
maar het is erg belangrijk dat je het doet.
(*Gandhi***)**

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Aan mijn lieve ouders en schoonouders

Manuscripts based on the studies presented in this thesis

Chapter 2

Visseren FLJ, Bouwman JJM, Bouter KP, Diepersloot RJA, de Groot PhG, Erkelens DW. Procoagulant activity of endothelial cells after infection with respiratory viruses.
Thromb Haemost 2000; 84: 319-24.

Chapter 3

Bouwman JJM, Visseren FLJ, Bosch MC, Bouter KP, Diepersloot RJA.
Procoagulant and inflammatory response of virus-infected monocytes
Eur J Clin Invest 2002; 32(10): 759-766.

Chapter 4

Bouwman JJM, Visseren FLJ, Bouter KP, Diepersloot RJA.
Azithromycin inhibits interleukin-6 but not fibrinogen production in hepatocytes infected with Cytomegalovirus and *Chlamydia pneumoniae*
J Lab Clin Med 2004; 144: 18-26.

Chapter 5

Bouwman JJ, Visseren FL, Bevers LM, van der Vlist WE, Bouter KP, Diepersloot RJ.
Azithromycin reduces Chlamydia pneumoniae-induced attenuation of eNOS and cGMP production by endothelial cells.
Eur J Clin Invest 2005; 35(9): 573-582.

Chapter 6

Kartikasari AE, Georgiou NA, de Geest M, van Kats-Renaud JH, Bouwman JJ, van Asbeck BS, Marx JJ, Visseren FL.
Iron enhances endothelial cell activation in response to Cytomegalovirus or Chlamydia pneumoniae infection.
Eur J Clin Invest 2006; 36(10):743-52.

Chapter 7

Bouwman JJM, Visseren FLJ, Bouter KP, Diepersloot RJA.
Infection induced inflammatory response of adipocytes *in vitro*.
Int J Obes (Lond) 2008; 32(6): 892-901.

Chapter 8

Bouwman JJM, Visseren FLJ, Diepersloot RJA.
Intracellular infections enhance IL-6 and PAI-1 production in co-cultivated human adipocytes and THP-1 monocytes.
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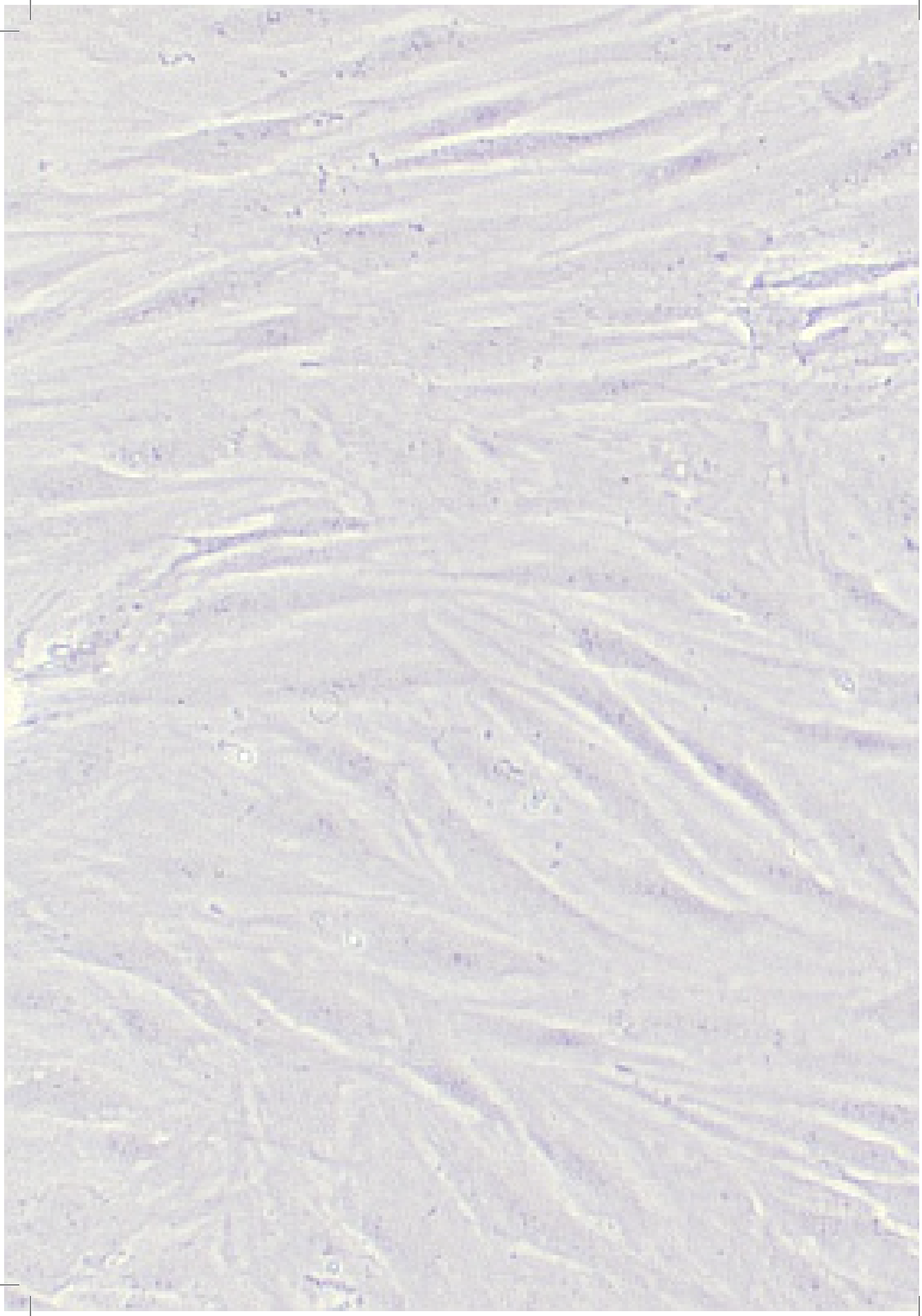

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General introduction

Inflammation

Inflammation is a process that develops in response to infection or injury. The inflammatory response usually protects tissues from infection or injury but may become harmful if misdirected. Inflammation is involved in destroying microorganisms and removing debris of damaged cells. Infiltration of leucocytes (acute inflammation) in the diseased tissue and production and release of inflammatory mediators by cells of key organs of the immune system are the first signs of inflammation. In chronic inflammation, predominantly mononuclear cells invade the infected or injured tissue. When an infectious agent has been able to penetrate the system the innate immune response is the next line of defense. Among neutrophils, dendritic cells and NK cells, macrophages play the most important role in this mechanism[1,2]. Should a pathogen still be able to escape the innate response, T cells and B cells activate the adaptive immune response. Adaptive immunity recognizes specific molecular structures (antigens) presented by major histocompatibility complex (MHC) classes II on antigen presenting cells such as macrophages. Once T cells recognize a presented antigen, an adaptive immune response against this specific antigen is initiated[3]. These responses include direct killing of antigen bearing cells by cytotoxic T lymphocytes, stimulation of B cells to produce antibodies against the antigen, and induction of an enhanced innate response in the area where the antigen is present[4]. CD8 expressing T cells are cytotoxic and kill the antigen bearing cells infected by virus or other intracellular organisms. Upon activation, CD4 positive cells may differentiate into T-helper (Th) cells or regulatory T-cells[5]. Th1 cells primarily secrete IFN-γ, IL-2 and TNF-α, which promote cellular immunity against intracellular bacteria and viruses. Th1 cytokines are generally referred to as pro-inflammatory and Th2 cytokines as anti-inflammatory. Common human diseases such as allergy, autoimmunity, chronic infections and sepsis are characterized by a deregulation of the pro- versus anti-inflammatory and Th1 versus Th2 cytokine balance[6]. The failure of these defensive measures suggests that the monocytic and/or T lymphocyte responses are either inadequate or that these inflammatory responses have been altered or subverted from performing their usual protective and reparative functions.

Monocytes and macrophages

Monocytes play a prominent role in inflammation, coagulation and atherosclerosis by their ability to produce tissue factor (TF) and cytokines[7-10]. The production of cytokines by monocytes may accelerate the chronic process of atherosclerosis and may cause coronary syndromes by eliciting plaque instability. In atherosclerosis, monocytes infiltrate the endothelial layer of the vessel wall and inflammatory mediators enhance uptake of modified lipoprotein particles and formation of lipid-filled macrophages[11,12]. Macrophages can not only be found in endothelial layers. For example, monocytes can migrate into various tissues and organs where they undergo a series of changes and differentiate into mature macrophages. Basically, two types of macrophages can be distinguished: resident (normal) and exudate (inflammatory) macrophages. Each type of resident macrophage, determined by its location, has a specific name[13] e.g. alveolar macrophages in the lung, "Kupffer cells" (liver)[14], Langerhans cells (skin), osteoclasts (bone) or ATM (adipose tissue macrophages). Macrophages are considered to be a heterogeneous cell population differing in their origin, development stage (differentiation) and local adaptation. Therefore macrophages, in their function and purpose, are responsible for numerous metabolic, immunological, and inflammatory processes in physiological and pathological conditions.

Atherosclerosis: an inflammatory disease

Monocytes adhere to the endothelium and can infiltrate the sub endothelial space. Eventually leading to foam cell formation and atherosclerotic lesions[15,16]. Atherosclerotic plaques contain inflammatory cells, cholesterol and cellular debris. The migration and infiltration of chronic inflammatory cells, i.e. monocytes and T-cells, reflect an "active" inflammatory atherosclerotic disease[17-19]. In turn, chronically inflamed tissues may perpetuate and even amplify the inflammatory state by releasing vasoactive and proinflammatory substances into the circulation. Inflammatory cell infiltrates are found only within or overlying atherosclerotic plaques. Monocytes are not found in normal intima that has no evidence of atherosclerosis[20,21]. Atherosclerosis, depending on its severity and the location of the artery it affects, may result in vessel obstruction and can cause a rupture of inflamed plaques[22]. Interleukin-6 (IL-6), a cytokine that can be measured in the circulation, initiates and accelerates the process of atherosclerosis. IL-6 has been identified as a marker of inflammation in coronary atherosclerotic plaques. Serum levels of IL-6 increase in response to acute

myocardial infarction, unstable angina, percutaneous coronary intervention and in late restenosis. IL-6 stimulates platelet aggregation and the expression of tissue factor, macrophage LDL receptors, CRP, and fibrinogen. IL-6 also regulates the expression of other inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF-α)[23]. It is conceivable that inflammatory cytokines play a causal role in the development of CHD. On the other hand it can be argued that these cytokines simply reflect an underlying disease process. C-reactive protein (CRP), one of many human acute-phase reactants, is produced in the liver in response to IL-6, IL-1ß and TNF-α. It activates the classic complement cascade, mediates phagocytosis, regulates inflammation, and is a nonspecific but sensitive marker of infection and tissue inflammation[24]. Because of their interrelationship, CRP could simply be a surrogate marker for IL-6 or some other factor[25]. Inflammatory cytokines directly stimulate the production of adhesion molecules. The expression of pro-inflammatory cytokines is decisive for the progression of atherosclerosis. Many pro-inflammatory cytokines, such as IL-12 and IFN-γ, and various chemokines exert their pro-atherosclerotic effects during all stages of lesion development[26,27]. Continued release of cytokines in the lesion by macrophages and T-cells not only perpetuates inflammation within the lesion but also modulates smooth muscle cell activity[28]. For example, cytokines such as TNF-α and IFN-γ can promote the uptake of modified lipoproteins that leads to smooth muscle cell derived foam cells *in vitro* and IL-10 inhibits smooth muscle cell accumulation in several animal models[29-31]. Also, many cytokines can influence tissue factor expression on monocytes and on endothelial cells *in vitro*. Tissue factor plays a central role in the initiation of coagulation activation. TNF-α, IL-1, IL-2, IL-6, and IL-12 are cytokines that have been found capable of activating the coagulation system in vivo, whereas IL-10 can act as an anticoagulant[32]. The presence of T-lymphocytes within an atherosclerotic plaque indicates that the immune system has been activated[18, 33-35]. Now that inflammation is considered the underlying pathophysiological cause and key process in atherosclerosis, the driving force behind chronic low-grade inflammation becomes a matter of question. The importance of macrophages normal function in wound healing has been noted before[7,36]. The chronic inflammatory process involving the arterial endothelium that ultimately results in the complications of atherosclerosis, may be caused by a response to the oxidative components of modified low-density lipoprotein (LDL), or to chronic infection, free radicals, classical risk factors such as diabetes, smoking, hypercholesterolemia and hypertension or to other factors[15,16].

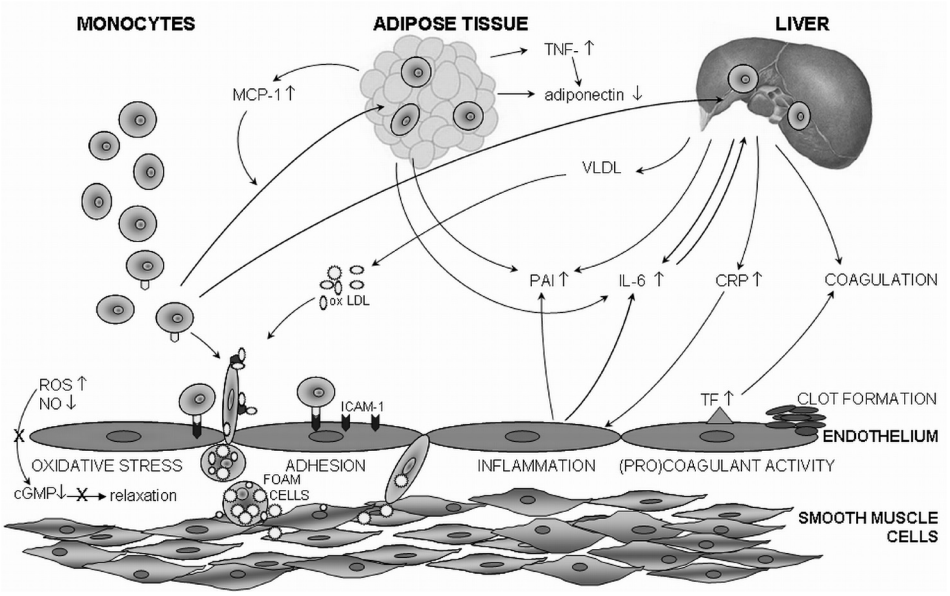

Figure 1. Mechansims involved in vascular injury and inflammation.

In atherosclerosis, monocytes infiltrate the endothelial layer of the vessel wall. Monocytes uptake low density lipoproteins (LDL) and differentiate into macrophages. Macrophages release cytokines that direct migration of monocytes from the blood to the sites of inflammation. The modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells. Excess LDL infiltrates the artery and is retained in the intima. Oxidative and enzymatic modifications lead to the release of inflammatory lipids that induce endothelial cells to express leukocyte adhesion molecules. Endothelial activation leads to the release of oxygen and nitrogen radicals, increased expression of adhesion molecules and inflammatory cytokines. Ultimately, this results in inflammation and tissue damage. The inflammatory response of adipocytes to infection may stimulate the production of inflammatory cytokines (TNF-alpha, IL-6). Visceral fat, in particular, contributes to endothelial dysfunction through the direct effect of adipokines, mainly adiponectin and TNF-α, which are secreted by fat tissue after macrophage recruitment (through monocyte chemoattractant protein-1, MCP-1). Indirect effects of TNF-α and IL-6 might influence inflammation (CRP) and endothelial function. By releasing hormones and cytokines into the portal circulation, abdominal adipose tissue is able to directly influence liver metabolism, potentially leading to accelerated inflammatory response, e.g. C-Reactive Protein (CRP) production, IL-6 production and leading to changes in hemostasis by the unbalanced production of coagulant factors and proteins involved in fibrinolysis (PAI-1).

Infection

The immune system is vital in defending the body against bacteria and viruses. Improper functioning can lead to a variety of diseases, ranging from immune deficiencies to chronic inflammation. In chronic infections, a limited number of cells (in the target organs) are infected. These infected cells may demonstrate a cytopathology effect, synthesize virus macromolecules, and release infectious virus. The spread of infection is limited by host factors such as humoral and cell-mediated immune responses. Viral affinity for specific body tissues (tropism) is determined by cell receptors for virus, cell transcription factors that recognize viral promoters and enhancer sequences, ability of the cell to support virus replication, physical barriers, local temperature, pH and digestive enzymes and bile in the gastrointestinal tract that may inactivate some viruses. Viruses enter the human body via the respiratory system, gastrointestinal tract, skin and genital routes although other routes can be used. The final outcome of infection may be determined by the dose and location of the virus as well as its infectivity and virulence. Most virus types spread among cells extracellular, but some may also spread intracellular. Establishment of local infection may lead to localized disease and localized shedding of virus. The most common route of systemic spread from the portal of entry is the circulation, which the virus reaches via the lymphatic system. Virus may enter the target organs from the capillaries by multiplying in endothelial cells or fixed macrophages, diffusing through gaps, and being carried in a migrating leukocyte. Dissemination via peripheral nerves usually occurs with rabies virus and sometimes with herpes virus and poliovirus infections. Depending on the balance between virus and host defenses, virus multiplication in the target organ may be sufficient to cause disease and death. Although the respiratory tract, alimentary tract, urogenital tract and blood are the most frequent sites of shedding, various viruses may be shed at virtually every site.

Infection causing 'non-infectious diseases'

Chronic infection and oncogenesis

Many chronic diseases are caused by pathogens or by the chronic inflammatory response of our own bodies to pathogens. Infections provoke an immune response from the body. It has recently been recognized that certain chronic infectious agents may contribute to carcinogenesis by inducing a state of persistent inflammation. Certain

persistent viral or bacterial infections can promote a chain of events by interfering with the regulation of cell proliferation or cell death or by inducing DNA damage. A feature common to all oncogenic infectious agents is their long term persistence in infected individuals. The infectious agents that have been recognized as human carcinogens include the viruses Epstein-Barr virus (EBV), Kaposi sarcoma herpes virus (KSHV), Human papillomavirus (HPV), Human T-cell lymphotropic virus (HTLV-I), Hepatitis B virus (HBV), Hepatitis C virus (HCV) and the Helicobacter pylori (HP) bacterium. EBV is associated with Non-Hodgkin's lymphoma in the lymph nodes[37,38] KSHV with Kaposi sarcomas[39,40] HPV with cervical cancer[41,42] and HBV and HCV with hepatocellular carcinoma[43-45]. The H. pylori bacterium is related to gastric cancer[46].

Chronic infection and atherosclerosis

The concept that chronic infection may contribute to the pathogenesis of atherosclerosis was already proposed in the early 1980's. With respect to the importance of inflammation in atherosclerosis, many have hypothesized that infectious agents may be the cause of chronic vascular inflammation. Although it remains not fully elucidated whether pathogens are etiological factors in atherosclerosis, pathogens can aggravate the inflammatory process in atherosclerosis[47-49]. The major organisms that have been studied with respect to vascular diseases are *Chlamydia pneumoniae (Cp)*, Cytomegalovirus (CMV), and Helicobacter pylori, but enterovirus, hepatitis A virus and herpes simplex virus type 1 and type 2 have also been implicated. An association is described between cytomegalovirus or *Cp* and an increased risk of vascular events[50]. These micro-organisms are able to infect endothelial cells and smooth muscle cells *in vivo* and *in vitro*, evoking pathophysiological reactions of these cells which may lead to atherosclerosis, arterial thrombosis and plaque rupture[50-54]. CMV, *Cp* and many other bacteria have been detected in human atherosclerotic lesions[51,55]. *Cp* in particular has been the focus of attention as a possible contributor to coronary atherosclerosis[56]. In an animal model, repeated infections with *Cp* resulted in endothelial dysfunction via impaired NO availability[57]. Elevated titers of antibodies against Chlamydia were found in patients with CAD[47] and it was speculated that this microbe causes atherosclerosis. Although *Cp*-infection did not cause atherosclerosis in animals it may stimulate disease progression and plaque activation[58,59]. This could be due either to a direct action in plaques or to remote signaling by inflammatory mediators[60]. Molecular mimicry between *Cp*-antigens and human molecules may contribute to the activation of inflammation[61]. However, several recent secondary-prevention trials, failed to prevent

acute coronary syndromes by administering antibiotics targeting *Cp*, suggesting that *Cp*-infection is not a predominant cause of these syndromes[62-64]. Herpes family viruses may also contribute to CAD. Infection with CMV and other herpes viruses, such as HSV, has been implicated in some, but not all studies[65-67]. Serum anti-CMV antibody levels are significantly elevated in patients with coronary disease when compared to control subjects[65,68] and in those with carotid intimal thickening[69]. In addition, CMV antigens, nucleic acid sequences, and DNA have been detected in smooth muscle cells of carotid artery plaques obtained from patients undergoing endarterectomy[70]. CMV is found in arterial lesions, can modulate immune-cell as well as vascular-cell activity, and increases experimental atherosclerosis[71]. Furthermore, *in vitro* infection of macrophages with CMV increases secretion of IL-1, TNF-α and M-CSF. Clinical data imply an important role for CMV in transplantation-related arteriosclerosis causing graft rejection[72]. There is an association between antibodies to CMV and elevated levels of markers of inflammation, including C-reactive protein (CRP) and interleukin (IL)-6. The combination of CMV-seropositivity and elevated serum CRP is a strong, independent predictor of mortality[56]; the data are similar for CMV-seropositivity and elevated serum IL-6 [73]. These findings suggest that CMV elicits a subclinical inflammatory response in certain individuals who are therefore susceptible to the development of atherosclerosis; those without an inflammatory response are resistant[74]. In contrast to CMV and *Cp*, influenza virus represents a micro-organism which causes more acute infections with severe (systemic) complications. Vaccination against influenza has been evaluated for a potential benefit in preventing cardiovascular disease[75-77]. Several studies have found a beneficial effect of influenza vaccination on cardiovascular events. While these studies are of interest and do document a beneficial effect of influenza vaccination in the elderly, they do not establish a causative relationship between influenza infection and the pathogenesis of atherosclerosis. It is possible that the observed beneficial effect of vaccination on the incidence of cardiac events relates to the prevention of clinical influenza, with consequent reduction in such complications as dehydration, hypoxemia, and demand ischemia. In a study of mice infected with influenza A, HDL lost its anti-inflammatory properties during the acute-phase response. *In vitro* analyses found that the ability of HDL to inhibit LDL oxidation and LDL-induced monocyte chemotactic activity in human arterial cell co-cultures decreased with time after infection. These changes were not associated with a direct effect of the virus on HDL, but were thought to result from a systemic response[78]. Regarding the possible role of infectious pathogens in atherosclerosis, it has been suggested that antimicrobial

therapy might reduce coronary risk. Results of clinical trials investigating anti-chlamydial antibiotics as an addition to standard therapy in patients with coronary artery disease have been inconsistent[79-81]. Although quinolone treatment seems to reduce adverse cardiac events in patients with acute coronary syndromes, the effect was independent of H. pylori or *Cp*-seropositivity[82]. The results of a meta-analysis of large randomized trials do not indicate that antibiotic therapy against *Cp* is beneficial for reducing cardiovascular coronary events[83].

Cellular dysfunction caused by infection

Chronic infection may contribute to the pathogenesis of cellular dysfunction by initiating and maintaining cellular injury leading to a local or systemic inflammatory response. In persistent viral infections, the host's immune system is challenged by the constant exposure to microbial antigens with no evidence of protein synthesis but still interference with cellular functions. Low-grade chronic inflammation that is persistent, as can be seen in obesity, diabetes, and the metabolic syndrome could result in acceleration of the cellular and humoral immunologic response[84]. An acute inflammatory response in normal subjects, as measured by elevated plasma concentrations of C-reactive protein, a marker of chronic inflammation, is an independent predictor of endothelial dysfunction. Even before clinical symptoms become evident, a complex of inflammatory and metabolic changes may have been initiated. In early stages of disease (pro-)inflammatory cytokines, adhesion molecules, oxygen radicals and impaired NO availability[57] can be detected as markers and predictors of cellular dysfunction. In other cases, the effect may simply be that of enhancing the pre-existing chronic inflammatory response of the body to standard risk factors such as hypercholesterolemia. Even though the infectious agent may not directly infect the arterial wall, it may perform its critical role from afar.

Endothelial dysfunction

Atherosclerosis is a major cause of morbidity and mortality worldwide. Inflammatory processes and endothelial dysfunction appear to play important roles in the etiology of cardiovascular diseases. Nevertheless, the mechanisms underlying these conditions are not fully understood, yet. A large number of risk factors for atherosclerosis have been proposed and specifically hypercholesterolemia, systemic hypertension, smoking and diabetes have been associated with endothelial dysfunction[85-87].

Endothelial dysfunction plays a pivotal role in atherogenesis and development of clinical evident vascular diseases. Under normal conditions the endothelium regulates vascular tone and has anti-atherogenic and anti-thrombotic properties. Dysfunctional endothelium may initiate and promote atherogenesis due to expression of adhesion molecules, tissue factor expression, platelet activation and release of pro-inflammatory cytokines. Enhanced expression of adhesion molecules leads to enhanced monocyte adhesion and subsequent migration across the endothelium into the vessel wall and accumulation of oxidized low density lipoproteins (LDL) leads to foam cell formation[88]. Reduced bioavailability of nitric oxide (NO) is the primary cause and/or result of endothelial dysfunction. Elevated plasma cholesterol concentrations and oxidized LDL in combination with high levels of radicals may inactivate NO and impair NO-synthesis by affecting the function of NO synthase[89-92]. A number of risk factors for atherogenesis, including infectious agents, have been shown to exert their influence via inflammatory actions[93]. Endothelial cell activation leads to expression of pro-inflammatory cytokines like IL-6, TNF-α and CRP. These inflammatory cytokines have local effects on neighboring endothelial cells and have systemic effects on the vessel wall and on insulin resistance. Infection and the resulting inflammatory response can produce endothelial dysfunction. In the last decade a role for infections in (chronic) inflammation in lesions and the pathogenesis of atherosclerosis has been proposed frequently[50,53,57,94]. Detection of microbial sequences in the vessel wall has proven the direct involvement of microorganisms in atherogenesis[51,55]. Direct infection of endothelial cells may cause acute and chronic activation of the endothelium and loss of its anti-atherogenic protection. In addition, there is now a growing recognition of non-traditional risk factors, like infections as potential modulators of the endothelial phenotype in obesity, including fat tissue production of pro-atherogenic adipokines, oxidative stress, and chronic inflammation[95-97].

Macrophage dysfunction

Macrophages are involved in all stages of the immune response and constitute an important cellular component of the immune responses against viruses. They serve as antigen presenting cells and also secrete inflammatory mediators to activate innate and adaptive immune cells. Resident tissue macrophages are relatively quiescent immunologically, but in response to invasive stimuli and inflammation they become activated and their numbers may increase dramatically. Mononuclear phagocytes (monocytes and macrophages) are central to inflammation, as they produce many

components that regulate the mediators of the inflammatory response. They are also actively phagocytic and are involved in microbial killing. Analysis of cytokine production during immune responses suggests that different macrophage populations participate at various stages, or that the changing conditions within the lesion differentially affect the functions of distinct macrophage populations. Recently the concept of macrophage polarization was described, indicating that macrophages are functionally polarized in response to microorganisms and host mediators. Polarized activation of cells of the monocyte-heterogeneous macrophage lineage into M1 and M2 cells reveals that M1 polarization can lead to tissue injury and contribute to pathogenesis. The so-called M2 macrophages play a critical role in the resolution of inflammation by producing anti-inflammatory mediators indicating a range of immune and inflammatory conditions[98-100]. In some conditions ineffective phagocytosis and macrophage dysfunction have been described[101] but activation of macrophages probably is a better reproduction of "macrophage dysfunction". In the patho-physiology of monocyte and macrophage dysfunction chronic inflammatory diseases play an important role. Dysfunctional macrophages feature oxidative stress, as reflected by increased levels of ROS, deregulated cytokine release and enhanced chemotaxis. Macrophages that have been modified by oxidized LDL release a variety of inflammatory substances, cytokines, and growth factors[102]. Among the many molecules that have been implicated are: monocyte chemotactic protein (MCP)-1, intercellular adhesion molecule (ICAM)-1)[103], granulocyte-macrophage colony stimulating factors[104], soluble CD40 ligand, interleukins[105] and TNF-α[106]. During infection with intracellular pathogens, macrophages serve a dual role as host and effector cells. Macrophage activation has to be tightly regulated to achieve effective anti-pathogen responses and at the same time avoid immunopathology. Down regulation of macrophages is mediated by multiple mechanisms, including inhibitory cytokines, induction of tolerance, cytokine antagonists, or inhibition of activating signaling pathways. Defects in these regulatory pathways can lead to autoimmunity and hyperinflammation; on the other hand, there is evidence that pathogens, especially those adapted to intracellular life within macrophages, exploit these endogenous inducers of deactivation[107]. Among numerous micro-organisms *Cp*, CMV and influenza are able to infect monocytes and macrophages. *Cp*-infection activates human vascular endothelium, smooth muscle cells and macrophages and affects TNF-α and MMP-production[108]. Growth kinetics of human cytomegalovirus may be altered in monocyte-derived macrophages[109]. Impaired viral replication indicated by non-lytic growth of

human cytomegalovirus in macrophages indicates a chronic persistent CMV-infection still enabling up regulation of cytokine production contributing to inflammatory processes[110, 111]. Influenza A virus-infected macrophages are primed for a high TNF-α release[112] and Influenza A infection in mice macrophages leads to increased arterial macrophage traffic and is associated with loss of anti-inflammatory properties of HDL [113].

Hepatocyte dysfunction

The liver is a key organ in inflammatory processes. Both local and systemic reactions are initiated. Hepatocytes respond by producing "acute phase proteins" like IL-6 and CRP. The intentional purpose of the acute phase reaction is protective aiming to limit tissue injury, infection or inflammation and restoration of homeostasis. Hepatocytes are responsible for a response to inflammation called the acute-phase reaction by production of "acute phase proteins". Positive acute-phase proteins serve different physiological functions for the immune system. These include pro-inflammatory factors like CRP and IL-6, coagulation factors like fibrinogen, prothrombin, factor VIII, von Willebrand factor and plasminogen but also complement factors, ferritin, ceruloplasmin, serum amyloid A and haptoglobin. The negative acute phase reactants like serpins (e.g. PAI-1) and alpha 2-macroglobulin give negative feedback on the inflammatory response. In response to injury, local inflammatory cells (neutrophil granulocytes and macrophages) secrete a number of cytokines into the bloodstream, most notable of which are the interleukins IL-1, IL-6 and IL-8, and TNF-α[114-116]. Under pathological conditions hepatocyte dysfunction can lead to lipid abnormalities and may elicit intensive production of inflammatory mediators and profound changes in the concentrations of proteins participating in coagulation and in fibrinolysis[117]. Infection and inflammation in the liver induce the acute-phase response (APR)[118,119]. This may lead to alterations in lipid and lipoprotein metabolism, oxidative stress, coagulation disorders and production of (pro-) inflammatory cytokines Microorganisms may also initiate and maintain chronic low-grade inflammation by infecting liver cells[120]. The most investigated liver infection is hepatitis caused by Hepatitis B or -C viruses (HBV, HCV). One of the most remarkable aspects of HCV infection is that it causes low-grade infection in the liver over many years[121,122]. Infection of the liver by CMV or *Cp* may induce a general inflammatory reaction contributing to accelerated atherogenesis. Infections with CMV and *Cp* increase fibrinogen production of hepatocytes *in vitro*. In addition, infections also increase IL-6 production. In addition to direct vascular wall infection by *Cp* and CMV, virus-related development of atherosclerosis might also be initiated by

chronic liver infection and subsequent production of inflammatory and procoagulant mediators released in the circulation. This may be another pathophysiological link for the observed relation between infections and the development of atherosclerosis[120]. On the other hand, intrahepatic cytokine production triggers the recruitment of mononuclear cells, which sustain acute and chronic liver damage[123]. A productive infection by *Cp* may take place in Kupffer cells and *Cp* might induce a local pro-inflammatory activity. *Cp* may therefore, be able to act as antigenic stimulus when localized in the liver. One could speculate that *Cp*-infection, involving cells of the innate immunity such as Kupffer cells, could also trigger pathological immune reactions involving the liver[124].

Adipocyte dysfunction

Adipose tissue is distributed over several locations in the body: subcutaneous, abdominal and visceral. Abdominal adipose tissue is correlated with cardiovascular diseases, hypertension and diabetes. Adipose tissue exists of adipocytes; lipid-filled cells which are embedded in an environment of collagen fibers. In addition to adipocytes, adipose tissue contains stromal-vascular cells including fibroblast-like cells, leukocytes, macrophages and pre-adipocytes. Adipose tissue thus appears as a complex tissue composed of different cell subsets that could vary according to the nature and the location of fat pads, or to the physiological or pathological status[125]. Obesity induces adipose tissue macrophage (ATM) infiltration in white adipose tissue in both humans and mice[126,127]. ATM content correlates with measures of adiposity and insulin resistance[128,129], based on the evidence that obesity is associated with infiltration[127] and activation[130] of macrophages in adipose tissue. Obese patients also have an increased risk for the development of atherosclerosis and diabetes mellitus type 2 and myocardial infarction[131]. Although the exact pathophysiological mechanisms are not yet clear, insulin resistance, closely related with obesity, and inflammation are key components[132,133]. Inflammatory and prothrombotic markers are associated with an increased risk for type 2 diabetes and subsequent CVD and obesity and the metabolic syndrome have been proposed to be a state of chronic inflammation, associated with elevated levels of CRP, IL-6, and plasminogen activator inhibitor (PAI)-1[134-136]. Consequently, adipose tissue appears to be important in vascular injury and adipocyte dysfunction can be the cause but also the consequence of insulin resistance. By producing (adiponectin) and inflammatory cytokines (TNF-α, IL-6), adipose tissue is likely to play an important role in the development of diabetes

and atherosclerosis[134,135,137]. Causes for adipocyte dysfunction are not well established but include abdominal obesity and elevated free fatty acid plasma concentration[138]. By releasing hormones and cytokines into the portal circulation, abdominal adipose tissue is able to directly influence liver metabolism, potentially leading to accelerated inflammatory response, e.g. C-reactive protein (CRP) production, and leading to changes in hemostasis by the unbalanced production of coagulant factors and proteins involved in fibrinolysis. Several microorganisms, hormones and inflammatory factors have already been related to diseases such as obesity, atherosclerosis and diabetes mellitus type 2. Diabetics have an increased propensity to develop morbidity from infections. In addition, the types of infections observed in patients with diabetes mellitus (DM) also are complex. Neutrophil chemotaxis and adherence to vascular endothelium, phagocytosis, intracellular bactericidal activity, opsonization, and cell-mediated immunity are all depressed in diabetics with hyperglycemia[139]. Vascular disease related to diabetes may impair the local inflammatory response, the bactericidal functions of leukocytes and the absorption of antibiotics. Although obesity has multiple causes, an often overlooked possibility is that of obesity due to an infection. The expression "infectobesity" describes the possibility that obesity may be, at least in part, the result of viral infections[140,141]. Among several other micro-organisms the human Adenovirus subtype 36, but also subtypes 5, 31 and 37 have been associated with adipogenesis and obesity[142,143]. Data from animal models suggest that the role of viral disease in the etiology of human obesity must be considered[144-146]. Ad-36 is associated with increased body weight and lower serum lipids in humans. Prospective studies need to indicate if Ad-36 plays a role in the etiology of human obesity[147]. The inflammatory response of adipocytes upon infection may also have paracrine effects by affecting the function of neighboring adipocytes and may attract macrophages into adipose tissue, increasing the capacity for the production of inflammatory mediators

Objectives of this thesis

In this thesis we aimed to investigate the role for microorganisms in inducing and maintaining cellular dysfunction causing pathophysiological processes potentially involved in the development of atherosclerosis and diabetes mellitus.

Outline of this thesis

In **chapter 2** we investigate the ability of respiratory viruses (influenza A, influenza B, parainfluenza-1, respiratory syncytial virus, adenovirus and CMV) to infect lung fibroblasts and human umbilical vein endothelial cells and the effect of infection on the procoagulant activity of these cells *in vitro*. Production of cytokines by monocytes may accelerate the chronic process of atherosclerosis and may contribute to coronary syndromes by eliciting plaque instability. Whether virus-infected monocytes initiate coagulation and produce (pro-) inflammatory cytokines was investigated in **chapter 3**.
In **chapter 4** we describe that human hepatocytes can be infected with the intracellular pathogens *Cp* and CMV. We measure the production of fibrinogen, IL-6, and PAI-1 after infection. Azithromycin will be added to establish whether biosynthesis of these parameters could be influenced by antibiotic treatment.
Nitric oxide (NO) is a key regulator of endothelial function. Under pathological conditions uncoupling of endothelial nitric oxide synthase (eNOS) leads to vessel damage as a result of production of oxygen radicals instead of NO, indicating endothelial dysfunction. In **chapter 5** we measured production of eNOS, cyclic guanosine monophosphate (cGMP) as a surrogate for NO and reactive oxygen species (ROS) by endothelial cells infected with *Cp* or CMV *in vitro*.
The labile forms of iron may play a role in the development of atherosclerotic vascular disease. Iron has been found accumulating in human atheroma and several studies have shown reduced formation of early atherosclerotic lesions by means of iron chelation or iron-deficient diets in experimental studies. In **chapter 6** we mimic a chronic low-grade vascular *Cp*-infection and observe the presumed modulating effects of iron on endothelial response towards *Cp*. Endothelial intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial selectin (E-selectin) expression are measured as an indication of endothelial activation. We also evaluate potential counteracting effects of iron chelation as well as radical scavenging on the effects of iron on endothelial cells.
In the study described in **chapter 7** we investigate the potential role for microorganisms in inducing and maintaining adipocyte dysfunction and investigate whether several viruses are able to infect adipocytes and pre-adipocytes *in vitro* and whether this leads to adipocyte dysfunction as measured by the altered production of the adipokines IL-6, TNF-α, adiponectin and PAI-1. The inflammatory response of adipocytes upon infection may also have paracrine effects by affecting the function of neighboring

adipocytes and may attract macrophages into adipose tissue, increasing the capacity for the production of inflammatory mediators. In **chapter 8** we measured adipokine production in an *in vitro* co-culture model of adipocytes and macrophages. Co-incubation of adipocytes and macrophages may synergistically amplify adipokine expression, presumably leading to an aggravated inflammatory response. Overproduction of adipokines may evoke potent proatherogenic effects in the vasculature. This may provide a pathological link between obesity and its associated cardiovascular complications. Finally, in **chapter 9** the results are summarized and discussed.

Reference List

1. Lien E, Ingalls RR. Toll-like receptors. Crit Care Med 2002 ;30 (1 Suppl):S1-11
2. Peiser L, Gordon S. The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. Microbes Infect 2001;3(2):149-59.
3. Heyzer-Williams MG, Heyzer-Williams LJ, Fanelli PJ, Bikah G, Pogue-Caley RR, Driver DJ, Eisenbraun MD. Antigen-specific immunity. Th cell-dependent B cell responses. Immunol Res 2000;22 (2-3):223-36
4. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res 2002 23;91(4):281-91.
5. Santana MA, Esquivel-Guadarrama F. Cell biology of T cell activation and differentiation. Int Rev Cytol 2006;250:217-74 .
6. Romagnani S. T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol 2000 ;85(1):9-18.
7. Leibovich SJ, Ross R. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. Am J Pathol 1975 ;78(1):71-100.
8. Osterud B. Tissue factor expression by monocytes: regulation and pathophysiological roles. Blood Coagul Fibrinolysis 1998;9 Suppl 1:S9-14.
9. Daniels AU, Barnes FH, Charlebois SJ, Smith RA. Macrophage cytokine response to particles and lipopolysaccharide *in vitro*. J Biomed Mater Res 2000 ;49(4):469-78.
10. Edwards RL, Rickles FR. Macrophage procoagulants. Prog Hemost Thromb 1984;7:183 -209.
11. Lucas AD, Greaves DR. Atherosclerosis: role of chemokines and macrophages. Expert Rev Mol Med 2001;3(25):1-18).
12. Linton MF, Fazio S. Macrophages, inflammation, and atherosclerosis. Int J Obes Relat Metab Disord 2003 ;27(3):S35-40.
13. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5(12):953-64

14. Naito M, Hasegawa G, Takahashi K. Development, differentiation, and maturation of Kupffer cells. Microsc Res Tech 1997;39 (4):350-64.
15. Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet 1994;344(8925):793-5.
16. Paoletti R, Gotto AM, Jr., Hajjar DP. Inflammation in atherosclerosis and implications for therapy. Circulation 2004;109(23):III20 -6.
17 Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340(2):115-26.
18. Frostegard J, Wu R, Giscombe R, Holm G, Lefvert AK, Nilsson J. Induction of T-cell activation by oxidized low density lipoprotein. Arterioscler Thromb 1992;12(4):461-7.
19. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994;89(1):36-44.
20. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis 1986;6(2):131-8.
21. Schwartz CJ, Mitchell JR. Cellular infiltration of the human arterial adventitia associated with atheromatous plaques. Circulation 1962;26:73-8.
22. Fuster V. Lewis A. Conner Memorial Lecture. Mechanisms leading to myocardial infarction: insights from studies vascular biology. Circulation 1994;90(4):2126-46.
23. Ikeda U, Ito T, Shimada K. Interleukin-6 and acute coronary syndrome. Clin Cardiol 2001;24 (11):701-4.
24. Mortensen RF. C-reactive protein, inflammation, and innate immunity. Immunol Res 2001;24(2):163-76.
25. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997;336(14):973-9.
26. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 2006;86(2):515-81
27. Mallat Z, Tedgui A. Cytokines as regulators of atherosclerosis in murine models. Curr Drug Targets 2007;8(12):1264-72.
28 Raines EW, Ferri N. Thematic review series: The immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. J Lipid Res 2005;46(6):1081-92
29. Li H, Freeman MW, Libby P. Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and *in vitro* by cytokines. J Clin Invest 1995;95(1):122-33.
30. Feldman LJ, Aguirre L, Ziol M, Bridou JP, Nevo N, Michel JB, Steg PG. Interleukin-10 inhibits intimal hyperplasia after angioplasty or stent implantation in hypercholesterolemic rabbits. Circulation 2000;101(8):908-16.
31. Mazighi M, Pelle A, Gonzalez W, Mtairag eM, Philippe M, Henin D, Michel JB, Feldman LJ. IL-10 inhibits vascular smooth muscle cell activation *in vitro* and in vivo. Am J Physiol Heart Circ Physiol 2004;287(2):H866-71

32. Veltrop MH, Langermans JA, Thompson J, Bancsi MJ. Interleukin-10 regulates the tissue factor activity of monocytes in an *in vitro* model of bacterial endocarditis. Infect Immun 2001;69(5):3197-202.
33. Libby P. Atheroma: more than mush. Lancet 1996;348 Suppl 1:s4-7.
34. Hansson GK. Immune mechanisms in atherosclerosis. Arterioscler Thromb Vasc Biol 2001;21(12):1876-90.
35. Hansson GK. Regulation of immune mechanisms in atherosclerosis. Ann N Y Acad Sci 2001;947:157-65.
36. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol 2005;15(11):599-607.
37. Kapatai G, Murray P. Contribution of the Epstein Barr virus to the molecular pathogenesis of Hodgkin lymphoma. J Clin Pathol 2007;60(12):1342-9.
38. Gandhi MK, Tellam JT, Khanna R. Epstein-Barr virus-associated Hodgkin's lymphoma. Br J Haematol 2004;125(3):267-81.
39. Krown SE, Lee JY, Dittmer DP. More on HIV-associated Kaposi's sarcoma. N Engl J Med 2008;358(5):535-6.
40. Engels EA, Biggar RJ, Hall HI, Cross H, Crutchfield A, Finch JL, et al. Cancer risk in people infected with human immunodeficiency virus in the United States. Int J Cancer 2008;123(1):187-94.
41. Woodman CB, Collins SI, Young LS. The natural history of cervical HPV infection: unresolved issues. Nat Rev Cancer 2007;7 (1):11-22.
42. Huang Y, Huang MN, Li N, Li XG, Li N, Wu LY. Association between human papillomavirus DNA load and development of cervical intraepithelial neoplasia and cervical cancer. Int J Gynecol Cancer 2008;18(4):755-60
43. Schafer DF, Sorrell MF. Hepatocellular carcinoma. Lancet 1999;353(9160):1253-7.
44. Gilliam JHI, Geisinger KR, Richter JE. Primary hepatocellular carcinoma after chronic non-A, non-B post-transfusion hepatitis. Ann Intern Med 1984;101(6):794-5.
45. Kiyosawa K, Akahane Y, Nagata A, Furuta S. Hepatocellular carcinoma after non-A, non-B posttransfusion hepatitis. Am J Gastroenterol 1984;79 (10):777-81.
46. Farinati F, Cardin R, Cassaro M, Bortolami M, Nitti D, Tieppo C, Zaninotto G, Rugge M. Helicobacter pylori, inflammation, oxidative damage and gastric cancer: a morphological, biological and molecular pathway. Eur J Cancer Prev 2008;7(3):195-200.
47. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK, Valtonen V. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 1988; 2(8618):983-6.
48. Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? Lancet 1997;350(9075):430-6.
49. Bell DS. Inflammation, insulin resistance, infection, diabetes, and atherosclerosis. Endocr Pract 2000;6(3):272-6.

50. de Boer OJ, van der Wal AC, Becker AE. Atherosclerosis, inflammation and infection. J Pathol 2000;190(3):237-43.
51. Prasad A, Zhu J, Halcox JP, Waclawiw MA, Epstein SE, Quyyumi AA. Predisposition to atherosclerosis by infections: role of endothelial dysfunction. Circulation 2002;106(2):184-90.
52. Capron L. [Role of infectious agents in the understanding of atherosclerosis]. Rev Med Interne 2002;23 Suppl 1:8s-9s.
53. Visseren FL, Erkelens DW. Atherosclerosis as an infectious disease. Ned Tijdschr Geneeskd 1999;143(6):291-5.
54. Kuo CC, Campbell LA, Rosenfeld ME. Chlamydia pneumoniae infection and atherosclerosis: methodological considerations. Circulation 2002;105(4):E34.
55. Ott SJ, El Mokhtari NE, Musfeldt M, et al. Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. Circulation 2006;113(7):929-37.
56. Muhlestein JB, Horne BD, Carlquist JF, Madsen TE, Bair TL, Pearson RR, Anderson JL. Cytomegalovirus seropositivity and C-reactive protein have independent and combined predictive value for mortality in patients with angiographically demonstrated coronary artery disease. Circulation 2000;102(16):1917-23.
57. Liuba P, Karnani P, Pesonen E, Paakkari I, Forslid A, Johansson L, Persson K, Wadstrom T, Laurini R. Endothelial dysfunction after repeated Chlamydia pneumoniae infection in apolipoprotein E-knockout mice. Circulation 2000;102(9):1039-44.
58. Caligiuri G, Rottenberg M, Nicoletti A, Wigzell H, Hansson GK. Chlamydia pneumoniae infection does not induce or modify atherosclerosis in mice. Circulation 2001;103(23):2834-8.
59. Hu H, Pierce GN, Zhong G. The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to Chlamydia pneumoniae. J Clin Invest 1999;103(5):747-53.
60. Kalayoglu MV, Libby P, Byrne GI. Chlamydia pneumoniae as an emerging risk factor in cardiovascular disease. Jama 2002;288 (21):2724-31.
61. Perschinka H, Mayr M, Millonig G, Mayerl C, van der Zee, Morrison SG, Morrison RP, Xu Q, Wick G. Cross-reactive B-cell epitopes of microbial and human heat shock protein 60/65 in atherosclerosis. Arterioscler Thromb Vasc Biol 2003;23(6):060-5.
62. Cannon CP, Braunwald E, Mccabe CH, Grayston JT, Muhlestein B, Giugliano RP, Cairns R, Skene AM. Antibiotic treatment of Chlamydia pneumoniae after acute coronary syndrome. N Engl J Med 2005;352(16):1646-54.
63. Cercek B, Shah PK, Noc M, Zahger D, Zeymer U, Matetzky S, Maurer G, Mahrer P. Effect of short-term treatment with azithromycin on recurrent ischaemic events in patients with acute coronary syndrome in the Azithromycin in Acute Coronary Syndrome (AZACS) trial: a randomised controlled trial. Lancet 2003;361(9360):809-13.

64. Grayston JT, Kronmal RA, Jackson LA, Parisi AF, Muhlestein JB, Cohen JD, Rogers WJ, Crouse JR, Borrowdale SL, Schron E, Knirsch C. Azithromycin for the secondary prevention of coronary events. N Engl J Med 2005;352(16):1637-45.

65. Blum A, Giladi M, Weinberg M, Kaplan G, Pasternack H, Laniado S, Miller H. High anti-cytomegalovirus (CMV) IgG antibody titer is associated with coronary artery disease and may predict post-coronary balloon angioplasty restenosis. Am J Cardiol 1998;81(7):866-8.

66. Rupprecht HJ, Blankenberg S, Bickel C, Rippin G, Hafner G, Prellwitz W, Schlumberger W, Meyer J. Impact of viral and bacterial infectious burden on long-term prognosis in patients with coronary artery disease. Circulation 2001;104(1):25-31

67. Sorlie PD, Nieto FJ, Adam E, Folsom AR, Shahar E, Massing M. A prospective study of cytomegalovirus, herpes simplex virus 1, and coronary heart disease: the atherosclerosis risk in communities (ARIC) study. Arch Intern Med 2000;160(13):2027-32.

68. Adam E, Melnick JL, Probtsfield JL, Petrie BL, Burek J, Bailey KR, McCollum CH, DeBakey ME. High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. Lancet 1987;2(8554):291-3.

69. Nieto FJ, Adam E, Sorlie P, Farzadegan H, Melnick JL, Comstock GW, Szklo M. Cohort study of cytomegalovirus infection as a risk factor for carotid intimal-medial thickening, a measure of subclinical atherosclerosis. Circulation 1996;94(5):922-7.

70. Melnick JL, Hu C, Burek J, Adam E, DeBakey ME. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. J Med Virol 1994 ;42(2):170-4.

71. Hsich E, Zhou YF, Paigen B, Johnson TM, Burnett MS, Epstein SE. Cytomegalovirus infection increases development of atherosclerosis in Apolipoprotein-E knockout mice. Atherosclerosis 2001;156(1):23-8.

72. Soderberg-Naucler C, Emery VC. Viral infections and their impact on chronic renal allograft dysfunction. Transplantation 2001;71 (11 Suppl):S24-30.

73. Blankenberg S, Rupprecht HJ, Bickel C, Espinola-Klein C, Rippin G, Hafner G, Ossendorf M, Steinhagen K, Meyer J. Cytomegalovirus infection with interleukin-6 response predicts cardiac mortality in patients with coronary artery disease. Circulation 2001;103(24):2915-21.

74. Zhu J, Quyyumi AA, Norman JE, Csako G, Epstein SE. Cytomegalovirus in the pathogenesis of atherosclerosis: the role of inflammation as reflected by elevated C-reactive protein levels. J Am Coll Cardiol 1999;34(6):1738-43.

75. Gurfinkel EP, de la Fuente RL, Mendiz O, Mautner B. Influenza vaccine pilot study in acute coronary syndromes and planned percutaneous coronary interventions: the FLU Vaccination Acute Coronary Syndromes (FLUVACS) Study. Circulation 2002;105 (18):2143-7.

76. Naghavi M, Barlas Z, Siadaty S, Naguib S, Madjid M, Casscells W. Association of influenza vaccination and reduced risk of recurrent myocardial infarction. Circulation 2000;102(25):3039-45.

77. Nichol KL, Nordin J, Mullooly J, Lask R, Fillbrandt K, Iwane M. Influenza vaccination and reduction in hospitalizations for cardiac disease and stroke among the elderly. N Engl J Med 2003;348(14):1322-32.
78. Van Lenten BJ, Wagner AC, Nayak DP, Hama S, Navab M, Fogelman AM. High-density lipoprotein loses its anti-inflammatory properties during acute influenza a infection. Circulation 2001;103(18):2283-8.
79. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Progression of early carotid atherosclerosis is only temporarily reduced after antibiotic treatment of Chlamydia pneumoniae seropositivity. Circulation 2004;109(8):1010-5.
80. Vainas T, Stassen FR, Schurink GW, Tordoir JH, Welten RJ, van den Akker LH, Kurvers HA, Bruggeman CA, Kitslaar PJ. Secondary prevention of atherosclerosis through chlamydia pneumoniae eradication (SPACE Trial): a randomised clinical trial in patients with peripheral arterial disease. Eur J Vasc Endovasc Surg 2005; 29(4):403-11.
81. Wiesli P, Czerwenka W, Meniconi A, Maly FE, Hoffmann U, Vetter W, Schulthess G. Roxithromycin treatment prevents progression of peripheral arterial occlusive disease in Chlamydia pneumoniae seropositive men: a randomized, double-blind, placebo-controlled trial. Circulation 2002;105(22):2646-52.
82. Stone AF, Mendall MA, Kaski JC, Edger TM, Risley P, Poloniecki J, Camm AJ, Northfield TC. Effect of treatment for Chlamydia pneumoniae and Helicobacter pylori on markers of inflammation and cardiac events in patients with acute coronary syndromes: South Thames Trial of Antibiotics in Myocardial Infarction and Unstable Angina (STAMINA). Circulation 2002;106(10):1219-23.
83. Andraws R, Berger JS, Brown DL. Effects of antibiotic therapy on outcomes of patients with coronary artery disease: a meta-analysis of randomized controlled trials. Jama 2005;293(21):2641-7.
84. Bistrian B. Systemic response to inflammation. Nutr Rev 2007;65(12 Pt 2):S170-2.
85. Gensini GF, Comeglio M, Colella A. Classical risk factors and emerging elements in the risk profile for coronary artery disease. Eur Heart J 1998;19 Suppl A:A53-61.
86. Lopes-Virella MF, Carter RE, Gilbert GE, Klein RL, Jaffa M, Jenkins AJ, Lyons TJ, Garvey WT, Virella G. Risk Factors related to Inflammation and Endothelial Dysfunction in the DCCT/EDIC Cohort and their Relationship with Nephropathy and Macrovascular Complications. Diabetes Care 2008 31(10):2006-12.
87. Schram MT, Chaturvedi N, Schalkwijk C, Giorgino F, Ebeling P, Fuller JH, Stehouwer CD. Vascular risk factors and markers of endothelial function as determinants of inflammatory markers in type 1 diabetes: the EURODIAB Prospective Complications Study. Diabetes Care 2003;26(7):2165-73.
88. Brevetti G, Martone VD, de CT, Corrado S, Silvestro A, Di Donato AM, Bucur R, Scopacasa F. High levels of adhesion molecules are associated with impaired endothelium-dependent vasodilation in patients with peripheral arterial disease. Thromb Haemost 2001;85(1):63-6.

89. Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation 1997;95(8):2068-74.

90. Mangin EL, Jr., Kugiyama K, Nguy JH, Kerns SA, Henry PD. Effects of lysolipids and oxidatively modified low density lipoprotein on endothelium-dependent relaxation of rabbit aorta. Circ Res 1993;72(1):161-6.

91. Quyyumi AA, Dakak N, Andrews NP, Husain S, Arora S, Gilligan DM, Panza JA, Cannon ROI. Nitric oxide activity in the human coronary circulation. Impact of risk factors for coronary atherosclerosis. J Clin Invest 1995;95(4):1747-55.

92. Vergnani L, Hatrik S, Ricci F, Passaro A, Manzoli N, Zuliani G, Brovkovych V, Fellin R, Malinski T. Effect of native and oxidized low-density lipoprotein on endothelial nitric oxide and superoxide production: key role of L-arginine availability. Circulation 2000;101(11):1261-6.

93. Mahmoudi M, Curzen N, Gallagher PJ. Atherogenesis: the role of inflammation and infection. Histopathology 2007; 50(5):53 -46.

94. Visseren FL, Verkerk MS, Bouter KP, Diepersloot RJ, Erkelens DW. Interleukin-6 production by endothelial cells after infection with influenza virus and cytomegalovirus. J Lab Clin Med 1999;134(6):623-30.

95. Meyers MR, Gokce N. Endothelial dysfunction in obesity: etiological role in atherosclerosis. Curr Opin Endocrinol Diabetes Obes 2007;14(5):365-9.

96. Stapleton PA, James ME, Goodwill AG, Frisbee JC. Obesity and vascular dysfunction. Pathophysiology 2008;15(2):79-89.

97. Taddei S, Ghiadoni L, Salvetti G, Virdis A, Salvetti A.Obesity and endothelial dysfunction. G Ital Cardiol 2006;7(11):715-23.

98. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004;25(12):677-86.

99. Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. J Immunol 2008;181(6):3733-9.

100. Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003;3(1):23-35.

101. Kirkham P. Oxidative stress and macrophage function: a failure to resolve the inflammatory response. Biochem Soc Trans 2007;35 (Pt 2):284-7.

102. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation 1995;91(9):2488-96.

103. Gawaz M, Neumann FJ, Dickfeld et al.. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation 1998;98(12):1164-71.

104. Takahashi M, Kitagawa S, Masuyama JI et al. Human monocyte-endothelial cell interaction induces synthesis of granulocyte-macrophage colony-stimulating factor. Circulation 1996;93(6):1185-93.

105. Szmitko PE, Wang CH, Weisel RD, de A, Jr., Anderson TJ, Verma S. New markers of inflammation and endothelial cell activation: Part I. Circulation 2003;108(16):1917-23.

106. Li H, Freeman MW, Libby P. Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and *in vitro* by cytokines. J Clin Invest 1995;95(1):122-33.

107. Rodriguez N, Lang R, Wantia N, Cirl C, Ertl T, Durr S, Wagner H, Miethke T. Induction of iNOS by Chlamydophila pneumoniae requires MyD88-dependent activation of JNK. J Leukoc Biol 2008;84(6):1585–93.

108. Kol A, Bourcier T, Lichtman AH, Libby P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J Clin Invest 1999;103(4):571-7.

109. Fish KN, Depto AS, Moses AV, Britt W, Nelson JA. Growth kinetics of human cytomegalovirus are altered in monocyte-derived macrophages. J Virol 1995;69(6):373743.

110. Fish KN, Britt W, Nelson JA. A novel mechanism for persistence of human cytomegalovirus in macrophages. J Virol 1996;70 (3):1855-62.

111. Dal MP, Bessia C, Ripalti A, Landini MP, Topilko A, Plachter B, Virelizier JL, Michelson S. Stably expressed antisense RNA to cytomegalovirus UL83 inhibits viral replication. J Virol 1996;70(4):2086-94.

112. Hinder F, Schmidt A, Gong JH, Bender A, Sprenger H, Nain M, Gemsa D. Influenza A virus infects macrophages and stimulates release of tumor necrosis factor-alpha. Pathobiology 1991;59(4):227-31.

113. Van Lenten BJ, Wagner AC, Anantharamaiah GM, Garber DW, Fishbein MC, Adhikary L, Nayak DP, Hama S, Navab M, Fogelman AM. Influenza infection promotes macrophage traffic into arteries of mice that is prevented by D-4F, an apolipoprotein A-I mimetic peptide. Circulation 2002;106(9):1127-32.

114. Streetz KL, Wustefeld T, Klein C, Manns MP, Trautwein C. Mediators of inflammation and acute phase response in the liver. Cell Mol Biol 2001;47(4):661-73.

115. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 1999; 340(6):448-54.

116. Moshage H. Cytokines and the hepatic acute phase response. J Pathol 1997;181(3):257-66.

117. Navab M, Fogelman AM, Berliner JA, Territo MC, Demer LL, Frank JS, Watson AD, Edwards PA, Lusis AJ. Pathogenesis of atherosclerosis. Am J Cardiol 1995;76(9):18C-23C.

118. Ceciliani F, Giordano A, Spagnolo V. The systemic reaction during inflammation: the acute-phase proteins. Protein Pept Lett 2002;9 (3):211-23.

119. Baumann H, Gauldie J. The acute phase response. Immunol Today 1994;15(2):74-80.

120. Verkerk MS, Visseren FL, Paul BK, Diepersloot RJ. Acute-phase response of human hepatocytes after infection with Chlamydia pneumoniae and cytomegalovirus. Eur J Clin Invest 2003;33(8):720-5.

121. Hiroishi K, Ito T, Imawari M. Immune responses in hepatitis C virus infection and mechanisms of hepatitis C virus persistence. J Gastroenterol Hepatol 2008;23(10):1473-82.

122 Pozzato G, Mazzaro C, Crovatto M, Modolo ML, Ceselli S, Mazzi G, Sulfaro S, Franzin F, Tulissi P, Moretti M. Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia. Blood 1994;84(9):3047-53.

123. Gehring AJ, Sun D, Kennedy PT, Nolte-'t HE, Lim SG, Wasser S, Selden C, Maini MK, Davis DM, Nassal M, Bertoletti A. The level of viral antigen presented by hepatocytes influences CD8 T-cell function. J Virol 2007;81(6):2940-9

124. Marangoni A, Donati M, Cavrini F, Aldini R, Accardo S, Sambri V, Montagnani M, Cevenini R. Chlamydia pneumoniae replicates in Kupffer cells in mouse model of liver infection. World J Gastroenterol 2006;12(40):6453-7.

125. Cousin B, Caspar-Bauguil S, Planat-Benard V, Laharrague P, Penicaud L, Casteilla L. [Adipose tissue: a subtle and complex cell system]. J Soc Biol 2006;200(1):51-7.

126. Apovian CM, Bigornia S, Mott M, Meyers MR, Ulloor J, Gagua M, McDonnell M, Hess D, Joseph L, Gokce N. Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects. Arterioscler Thromb Vasc Biol 2008;28(9):1654-9.

127. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112(12):1796-808.

128. Di Gregorio GB, Yao-Borengasser A, Rasouli N, Varma V, Lu T, Miles LM, Ranganathan G, Peterson CA, McGehee RE, Kern PA. Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin resistance, and reduction by pioglitazone. Diabetes 2005;54(8):2305-13.

129. Cancello R, Henegar C, Viguerie N et al. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes 2005;54(8):2277-86.

130. Lumeng CN, Deyoung SM, Bodzin JL, Saltiel AR. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes 2007;56(1):16-23.

131. Yusuf S, Hawken S, Ounpuu S, Bautista L, Franzosi MG, Commerford P, Lang CC, Rumboldt Z, Onen CL, Lisheng L, Tanomsup S, Wangai P, Jr., Razak F, Sharma AM, Anand SS. Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. Lancet 2005;366(9497):1640-9.

132. Lindsay RS, Howard BV. Cardiovascular risk associated with the metabolic syndrome. Curr Diab Rep 2004;4(1):63-8.

133. Koh KK, Han SH, Quon MJ. Inflammatory markers and the metabolic syndrome: insights from therapeutic interventions. J Am Coll Cardiol 2005;46(11):1978-85.

134. Duncan BB, Schmidt MI, Pankow JS et al. Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. Diabetes 2003;52(7):1799-805.

135. Festa A, D'Agostino R, Jr., Tracy RP, Haffner SM. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes 2002 ;51(4):1131-7.

136. Jialal I, Devaraj S, Venugopal SK. C-reactive protein: risk marker or mediator in atherothrombosis? Hypertension 2004;44(1):6-11.

137. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. Nature 2006;444(7121):875-80.

138. Gasteyger C, Tremblay A. Metabolic impact of body fat distribution. J Endocrinol Invest 2002;25(10):876-83.

139. Geerlings SE, Hoepelman AI. Immune dysfunction in patients with diabetes mellitus (DM). FEMS Immunol Med Microbiol 1999;26 (3-4):259-65.

140. Desruisseaux MS, Trujillo ME, Trujillo ME, Tanowitz HB, Scherer PE. Adipocyte, adipose tissue, and infectious disease. Infect Immun 2007;75(3):1066-78.

141. Dhurandhar NV. Infectobesity: obesity of infectious origin. J Nutr 2001;131(10):2794S-2797S.

142. Dhurandhar NV. Contribution of pathogens in human obesity. Drug News Perspect 2004;17(5):307-13.

143. Whigham LD, Israel BA, Atkinson RL. Adipogenic potential of multiple human adenoviruses in vivo and *in vitro* in animals. Am J Physiol Regul Integr Comp Physiol 2006;290(1):R190-4.

144. Dhurandhar NV, Whigham LD, Abbott DH, Schultz-Darken NJ, Israel BA, Bradley SM, Kemnitz JW, Allison DB, Atkinson RL. Human adenovirus Ad-36 promotes weight gain in male rhesus and marmoset monkeys. J Nutr 2002;132(10):3155-60.

145. Dhurandhar NV, Israel BA, Kolesar JM, Mayhew G, Cook ME, Atkinson RL. Transmissibility of adenovirus-induced adiposity in a chicken model. Int J Obes Relat Metab Disord 2001;25(7):990-6.

146. Dhurandhar NV, Israel BA, Kolesar JM, Mayhew GF, Cook ME, Atkinson RL. Increased adiposity in animals due to a human virus. Int J Obes Relat Metab Disord 2000;24(8):989-96.

147. Atkinson RL, Dhurandhar NV, Allison DB, Bowen RL, Israel BA, Albu JB, Augustus AS. Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. Int J Obes (Lond) 2005;29(3):281-6.

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2

Procoagulant Activity of Endothelial Cells after Infection with Respiratory Viruses

Summary

Influenza virus epidemics are associated with excess mortality due to cardiovascular diseases. There are several case reports of excessive coagulation during generalized influenza virus infection. In this study, we demonstrate the ability of respiratory viruses (influenza A, influenza B, parainfluenza-1, respiratory syncytial virus, adenovirus, cytomegalovirus) to infect lung fibroblasts and human umbilical vein endothelial cells in culture. All viral pathogens induced procoagulant activity in infected endothelial cells, as determined in a one-stage clotting assay, by causing an average 55% reduction in the clotting time. When factor VII deficient plasma was used clotting time was not reduced. The induction of procoagulant activity was associated with a 4- to 5-fold increase in the expression of tissue factor, as measured by the generation of factor Xa. Both experiments indicate that the procoagulant activity of endothelial cells in response to infection with respiratory viruses is caused by up regulation of the extrinsic pathway. Although both enveloped viruses and a non-enveloped virus (adenovirus) induced procoagulant activity in endothelial cells by stimulating tissue factor expression, the role of the viral envelope in the assembly of the prothrombinase complex remains uncertain.

We conclude that both enveloped and non-enveloped respiratory viruses are capable of infecting cultured human endothelial cells and causing a shift from anticoagulant to procoagulant activity associated with the induction of tissue factor expression.

Introduction

Endothelial cells form a functional and dynamic non-thrombogenic surface in the vascular lumen, and damage to these cells may be a starting point for inflammation, thrombosis and eventually atherosclerosis. There is increasing evidence that viral pathogens, in particular members of the herpes virus family (cytomegalovirus (CMV) and herpes simplex virus (HSV)) can damage the endothelium[1-4]. Herpes viruses[5-7] and measles virus[8] induce procoagulant activity in endothelial cells and cause increased adherence of polymorph nuclear leukocytes[9] and monocytes[10] to endothelial cell monolayers. In an overview Hajjar describes the potential mechanisms of viral activation of coagulation by herpes viruses[11].

Influenza epidemics are associated with excess mortality and morbidity due to cardiovascular diseases[12-15]. Although age and background diseases appeared to be associated risk factors for influenza-related morbidity and mortality, the exact

mechanism by which influenza has this effect is not known. An influenza-like syndrome resembling acute respiratory infection often precedes myocardial infarction[16, 17]. Also, symptoms of chronic upper airway infection predicted the risk of coronary disease independent of the known major cardiovascular risk factors[18]. Influenza virus is able to invade myocardial and pericardial tissue[19-23] and enhances platelet aggregation *in vitro* [24-26] and *in vivo*[27]. In a clinical survey, Bogomolov et al. demonstrated a pronounced acceleration of coagulation in elderly patients with an influenza virus infection[27]. During convalescence, these patients had low concentrations of antithrombin III. A pregnant female is reported to have generalized influenza infection resulting in disseminated intravascular coagulation (DIC)[28]. Multiple thrombosis was observed in the microcirculation. In an animal model, the most pronounced changes observed after infection with influenza were found in the vessels of the lung and brain microcirculation. These changes included alteration of the luminal surface profile and damage to the super membrane layer[26]. In some studies human endothelial cells were infected with influenza virus *in vitro*[29-32]. Infected cells produced interferon-α and were thus able to up regulate the immune system[29]. Moreover, leukocytes adhered to human endothelial cells infected with influenza virus[31]. In order to investigate the thrombogenic effects of viral infection of endothelial cells and the potential role of virus infection in initiating atherothrombosis, we investigated the ability of respiratory viruses, including influenza viruses, to infect human endothelial cells and cells of other cell lines *in vitro* and the response of endothelial cells, in terms of procoagulant activity, to infection.

Methods

Cell culture

Endothelial cells. Human umbilical vein endothelial cells were harvested and cultured as described by Jaffe et al[33] with minor modifications. Briefly, the umbilical veins were canulated at each end and washed through with phosphate buffered saline (PBS). A solution of 0.05% trypsin-0.02% EDTA (Trypsin-EDTA, Life Technologies Inc., Paisley, Scotland) was introduced into the vessel lumen and left at 37 ^{0}C for 20 minutes. The cells were collected by flushing the vein with sterile PBS and centrifuging the suspension at 1000 rpm for 10 minutes. The pellet was resuspended in endothelial cell growth medium (EGM, Clonetics, San Diego, USA), supplemented with 2% fetal bovine serum, 10 mg/L epidermal growth factor, 12 mg/L bovine brain extract, 1 mg/L hydrocortisone, 50 mg/L gentamycin sulphate and 50 mg/L amphotericin-B. Cells were

cultured in tissue culture flasks (Nunc, Roskilde, Denmark) in a humidified incubator at 37 ^{0}C with 5% CO_2. The growing surfaces of the tissue culture flasks were coated with fibronectin (a generous gift from dr. J.A. van Mourik, CLB, Amsterdam, the Netherlands). For all experiments cultures of passage 3 were used. For procoagulant studies cells were cultured in coagulometer cups (Amelung, Lemgo, Germany) pretreated with fibronectin or for immunocytochemical studies in 24-well tissue culture plates (Nunc, Roskilde, Denmark). The purity of the cultures was verified by morphological analysis as well as by immunostaining (Dako, Glostrup, Denmark) for human Von Willebrand factor antigen (> 97% staining).

Fibroblasts. Human embryonic lung fibroblasts were cultured in Eagle Minimal Essential Medium (EMEM) (Life Technologies Ltd., Paisley, Scotland) supplemented with 10% fetal calf serum (Life Technologies Ltd., Paisley, Scotland), 4 mg/L amphotericin-B (Bristol-Myers-Squibb, Epernon, France), 10 mg/L gentamycin (Schering-Plough, Heist op den Berg, Belgium), 10 mg/L vancomycin (Eli Lilly, Indianapolis, USA), 2 mM L-glutamine (Life Technologies Ltd., Paisley, Scotland) and 1% non-essential amino acids (Life Technologies Ltd., Paisley, Scotland) at 37 ^{0}C up to passage 4 in 80-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) and stored in liquid nitrogen until further use. Cell passages 5 to 20 were used for experiments.

Kidney cells. Rhesus monkey kidney cells (LLC-MK_2; ICN Biomedical Inc., Costa Mesa, USA) were cultured in tissue culture flasks and maintained in EMEM (described above). Cells were stored in liquid nitrogen.

Larynx carcinoma cells. Human epidermoid larynx carcinoma cells (HEP-2) (Boehringer Ingelheim/Bio-whittaker, Verviers, Belgium) were cultured in the same way as LLC-MK_2 cells described above.

Virus preparations

The influenza A clinical isolates H1N1 (A/Singapore/6/86-like), H3N2 (A/Beijing/32/92-like), Influenza B (B/Panama/45/90) and Parainfluenza type 1 were used. In the experiments with factor deficient plasma, the influenza strains H1N1 (A/Taiwan/1/81), H3N2 (A/Beijing/32/92-like) and B/Netherlands/580/89 were used. Virus strains were identified by immunofluorescence staining with monoclonal antibodies against influenza A and B (Imagen K6105), parainfluenza (Imagen K6103), adenovirus type 2 (Imagen K6100), respiratory syncytial virus (RSV; Imagen K6102), and cytomegalovirus (CMV; 12-003 CMV IEA clone E13). The antibody against CMV was from Argene, Varilhes, France and the other antibodies were from Dako, Glostrup, Denmark. Parainfluenza

and influenza strains were propagated in a monkey kidney cell line (LLC-MK_2) with M199 medium (Life Technologies Ltd., Paisley, Scotland) free of serum and supplemented with 0.25‰ trypsin, 0.2% glucose, 0.1% bovine serum albumin (Behring, Marburg, Germany) and 25 mM Hepes. Clinical isolates of adenovirus type 2 and respiratory syncytial virus (RSV) were propagated in a HEP-2 cell line with EMEM (Life Technologies Ltd., Paisley, Scotland) supplemented with 4 mg/L amphotericin-B, 10 mg/L gentamycin, 10 mg/L vancomycin, 2 mM L-glutamine, 1% non-essential amino acids, 2% fetal calf serum and 25 mM Hepes. Cytomegalovirus was isolated from a newborn infant with a congenital infection and was propagated in human embryonic lung cells in order to get a pool of CMV. After more than 80% of all cells showed changes in morphology due to a virus infection e.g. cytopathologic effect (CPE), as determined by light microscopy evaluation, all virus suspensions were frozen at -90 ^{0}C after addition of GLY-medium (1:1 dilution). To determine the 50% tissue culture infecting dose ($TCID_{50}$) virus stocks were titrated against human embryonic lung cells (CMV) and HEP-2 cells (adenovirus and RSV) according to the method of Reed and Muench [34]. For the influenza strains H1N1, H3N2, influenza B and parainfluenza, hemagglutinin (HA) titers were determined by titration. To do this, turkey erythrocytes (0.5%) obtained from the National Institute for Public Health (RIVM, Bilthoven, the Netherlands) were suspended in McIlvaine buffer with 0.2% bovine albumin and 0.01% sodiumazide. Twenty-five microlitres of erythrocyte suspension and 25 μL virus suspension in two-fold dilutions were added to microtiter plates with V-shaped wells (Nunc, Denmark), shaken for 1 minute and incubated for 1 hour at 4 ^{0}C. The highest dilution which induced agglutination was considered the HA titer. Influenza growth medium was used as negative control and did not have a detectable HA titer. As a positive control, egg suspensions infected with a known HA titer were used. These suspensions were obtained from the National Institute for Public Health (RIVM, Bilthoven, the Netherlands).

Viral inoculation of cell monolayers

Endothelial cell monolayers grown to confluence were used for infection experiments. The growth medium was removed from the monolayers and maintenance medium (EMEM, 2% fetal calf serum, 25mM Hepes) was added as described previously. For the procoagulant assay endothelial cell monolayers were incubated with 200 μL virus suspension for 3 and 24 hours. For comparison of infectivity in different cell lines, 200 μL virus suspension was added to cell monolayers grown in 24 well tissue culture plates, centrifuged at 800*g* for 60 minutes and incubated for 24 hours. For quantifi-

cation of infection, cells were fixed for 30 minutes in absolute methanol/acetone, air dried, and stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies for 30 minutes at 37 ^{0}C. Infected monolayers from different cell lines were examined by fluorescence microscopy. Each virus-infected cell showing apple green fluorescence was scored as one fluorescent unit. Fluorescent units were counted in 10 random fields at 200x magnification. The number of infected cells was expressed as percentage of the infection of the control cell line (the standard cell line was assigned to an infection-% of 100%). The actual infection percentage of the standard cell line was approximately 20%. Infection with all viral strains did not influence cell viability or integrity, as judged by trypan blue exclusion after the 24-hours incubation.

Cell procoagulant assay

Procoagulant activity was measured on the surface of intact endothelial cell monolayers in coagulometer cups (Amelung, Germany), using a one-stage clotting assay. After different incubation times (3 and 24 hours) culture medium was removed and cells were washed with PBS. Monolayers were incubated with 100 μL Michaelis buffer (sodium-acetate 28.5 mM, sodium-barbital 28.5 mM, NaCl 50 mM, $CaCl_2$ 33 mM, pH 7.35) at 37 ^{0}C for 60 seconds after which 100 μL human pooled citrated plasma or factor VII or XI deficient plasma (Biopool, Burlington, Canada) was added. The time necessary for fibrin to form was measured on the coagulometer. Endothelial cells pre-treated with 200 pM/well tumor necrosis factor-α (TNF-α; gift from Department of Hematology, University Hospital Utrecht, the Netherlands) were used as positive control; uninfected endothelial cells served as negative control. The human plasma pool was obtained from six healthy male volunteers. Clotting times in this assay were converted to percentage thromboplastin by relating clotting times to a standard thromboplastin dilution curve as described by Pronk et al[35]. Briefly, various dilutions of a rabbit brain thromboplastin suspension (Neoplastin, Boehringer Mannheim, Mannheim, Germany) in Ca^{2+}-Michaelis buffer were pre-warmed for 1 minute at 37 ^{0}C in the cups of a coagulometer. Coagulation was started by addition of an equal volume of human pooled citrated plasma. The relation between clotting times and thromboplastin dilutions was used as a calibration curve. The procoagulant activities of the endothelial cell monolayers were read from the curve and expressed as percentages of thromboplastin time.

Factor Xa generation assay

All studies were performed in 48-well tissue culture plates with confluent endothelial cell monolayers at 37 ^{0}C. Confluent endothelial cell monolayers were inoculated as previously described and incubated for 3 and 24 hours. The cells pre-treated with 200 pM/well TNF-α for 6 hours served as positive control. The cells were washed with Hepes buffer (Hepes 25 mM, NaCl 135 mM, KCl 4.5 mM, glucose 4.5 mM, pH 7.4, 0.3% BSA), and then washed twice with the same buffer containing $CaCl_2$ (5 mM). Factor VII and X were purified from plasma essentially as described by Bajaj[36] and Miletich [37]. They had a specific activity of 2000 U/mg (factor VII) and 100 U/mg (factor X) and were diluted in the Hepes-$CaCl_2$ buffer. Endothelial cells were pre-incubated with 100 µL factor VII (2.0 nM) for 5 minutes, after which 50 µL factor X (168 nM) was added and the cells were incubated for 15 minutes under gentle shaking. The reaction was terminated by addition of 25 µL of the sample to 50 µL EDTA (25 mM in Hepes buffer) in a 96-well plastic assay plate. To determine the total amount of activated factor X (factor Xa) formed, 100 µL 0.2 mM chromogenic substrate S2765 (Chromogenics, Mondal, Sweden) was added and the absorbance at 405 nm was measured for 1 minute at 5 second intervals in a V-max ELISA reader (Molecular Devices, Menlo Park, CA, USA). The concentration of factor Xa was calculated by using a standard curve for purified factor Xa[38]. Tissue factor activity was expressed as pmol factor Xa formed per minute by $1*10^5$ endothelial cells. The quantitative determination of tissue factor was performed using the Imubind Tissue Factor ELISA Kit (American diagnostics Inc, Greenwich, USA) recognizing TF-apo, TF and TF-VII complexes. Infected endothelial cell monolayers were lysed with 1 % triton-X-100 before measuring TF antigen.

Data analysis

Results are expressed as means $\pm$ standard error of mean (SEM). All data were analyzed by using the Statistical Package of Social Sciences (SPSS). Differences in quantitative measures were tested for significance by using the unpaired two-tailed Student's t-test, unless otherwise stated.

Results

Hemagglutinin, virus titers

Virus titrations were performed with fibroblasts and larynx carcinoma cells (HEP-2). The highest dilution of virus at which ≥50% of 10 separate cell monolayers showed morphological changes due to infection (tissue culture infecting dose; $TCID_{50}$) was determined for each pathogen. The $TCID_{50}$ values were 10^{-1} for RSV; $10^{-2.7}$ for CMV and $10^{-5.4}$ for adenovirus. For the influenza strains the hemagglutinin titer was measured: 1:32 for influenza A (H1N1), 1:64 for influenza A (H3N2), 1:32 for influenza B and 1:16 for parainfluenza 1.

Susceptibility of cell lines to virus infection

We assessed the *in vitro* susceptibility of different cell monolayers to infection with respiratory viruses. Endothelial cells, fibroblasts, monkey kidney cells and larynx carcinoma cells were all susceptible to infection with influenza A (H1N1, H3N2), influenza B, parainfluenza type 1, CMV, RSV and adenovirus (**Table 1**). Inoculated cell monolayers were centrifuged at 200g for 60 minutes, to increase virus infectivity. After infection all monolayers were intact as determined by phase contrast microscopy. Cell viability was ≥98 % as judged by trypan blue exclusion and the release of lactate dehydrogenase (LDH) after 24 hours' incubation with virus. This was of special concern because influenza viruses A and B are lytic viruses and destroy their host cell after infection.

Table 1 Susceptibility of different cell lines to infection by respiratory viruses

	LLC-MK2	HEL	HEP-2	HUVEC
Inf A (H1N1)	100[a]	108 + 21.0		67 + 19.2
Inf A (H3N2)	100[a]	64 + 3.2		36 + 7.3
Inf B	100[a]	70 + 15.2		78 + 11.7
Para	100[a]	91 + 5.5		83 + 10.1
CMV[b]		100[a]		72 + 2.8
RSV		61 + 5.8	100[a]	69 + 7.1
Adeno		68 + 10.3	100[a]	31 + 0.6

Susceptibility % ± SD after a 24-hour incubation. a Cells were assigned a susceptibility of 100%. Actual infection percentage of the standard cells is approximately 20%. b 48-hour incubation. In each experiment 10 fields of the monolayer were examined. The values are the mean of 3 experiments in duplicate. Abbreviations: LLC-MK2, Rhesus monkey kidney cells; HEL, Human embryonic lung fibroblasts; HEP-2, Human epidermoid larynx carcinoma cells; HUVEC, Human umbilical vein endothelial cells.

In a pilot study, influenza virus infection caused major damage to all cell monolayers after 36 to 48 hours (data not shown). Endothelial cells were highly susceptible to infection with most strains of virus. Influenza A and B and parainfluenza also infected fibroblasts *in vitro*.

Procoagulant activity

We determined the effect of infection with various respiratory viruses on the procoagulant activity of confluent, intact endothelial cell monolayers. Procoagulant activity was measured 3 and 24 hours after infection. The conversion of fibrinogen to fibrin was measured in a one-stage clotting assay with human pooled plasma or plasma deficient in factor VII or XI. After 3 hours' incubation all strains reduced the clotting time compared to that of uninfected cells (**Figure 1**), with the influenza strains reducing the clotting time by 55%. After 24 hours' incubation (**Figure 2**) the reduction in clotting time was even more pronounced (66% reduction) except for adenovirus. When factor VII deficient plasma was used, the clotting time was not reduced. Results showed that the reduction in clotting time was factor VII dependent and therefore mediated by the extrinsic coagulation pathway.

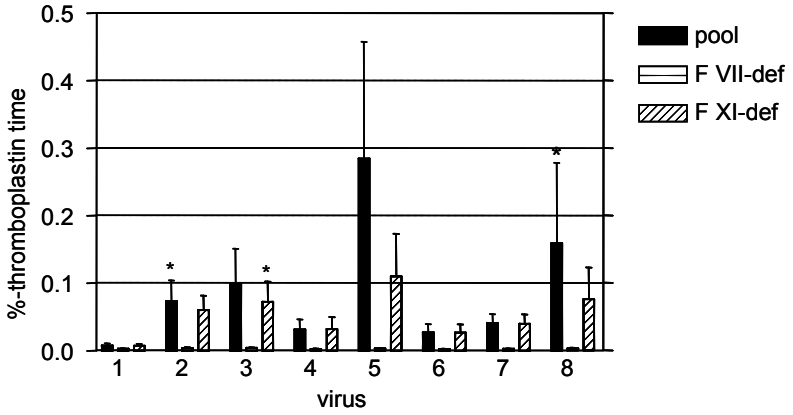

Figure 1. Procoagulant activity of human umbilical vein endothelial cells after incubation with respiratory viruses for 3 hours. Procoagulant activity was measured in a coagulometer with pooled human plasma (*pool*), factor VII-deficient plasma (*F VII-def*) or factor XI-deficient plasma (*F XI-def*). The procoagulant activity of the endothelial cell monolayers is expressed as a percentage of the thromboplastin time. X-axis: **1**: uninfected confluent endothelial cell monolayer, **2**: influenza A/H1N1/Taiwan/1/81, **3**: influenza A/H3N2/Beijing/32/92-like, **4:** influenza B/Netherlands/580/89, **5**: parainfluenza-1, **6**: cytomegalovirus, **7**: respiratory syncytial virus, **8**: adenovirus type 2. Levels are expressed as means $\pm$ S.E., based on 4 separate experiments in duplicate. * $P < 0.05$ compared with uninfected cells; paired t-test.

This indicates an increased expression of tissue factor (TF) on the endothelial cell membrane. There was no relationship between the percentage of endothelial cells infected by the different strains of virus and the reduction in clotting time.

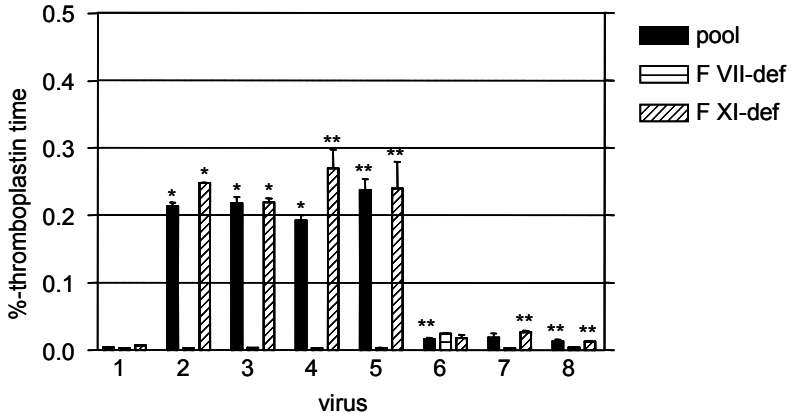

Figure 2. Procoagulant activity of human umbilical vein endothelial cells after incubation with respiratory viruses for 24 hours. Procoagulant activity was measured in a coagulometer with pooled human plasma (*pool*), factor VII-deficient plasma (*F VII-def*) or factor XI-deficient plasma (*F XI-def*). The procoagulant activity of the endothelial cell monolayers is expressed as a percentage of the thromboplastin time. X-axis: **1**: uninfected confluent endothelial cell monolayer, **2**: influenza A/H1N1/Taiwan/1/81, **3**: influenza A/H3N2/Beijing/32/92-like, **4:** influenza B/Netherlands/580/89, **5**: parainfluenza-1, **6**: cytomegalovirus, **7**: respiratory syncytial virus, **8**: adenovirus type 2. Levels are expressed as means $\pm$ S.E., based on 4 separate experiments in duplicate. * $P < 0.001$ compared with uninfected cells, ** $P < 0.05$ compared with uninfected cells; paired t-test.

Factor Xa generation

To establish whether the reduction in clotting time was mediated by the production of tissue factor by endothelial cells, we decided to measure TF production as the generation of factor Xa, using a chromogenic substrate technique. Experiments were performed after incubation with virus for 3 and 24 hours. These time points were chosen to evaluate the effect of a 'full blown' infection without morphological damage to the monolayer seen with longer (36 hours) incubation. After 3 hours' incubation low levels of TF expression was observed on infected as well as on uninfected monolayers (**Figure 3**). After 24 hours' incubation all virus strains induced TF expression at different levels. Compared with the negative (uninfected) control there was a 3 to 4-fold increase in TF production. The confluent endothelial cell monolayers were morphologically intact after the 3- and 24-hour incubations with viruses.

Tissue factor antigen was measured in order to determine whether increased TF activity was the result of increased TF antigen expression on the endothelial cell surface. After 3 and 24 hours' infection increased TF antigen concentrations were measured in the endothelial cell homogenate (**Figure 4**).

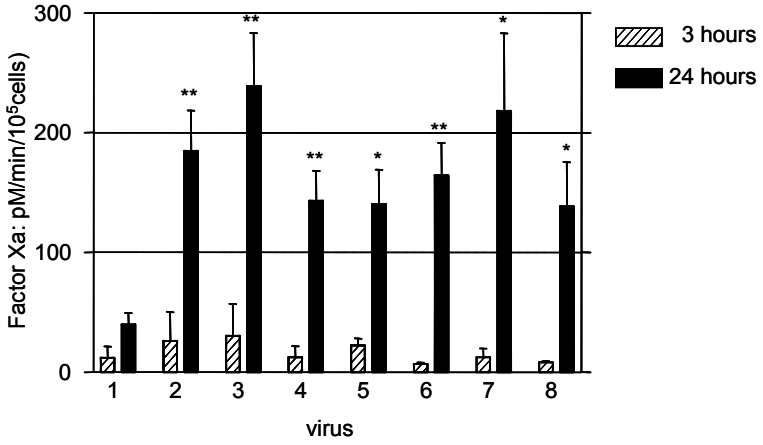

Figure 3. Generation of factor Xa by human umbilical vein endothelial cells after incubation with respiratory viruses and incubation for 3 and 24 hours. X-axis: 1: uninfected confluent endothelial cell monolayer, 2: influenza A/H1N1/Taiwan/1/81, 3: influenza A/H3N2/Beijing/32/92-like, 4: influenza B/Netherlands/580/89, 5: parainfluenza-1, 6: cytomegalovirus, 7: respiratory syncytial virus, 8: adenovirus type 2. Levels are expressed as means + S.E., based on 5 separate experiments in duplicate. * $P < 0.001$ compared with uninfected cells, ** $P < 0.05$ compared with uninfected cells; paired t-test.

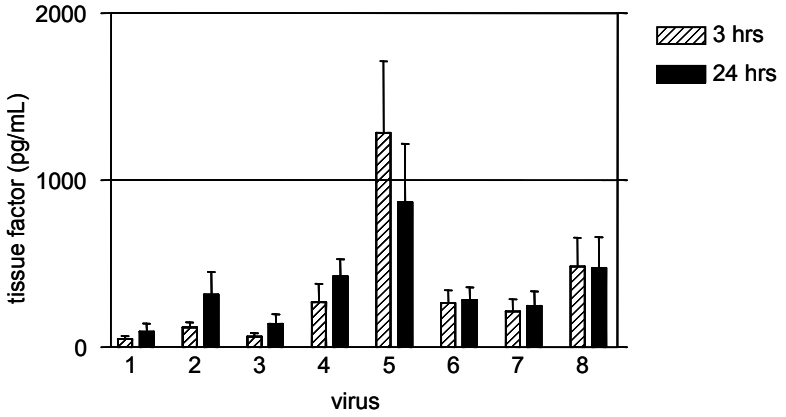

Figure 4. Tissue factor antigen expression by endothelial cells after infection with viruses and incubation for 3 and 24 hours. X-axis: 1: uninfected confluent endothelial cell monolayer, 2: influenza A/H1N1/Taiwan/1/81, 3: influenza A/H3N2/Beijing/32/92-like, 4: influenza B/Netherlands/580/89, 5: parainfluenza-1, 6: cytomegalovirus, 7: respiratory syncytial virus, 8: adenovirus type 2. Levels are expressed as means + S.E., based on 5 separate experiments in duplicate.

Discussion

The mechanism by which infection with viruses contributes to increased cardiovascular morbidity and mortality is not known but may include alterations in the haemostatic balance. Endothelial cells may be a target for viruses *in vivo*, and infection may result in changes in endothelial cell functions. The association between infection with respiratory viruses and cardiovascular disease has not been confirmed in serological studies, but there is circumstantial evidence that these viruses may be a trigger for cardiovascular disease[14,16,17]. In this study, we found that viruses (influenza A, influenza B, parainfluenza, RSV, adenovirus and CMV) were able to infect different cell monolayers including human umbilical vein endothelial cells and human embryonic lung fibroblasts *in vitro*. Infection rates for CMV were similar to those previously published[5]. The susceptibility of endothelial cells to infection with respiratory viruses is less well documented. The observation that most respiratory viruses surveyed in this study were able to infect lung fibroblasts as well as endothelial cells may have implications for the *in vivo* situation where infection of the respiratory tract by respiratory viruses subsequently leads to infection of lung fibroblasts. Respiratory viruses then enter the vascular system and cause a more general infection, or viremia. In this way respiratory viruses can spread throughout the body, infecting other organs and other sites of the vascular tree. Endothelial cells, which line the lumen of blood vessels, can be targets for infection with these viruses.

Tissue factor is the physiological activator of the extrinsic coagulation pathway. Several stimuli (e.g. thrombin, IL-1ß, TNF-α) are known to induce the production of TF in endothelial cells, with detectable levels being reached after 2-4 hours and peak expression after 4-6 hours[39]. High concentrations of TF antigen and activity are found in coronary arteries from patients with unstable angina or myocardial infarction[40]. In this study viruses induced endothelial cell procoagulant activity *in vitro*. All viruses tested reduced the clotting time in a time-dependent manner. By different experimental set-ups, we could show that this reduction in clotting time was a result of TF expression on endothelial cells. Low levels of TF activity were detectable on infected as well as on uninfected cell monolayers after 3 hours' incubation (**Figure 3**), and there was a factor VII-dependent reduction in the clotting time at this time point (**Figure 1**). Tissue factor antigen levels were already increased after 3 hours' incubation (**Figure 4**). At 24 hours, TF antigen levels were similarly increased as at the 3 hours time point, but this was accompanied with a marked increase in TF activity. Apparently, the

increased activity of TF was not the result of increased antigen expression only. Most likely changed phospholipid expression on the cell membrane, as a result of the virus infection, leads to increased TF activity. Increased TF activity due to elevated levels of phosphatidylserine[41] on the cell membrane was not likely, because apoptosis was not observed after infection as determined by the lack of annexin V binding to infected endothelial cells. Another explanation for the discrepancy between levels of TF activity and TF antigen may be the fact that TF antigen was measured in the cell homogenate. After 3 hours' incubation TF antigen was probably still intracellular and at the 24 hours measurement TF was expressed on the cell membrane which subsequently resulted in the measured increase in TF activity. Thus, the clotting time reduction on endothelial cells after virus infection is due to increased TF activity as a result of up regulation of TF antigen expression in combination with increased activity of the expressed TF possibly by changed co-expression of phospholipids on the cell outer membrane. Our results are in line with the studies by Key et al[42,43]. They showed TF expression on the cell surface of herpes simplex infected endothelial cells. In contrast to our study, they observed that TF activity returned to baseline after 20 hours' infection. However, an other study has shown that TF does not play a major role at all in endothelial cell procoagulant activity induction by CMV[5]. In that study, the facilitated formation of the prothrombinase complex on endothelial cells was considered important. Key et al[43] demonstrated TF dependent procoagulant activity in endothelial cells infected with herpes simplex virus-1 (HSV-1) by using a neutralizing antibody to human TF. In both studies[5, 43] and in a study by Pryzdal and coworkers[44] the facilitated assembly of the prothrombinase complex could be related to the viral envelope of HSV-1 and CMV. Presentation of antigens of the viral envelope on the surface of endothelial cells promotes procoagulant activity. However, we found that adenovirus, a non-enveloped virus, could also induce procoagulant activity in endothelial cells in conjunction with TF expression. Viral antigens may enhance prothrombinase complex assembly on the surface of endothelial cells which in turn facilitates thrombin generation[45]. This in turn stimulates endothelial cells to express TF. Moreover, the endogenous production of IL-1ß by endothelial cells, in response to viral infection, might induce TF expression of the same or neighboring cells.

The observation that influenza viruses, due to their lytic properties, caused major damage to the endothelium after a 36-hour incubation may be of importance. A dying cell expresses phosphatidylserine on its surface, and after lysis the prothrombotic extracellular matrix is exposed to the vascular lumen. We could not demonstrate

annexin V binding to infected endothelial cells but cell lysis may be a mechanism by which respiratory infections could contribute to alterations in the procoagulant-anticoagulant balance. We did not find a relationship between the proportion of endothelial cells infected with virus and the induction of procoagulant activity. A linear relationship has been described between the number of endothelial cells infected with HSV-1 and TF production[42]. It is not possible to compare the infectivity of different strains of virus as long as the mechanism of TF activity induction is not known. It is possible that the viruses induce IL-1ß or TNF-α production in the endothelial cells. In turn, is IL-1ß a mediator of TF expression on the endothelium. However, we could not detect TNF-α or IL-1ß in the supernatant after infection of endothelial cells by the different viruses (data not shown) but it is possible that very low (undetectable) TNF-α and IL-1ß concentrations are responsible for TF expression on endothelial cells.

In conclusion, we have shown that respiratory viruses can infect human endothelial cells and fibroblasts *in vitro*. Both enveloped and non-enveloped respiratory viruses induce procoagulant activity on cultured endothelial cells by stimulating the expression of TF antigen and enhancing the activity of expressed TF antigen. Also, facilitated prothrombinase complex assembly may contribute to procoagulant activity of infected endothelial cells. If similar processes occur *in vivo*, then infections with respiratory viruses give rise to endothelial procoagulant activity and hence contribute to an increased risk of atherothrombosis.

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References

1. Hendrix MGR, Dormans PHJ, Kitslaar P, Bosman F, Bruggeman CA. The presence of CMV nucleic acids in arterial walls of atherosclerotic and non-atherosclerotic patients. Am J Pathol 1989;134:1151-1157.
2. Hendrix MGR, Salimans MMM, Boven v CPA, Bruggeman CA. High prevalence of latently present cytomegalovirus in arterial wall of patients suffering from grade III atherosclerosis. Am J Pathol 1990;136:23-28.
3. Adam E, Melnick JL, Probstfield JL, Petrie BL, Burek J, Bailey KR, McCollum CH, DeBakey ME. High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. Lancet 1987;2:291-293.
4. Melnick JL, Hu C, Burek J, Adam E, DeBakey ME. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. J Med Virol 1994; 42: 170-174
5. van Dam-Mieras MCE, Muller AD, van Hinsbergh VWM, Mullers WJHA, Bomans PHH, Bruggeman CA. The procoagulant response of cytomegalovirus infected endothelial cells. Thromb Haemost 1992;68(3):364-370.
6. Etingin OR, Silverstein RL, Friedman HM, Hajjar DP. Viral activation of the coagulation cascade: molecular interactions at the surface of infected endothelial cells. Cell 1990;61:657-662.
7. Vercellotti GM. Proinflammatory and procoagulant effects of herpes simplex infection on human endothelium. Blood Cells 1990;16:209-216.
8. Mazure G, Grundy JE, Nygard G, Hudson M, Khan K, Srai K, Dhillon AP, Pounder RE, Wakefield AJ. Measles virus induction of human endothelial cell tissue factor procoagulant activity in vitro. J Gen Virol 1994;75:2863-2871.
9. Span AHM, van Boven CPA, Bruggeman CA. The effect of cytomegalovirus infection on the adherence of polymorph nuclear leukocytes to endothelial cells. Eur J Clin Invest 1989;19:542-548.
10. Etingin OR, Silverstein RL, Hajjar DP. Identification of a monocyte receptor on herpes virus-infected endothelial cells. Proc Natl Acad Sci USA 1991;88:7200-7203.
11. Hajjar DP, Nicholson AC. Viral activation of coagulation: implications for thrombosis and atherosclerosis. Ann N Y Acad Sci 1997;811:155-165.
12. Tillet HE, Smith JWG, Gooch CD. Excess deaths attributable to influenza in England and Wales: age and dead and artificial course. Int J Epidemiol 1983;12:344-356.
13. Sprenger MJW, Diepersloot RJA, Beyer WEP, Masurel N. Influenza related excess mortality in The Netherlands. Lancet 1990;336:382.
14. Sprenger MJW, Naelten MAMG van, Mulder PGH. Influenza mortality and excess deaths in the elderly, 1967-1982. Epidem Inf 1989;103:633-641.

15. Bainton D, Jones GR, Hole D. Influenza and ischaemic heart disease as a possible trigger for acute myocardial infarction? Int J Epidem 1978;7:231-239.
16. Mattila KJ. Viral and bacterial infections in acute myocardial infarction. J Intern Med 1989;225:293-296.
17. Spodick DH, Flessas AP, Johnson MM. Association of acute respiratory symptoms with onset of acute myocardial infarction: prospective investigation of 150 consecutive patients and matched control patients. Am J Cardiol 1984;53:481-482.
18. Jousilahti P, Vartiainen E, Tuomilehto J, Puska P. Symptoms of chronic bronchitis and the risk of coronary disease. Lancet 1996;348:567-572.
19. Engblom E, Ekfors TO, Meurman OH, Toivuren A, Nikoskelainen J. Fatal influenza A myocarditis with isolation of virus from the myocardium. Acta Med Scand 1982;213(1):75-78.
20. Witzleb W, Witzleb H, Mehllorn J, Sprossig M, Wutzler P. Demonstration of influenza virus A in human heart by semiquantitative virus assay and immunofluorescence. Acta Virol 1976;20:168.
21. Edelen JS, Bender TR, Chin TDY. Encephalopathy and pericarditis during an outbreak of influenza. Am J Epidl 1974;100:79-84.
22. Middleton PJ Alexander RM, Szymanski MT. Severe myositis during recovery from influenza. Lancet 1970;2:533-535.
23. Kessler HA, Trenholme GM, Harris AA, Levin SL. Acute myopathy associated with influenza A/Texas/1/177 infection. JAMA 1980;243:461-462.
24. Tevada H. Studies on platelets and interaction with particular reference to the interaction of influenza virus with blood platelets. Acta Hematol Jap 1969;32:45-49.
25. Tevada H, Baldini M, Ebbe S, Madoff MA. Interaction of influenza virus with blood platelets. Blood 1966;28:213-216.
26. Barshtein IuA, Frolov AF, Persidskii IuV, Gavrilov SV. Characteristics of the action of the influenza virus on the microcirculatory vessels in an experiment. Mikrobiol Zh 1989;51(4):44-50.
27. Bogomolov BP, Barinov VG, Dviatkin AV, Shvedova LP, Arkharova MN Frolova IN, Isaeva NP, Karelina LA. Hemostasis in influenza and acute respiratory viral infections in the middle-aged and elderly. Ter Arkh 1990;62(7):98-102.
28. Anisimova IuN, Trushnikova GV. Infectious-toxic shock and disseminated intravascular coagulation in a pregnant women with influenza. Arkh Patol 1994;56(3):79-82.
29. Shcheglovitova ON, Antonov AS, Kabaeva NV, Orlova TG. Interferon production by primary culture of human endothelial cells. Biull Eksp Biol Med 1989;108(11):580-583.
30. Colden-Stanfield M, Cramer EB, Gallin EK. Comparison of apical and basal surfaces of confluent endothelial cells: patch-clamp and viral studies. Am J Physiol 1992;263(3):C573-583.
31. Colden-Stanfield M, Ratcliffe D, Cramer EB, Gallin EK. Characterization of influenza virus-induced leukocyte adherence to human umbilical vein endothelial cell monolayers. J Immunol 1993;151(1):310-321.

32. Colden-Stanfield M, Kalinich JF, Gallin EK. Ionizing radiation increases endothelial and epithelial cell production of influenza virus and leukocyte adherence. J Immunol 1994;153(11):5222-5229.
33. Jaffe EA, Nachman RL, Becker CG, Minick RC. Culture of human endothelial cells derived from umbilical veins. J Clin Invest 1973;52:2745-2756.
34. Reed LJ, Muench J. A simple method of estimating fifty per cent endpoints. Am J Hyg 1938;27:493.
35. Pronk A, Groot de PhG, Hoynck van Papendrecht AAGM, Verbrugh HA, Leguit P, Vroonhoven van ThJMV, Sixma JJ. Thrombogenicity and procoagulant activity of human mesothelial cells. Arter Thromb 1992;12:1428-1436.
36. Bajaj SP, Papaport SI, Brown SF. Isolation and characterisation of human factor VII. J Biol Chem 1981;256:253-259.
37. Miletich JP, Jackson CM, Majerus PW. Properties of the factor Xa binding site on human platelets. J Biol Chem 1978;253:6908-6916.
38. Meijers JCM, Tijburg PNM, Bouma BN. Inhibition of human blood coagulation factor Xa by alpha-2-macroglobulin. Biochem 1987;26:5932-5937.
39. Cotran RS. New roles for the endothelium in inflammation and immunity. Am J Pathol 1987;129:407-413.
40. Aridssino D, Merlini PA, Ariens R, Coppola R, Bramucci E, Mannucci PM. Tissue-factor antigen and activity in human coronary atherosclerotic plaques. Lancet 1997;349:769-771.
41. Homburg CH, de Haas M, von dem Borne AE, Verhoeven AJ, Reutelingsperger CP, Roos D. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. Blood 1995;85(2):532-540.
42. Key NS, Vercellotti GM, Winkelmann JC et al. Infection of vascular endothelial cells with herpes simplex virus enhances tissue factor activity and reduces thrombomodulin expression. Proc Natl Acad Sci USA 1990;87:7095-7099.
43. Key NS, Bach RR, Vercellotti GM, Moldow CF. Herpes simplex virus type I does not require productive infection to induce tissue factor in human umbilical vein endothelial cells. Lab Invest 1993;68(6):645-651.
44. Pryzdial ELG, Wright JF. Prothrombinase assembly on an enveloped virus: evidence that the cytomegalovirus surface contains procoagulant phospholipid. Blood 1994;84(11):3749-3757.
45. Visser MR, Tracy PB, Vercellotti GM, Goodman JL, White JG, Jacob HS. Enhanced thrombin generation and platelet binding on herpes simplex virus-infected endothelium. Proc Natl Acad Sci USA 1988;85:8227-8230.

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3

Procoagulant and inflammatory response of virus-infected monocytes

Abstract

Background

Monocytes play a prominent role in inflammation, coagulation and atherosclerosis by their ability to produce tissue factor (TF) and cytokines. The aim of the present study was to establish whether virus-infected monocytes initiate coagulation. In addition, the production of cytokines by monocytes may accelerate the chronic process of atherosclerosis and may contribute to coronary syndromes by eliciting plaque instability.

Materials and methods

Monocytes were isolated by Vacutainer®, BD Biosciences, Alphen aan den Rijn, Netherlands and subsequent magnetic cell sorting (MACS®, Miltenyi Biotec, Bergish Gladbach, Germany). Coagulation times in normal pooled plasma and Factor VII-deficient plasma were measured after infection with cytomegalovirus (CMV), *Chlamydia pneumoniae (Cp)* and influenza A\H1N1. Anti-TF antibodies were added to neutralize TF expressed on monocytes. Interleukins (IL) 6, 8 and 10 were measured in the supernatants.

Results

Chlamydia pneumoniae- and CMV-infected monocytes decreased the clotting time by 60% and influenza-infected monocytes by 19%, as compared to uninfected monocytes. Procoagulant activity was absent when Factor VII-deficient plasma or anti-TF antibodies were used. Monocytes produced both IL-6 and IL-8 after infection with CMV (317 pg/mL and 250 pg/mL) or *Cp* (733 pg/mL and 268 pg/mL). Similar results were obtained for influenza virus-infected monocytes, but the levels of both cytokines were 3-5 fold higher (1797 pg/mL and 725 pg/mL). Interleukin-10 was not produced by infected monocytes.

Conclusion

The procoagulant activity of virus-infected monocytes is TF-dependent. Although influenza infection did not generate a significant reduction in clotting time, the pronounced expression of IL-6 and IL-8 may induce local and/or systemic inflammatory reactions, which may be associated with plaque rupture and atherosclerosis. The lack of production of the anti-inflammatory cytokine IL-10 may even accelerate these processes.

Keywords: *Chlamydia pneumoniae*, cytomegalovirus, influenza A virus, interleukins, monocytes, tissue factor.

Introduction

Atherosclerosis is thought to be an inflammatory disease[1–3]. The cause of this chronic low-grade inflammation is not unambiguous, but may well be a combination of classical risk factors or novel risk factors such as micro-organisms. There is increasing evidence that viruses have a role in the etiology of atherosclerosis[4,5] and several studies have identified viruses and bacteria as potential agents contributing to atherogenesis[6–10]. Cytomegalovirus (CMV) and *Chlamydia pneumoniae (Cp)* have been found in atherosclerotic plaques and smooth muscle cells[11–13]. Also, several sero-epidemiological studies have shown association between seropositivity for *Cp* and increased risk of cardiovascular diseases[14,15]. At present, controversy exists about the role of chronic CMV infection in relation to the risk of cardiovascular disease. In general predisposition of CMV for inflammation and subsequent susceptibility for chronic coronary artery disease (CAD) is well accepted, but seropositivity for CMV alone is unlikely to be a strong risk factor for CAD[16–19]. Cytomegalovirus-IgG titers rather than seropositivity, seropositivity for more than one agent, and pathogen burden, are associated with enhanced inflammatory responses, potentially accelerating the atherothrombotic process [20–22]. However, most predictive indicators for the risk of CAD are seropositivity for CMV with increased IgG titers in correlation with elevated IL-6 or CRP levels, reflecting a (subclinical) inflammatory response[23–26]. Infection of the endothelium shifts endothelial properties from anticoagulant to procoagulant as a result of the production and expression of tissue factor (TF)[27,28]. Although normally absent from all intravascular cell types, TF can be synthesized by endothelial cells, smooth muscle cells and macrophages in response to inflammatory stimuli, endotoxins or immunological compounds such as lipopolysaccharides (LPS), interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [29,30]. Elevated expression of TF on monocytes/macrophages in atherosclerotic plaques suggests a role for TF in fatal thrombosis associated with plaque rupture[31,32]. Virus infection of the endothelium results in the promotion of monocyte adhesion[33–35] as well as cytokine production[36–38]. The cytokines IL-6 and IL-8 may induce monocyte procoagulant activity by stimulating TF expression. This may link inflammation to thrombotic events[39]. Undoubtedly cross-talk between cells of the immune system and the vessel wall mediated by cytokines and other pro-inflammatory substances is responsible for initiating cascade effects leading to vascular disorders[40]. Studies conducted on the effects of infection on monocyte activation reveal inflammatory responses by production of tissue factor[41,42] and various

proinflammatory cytokines like IL-1 and IL-6, but also potent chemo-attractants like monocyte chemo attractant protein1 (MCP-1) are readily produced[43-45]. Next to this, foam cell formation and lipid oxidation are induced and loss of anti-inflammatory properties of high-density lipoproteins has been recorded[46,47]. In combination with up regulation of adhesion molecules by endothelial cells[48], monocytes harboring virus and oxidized low-density lipoproteins eventually infiltrate the vessel wall[46,49]. In the present study, we investigated the response of monocytes to infection with *Cp*, CMV and influenza A *in vitro*. *Chlamydia pneumoniae* and CMV cause more chronic events while influenza represents an agent responsible for acute cardiovascular complications. Considering the distinct properties of these viruses, distinct modes of action by monocytes infected with these viruses may be observed.

Methods

Isolation of monocytes

For isolation of the mononuclear fraction, fresh blood from healthy volunteers was drawn by venapuncture either into citrated tubes for Ficoll/Hypaque isolation or into mononuclear cell preparation tubes (Vacutainer CPT®, BD Biosciences, Alphen aan den Rijn, Netherlands). Ficoll/ Hypaque isolation was performed according to the well defined protocol of Boyum [50]. Vacutainer isolation tubes were inverted a few times and centrifuged at 1400g for 20 min. At this speed a gel barrier separates the mononuclear fraction from plasma and the fraction containing erythrocytes and polymorph nuclear neutrophils. Plasma from each donor was stored at - 70 °C. Subsequently, monocytes were purified from the mononuclear fraction by means of a magnetic-activated cell sorting protocol (MACS®, Miltenyi Biotec, Germany). Monocytes can be isolated by two protocols [1]: depletion of non-monocytes with a cocktail of CD3, CD7, CD19, CD45RA, CD56 and anti-IgE antibodies, leaving an untouched monocyte fraction or [2] positive selection of CD14+ cells (monocytes). For reasons discussed later in this paper, positive selection was our method of choice. For the isolation of monocytes by the MACS positive-selection protocol, 10^7 cells were suspended in 80 µL cold phosphate-buffered saline (PBS) pH 7.2 supplemented with 0.5% fetal calf serum (FBS) and 2 mM EDTA. Cells were magnetically labeled with 20 µL CD14-microbeads® (Miltenyi Biotec) per 10^7 cells, mixed well and incubated at 4 °C for 15 min. Then cells were washed with 10 volumes of cold buffer and centrifuged at 300g and 4 °C for 10 min. The cell pellet was resuspended in buffer (500 µL buffer/10^8 cells) and applied to a prewashed LS+

positive selection column (Miltenyi Biotec), which was placed in the magnetic field of a MACS separator® (Miltenyi Biotec). Unlabelled (CD14-) cells passed through the column and were discarded with the effluent. The column was rinsed three times with PBS. After the column was removed from the magnetic field, the retained CD14+ cells were eluted in cold PBS. The yield and purity of the isolated cells were determined by flow cytometry. Cells were kept on ice until experiments were performed.

Preparation of virus stocks

Human embryonic lung (HEL), human epidermoid larynx carcinoma (HEP-2) and LLC-MK2 cells were cultured at 37 °C and 5% CO_2 in Eagle's minimum essential medium with Earle's salts (EMEM, Life Technologies Ltd, Paisley, Scotland) with 10% FBS (Life Technologies Ltd). This culture medium was supplemented with 2 mmol/L L-glutamine (Life Technologies Ltd), 5 mL nonessential amino acids (Life Technologies Ltd), 10 mg/L vancomycin (Faulding Pharmaceuticals, Brussels, Belgium), 4 mg/L fungizone (Bristol-Meyers Squibb, Woerden, the Netherlands) and 10 mg/L gentamycin (Schering Plough, Maarssen, the Netherlands). The same supplements were also added to the media used for the propagation of the virus strains. A clinical isolate of CMV was propagated in HEL cells in EMEM containing 2% FBS, 20 mM Hepes and supplements. At >80% cytopathologic effect (CPE), CMV-infected HEL cells were detached with trypsin/EDTA solution (Life Technologies Ltd) and centrifuged. The cell pellet was resuspended in EMEM containing 2% FBS, 10% DMSO (Sigma-Aldrich, Zwijndrecht, the Netherlands) and supplements. Cytomegalovirus stock was aliquoted and stored at -70 °C.
Chlamydia pneumoniae-strain AR 39 was propagated in HEP-2 cells in EMEM 10% FBS with 0.1% cycloheximide (Sigma-Aldrich) and supplements at 37 °C / 5% CO_2. After 72 h cells were frozen and thawed to release the elementary bodies. After a short centrifugation step, cell debris was discarded and 0.2 M SPG medium (2.088 g/L K_2HPO_4, 1.088 g/L KH_2PO_4, 68.46 g/L saccharose, 7.16 g/L L-glutamine, 10% FBS, 2.5 mg/L fungizone, 23 mg/L vancomycin and 18 mg/L gentamycin) was added (1:1 v/v). *Chlamydia pneumoniae* stock was aliquoted and stored at - 70 °C. Influenza A/H1N1/1/86/Singapore was propagated in LLC-MK2 cells in serum-free medium M199 with 20 mM Hepes and supplements. At > 80% CPE, the virus was harvested by freezing and thawing the cells. After centrifugation, the pellet was discarded and 0.2 M sucrose phosphate medium was added to the supernatant (1:1 v/v). Influenza A stock was aliquoted and stored at -70 °C. The 50% tissue culture infective dose ($TCID_{50}$) of each virus stock was calculated using the method of Reed & Muench[51].

For CMV and influenza A reading CPE was used to calculate $TCID_{50}$ titers. In the case of *Cp*, the $TCID_{50}$ titer was calculated based on the number of immunofluorescent units per field in the infected HEP-2 cells after staining with Chlamydia culture confirmation monoclonal antibodies (de Beer, Diessen, the Netherlands).

Infection of monocytes

Isolated monocytes were counted and brought to a concentration of 10^5 cells/mL in RPMI medium with 10% autologous plasma. Coagulation tubes (P1000 sample cups, Amelung; Boom BV, Meppel, the Netherlands) were precoated, for at least 1 h, with fibronectin (CLB, Amsterdam, the Netherlands) diluted 1:100 in PBS. Aliquots of 1 mL (10^5 monocytes) were distributed over the tubes. Subsequently, 100 mL of each virus stock was added to one tube. The multiplicity of infection (MOI) was 0.4 for CMV, 0.1 for *Cp* and 0.04 for Influenza A. As negative control, 100 µL of RPMI instead of the virus was added. For the positive controls, 10^5 monocytes were incubated with 1 µg/mL LPS (prepared in endotoxin-free water; Sigma-Aldrich). Tubes were incubated at 37 °C and 5% CO_2 for 4 h and then centrifuged at 300g and 4 °C for 5 min. Supernatants were collected and frozen at -70 °C for cytokine assays. Monocytes were washed immediately in cold PBS, refreshed with 100 µL PBS, and kept on ice until clotting assays were performed.

Coagulation assay

Normal pooled plasma was prepared from the blood of nine healthy donors who did not use aspirin or oral anticonception. Freeze-dried Factor VII-deficient plasma (Kordia Biopool, Leiden the Netherlands) was reconstituted for use with 1 mL of distilled water. Clotting assays were performed at 37 °C in a coagulometer (KC4A Amelung/Boom BV), which records the time taken for fibrin formation. To 100 µL (10^5 cells) of infected monocytes, incubated in a coagulation tube, were added: 100 µL of Michaelis buffer (28.5 mM sodium barbital, 28.5 mM sodium acetate, 50 mM sodium chloride and 33 mM calcium dichloride), one metal bead, and 100 µL of normal pooled plasma or Factor VII-deficient plasma. All components were kept cold until use. Clotting times at 37 °C were measured as described. To investigate whether TF played a central role in the reduction of clotting time, samples in normal pooled plasma were also incubated with 1:100 diluted neutralizing antibodies against TF (American Diagnostica Greenwich, CT).

Cytokine assays

Interleukins IL-6, IL-8 and IL-10 were measured in the supernatants of virus-infected monocytes. Sandwich-Elisa's were used (IL-6, IL-8 and IL-10 Pelikine compact human ELISA kits; CLB the Netherlands) and performed as prescribed by the manufacturer. Before the assay, the frozen supernatants were thawed quickly in a 37 °C water bath and brought to room temperature. Samples were prediluted in buffer, at least 1:2 or more, depending on the expected IL titer. A standard curve (serial dilution of the human recombinant cytokine) was included in each experiment. All experiments were performed at least three times in duplicate. The absorbance of standards and samples was read at 450 nm using an ELISA reader (ELx800, Biotek Instruments Inc). The cytokine concentration of the samples was calculated by interpolation from the standard curve and corrected for the dilution factor.

Statistics

Results are expressed as means ± standard errors (SE) of three experiments. ANOVA analysis of variances was performed to check for variances among means in the test populations. P-values of means ± SE were calculated by comparing all samples to the negative control using Dunnett's multiple comparisons test, which corrects for unequal variances.

Results

Infection of monocytes

After infection, $TCID_{50}$-titres of the virus stocks in standard cell lines and MOI values in monocytes were calculated for each virus strain.$TCID_{50}$ values were: $10^{-4.25}$ for CMV, $10^{-3.75}$ for *Cp* and $10^{-3.25}$ for influenza A. The *in vitro* susceptibility of monocytes to the virus strains was determined with immunofluorescent staining for the presence of virus in monocytes. Cytomegalovirus-infected monocytes demonstrated the typical nuclear staining of infected cells (**Figure 1a**), whereas influenza- or *Cp*-infected monocytes showed cytoplasmatic fluorescence (**Figure 1b, c**). It appeared that all strains could infect monocytes, but in all cases the infection percentage was below 5% when undiluted virus stocks were used (MOI: 0.4, 0.1 and 0.04 for CMV, *Cp* and influenza virus, respectively).

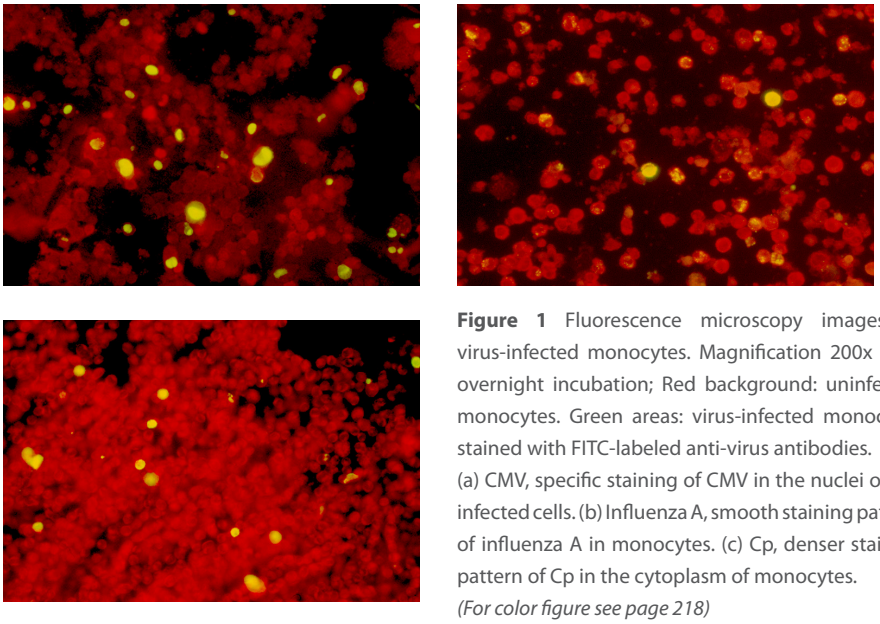

Figure 1 Fluorescence microscopy images of virus-infected monocytes. Magnification 200x after overnight incubation; Red background: uninfected monocytes. Green areas: virus-infected monocytes stained with FITC-labeled anti-virus antibodies.
(a) CMV, specific staining of CMV in the nuclei of the infected cells. (b) Influenza A, smooth staining pattern of influenza A in monocytes. (c) Cp, denser staining pattern of Cp in the cytoplasm of monocytes.
(For color figure see page 218)

Isolation of monocytes

In both (Ficoll and Vacutainer) fractions, obtained by MACS-depletion, large amounts of debris were present and some granulocytes were left. In both Ficoll and Vacutainer depletion prepared suspensions, 80% of the monocytes were covered with activated (CD41+/CD42+) platelets. Practically no free platelets were present in the suspensions. In suspensions prepared by the positive selection method little debris was present and 78% of all isolated cells were monocytes with only a few platelets (3%) attached (**Table 1**).

Table 1 Immunophenotyping of isolated leucocytes

	Ficoll + depletion *	VPT + depletion †	VPT + positive selection ‡
CD14+/CD45+	78%	95%	78%
CD42+/CD14+	77%	83%	2%
CD42-/CD14+	22%	16%	97%
CD41+	47%	78%	3%
CD41+/CD42+	46%	78%	3%

FACS-analysis of isolated monocyte fractions; determination of yield and purity of monocyte fractions obtained from three different isolation protocols: * Ficoll isolation followed by MACS depletion of non-monocytes; † Vacutainer CPT isolation followed by MACS depletion of non-monocytes; ‡ Vacutainer CPT isolation followed by MACS CD14-isolation protocol (positive selection). CD45+: leucocytes; CD14+: monocytes; CD41+: platelets; CD42+: activated platelets.

Procoagulant activity

Infection of monocytes with *Cp* or CMV for 4 h resulted in a significant reduction of the clotting time by approximately 60% (140 ± 3.8 and 134 ± 8.9 s, respectively; $p<0.05$) compared with that of uninfected monocytes (333 ± 22.1 s) [**Figure 2**]. The clotting time of monocytes infected with influenza A/H1N1 virus (273 ± 81 s) was reduced by 19% ($p>0.05$). The same experiments were performed with Factor VII-deficient plasma. In these experiments no differences between clotting times were recorded ($p>0.05$). Moreover, addition of anti-TF antibodies during incubation of the monocyte suspensions with virus completely blocked the reduction in clotting time to the level of the negative control. ANOVA analysis on variances showed that the variances between test samples in the normal pooled plasma were significantly different (F=6.4/ P=0.0079). In contrast, the analysis of variances among Factor VII-deficient- and anti-TF neutralized sample populations no longer indicated any differences (F=0.75/ p=0.58 and F=0.96/p=0.47, respectively).

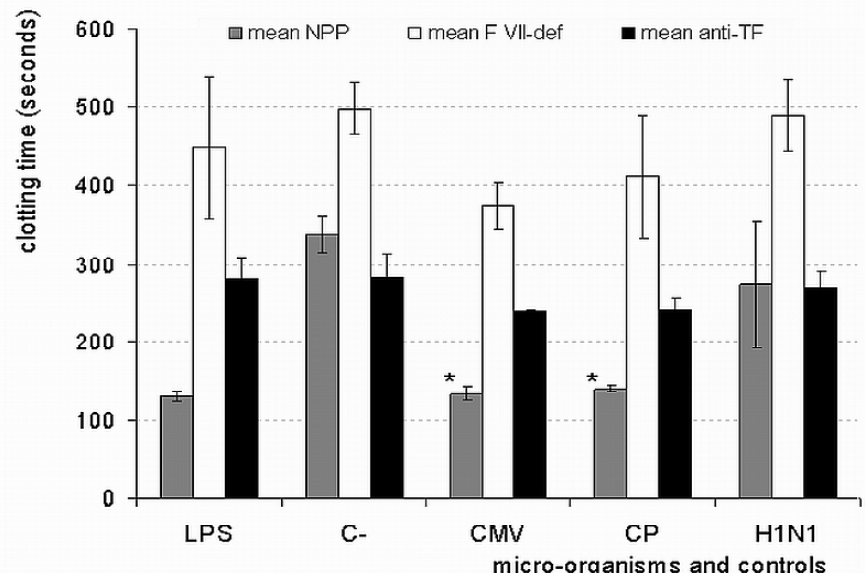

Figure 2 Procoagulant activity of infected monocytes.

Mean clotting times of virus-infected monocytes in seconds ± SE based on three experiments in duplicate. LPS: positive control (1 µg/mL), C-: negative control (culture medium/no virus); H1N1: Influenza A/ H1N1/1/86/Singapore; *Cp: Chlamydia pneumoniae*, CMV: Cytomegalovirus; NPP: normal pooled plasma; F VII-def: Factor VII-deficient plasma; anti-TF: with anti-tissue factor antibodies pre-incubated samples in NPP. *P < 0.05 indicating significant difference from the negative control (Dunnett's multiple comparisons test).

Cytokine assays

Results from IL-6 and IL-8 experiments in the supernatants of the infected monocytes were more or less identical. *Chlamydia pneumoniae*-infected monocytes produced significantly more IL-6 (733 ± 84 pg/mL; p<0.01) than did the uninfected monocytes (90 ± 61 pg/mL), whereas the difference was not significant for CMV-infected monocytes (317 ± 94 pg/mL IL-6; p>0.05). IL-8 production by *Cp*- and CMV-infected monocytes was marginally elevated (268 ± 58 pg/mL and 250 ± 48 pg/mL, respectively; p>0.05). Influenza A/H1N1-infected monocytes produced high levels of IL-6 (1797 ± 244 pg/mL; p<0.001) and IL-8 (725 ± 92 pg/ mL; P <0.001) [**Figure 3**]. Interleukin 10 was not detected in the supernatants or in the LPS control.

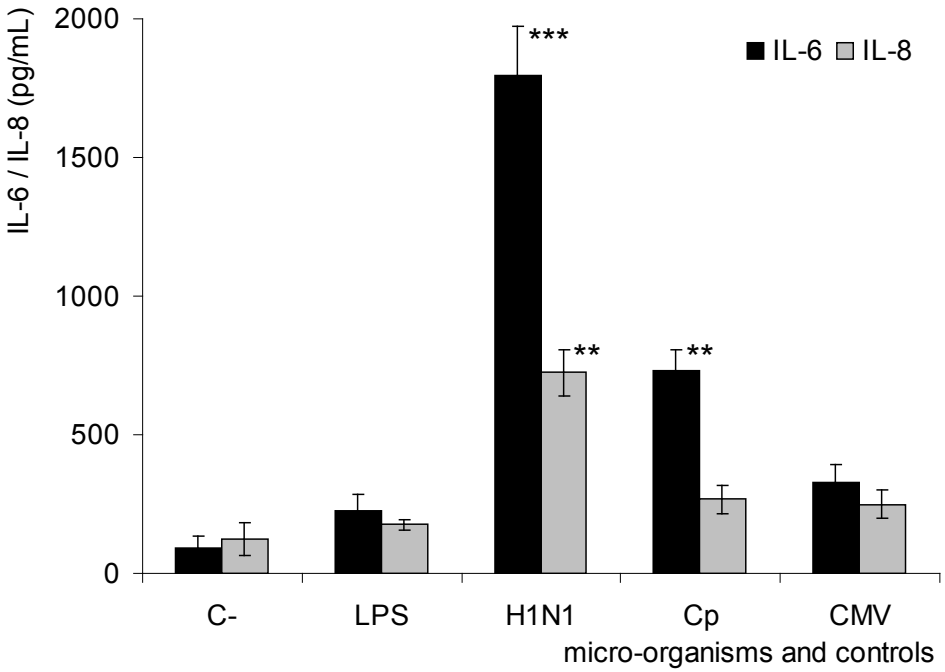

Figure 3 Interleukine production of infected monocytes.
Mean ± SE concentrations of interleukin-6 (IL-6) and interleukin 8 (IL-8) in the supernatants of virus-infected monocytes, based on three experiments in duplicate. LPS: positive control (1 µg/mL), C-: , negative control (culture medium/no virus); CMV: cytomegalovirus; H1N1: Influenza A/H1N1/1/86/Singapore; *Cp: Chlamydia pneumoniae*. ***P < 0.001, indicating extremely significant difference from the negative control (Dunnett's multiple comparisons test). **P < 0.01, indicating very significant difference from the negative control (Dunnett's multiple comparisons test).

Discussion

Isolation of monocytes

Ficoll isolation is the most commonly used method for isolation of peripheral blood mononuclear cells[50,52,53]. Care must be taken to prevent monocytes becoming activated during isolation procedures and to obtain platelet-free monocytes. Platelets adherent to monocytes substantially contribute to procoagulant activity by expressing glycoprotein IIb/IIIa complexes; the receptor complexes for fibrinogen. Moreover, P-selectin on activated platelets is able to up-regulate TF expression. Several authors have emphasized the importance of obtaining platelet-free monocyte preparations[54-58]. After the MACS depletion procedure, a large number of monocytes had platelets attached to them. This is probably because the cocktail of haptene conjugated antibodies, which was used in the depletion protocol, contains antibodies against almost all cell types (CD3, CD7, CD19, CD45RA, CD56 and IgE) but not against platelets (CD41 or CD61). Fluorescence-activated cell sorter analysis showed that platelets were activated and attached to 80% of the monocytes. Compared with the depletion protocol, the positive selection method yielded monocyte suspensions of greater purity and with only marginal interference from platelets; after all, only 3% of monocytes had platelets on their membrane. In our hands, Vacutainer CPT® isolation combined with MACS® CD14-positive selection provided a reliable technique for the isolation of unstimulated monocytes from peripheral blood.

Procoagulant activity and acute-phase response

In previous research we described the procoagulant activity of endothelial cells after infection with respiratory viruses. Viral pathogens induced a TF-dependent reduction of clotting time by approximately 55% [27]. Thiruvikraman et al. found TF to be localized extracellular in high levels in atherosclerotic plaques[31]. Thrombosis can be initiated rapidly when arterial plaques rupture and expose their contents to the bloodstream.
Co-culturing of endothelial cells with monocytes revealed that TF induction occurred in monocytes rather than in endothelial cells[59]. Levels of TF are already detectable 2-4 h after infection/stimulation and TF expression reaches a maximum at 4-6 h after infection. In our coagulation experiments, CMV and *Cp* as well as influenza reduced the clotting time by 19-60%. The initiation of coagulation by virus-infected monocytes was a result of the expression of TF. By using Factor VII-deficient plasma and anti-TF

antibodies, we showed that TF plays an important role in virus-induced monocytic procoagulant activity. Infection with CMV and *Cp* induced the production of modest levels of IL-6 and IL-8, whereas infection with influenza A strongly stimulated the production of IL-6 and IL-8. Our results indicate that only small doses of an infectious virus are needed to stimulate monocytes to exert considerable immunological effects. There have been conflicting reports about whether influenza virus is able to infect monocytes. It is imaginable that viral proteins are synthesized de novo, but that there is no or little productive infection. Using immunofluorescence, we showed that influenza virus did infect monocytes (Fig. 1b), although the infection percentage was very low (< 5%). Secondary bacterial infections are not uncommon after influenza infections. It was assumed that increased sensitivity to bacterial products is the result of 'priming' of cells[60] because low amounts of LPS can accelerate cytokine production substantially[61,62]. As described earlier, TF-expression is elevated after infection, which may be a direct virus effect but which may also be triggered by IL-6. By producing IL-6, infected monocytes stimulate uninfected cells to express TF. Continuing viral infection may sustain excess TF production and exhaust the inhibitory effects of tissue factor pathway inhibitor in vivo[63]. We did not detect IL-10 production by virus-infected monocytes, not even in the LPS control. However, because cytokine production is maximal at 24 h after infection, it is possible that the infection times we used were too short to stimulate IL-10 production. After 4 h IL-10 production may still be minimal or only detectable at the mRNA level. During viral infections monocytes predominantly produce the inflammatory cytokines IL-6 and IL-8. Interleukin-10 is detected predominantly in atherosclerotic lesions, but monocytes may produce this cytokine transiently. Nevertheless detection of IL-10 is of utmost importance because it is a potent down regulator of pro-inflammatory cytokine production, thus being a potent immunosuppressor with potential for therapeutic use. The balance between pro-inflammatory and anti-inflammatory cytokines in the atherosclerotic plaque may be decisive for the progression of the lesion. This concept is confirmed by a recent study showing that patients with unstable angina had significantly lower serum IL-10 concentrations compared with patients with chronic stable angina[64]. Moreover experimental work in animals confirms the protective role of IL-10 in atherosclerosis[65, 66]. We conclude that virus infection of monocytes triggers pro-atherosclerotic and pro-thrombotic processes in vitro.

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References

1. Ross R. The pathogenesis of atherosclerosis; a perspective for the 1990s. Nature 1993; 362:801–9.
2. Whicher J, Biasucci L, Rifai N. Inflammation, the Acute Phase Response and Atherosclerosis. Clin Chem Lab Ned 1999; 37 (5): 495-503.
3. Buja LM. Does atherosclerosis have an infectious etiology? Circulation 1996; 94:872.
4. Morré SA, Stooker W, Lagrand WK, van de Brule AJC, Niessen HWM. Micro-organisms in the etiology of atherosclerosis. J Clin Pathol 2000;53:647-54.
5. Ross R. Atherosclerosis, an inflammatory disease. N Engl J Med 1999;340(2):115-26.
6. Friedman HM, Macarak EJ, MacGregor RR, Wolfe J, Kefalides NA. Virus infection of endothelial cells. J Infect Dis 1981;143(2):266-73.
7. Minick CR, Fabricant CG, Fabricant J, Litrenta MM. Atheroarteriosclerosis induced by infection with a herpes virus. Am J Pathol 1979;96:673-706.
8. Campbell AE, Loria RN, Madge GE. Coxsackie virus B cardiopathy and angiopathy in the hypercholesteremic host. Atherosclerosis 1978;31:295-306.
9. Burch GE, Tsui CY, Harb JM. Pathologic changes of aorta and coronary arteries of mice infected with Coxsackie B virus. Proc Soc Exp Biol Medl 1971;137:657-61.
10. Burch GE, Harb JM, Hiramoto Y, Shewey L. Viral infection of the aorta of man associated with early atherosclerotic changes. Am Heart J 1973;86:523-34.
11. Libby P, Egan D, Skarlatos S. Roles of infectious agents in atherosclerosis and restenosis; an assessment of the evidence and the need for future research. Circulation 1997;96:4095-103.
12. Mosorin et al. Detection of Chlamydia pneumoniae-Reactive T lymphocytes in human atherosclerotic plaques of the carotid artery. Arterioscl Thromb Vasc Biol 2000;20:1061-7.
13. Quinn TC, Gaydos CA. In vitro infection and pathogenesis of Chlamydia pneumoniae in endovascular cells. Am Heart J 1999;138:S507-S511.
14. Saikku P, Leinonen M, Matilla K et al. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 1988;2:983-6.
15. Saikku P, Leinonen M, Tenkanen L et al. Chronic Chlamydia pneumoniae infection as a risk factor for coronary heart disease in the Helsinki heart study. Ann Intern Med 1992;116:372-8.
16. Zhu J, Shearer GM, Norman JE, Pinto LA, Marincola FM et al. Host response to Cytomegalovirus infection as a determinant of susceptibility to coronary artery disease: sex based differences in inflammation and type of immune response. Circulation 2000;102(20):2491-6.

17. Strachan DP, Carrington D, Mendall MA, Butland BK, Sweetnam PM, Elwood PC. Cytomegalovirus seropositivity and incident ischaemic heart disease in the Caerphilly prospective heart disease study. Heart 1999;81(3):248-51.
18. Ridker PM, Hennekens CH, Buring JE, Kundsin R, Shih J. Baseline IgG antibody titers to Chlamydia pneumoniae, Helicobacter pylori, Herpes simplex virus and Cytomegalovirus and the risk for cardiovascular disease in women. Ann Intern Med 1999;131(8):573-7.
19. Ridker PM, Hennekens CH, Stampfer MJ, Wang F. Prospective study of herpes simplex virus, Cytomegalovirus and the risk of future myocardial infarction and stroke. Circulation 1998;98(25):2796-9.
20. Gattone M, Iacoviello L, Colombo M, Castelnuovo AD, Soffiantino F et al. Chlamydia pneumoniae and Cytomegalovirus seropositivity, inflammatory markers and the risk of myocardial infarction at young age. Am Heart J 2001;142(4):633-40.
21. Zhu J, Quyyumi AA, Norman JE, Csako G, Waclawiw MA, Shearer GM, et al. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. Am J Cardiol 2000;85(2):140-6.
22. Visseren FL, Bouter KP, Pon MJ, Hoekstra JB, Erkelens DW, Diepersloot RJ. Patients with diabetes mellitus and atherosclerosis; a role for Cytomegalovirus? Diabetes Res Clin Pract 1997;36(1):49-55.
23. Blankenberg S, Rupprecht HJ, Bickel C, Espinola-Klein C, Rippin G et al. Cytomegalovirus infection with interleukine 6 response predicts cardiac mortality in patients with coronary artery disease. Circulation 2001;103(24):2915-21.
24. Muhlestein JB, Horne BD, Carlquist JF, Madsen TE, Blair TL et al. Cytomegalovirus seropositivity and C-reactive protein have independent and combined predictive value for mortality in patients with angiographically demonstrated coronary artery disease. Circulation 2000;102(16):1917-23.
25. Zhu J, Quyyumi AA, Norman JE, Csako G, Epstein SE. Cytomegalovirus in the pathogenesis of Atherosclerosis: the role of inflammation as reflected by elevated C-reactive protein levels. J Am Coll Cardiol 1999;34(6):1738-43.
26. Kaftan HA, Kaftan O, Kilic M. Markers of chronic inflammation. Are they important in cases with chronic coronary heart disease? Jpn Heart J 1999;40(3):275-80.
27. Visseren FLJ, Bouwman JJM, Bouter KP, Diepersloot RJA, de Groot Ph, Erkelens DW. Procoagulant activity of endothelial cells after infection with respiratory viruses. Thromb Haemost 2000;84(2):319-24.
28. Van Dam-Mieras M, Muller AD, van Hinsbergh V, Mullers W, Bomans P, Bruggeman C. The procoagulant response of Cytomegalovirus infected endothelial cells. Thromb Haemost 1992;68(3):364-70.
29. Drake TA, Morrissey JH, Edgington TS. Selective expression of tissue factor in human tissues. Am J Pathol 1989;134:1087-97.
30. Rivers RPA, Cattermole HEJ, Wright I. The expression of surface tissue factor apoprotein by blood monocytes in the course of infections in early infancy. Pediatr Res 1992;31(6):567-73.

31. Thiruvikraman SV, Guha A, Roboz J, Taubman MB, Nemerson F, Fallon JT. In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin labeled factors VIIa and X. Lab Invest 1996;75:451-61.
32. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci U S A 1989;86:2839-45.
33. Colden-Stansfield M, Ratcliffe D, Cramer E, Gallin E. Characterization of influenza virus-induced leukocyte adherence to human umbilical vein endothelial cell monolayers. J Immunol 1993;151:310-21.
34. Span A, van Dam-Mieras Mullers W, Endert J, Muller A, Bruggeman C. The effect of virus infection on the adherence of leucocytes or platelets to endothelial cells. Eur J Clin Invest 1991;21:331-8.
35. Span A, Endert J, van Boven C, Bruggeman C. Virus induced adherence of monocytes to endothelial cells. FEMS Microbiol 1989;47:237-44.
36. Kaukoranta-Tolvanen S, Teppo A, Laitinen K, Saikku P, Linnavuori K, Lainonen M. Growth of Chlamydia pneumoniae in peripheral blood monocytes and induction of a cytokine response. Microb Pathog 1996;21:215-21.
37. Visseren FLJ, Verkerk MS, Bouter KP, Diepersloot RJA, Erkelens DW. Interleukine IL-6 production by endothelial cells after infection with influenza virus and Cytomegalovirus. J Lab Clin Med 1999;134(6):623-30.
38. Frostegard J, Ulfgren A, Nyberg P, Hedin U, Swedenborg J et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage stimulating cytokines. Atherosclerosis 1999;145:33-43.
39. Neumann FJ, Ott I, Marx N, Luther T, Kenngott S et al. Effect of human recombinant Interleukin-6 and Interleukin-8 on Monocyte Procoagulant activity. Arteriosclerosis Thromb Vasc Biol 1997;17:3399-405.
40. Mahoney JB, Coombes BK. Chlamydia pneumoniae and atherosclerosis: does the evidence support a causal or contributory role? FEMS Microbiol Lett 2001;197(1):1-9.
41. Osterud B. Tissue factor expression by monocytes: regulation and pathophysiological roles. Review. Blood Coagul Fibrinolysis 1998;9(1):9-14.
42. Carson SD, Johnson DR, Tracy SM. Tissue factor and the extrinsic pathway of coagulation during infection and vascular inflammation. Eur Heart J 1993;14 (K):98-104.
43. Froberg MK, Adams A, Seacotte N, Parker-Thornberg J, Kolattukudy P. Cytomegalovirus infection accelerates inflammation in vascular tissue overexpressing monocyte chemoattractant protein 1. Circ Res 2001;89(12):1224-30.
44. Denger S, Jahn L, Wende P, Watson L, Gerber SH et al. Expression of chemo attractant protein cDNA in vascular smooth muscle cells: induction of the synthetic phenotype: a possible clue to SMC differentiation in the process of atherogenesis. Atherosclerosis 1999;144(1):15-23.
45. Julkunen I, Melen K, Nyqvist M, Pirhonen J, Sareneva T, Matikainen S. Inflammatory responses in influenza A virus infection. Vaccine 2000;19(Suppl. 1):S32-7.

46. Byrne GI, Kaloyoglu MV. Chlamydia pneumoniae and atherosclerosis: links to the disease process. Am Heart J 1999;138 (5 Part 2):S488-90.
47. Van Lenten BJ, Wagner AC, Nayak DP, Hama S, Navab M, Fogelman AM. High-density lipoprotein loses its anti inflammatory properties during acute influenza A infection. Circulation 2001;103(18):2283-8.
48. Kalayoglu MV, Perkins BN, Byrne GI. Chlamydia pneumoniae infected monocytes exhibit increased adherence to human aortic endothelial cells. Microbes Infect 2001;3(12):963-9.
49. Guetta E, Guetta V, Shibutani T, Epstein SE. Monocytes harboring Cytomegalovirus: interactions with endothelial cells, smooth muscle cells and oxidized low-density lipoprotein. Possible mechanisms for activating virus delivered by monocytes to sites of vascular injury. Circ Res 1997;81(1):8-16.
50. Boyum A. Separation of leucocytes from blood and bone marrow. Scand. J Clin Lab Invest Suppl 1968;97:77.
51. Reed LJ, Muench J. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493.
52. Boyum A, Lovhaug D, Tresland L, Nordlie EM. Separation of leucocytes. improved cell purity by fine adjustments of gradient medium density and osmolality. Scand J Immunol 1991;34(6):697-712.
53. Stoll HP, Kramer S, Oberhausen E. A new method of preparing monocyte suspensions from human whole blood. Blood Cells 1986;11(3):421-8.
54. Clemetson KJ, McGregor JL, McEver RP, Jacques YV, Bainton DF et al. Absence of platelet membrane glycoproteins IIb/IIIa from monocytes. J Exp Med 1985;161:972-83.
55. Levene RB, Rabellino EM. Platelet glycoproteins IIb and IIIa associated with blood monocytes are derived from platelets. Blood 1986;67(1):207-13.
56. Celi A, Pellegrini G, Lorenzet R, De Blasi A, Ready N et al. P selectin induces the expression of tissue factor on monocytes. Proc Natl Acad Sci USA 1994;91(19):8767-71.
57. Perussia B, Jankiewicz J, Trinchieri G. Binding of platelets to human monocytes: a source of artifacts in the study of the specificity of antileukocyte antibodies. J Immunol Meth 1982;50(3):269-76.
58. Osterud B. Tissue factor expression by monocytes: regulation and pathophysiological roles. Review. Blood Coagul Fibrinolysis 1998;9(1):S9-S14.
59. Collins P, Noble K, Reittie J, Hoffbrand K, Pasi K, Young K. Induction of tissue factor expression in human monocyte/endothelium cocultures. Br J Haematol 1995;91:963-70.
60. Shaw R, Clark R. Interleukines and their interactions in inflammation. Handbook of inflammation. Mediators Inflamm 1989;6:245-58.
61. Nain M, Hinder F, Gong JH, Schmidt A, Bender A et al. Tumor necrosis factor-alpha production of influenza A virus-infected macrophages and potentiating effect of lipopolysaccharides. J Immunol 1990;145(6):1921-8.
62. Lehmann C, Sprenger H, Nain M, Bacher M, Gemsa D. Infection of macrophages by influenza A virus; characteristics of tumor necrosis factor-alpha (TNF-alpha) gene expression. Res Virol 1996;147(2-3):123-30.

63. Osterud B, Bjorklid E. The tissue factor pathway in disseminated intravascular coagulation. Semin Thromb Hemost 2001;27(6):605-17.

64. Smith DA, Irving SD, Sheldon J, Cole D, Kaski JC. Serum levels of the anti-inflammatory cytokine Interleukin-10 are decreased in patients with unstable angina. Circulation 2001;104:746-9.

65. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F et al. Protective role of interleukine-10 in atherosclerosis. Circ Res 1999;85(8):e17-24.

66. Pinderski Oslund LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M et al. Interleukine-10 blocks atherosclerotic events in vitro and in vivo. Arterioscl Thromb Vasc Biol 1999;19:2847–53.

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Azithromycin inhibits interleukin-6 but not fibrinogen production in hepatocytes infected with Cytomegalovirus and *Chlamydia pneumoniae*

Summary

Chlamydia pneumoniae and cytomegalovirus (CMV) have been associated with the development of atherosclerosis. Inflammatory stimuli initiate the biosynthesis of fibrinogen, interleukin (IL)-6 and plasminogen activator inhibitor 1 (PAI-1) in the liver. Chronic infection may perpetuate the inflammatory status. We hypothesized that infection of human hepatocytes with the intracellular pathogens *C. pneumoniae a*nd CMV accelerates biosynthesis of fibrinogen, IL-6, and PAI-1 but that this biosynthesis can be reduced with the use of azithromycin.

HepG2 human hepatocytes were infected with *C. pneumoniae* and CMV *in vitro* in the presence of 0, 0.016, 0.125, or 1 µg/mL azithromycin. We measured IL-6, PAI-1, and fibrinogen after 24, 48, 72, and 96 hours. *C. pneumoniae*–infected hepatocytes produce IL-6 (2667 ± 309 pg/mL vs. 137 ± 120 pg/mL in uninfected cells after 96 hours. Incubation with 0.016 µg/mL azithromycin decreased IL-6 levels to a mean of 1516 ± 402 pg/mL, and incubation with 0.125 and 1 µg/mL azithromycin decreased IL-6 to 871 ± 364pg/mL and 752 ± 403 pg/mL, respectively. *C pneumoniae*–induced IL-6 production was time- and dose dependent.

The interaction of *C. pneumoniae* with azithromycin treatment was significant, indicating an inhibitory effect of azithromycin on *C. pneumoniae*–induced IL-6 production. CMV infection did not lead to IL-6 production by hepatocytes.

C. pneumoniae and CMV infection did not induce any changes in PAI-1 production. Fibrinogen production was increased by CMV infection after 72 hours (838 ± 88 ng/mL; $p<0.01$) and after 96 hours by infection with both *C. pneumoniae* and CMV (765 ± 100 ng/mL and 846 ± 123 ng/mL, respectively; $p<0.05$). Azithromycin did not suppress CMV- or *C. pneumoniae*–induced fibrinogen production. Moreover, we could not confirm an anti-inflammatory effect of azithromycin in experiments with cross-titrations of azithromycin against either IL-1 or IL-6 ($p>0.05$). Azithromycin reduces *C. pneumoniae*–induced IL-6 production, but not fibrinogen production, by human hepatocytes. This is a result of the antimicrobial properties of azithromycin and not a direct anti-inflammatory effect.

Abbreviations

ANOVA: analysis of variance; APP: acute-phase protein; APR: acute phase reaction; BGM: Buffalo green monkey kidney cells; CMV: cytomegalovirus; *Cp: Chlamydia pneumoniae*; CRP: C-reactive protein; EMEM Earle's minimal essential medium; FBS: fetal bovine serum; HEL: human embryonic lung cells: Hep: human epithelioma; IL-6: interleukin 6; Lp(a): lipoprotein A; MBC: minimal bactericidal concentration; MIC: minimal inhibitory concentration; MOI: multiplification of infection; PAI: plasminogen-activator inhibitor; $TCID_{50}$: 50% tissue culture infective dose; TNF: tumor necrosis factor

Introduction

Chronic low-grade inflammation plays an important role in the development of atherosclerosis[1]. Whether infectious agents play a causative role in atherogenesis is still a matter of debate. Several studies have indicated that *Chlamydia pneumoniae* and CMV are associated with the development of atherosclerosis[2-4]. Direct vessel-wall infection may trigger atherogenic processes such as procoagulant activity, local inflammation, and endothelial dysfunction, leading to the development of atherosclerotic plaque and eventually to plaque rupture, exposing the thrombogenic plaque content to the bloodstream[5,6]. The liver is able to produce a variety of APP's such as IL-6, CRP, TNF-alpha, fibrinogen, haptoglobin, PAI-1, and complement factors C3 and C4, leading to plasma concentrations as high as 2 to 100 times normal[8–11]. In pathophysiological conditions, plasma levels can remain increased during chronic inflammatory processes such as atherosclerosis[12–15]. Fibrinogen is synthesized by hepatocytes under control of inflammatory cytokines. Oncostatin M and the pleiotropic cytokine IL-6 are considered the most potent stimulators of fibrinogen expression. PAI-1 is a major physiologic inhibitor of plasminogen activation. High plasma levels of PAI-1 have been associated with thrombotic and arterial disease. Increased synthesis of PAI-1 in the liver and cellular components of the atherosclerotic plaque may contribute to the thrombotic complications associated with plaque rupture and the accumulation of extracellular matrix deposits [26,27]. Macrolide antibiotics are effective in the treatment of intracellular infections because of their high cellular accumulation and tissue retention[28–30]. The effects of neomacrolides such as roxithromycin and azithromycin on inflammation have been delineated in animal studies identifying anti-inflammatory activities on top of the expected antibiotic properties of these antibiotics[31,32]. Treatment of *C. pneumoniae* infection with macrolide antibiotics has been shown to lead to decreased plasma concentrations of CRP, TNF-α, and IL-6 but did not reduce the incidence of cardiovascular events[19,33-35]. This may be due to insufficient dose regimens, short duration of treatment and follow-up, underpowered studies, or ineffective treatment of *C. pneumoniae* in monocytes.

In this *in vitro* study we investigated whether azithromycin was able to inhibit *C. pneumoniae* infection of human hepatocytes and reduce the inflammatory response of hepatocytes to infection.

Methods

C pneumoniae and CMV

We propagated *C. pneumoniae* strain AR 39 and a clinical isolate of CMV in Buffalo green monkey kidney cells (BGM) and human embryonic lung cells (HEL), respectively. Both strains were harvested at greater than 80% CPE(cytopathologic effect) and virus stocks were aliquoted and frozen at -90 °C until the time of infection. In an attempt to determine the infectious doses for the experiments, we calculated the $TCID_{50}$ of each strain in accordance with the method of Reed and Muench.

Hep-G2 cell culture

We grew 2 cell lines of Hep-G2 human hepatoma cells (1 donated by the oncology department of the University Hospital of Utrecht and the other obtained from the European Collection of Cell Cultures no. 85011430) in 80 cm^2 tissue-culture flasks (Nunc, Roskilde, Denmark) using EMEM (Life Technologies Ltd, Paisley, Scotland) supplemented with 10 % FBS (Life Technologies) and 2 mM L-glutamine (Life Technologies), 5 mL of nonessential amino acids (Life Technologies) and 4 mg/L fungizone (Bristol-Meyers Squibb, Woerden, the Netherlands). Before the experiments, we propagated HepG2 cells and maintained them for at least 3 passages in EMEM without any antibiotics. In the infection experiments, cells were seeded in 48-well plates (Corning Costar, Corning Inc, Corning, NY) and grown to confluence at 37 °C under 5% CO_2, also in an antibiotic-free medium. To determine the number of cells per well, we detached cells using trypsin solution and counted cells in suspensions using a Bürker-Türk chamber.

Azithromycin

We pre-dissolved 262.5 mg of purified azithromycin (Zithromax; 94.6% azithromycin dihydrate, a kind gift from Pfizer BV) in 5 mL of ultra pure water in the presence of 192.3 mg of sodium citrate (approximate pH 3.0). We also prepared a stock solution of 25 mg/mL azithromycin by adding 99.15 mg NaOH and ultrapure water for a final volume of 10 mL (approximate final pH 6.3). We stored this solution at -20 °C until it was needed.

MIC/MBC

We determined the MIC and MBC of azithromycin for *C. pneumoniae* in HepG2 cells against a 100*$TCID_{50}$ dose of *C pneumoniae*. MIC and MBC were calculated on the basis of immunofluorescence for *C. pneumoniae* on the HepG2 cells.

Infection experiments

Twenty-four hours before inoculation, we refreshed the cells and pre-treated them with 10% EMEM supplemented with 1, 0.125, 0.016 or 0 µg/mL azithromycin. Immediately before inoculation, we performed this procedure again and added 0.4 mL of fresh medium to each well. Cells were infected with 0.1 mL of 2 or 3 doses from both the *C. pneumoniae* AR39 strain and the CMV strain (MOI 0.6, 0.06, and 0.006, respectively). To the uninfected cells we added 0.1 mL of EMEM with the corresponding azithromycin concentration. IL-1 (100 pg/mL for IL-6 experiments) or IL-6 (1 ng/mL for fibrinogen and PAI-1 experiments) was added to the positive control wells. We conducted all incubations 3 times in duplicate. After inoculation, the cells were centrifuged for 1 hour at 800g and incubated for 24, 48, 72, or 96 hours. At each time point, we harvested supernatants and froze them at -35 °C until measurements could be taken. We verified infection rates by immunostaining the infected cells with antibodies to *C. pneumoniae* (30701 Pathfinder *Chlamydia* Culture Confirmation System, BioRad, Hercules, Calif) and CMV (anti-CMV immediate early antigen clone E13, no.12-003; Argene, Varilhes, France).

Cytokine incubations

To investigate the anti-inflammatory effects of azithromycin, we incubated HepG2 cells with panels of IL-1 and IL-6 (200-01B and 200-06; Peprotech Inc, Rocky Hill, NJ) in low to (sub)maximal stimulating concentrations of these cytokines (0, 10 or 100 pg/mL; 1, 10, or 50 ng/mL). Cells were pre-treated in the same fashion as those used in the infection experiments.

APR assays

We conducted sandwich Elisa's for Fibrinogen (FG-EIA Kordia; Affinity Biologicals, Inc, Leiden, the Netherlands), PAI-1 (Imulyse PAI-1 no. 211000; Kordia/ Biopool), and IL-6 (Pelikine IL-6 assay M1916; CLB, Amsterdam, the Netherlands) in accordance with the manufacturer's instructions. For fibrinogen, we constructed a standard curve from

normal haemostasis reference plasma (no. 50720; Kordia/Biopool). Standard curves for PAI-1 and IL-6 were supplied with the kits. For PAI-1, we used a standard fibrinolysis reference plasma (no. 210540; Kordia /Biopool) as a positive control.

Statistical analysis

In all infection experiments we compared the production levels of infected hepatocytes with those of the negative controls (uninfected HepG2) at each time point. We analyzed data using Dunnett's multiple-comparisons q-test for differences between pairs of columns, which corrects for unequal variances. P values of less than 0.05 were taken to indicate a significant difference between production of IL-6, PAI-1, or fibrinogen by infected hepatocytes and that by uninfected hepatocytes. We also conducted analysis of linear trends in the IL-6 data to establish whether the effects of treatment were time- or dose-dependent. Linear regression was used to analyze the effects of *C. pneumoniae*-infection, time, and azithromycin on IL-6 production after transformation of the response variable on the log scale. On the other hand, our objective in the anti-inflammatory experiments was to assess whether azithromycin was capable of inhibiting production of IL-6, PAI-1 or fibrinogen by interleukin-stimulated hepatocytes, regardless of infection. For this reason it was necessary to compare the levels of production in all incubations with different concentrations of azithromycin and IL's among themselves. For this purpose we used Bonferroni's multiple-comparisons t-test. Beforehand, we performed ANOVA to rule out the possibility that any effect might have been due to a difference in variations instead of azithromycin alone. p-values greater than 0.05 were considered not significant.

Results

C. pneumoniae and CMV infection in human hepatocytes

Infection rates of HepG2 cells were assessed by determining the $TCID_{50}$-titres for CMV and *Cp* and the number of cells counted on the growth areas in each well of a 48-well TC-plate. $TCID_{50}$-values of the virus stocks were $10^{5.5}$/50 µL and $10^{4.5}$/50 µL for *C. pneumoniae* and CMV, respectively, and 10^5 cells were counted on the growth surfaces. For *C. pneumoniae*, 100 µL of a 10x, 100x and a 1000x dilution was inoculated on the HepG2 cells and for CMV 100 µL of undiluted and 10x-diluted virus stock. Therefore actual MOI's were 0.6, 0.06, and 0.006 for *C. pneumoniae* and 0.6 and 0.06 for CMV (**Figure 1 and 2**). The MIC and MBC values for azithromycin on *C. pneumoniae*–infected

HepG2 cells were assessed at 0.063 and 0.125 µg/mL, respectively. Hence, on the basis of these MIC and MBC values, we selected azithromycin concentrations of 0.016, 0.125, and 1 µg /mL to be added to the culture medium in all experiments.

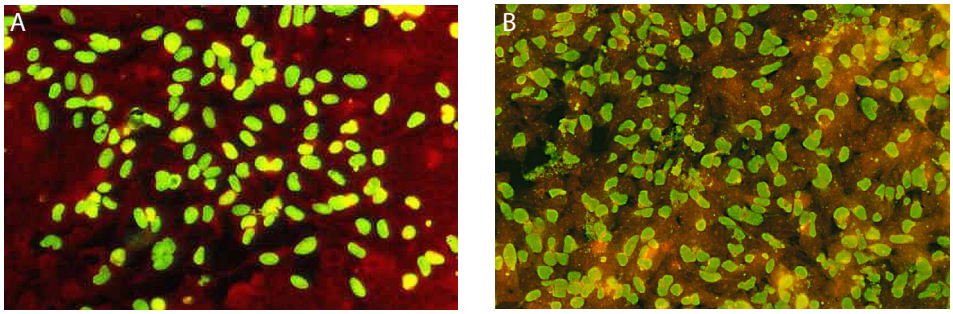

Figure 1 Infection of HepG2 cells. **A**: Fluorescence staining of CMV-infected HepG2 cells. Note apple-green fluorescence of CMV immediate early antigen in the nuclei of infected cells. **B**: Fluorescence staining of C. pneumoniae–infected HepG2 cells. Apple-green fluorescence (genus-specific) denotes Chlamydia antigens in the cytoplasm of infected cells. Background of each photo contains uninfected cells counterstained with Evans blue (red). Original magnification 100x. (*For color figure see page 218)*

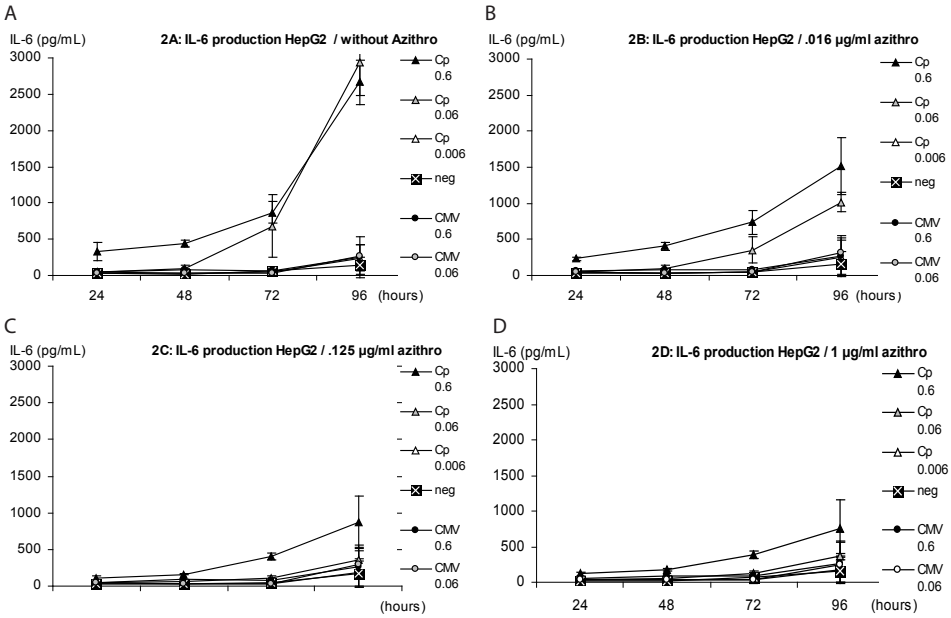

Figure 2 IL-6 production by infected HepG2 cells. *Cp* = C pneumoniae; *neg* = uninfected HepG2 cells. Values of 0.6, 0.06, and 0.006 for *C pneumoniae* and CMV refer to the MOI. **A**, Incubations without azithromycin. **B**, HepG2 cells incubated with 0.016 µg/mL azithromycin. **C**, HepG2 cells incubated with 0.125 µg/mL azithromycin. **D**, HepG2 cells incubated with 1 µg/mL azithromycin. *Y-axis* denotes IL-6 levels in picograms per milliliter (mean ± SEM); *x-axis* denotes incubation time in hours. All data are from 3 experiments performed in duplicate.

IL-6, PAI-1, and fibrinogen production by hepatocytes after infection and effects of azithromycin.

The basal secretion of IL-6 by uninfected hepatocytes ranged from 25 ± 5 pg/mL after 24 hours to 137 ± 120 pg/mL after 96 hours. Immediately after inoculation, no IL-6 (<20 pg/mL) was detected in the supernatants (data not shown). CMV infection did not induce IL-6 production by HepG2 cells at any point. *C. pneumoniae* infection with the highest dose of *C. pneumoniae* resulted in IL-6 production at all time points up to 2667 ± 309 pg/mL IL-6 after 96 hours, whereas infection with a lower concentration of *C. pneumoniae* did not induce IL-6 production until 96 hours had elapsed (2930 ± 447 pg/mL IL-6). After 96 hours of high-dose *C. pneumoniae* infection, 0.125 and 1 µg/mL, azithromycin suppressed IL-6 production (871±364 and 752±403 pg/mL, respectively) although azithromycin did not entirely reduce IL-6 production to the level of the uninfected HepG2 cells. *C. pneumoniae*-infected HepG2 cells incubated without azithromycin and with 0.016 µg/mL azithromycin still produced IL-6 (2667 ± 309 and 1516 ± 402 pg/mL, respectively (**Figure 2, A–D**). To substantiate the effects of *C. pneumoniae* on IL-6 production and the inhibitory effects of azithromycin, we subjected IL-6 responses to analysis for linear trends. After log transformation of the data, analysis for linear trends in a regression model showed dose-dependent effects on IL-6 production by *C. pneumoniae* doses with MOI's of 0.6 and 0.06 ($p<0.001$) but not for the lowest dose (MOI 0.006; p:0.39). The effects of time and azithromycin concentration on IL-6 production were also significant ($p<0.001$). After azithromycin treatment, *C. pneumoniae*-induced IL-6 production was no longer significant (p-values 0.23, 0.21, and 0.32, respectively). ANOVA revealed significant interaction of *C. pneumoniae* with time ($p<0.001$), indicating a time dependent effect of *C. pneumoniae* infection on IL-6 production. Interaction of *C. pneumoniae* with azithromycin was also significant (p:0.02) establishing the inhibitory effect of azithromycin on *C. pneumoniae*–induced IL-6 production. The interaction of azithromycin with time was not significant (p:0.20), indicating that azithromycin did not have additional effects on log IL-6 responses over time.

Although PAI-1 levels gradually increased over time, PAI-1 production by hepatocytes was not influenced by infection (**Figure 3**). Average PAI-1 production ranged from 10.3 ± 1.7, 7.7 ± 1.2 and 9.7 ± 1.2 ng/mL after 24 hours to 28.7 ± 0.5, 20.3 ± 0.5 ng/mL and 21.3 ± 1.7 ng/mL after 96 hours for uninfected cells, *C pneumoniae*–infected cells and CMV-infected cells, respectively (all $p>0.05$). Production of PAI by IL-6 stimulated hepatocytes was quite significant ($p<0.01$), ranging from 12 ng/mL at 24 hours to 60 ng/

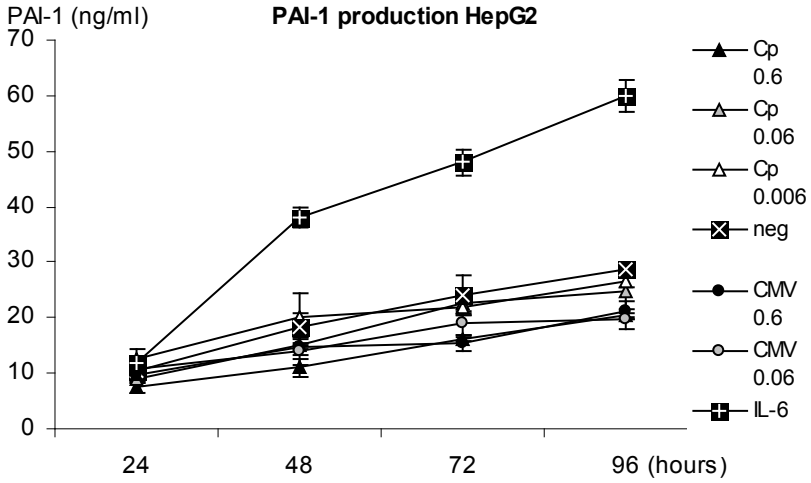

Figure 3 PAI-1 production by infected HepG2 cells. *Cp: C. pneumoniae*; *neg:* uninfected HepG2 cells; *IL-6:* HepG2 cells incubated with 1 ng/mL IL-6 (positive control). Values of 0.6. 0.06, and 0.006 for *C. pneumoniae* and CMV refer to the MOI. *Y-axis* denotes PAI levels in nanograms per milliliter (mean±SEM); *x-axis:* incubation time in hours. All data are from 3 experiments performed in duplicate.

mL at 96 hours. Incubation with azithromycin did not alter these findings (p>0.05).

HepG2 cells constitutionally produce fibrinogen

After 24 and 48 hours, we found no significant differences among fibrinogen levels in infected cells, uninfected cells, and IL-6–stimulated cells. Seventy-two hours stimulation with IL-6 and both CMV doses induced significant increases in fibrinogen production by hepatocytes compared with that in the negative control (838 ± 88 and 794 ± 98 ng/mL for both CMV concentrations (p<0.01) and 818 ± 103 ng/mL (p:0.02) for IL-6, compared with 442 ± 44 ng/mL for the negative control. After 96 hours, both *C. pneumoniae* and CMV infections induced significant fibrinogen production: 765 ± 100 and 741 ± 155 ng/mL (both p<0.05) for *C. pneumoniae* and 846 ± 123 ng/mL (p<0.01) and 804 ± 106 ng/mL (p<0.05) for CMV, compared with the negative control (460 ± 16 ng/mL). Ninety-six hours incubation with IL-6 induced 766 ± 99 ng/mL fibrinogen (p:0.03). Fibrinogen production after 72 (CMV) and 96 hours (CMV and *C. pneumoniae*) was not changed by the addition of 0.016, 0.125, or 1 µg/mL azithromycin, indicating that azithromycin did not suppress fibrinogen production (Bonferroni tests, all p>0.05). ANOVA showed no significant differences in variance among these groups (all p>0.05;

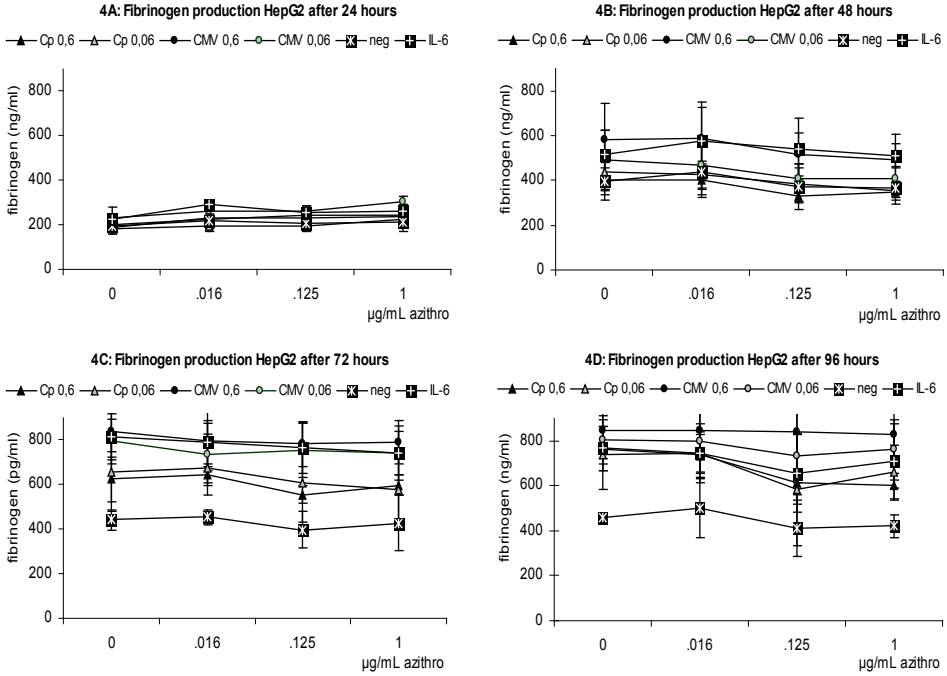

Figure 4 Fibrinogen production by infected HepG2 cells. *Cp:* C pneumoniae; *neg:* uninfected HepG2 cells; *IL-6:* HepG2 cells incubated with 1 ng/mL IL-6 (positive control). Values of 0.6 and 0.06 for *C pneumoniae* and CMV refer to the MOI. *Y-axis* denotes fibrinogen levels in nanograms per milliliter (mean ± SEM); *X-axis* denotes azithromycin concentrations (micrograms per milliliter) added to the incubations. All data are from 3 experiments performed in duplicate. **A:** Fibrinogen production by HepG2 cells after 24 hours. **B:** Fibrinogen production by HepG2 cells after 48 hours. **C:** Fibrinogen production by HepG2 cells after 72 hours. **D:** Fibrinogen production by HepG2 cells after 96 hours.

Figure 4, A–D).

Anti-inflammatory effects of azithromycin

We also investigated the possibility of anti-inflammatory action on the part of azithromycin by performing cross-titrations of azithromycin against dilution panels (0, 10, and 100 pg/mL; 1, 10, and 50 ng/mL) of both IL-1 ß and IL-6. Hepatocytes did not produce significant concentrations of IL-6 at any point during incubation with a low concentration of IL-1ß (10 pg/mL). Incubation with 100 pg/mL IL-1β, however, had induced IL-6 concentrations of as much as 1360 pg/mL after 96 hours, and the higher IL-1β concentrations of 1, 10, and 50 ng/mL had induced maximal IL-6 levels of 2299,

Table 1 Anti-inflammatory effects of azithromyci

Azithromycine dosage (µg/mL)	Unstimulated	10 pg/mL IL	100 pg/mL IL	1 ng/mL IL	10 ng/mL IL	50 ng/mL IL
Fibrinogen (ng/mL)						
0	616 (100%)	780 (100%)	758 (100%)	781 (100%)	761 (100%)	775 (100%)
.016	615 (100%)	777 (100%)	739 (97%)	740 (95%)	734 (96%)	766 (99%)
.125	577(94%)	744 (95%)	787 (104%)	757 (97%)	720 (95%)	728 (94%)
1	621 (101%)	100 (95%)	753 (99%)	748 (96%)	752 (99%)	745 (96%)
IL-6 (pg/mL)						
0	138 (100%)	268 (100%)	1360 (100%)	2299 (100%)	2617 (100%)	3403 (100%)
.016	129 (94%)	257 (96%)	1261 (93%)	2301 (100%)	2370 (91%)	3416 (100%)
.125	118 (86%)	257 (96%)	1278 (94%)	2312 (101%)	2656 (101%)	3609 (106%)
1	126 (91%)	264 (98%)	1226 (90%)	2411 (105%)	2581 (99%)	3618 (106%)
PAI-1 (ng/mL)						
0	32.7 (100%)	37.7 (100%)	54.3 (100%)	55.3 (100%)	25.6 (100%)	39.2 (100%)
.016	33.3 (102%)	35.0 (93%)	50.4 (93%)	51.1 (92%)	18.3 (71%)	28.7 (73%)
.125	28.1 (86%)	27.5 (73%)	43.7 (81%)	51.4 (93%)	22.3 (87%)	34.3 (88%)
1	35.0 (107%)	31.9 (85%)	52.5 (97%)	55.4 (100%)	25.2 (98%)	31.4 (80%)

Overview of fibrinogen and PAI-1 production by IL-6 stimulated hepatocytes incubated with azithromycin. For IL-6 hepatocytes were stimulated with IL-1 and incubated with azithromycin. IL: dilution series of IL-1 or IL-6 from 0 (unstimulated) – 50 ng/ml. Unstimulated: HepG2 cells in medium without azithromycin and IL-1 or IL-6 (negative control). First row: azithromycin concentrations in µg/ml. Results are means of 3 experiments in duplicate and expressed in ng/ml (fibrinogen and PAI) or pg/ml (IL-6). Between brackets: each incubation without azithromycin (0 µg/ml Azithro) was set at 100%. For each azithromycin concentration expressions were related to expressions without Azithromycin incubation and expressed as a percentage of these values.

2617, and 3403 pg/mL, respectively, after 96 hours. Co-incubation with azithromycin did not affect IL-6 production by hepatocytes (Bonferroni tests, all p>0.05; **Table 1**).

IL-6 is a stimulus for hepatocytes to produce PAI-1

Maximal PAI production in hepatocytes was induced by 100 and 1 ng/mL IL-6, resulting in PAI-1 levels of 18.0 ± 7.2 ng/mL (p<0.05) and 20.6 ± 6.2 ng/ml (p<0.05), respectively, after 24 hours, compared with 8.6 ± 3.5 ng/mL for unstimulated hepatocytes. After 96 hours, PAI-1 levels had increased to 54.3 ± 7.5 ng/mL (p<0.05) and 55.3 ± 6.1 ng/mL (p<0.05) after incubation with 100 pg/mL and 1 ng/mL IL-6, respectively and to 32.7 ± 11.6 ng/mL in unstimulated cells. Nevertheless, co-incubation with azithromycin had no effect on PAI levels (Bonferroni test, p >0.05; **Table 1**).

Basal production of fibrinogen by hepatocytes ranged from 466 ± 31 ng/mL after 24 hours to 616 ± 20 ng/mL after 96 hours. Stimulation of hepatocytes with IL-6 concentrations of up to 1 ng/ml slightly increased fibrinogen production from 584 ± 19 ng/mL after 24 hours (p:0.03) to 664 ± 42 ng/mL (p:0.04), 766 ± 12 ng/mL (p<0.01), and 781 ± 18 ng/mL (p<0.01) after 48, 72, and 96 hours, respectively. Co-incubation with azithromycin did not affect fibrinogen synthesis by HepG2 (Bonferroni multiple-comparisons tests, all p<0.05; **Table I**).

Discussion

In this study we designed an *in vitro* model for infection of hepatoma cells and the effect of azithromycin in the outcome of these infections. We have shown that *in vitro* infection of hepatocytes with *C. pneumoniae* leads to time and dose-dependent production of IL-6 but only marginal production of fibrinogen. Co-incubation with azithromycin strongly inhibited this inflammatory response.

Now that inflammation is considered the underlying pathophysiological cause of atherosclerosis, the driving force behind chronic low-grade inflammation becomes a matter of question. The liver is the most important production site for inflammatory mediators such as IL-6 and CRP. IL-6 is the major regulator of hepatic CRP production and is an inducer of proatherogenic processes. We have previously shown that infection of liver cells contributes to the production of inflammatory proteins *in vitro*[7] possibly leading to increased plasma concentrations.

In vivo studies have revealed an anti-inflammatory effect of azithromycin, as measured on the basis of reduced plasma concentrations of IL-6 and CRP after treatment[36,37].

In this study we showed that azithromycin reduces the inflammatory response of infected hepatocytes *in vitro*. We conclude that this reduction is a manifestation of an antimicrobial effect because a cytokine-induced inflammatory response could not be inhibited by azithromycin. Similarly, azithromycin did not affect IL-6 production by CMV-infected hepatocytes. The findings of several studies however, suggest anti-inflammatory potential for macrolides. Although the exact mechanisms seem quite heterogeneous and still must be elucidated, it is recognized that macrolides can prevent or inhibit the production of proinflammatory mediators independent of their antibacterial activities[31,32,35]. Whether these effects have therapeutic consequences in vivo is still under investigation.

The addition of 0.016 mg/L azithromycin to *C pneumoniae*–infected hepatocytes inhibited IL-6 production, but not entirely ($p<0.05$). Concentrations of 0.125 and 1 mg/L azithromycin reduced IL-6 production to almost the same level seen in the negative control ($p>0.05$). Nevertheless, even 1 µg/mL azithromycin was not sufficient to inhibit IL-6 production in the *C pneumoniae*–infection experiment entirely, possibly indicating an alternative IL-6–stimulating mechanism insensitive to the action of azithromycin.

In contrast to IL-6 production, fibrinogen production by hepatocytes could not be suppressed with azithromycin treatment. Taking into account that the inoculation materials did not contain IL-6 (<20 pg/mL; data not shown) and that we used azithromycin concentrations well above the MIC and MBC values for *C. pneumoniae*, this suggests that fibrinogen production by azithromycin-treated infections was induced by factors other than *C. pneumoniae* infection alone. It is possible that IL-6 produced by neighboring cells led to paracrine effects stimulating fibrinogen expression. These findings are supported by the results of other studies describing alternative pathways in fibrinogen synthesis. Northemann et al[38] suggested that different rat hepatoma lines each secrete different characteristic sets of hepatocyte stimulating factor activities and establish unambiguously that IL-6 is secreted by at least some of these lines. Another consideration is the fact that hepatoma cell lines may be quite heterogeneous and may contain distinct subsets of cells[39]. Zuraw and Lotz also described a striking phenomenon in studies of cultured HepG2 cells incubated with supernatants from previous HepG2 cultures. The stimulatory effect of these supernatants was neutralized by IL-6 antibodies, indicating that IL-6 may be active in the same culture in which it was produced, confirming its role as the major hepatocyte stimulating factor[40]. Paysant et al[41] used fibrinogen production in HepG2 cells as an index of IL-6 responsiveness; a concentration range of 1 to 5 ng/mL IL-6 was considered to yield the maximal response

of fibrinogen biosynthesis. In our experiments, hepatocytes still produced IL-6 levels approaching this range (~800 pg/mL after 72 and 96 hours), even in the presence of azithromycin. This IL-6 activity in our cultures may represent an alternative pathway for fibrinogen production, accounting for the failure of azithromycin to inhibit fibrinogen production. Apparently this is due to underlying processes other than infection alone.

On the basis of the concept of atherosclerosis being an inflammatory disease, a role of increasing importance has been assigned to *C. pneumoniae*[3,42]. Increasing evidence for a possible causal role of *C. pneumoniae* in atherogenesis prompted studies with macrolide antibiotics in patients at high risk for cardiovascular complications[43]. Several recent clinical studies, including WIZARD[44], ACES[45], AZACS[46], and ACADEMIC[47], in addition to animal studies[33,48] have addressed the properties and possible benefits of azithromycin in particular, as well as other macrolides[49,50]. Although some investigators foresee improvement in clinical outcome after macrolide treatment [33,43,49,51] most of these trials demonstrate no significant or only marginally beneficial effects of azithromycin in the treatment of coronary-artery disease. Recently the limitations of these studies were reviewed extensively by Grayston[52]. From the findings of 12 published and 2 ongoing clinical studies of antibiotic treatment for the secondary prevention of atherosclerotic cardiovascular disease, it was concluded that a "substantial number of these studies were performed on an inadequate number of subjects and length of observation to provide convincing results". Treatment of *C. pneumoniae* for just 7 days was considered entirely inadequate for a chronic infection. Unsuccessful treatment can also be explained from the life cycle of *C. pneumoniae*. Elementary bodies of *C. pneumoniae* may be unsusceptible to antibiotics, and the initially susceptible reticulate bodies may be able to enter a persistent phase[52]. This concept was also supported by Gieffers et al[53]. In this study we confirm the antibacterial properties of azithromycin on *C. pneumoniae*. We could not show direct anti-inflammatory properties of this drug *in vitro*.

Acknowledgements

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References

1. Ross R. Atherosclerosis: an inflammatory disease. N Engl J Med 1999;340:115-26.
2. Zhu J, Quyyumi AA, Norman JE, Csako G, Epstein SE. Cytomegalovirus in the pathogenesis of atherosclerosis: the role of inflammation as reflected by elevated C-reactive protein levels. J Am Coll Cardiol 1999;34:1738-43.
3. Mlot C. Chlamydia linked to atherosclerosis. Science 1996;272:1422.
4. Capron L. Role of infectious agents in the understanding of atherosclerosis. Rev Med Interne 2002;23 (suppl 1):8s-9s.
5. Visseren FL, Bouwman JJ, Bouter KP, Diepersloot RJ, de Groot PH, Erkelens DW. Procoagulant activity of endothelial cells after infection with respiratory viruses. Thromb Haemost 2000;84:319-24.
6. Visseren FL, Verkerk MS, Bouter KP, Diepersloot RJ, Erkelens DW. Interleukin-6 production by endothelial cells after infection with influenza virus and cytomegalovirus. J Lab Clin Med 1999;134:623-30.
7. Verkerk MS, Visseren FL, Bouter KP, Diepersloot RJ. Acute phase response of human hepatocytes after infection with Chlamydia pneumoniae and cytomegalovirus. Eur J Clin Invest 2003;33:720-5.
8. Darlington GJ, Wilson DR, Revel M, Kelly JH. Response of liver genes to acute phase mediators [discussion]. Ann N Y Acad Sci 1989;557:310-5.
9. Ceciliani F, Giordano A, Spagnolo V. The systemic reaction during inflammation: the acute-phase proteins. Protein Pept Lett 2002;9:211-23.
10. Baumann H, Gauldie J. The acute phase response. Immunol Today 1994;15:74-80.
11. Fey GH, Gauldie J. The acute phase response of the liver in inflammation. Prog Liver Dis 1990;9:89-116.
12. Vasse M, Paysant J, Soria J, Collet JP, Vannier JP, Soria C. Regulation of fibrinogen biosynthesis by cytokines, consequences on the vascular risk. Haemostasis 1996; 26(suppl 4):331-9.
13. Vasse M, Paysant I, Soria J, Mirshahi SS, Vannier JP, Soria C. Down-regulation of fibrinogen biosynthesis by IL-4, IL-10 and IL-13. Br J Haematol 1996;93:955-61.
14. Niessen RW, Lamping RJ, Jansen PM, Prins MH, Peters M, Taylor FB Jr, et al. Antithrombin acts as a negative acute phase protein as established with studies on HepG2 cells and in baboons. Thromb Haemost 1997;78:1088-92.
15. Gabay C, Porter B, Guenette D, Billir B, Arend WP. Interleukin-4 (IL-4) and IL-13 enhance the effect of IL-1beta on production of IL-1 receptor antagonist by human primary hepatocytes and hepatoma HepG2 cells: differential effect on C-reactive protein production. Blood 1999;93:1299-307.
16. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, et al. IL-6 is an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest 1998;101:311-20.
17. Gauldie J, Richards C, Baumann H. IL6 and the acute phase reaction. Res Immunol 1992;143:755-9.

18. Richards C, Gauldie J, Baumann H. Cytokine control of acute phase protein expression. Eur Cytokine Netw 1991;2:89-98.
19. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J 1990;265:621-36.
20. Naka T, Nishimoto N, Kishimoto T. The paradigm of IL-6: from basic science to medicine. Arthr Res 2002;4(suppl 3):S233-42.
21. Kerr R, Stirling D, Ludlam CA. Interleukin 6 and haemostasis. Br J Haematol 2001;115:3-12.
22. Luc G, Bard JM, Juhan-Vague I, Ferrieres J, Evans A, Amonyel P, et al. C-reactive protein, interleukin-6, and fibrinogen as predictors of coronary heart disease: the PRIME study. Arterioscler Thromb Vasc Biol 2003;23:1255-61.
23. Bouwman JJ, Visseren FL, Bosch MC, Bouter KP, Diepersloot RJ. Procoagulant and inflammatory response of virus-infected monocytes. Eur J Clin Invest 2002;32:759-66.
24. Dechend R, Maass M, Gieffers J, Dietz R, Scheidereid C, Lentz A, et al. Chlamydia pneumoniae infection of vascular smooth muscle and endothelial cells activates NF-kappaB and induces tissue factor and PAI-1 expression: a potential link to accelerated arteriosclerosis. Circulation 1999;100:1369-73.
25. Rodel J, Woytas M, Groh A, Schmidt KH, Hartmann M, Lehmann M et al. Production of basic fibroblast growth factor and interleukin 6 by human smooth muscle cells following infection with Chlamydia pneumoniae. Infect Immun 2000;68:3635-41.
26. Lupu F, Bergonzelli GE, Heim DA, Cousin E, Genton CY, Bachmann F, et al. Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. Arterioscler Thromb 1993;13:1090-100.
27. Healy AM, Gelehrter TD. Induction of plasminogen activator inhibitor-1 in HepG2 human hepatoma cells by mediators of the acute phase response. J Biol Chem 1994;269:19095-100.
28. Ballow CH, Amsden GW. Azithromycin: the first azalide antibiotic. Ann Pharmacother 1992;26:1253-61.
29. Peters DH, Friedel HA, McTavish D. Azithromycin. A review of its antimicrobial activity, pharmacokinetic properties and clinical efficacy. Drugs 1992;44:750-99.
30. Van Bambeke F, Tulkens PM. Macrolides: pharmacokinetics and pharmacodynamics. Int J Antimicrob Agents 2001;18(suppl 1): S17-23.
31. Ianaro A, Ialenti A, Maffia P, Sautebin L, Rombola L, Carnuccio R et al. Anti-inflammatory activity of macrolide antibiotics. J Pharmacol Exp Ther 2000;292:156-63.
32. Scaglione F, Rossoni G. Comparative anti-inflammatory effects of roxithromycin, azithromycin and clarithromycin. J Antimicrob Chemother 1998;41(suppl B):47-50.
33. Muhlestein JB, Anderson JL, Hammond EH, Zhao L, Trehan S, Schwobe EP, et al. Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. Circulation 1998;97: 633-6.

34. Muhlestein JB. Chlamydia pneumoniae–induced atherosclerosis in a rabbit model. J Infect Dis 2000;181(suppl 3):S505-7.
35. Uriarte SM, Molestina RE, Miller RD, Bernabo J, Farinati A, Iguchi K, et al. Effect of macrolide antibiotics on human endothelial cells activated by Chlamydia pneumoniae infection and tumor necrosis factor-alpha. J Infect Dis 2002;185:1631-6.
36. Culic O, Erakovic V, Cepelak I, Barisic F, Brajsa K, Ferencic Z, et al. Azithromycin modulates neutrophil function and circulating inflammatory mediators in healthy human subjects. Eur J Pharmacol 2002;450:277-89.
37. Parchure N, Zouridakis EG, Kaski JC. Effect of azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of Chlamydia pneumoniae infection. Circulation 2002;105:1298-303.
38. Northemann W, Hattori M, Baffet G, Braciak TA, Fletcher RG, Abraham LJ, et al. Production of interleukin 6 by hepatoma cells. Mol Biol Med 1990;7:273-85.
39. Lotz M, Zuraw BL, Carson DA, Jirik FR. Hepatocytes produce interleukin-6. Ann N Y Acad Sci 1989;557:509–11.
40. Zuraw BL, Lotz M. Regulation of the hepatic synthesis of C1 inhibitor by the hepatocyte-stimulating factors interleukin 6 and interferon gamma. J Biol Chem 1990;265:12664.
41. Paysant J, Blanque R, Vasse M, Soria C, Soria J, Gardner CR. Factors influencing the effect of the soluble IL-6 receptor on IL-6 responses in HepG2 hepatocytes. Cytokine 2000;12:774-9.
42. Campbell LA, Kuo CC. Chlamydial infections of the cardiovascular system. Front Biosci 2003;8:e36-43.
43. Gupta S, Leatham EW, Carrington D, Mendall MA, Kaski JC, Camm AJ. Elevated Chlamydia pneumoniae antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. Circulation 1997;96:404-7.
44. Dunne MW. Rationale and design of a secondary prevention trial of antibiotic use in patients after myocardial infarction: the WIZARD (Weekly Intervention with Zithromax [azithromycin] for Atherosclerosis and its Related Disorders) trial. J Infect Dis 2000;181(suppl 3):S572-8.
45. Jackson LA. Description and status of the Azithromycin and Coronary Events Study (ACES). J Infect Dis 2000;181(suppl 3):S579-81.
46. Cercek B, Shah PK, Noc M, Zagher D, Zeymer U, Matetsky S, et al. Effect of short-term treatment with azithromycin on recurrent ischaemic events in patients with acute coronary syndrome in the Azithromycin in Acute Coronary Syndrome (AZACS) trial: a randomised controlled trial. Lancet 2003;361:809-13.
47. Anderson JL, Muhlestein JB, Carlquist J, Allen A, Trehan S, Nielson C, et al. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for Chlamydia pneumoniae infection: The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study. Circulation 1999;99:1540-7.

48. Rothstein NM, Quinn TC, Madico G, Gaydos CA, Lowenstein CJ. Effect of azithromycin on murine arteriosclerosis exacerbated by Chlamydia pneumoniae. J Infect Dis 2001;183:232-8.
49. Gurfinkel E, Bozovich G, Beck E, Testa E, Livellara B, Mautner B. Treatment with the antibiotic roxithromycin in patients with acute non–Q-wave coronary syndromes. The final report of the ROXIS Study. Eur Heart J 1999;20:121-7.
50. Zahn R, Schneider S, Frilling B, Zeidl K, Tebbe U, Weber M, et al. Antibiotic therapy after acute myocardial infarction: a prospective randomized study. Circulation 2003;107:1253-9.
51. Wiesli P, Czerwenka W, Meniconi A, Maly FE, Hoffmann U, Vetter W, et al. Roxithromycin treatment prevents progression of peripheral arterial occlusive disease in Chlamydia pneumoniae–seropositive men: a randomized, double-blind, placebo-controlled trial. Circulation 2002;105:2646-52.
52. Grayston JT. Antibiotic treatment of atherosclerotic cardiovascular disease. Circulation 2003;107:1228-30.
53. Gieffers J, Fullgraf H, Jahn J, Flinger M, Dahlhoff K, Fatus HA, et al. Chlamydia pneumoniae infection in circulating human monocytes is refractory to antibiotic treatment. Circulation 2001;103:351-6.
54. Gelfand EV, Cannon CP. Antibiotics for secondary prevention of coronary artery disease: An ACES hypothesis but we need to PROVE IT. Am Heart J 2004;147:202-9.

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Azithromycin reduces *Chlamydia pneumoniae*-induced attenuation of eNOS and cGMP production by endothelial cells

Summary

Background

Intracellular infections with cytomegalovirus (CMV) or *Chlamydia pneumoniae (Cp)* may play a role in the etiology of atherosclerosis. Nitric oxide (NO) is a key regulator of endothelial function. Under pathological conditions uncoupling of endothelial nitric oxide synthase (eNOS) leads to vessel damage as a result of production of oxygen radicals instead of NO. We hypothesized that infection-induced atherosclerosis is initiated by changes in NO metabolism and may be reversed by azithromycin treatment.

Methods

Confluent human umbilical vein endothelial cells (HUVECs) were infected with *Cp* or CMV. After 48 h of infection, production of eNOS, cyclic guanosine monophosphate (cGMP) and reactive oxygen species (ROS) was measured. Detection of cGMP was used as a reporter assay for the bioavailability of NO. Subsequently, *Cp*- and CMV-infected HUVECs were co-incubated with 0.016 mg/Land 1 mg/L azithromycin.

Results

Infection with *Cp* (MOI 1 and MOI 0.1) and CMV (MOI 1) caused a dose- and time-dependent reduction of eNOS production in the HUVECs: *Cp* MOI 1: 1141 ± 74 pg/mL ($p< 0.01$); *Cp* MOI 0.1: 3189 ± 30 pg/mL ($p< 0.01$); CMV: 3213 ± 11 pg/mL ($p< 0.01$) vs. 3868 ± 83 pg/mL for uninfected HUVECs. *Chlamydia pneumoniae*- but not CMV-infection also reduced cGMP-production (Cp: 0.195 ± 0.030 pmol/mL ($p< 0.01$); CMV: 0.371 ± 0.027 pmol/L ($p>0.05$) vs. 0.378 ± 0.019 pmol/mL for uninfected HUVECs.
CMV-infection did not affect ROS production either, but *Cp*-infection reduced ROS-production by 21% ($p>0.05$; Cp MOI 0.1) to 68% (p 0.01; Cp MOI 1). Azithromycin treatment restored *Cp*-induced eNOS, cGMP and ROS production in a dose-dependent manner.

Conclusions

Infection with *Cp* in endothelial cells *in vitro* attenuates eNOS, cGMP and ROS production in HUVECs and azithromycin reverses *Cp*-induced effects on eNOS, cGMP and ROS-production. The results from our *in vitro* research support the role of antibiotic therapy for infection-induced atherosclerosis by indicating that azithromycin does actually improve endothelial function.

Introduction

Chronic infection may contribute to the pathogenesis of atherosclerosis. The major organisms that have been studied are *Chlamydia pneumoniae (Cp)* and cytomegalovirus (CMV)[1-3]. Chronic infection could act by a number of mechanisms including direct vascular injury and induction of a systemic inflammatory state; both leading to endothelial dysfunction[4,5]. Early evidence for involvement of *Cp* in atherogenesis came from sero-epidemiological studies[6] but also from animal models and histopathological studies[7,8]. Incidentally, even viable *Cp* was isolated from atherosclerotic vascular tissue[9,10]. Nevertheless, the relationship between *Cp* and atherosclerosis is still under debate. It now appears that some of the inconsistency of results from study to study may be owing, in part, to a lack of standardized methods. In addition to the lack of consistent serologic criteria, recent evaluations have demonstrated inherent problems with the performance of the most widely used serologic methods. Most importantly, there is no gold standard nor a reliable serologic marker for chronic or persistent *Cp* infection[11-13]. Plasma concentrations of CMV antibodies in patients with coronary disease are higher compared with control subjects[14,15] and those with carotid intimal thickening (a measure of subclinical atherosclerosis)[16]. Patients with antibodies directed against CMV more frequently have endothelial dysfunction and impaired responses to NO[17]. In addition, CMV antigens, nucleic acid sequences and DNA have been detected in smooth muscle cells of carotid artery plaques obtained from patients undergoing endarterectomy[18]. There is also an association between antibodies to CMV and elevated markers of inflammation, including C-reactive protein and interleukin-6[19,20]. Impaired endothelial function, induced by vascular risk factors such as dyslipidemia, hypertension and diabetes and by factors such as oxidative stress and inflammation, is a key process in the initiation and progression of atherosclerosis. Impaired vasoreactivity has already been documented in asymptomatic young adults with a family history of premature coronary disease but no other risk factors[21]. Nitric oxide (NO) is a multifunctional molecule with effects on vessel dilatation, inhibition of platelet adhesion/aggregation and monocyte adhesion to the vessel wall. In this way NO has anti-atherosclerotic properties[22]. Nitric oxide activates guanylate cyclase, resulting in an increase in the concentration of intracellular cGMP. Cyclic GMP is a second messenger, mediating the vasodilatating effects of NO. Under normal conditions, NO is synthesized in endothelial cells from L-arginin in a reaction catalyzed by endothelial nitric oxide synthase (eNOS). Under the influence of cardiovascular risk

factors this process can be disturbed, for instance by suppression or uncoupling of eNOS leading to vessel damage as a result of production of oxygen radicals instead of NO. Excessive formation of reactive oxygen species (ROS) leads to inflammation, endothelial barrier dysfunction and increased adhesion for leucocytes[23]. In the present study we investigated the effects of intracellular infections with CMV or *Cp* on the production of cGMP, eNOS and ROS by human endothelial cells. In addition, the effects of azithromycin on infection induced endothelial dysfunction were also studied.

Methods

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical veins by canulating the veins at both ends. Subsequently, veins were rinsed with warm phosphate-buffered saline solution. Then warm trypsin/EDTA solution (trypsin 0.05%/EDTA 0.02%, Life Technologies Inc, Paisley, UK) was injected in the vein and incubated for 30 min at 37 °C. After gently massaging the vein, the cell suspension was collected in a 50 mL centrifugation tube and 10% fetal calf serum was added for neutralization. Cells were spun for 10 min at 500g. The pellet was resuspended in endothelial growth medium, consisting of endothelial basal medium (CC-3121, Clonetics, Cambrex Corporation, East Rutherford, NJ) and an EGM Bullet kit (CC-4133, Clonetics). The obtained cell suspension was brought into a 75 cm^2 tissue culture flask (3376, Costar®, Corning Inc., Corning, NY), which was precoated with fibronectin-solution (1:200 in PBS, a generous gift from M. Mul, CLB, Amsterdam, the Netherlands). Cells were cultured in a humidified CO_2-incubator at 37 °C and 5% CO_2. At confluence the cells were detached with trypsin-EDTA solution and seeded into new tissue culture flasks (split ratio 1:2-1:3, passages 1 and 2) or 48-well tissue culture plates (passage 3) precoated with fibronectin. Cell viability was regularly checked by trypan blue staining. For all experiments cultures of passage 3 were used unless otherwise stated. Human umbilical vein endothelial cells were identified by immunostaining with antibodies against factor VIII-related antigen (anti-von Willebrand, M0616 DAKO, Glostrup, Denmark).

Preparation of Cp- and CMV-stock

Human embryonic lung (HEL) and Buffalo Green Monkey cells (BGM) were cultured at 37 °C and 5% CO_2 in Minimal Essential Medium Eagle with Earle's Salts (EMEM, Gibco) with 10% fetal calf serum (Gibco). This culture medium was supplemented with 2 mM

L-glutamine (Gibco), 5 mL non-essential amino acids (Gibco), 10 mg/Lvancomycin (Faulding Pharmaceuticals, Brussels, Belgium), 4 mg/L amphothericin B (Fungizone; Bristol-Meyers Squibb, Woerden, the Netherlands) and 10 mg/L gentamicin (Schering Plough, Maarssen, the Netherlands). The same supplements were also added to the media used for the propagation of the virus strains. A clinical isolate of CMV was propagated in HEL cells with minimal essential medium containing 2% FBS, 20 mM Hepes and supplements. At > 80% cytopathologic effect, CMV-infected HEL cells were detached with trypsin/EDTA solution (Gibco) and centrifuged. The cell pellet was resuspended in the same medium containing 2% fetal calf serum and 10% DMSO (Sigma-Aldrich, Zwijndrecht the Netherlands) and supplements. *Cp*-strain AR 139 was propagated in BGM cells at 37 °C/5% CO_2 in EMEM with 10% fetal calf serum and 0.1% cycloheximide (Sigma-Aldrich Zwijndrecht, the Netherlands) and supplements. After 72 h of growth, infected cells were frozen and thawed to release the elementary bodies. After a short centrifugation step, cell debris was discarded and 0.2M SPG-medium (2.088 g/L K_2HPO_4, 1.088 g/L KH_2PO_4, 68.46 g/L saccharose, 7.16 g/L L-glutamine, 10% FBS, 2.5 mg/L amphothericin B, 23 mg/L vancomycin and 18 mg/L gentamicin) was added (1:1 v/v). *Chlamydia pneumoniae* and CMV stock suspensions were aliquoted and stored at -80 °C until further use. Also, aliquots of inactivated CMV and *Cp* were made by overnight UV-inactivation. The 50% tissue culture infective doses ($TCID_{50}$) of live virus stocks were calculated using the method of Reed & Muench[24]. For calculation of $TCID_{50}$-titres for CMV the HEL cells were examined daily for cytopathologic effects during 1 week. The $TCID_{50}$-titre of the Cp stock was calculated otherwise, based on the number of immunofluorescent units per field in the infected BGM cells after staining with Chlamydia culture confirmation monoclonal antibodies (de Beer, Diessen, the Netherlands). Infection concentrations were chosen to mimic chronic low-grade infection (MOI 1 and 0.1; **Figure 1**).

MIC and MBC concentrations of azithromycin

Minimal inhibitory (MIC) and bactericidal (MBC) concentrations of azithromycin concentrations in *Cp*-infected HUVECs cells were calculated with minor changes according to the protocol of Suchland et al[25]. Shortly, a twofold dilution series with final concentrations of azithromycin from 4.096 mg/L to 0.008 mg/L of azithromycin in antibiotic-free medium was made and brought onto HUVEC monolayers. The cells were infected with a 100x $TCID_{50}$ dose of *Cp* and incubated for 48 h. The azithromycin concentration in which no or morphologically changed inclusions were formed was recorded as the MIC-value. For MBC assessment, all cells were freeze-thawed and passed

onto new HUVEC monolayers. After 48 h the MBC-value was read as the concentration of azithromycin in which no inclusions were formed. Accumulation of azithromycin, associated with high tissue retention and a half-life time of 50-90 h, results in tissue concentrations up to 100-fold higher than plasma levels. Mean peak plasma levels after a 500mg dose of azithromycin approximate 0.40–0.45 mg/L after 2.5 h [26]. Azithromycin concentrations in tissue usually peak between 48 and 96 h after administration. Baldwin et al. determined mean peak tissue and serum concentrations after 48 h in bronchial epithelial lining fluid (1.56 mg/L vs. 0.03 mg/L; ratio 52:1) and broncho-alveolar macrophages (23 mg/L vs. 0.02 mg/L; ratio 1150:1) [27]. In 2000, Schneider et al. described the in vivo uptake of azithromycin in human coronary plaques. The median concentration in all plaques was 0.284 mg/L (CI: 0.163-517 mg/L; n=10)[28]. Recently, Reveneau et al. described the superior bactericidal effect of azithromycin on persistent chlamydial infection in interferon gamma-treated epithelial cells, which was correlated with an enhanced uptake of the drug[29]. Azithromycin concentrations found in these studies are within the *in vitro* susceptibility range. In general, MICs and MBCs of azithromycin for *Cp* infections range from ≤ 0.03 to 1 mg/L[30-32]. Hence, in this study we chose to perform neutralization experiments with 0.016 mg/L (MIC) and 1 mg/L azithromycin doses.

Set-up of experiments

Based on the outcome of the first eNOS and cGMP experiments (Figs 2 and 3) incubation times of 48 h were chosen for all (neutralization) experiments. In previous optimization experiments the influence of the HUVEC passage number on eNOS production was only marginal, in contrast to cGMP production for which cells in passage 3 produced 50%-90% greater cGMP than in previous or higher passages, indicating an optimum production in passage 3 cells (data not shown).

Inoculation of endothelial cells

For all experiments HUVEC cells were subcultured at passage 3 in 48-well plates (Costar® Corning Inc.) until confluence was reached. All experiments were performed at least three times in duplicate on cells obtained from different donors. One day before inoculation, cells were refreshed with endothelial growth medium.

Purified azithromycin 262.5 mg (94.6% azithromycin dihydrate; a kind gift from Pfizer BV.) was predissolved in 5 mL ultra pure water in the presence of 192.3 mg sodium

citrate (pH approx. 3.0). A stock solution of 25 mg/mL azithromycin was prepared further by adding 99.15 mg NaOH and ultra pure water to a final volume of 10 mL (final pH approximately 6.3). This stock solution was stored at -20 °C until further use. For neutralization experiments, concentrations of 1 mg/L and 0.016 mg/L azithromycin were added to the endothelial growth medium. *Chlamydia pneumoniae* and CMV were prediluted in endothelial growth medium and added at a multiplicity of infectious units per cell (MOI) of 1 and 0.1 for both *Cp* and CMV. Uninfected cells and inactivated virus were used as negative controls. After inoculation, cell cultures were centrifuged for 1 h at 800 g and re-fed with the corresponding culture medium. 48 hours after infection, production of eNOS, cGMP and ROS was measured. All infections were monitored by immunostaining for either *Cp* or CMV (Fig 1b-d). Cell-free supernatants were collected and frozen at -80 °C for cytotoxicity testing. For eNOS and cGMP testing, cells were washed with PBS. For cell lysis 200 µL of lysis buffer (eNOS-assay) or 200 µL of 0.1 M HCl (cGMP-assay) was added to each well. After 30 min on an orbital shaker the cells were inspected microscopically to verify complete cell lysis. Lysates were stored at -80 °C until the assay was scheduled. ROS-detection was performed immediately.

Immunoassays

In order to obtain valid results for eNOS, cGMP and ROS production it was mandatory to assess the extent of cytotoxicity beforehand. The viability of the HUVECs cells was measured using a LDH activity assay (cytotoxicity detection kit 1644793, Roche Applied Science, Germany). For eNOS detection a quantitative solid-phase sandwich enzyme immunoassay (Quantikine Human eNOS assay, DEN00, R & D Systems Europe Ltd., Abingdon, UK) was used, and cGMP was measured in a competitive immunoassay (Correlate-EIA #900-014, Assay Design Inc., Ann Arbor, MI).

For ROS measurement HUVEC were grown in 96-microwell plates (Nunclon 167008, NUNC A/S, Roskilde, Denmark). Cells were washed once with 100 µL PBS+++ (1 mM CaCl2, 0.5 mM $MgCl_2$, 0.1% (w/v) glucose in phosphate-buffered saline). Cells were loaded with 75 µL of 10 µM dichlorodihydrofluorescein diacetate probe (CM-H2DCFDA; cat nr C6827, Molecular Probes Europe, Leiden, the Netherlands) for 30 min at 37 °C in the dark. The DCF-probe is a cell-permeant indicator for reactive oxygen species that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Cells were washed twice with 100 µL PBS+++ and 190 µL PBS+++ was added to each well. Subsequently, 10 µL 20 mM H_2O_2 (final concentration 1 mM) or 10 µL 2 mM Angiotensin II (final concentration 100 µM) was

added. Fluorescence (DCF-signal) was read every 5 min during 70 min at 485 nm excitation and 538 nm emission. ROS-values were expressed as fluorescent signals from the DCF-probe we used without an indication of units. In general, the signals would be low to such a degree that relating them to the signals of controls (Angiotensin and H_2O_2) would have led to marginal values and that any effects, if present, might remain unnoted. Alternatively, incubation with inactivated *Cp* and CMV was considered to be the most accurate (negative) infection control and fluorescence values of these incubations were fixed at 100%. Subsequently, all other fluorescence values were related to this and expressed as a percentage of these controls to give an impression of the relative ROS-production after infection with or without azithromycin treatment.

Statistical analysis

The GraphPad Instat™ software package version 2.05a (Graphpad Software Inc., San Diego, CA) was used for data analysis. After anova-analysis on variation between means, the production levels of infected HUVECs were compared with those of uninfected HUVEC cells (or cells incubated with inactivated virus) at each separate time point. Data were analyzed using Dunnett's multiple comparisons q-test (which corrects for possibly unequal variances) for differences between controls and incubations; p-values less than 0.05 indicate a significant difference between results. Analyses on linear trends were performed to warrant that the decrease of eNOS-production in time was owing to infection rather than merely a time effect. The null hypothesis assumes that there is no correlation between means and time points. Therefore, low p-values suggest a linear trend.

Results

Chlamydia pneumoniae and Cytomegalovirus infection of endothelial cells

Log $TCID_{50}$/mL-values of the virus- and Chlamydia stocks were calculated on 5.3 and 4.3 for *Cp* and CMV, respectively. At confluence the mean density of cells grown in 48-well tissue culture plates was 4×10^4 per well. One hundred µL of each dilution (*Cp*: ×10 and ×100; CMV: undiluted and ×10) was inoculated on HUVECs, thus obtaining MOI values of 1 and 0.1 for both *Cp* and CMV. In addition, HUVEC cells of each donor were identified as such, by immunostaining on von Willebrand factor (**Figure 1a**). The minimal inhibitory (MIC) and bactericidal (MBC) concentrations of azithromycin in *Cp*-infected HUVEC cells were calculated to be 0.016 mg/L and 0.125 mg/L, respectively.

Cytotoxicity testing

Human umbilical vein endothelial cell suspensions used for seeding the plates did not show any loss of viability (≥ 99% trypan blue exclusion); neither did HUVEC monolayers after visual examination by phase-contrast microscopy before infection (data not shown). In addition, infections were monitored by immunostaining. Care was taken to obtain low-grade infections with minimal cell damage (**Figure 1b-d**). Cell injury was determined by measuring LDH leakage in the supernatants. Relative cytotoxicity in uninfected cells ranged from 1.3 to 6.4%. Cytotoxicity in HUVEC cells incubated with inactive or infectious Cp and CMV was approximately 2-3-fold higher than in uninfected cells (data not shown).

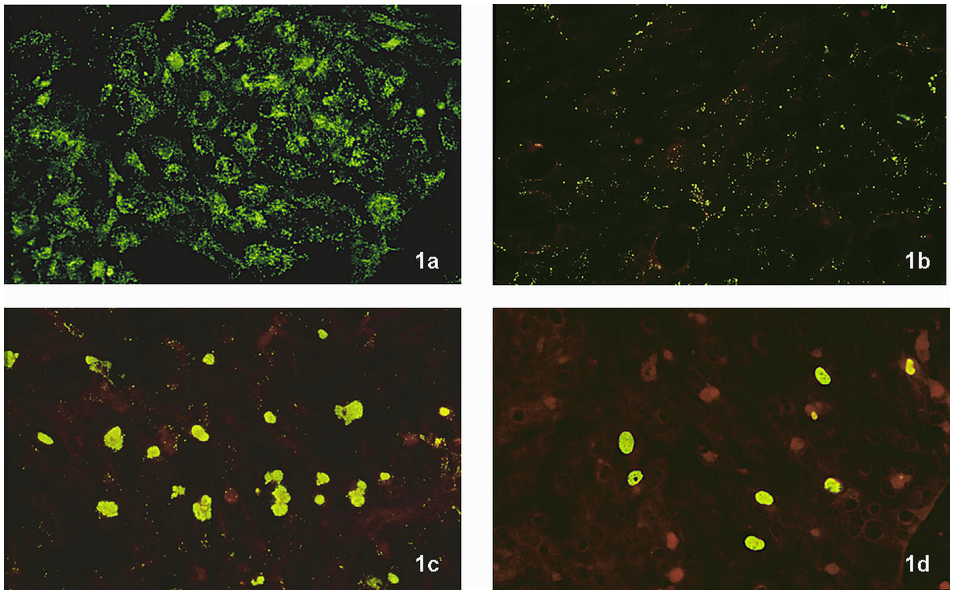

Figure 1. Fluorescence microscopy images (×200) of human umbilical vein endothelial cells (HUVECs) 48 h after inoculation. Indirect immunostaining with antihuman von Willebrand factor (a), anti-Cp (b,c) and anti-CMV (d) antibodies. Secondary antibody: FITC-labelled rabbit antimouse conjugate; counterstaining: Evans Blue. Red background: uninfected cells. Apple green fluorescence: positive identification of von Willebrand factor (a), inactivated *Cp* (b), *Cp* (MOI = 1) (c) and CMV (MOI = 1) (d). (*For color figure see page 219)*

eNOS and cGMP production after infection with Cp or CMV

In uninfected HUVECs, eNOS production gradually increased from 3156 ± 19 pg/mL to 7350 ± 621 pg/mL after 6 days, indicating a linear trend in eNOS production in time ($p<0.0001$). After 1 day eNOS production did not differ from time point zero, but as from 48 h eNOS production was significantly different from time point zero (Dunnet: $p<0.01$). Infection with the lowest CMV concentration (MOI 0.1) did not alter eNOS expression at any time point (Dunnett: all $p>0.05$) and increase of eNOS in time was still linear, also indicating that there was no effect of this infection on eNOS-production ($p<0.001$). On the other hand, incubation of 24 h or longer with *Cp* MOI 1, 0.1 and CMV MOI 1 suppressed eNOS expression significantly (1141 ± 74 pg/mL, 3189 ± 30 pg/mL and 3213 ± 11 pg/mL after 24 h, respectively; Dunnett: all $p<0.01$) when compared with uninfected HUVECs (3868 ± 83 pg/mL after 24 h). There was no longer a linear relation between eNOS and time ($p>0.05$), also indicating the suppressive effect of infection on eNOS production (**Figure 2**).

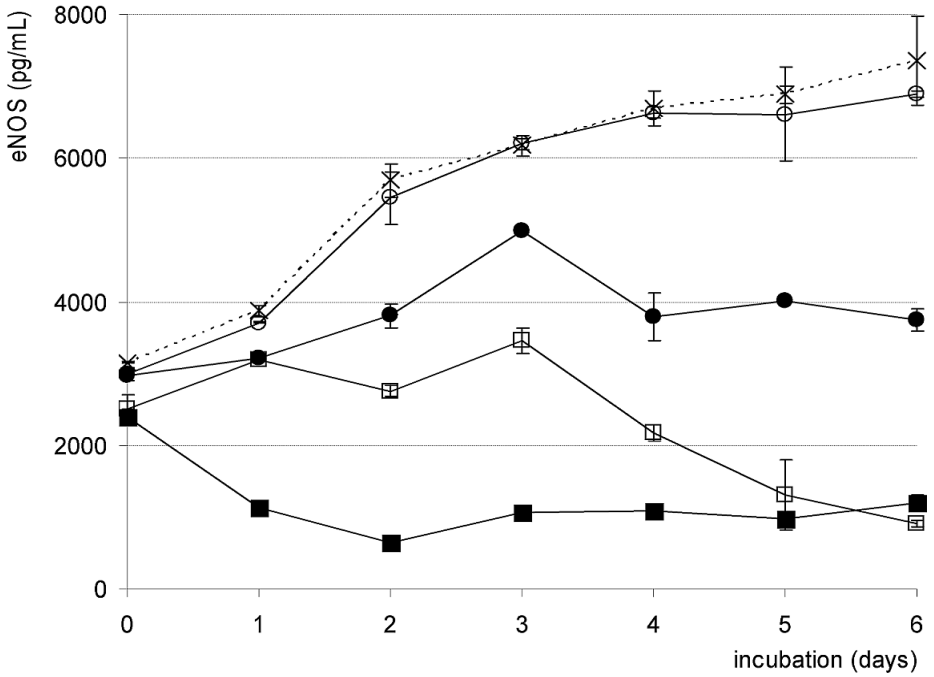

Figure 2 eNOS expression in infected human umbilical vein endothelial cells (HUVECs). Means ± standard error (SE) of eNOS expression in three experiments in duplicate (n = 3) and incubation times from 0 to 6 days. X-axis: incubation time in days; Y-axis: eNOS expression in pg/mL; -- x -- uninfected HUVECs; ■ infection with *Cp* MOI 1; □ infection with *Cp* MOI 0.1; • infection with CMV MOI 1; ○ infection with CMV MOI 0.1.

Cyclic guanosine monophosphate production in *Cp*-infected HUVECs did not differ from cGMP production in uninfected cells during the first 48 h of incubation ($p>0.05$). After 48 h, infection with both *Cp*-doses suppressed cGMP-production significantly (MOI 1: 0.195 ± 0.030 pmol/mL, $p< 0.01$; MOI 0.1: 0.216 ± 0.064 pmol mL, $p<0·05$) compared with uninfected HUVECs (0.378 ± 0.019 pmol/mL). Cytomegalovirus infection did not alter cGMP-production at all (0.371 ± 0.027 and 0.380 ± 0.023 pmol mL, $p>0.05$). After 72 h, infection with *Cp* MOI 1 still gave a significant reduction of cGMP-production (0.210 ± 0.099 vs. 0.322 ± 0014 pmol/mL for uninfected cells; $p<0.05$) but after incubation times of 96 h and longer overall cGMP-production was decreased and was only marginal, indicating abolished cGMP-synthesis (**Figure 3**). Cyclic guanosine monophosphate production-synthesis in this culture model seems self-aborting, probably owing to exhaustion of one more components in the eNOS-NO-cGMP pathway. Consequently, an incubation time of 48 h was kept for all upcoming azithromycin experiments.

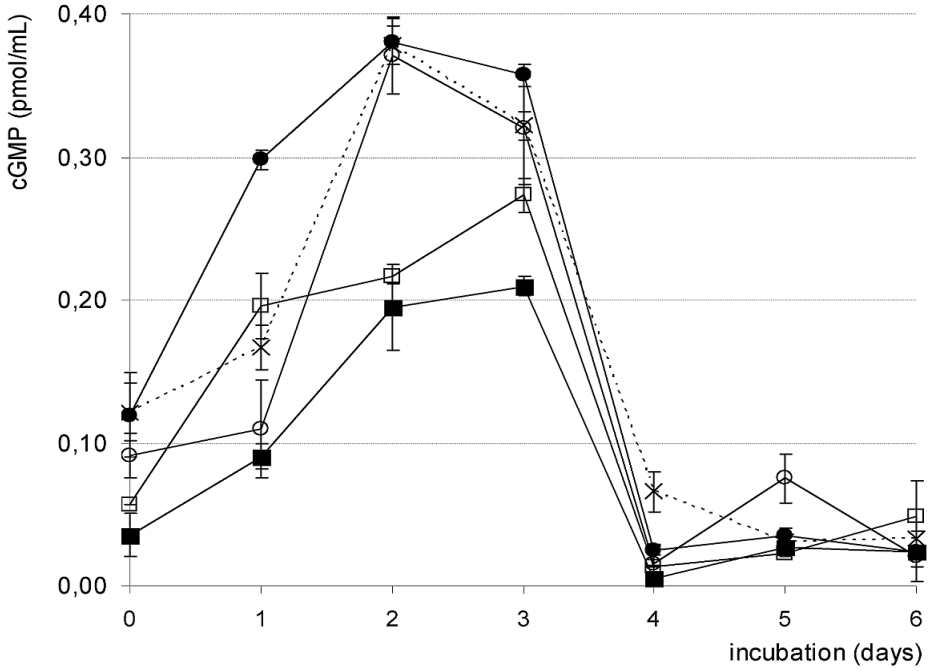

Figure 3 Cyclic guanosine monophosphate (cGMP) production of infected human umbilical vein endothelial cells (HUVECs). Means ± standard error (SE) of cGMP production in three experiments in duplicate (n = 3) and incubation times from 0 to 6 days. X-axis: incubation time in hours; Y-axis: cGMP production in pmol/mL; -- x -- uninfected HUVECs; ■ infection with *Cp* MOI 1; □ infection with *Cp* MOI 0.1; • infection with CMV MOI 1; ○ infection with CMV MOI 0.1.

Effect of azithromycin on ROS production by infected HUVECs

After 48 h of incubation with infectious and inactivated CMV (MOI 1), ROS-production in HUVEC was unchanged compared with uninfected HUVEC (DCF signals for infectious CMV: 1.128 ± 0.117 (p>0·05); inactivated CMV: 0.863 ± 0.266 (p>0.05) vs. 0.883 ± 0.129 for uninfected HUVEC.

In contrast, infectious *Cp* (MOI 1), but not inactivated *Cp*, suppressed ROS-production significantly (infectious *Cp*: 0.342 ± 0.029; p<0.05, inactivated *Cp*: 1.080 ± 0.19; p>0.05) compared with uninfected HUVECs (0.883 ± 0.129), indicating a reduction of 61% in ROS-production.

Co-incubation with azithromycin restored relative ROSproduction of *Cp*-infected HUVECs (MOI 1) compared with HUVECs with inactivated *Cp* from 31.7% without azithromycin (342 ± 29; p<0.01) to 46.2% with 0.016 mg/L azithromycin (480 ± 24; p<0.05) and 83.4% with 1 mg/L azithromycin (723 ± 54; p>0.05), indicating a dose-dependent effect of azithromycin on ROS-production. Infection with *Cp* MOI 0.1 still reduced ROS-production by 21.2% compared with inactivated *Cp*-incubation (0.852 ± 0.176 vs. 1.080 ± 0.197 for inactivated *Cp*) but this reduction was not statistically significant (p>0.05). Although not significant, azithromycin was still able to enhance ROS-production of MOI 0.1 *Cp*-infected cells from 78.8% (DCF: 0.852 ± 0.176) to 91.5% for 0.016 mg/L azithromycin (DCF: 0.951 ± 0.060) and 96.4% for 1 mg/L azithromycin (DCF: 0.836 ± 0.060), respectively (**Figure 4**).

Effect of azithromycin on eNOS-expression by infected HUVECs

After 48 h, eNOS-expression in HUVECs was 4970 ± 128 pg/mL. Incubation with either infectious or inactivated CMV did not alter eNOS-expression (4940 ± 222 pg/mL and 5010 ± 262 pg/mL resp. both p>0.05; (**Figure 5**). *Chlamydia pneumoniae*-infection, however, did suppress eNOS-expression significantly. Infection with *Cp* MOI 1 and MOI 0.1 *Cp* reduced eNOS-expression to 2649 ± 381 pg/mL (p< 0.001) and 4214 ± 87 pg/mL (p<0.05), respectively. eNOS-expression after incubation with both inactivated *Cp* stocks was not different (p>0.05) from eNOS-expression in uninfected HUVECs (4425 ± 298 pg/mL, p>0.05 for inactivated *Cp* MOI 1 and 4640 ± 489 pg/mL (p>0.05) for inactivated Cp MOI 0.1 vs. 4970 ± 128 pg/mL for uninfected HUVECs). Addition of azithromycin to the *Cp*-infected cells restored eNOS-expression. Co-incubation with 0.016 mg/L azithromycin increased eNOS-expression only moderately from 2649 ± 381 pg/mL (p<0.001) to 3018 ± pg/mL for *Cp* MOI 1 (p<0.001) and from

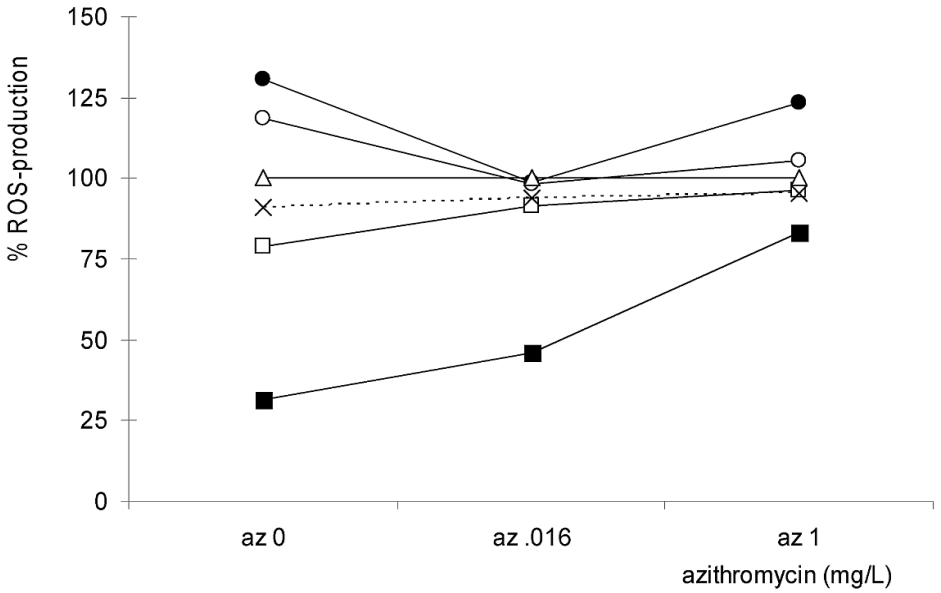

Figure 4: ROS production of infected human umbilical vein endothelial cells (HUVECs) and effect of azithromycin. Means ± standard error (SE) of ROS production in three experiments in duplicate (n = 3); incubation time 48 h. ROS = reactive oxygen species; Cp = Chlamydia pneumoniae; CMV = cytomegalovirus; MOI = multiplification of infection. X-axis: azithromycin concentration (mg/L); Y-axis: relative ROS-production in percentage of inactivated virus control; incubations with inactivated Cp and inactivated CMV were both adjusted to 100% (control). ◊ inactivated Cp/CMV; -- x -- uninfected HUVECs; ■ infection with Cp MOI 1; □ infection with Cp MOI 0.1; • infection with CMV MOI 1; ○ infection with CMV MOI 0.1.

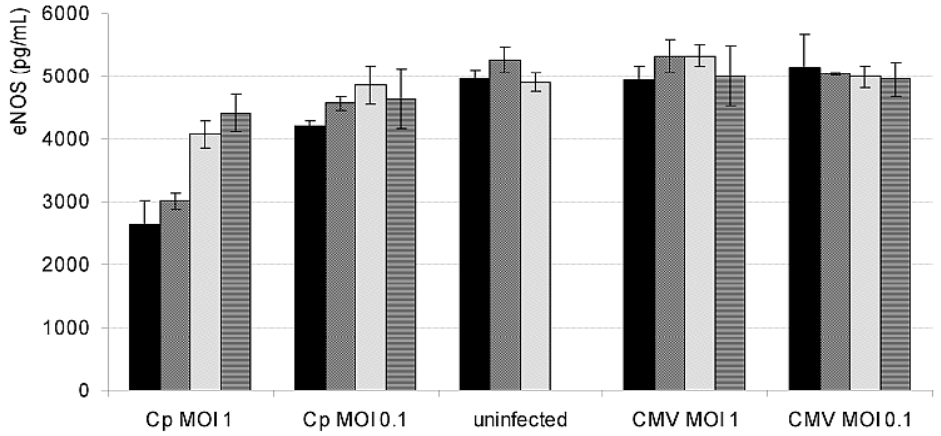

Figure 5. Effect of azithromycin on eNOS-expression in human umbilical vein endothelial cells (HUVECs). Means ± standard error (SE) of eNOS expression in three experiments in duplicate (n = 3); Incubation time 48 h; *Cp* = *Chlamydia pneumoniae*; CMV = cytomegalovirus; uninfected: uninfected HUVECs cells; MOI = multiplification of infection; Y-axis: eNOS expression in pg/mL; X-axis: incubation type; ▮ infection without azithromycin; ▒ 0·016 mg/L azithromycin; ▒ 1 mg/L azithromycin; ▤ incubation with inactivated Cp or CMV.

4214 ± 87 pg/mL (p<0.05) to 4575 ± 108 mg/L (p<0.05) for *Cp* MOI 0.1 infected HUVECs compared with azithromycin-treated uninfected HUVECs (5262 ± 197 pg/mL). On the other hand, co-incubation with 1 mg/L azithromycin almost completely restored eNOS-expression to 4084 ± 219 pg/mL (p>0.05) for *Cp* MOI and 4857 ± 294 pg/mL (p>0.05) for 0.1 MOI when compared with azithromycin-treated uninfected HUVECs (4909 ± 151 pg/mL) (**Figure 5**).

Effect of azithromycin on cGMP-production by infected HUVECs

After 48 h, cGMP-expression in uninfected HUVECs cells was 0.280 ± 0.077 pmol/mL. Addition of azithromycin did not alter cGMP-production of uninfected HUVECs. Initially, CMV-infection did not affect cGMP-production in HUVECs (0.241 ± 0.089 pmol/mL; p>0.05). Therefore, addition of azithromycin to CMV-infected HUVECs did not induce any changes in cGMP-production either. *Chlamydia pneumoniae*-infection however, did suppress cGMP-production (0.096 ± 0.037 pmol mL). Co-incubation of azithromycin (0.016 and 1 mg/L) with the *Cp*-infected HUVEC cells blunted cGMP-suppression (0.164 ± 0.038 pmol/mL and 0.249 ± 0.049 pmol/mL for co-incubation with 0.016 mg/L and 1 mg/L azithromycin, respectively). Although these results were not statistically significant, there was an upward trend in cGMP-production after co-incubation with azithromycin (**Figure 6**).

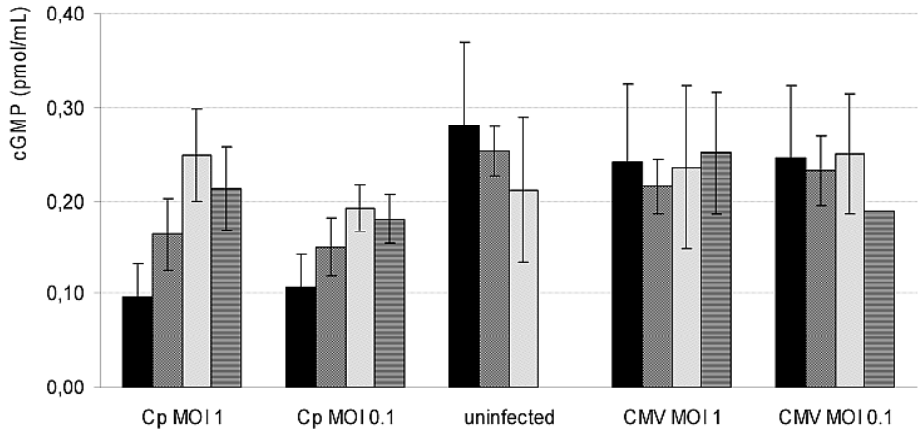

Figure 6. Effect of azithromycin on Cyclic guanosine monophosphate (cGMP)-production in human umbilical vein endothelial cells (HUVECs). Means ± standard error (SE) of cGMP production in three experiments in duplicate (n = 3); incubation time 48 h; *Cp* = *Chlamydia pneumoniae*; CMV = cytomegalovirus; uninfected: uninfected HUVECs cells; MOI = multiplification of infection;
Y-axis: cGMP production in pmol/mL; X-axis: incubation type; infection without azithromycin; 0·016 mg/L azithromycin; 1 mg/L azithromycin; incubation with inactivated Cp or CMV.

Discussion

Endothelial dysfunction plays a pivotal role in atherogenesis and development of vascular complications. Classical risk factors contribute to endothelial function, but also non classical risk factors like oxidation products and inflammatory mediators may lead to endothelial dysfunction. Also, several studies indicate a role for infections with *Cp* and CMV in the pathogenesis of atherosclerosis[33-35]. Infection of the endothelium causes continuous activation and loss of anti-atherogenic cellular mechanisms like a decrease in NO-bioavailability[36,37]. In the present study we investigated the effects of intracellular endothelial infection with CMV and *Cp* on NO-metabolism. Endothelial nitric oxide synthase, cGMP and ROS production were attenuated in *Cp*-infected HUVECs. CMV-infection did not induce any changes in cGMP- or ROS-production and only marginally decreased eNOS-production. Subsequent co-incubations with azithromycin restored eNOS, cGMP and ROS-production of *Cp*-infected HUVECs to levels comparable with those in uninfected HUVECs.

Inflammation is considered to be a major underlying mechanism contributing to endothelial dysfunction. Uncoupling of eNOS and subsequent vascular production of ROS may be the result of inflammatory mediators[38]. Reports about the relationship between CMV and *Cp* with atherosclerosis and endothelial dysfunction are quite inconsistent. Several studies show an association between *Cp* and CMV and endothelial dysfunction[39,40] and other studies do not[41-43]. In our study, CMV-infection of endothelial cells did not influence eNOS, cGMP or ROS-production, suggesting that CMV does not induce direct effects on vascular function by influencing eNOS-dependent NO-production as determined by cGMP levels. We did not demonstrate involvement of CMV in decreased NO-production; this may indicate that involvement in atherogenesis is mediated by other mechanisms. Infection with CMV may lead to atherosclerosis by other mechanisms, including production of inflammatory cytokines in the liver, expression of endothelial adhesion molecules and production of tissue factor on endothelial cells[44-46]. Other investigators suggested that CMV elicits a general subclinical inflammatory response in certain individuals who are therefore susceptible to the development of atherosclerosis; those without an inflammatory response are expected to be resistant[47].

In this study *Cp*-infection attenuated both eNOS- and cGMP-production in HUVECs significantly, indicating decreased NO-bioavailability. Inactivated *Cp* did not alter eNOS or cGMP-expression, suggesting that active infection is involved in reduced eNOS

production. Uncoupling of eNOS should lead to elevated production of ROS instead of NO[48]. Surprisingly enough, ROS-production by Cp-infected HUVECs was also reduced but this was not caused by cytotoxicity owing to infection. These findings are in contradiction with most studies in which *Cp*-infection generates ROS and subsequent activation of the NF-kappa B pathway, resulting in synthesis of pro-inflammatory cytokines and procoagulant activity. Our group previously demonstrated that co-incubation of Cp-infected HUVECs with radical scavengers elevated IL-6 production, indicating NF-kappa B activation[49]. In the present infection-experiments eNOS may have become degraded, inactivated or uncoupled. Subsequently, in the tissue culture environment the dual action of eNOS may have resulted in production of NO as well as superoxide. The superoxide radical is frequently involved in oxidative inactivation of NO. Likely, enhanced degradation of NO occurs by reaction of NO with superoxide. This reaction kinetically exceeds other NO-reactions and leads to formation of peroxynitrite[50]. Nevertheless, the used DCF-probe should be sensitive to peroxynitrite as well as other radical oxygen species (e.g. superoxide, peroxynitrite, hydroxyl radical and hydrogen peroxide). However, in the process of degrading NO, ROS themselves might have been consumed. Addition of radical scavengers might reveal which radicals are involved or not, but seems unrealistic if ROS levels appear to be reduced. In addition, development of *Cp*-infection inside the cytoplasm of cultured HUVEC cells may have down-regulated cell metabolism, possibly leading to unstable eNOS and diminished NO and ROS-synthesis.

The suppressive effect of *Cp*-infection on eNOS-expression in human endothelial cells and loss of functionality leads to decreased NO-bioavailability and may trigger atherogenic processes. The results of the present *in vitro* study support the feasibility of an infectious etiology in atherogenesis and confirm a link between *Cp*-infection and endothelial dysfunction. In vivo this hypothesis is supported by clinical studies on the relation between inflammation and vascular function in patients with documented (coronary) artery disease. Impaired vasodilator function as measured by plethysmography demonstrated CRP to be an independent predictor for endothelial function [51,52]. Similar studies have shown that macrolide therapy effectively improved flow-mediated vasodilatation in *Cp*-seropositive patients[53,54].

Endothelial nitric oxide synthase expression was measured in a protein assay, which does not provide any information about the functionality of the eNOS enzyme. Actual detection of NO provides information about eNOS activity. However, detection of NO *in vitro* is cumbersome. The NO molecule itself is a very reactive gas with short half-

life time and is present in endothelial cells only at a nanomolar level lying far beyond detection levels of current (commercial) detection methods. We acknowledge this as one of the potential pitfalls in our study. Alternative methods for detection of NO may be detection of degradation products like nitrate or nitrite; by-products like citruline or second messenger products like cGMP. Furthermore, the relationship between local NO concentration and the guanylate cyclase activity might be more complex than a straightforward 1:1 relation[55]. Moreover, cGMP formation might be influenced by other stimuli than NO. In the experimental setup it appeared that cell culture itself was of great influence, particularly on cGMP expression. Apart from individual variation in cell donors we found that cGMP expression was optimal in HUVEC cells of passage 3; in lower and higher passages cGMP levels were at least 50% lower (data not shown). Pooling of results obtained from cells in different passages would subdue cGMP measurement. As **Figure 3** shows, cGMP production was completely abolished after 3 days. A plausible explanation for this phenomenon may be exhaustion of cofactors or substrate (L-arginin), required for NO-synthesis in the tissue culture environment, leading to uncoupling of eNOS. Preventing this from happening, all incubation times for the azithromycin experiments were set at 48 h. Finally, the low dynamic range of the cGMP-assay should be mentioned. With maximal absorbance values of 0.500 for this assay, small variations in culture and detection procedures may yield undesirable uncertainty in calculated cGMP levels.

At present, there are several therapeutic options for increasing the bioavailability of NO, aiming to improve endothelial function. Drugs like statins, ACE inhibitors and antioxidants seem to have beneficial effects on endothelial dysfunction. Sildenafil (Viagra®) therapy has been shown to be a clinically effective treatment for erectile dysfunction. With respect to the possible role of infectious pathogens in atherosclerosis it has been suggested that antimicrobial therapy might be beneficial for vascular function and therefore protective for the development of atherosclerotic complications. Application and effects of quinolone- and neomacrolide antibiotics like roxithromycin and azithromycin on inflammation have been evaluated in prospective clinical trials[56-59]. Until today results from studies upon antibiotics, that might be beneficial in preventing CHD, remain inconclusive. As for azithromycin, some even tend to indicate that this drug is ineffective for secondary coronary heart disease prevention. Results from the WIZARD and ACES & PROVE IT trials showed no benefit of long-term antibiotic use after myocardial infarction[60,61]. In previous research other reservations were made about the effectiveness of antibiotic therapy; Gieffers et al.

postulated that intracellular *Cp* in circulating monocytes may be refractory to antibiotic therapy because of the inability of antibiotics to eliminate *Cp* from the circulation[62]. In addition, nonviable *Cp* present in atherosclerotic lesions may still be potentially immunogenic, thus maintaining an inflammatory status[63]. Still, antimicrobial drugs may contribute to improvement of endothelial dysfunction[64,65] although it remains debatable whether any observed benefits of antibiotic drugs should be contributed to antimicrobial or anti-inflammatory effects. In a previous study we already demonstrated that azithromycin suppressed inflammatory responses to infection of hepatocytes although the observed benefits should be contributed to its antimicrobial effects rather than anti-inflammatory properties[66]. In addition, the results from our present research support the role of antibiotic therapy for infection-induced atherosclerosis by indicating that azithromycin actually does improve endothelial function.

In conclusion we demonstrated that infection with *Cp* in endothelial cells *in vitro* attenuates eNOS, cGMP and ROS production in HUVECs and that azithromycin reversed *Cp*-induced effects on eNOS, cGMP and ROS-production.

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References

1. Blum A, Peleg A, Weinberg M. Anti-cytomegalovirus (CMV) IgG antibody titer in patients with risk factors to atherosclerosis. Clin Exp Med 2003; 3(3):157-60.
2. Campbell LA, Kuo CC. Chlamydia pneumoniae and atherosclerosis. Semin Respir Infect 2003;18(1):48-54.
3. Kalayoglu MV, Libby P, Byrne GI. Chlamydia pneumoniae as an emerging risk factor in cardiovascular disease. JAMA 2002; 288 (21): 2724-31.
4. Muhlestein JB, Anderson JL. Chronic infection and coronary artery disease. Cardio Clin 2003; 21(3): 333-62.
5. Becker AE, de Boer OJ, van der Wal AC. The role of inflammation and infection in coronary artery disease. Ann Rev Med 2001; 52: 289-97.
6. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH et al. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 1988; 2(8618):983-6.

7. Kuo CC, Shor A, Campbell LA, Fukushi H, Patton DL, Grayston JT. Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. J Infect Dis 1993;4:841-9.
8. Muhlestein JB, Hammond EH, Carlquist JF, Radicke E, Thomson MJ, Karagounis LA et al. Increased incidence of Chlamydia species within the coronary arteries of patients with symptomatic atherosclerotic versus other forms of cardiovascular disease. J Am Coll Cardiol 1996;7:1555-61.
9. Jackson LA, Campbell LA, Kuo CC, Rodriguez DI, Lee A, Grayston JT. Isolation of Chlamydia pneumoniae from a carotid endarterectomy specimen. J Infec Dis 1997;76(1):292-5.
10. Ramirez JA. Isolation of Chlamydia pneumoniae from the coronary artery of a patient with coronary atherosclerosis. The Chlamydia pneumoniae/Atherosclerosis Study Group. Ann Intern Med 1996;125(12):979-82.
11. Boman J, Hammerschlag MR. Chlamydia pneumoniae and atherosclerosis: critical assessment of diagnostic methods and relevance to treatment studies. Clin Microbiol Rev 2002;15(1) 1-20.
12. Dowell SF, Peeling RW, Boman J, Carlone GM, Fields BS, Guarner J et al. Standardizing Chlamydia pneumoniae assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). Clin Infect Dis 2001;33(4):492-503.
13. Ieven MM, Hoymans VY. Involvement of Chlamydia pneumoniae in atherosclerosis: more evidence for lack of evidence. J Clin Microbiol 2005; 43(1):19-24.
14. Adam E, Melnick JL, Probtsfield JL, Petrie BL, Burek J, Bailey KR et al. High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. Lancet 1987;2(8554):291-3.
15. Blum A, Giladi M, Weinberg M, Kaplan G, Pasternack H, Laniado S et al. High anti-cytomegalovirus (CMV) IgG antibody titer is associated with coronary artery disease and may predict post-coronary balloon angioplasty restenosis. Am J Cardiol 1998; 81(7):866-8.
16. Nieto FJ, Adam E, Sorlie P, Farzadegan H, Melnick JL, Comstock GW et al. Cohort study of cytomegalovirus infection as a risk factor for carotid intimal-medial thickening, a measure of subclinical atherosclerosis. Circulation 1996;94(5):922-7.
17. Grahame-Clarke C, Chan NN, Andrew D, Ridgway GL, Betteridge DJ, Emery V et al. Human cytomegalovirus seropositivity is associated with impaired vascular function. Circulation 2003;108(6):678-83.
18. Melnick JL, Hu C, Burek J, Adam E, DeBakey ME. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. J Med Virol 1994;42(2):170-4.
19. Muhlestein JB, Horne BD, Carlquist JF, Madsen TE, Bair TL, Pearson RR et al. Cytomegalovirus seropositivity and C-reactive protein have independent and combined predictive value for mortality in patients with angiographically demonstrated coronary artery disease. Circulation 2000;102(16):1917-23.
20. Blankenberg S, Rupprecht HJ, Bickel C, Espinola-Klein C, Rippin G, Hafner G et al. Cytomegalovirus infection with interleukin-6 response predicts cardiac mortality in patients with coronary artery disease. Circulation 2001;103(24):2915-21.

21. Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM, Deanfield JE. Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. Circulation 1997;96(10):3378-83.
22. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329(27):2002-12.
23. Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. Am J Physiol Cell Physiol 2001;280(4):C719-41.
24. Reed LJ, Muench MJ. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493.
25. Suchland RJ, Geisler WM, Stamm WE. Methodologies and cell lines used for antimicrobial susceptibility testing of Chlamydia spp. Antimicrob Agents Chemother 2003;47(2):636-42.
26. Foulds G, Shepard RM, Johnson RB. The pharmacokinetics of azithromycin in human serum and tissues. J Antimicrob Chemother 1990;25(Suppl A):73-82.
27. Baldwin DR, Wise R, Andrews JM, Ashby JP, Honeybourne D. Azithromycin concentrations at the sites of pulmonary infection. Eur Respir J 1990;3(8):886-90.
28. Schneider CA, Diedrichs H, Riedel KD, Zimmermann T, Hopp HW. In vivo uptake of azithromycin in human coronary plaques. Am J Cardiol 2000;86(7):789-91.
29. Reveneau N, Crane DD, Fischer E, Caldwell HD. Bactericidal activity of first-choice antibiotics against gamma interferon-induced persistent infection of human epithelial cells by Chlamydia trachomatis. Antimicrob Agents Chemother 2005;49(5):1787-93.
30. Chirgwin K, Roblin PM, Hammerschlag MR. In vitro susceptibilities of Chlamydia pneumoniae (Chlamydia sp. strain TWAR). Antimicrob Agents Chemother 1989; 33(9):1634-5.
31. Agacfidan A, Moncada J, Schachter J. In vitro activity of azithromycin (CP-62,993) against Chlamydia trachomatis and Chlamydia pneumoniae. Antimicrob Agents Chemother 1993;37(9):1746-8.
32. Donati M, Rodriguez FM, Olmo A, D'Apote L, Cevenini R. Comparative in-vitro activity of moxifloxacin, minocycline and azithromycin against Chlamydia spp. J Antimicrob Chemother 1999;43(6):825-7.
33. High KP. Atherosclerosis and infection due to Chlamydia pneumoniae or cytomegalovirus: weighing the evidence. Clin Infect Dis 1999;28(4):746-9.
34. Spence JD, Norris J. Infection, inflammation, and atherosclerosis. Stroke 2003;34(2):333-4.
35. Leinonen M, Saikku P. Evidence for infectious agents in cardiovascular disease and atherosclerosis. Lancet Infect Dis 2002;2(1): 11-7.
36. Hippenstiel S, Suttorp N. Interaction of pathogens with the endothelium. Thromb Haemost 2003;89(1):18-24.
37. Keller TT, Mairuhu AT, de Kruif MD, Klein SK, Gerdes VE, ten Cate XX et al. Infections and endothelial cells. Cardiovasc Res 2003; 60(1):40-8.
38. Cardaropoli S, Silvagno F, Morra E, Pescarmona GP, Todros T. Infectious and inflammatory stimuli decrease endothelial nitric oxide synthase activity in vitro. J Hypertens 2003;2 (11) 2103-10.

39. Nerheim PL, Meier JL, Vasef MA, Li WG, Hu L, Rice JB et al. Enhanced cytomegalovirus infection in atherosclerotic human blood vessels. Am J Pathol 2004;164(2):589-600.
40. Prasad A, Zhu J, Halcox JP, Waclawiw MA, Epstein SE, Quyyumi AA. Predisposition to atherosclerosis by infections: role of endothelial dysfunction. Circulation 2002;010(2):184-90.
41. Caligiuri G, Rottenberg M, Nicoletti A, Wigzell H, Hansson GK. Chlamydia pneumoniae infection does not induce or modify atherosclerosis in mice. Circulation 2001;103(23):2834-8.
42. Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. Circulation 1999;100(4):e20-8.
43. Khairy P, Rinfret S, Tardif JC, Marchand R, Shapiro S, Brophy J et al. Absence of association between infectious agents and endothelial function in healthy young men. Circulation 2003;107(15):1966-71.
44. Visseren FL, Verkerk MS, Bouter KP, Diepersloot RJ, Erkelens DW. Interleukin-6 production by endothelial cells after infection with influenza virus and cytomegalovirus. J Lab Clin Med 1999;134(6): 623-30.
45. Bouwman JJ, Visseren FL, Bosch MC, Bouter KP, Diepersloot RJ. Procoagulant and inflammatory response of virus-infected monocytes. Eur J Clin Invest 2002;32(10):759-66.
46. Knight DA, Waldman WJ, Sedmak DD. Cytomegalovirus-mediated modulation of adhesion molecule expression by human arterial and microvascular endothelial cells. Transplantation 1999;68(11):1814–8.
47. Zhu J, Quyyumi AA, Norman JE, Csako G, Epstein SE. Cytomegalovirus in the pathogenesis of atherosclerosis: the role of inflammation as reflected by elevated C-reactive protein levels. J Am Coll Cardiol 1999;34(6):1738-43.
48. Kawashima S. The two faces of endothelial nitric oxide synthase in the pathophysiology of atherosclerosis. Endothelium 2004;11 (2):99-107.
49. Visseren FL, Verkerk MS, van der Bruggen T, Marx JJ, van Asbeck BS, Diepersloot RJ. Iron chelation and hydroxyl radical scavenging reduce the inflammatory response of endothelial cells after infection with Chlamydia pneumoniae or influenza A. Eur J Clin Invest 2002;32(Suppl 1):84-90.
50. Thomas SR, Chen K, Keaney JF Jr. Oxidative stress and endothelial nitric oxide bioactivity. Antioxid Redox Signal 2003; 5(2):18-94.
51. Fichtlscherer S, Zeiher AM. Endothelial dysfunction in acute coronary syndromes: association with elevated C-reactive protein levels. Ann Med 2000;32(8):515-8.
52. Fichtlscherer S, Breuer S, Schachinger V, Dimmeler S, Zeiher AM. C-reactive protein levels determine systemic nitric oxide bioavailability in patients with coronary artery disease. Eur Heart J 2004;25(16):1412-8.
53. Parchure N, Zouridakis EG, Kaski JC. Effect of azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of Chlamydia pneumoniae infection. Circulation 2002;105(11): 1298-303.

54. Wiesli P, Czerwenka W, Meniconi A, Maly FE, Hoffmann U, Vetter W et al. Roxithromycin treatment prevents progression of peripheral arterial occlusive disease in Chlamydia pneumoniae seropositive men: a randomized, double-blind, placebo-controlled trial. Circulation 2002;105(22):2646-52.
55. Ballou DP, Zhao Y, Brandish PE, Marletta MA. Revisiting the kinetics of nitric oxide (NO) binding to soluble guanylate cyclase: the simple NO-binding model is incorrect. Proc Natl Acad Sci USA 2002;99(19):12097–101.
56. Muhlestein JB. Antibiotic treatment of atherosclerosis. Curr Opin Lipido 2003;14(6):605-14.
57. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Reduced progression of early carotid atherosclerosis after antibiotic treatment and Chlamydia pneumoniae seropositivity. Circulation 2002;106(19):2428-33.
58. Wells BJ, Mainous AGI, Dickerson LM. Antibiotics for the secondary prevention of ischemic heart disease: a meta-analysis of randomized controlled trials. Arch Intern Med 2004;164(19):2156-61.
59. Grayston JT. Antibiotic treatment of atherosclerotic cardiovascular disease. Circulation 2003;107(9):1228-30.
60. Cleland JG, Huan LP, Freemantle N, Clark AL, Coletta AP. Clinical trials update from the European Society of Cardiology: SENIORS, ACES, PROVE-IT, ACTION, and the HF-ACTION trial. Eur J Heart Fail 2004;6(6):787-91.
61. O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S et al. Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. JAMA 2003;290(11):1459-66.
62. Gieffers J, Fullgraf H, Jahn J, Klinger M, Dalhoff K, Katus HA et al. Chlamydia pneumoniae infection in circulating human monocytes is refractory to antibiotic treatment. Circulation 2001;103(3):351-6.
63. Hoymans VY, Bosmans JM, Ieven M, Vrints CJ. Chlamydia pneumoniae and atherosclerosis. Acta Chir Belg 2002;102(5):317-22.
64. Kuvin JT, Gokce N, Holbrook M, Hunter LM, Patel AR, Sliney KA et al. Effect of short-term antibiotic treatment on Chlamydia pneumoniae and peripheral endothelial function. Am J Cardiol 2003;91(6):732-5.
65. SoRelle R. Treatment with azithromycin improves endothelial function. Circulation 2002;105(11):e9083-4.
66. Bouwman JJ, Visseren FL, Bouter PK, Diepersloot RJ. Azithromycin inhibits interleukin-6 but not fibrinogen production in hepatocytes infected with cytomegalovirus and Chlamydia pneumoniae. J Lab Clin Med 2004;144(1):18-26

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6

Iron enhances endothelial cell activation in response to Cytomegalovirus or *Chlamydia pneumoniae*-infection

Summary

Background

Chronic inflammation has been implemented in the pathogenesis of inflammatory diseases like atherosclerosis. Several pathogens like *Chlamydia pneumoniae (Cp)* and cytomegalovirus (CMV) result in inflammation and thereby are potentially atherogenic. Those infections could trigger endothelial activation, the starting point of the atherogenic inflammatory cascade. Considering the role of iron in a wide range of infection processes, the presence of iron may complicate infection-mediated endothelial activation.

Materials and methods

Endothelial intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial selectin (E-selectin) expression were measured using flow cytometry, as an indication of endothelial activation. Cytotoxicity was monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Immunostaining was applied to measure *Cp* and CMV infectivity to endothelial cells.

Results

An increased number of infected endothelial cells in a monolayer population leads to a raised expression of adhesion molecules of the whole cell population, suggesting paracrine interactions. Iron additively up-regulated *Cp*-induced VCAM-1 expression, whereas synergistically potentiated *Cp*-induced ICAM-1 expression. Together with CMV, iron also enhanced ICAM-1 and VCAM-1 expression. These iron effects were observed without modulation of the initial infectivity of both microorganisms. Moreover, the effects of iron could be reversed by intracellular iron chelation or radical scavenging, conforming modulating effects of iron on endothelial activation after infections.

Conclusions

Endothelial response towards chronic infections depends on intracellular iron levels. Iron status in populations positive for *Cp* or CMV infections should be considered as a potential determinant for the development of atherosclerosis.

Keywords: Adhesion molecules, atherosclerosis, infection.

Introduction

Chronic inflammation plays a crucial role in coronary artery disease (CAD) and other manifestations of atherosclerosis[1]. The pathogenic inflammatory event is characterized by over-recruitment of leukocytes to the sites of inflammation. This event is mediated by activation of vascular endothelial cells. Endothelial cell activation, the key mechanism of atherosclerotic inflammation, is characterized by up-regulation of adhesion molecule expression, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and endothelial selectin (E-selectin)[2]. These adhesion molecules have been found in human atherosclerotic lesions[3–6]. *Chlamydia pneumoniae* (Cp), the gram-negative obligate intracellular bacterium, is capable of infecting endothelial cells[7–10] as well as inducing the expression of adhesion molecules on these cells[11,12]. *Cp*-infection also leads to increased soluble adhesion molecules in human[13]. Differential induction of adhesion molecule expression by cytomegalovirus (CMV) has also been demonstrated[14–18]. The up-regulation of adhesion molecules by *Cp* and CMV infections suggests a mechanism whereby infections could induce arterial disease. In addition, CMV and at a lesser extent *Cp* show high infection prevalence in the community[19,20]. *Cp* has been established as a respiratory pathogen and contributed 10–20% of community-acquired pneumonia[19], whereas the herpes virus CMV is associated with persistent, latent and recurrent infections due to reactivation of latent virus, with a prevalence of 50–90% in adults[20].

Coronary artery disease (CAD) risk has been linked to certain persistent microorganism infections, like *Chlamydia pneumoniae (Cp)* and cytomegalovirus (CMV). Lack of correlations between *Cp*-serology and atherosclerotic lesion, however, has been observed in several studies[21]. Moreover, the evidence for the role of CMV in atherogenesis is conflicting[22]. Further studies are therefore warranted especially to unravel the pathological mechanisms of infections in arterial disease. Recently, we and other researchers have shown that iron status influences the endothelial activation state[23–35].

There are many abnormal conditions that may cause increased body iron stores, and the formation of low molecular weight labile forms of iron that are capable of freely entering cells with no feedback-regulated process[36]. These conditions include hereditary haemochromatosis and secondary iron overload like in thalassaemia with frequent blood transfusions[37]. The labile forms of iron may play a role in the development of atherosclerotic vascular disease[38,39]. Coincidently, iron has been found accumulating

in human atheroma[40]. Several other studies show reduced formation of early atherosclerotic lesions by means of iron chelation or iron-deficient diets in experimental animals[41-44]. In this study, we investigated the effects of iron-rich and iron-withholding conditions during *Cp*- or CMV-infection on endothelial cells, in particular on the expression levels of VCAM-1, ICAM-1 and E-selectin. The possible involvement of iron-catalyzed oxygen-derived radical formation was also investigated. In light of the high frequencies of *Cp*- and CMV- infections in the population, together with the tendency of having increased body iron stores in conditions like hereditary haemochromatosis and secondary iron overload, this study provides important new insights and advances to the knowledge of the pathological mechanism of infections in atherosclerotic artery disease.

Methods

Baseline iron level

The iron content of the endothelial growth medium-2 (EGM-2, Clonetics, Wallkersville, MD, USA) was measured by Vitros® 950 Chemistry System (Ortho-Clinical Diagnostics, Tilburg, the Netherlands) to monitor the baseline iron level in all of the experiments involving human umbilical cord endothelial cells (HUVECs). To avoid any external iron contamination, plastic materials having an affinity for iron lower than glass were used in all experiments.

HUVEC isolation and culture

HUVECs were isolated and cultured as described by Jaffe *et al*.[45]. To minimize donor-to-donor variability, HUVECs were pooled from three to four donors for each experiment. Experiments were performed at least three times on cells from sets of different donors. Moreover, the cells were always used and maintained at a cobblestone confluent density for all conducted experiments.

Propagation of microorganisms

Human embryonic lung (HEL) and buffalo green monkey (BGM) cells were cultured at 37 °C and 5% CO_2 in minimal essential medium Eagle with Earle's salts (EMEM, Gibco, Breda, the Netherlands) containing 10% Fetal Bovine Serum (FBS, Gibco). This culture medium was supplemented with 2 mmol/L L-glutamine (Gibco), 5 mL non-essential amino acids (Gibco), 10 mg/L vancomycin (Faulding Pharmaceuticals, Brussels,

Belgium), 4 mg/L amphotericin B (Fungizone; Bristol-Meyers Squibb, Woerden, the Netherlands) and 10 mg/L gentamycin (Schering Plough, Maarssen, the Netherlands). The same supplements were also added to the media used for the propagation of the virus strains. A clinical isolate of CMV was propagated in HEL cells with EMEM containing 2% FBS, 20 mmol/L Hepes and supplements. At > 80% cytopathologic effect, CMV-infected HEL cells were detached with trypsin/EDTA solution (Gibco) and centrifuged. The cell pellet was re-suspended in the same medium containing 2% FBS, 10% dimethyl sulphoxide (DMSO, Sigma-Aldrich, Zwijndrecht, the Netherlands) and supplements.

Cp-strain AR39 was propagated in BGM cells at 37 °C, 5% CO_2 in EMEM containing 10% FBS and 0.1% cycloheximide (Sigma-Aldrich) and supplements. After 72 h of growth, infected cells were frozen and thawed to release the elementary bodies. After a short centrifugation step, cell debris was discarded and 0.2 mol/L sucrose phosphate-glutamic acid (SPG) medium (2.088 g/L K2HPO4, 1.088 g/L KH2PO4, 68.46 g/L saccharose, 7.16 g/L L-glutamine, 10% FBS, 2.5 mg/L amphotericin B, 23 mg/L vancomycin and 18 mg/L gentamycin) was added (1:1 v/v). Both *Cp* and CMV stock suspensions were aliquoted and stored at –80 °C until further use. The tissue culture infective dose ($TCID_{50}$) of CMV was determined by daily examination of the infected HEL cells for cytopathologic effects during 1 week, whereas the $TCID_{50}$ of the *Cp* stock was calculated based on the number of immunofluorescent units per field in the infected BGM cells after staining with chlamydia culture confirmation monoclonal antibodies (de Beer, Diessen, the Netherlands)[10].

Inoculation and immunostaining of endothelial cells

Cp and CMV were pre-diluted in endothelial EGM-2 medium and added at a multiplicity of infection (MOI) of 0.1 for both *Cp* and CMV. Uninfected cells and filtrate of microorganisms through a 100 kDa Microcon filter (Millipore, Bedford, MA, USA) were used as negative controls. 2 days after *Cp*- inoculation or 4 days after CMV inoculation, cells were harvested for flow cytometry. The infectivity of *Cp* and CMV to HUVECs was verified by immunostaining the infected cells with antibodies to *Cp* (30701 pathfinder chlamydia culture confirmation system, Bio-Rad, Redmond, WA, USA) and CMV (anti-CMV immediate early antigen clone E13, no. 12-003, Argene, Varilhes, France).

Confocal scanning laser microscopy

For visualization purpose, infected immunostained cells were fixed in phosphate buffered saline (PBS) containing 3% paraformaldehyde (Polysciences, Warrington, PA, USA) and 0.02% glutaraldehyde (Merck, Darmstadt, Germany) and visualized using a Leica TCS-SP2 confocal scanning laser microscope and Leica confocal software (Leica Microsystems, GmbH, Heidelberg, Germany).

Preparation of iron, iron chelators and radical scavengers

A 10 mmol/L Fe(III)citrate (1:6 iron-citrate molar ratio; Sigma-Aldrich) solution was made by dissolving the iron crystals in distilled water at 56 °C for 30 min. Iron solutions were always freshly prepared and filter-sterilized prior to use. The iron chelators were prepared as stocks in PBS; 10 mmol/L deferoxamine (Novartis, Arnhem, the Netherlands) and 30 mmol/L deferiprone (Duchefa Biochemie, Haarlem, the Netherlands), and stored at −20 °C prior to use. The final pH in incubation medium was maintained at 7.8. Several radical scavengers were used in some experiments, including tempol (Sigma-Aldrich), thiourea (OPG, Utrecht, the Netherlands) and 1,3-dimethyl-2-thiourea (DMTU, Aldrich Chemical, Milwaukee, WI, USA).

Viability assay

Cellular viability of HUVECs was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich) method[46]. Compound cytotoxicity was expressed as a TC_{50} denoting the concentration resulting in 50% loss of cell viability, as calculated by Calcusyn[47].

Fluorescence-activated cell sorting (FACS)

Human umbilical cord endothelial cells (HUVECs) were harvested by incubating with 0.2% trypsin-EDTA at 37 °C for 3 min. The cells were then incubated with fluorescence labelled monoclonal antibodies against the surface proteins, fluorescein isothiocyanate (FITC)-conjugated ICAM-1 antibody (R&D systems, Minneapolis, MN, USA), phycoerythrin (PE)-conjugated VCAM-1 antibody (BDBiosciences, San Diego, CA, USA), or Cychrome-conjugated E-selectin antibody (BDBiosciences), for 30 min at 4 °C. Each flow cytometric measurement was performed using a Becton Dickinson (San Jose, CA, USA) FACScan and 10.000 events were analyzed.

Measurement of oxidative stress

2,7-Dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) is a non-polar compound that is converted into a membrane-impermeable non-fluorescent polar derivative, 2,7-dichlorofluorescein (DCFH) by cellular esterase after incorporation into cells. The trapped DCFH is rapidly oxidized to fluorescent 2,7-dichlorofluorescein (DCF) by intracellular hydrogen peroxide and hydroxyl radicals [48]. Human umbilical cord endothelial cells (HUVECs) were harvested by incubating with 0.2% trypsin-EDTA at 37 °C for 3 min. Cells were then re-suspended in DCFHDA at a final concentration of 5 µM, incubated for 30 min at room temperature and washed. The emission of the trapped, oxidized DCF in 10.000 cells was analysed on a FACScan.

Calcein assay

In this assay[49], cells were incubated with 0.125 µM calcein-AM (30 min at 37 °C). The cells were washed twice to remove the remaining extracellular calcein-AM before fluorescence signal of calcein (excitation = 485 nm; emission = 530 nm) was followed using the Flexstation (Molecular Devices, Workingham, UK) at 37 °C. After a stable basal fluorescence signal was observed, iron was added to the incubation medium. Addition of iron quenches the fluorescence intensity of calcein signal. The accumulation of labile iron within cells due to addition of iron was expressed as the level of quenched calcein fluorescence adjusted to untreated controls at indicated time periods.

Data analysis

Results are expressed as means ± standard error of the mean (SEM). Differences in quantitative measures were tested for significance by using the unpaired two-tailed Student's *t*-test, unless otherwise stated. Significance was established when $p < 0.05$.

Results

Cp and CMV infections of endothelial cells

The log$TCID_{50}$ values of the stock *Cp* and CMV were calculated as 5.5 and 4, respectively, indicating titers of $6x10^6$ *Cp*/mL and $2x10^5$ CMV/mL. As HUVEC density was 10^5 cells/cm^2 at a cobblestone confluence, in order to obtain an MOI of 0.1 for *Cp* and CMV, *Cp* was diluted 300-fold, whereas CMV was diluted 10-fold before being used for HUVEC inoculation.

Cytotoxicity testing

The viability of HUVECs, after *Cp* (**Figure 1a**) or CMV infection (**Figure 1b**) at an MOI of 0.1, was > 85%.

Cp and CMV infectivity

Infectivity of *Cp* and CMV to HUVECs were counted using fluorescence microscopy on a random and blind basis after immunostaining procedure, using monoclonal antibodies specific towards Cp or CMV. The complete developmental cycle of *Cp* in cell culture models is between 48 and 72 h[50], whereas the slow-replicating CMV enters the early stage of infection at 72–96 h post-infection, and reaching the late stage of

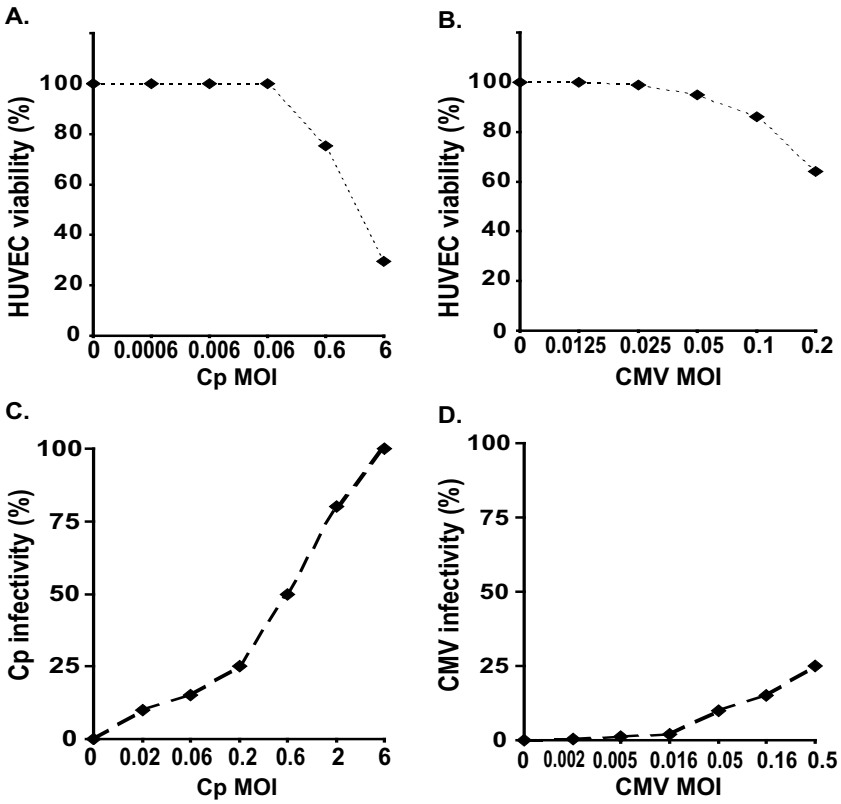

Figure 1 Endothelial viability and microorganism infectivity. (a) Shows the percentage of human umbilical vein endothelial cell (HUVEC) viability determined by the 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cytotoxicity assay, 2 days after *Chlamydia pneumoniae* (Cp) infection at an indicated multiplicity of infection (MOI), whereas (b) shows HUVEC viability 4 days after cytomegalovirus (CMV) infection. (c) shows the percentage of Cp infectivity towards HUVECs determined by immunostaining, and (d) shows CMV infectivity. The interpolated values at MOI of 0.1 are indicated by arrows (all data, $n = 3$).

infection at the 7th day post-infection[51]. To mimic the conditions of chronic steady-grade infections without having secondary infections, HUVECs were inoculated for 2 days with *Cp* or 4 days with CMV. *Cp* at an MOI of 0.1 resulted in < 20% infection (**Figure 1c**). Additionally, CMV at an MOI of 0.1, gave rise to < 10% infection (**Figure 1d**).

Low-grade *Cp*- and CMV-infections induced endothelial adhesion molecule expression without an increased oxygen-derived radical production

Chlamydiapneumoniae (Cp) infection markedly induced ICAM-1 (**Figure 2a**) and VCAM-1 expression (**Figure 2b**). A two-fold increase in ICAM-1 expression was observed with *Cp*-infection at an MOI of as low as 0.0006. E-selectin was significantly up-regulated by *Cp* starting at an MOI of 0.6 (**Figure 2c**).

Cytomegalovirus (CMV) at an MOI of as low as 0.05 induced a more than two-fold increase in both ICAM-1 and VCAM-1 expression, whereas E-selectin up-regulation was negligibly noticed (**Figure 2e-g**). The filtrate of microorganisms through a 100 kD Microcon filter did not result in up regulation of adhesion molecule expression (not shown), confirming the specific effects of *Cp* and CMV infections on the induction of endothelial adhesion molecule expression. No increase in intracellular oxygen-derived radicals was observed in both *Cp*- (**Figure 2d**) and CMV-infected HUVECs (**Figure 2h**), indicating that the induction of ICAM-1 and VCAM-1 in infected HUVECs could not primarily be attributed to radical formation.

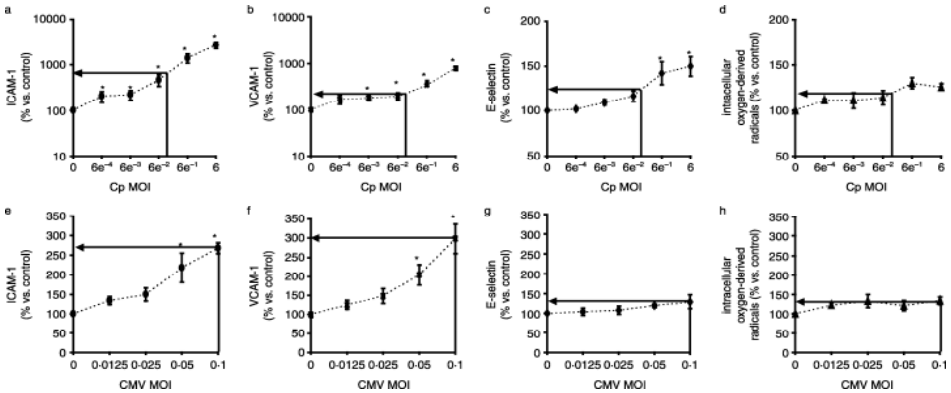

Figure 2 Induction of adhesion molecule expression by Cp or CMV infection. The expression of (a) intercellular adhesion molecule-1 (ICAM-1); (b) vascular cell adhesion molecule-1 (VCAM-1); (c) endothelial selectin (E-selectin); and (d) the levels of intracellular oxygen derived radicals in human umbilical vein endothelial cells (HUVECs) after *Chlamydia pneumoniae* (Cp) infection at an indicated multiplicity of infection (MOI) (mean ± SEM, $n = 4$, $^*P < 0.05$). The expression of (e) ICAM-1; (f) VCAM-1; (g) E-selectin; and (h) the levels of intracellular oxygen-derived radicals in HUVECs after cytomegalovirus (CMV) infection at an indicated MOI. The interpolated values at MOI of 0.1 are indicated by arrows (mean ± SEM, $n = 4$, $^*P < 0.05$).

Increased level of endothelial intracellular iron due to the addition of low molecular weight iron

With no external iron addition, the baseline iron level in the basal cell culture growth medium, EGM-2, was 0.36 µmol/L. Accumulation of intracellular labile iron within HUVECs was monitored by following the fluorescence of calcein for up to 15 h (**Figure 3**). An iron concentration of as low as 1 µmol/L was able to quench 5% of calcein signal after 2 h and 10% after 5 h, indicating an increase in the intracellular labile iron level. This result indicates that the addition of low molecular weight iron augmented the level of endothelial cytoplasmatic labile iron.

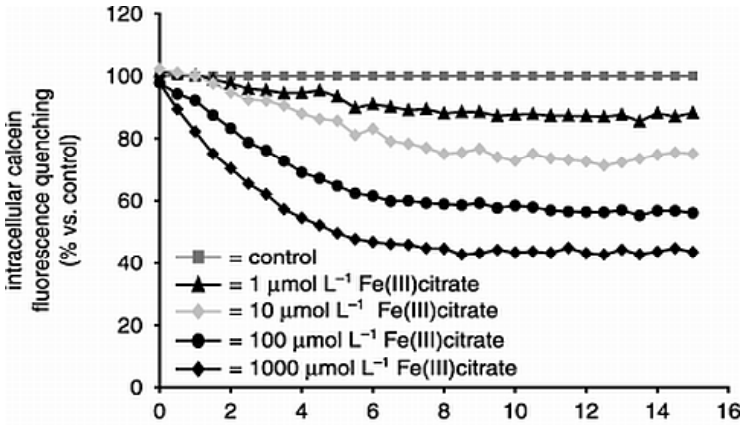

Figure 3 Endothelial intracellular iron level is modulated by addition of low molecular weight iron. Data represent the normalized mean fluorescence of intracellular calcein in human umbilical vein endothelial cells (HUVECs) in the presence of specified concentrations of Fe(III)citrate, monitored up to 15 h (n = 3). Quenching indicates the presence of intracellular labile iron.

Cp-induced endothelial adhesion molecule expression was markedly potentiated by iron

At an MOI of 0.1, *Cp*-induced VCAM-1 expression was additively up-regulated by iron, whereas ICAM-1 expression was synergistically up-regulated by iron (**Figure 4a-c**). Iron of 30 µmol/L significantly enhanced *Cp*-induced VCAM-1 expression two-fold and ICAM-1 expression almost threefold.

Infectivity and the size of inclusions of *Cp* in HUVECs were not affected by various concentrations of iron, ranging between 0 and 1000 µmol/L. This result was obtained by examination using fluorescence microscopy after immunostaining with monoclonal

antibody against *Cp* (**Figure 4d**) on a blind and random basis. This finding indicates that iron could modulate endothelial response towards chronic *Cp*-infection without affecting initial infectivity and the growth of *Cp*.

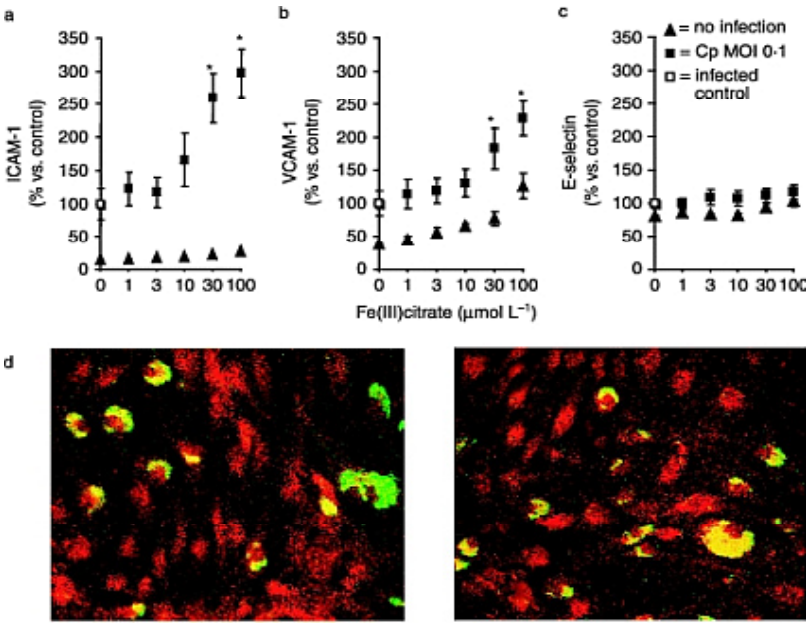

Figure 4 Iron modulates Cp-induced adhesion molecule expression. The expression of (a) intercellular adhesion molecule-1 (ICAM-1); (b) vascular cell adhesion molecule-1 (VCAM-1); and (c) endothelial selectin (E-selectin) on human umbilical vein endothelial cells (HUVECs) 2 days after *Chlamydia pneumoniae* (Cp) infection in the presence of increasing iron concentrations (mean ± SEM, $n = 4$, $^*P < 0.05$). (d) Confocal laser micrographs, representing four different slides, visualize the infectivity of Cp (green) on HUVECs (red) in the absence and presence of iron. (*For color figure see page 220)*

CMV-induced endothelial adhesion molecule expression was enhanced by iron

Both CMV-induced VCAM-1 and ICAM-1 expression were additively up-regulated by iron (**Figure 5a-b**), whereas E-selectin was not affected (**Figure 5c**). Iron of 30 µmol/L significantly enhanced CMV-induced VCAM-1expression 1.5-fold, and CMV-induced ICAM-1 expression 1.3-fold. As noted for *Cp*, the infectivity of CMV towards HUVECs and the size of CMV inclusions were not affected by various concentration of iron, ranging between 0 and 1000 µmol/L (**Figure 5d**). This result indicates that iron could also modulate endothelial response towards chronic CMV-infection, without affecting initial infectivity and the growth of CMV.

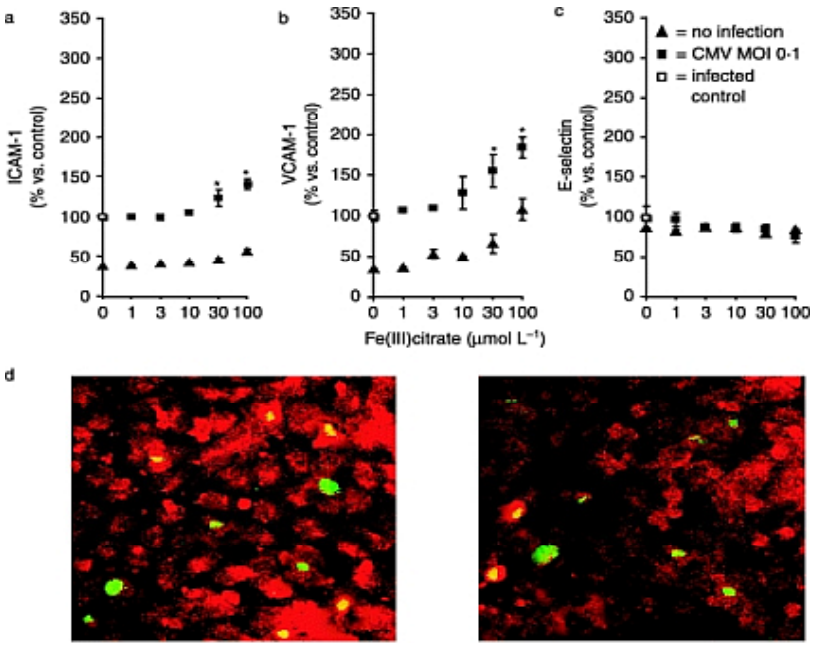

Figure 5 Iron modulates CMV-induced adhesion molecule expression. The expression of (a) intercellular adhesion molecule-1 (ICAM-1); (b) vascular cell adhesion molecule-1 (VCAM-1); and (c) endothelial selectin (E-selectin) on human umbilical vein endothelial cells (HUVECs) 4 days after cytomegalovirus (CMV) infection in the presence of increasing iron concentrations. (mean ± SEM, $n = 4$, *$p < 0.05$). (d) Confocal laser micrographs, representing four different slides, visualize the infectivity of CMV (green) on HUVECs (red) in the absence and presence of iron. (*For color figure see page 220)*

Iron chelation and radical scavenging could counteract potentiating effects of iron on infections

Addition of chelator-bound iron no longer modulated *Cp*- or CMV-induced endothelial adhesion molecule expression (**Figure 6a-b** for ICAM-1 expression), confirming the specific modulating effects of iron on endothelial response towards infections. The scavengers, including tempol, thiourea and DMTU, were also able to counteract the modulating effects of iron on *Cp*- or CMV-infection, indicating the involvement of oxygen-derived radicals in this process (**Figure 6a-b**). Furthermore, addition of iron chelator alone did not significantly down-regulate *Cp*- or CMV-induced adhesion molecule expression (**Figure 6a-b**), suggesting that infections may induce endothelial activation through a distinct pathway than iron. Concentrations of 10 µmol/L for

deferoxamine and 30 µmol/L for the bidentate deferiprone were chosen to cover the possible highest level of iron in HUVECs. These concentrations are below the TC_{50} values (deferoxamine = 15 ± 2.3 µmol/L, deferiprone = 100 ± 11.3 µmol/L) [24].

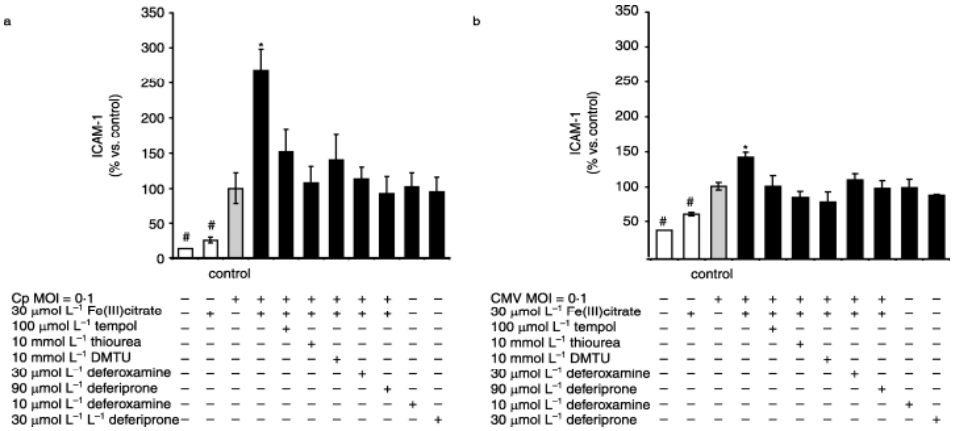

Figure 6 Effects of iron chelation and radical scavenging on infections. The expression of intercellular adhesion molecule-1 (ICAM-1) on (a) *Chlamydia pneumoniae (Cp)* or (b) cytomegalovirus (CMV) infected human umbilical vein endothelial cells (HUVECs) in the presence of indicated compounds. (mean ± SEM, n=3, *higher than control p<0·05; #lower than control p<0·05).

Discussion

Endothelial dysfunction plays an important role, not only at the initial step in the development of atherosclerosis, but also at a critical late step of thrombosis that leads to vessel occlusion and acute cardiovascular events[1]. As serological associations were found between *Cp* or CMV infections and acute myocardial infarctions as well as chronic coronary heart disease, there has been much effort on determining how endothelial infection by *Cp* or CMV causes endothelial dysfunction. One well-characterized phenotype of endothelial dysfunction is increased expression of the endothelial adhesion molecules, E-selectin, ICAM-1 and VCAM-1 [2]. In this study, we analysed endothelial activation after low-grade *Cp*- or CMV-infections that resulted in less than 20% infection of an endothelial cell population. Both low-grade chronic *Cp*- and CMV-infections had readily up-regulated the expression of adhesion molecules. The whole endothelial cell population showed relatively homogenous induction of adhesion molecule expression despite a low number of infections. This finding indicates that paracrine interactions such as through secretion of pro-inflammatory

cytokine interleukin-6 by the infected cells are crucial to generate response towards infections[52]. Our results therefore support the hypothesis that *Cp*- or CMV- infections are likely to contribute to the chronic inflammatory events in the vasculature associated with atherosclerosis.

In this study, we show that the expression of adhesion molecules in HUVECs infected with *Cp* was further enhanced when iron-rich medium was used during incubation. This iron-rich medium modulated intracellular iron level. It has been previously described that the infectivity and the growth of a relatively high-grade *Cp*-infection with a long incubation time could be restricted by iron chelation in epithelial cell line[53,54]. Using the current experimental settings mimicking a chronic low-grade vascular *Cp* infection, we observed modulating effects of iron on endothelial response towards *Cp*, before the infectivity or the growth of the microorganism were noticed. We also observed counteracting effects of iron chelation as well as radical scavenging on the effects of iron. These findings suggest that the modulating role of iron in endothelial response towards chronic infection is not by way of increasing the infectivity or the growth of the microorganism. Instead, our findings suggest that iron primarily exerted its effects through priming of the endothelial cells by generating oxidative stress. These iron-primed endothelial cells may consequently be more responsive towards the paracrine effects of infection. Additionally, it is known that for *Cp*, attachment is sufficient to initiate an endothelial response, while uptake may not be required[11]. Furthermore, we observed additive effects of iron on *Cp*-induced VCAM-1 expression and synergistic effects on *Cp*-induced ICAM-1 expression. This could be due to the differential signal transduction activated by *Cp* on endothelial cells[11,12] that in turn was potentiated by iron [30]. Iron chelators alone, however, did not down-regulate *Cp* or CMV-induced adhesion molecule expression, because there were no changes in the initial *Cp* or CMV infectivity to HUVECs due to addition of iron chelators. This could be because the resting iron levels in HUVECs passages 2-3 used in this study were already low, that is, in the order of 10 folds less than in freshly isolated cells [26,28] suggesting that further chelation probably would give little or no effects. Additionally, the level of intracellular oxygen-derived radicals was not changed due to infections. This may explain the absence of iron chelator effects as antioxidants in reducing infection enhanced endothelial adhesion molecule expression. This finding also suggests that infections may exert their effects on endothelial activation through a pathway that is different from the formation of oxygen-derived radicals.

Cytomegalovirus (CMV) infection has a relatively slow development cycle[51]. The early

stage of infection is started at 72-96 h post-infection[51]. During this time CMV has produced the immediate early gene products that are capable of inducing endothelial activation[15,17]. In this study we harvested HUVECs at the 4th day post infection, to allow CMV-induced endothelial activation to take place without the propagation of the microorganism. Up-regulation of both ICAM-1 and VCAM-1 was observed, whereas E-selectin was not affected significantly. This result confirms previous studies[14-16] showing differential induction of endothelial adhesion molecule expression by CMV. Furthermore, compared with controls, the iron primed endothelial cells generated higher levels of CMV-induced ICAM-1 and VCAM-1 expression without affecting the initial infectivity of CMV. These results again demonstrate the potentiating effects of iron on endothelial response towards infections without modulation of the initial infectivity of the virus.

The role of infections in promoting or accelerating atherosclerosis has been extensively demonstrated, although some studies showed lack of evidence[21,22]. Our *in vitro* study demonstrated modulating effects of iron on the endothelial response towards chronic infections of *Cp* and CMV. These findings imply that iron status in populations positive for *Cp*- or CMV- infections could be a potential determinant for the development of atherosclerosis. Further studies showing functional consequences of these recent findings such as the extent of leukocyte infiltration through endothelial cells are indeed warranted, in order to clarify the true role of iron in atherosclerosis. Furthermore, this study also implies that populations with increased body iron levels such as hereditary haemochromatosis and thalassaemia with frequent blood transfusions [37], and populations with disturbed iron homeostasis such as chronic renal insufficiency with iron substitution[55] may have an aggravated susceptibility towards atherosclerotic vascular disease when they are positive for *Cp*- or CMV-infections.

Acknowledgements

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References

1. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352(16):1685-95.
2. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002;105(9):1135-43.
3. Printseva OY, Peclo MM, Gown AM. Various cell types in human atherosclerotic lesions express ICAM-1. Further immunocytochemical and immunochemical studies employing monoclonal antibody 10F3. Am J Pathol 1992;140(4):889-96.
4. van der Wal AC, Das PK, Tigges AJ, Becker AE. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. Am J Pathol 1992;141(6):1427-33.
5. O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers CE. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. Circulation 1996;93 (4):672-82.
6. O'Brien KD, Allen MD, McDonald TO, Chait A, Harlan JM, Fishbein D et al. Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques. Implications for the mode of progression of advanced coronary atherosclerosis. J Clin Invest 1993;92(2):945-51.
7. Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC. Replication of hlamydia pneumoniae in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. Infect Immun 1996;64 (5):1614-20.
8. Kaukoranta-Tolvanen SS, Laitinen K, Saikku P, Leinonen M. Chlamydia pneumoniae multiplies in human endothelial cells in vitro. Microb Pathog 1994;16(4):313-19.
9. Godzik KL, O'Brien ER, Wang SK, Kuo CC. In vitro susceptibility of human vascular wall cells to infection with Chlamydia pneumoniae. J Clin Microbiol 1995;33(9):2411-14.
10. Bouwman JJ, Visseren FL, Bevers LM, van der Vlist WE, Bouter KP, Diepersloot RJ. Azithromycin reduces Chlamydia pneumoniae-induced attenuation of eNOS and cGMP production by endothelial cells. Eur J Clin Invest 2005;35(9):573-82.
11. Krull M, Klucken AC, Wuppermann FN, Fuhrmann O, Magerl C, Seybold J et al. Signal transduction pathways activated in endothelial cells following infection with Chlamydia pneumoniae. J Immunol 1999;162(8):4834-41.
12. Kaukoranta-Tolvanen SS, Ronni T, Leinonen M, Saikku P, Laitinen K. Expression of adhesion molecules on endothelial cells stimulated by Chlamydia pneumoniae. Microb Pathog1996;21(5):407-11.
13. Schumacher A, Seljeflot I, Lerkerod AB, Sommervoll L, Otterstad JE, Arnesen H. Does infection with Chlamydia pneumoniae and/or Helicobacter pylori increase the expression of endothelial cell adhesion molecules in humans? Clin Microbio lInfect 2002;8 (10):654-61.

14. Shahgasempour S, Woodroffe SB, Garnett HM. Alterations in the expression of ELAM-1, ICAM-1 and VCAM-1 after in vitro infection of endothelial cells with a clinical isolate of human cytomegalovirus. Microbiol Immunol 1997;41(2):121-9.
15. Guetta E, Scarpati EM, DiCorleto PE. Effect of cytomegalovirus immediate early gene products on endothelial cell gene activity. Cardiovasc Res 2001;50(3):538-46.
16. Sedmak DD, Knight DA, Vook NC, Waldman JW. Divergent patterns of ELAM-1, ICAM-1, and VCAM-1 expression on cytomegalovirus-infected endothelial cells. Transplantation 1994;58(12):1379-85.
17. Burns LJ, Pooley JC, Walsh DJ, Vercellotti GM, Weber ML, Kovacs A. Intercellular adhesion molecule-1 expression in endothelial cells is activated by cytomegalovirus immediate early proteins. Transplantation 1999;67(1):137-44.
18. Knight DA, Waldman WJ, Sedmak DD. Cytomegalovirus mediated modulation of adhesion molecule expression by human arterial and microvascular endothelial cells. Transplantation 1999;68(11):1814-18.
19. Ewig S, Torres A. Is Chlamydia pneumoniae an important pathogen in patients with community-acquired pneumonia? Eur Respir J 2003;21(5):741-2.
20. Forbes BA. Acquisition of cytomegalovirus infection: an update. Clin Microbiol Rev 1989;2 (2):204–16.
21. Ieven MM, Hoymans VY. Involvement of Chlamydia pneumoniae in atherosclerosis: more evidence for lack of evidence. J Clin Microbiol 2005;43(1):19-24.
22. Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? Lancet 1997;350(9075):430-6.
23. Kartikasari AER, Georgiou NA, Visseren FLJ, van Kats-Renaud H, van Asbeck BS, Marx JJM. Iron and atherosclerosis: Iron chelators decrease adhesion of monocytes to vascular endothelium. Biomarkers Environ 2003;6(Suppl. 1):34-8.
24. Kartikasari AER, Georgiou NA, Visseren FLJ, Kats-Renaud H, van Asbeck BS, Marx JJM. Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. Arterioscler Thromb Vasc Biol 2004;24(12):2257-62.
25. Visseren FLJ, Verkerk MS, van der Bruggen T, Marx JJM, van Asbeck BS, Diepersloot RJ. Iron chelation and hydroxyl radical scavenging reduce the inflammatory response of endothelial cells after infection with Chlamydia pneumoniae or influenza A. Eur J Clin Invest 2002;32(Suppl. 1):84-90.
26. Varani J, Dame MK, Gibbs DF, Taylor CG, Weinberg JM, Shayevitz J et al. Human umbilical vein endothelial cell killing by activated neutrophils: loss of sensitivity to injury is accompanied by decreased iron content during in vitro culture and is restored with exogenous iron. Lab Invest 1992;66(6):708-14.
27. Ryter SW, Si M, Lai CC, Su CY. Regulation of endothelial heme oxygenase activity during hypoxia is dependent on chelatable iron. Am J Physiol Heart Circ Physiol 2000;279(6):H2889-97.

28. Sasaki K, Hashida K, Michigami Y, Bannai S, Makino N. Restored vulnerability of cultured endothelial cells to high glucose by iron replenishment. Biochem Biophys Res Commun 2001;289(3):664-9.
29. Satoh T, Tokunaga O. Intracellular oxidative modification of low density lipoprotein by endothelial cells. Virchows Arch 2002;440 (4):410-17.
30. Zhang WJ, Frei B. Intracellular metal ion chelators inhibit TNFalpha-induced SP-1 activation and adhesion molecule expression in human aortic endothelial cells. Free Radic Biol Med 2003;34(6):674-82.
31. Koo SW, Casper KA, Otto KB, Gira AK, Swerlick RA. Iron chelators inhibit VCAM-1 expression in human dermal microvascular endothelial cells. J Invest Dermatol 2003;120(5):871-9.
32. Ramachandran A, Ceaser E, Darley-Usmar VM. Chronic exposure to nitric oxide alters the free iron pool in endothelial cells: role of mitochondrial respiratory complexes and heat shock proteins. Proc Natl Acad Sci USA 2004;101(1):384-9.
33. Dhanasekaran A, Kotamraju S, Kalivendi SV, Matsunaga T, Shang T, Keszler A et al. Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxideinduced mitochondrial iron uptake, oxidative damage, and apoptosis. J Biol Chem 2004;279(36):37575-87.
34. Soares MP, Seldon MP, Gregoire IP, Vassilevskaia T, Berberat PO, Yu J et al. Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation. J Immunol 2004;172(6):3553-63.
35. Kartikasari AER, Georgiou NA, Visseren FLJ, van Kats-Renaud H, van Asbeck BS, Marx JJM. Endothelial activation and induction of monocyte adhesion by nontransferrin-bound iron present in human sera. FASEB J 2006;20(2):353-5.
36. Kaplan J, Jordan I, Sturrock A. Regulation of the transferring independent iron transport system in cultured cells. J Biol Chem 1991;266(5):2997-3004.
37. Marx JJM. Primary and secondary haemochromatosis. Transfus Sci 2000;23(3):183-4.
38. Sullivan JL. Iron and the sex difference in heart disease risk. Lancet 1981;1(8233):1293-4.
39. Kartikasari AER, Georgiou NA, Marx JJM. Iron intake and cardiovascular disease. In: Arnoldi A, editor. Functional Foods, Cardiovascular Disease and Diabetes. Abington, Cambridge, UK: Woodhead Publishing Ltd and Boca Raton, FL, USA: CRC Press LLC; 2004, pp. 99-125.
40. Stadler N, Lindner RA, Davies MJ. Direct detection and quantification of transition metal ions in human atherosclerotic plaques: evidence for the presence of elevated levels of iron and copper. Arterioscler Thromb Vasc Biol 2004;24(5):949-54.
41. Lee TS, Shiao MS, Pan CC, Chau LY. Iron-deficient diet reduces atherosclerotic lesions in apoE-deficient mice. Circulation 1999;99 (9):1222-9.
42. Matthews AJ, Vercellotti GM, Menchaca HJ, Bloch PH, Michalek VN, Marker PH et al. Iron and atherosclerosis: inhibition by the iron chelator deferiprone (L1). J Surg Res 1997;73(1):35-40.

43. Minqin R, Rajendran R, Pan N, Tan BK, Ong WY, Watt F et al. The iron chelator desferrioxamine inhibits atherosclerotic lesion development and decreases lesion iron concentrations in the cholesterol-fed rabbit. Free Radic Biol Med 2005;38(9):1206-11.
44. Minqin R, Watt F, Huat BT, Halliwell B. Correlation of iron and zinc levels with lesion depth in newly formed atherosclerotic lesions. Free Radic Biol Med 2003;34(6):746-52.
45. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria.J Clin Invest 1973;52(11):2745-56.
46. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983;65(1-2):55-63.
47. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27-55.
48. Vanden Hoek TL, Li C, Shao Z, Schumacker PT, Becker LB. Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. J Mol Cell Cardiol 1997;29 (9):2571-83.
49. Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik ZI. Fluorescence analysis of the labile iron pool of mammalian cells. Anal Biochem 1997;248(1):31-40.
50. Beatty WL, Morrison RP, Byrne GI. Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. Microbiol Rev 1994;58(4):686-99.
51. Halwachs-Baumann G, Wilders-Truschnig M, Desoye G, Hahn T, Kiesel L, Klingel K et al. Human trophoblast cells are permissive to the complete replicative cycle of human cytomegalovirus. J Virol 1998;72(9):7598-602.
52. Visseren FLJ, Verkerk MS, Bouter KP, Diepersloot RJ, Erkelens DW. Interleukin-6 production by endothelial cells after infection with influenza virus and cytomegalovirus. J Lab Clin Med 1999;134(6):623-30.
53. Freidank HM, Billing H, Wiedmann-Al-Ahmad M. Influence of iron restriction on Chlamydia pneumoniae and C. trachomatis. J Med Microbiol 2001;50(3):223-7.
54. Al-Younes HM, Rudel T, Brinkmann V, Szczepek AJ, Meyer TF. Low iron availability modulates the course of Chlamydia pneumoniae infection. Cell Microbiol 2001;3(6):427-37.
55. Kooistra MP, Kersting S, Gosriwatana I, Lu S, Nijhoff-Schutte J, Hider RC et al. Nontransferrin-bound iron in the plasma of haemodialysis patients after intravenous iron saccharate infusion. Eur J Clin Invest 2002;32(Suppl. 1):36-41.

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7

Infection-induced inflammatory response of adipocytes *in vitro*

Abstract

Background

Abdominal obesity plays an important role in the development of insulin resistance, diabetes mellitus and atherosclerosis. The exact pathophysiological mechanisms are unclear but adipocyte dysfunction is thought to be crucial. Infections are associated with the development of atherosclerosis as well as diabetes. In this study we investigated whether adipocytes can be infected and whether this results in production of inflammatory cytokines relevant for the development of atherosclerosis and diabetes.

Methods

Pre-adipocytes were cultured and differentiated into mature adipocytes *in vitro*. Adipocytes and pre-adipocytes were incubated with infective and heat inactivated *Chlamydia pneumoniae (Cp)*, Cytomegalovirus (CMV), Adenovirus (Ad) subtypes 2 & 36, Influenza A (Inf A) and Respiratory Syncitial Virus (RSV). After 48 hours, adiponectin, Interleukin-6 (IL-6), Tumor necrosis factor-alpha (TNF-α) and Plasminogen Activator Inhibitor-1 (PAI-1) were measured in supernatants.

Results

Infection of adipocytes with Ad 36, CMV and RSV resulted in increased IL-6 production from 192±22 pg/mL (uninfected) to 1030±86 pg/mL, 838±59 pg/mL and 1241±191 pg/mL respectively (all $p<0.01$ vs. control). In addition, Ad 36 infection slightly reduced PAI-production in adipocytes (285±26.8 ng/mL vs. uninfected: 477±71.2 ng/mL; $p=0.05$) and pre-adipocytes (709±43.3 ng/mL vs. uninfected: 1071±71.8 ng/mL; $p<0.01$). In contrast, human adenovirus type 2 did not exert any effect on IL-6 or PAI-production. None of the micro organisms induced significant changes in adiponectin and/or TNF-α production.

Conclusions

Adipocytes can be infected with several micro organisms *in vitro*. Infection of adipocytes with Ad 36, but not Ad 2 leads to increased production of IL-6 which might contribute to chronic low-grade inflammation, a process known to be involved in the development of cardiovascular diseases and type 2 diabetes.

Introduction

Obese patients have an increased risk for the development of atherosclerosis and diabetes mellitus type 2 and myocardial infarction[1]. Although the exact pathophysiological mechanisms are not yet clear, insulin resistance, closely related with obesity, and inflammation are key components [2,3]. Abdominal adipose tissue not only absorbs and stores free fatty acids, but is also able to produce and secrete adipokines such as IL-6, Tumor Necrosis Factor (TNF-α), Plasminogen Activator Inhibitor-1 (PAI-1) and adiponectin. By producing hormones and inflammatory cytokines, adipose tissue is likely to play an important role in the development of diabetes and atherosclerosis [4-7].

Adipocyte dysfunction can be the cause but also the consequence of insulin resistance. Causes for adipocyte dysfunction are not well established but include abdominal obesity and elevated free fatty acid plasma concentration [8].

Cytomegalovirus (CMV) en *Chlamydia pneumoniae (Cp)* are thought to be involved in the pathogenesis of atherosclerosis and cardiovascular disorders [9-14]. It has been shown before that adenoviruses are able to infect adipocytes [15,16] and that this is possibly related to the development of obesity [17,18]. Data from studies in animals indicate a possible role for virus infections in the etiology of human obesity [19-21]. Moreover, Adenovirus-36 is associated with increased body weight and paradoxically lower cholesterol and triglyceride levels in Ad-36 antibody-positive vs. -negative subjects [22]. Therefore it can be hypothesized that (chronic) virus infections induce adipocyte dysfunction thus leading to the development of diabetes mellitus and/or cardiovascular diseases.

In the present study we investigated whether several viruses are able to infect adipocytes *in vitro* and whether this leads to adipocyte dysfunction as measured by the altered production of IL-6, TNF-α, adiponectin and PAI-1.

Methods

Culturing of adipocytes

Cryopreserved human subcutaneous pre-adipocytes from an obese donor (BMI >30; SP-F3 passage 2, ZenBIo Inc, Research Triangle Park, NC, USA) were cultured in pre-adipocyte medium (PM-1, ZenBio Inc) in 75 cm^2 flasks (Nunc, Roskilde Denmark) at 37° C with 5% CO_2. Subsequently, cells were seeded in 24-well plates (Nunc, Roskilde Denmark) and grown to confluence (passage 4). When cells were grown confluent, differentiation was started by replacing pre-adipocyte medium by differentiation

medium (DM-2, ZenBio Inc) which is PM-1 supplemented with 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin and a PPAR-γ agonist. After one week the differentiation medium was replaced by adipocyte medium (AM-1, ZenBio Inc). Cells were refed with fresh AM-1 every 3-4 days until experiments were performed. Control pre-adipocytes were kept and refreshed with PM all the time. The progression of lipid droplet accumulation and cell morphology during differentiation was monitored using an inverted microscope or Oil Red O staining.

Infectious and inactivated micro-organisms

Adenovirus subtypes 2 (Ad2), 36 (Ad36) and Respiratory Syncitial Virus (RSV) were propagated on human Hep-2 larynx carcinoma cells (#03-108/ATCC #CCL 23; Flow laboratories/Amstelstad BV, Zwanenburg, The Netherlands). The Ad36 isolate was kindly provided by E. Fries, Erasmus University Rotterdam. Influenza strain A/H1N1/Nederland/300/00 (Inf) was propagated on LLC-MK2 Rhesus monkey kidney cells (#03-200/ATCC #CCL7; Flow laboratories/Amstelstad BV, Zwanenburg, The Netherlands) and Cytomegalovirus (CMV) was cultured on a human embryonic lung (HEL) cell line. The non-adipogenic Ad2 subtype (control virus for Ad36), RSV, Inf and CMV strains were clinical isolates selected from patients with common respiratory infections (Dept. of Virology, Diakonessen Hospital Utrecht, The Netherlands). The intracellular bacterium Chlamydia pneumoniae AR 39 (Cp; ATCC #535920) was grown on Buffalo Green Monkey (BGM) kidney cells (ECACC no 90092601).

Ad2, Ad36, RSV and Inf infections in cell culture were identified by indirect immunofluorescent staining with a pooled specimen screening reagent containing affinity purified mouse monoclonal antibodies directed against adenovirus; influenza A and B; parainfluenza types 1, 2, and 3 and RSV (Bartels VRK Anti-Viral Screening Reagent, #B1029-86A; Trinity Biotech, Bray Ireland). Cytomegalovirus infection was identified by indirect immunofluorescent staining with anti CMV immediate early antigen antibodies (clone E13, #11-003; Argene Varilhes, France). Apple-green fluorescence in the cytoplasm (Ad, RSV, Inf) or nuclei (CMV, Inf) is observed in a positive stain while uninfected cells (counterstained with Evan's blue) appear red. Cp-infection was identified by direct immunofluorescent detection using the Pathfinder® Chlamydia Culture Confirmation System (#30701 Biorad, Veenendaal, The Netherlands). This assay detects all known serovars of *Chlamydia trachomatis, Chlamydia psittaci* and *Chlamydia pneumoniae*. Cells infected with *Cp* will show a characteristic apple-green fluorescence of cytoplasmic inclusions against a red background. After propagation on

the appropriate cell lines in tissue culture bottles, the upscaled inoculation materials were harvested, aliquoted and subsequently frozen at -80 °C. Infective doses ($TCID_{50}$) of each isolate were determined using corresponding cell lines according to the method of Reed and Muench [23]. As infection controls for the main experiments, aliquots of all isolates were inactivated using one standard procedure: incubation at 56° C for one hour followed by overnight UV-irradiation. Effectiveness of the inactivation procedure was checked by incubating undiluted inactivated isolates on the cell lines on which they were propagated initially for 48 hours.

Infection experiments

Cultures with more than 75% differentiated adipocytes were infected 13-15 days after the start of the differentiation. For each inoculation of cultures with differentiated adipocytes, cultures with pre-adipocytes were inoculated simultaneously. Infection rates were determined by calculating the number of infectious virus per cell. This was expressed as the multiplification of infection (MOI). For each inoculation with infective virus, inactivated virus was inoculated as an infection control. As a negative control, cells were incubated with culture medium only. All incubations were monitored by the corresponding immunofluorescent staining for each virus type. Optimal infection rates for the main experiments were determined in preliminary infection experiments with serial dilutions of each stock (data not shown). The rationale for using fairly low MOIs was to mimic chronic infection *in vivo* and to avoid unwanted cell death. Therefore infection rates of maximum 25% (fluorescence) were chosen.

Prior to infection, adipocytes were refed with fresh AM and pre-adipocytes with PM. Adipocytes and pre-adipocytes were inoculated with 200 µL of both infective and inactivated virus; multiplifications of infections (MOI) were: 0.01 and 0.001 for CMV, Ad2 and Ad 36 and MOI 0.02 and 0.002 for *Cp*, Influenza and RSV.

In addition to the infections, the ability of cells to produce adipokines was measured by incubating the cells with 1 or 10 ng/mL IL-1β or IL-6 or 100 µg/mL TNF-α.

All experiments were performed 3 times in duplicate on cells at passage 4, grown in 24-well plates. After inoculation, plates were centrifuged 1 hour at 500g. All incubations were kept for 48 hours at 37 °C and 5% CO_2. After 48 hours supernatants were collected and frozen at -80 °C until adipokines were measured. Infections and incubations with inactivated virus were checked by immunofluorescence.

Measurement of adipokines

Commercially available ELISA kits were used for measuring IL-6 (Pelikine compact human IL-6 kit, # M1916 Sanguin Reagents, The Netherlands), (Pelikine compact human TNF-α kit, # M1923 Sanguin Reagents, The Netherlands), PAI (PAI-1 antigen Elisa reagent kit, #TC11070, Technoclone GmbH Vienna, Austria) and adiponectin (human Adiponectin/Acrp30 ELISA kit, (#ELH-ACRP30-001, RayBiotech Inc. Norcross GA, USA). All assays were performed three times in duplicate. Optical densities were measured with an automated plate reader (Elx800, Biotek Instruments Inc.) at 450/630 nm and data were processed in analytical curve fitting software (KC Junior, Biotek instruments Inc.).

Statistical analysis

ELISA results were processed using the GraphPad Instat™ statistical software package (V2.05a). After infection, adipokine levels in the supernatants of infected adipocytes were compared with those in pre-adipocytes and in uninfected control cells or cells incubated with inactivated virus. In addition, adipokine production after incubation with IL-1β, IL-6 or TNF-α was compared to the adipokine levels in uninfected cells. An unpaired Student t-test was performed on all results unless standard error of means were significantly different ($p < 0.05$). On those results where standard errors of means did differ, a Welch t-test, correcting for these differences was performed.

Results

(Pre-) adipocyte culture

After seeding in 24-well plates, cultured pre-adipocytes (passage 4) needed 3-5 days to grow to confluence (**Figure 1a**). During differentiation cells transformed from spindle shaped "fibroblast-like" to more rounded cells which accumulated lipid droplets in their cytoplasm (**Figure 1b**). The differentiation grade was at least 75% after 13 days.

Virus infection of adipocytes

All viruses (CMV, Ad2, Ad 36, Influenza A, RSV) and Cp were able to infect adipocytes *in vitro* (**Figure 2 a,b,c,d,e,f**). The corresponding MOIs were 0.01 and 0.001 for CMV, Ad2 and Ad 36 and MOI 0.02 and 0.002 for *Cp*, Influenza A and RSV.

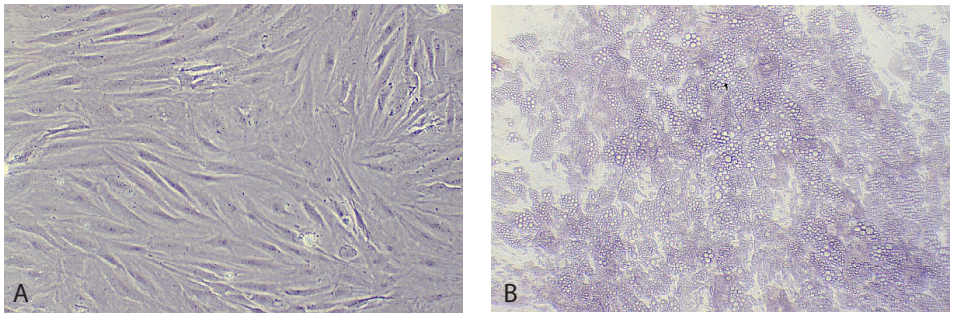

Figure 1 (Pre-) Adipocytes in culture (200x)

A Pre-adipocytes: spindle-shaped "fibroblast-like" cells grown to confluence.

B Adipocytes: differentiated cells 13 days after start of differentiation. Cells are rounded and have accumulated lipid droplets in their cytoplasm. (*For color figure see page 221)*

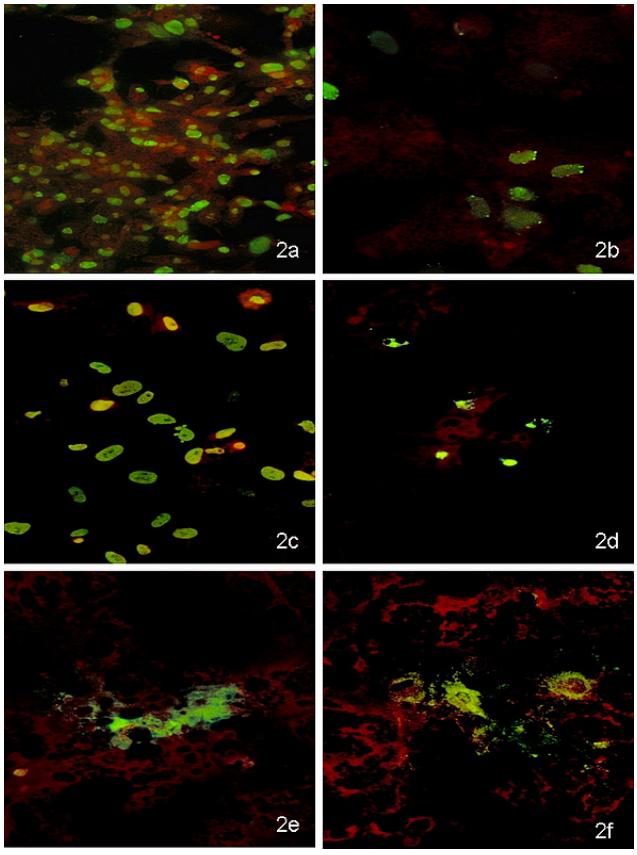

Figure 2. Fluorescent images of infected adipocytes (200x, 90% differentiated) after 48 hours infection with: Ad2, Ad 36, Cp, CMV, Inf, and RS-virus. Background (red): uninfected cells. Infected cells: apple green fluorescence either condensed in the nuclei (CMV;2c) or diffuse in the cytoplasm (Ad 2, Ad 36, Inf, RSV; 2a,b,d,e,f respectively). Cp-infected cells: compact fluorescent vacuoles in the cytoplasm (2d). (*For color figure see page 221)*

IL-6 production by uninfected pre-adipocytes and adipocytes

During the differentiation process, adiponectin production increased from 159±155 pg/mL at day 0 (start of differentiation) to 259±110 pg/mL on day 3 and 8972±3228 pg/mL on day 7. Pre-adipocytes did not produce adiponectin at all. The IL-6 production by uninfected adipocytes decreased from 3058±550 pg/mL at day 0 (start of differentiation) to 1433±485 pg/mL (day 3), 1146±285 pg/mL (day 7) and 71±32 pg/mL (day 9) and increased from day 13: (201±66 pg/mL) to day 15: (177± 24 pg/mL) and day 19 (257±57 pg/mL). IL-6 production in pre-adipocytes remained at the same level as was measured at the start of the differentiation phase.

Adipokine production by infected adipocytes

After 48 hours, IL-6 production of adipocytes was 1030±86 pg/mL ($p<0.01$) after incubation with infective Ad36 and 744±91 pg/mL ($p <0.01$) after incubation with inactivated Ad36 respectively, both compared to uninfected adipocytes (192±22 pg/mL). However, there was not a significant difference between IL-6 production by heat inactivated an infective Ad36 ($p=0.08$). After incubation with lower doses of A36, IL-6 levels in adipocytes were not elevated anymore ($p<0.05$). Therefore the effect was considered to be virus-dose dependent. In contrast to the clearly elevated IL-6 production by Ad 36, IL-6 production in Ad2 infected adipocytes was not affected (171±30 pg/mL; $p>0.05$).

Compared to uninfected adipocytes, infection of adipocytes with CMV or RSV also induced significant increase in IL-6 production (838±59 pg/mL and 1241±191 pg/mL respectively vs. uninfected adipocytes (192±22 pg/mL; $p<0.01$) (**Figure 3a**).

Baseline production of IL-6 by pre-adipocytes was considerably higher than in adipocytes (5234±837 pg/mL vs. 192±22 pg/mL) but quite similar to infected adipocytes, infection of pre-adipocytes with Ad36 or RSV resulted in a more than 2-fold increase in IL-6 from baseline (10957±1945 pg/mL ($p<0.05$) and 12079±2302 pg/mL ($p<0.01$) respectively vs. 5234±837 pg/mL in uninfected cells) CMV-infection did not lead to elevated IL-6 production by pre-adipocytes (**Figure 3b**).

Mean TNF-α productions of uninfected adipocytes and pre-adipocytes were 52.9 ± 3.9 pg/mL and 55.4 ± 11.3 pg/mL respectively as measured after 15 days incubation. None of the micro-organisms were able to increase TNF-α secretion by adipocytes (**Figure 4a**). In contrast, pre-adipocytes infected with the highest doses of Ad 36, CMV and RS-virus produced approximately 2-fold more TNF-α than uninfected pre-adipocytes

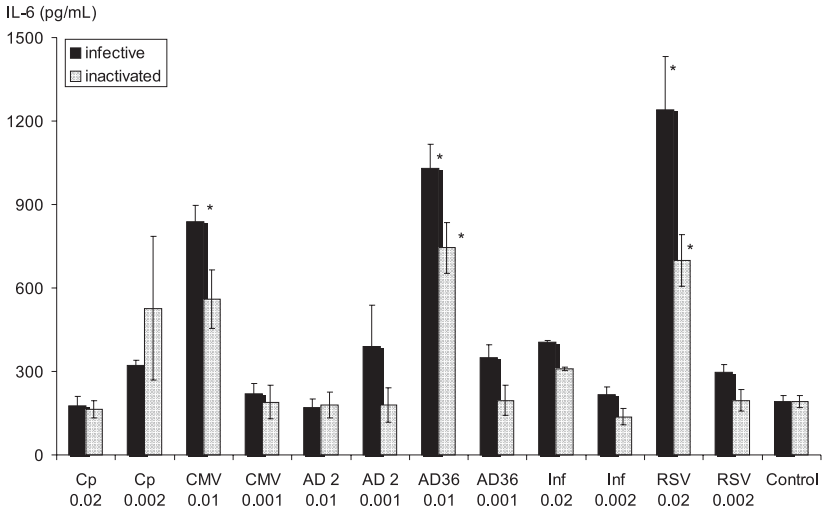

Figure 3a. IL-6 production in infected adipocytes.

Means ± SE values of 3 experiments in duplicate (n=3). ▇: IL-6 production in adipocytes incubated with infective micro-organisms. ▒: IL-6 production in adipocytes incubated with inactivated micro-organisms. Y-axis: IL-6 production in pg/mL. X-axis: Micro-organisms used for incubations with corresponding MOI (multiplification of infection) values. Baseline: Uninfected cells; Cp: Chlamydia pneumoniae; CMV: Cytomegalovirus; Ad: Adenovirus; Inf: Influenza A virus; RSV: Respiratory syncytial virus; * p <0.01 (t-test).

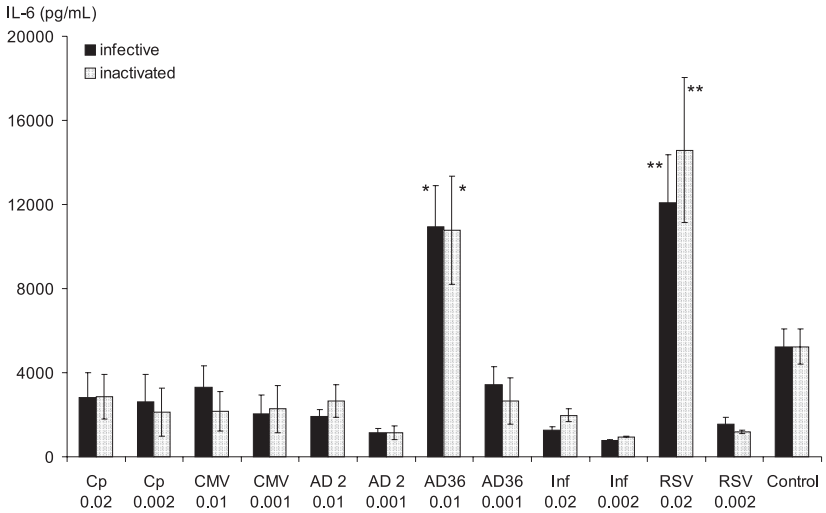

Figure 3b. IL-6 production in infected pre-adipocytes.

Means ± SE values of 3 experiments in duplicate (n=3). ▇: IL-6 production in pre-adipocytes incubated with infective micro-organisms. ▒: IL-6 production in pre-adipocytes incubated with inactivated micro-organisms. Y-axis: IL-6 production in pg/mL. X-axis: Micro-organisms used for incubations with corresponding MOI (multplification of infection) values. Baseline: Uninfected cells; Cp: Chlamydia pneumoniae; CMV: Cytomegalovirus; Ad: Adenovirus; Inf: Influenza A virus; RSV: Respiratory syncytial virus; * p<0.01, ** p<0.05 (t-test).

(120.3±38.2 pg/mL, 102.9±17.2 pg/mL and 122.1±54.5 pg/mL, respectively vs. 55.4 ± 11.3 pg/mL in uninfected cells), but this increase was not statistically significant ($p > 0.05$; Welch t-test). There was also a tendency towards increased TNF-α production by CMV, Ad36 and RS-infected pre-adipocytes compared with pre-adipocytes incubated with the same, but inactivated, micro-organisms (102.9±17.2 vs. 45.8±18.1 pg/mL for CMV, 120.3±38.2 vs. 37.1±18.6 pg/mL for Ad36 and 122.1±54.5 vs. 47.6±1.6 pg/mL for RS), but again these differences were not statistically significant ($p > 0.05$; Welch t-test) (**Figure 4b**).

Only incubation with the highest infective dose of Ad36 led to decreased PAI-1 production in pre-adipocytes (709±43.3 ng/mL), compared to uninfected pre-adipocytes (1071±71.8 ng/mL; $p<0.01$) but not in adipocytes: 285±26.8 ng/mL; $p=0.05$ vs. 477±71.2 ng/mL in uninfected adipocytes. None of the other micro-organisms affected PAI-production (**Figure 5a,b**).

After 48 hours, the adiponectin production by uninfected adipocytes was 9325±3029 pg/mL. Compared to adiponectin production by uninfected adipocytes, the adiponectin production by adipocytes that were incubated with either infective or inactivated micro-organisms, tended to decrease. (Overall -35%; Cp -37%, CMV -52%, Ad2 -30%, Ad-36 -30%, Inf A -26%, RSV -36%). This effect however, was not statistically significant (**Figure 6**). Pre-adipocytes did not produce adiponectin at all (data not shown).

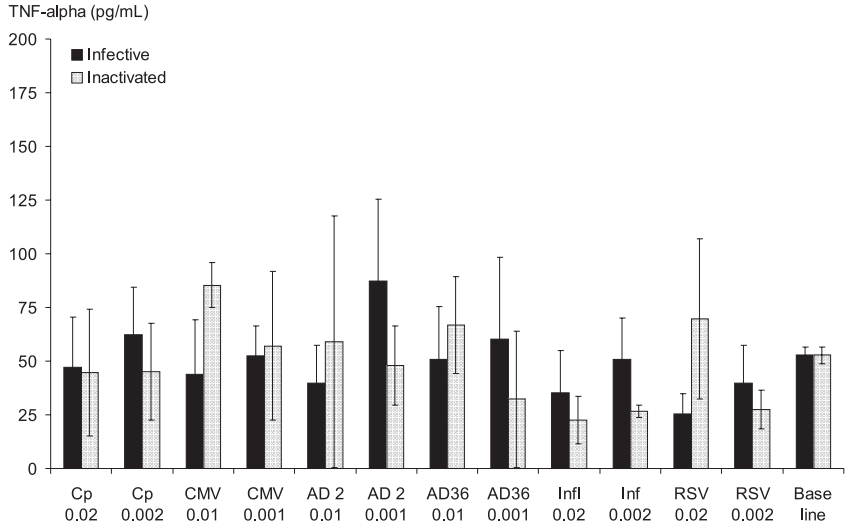

Figure 4a. TNF-alpha production in infected adipocytes.

Means ± SE values of 3 experiments in duplicate (n=3). ▓: TNF-alpha production in adipocytes incubated with infective micro-organisms. ▒: TNF-alpha production in adipocytes incubated with inactivated micro-organisms. Y-axis: TNF-alpha production in pg/mL. X-axis: Micro-organisms used for incubations with corresponding MOI (multiplification of infection) values. Baseline: Uninfected cells; Cp: Chlamydia pneumoniae; CMV: Cytomegalovirus; Ad: Adenovirus; Inf: Influenza A virus; RSV: Respiratory syncytial virus.

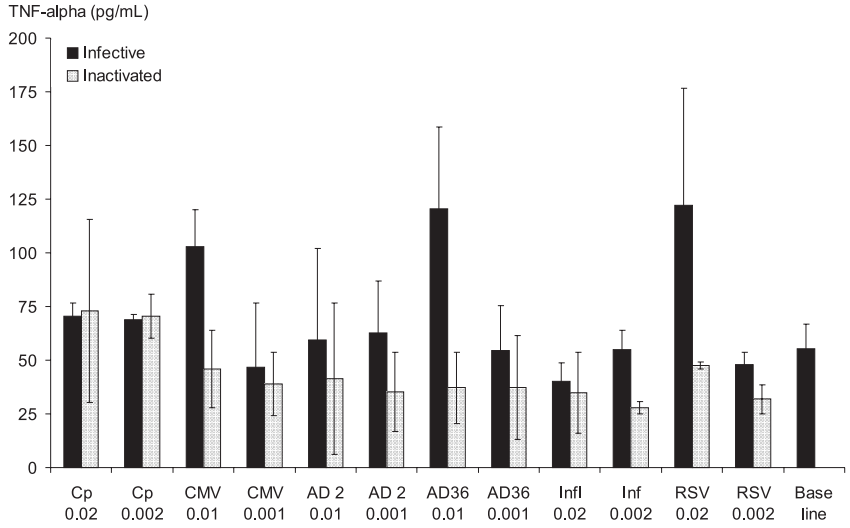

Figure 4b. TNF-alpha production in infected pre-adipocytes.

Means ± SE values of 3 experiments in duplicate (n=3). ▓: TNF-alpha production in pre-adipocytes incubated with infective micro-organisms. ▒: TNF-alpha production in pre-adipocytes incubated with inactivated micro-organisms. Y-axis: TNF-alpha production in pg/mL. X-axis: Micro-organisms used for incubations with corresponding MOI (multplification of infection) values. Baseline: Uninfected cells; Cp: Chlamydia pneumoniae; CMV: Cytomegalovirus; Ad: Adenovirus; Inf: Influenza A virus; RSV: Respiratory syncytial virus.

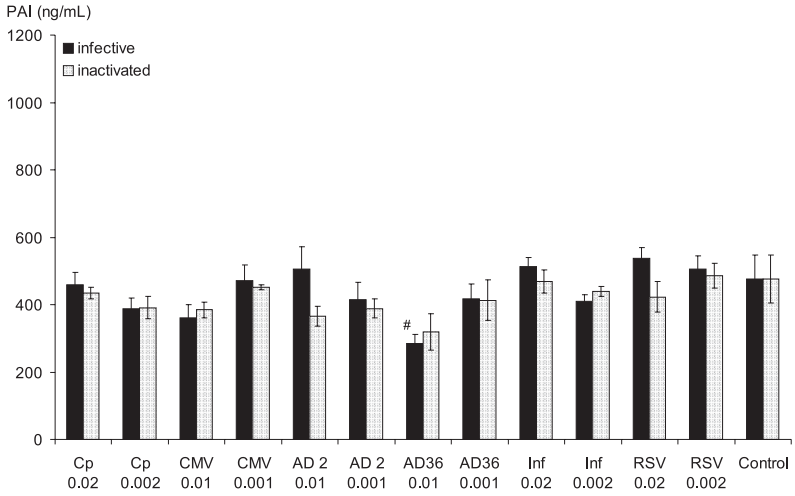

Figure 5a. PAI-1 production in infected adipocytes.

Means ± SE values of 3 experiments in duplicate (n=3). ▓: PAI-1 production in adipocytes incubated with infective micro-organisms. ▒: PAI-1 production in adipocytes incubated with inactivated micro-organisms. Y-axis: PAI-1 production in ng/mL. X-axis: Micro-organisms used for incubations with corresponding MOI (multiplification of infection) values. Baseline: Uninfected cells; Cp: Chlamydia pneumoniae; CMV: Cytomegalovirus; Ad: Adenovirus; Inf: Influenza A virus; RSV: Respiratory syncytial virus; # p=0.05 (t-test).

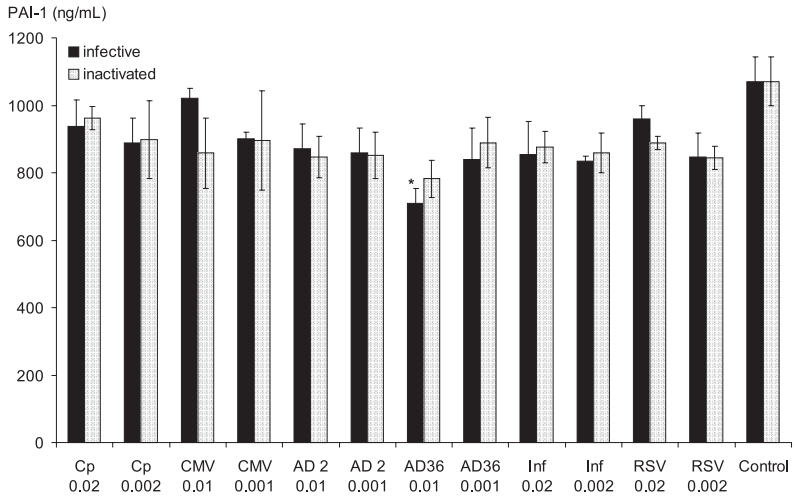

Figure 5b. PAI-1 production in infected pre-adipocytes.

Means ± SE values of 3 experiments in duplicate (n=3). ▓: PAI-1 production in pre-adipocytes incubated with infective micro-organisms. ▒: PAI-1 production in pre-adipocytes incubated with inactivated micro-organisms. Y-axis: PAI-1 production in ng/mL. X-axis: Micro-organisms used for incubations with corresponding MOI (multiplification of infection) values. Baseline: Uninfected cells; Cp: Chlamydia pneumoniae; CMV: Cytomegalovirus; Ad: Adenovirus; Inf: Influenza A virus; RSV: Respiratory syncytial virus; * p <0.05 (t-test).

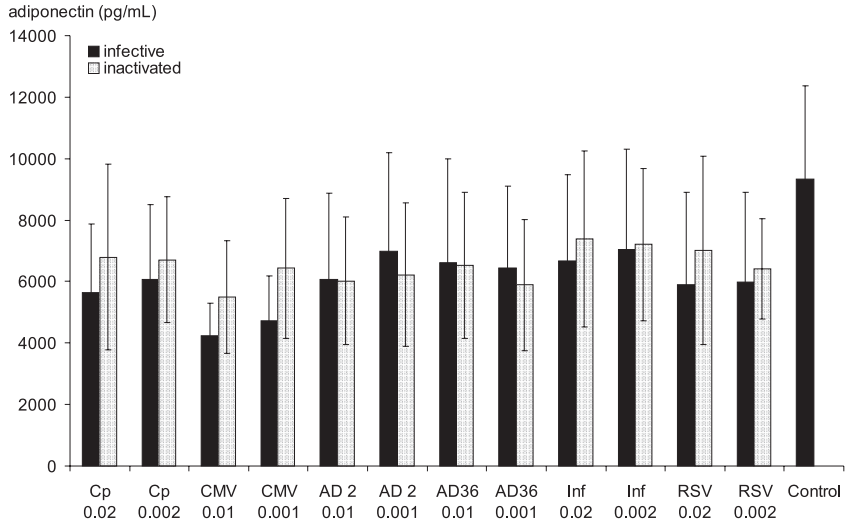

Figure 6. Adiponectin production in infected adipocytes.
Means ± SE values of 3 experiments in duplicate (n=3). ▉: Adiponectin production in adipocytes incubated with infective micro-organisms. ▒: Adiponectin production in adipocytes incubated with inactivated micro-organisms. Y-axis: adiponectin production in pg/mL after log transformation. X-axis: Micro-organisms used for incubations with corresponding MOI (multiplification of infection) values. Baseline: Uninfected cells; Cp: Chlamydia pneumoniae; CMV: Cytomegalovirus; Ad: Adenovirus; Inf: Influenza A virus; RSV: Respiratory syncytial virus.

Discussion

The present study shows that various microorganisms are able to infect adipocytes *in vitro*. Adipocyte infection resulted in an increased production of IL-6. Adenovirus infection of pre-adipocytes leads to altered PAI-1 production. These results can be viewed in the context of involvement of adipocytes in the pathogenesis of cardiovascular diseases and in relation with the development of diabetes mellitus. Compared with endothelial cells [9,13], monocytes [10] and hepatocytes [24] adipocytes react in a similar way to infection with a variety of microorganisms by producing inflammatory cytokines. Abdominal adipose tissue was thought to be just a storage depot for fat, but is now considered to be an active endocrine organ able to produce several hormones and inflammatory cytokines. These hormones and inflammatory cytokines have systemic effects on the vessel wall, vascular risk factors and on insulin resistance. By the portal circulation, these hormones and inflammatory cytokines are also able to directly influence liver metabolism, potentially leading to accelerated inflammatory response, e.g. C-Reactive Protein (CRP) production, and leading to

changes in hemostasis by the production of coagulant factors and proteins involved in fibrinolysis. Specific causes for changes in adipocyte function are not well known. We propose a role for microorganisms in inducing and maintaining adipocyte dysfunction.

At present, it is commonly accepted that (chronic) inflammation plays a key role in atherogenesis and atherosclerosis [25-27]. Elevated plasma concentrations of CRP are associated with increased cardiovascular risk and are seen in patients with obesity, metabolic syndrome or type 2 diabetes [28-30]. Microorganisms are thought to play a role in the development of atherosclerosis [31,32] although treatment of *Cp* with antibiotics did not lead to a reduction in the occurrence of vascular events [33]. The absence of an effect of antibiotic treatment on vascular events may be due to ineffective treatment strategies not affecting *Cp* in circulating monocytes [34]. Also, it can still be argued that viral infections, not affected by macrolide treatment, are involved in atherogenesis, arterial thrombosis and plaque rupture [35,36].

Recently, adenovirus infections were considered to play a role in the development of obesity and are associated with plasma lipid abnormalities [15,17,18,37,38]. In the present study it is shown that a range of different viral and bacterial micro organisms are able to infect human adipocytes and induce an inflammatory response. The production of IL-6 by adipocytes increased in a dose and infection dependent manner.

Circulating TNF-α and IL-6 plasma concentrations are mildly elevated in obesity. Both TNF-α and IL-6 modify insulin sensitivity by interfering with intracellular insulin signaling pathways [39]. The production of TNF-α and IL-6 by infected abdominal adipocytes and their release into the portal circulation, may lead to increased production of CRP by the liver and influence lipid metabolism leading to an increased cardiovascular risk [40,41] and increased risk for the development of type 2 diabetes [42,43]. The inflammatory response of adipocytes upon infection may also have paracrine effects by affecting the function of neighboring adipocytes and may attract macrophages into adipose tissue, increasing the capacity for the production of inflammatory mediators. The production of the proinflammatory cytokines IL-6, TNF-α and MCP-1 is unregulated in a co culture with 3T3-L1 adipocytes and a macrophage cell line RAW264 [44]. It has been suggested that one third of circulating plasma IL-6 is secreted by adipose tissue [45,46] reaching systemic concentrations high enough to elicit biological effects [47,48].

Adenoviruses were the first pathogens associated with human obesity. SMAM-1 was the first (avian) adenovirus described to be positively related to obesity [37], and Ad36 was the major adenovirus subtype reported to enhance adipocyte differentiation and having an association with obesity [15,37]. The human adenovirus types 5, 31 and 37 also appear to

increase pre-adipocyte differentiation, although their association with obesity is still unclear [16,22,38]. In the present study we showed that the Ad36 virus was able to increase IL-6 production by pre-adipocytes and adipocytes. Interestingly, the Ad2 subtype did not exert any functional effects on (pre-) adipocytes, indicating a specific mechanism of action in adipocyte signaling for the Ad36 virus type.

It has been suggested that enhanced differentiation of cultured pre-adipocytes by Ad36, but not Ad2, may contribute to adipogenesis [49,50] This would implicate that Ad-36 is associated with increased body weight. This was confirmed by showing that the antiviral agent cidofovir attenuated differentiation induced by Ad-36 through altering gene expression in adenovirus infected 3T3-L1 cells [49]. In mice, treatment with cidofovir significantly suppressed plasma IL-6 concentrations after intraperitoneal infection with cowpox [51]. Still, it remains unclear whether reduction of IL-6 concentrations by cidofovir treatment also leads to subsequent weight loss.

We acknowledge some limitations of the present study. For all experiments a stable culture of human subcutaneous cells from one obese donor was used. This culture was not continuous. It is not known whether the results from these adipocytes can be extrapolated to cells derived from other donors and from different body depots. Adipocytes beyond passage 4 had a reduced capacity for differentiation and had changed capacity for producing adipokines. This indicates that adipocyte cultures are very sensitive to functional changes. In our experiments a differentiation grade of approximately 75% was reached. Not reaching full differentiation was not considered to be an issue because adipose tissue in vivo consists of 30-60% adipocytes. Others already suggested differences in the capacity of adipokine production by pre-adipocytes compared to mature adipocytes [52-54]. We demonstrated that adiponectin was mainly produced by adipocytes, while IL-6 and PAI were produced by both adipocytes and pre-adipocytes, but pre-adipocytes produced more IL-6 and PAI than adipocytes.

In conclusion, various microorganisms are able to infect human adipocytes in vitro. Infection of adipocytes elicited increased IL-6 production. This pro-inflammatory effect of adipocyte infection may contribute to the development of cardiovascular diseases and/or the development of diabetes mellitus.

References

1. Yusuf S., Hawken S., Ounpuu S. et al. Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. *Lancet* 2005; 366 (9497):1640 -9.
2. Lindsay R.S., Howard B.V. Cardiovascular risk associated with the metabolic syndrome. *Curr Diab Rep* 2004; 1: 63 -8.
3. Koh K.K., Han S.H., Quon M.J. Inflammatory markers and the metabolic syndrome: insights from therapeutic interventions. *J Am Coll Cardiol* 2005; 46 (11): 1978 -85
4. Duncan B.B., Schmidt M.I., Pankow J.S. et al. Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes* 2003; 52 (7):1799 -805.
5. Festa A., D'Agostino R., Jr., Tracy R.P., Haffner S.M. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study.*Diabetes* 2002; 51(4):1131-7.
6. Jialal I., Devaraj S., Venugopal S.K. C-reactive protein: risk marker or mediator in atherothrombosis? *Hypertension* 2004; 44 (1): 6 -11
7. Festa A., D'Agostino R., Jr., Williams K. et al. The relation of body fat mass and distribution to markers of chronic inflammation. *Int J Obes Relat Metab Disord* 2001; 25 (10): 1407 -15.
8. Gasteyger C., Tremblay A. Metabolic impact of body fat distribution. *J Endocrinol Invest* 2002; 25 (10): 876 -83.
9. Bouwman J.J., Visseren F.L., Bevers L.M., van d., V, Bouter K.P., Diepersloot R.J. Azithromycin reduces Chlamydia pneumoniae-induced attenuation of eNOS and cGMP production by endothelial cells. *Eur J Clin Invest* 2005; 35 (9): 573 -82.
10. Bouwman J.J., Visseren F.L., Bosch M.C., Bouter K.P., Diepersloot R.J. Procoagulant and inflammatory response of virus-infected monocytes. *Eur J Clin Invest* 2002; 32 (10): 759 -66.
11. Campbell L.A., Kuo C.C. Chlamydia pneumoniae--an infectious risk factor for atherosclerosis? *Nat Rev Microbiol* 2004; 2(1): 23-32.
12. Grayston J.T., Kuo C.C., Campbell L.A., Benditt E.P. Chlamydia pneumoniae, strain TWAR and atherosclerosis. *Eur Heart J* 1993; 14 Suppl K : 66 -71.
13. Visseren F.L., Bouwman J.J., Bouter K.P., Diepersloot R.J., de Groot P.H., Erkelens D.W. Procoagulant activity of endothelial cells after infection with respiratory viruses. *Thromb Haemost* 2000; 84(2): 319 -24.
14. Zhu J., Quyyumi A.A., Norman J.E., Csako G., Epstein S.E. Cytomegalovirus in the pathogenesis of atherosclerosis: the role of inflammation as reflected by elevated C-reactive protein levels. *J Am Coll Cardiol* 1999; 34 (6): 1738 -43.
15. Dhurandhar N.V. Contribution of pathogens in human obesity. *Drug News Perspect* 2004; 17(5): 307 -13.

16. Whigham L.D., Israel B.A., Atkinson R.L. Adipogenic potential of multiple human adenoviruses in vivo and in vitro in animals. *Am J Physiol Regul Integr Comp Physiol* 2006; 290 (1): R190 -4 Epub
17. Desruisseaux M.S., Trujillo M.E., Trujillo M.E., Tanowitz H.B., Scherer P.E. Adipocyte, adipose tissue, and infectious disease. *Infect Immun* 2007 ; 75 (3): 1066 -78
18. Dhurandhar N.V. Infectobesity: obesity of infectious origin. *J Nutr* 2001; 131 (10): 2794S -2797S.
19. Dhurandhar N.V., Whigham L.D., Abbott D.H. et al. Human adenovirus Ad-36 promotes weight gain in male rhesus and marmoset monkeys. *J Nutr* 2002; 132 (10): 3155 -60.
20. Dhurandhar N.V., Israel B.A., Kolesar J.M., Mayhew G., Cook M.E., Atkinson R.L. Transmissibility of adenovirus-induced adiposity in a chicken model. *Int J Obes Relat Metab Disord* 2001; 25 (7): 990 -6.
21. Dhurandhar N.V., Israel B.A., Kolesar J.M., Mayhew G.F., Cook M.E., Atkinson R.L. Increased adiposity in animals due to a human virus. *Int J Obes Relat Metab Disord* 2000; 24 (8): 989 -96.
22. Atkinson R.L., Dhurandhar N.V., Allison D.B. et al. Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. *Int J Obes (Lond)* 2005; 29 (3): 281 -6 2005.
23. Reed LJ and Muench J. A simple method for estimating fifty percent endpoints. Am J Hyg 27; 493. 1938. Ref Type: Generic
24. Bouwman J.J., Visseren F.L., Bouter P.K., Diepersloot R.J. Azithromycin inhibits interleukin-6 but not fibrinogen production in hepatocytes infected with cytomegalovirus and chlamydia pneumoniae. *J Lab Clin Med* 2004; 144 (1): 18 -26.
25. Ross R. Atherosclerosis is an inflammatory disease. *Am Heart J* 1999; 138 (5 Pt 2): S419 -20.
26. Altman R. Risk factors in coronary atherosclerosis athero-inflammation: the meeting point. *Thromb J 2003; 1(1): 4*.
27. Wu J.T., Wu L.L. Linking inflammation and atherogenesis: Soluble markers identified for the detection of risk factors and for early risk assessment. *Clin Chim Acta 2006; 366 (1-2): 74 -80*
28. Erdmann E. Diabetes and cardiovascular risk markers. *Curr Med Res Opin* 2005 ; 21 Suppl 1: S21 -8 2005.
29. Basta G., Schmidt A.M., De C.R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovasc Res* 2004; 63 (4): 582 -92.
30. de Larranaga G.F., Bocassi A.R., Puga L.M., Alonso B.S., Benetucci J.A. Endothelial markers and HIV infection in the era of highly active antiretroviral treatment. *Thromb Res* 2003; 110 (2-3): 93 -8.
31. Leinonen M., Saikku P. Evidence for infectious agents in cardiovascular disease and atherosclerosis. *Lancet Infect Dis* 2002; 2(1): 11-7.
32. Muhlestein J.B. Infectious Agents, Antibiotics, and Coronary Artery Disease. *Curr Interv Cardiol Rep* 2000; 2(4): 342 -348.
33. Cannon C.P., Braunwald E., Mccabe C.H. et al. Antibiotic treatment of Chlamydia pneumoniae after acute coronary syndrome. *N Engl J Med* 2005; 352 (16): 1646 -54.

34. Gieffers J., Fullgraf H., Jahn J. et al. Chlamydia pneumoniae infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation* 2001; 103 (3): 351 -6.
35. Chiu B., Viira E., Tucker W., Fong I.W. Chlamydia pneumoniae, cytomegalovirus, and herpes simplex virus in therosclerosis of the carotid artery. *Circulation* 1997; 96 (7): 2144-8.
36. Watt S., Aesch B., Lanotte P., Tranquart F., Quentin R. Viral and bacterial DNA in carotid atherosclerotic lesions. *Eur J Clin Microbiol Infect Dis* 2003; 22 (2): 99 -105
37. Dhurandhar N.V., Kulkarni P.R., Ajinkya S.M., Sherikar A.A., Atkinson R.L. Association of adenovirus infection with human obesity. *Obes Res* 1997; 5(5): 464 -9.
38. So P.W., Herlihy A.H., Bell J.D. Adiposity induced by adenovirus 5 inoculation. *Int J Obes (Lond)* 2005 ; 29 (6): 603 -6
39. Bastard J.P., Jardel C., Delattre J., Hainque B., Bruckert E., Oberlin F. Evidence for a link between adipose tissue interleukin-6 content and serum C-reactive protein concentrations in obese subjects. *Circulation* 1999; 99 (16): 2221 -2.
40. Esteve E., Castro A., Lopez-Bermejo A., Vendrell J., Ricart W., Fernandez-Real J.M. Serum interleukin-6 correlates with endothelial dysfunction in healthy men independently of insulin sensitivity. *Diabetes Care* 2007; 30 (4): 939 -45.
41. Paik J.K., Kim O.Y., Koh S.J. et al. Additive effect of interleukin-6 and C-reactive protein (CRP) single nucleotide polymorphism on serum CRP concentration and other cardiovascular risk factors. *Clin Chim Acta* 2007; 380 (1-2): 68 -74
42. Roytblat L., Rachinsky M., Fisher A. et al. Raised interleukin-6 levels in obese patients. *Obes Res* 2000; 8 (9): 673 -5.
43. Klover P.J., Zimmers T.A., Koniaris L.G., Mooney R.A. Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. *Diabetes* 2003 ; 52 (11): 2784 -9.
44. Suganami T., Nishida J., Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol* 2005; 25 (10): 2062 -8
45. Yudkin J.S., Kumari M., Humphries S.E., Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 2000; 148 (2): 209 -14.
46. Mohamed-Ali V., Goodrick S., Rawesh A. et al. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997; 82 (12): 4196 -200.
47. Greenberg A.S., Nordan R.P., McIntosh J., Calvo J.C., Scow R.O., Jablons D. Interleukin 6 reduces lipoprotein lipase activity in adipose tissue of mice in vivo and in 3T3-L1 adipocytes: a possible role for interleukin 6 in cancer cachexia. *Cancer Res* 1992; 52 (15): 4113 -6.

48. Fruhbeck G., Gomez-Ambrosi J., Muruzabal F.J., Burrell M.A. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 2001 ; 280 (6): E827 -47.
49. Rathod M., Vangipuram S.D., Krishnan B., Heydari A.R., Holland T.C., Dhurandhar N.V. Viral mRNA expression but not DNA replication is required for lipogenic effect of human adenovirus Ad-36 in preadipocytes. *Int J Obes (Lond)* 2007; 31 (1): 78 -86.
50. Vangipuram S.D., Sheele J., Atkinson R.L., Holland T.C., Dhurandhar N.V. A human adenovirus enhances preadipocyte differentiation. *Obes Res* 2004; 12 (5): 770 -7.
51. Knorr C.W., Allen S.D., Torres A.R., Smee D.F. Effects of cidofovir treatment on cytokine induction in murine models of cowpox and vaccinia virus infection. *Antiviral Res* 2006 ; 72 (2): 125 -33
52. Chung S., Lapoint K., Martinez K., Kennedy A., Boysen S.M., McIntosh M.K. Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. *Endocrinology 2006; 147 (11): 5340 -51*
53. Hube F., Lee Y.M., Rohrig K., Hauner H. The phosphodiesterase inhibitor IBMX suppresses TNF-alpha expression in human adipocyte precursor cells: a possible explanation for its adipogenic effect. *Horm Metab Res* 1999; 31(6): 359 -62.
54. Harkins J.M., Moustaid-Moussa N., Chung Y.J. et al. Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice. *J Nutr* 2004; 134 (10): 2673 -7.

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Intracellular infections enhance IL-6 and PAI-1 production by co-cultivated human adipocytes and THP-1 monocytes

Abstract

Introduction

Obesity is associated with a chronic inflammatory state, and adipocyte dysfunction is thought to play a crucial role in this. Infection of adipose tissue may trigger the production of inflammatory cytokines, leading to increased recruitment of macrophages into adipose tissue, which in turn may exacerbate the inflammatory state in obesity.

Methods

Low-grade inflammation was mimicked in an *in vitro* co-culture model with human adipocytes and THP-1 monocytes. Adipocytes and monocytes were infected with adenovirus, cytomegalovirus (CMV), or influenza A (inf A) virus. After 48 hours, transinfection was evaluated and interleukin (IL)-6, tumor necrosis factor (TNF)-α, adiponectin and plasminogen activator inhibitor (PAI)-1 were measured.

Results

IL-6 production was up regulated in co-cultures of uninfected adipocytes and THP-1 macrophages in a THP-1 cell number-dependent fashion. IL-6 production by CMV-infected adipocytes was increased relative to that of uninfected adipocytes ($p<0.01$). IL-6 production by CMV-infected co-cultures, was 16-37 fold higher than that of uninfected adipocytes,($p<0.001$). IL-6 production in influenza A-infected co-cultures was increased 12- to 20-fold ($p<0.05$). Only CMV-infection increased levels of PAI-1 in co-cultures (4-fold, $p<0.05$). Soluble factors produced by THP-1 macrophages rather than by adipocytes were responsible for the increased production of IL-6 in co-cultures.

Conclusions

Infection of co-cultivated human adipocytes and THP-1 monocytes with CMV or influenza A led to increased production of IL-6 and PAI-1. Thus infection of adipose tissue evokes an inflammatory response, leading to adipose tissue dysfunction and subsequent overproduction of IL-6 and PAI-1. This may further compound the atherogenic effects of obesity.

Key words: *adipocytes, macrophages, co-culture, inflammation, IL-6, plasminogen activator inhibitor, cytomegalovirus, influenza.*

Introduction

Abdominal obesity is an important risk factor for the development of insulin resistance, metabolic syndrome, diabetes mellitus type 2, and atherosclerosis [1, 2]. Adiposity is also associated with a state of low-grade chronic inflammation. Adipose tissue is composed of different cell types, including adipocytes and macrophages [3], both of which are involved in lipid storage and cytokine secretion. Inflamed adipose tissue that has been invaded by macrophages produces interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α, thereby contributing to the development of insulin resistance and cardiovascular diseases [4-7]. Moreover, the increase in adipose tissue mass may lead to hypoxia, which may stimulate the invasion of T-lymphocytes [8] and macrophages [9, 10]. Although the trigger for adipose tissue inflammation is not yet known, it may be viral infection. The inflammatory response of adipocytes to infection may stimulate the production of inflammatory cytokines, which in turn may exert paracrine effects on neighboring cells and attract more macrophages into adipose tissue. This further increases the capacity of adipose tissue to produce inflammatory mediators [11, 12]. In addition, viruses and bacteria can also exert direct atherogenic effects following infection of the vascular wall [13-16].

Although a viral etiology of obesity remains unproven and controversial, adenoviruses may be causally related to obesity, suggesting a role for these pathogens in the etiology of obesity [17-21]. Previously, we showed that human adipocytes could be infected by several microorganisms *in* vitro, and that infection led to an increased production of IL-6. These results suggested that viral infections could contribute to the development of type 2 diabetes and atherosclerosis [22]. In the present study, we investigated the effects of infection of co-cultures of human adipocytes and macrophages by cytomegalovirus, influenza A virus, and adenovirus subtypes 2 and 36 on the production of inflammatory cytokines and plasminogen activator inhibitor-1 (PAI-1), and whether transinfection of the two types of cells occurred.

Methods

Preparation of virus stocks

Adenovirus subtypes 2 and 36 were propagated on human Hep-2 larynx carcinoma cells (#03-108/ATCC #CCL 23; Flow laboratories/Amstelstad BV, Zwanenburg, the Netherlands). The adenovirus 36 isolate was kindly provided by the Department of

Microbiology of the Erasmus University Rotterdam, the Netherlands. Influenza A/H1N1/Netherlands/300/00 was propagated on LLC-MK2 Rhesus monkey kidney cells (#03-200/ATCC #CCL7; Flow laboratories/Amstelstad BV, Zwanenburg, the Netherlands) and Cytomegalovirus (CMV) was cultured on a human embryonic lung cell line. The non-adipogenic adenovirus 2 subtype (control virus for adenovirus 36), influenza A, and CMV strains were clinical isolates from patients with common respiratory infections (Department of Virology, Diakonessen Hospital Utrecht, the Netherlands). Infection of cells in cultures with adenoviruses 2, 36 and influenza A was established by indirect immunofluorescence staining with a pooled specimen screening reagent containing affinity purified mouse monoclonal antibodies directed against respiratory viruses, among which adenovirus and influenza A/B. (Bartels VRK Anti-Viral Screening Reagent, #B1029-86A; Trinity Biotech, Bray Ireland). CMV infection was identified by indirect immunofluorescent staining with anti-CMV immediate early antigen antibodies (clone E13, #11-003; Argene, Varilhes, France). The cytoplasm (RSV, influenza A) and nuclei (adenoviruses, CMV, influenza A) of infected cells display apple-green fluorescence whereas the cytoplasm of uninfected cells is red (counterstained with Evan's blue). After propagation on the appropriate cell lines in tissue culture bottles, the inoculation materials were harvested, aliquoted, and frozen at -80°C. The 50% tissue culture infective dose ($TCID_{50}$) of each isolate was determined using corresponding cell lines according to the method of Reed and Munch [23].

Cell culture

Adipocyte precursor cells were grown and stimulated to differentiate into mature adipocytes as described previously [22]. Briefly, human subcutaneous pre-adipocytes (ZenBIo Inc. SP-F3, Tebu-Bio BV Heerhugowaard, the Netherlands) were seeded at passage 4 in 24-well plates (Nunc, Roskilde Denmark) and grown to semi-confluence in pre-adipocyte medium (PM-1, ZenBio Inc. Tebu-Bio BV Heerhugowaard, the Netherlands) at 37 °C and 5% CO_2. Cells were stimulated to differentiate by replacing PM-1 by differentiation medium (DM-2, ZenBio Inc. Tebu-Bio BV Heerhugowaard, the Netherlands), which is PM-1 supplemented with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, and a PPAR-γ agonist. After 1 week, the differentiation medium was replaced by adipocyte medium (AM-1, ZenBio Inc. Tebu-Bio BV Heerhugowaard, the Netherlands). The AM-1 medium was replaced every 3-4 days until experiments were performed.

Cells of the human leukemic monocyte cell line THP-1 (ECACC 88081201) were grown in suspension cultures in RPMI 1640-medium (21875 Gibco, Invitrogen Breda, the Netherlands) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. Cell densities were kept between $2*10^5$ cells/mL and $8*10^5$ cells/mL. Five days prior to infection, monocytes were driven to differentiate into macrophage-like cells by addition of 50 ng/mL phorbol-12-myristate-13-acetate (PMA; Fluka 79346, Sigma Zwijndrecht, the Netherlands).

Infection of adipocytes

Cultures containing more than 75% differentiated adipocytes were infected 13-15 days after the start of differentiation. All infections were monitored by fluorescent immunostaining, and the severity or grade of infection was determined by calculating the number of cells infected, expressed as a proportion of the total cell count. Optimal infection grades, expressed as the multiplication of infection (MOI), were determined in preliminary experiments with serial dilutions of each stock (data not shown). The rationale for using fairly low MOI values was to mimic chronic infection *in vivo* and to avoid causing cell death. One day prior to infection, the AM-1 medium was replaced and adipocytes and macrophages were incubated with 100 µl of each virus at MOI 0.01. As a negative control, cells were incubated with culture medium only. In addition to infection, the ability of cells to produce adipokines was determined by incubating the cells with 1 ng/mL IL-1β. All experiments were performed three times in triplicate on cells at passage 4, grown in 24-well plates. After the addition of virus, the plates were centrifuged for 1 hour at 500g and then incubated at 37 °C and 5% CO_2 for 48 hours, after which the culture medium was collected and frozen at -80 °C until adipokines were measured.

Infection of co-cultivated adipocytes and THP-1

Twenty-four hours before the adipocytes and THP-1 cells were infected, the AM-1 medium was replaced. Adipocytes and THP-1 monocytes were co-cultivated in fixed monocyte:adipocyte ratios of 1:2.5, 1:12.5, and 1:50, achieved by incubating 50,000, 10,000 and 2,500 THP-1 monocytes with 125,000 adipocytes per well. In contrast to the method of cell infection described above, in this experiment adipocytes or monocytes were infected separately by pre-incubating the cells with virus for 4 hours at 37 °C, after which the cells were washed and 1 mL of fresh AM-1 medium was added, followed by

the other cell type. Controls were uninfected adipocytes, THP-1 cells, and co-cultivated cell cultures. After 48 hours, the culture medium was harvested and frozen at -80 °C. Adipocytes and THP-1 monocytes were separated and fixed in acetone/methanol (1:1 vol/vol). To assess whether viruses are able to migrate from adipocytes to monocytes and vice versa (transinfection), viral infections in fixed cells were monitored with the appropriate fluorescent antibodies as described above. The number of fluorescent cells in four fields per view was counted and expressed as a percentage of the total cell count.

IL-6 production after incubation with conditioned medium

Incubations of fresh adipocytes and THP-1 cells with medium recovered from previously infected THP-1 or adipocytes were conducted to determine the relative contribution of THP-1 cells vs. adipocytes to increased IL-6 levels in co cultures. Thus 250 µL of conditioned medium was added to 750 µL of fresh AM-1 medium and added to freshly cultured cells, and 48 hours later IL-6 was measured in the culture medium, as an indicator of adipokine production. To rule out the possibility of virus being present in the conditioned media we performed immunofluorescence on the cells that were inoculated with the conditioned media.

Adipokine assays

Commercially available ELISA kits were used for measuring IL-6 (Pelikine compact human IL-6 kit, # M1916 Sanguin Reagents, the Netherlands), TNF-α (Pelikine compact human TNF-alpha kit, # M1923 Sanguin Reagents, the Netherlands), PAI (PAI-1 antigen Elisa reagent kit, #TC11070, Technoclone GmbH Vienna, Austria) and adiponectin (human Adiponectin/Acrp30 ELISA kit, (#ELH-ACRP30-001, RayBiotech Inc. Norcross GA, USA / Tebu-Bio BV Heerhugowaard, the Netherlands). All assays were performed three times in duplicate. Optical density was measured with an automated plate reader (Elx800, Biotek Instruments Inc.) at 450/630 nm and data were processed with analytical curve fitting software (KC Junior, Biotek instruments Inc).

Cytotoxicity testing

Lactate dehydrogenase (LDH) was measured in culture medium to monitor cytotoxicity in infected and uninfected (control) cultures of adipocytes and/or THP-1 macrophages, using a colorimetric cytotoxicity detection assay (Cytotoxicity Detection Kit ^{PLUS} (LDH)

#04744926001, Roche Diagnostics BV, Almere, the Netherlands). The amount of color formed is proportional to the number of lysed cells and is presented as a percentage of maximal LDH release following cell lysis.

Data analysis

Means and corresponding standard errors of adipokine levels in the culture medium of infected adipocytes and monocytes were compared with those in the culture medium of uninfected co-cultures. One-way ANOVA with Tukey-Kramer Multiple Comparison post tests were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA). Two-way ANOVA with Bonferroni post tests on co-cultivation results were performed to evaluate differences in adipokine concentrations.

Results

Infection of adipocytes and THP-1 cells

The percent of infected adipocytes cells after 48 hours incubation with adenoviruses 2 and 36, CMV, and influenza A was 1.4±0.2%, 2.8±0.3%, 2.5±0.1%, and 0.2±0.1%, respectively. The percent of infected THP-1 cells after 48 hours incubation with adenovirus 36, CMV, and influenza A was 4.8±0.8%, 5.2±0.4%, and 4.0±0.6% respectively. THP-1 cells were refractory to adenovirus 2 [**Table 1**]. Transinfection occurred from adipocytes to THP-1 cells and vice versa. Addition of THP-1 cells to infected adipocytes resulted in infection of THP-1 cells by adenovirus 36 (4.6±1.0%),

Table 1 Transinfection in co-cultivated adipocytes and THP-1 monocytic cells

			pre-incubated adipocytes		pre-incubated THP-1	
	Adipocytes	**THP-1**	**adipocytes**	**THP-1**	**adipocytes**	**THP-1**
Ad 2	1.4 ± 0.2	nd	1.5 ± 0.1	nd	0.1 ± 0.0	nd
Ad 36	2.8 ± 0.3	4.8 ± 0.8	2.8 ± 0.5	4.6 ± 1.0	1.4 ± 0.3	2.6 ± 0.4
CMV	2.5 ± 0.1	5.2 ± 0.4	2.6 ± 0.2	1.8 ± 0.7	1.4 ± 0.5	2.3 ± 0.3
Inf A	0.2 ± 0.1	4.0 ± 0.6	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	2.5 ± 1.1

Percentages of positive cells from total in adipocytes and THP-1 cells as determined by specific immunofluoresence for each virus type. For co-cultivation either adipocytes or THP-1 were pre-incubated during 4 hours with virus before adding the other cells. All percentages derived from 4 fields per view, determined in 3 different infection experiments. MOI for each virus type: 0.01. Ad2: Adenovirus type 2; Ad36 Adenovirus type 36, CMV: Cytomegalovirus, Inf A: Influenza A virus, nd: not detected

CMV (1.8±0.7%), and influenza A (0.2±0.1%); adenovirus 2 infection did not proceed to infect THP-1 cells. Addition of uninfected adipocytes to infected THP-1 cells led to subsequent infection of adipocytes by adenovirus 36 (1.4±0.3%), CMV (1.4±0.5%), and influenza A (0.2±0.1%).

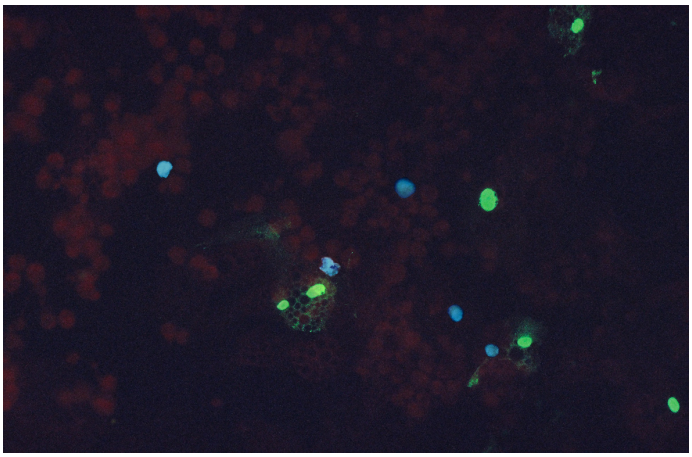


Figure 1 Dual color immunofluorescence image of transinfection of influenza A in a co-culture of adipocytes and THP-1 cells. Influenza infected THP-1 cells were co-cultured with uninfected adipocytes for 48 hours. THP-1 cells infected with influenza A demonstrate blue fluorescence (AMCA label). After 48 hours, influenza infection was transmitted from THP-1 cells to adipocytes, as indicated by apple green fluorescence (FITC label). Background: uninfected adipocytes and THP-1 cells (round) counterstained with Evans Blue (red). Lipid droplets in adipocytes are clearly visible as dark inclusions in the cytoplasm of adipocytes.
(*For color figure see page 222)*

Cytotoxicity

Viral infection of co-cultures did not evoke a substantial cytotoxic reaction, as determined by the release of LDH into the culture medium. Overall cytotoxicity in infected cell cultures relative to uninfected (co-) cultures varied between 0 % and 7.4%. In cell cultures incubated for 48 hours with 1 ng/mL IL-1, cytotoxicity was maximally 11.3%.

PAI-1 production in adipocyte/THP-1 co-cultures

After 48 hours, PAI-1 production by uninfected adipocytes was 199.8 ± 82.6 ng/mL. Neither uninfected THP-1 monocytes nor virus infected THP-1 cells alone, produced PAI-1 (<2 pg/mL; data not shown). In uninfected co-cultures with high THP-1 numbers, PAI-1 production increased, but was not significant (406±74,9 ng/mL; $p>0.05$). There were no significant effects of infection on PAI-1 production by adipocytes, THP-1 cells

or co-cultures except in the co-cultures infected with CMV and a high THP-1 cell/ adipocyte ratio (788±167.3 ng/mL; p<0.05) [**Figure 3**]. Considering the fact that THP-1 cells did not produce PAI-1, it seems obvious that adipocytes were accountable for the increased PAI-1 production, induced by CMV-infection in combination with a high number of THP-1 cells.

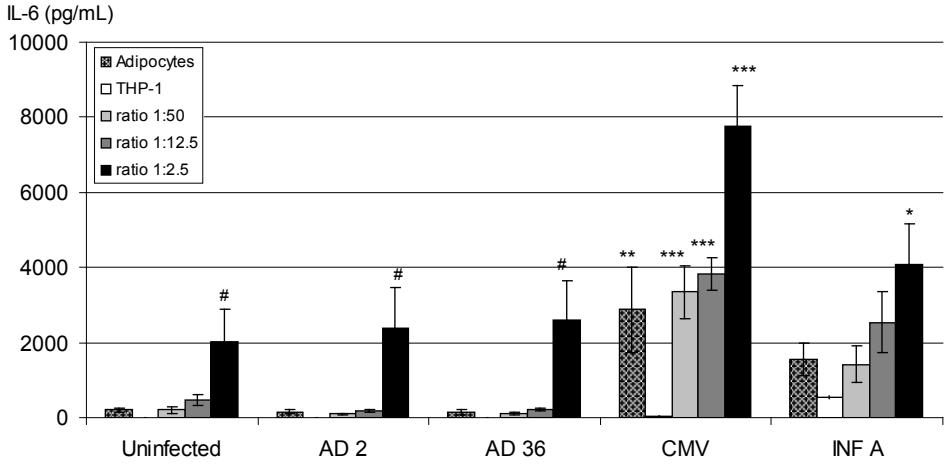


Figure 2 IL-6 production by infected adipocytes and THP-1 cell co-cultures.
Y-axis: IL-6 production in pg/mL. Bars represent control adipocytes without THP-1 cells (▒), control THP-1 cells without adipocytes (□) and adipocytes co-cultured with THP-1 cells. Number of THP-1 cells added to 125,000 adipocytes per well and THP-1 cells/adipocytes ratio (between brackets): ■ 2500 (ratio 1:50), ■ 10,000 (ratio 1: 12.5), ■ 50,000 (ratio 1:2.5). X-axis: Adipocytes co-cultivated with THP-1 cells and infected with adenovirus type 2 (AD2), adenovirus type 36 (AD36), Cytomegalovirus (CMV) and influenza A (INF A) and uninfected adipocytes (uninfected). # p <0.05 compared to uninfected adipocytes. * p <0.05, ** p <0.01, *** p <0.001 compared to uninfected (co-)cultures.

Adiponectin and TNF-α production in adipocyte/THP-1 co-cultures

Adiponectin production by uninfected adipocytes was 13917±850 pg/mL after 48 hours. As expected, THP-1 monocytes did not produce adiponectin. Adiponectin production by adipocytes and monocytes alone or in co-culture was not affected by infection (data not shown). TNF-α production by uninfected adipocytes was 24±6.3 pg/mL; it was 5±1.9 pg/mL by uninfected THP-1 monocytes. TNF-α production by adipocytes and monocytes alone or in co-culture was not affected by infection (data not shown).

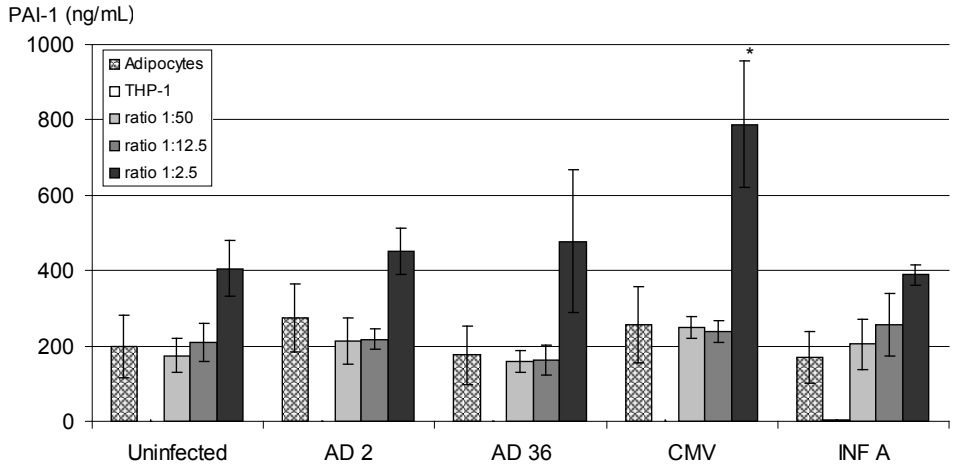


Figure 3 PAI-1 production by infected adipocytes and THP-1 cell co-cultures.
Y-axis: PAI-1 production in ng/mL. Bars represent control adipocytes without THP-1 cells (▒), control THP-1 cells without adipocytes (□) and adipocytes co-cultured with THP-1 cells. Number of THP-1 cells added to 125,000 adipocytes per well and THP-1 cells/adipocytes ratio (between brackets): ■ 2500 (ratio 1:50), ■ 10,000 (ratio 1: 12.5), ■ 50,000 (ratio 1:2.5). X-axis: Adipocytes co-cultivated with THP-1 cells and infected with adenovirus type 2 (AD2), adenovirus type 36 (AD36), Cytomegalovirus (CMV) and influenza A (INF A) and uninfected adipocytes (uninfected). *: $p < 0.05$.

IL-6 production in THP-1 cultures

After 48 hours, uninfected THP-1 cells did not produce IL-6, and neither did adenovirus 2 and 36 infected THP-1. CMV-infected THP only marginally produced IL-6: 40±6 pg/mL, 45±1 pg/mL and 43±1 pg/mL by 2,500, 12,500 and 50,000 THP-1 cells, respectively. IL-6 production by influenza infected THP-1 was 558±128 pg/mL, 627±2 pg/mL and 551±43 pg/mL by 2,500, 12,500 and 50,000 THP-1 cells, respectively (**Figure 2**).

IL-6 production in adipocyte cultures

IL-6 production by adipocytes infected with adenoviruses 2 (148±59 pg/mL, $p>0.05$) and 36 (133±67 pg/mL, $p>0.05$) was similar to that of uninfected adipocytes (210±49 pg/mL). Although IL-6 production by CMV- and influenza infected adipocytes increased to 2877±1130 pg/mL and 1559±442 pg/mL this tendency was not statistically significant (both $p>0.05$ compared to uninfected adipocytes) (**Figure 2**).

IL-6 production in adipocyte/THP-1 co-cultures

IL-6 production in uninfected co-cultures with high THP-1 cells/adipocytes ratio's (1:2.5) was 2004±882 pg/mL ($p<0.05$) compared to IL-6 production in uninfected adipocytes

(210±49 pg/mL). IL-6 production by adenovirus-infected co-cultures with high THP-1 cell numbers (THP cell/adipocyte ratio 1:2.5) was higher, but not significantly so, than in uninfected co-cultures: 2400±1075 pg/mL (adeno 2), and 2608±1056 pg/mL (adeno 36) vs. 2004±882 pg/mL (uninfected); both $p>0.05$). In contrast, IL-6 production was significantly increased in CMV-infected co-cultures of adipocytes and THP-1 cells (3345±715 pg/mL; $p<0.01$ (ratio 1:50), 3838±430 pg/mL; $p<0.01$ (ratio 1:12.5) and 7768±1073 pg/mL; $p<0.001$ (ratio 1:2.5). Influenza A infection only increased IL-6 production in co-cultures with high THP-1 numbers (4081±1073 pg/mL; $p<0.05$) [**Figure 2**].

The increased IL-6 production in these co cultures could be attributable to either adipocytes or THP-1 cells. To determine the relative contribution of THP-1 cells vs. adipocytes to increased IL-6 levels in co cultures, fresh adipocytes and THP-1 cells were incubated with medium recovered from previously infected THP-1 or adipocytes (conditioned media).

IL-6 production in co-cultures after incubation with conditioned media

In **table 2** ratios are given for the relative contribution of THP-1 cells vs. adipocytes to increased IL-6 levels in co cultures. Each ratio was calculated by dividing the measured IL-6 concentration after 48 hours incubation with conditioned media by the input concentrations of the conditioned media themselves. Because the IL-6 contents of the conditioned media were very different, we corrected for these differences by expressing the quotients as a ratio indicative for the relative "de novo IL-6 production". Therefore the ratio's given in table 2 must be interpreted as the active increase of IL-6 after incubation of fresh adipocytes or THP-1 (alone or in co-culture) with conditioned media from previously infected cells [**Table 2**]. The IL-6 concentration in the conditioned medium of CMV-infected adipocytes was 870±238 pg/mL and 370±95 pg/mL in influenza A infected adipocyte conditioned medium. After a 48-hour incubation of THP-1 cells with adipocyte CMV conditioned medium, the measured IL-6 concentration in the culture medium was 1762±1042 pg/mL ($p=0.36$, ratio 2.0); it was 550±177 pg/mL ($p=0.33$; ratio 1.4) after incubation of THP-1 cells with adipocyte influenza A conditioned medium [**Table 2**].

The IL-6 concentration in the conditioned medium of CMV-infected THP-1 cells was 11±2 pg/mL and 117±11pg/mL in influenza A infected THP-1 conditioned medium. After a 48-hour incubation of adipocytes with THP-1 CMV conditioned medium, the measured IL-6 concentration in the culture medium was 1038±204 pg/mL ($p=0.01$;

ratio 76.5); it was 639±149 pg/mL (p=0.03; ratio 3.8) after incubation of THP-1 cells with adipocyte influenza A conditioned medium [**Table 2**]. Conditioned medium from adenovirus 2 or 36 infected adipocytes and THP-1 did not induce any changes in IL-6 production.

Immunofluorescence on cells that were incubated with the respective conditioned media, did not demonstrate any viable virus, ruling out the possibility that viral presence could have accounted for the observed effects.

Table 2 IL-6 production after incubation with conditioned media

	uninfected	Ad 2	Ad 36	CMV	inf A
AD-CM THP	59 ± 12	66 ± 14	73 ± 11	1762 ± 1042	550 ± 177
Ratio	0.6	1.3	1.6	2.0	1.4
AD-CM co-culture	361 ± 4	310 ± 27	364 ± 31	1490 ± 583	772 ± 79
Ratio	0.4	-1.0	0.8	1.3	1.2
THP-CM adipocytes	229 ± 38	266 ± 62	242 ± 41	1038 ± 204	639 ± 149
Ratio	2.4	4.7	8.6	76.5 *	3.8 *
THP-CM co-culture	382 ± 65	388 ± 71	365 ± 22	1224 ± 509	802 ± 191
Ratio	2.5	2.9	4.0	79.5 *	3.9 *

IL-6 concentrations (mean ± SE in pg/mL, n=2) after incubation with conditioned medium from adipocytes and THP-1 cells. Ad2: adenovirus subtype, Ad36: adenovirus subtype 36, CMV: cytomegalovirus, inf A: influenza A. AD-CM THP: adipocyte conditioned medium incubated with THP-1 cells, AD-CM co-culture: adipocyte conditioned medium added to co-cultures, THP-CM adipocytes: THP-1 cell conditioned medium added to adipocytes, THP-CM co-culture: THP-1 cell conditioned medium added to co-cultures of adipocytes and THP-1 cells. *Ratio*: de novo IL-6 synthesis expressed as ratio of measured IL-6 concentrations against IL-6 present in the conditioned medium used for incubation after subtraction of baseline values. * indicates statistically significant (P<0.05) increase in de novo IL-6 production.

Discussion

Obesity induces an inflammatory state in adipose tissue [6, 10, 24]. Elevated plasma concentrations of inflammatory cytokines are associated with an increased risk of metabolic syndrome, type 2 diabetes, and cardiovascular diseases [25-27]. The production of various (pro-atherogenic) chemokines and other factors, including angiotensin, free fatty acids, leptin, and PAI-1, by adipose tissue may contribute to the pathogenesis of these diseases. In the present study, we showed that both human adipocytes and THP-1 monocytes can be infected by adenoviruses 36, CMV, and influenza A viruses.

Adipocytes are also susceptible to adenovirus type 2, but THP-1 monocytes are refractory to this virus type. The percent of infection in monocytes by adenovirus 36 and CMV was 2-fold higher than that in adipocytes. This difference was even more pronounced for influenza A, with virus infecting more THP-1 cells than adipocytes (4.0±0.6% in THP-1 vs. 0.2±0.1% in adipocytes). Infected adipocytes transmitted the infection to monocytes and vice versa (transinfection). In general, the rate of transinfection was 50% of that of direct infection.

In this study, infections with CMV and influenza evoke an inflammatory response in co-cultivated adipocytes and monocytes, as measured by enhanced IL-6 and PAI-production.. Circulating TNF-α and IL-6 plasma concentrations are mildly elevated in obesity [28]. IL-6 not only predisposes cells to become insulin resistant but also enhances the hepatic production of acute-phase proteins, such as C-reactive protein or fibrinogen [29, 30]. Therefore, obesity may represent a state of chronic low-grade inflammation accompanied by adipocyte dysfunction, as reflected by enhanced production of IL-6. This may also provide a link between obesity and the development of associated vascular complications such as atherosclerosis [31-33]. Previously we showed that viral infection adipocytes stimulated IL-6 production [22]. In the present study, infection with CMV and influenza further accelerates IL-6 production in co-cultures of adipocytes and THP-1 cells. This extensive IL-6 production may be explained by the ability of infected adipocytes as wells as monocytes to produce IL-6 and that both infection and monocyte numbers significantly contributed to this. Monocytes do not constitutively produce PAI-1. Nevertheless, IL-6 and other soluble factors produced by monocytes may exert paracrine actions on adipocytes, suggesting a pathway by which adipocytes *in vitro* can be stimulated to produce elevated PAI-1 levels.

Our results are consistent with those of recent studies with animal cell models. In a co-culture model with murine 3T3-L1 adipocytes and murine macrophage-like cell line RAW264 (peritoneal macrophages), the production of IL-6, TNF-α, and monocyte chemotactic protein-1 (MCP-1) was up regulated whereas that of adiponectin was down regulated [34]. Also, the duration of co-culture influences the increase in TNF-α mRNA-expression in co-cultures of 3T3-L1 and THP-1 [35]. Incubation with bacterial lipopolysaccharide in a co-culture of 3T3-L1 and RAW264 did not influence TNF-α production, but increased IL-6 production 100-fold, suggesting an exaggerated biological interaction between macrophages and adipocytes in persistent low-grade

infection by gram-negative bacteria [36].

The increased production of IL-6 in co-cultures may be mediated by cell-cell contact, because the production of IL-6 increased with the number of THP-1 monocytes added to the co-cultures. However, it is more likely that locally produced inflammatory substances produced by dysfunctional adipocytes and adipose tissue macrophages exerted autocrine/paracrine effects on neighboring cells. Conditioned media from uninfected or virus-infected adipocytes did not stimulate IL-6 production by THP-1 cells or co-cultures, whereas incubation of adipocytes and co-cultures with conditioned medium from CMV-infected THP-1 led to a nearly 80-fold increase in IL-6 production. Infective virus was not detected in the conditioned media, indicating that there was no lytic infection and that *de novo* IL-6 synthesis was not a direct effect of infection. Moreover, this suggests that the observed effects depended on cross-talk between cells that was orchestrated by soluble factors produced by infected cells in the culture environment. This is consistent with the observation that the release of inflammatory cytokines in adipose tissue samples from obese subjects is mainly due to the production of cytokines by cells other than adipocytes [37]. Adipose tissue macrophages are able to produce extensive amounts of pro-inflammatory mediators, which could contribute to the development of insulin resistance [12]. Here, we conclude that the paracrine effects of soluble factors (such as IL-6 or TNF-α) released by infected macrophages, rather than by infected adipocytes, were responsible for the increased production of IL-6 in co-cultures. The transinfection phenomenon we demonstrated may explain how infectious agents invade several parts of the body and infect cells of different organ systems, such as vessel walls and adipose tissue, thus contributing to local and systemic inflammatory states.

Microorganisms, such as CMV or influenza A, that infect adipose tissue may initiate and perpetuate a chronic inflammatory response, attracting more macrophages into the already inflamed tissue and exacerbating the total inflammatory response to the infection. The results of the present study further support the concept that both adipocytes and adipose tissue macrophages are involved in adipokine production and that their concerted actions synergistically contribute to increased production of IL-6 and PAI-1, reflecting the inflammatory state associated with obesity. Although adipocytes are the most abundant cell type in adipose tissue, the extent or recruitment of (infected) macrophages is likely to be decisive for the inflammatory state of adipose tissue. It is also conceivable that infected monocytes invade adipose tissue.

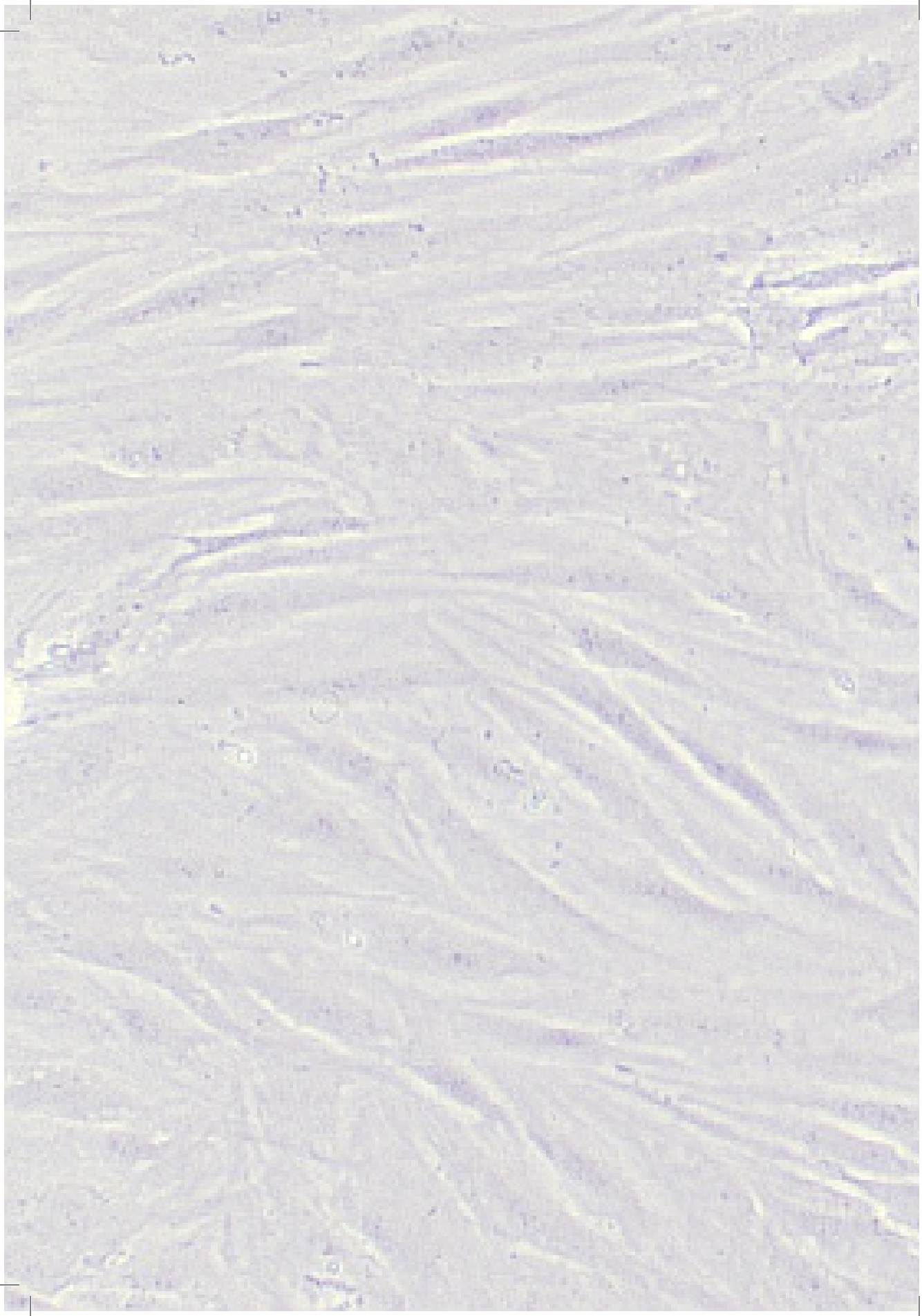
In conclusion, adipocytes and monocytes can become infected by several viruses *in vitro*, leading to an inflammatory response. Transinfection occurs from adipocytes to monocytes and vice versa. The relative contribution of monocytes to the production of IL-6 in co-cultures is likely to be higher than that of adipocytes.

Reference List

1. Hutley L, Prins JB. Fat as an endocrine organ: relationship to the metabolic syndrome. Am J Med Sci 2005;330(6):280-9.
2. Rajala MW, Scherer PE. Minireview: The adipocyte-at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 2003;144(9):3765-73.
3. Cousin B, Caspar-Bauguil S, Planat-Benard V, Laharrague P, Penicaud L, Casteilla L. [Adipose tissue: a subtle and complex cell system]. J Soc Biol 2006;200(1):51-7.
4. Bastard JP, Maachi M, Lagathu C et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw 2006;17(1):4-12).
5. Xu H, Barnes GT, Yang Q et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003;112(12):1821-30.
6. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest 2003;112(12):1785-8.
7. Matsuzawa Y. The metabolic syndrome and adipocytokines. FEBS Lett 2006;580(12):2917-21.
8. Kintscher U, Hartge M, Hess K et al. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arterioscler Thromb Vasc Biol 2008;28(7):1304-10.
9. Cinti S, Mitchell G, Barbatelli G et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 2005;46(11):2347-55.
10. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112(12):1796 -808.
11. Zeyda M, Stulnig TM. Adipose tissue macrophages. Immunol Lett 2007;112(2):61-7.
12. Zeyda M, Farmer D, Todoric J et al. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. Int J Obes (Lond) 2007;31(9):1420-8.
13. Nieto FJ. Infective agents and cardiovascular disease. Semin Vasc Med 2002;2(4):401-15.
14. Prasad A, Zhu J, Halcox JP, Waclawiw MA, Epstein SE, Quyyumi AA. Predisposition to atherosclerosis by infections: role of endothelial dysfunction. Circulation 2002;106(2):184-90.

15. Gurfinkel E. Link between intracellular pathogens and cardiovascular diseases. Clin Microbiol Infect 1998:Suppl 4:S33-S36.
16. Morre SA, Stooker W, Lagrand WK, van den Brule AJ, Niessen HW. Microorganisms in the aetiology of atherosclerosis. J Clin Pathol 2000;53(9):647-54.
17. Dhurandhar NV, Kulkarni PR, Ajinkya SM, Sherikar AA, Atkinson RL. Association of adenovirus infection with human obesity. Obes Res 1997;(5):464-9.
18. Dhurandhar NV. Infectobesity: obesity of infectious origin. J Nutr 2001;131(10):2794S-2797S..
19. Dhurandhar NV. Contribution of pathogens in human obesity. Drug News Perspect 2004;17(5):307-13.
20. Atkinson RL. Viruses as an etiology of obesity. Mayo Clin Proc 2007;82(10):1192-8.
21. So PW, Herlihy AH, Bell JD. Adiposity induced by adenovirus 5 inoculation. Int J Obes (Lond) 2005;29(6):603-6.
22. Bouwman JJ, Visseren FL, Bouter KP, Diepersloot RJ. Infection-induced inflammatory response of adipocytes in vitro. Int J Obes (Lond) 2008;32(6):892-901..
23. Reed LJ, Muench J. A simple method for estimating fifty percent endpoints. Am J Hyg 27;493. 1938.
24. Cancello R, Clement K. Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. Bjog 2006;113(10):1141-7.
25. Duncan BB, Schmidt MI, Pankow JS et al. Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. Diabetes 2003;52(7):1799-805.
26. Festa A, D'Agostino R, Jr., Tracy RP, Haffner SM. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes 2002;51(4):1131-7.
27. Jialal I, Devaraj S, Venugopal SK. C-reactive protein: risk marker or mediator in atherothrombosis? Hypertension 2004;44(1):6-11.
28. Roytblat L, Rachinsky M, Fisher A et al. Raised interleukin-6 levels in obese patients. Obes Res 2000;8(9):673-5.
29. Esteve E, Castro A, Lopez-Bermejo A, Vendrell J, Ricart W, Fernandez-Real JM. Serum interleukin-6 correlates with endothelial dysfunction in healthy men independently of insulin sensitivity. Diabetes Care 2007;30(4):939-45.
30. Paik JK, Kim OY, Koh SJ et al. Additive effect of interleukin-6 and C-reactive protein (CRP) single nucleotide polymorphism on serum CRP concentration and other cardiovascular risk factors. Clin Chim Acta 2007;380(1-2):68-74.
31. Calabro P, Yeh ET. Obesity, inflammation, and vascular disease: the role of the adipose tissue as an endocrine organ. Subcell Biochem 2007;42:63-91.
32. Juge-Aubry CE, Henrichot E, Meier CA. Adipose tissue: a regulator of inflammation. Best Pract Res Clin Endocrinol Metab 2005;19(4):547-66.

33. Mehta S, Farmer JA. Obesity and inflammation: a new look at an old problem. Curr Atheroscler Rep 2007;9(2):134-8.
34. Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. Arterioscler Thromb Vasc Biol 2005;25(10):2062-8.
35. Unoki H, Bujo H, Jiang M, Kawamura T, Murakami K, Saito Y. Macrophages regulate tumor necrosis factor-alpha expressio! in adipocytes through the secretion of matrix metalloproteinase-3. Int J Obes (Lond) 2008;32(6):902-11.
36. Yamashita A, Soga Y, Iwamoto Y et al. Macrophage-adipocyte interaction: marked interleukin-6 production by lipopolysaccharide. Obesity (Silver Spring) 2007;15(11):2549-52.
37. Fain JN. Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. Vitam Horm 2006;74:443-77.



Discussion

Summary

Samenvatting

Dankwoord

Curriculum vitae

Color figures

Inflammation, infection, and atherosclerosis

Chronic low-grade inflammation, as is often seen in atherosclerosis, obesity, diabetes and the metabolic syndrome, is a component of many diseases. The presence of inflammation in atherosclerosis is evidenced by elevated plasma concentrations of a variety of markers, including C-reactive protein (CRP), tumor necrosis factor (TNF)-α, and several interleukins and adhesion molecules. Inflammation is critically involved in the destabilization of atheroma in the vascular wall, with the stability of arterial plaques being determined by the severity of inflammation. The origin of the inflammation, however, is unclear. Vascular damage may trigger inflammation, but most likely inflammation originates from (excessive) abdominal adipose tissue. The release of cytokines exacerbates the existing insulin resistance and endothelial dysfunction, which play a central role in the pathogenesis and development of atherosclerosis and thus promote the atherogenic process. Potential triggers for local vascular inflammation are traditional risk factors for artery disease, such as hypercholesterolemia, hypertension, diabetes, free radicals, low levels of high-density lipoprotein cholesterol, and elevated or modified LDL. Chronic or repetitive infections may be triggers for chronic inflammation[1,2]. However, atherosclerosis is a multifactorial process and cannot be explained solely in terms of the traditional cardiovascular risk factors, and intracellular infections have been suggested as a direct cause of atherosclerosis. Several clinical and experimental studies suggest that various infectious agents such as *Chlamydia pneumoniae* (*Cp*) or herpes viruses may be the cause of chronic inflammation in lesions, but to date data about a direct causal relation between infections with these pathogens and atherosclerosis are conflicting. In the *in vitro* studies presented in this thesis, we investigated the mechanisms by which pathogens may function as etiological factors for atherosclerosis.

Pathogens involved in atherogenesis

Cytomegalovirus (CMV) and *Cp,* in particular, have been associated with the presence and development of cardiovascular diseases[3-5]. Results from animal studies and *in vitro* studies show that *Cp* can modulate atheroma biology, including lipid- and inflammatory-related processes[6]. CMV infection of endothelial cells and macrophages has an important role in the establishment of latency and persistence, which are critical for the maintenance of CMV in the host. Infection with CMV can stimulate processes related to the development of atherosclerosis[7]. The replication of CMV,

however, appears to be dependent on the specific origin and tropism of endothelial cells and macrophages[8]. In patients with documented coronary artery disease and elevated levels of interleukin 6 (IL-6), CMV seropositivity was independently associated with future cardiac mortality. These data support the hypothesis that the atherosclerotic effects of CMV are mediated through an underlying inflammatory response[9]. In contrast to CMV and *Cp*, influenza virus causes more acute infections with severe (systemic) complications. Influenza induces substantial inflammation of the vessel wall and may trigger plaque destabilization, which leads to acute coronary syndromes[10]. In the studies presented in this thesis, we infected endothelial cells, hepatocytes, adipocytes and monocytes with *Cp*, CMV, and influenza A *in vitro*. We investigated which of the effects caused by these infections could account for the impairment of vascular function and atherogenesis.

It has been proposed that obesity represents a chronic inflammatory state that has multiple implications for cardiovascular morbidity and mortality[11,12]. Because adenoviruses have been associated with obesity[13] and may indirectly contribute to the development of cardiovascular diseases, we also infected adipocytes with adenovirus. We found that infection with adenovirus 36 increased the production of pro-inflammatory IL-6.

Properties of intracellular pathogens

The microorganisms we used in most of our studies (CMV, *Cp,* and influenza virus) have repeatedly been shown to be associated with cardiovascular disorders. Nevertheless, these microorganisms have their own distinct properties. Although most of these viruses cause infection, we found that the effects of intracellular infections differed by virus type, indicating that infection-dependent mechanisms are virus specific. To test the specificity of the association of these viruses with cardiovascular disease, we also tested other viruses not associated with cardiovascular disease, such as parainfluenza (chapter 2), RSV (chapters 2,7), and adenovirus type 2 (chapters 7,8). We also used heat-inactivated viruses to determine whether the measured effects really were infection dependent. In some studies we found striking differences. For instance, infection of human umbilical vein endothelial cells (HUVEC) and monocytes with CMV and *Cp*, but not with influenza virus, significantly reduced the clotting time. In contrast, interleukin production was higher after infection with influenza virus than after infection with CMV or *Cp*. (chapters 2,3). In studies with adipocytes,

we demonstrated that infection with Ad36, but not Ad2, increased IL-6 production by pre-adipocytes and adipocytes (chapter 7). We also demonstrated that Ad36 readily infected monocytes whereas Ad2 was not capable of infecting monocytes (chapter 8). This may explain why Ad2 is associated with more superficial (respiratory) infections and not with systemic infections, which supports our choice of using Ad2 as a 'control virus' in this study. These differences show that infecting microorganisms adversely affect cell function via distinct mechanisms in a tissue-dependent manner.

Infective progeny virus may invade cells involved in atherogenesis and be disseminated to other cells. Intracellular infections *in vivo* may become latent or even persistent if the viral genome integrates into a host chromosome and only few viral proteins are synthesized because viral genes are not, or poorly, expressed. Even in such situations, viruses may still be capable of having a deleterious effect on cells. For example, CMV mimics host proteins and may behave as a molecular pirate, using clever strategies to gain cell entry, manipulate host gene regulation, and cause immune evasion[14]. Highjacking the cellular machinery alters cellular function and may result in cellular dysfunction and inflammatory reactions, which are a hallmark of atherosclerosis.

Infection of various cells by various pathogens leading to different cellular responses: implications for atherogenesis

Infections with the microorganisms used in the experiments described in this thesis are common. One of the ports of entry is the respiratory tract, and community-acquired respiratory infections with multiple microorganisms are common. Even though respiratory infections may be seasonal, clinical studies suggest that acute respiratory tract infection may be a risk factor for acute coronary syndromes[15,16]. During influenza epidemics, the mortality rate for cardiovascular syndromes increases[17,18]. The number of deaths due to ischemic heart disease, hypertension, and cerebrovascular disease peaks 2 weeks after the influenza infection peaks[10,19]. Atherosclerosis however, is a chronic disease that begins in early life with the formation of fatty streaks and may progress to cause severe adverse effects in adult life. Individuals are infected with a variety of microorganisms during their lifetime. Viruses replicate in the epithelial cells of the respiratory tract and release infective virus in the vicinity of alveoli. In this way, neighboring endothelial cells in vessel walls aligning lung tissue may become infected. It is highly likely that virus can spread hematogenously, either as free virus (viremia) or in macrophages. In this way, a virus can be transported to remote tissues

and directly infect healthy endothelium and atherosclerotic plaques. It is not known whether viruses infect plaques but several studies have shown the presence of viral sequences in cells of atherosclerotic plaques[20-25]. An important question is whether microorganisms need to enter the vessel wall in order to elicit immune responses. Local inflammatory effects could induce arterial wall inflammation and thereby trigger plaque destabilization or even rupture of vulnerable plaques. In this way, what starts as local vessel wall inflammation could develop into systemic (low grade) inflammation. In our studies, we demonstrated that various microorganisms are able to infect endothelial cells (chapters 2, 5 and 6), monocytes (chapters 3 and 8), hepatocytes (chapter 4), and (pre-)adipocytes (chapters 7, 8). Intracellular infection of these cells *in vitro* leads to cellular dysfunction and initiates procoagulant activity, hypofibrinolysis, impaired cellular function, and inflammation, as reflected by increased expression of pro-inflammatory cytokines. This may contribute to local and/or systemic inflammation. There was a remarkable resemblance in how monocytes, hepatocytes, endothelial cells, and adipocytes reacted to infection, with the activation of processes that could be involved in atherogenesis. This suggests that not only direct infection of the vessel wall but also indirect infection by transmission of viruses to tissues elsewhere in the body may initiate a systemic inflammatory and procoagulant state.

In a co-culture model, we demonstrated that viruses were able to disseminate from infected adipocytes to monocytic cells and vice versa (chapter 8), a phenomenon that we termed 'transinfection'. This phenomenon may be quite similar to the ability of viruses to infect other cell types after a primary infection. This is in line with the demonstration that monocytes were able to transmit infectious CMV in a co-culture model of peripheral blood mononuclear cells and CMV-infected endothelial cells. Thus the endothelium and circulating monocytes have an interactive role in the dissemination of virus[26]. Taken together, these findings provide a mechanism by which viruses are able to reach various tissues. Moreover, they show that infectious agents can invade several parts of the body and infect cells of different organs, such as the vessel wall and adipose tissue, contributing to local and systemic inflammatory states (**Figure 1**).

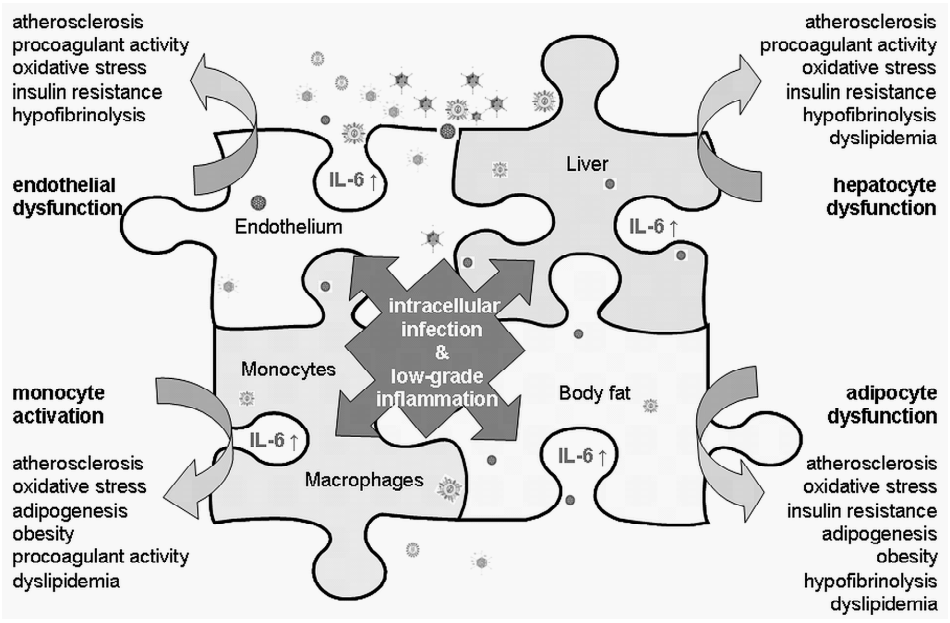


Figure 1 The concept of intracellular infections in the etiology of vascular diseases.
A variety of microorganisms are able to infect cells and organs *in vitro*. Intracellular infection of endothelial cells, liver cells, fat cells and monocytes/macrophages leads to cellular dysfunction, as reflected by procoagulant activity, hypofibrinolysis, and (chronic) local or systemic low-grade inflammation. Impaired cellular function with subsequent inflammation is reflected by increased expression of pro-inflammatory cytokines. Increased IL-6 production is a predominant key feature of an altered secretion profile in infected cells and may be considered as a marker for cellular dysfunction. Endothelial, monocyte, hepatocyte, and adipocyte dysfunction caused by intracellular infections may contribute to local and systemic inflammation, with subsequent effects on vascular function that may contribute to the development of cardiovascular diseases. In addition, cross-talk between cells of the immune system, cells of remote tissues (liver, fat), and the vessel wall, mediated by cytokines and other pro-inflammatory substances, is responsible for the cascade effects leading to exacerbation of vascular disorders.

Aspects of *in vitro* culture experiments

The methods we used to infect cells *in vitro* may not mimic the mechanisms by which cells become infected *in vivo*. We acknowledge this as a limitation of our studies but our aim was to mimic chronic low-grade intracellular infection, consistent with the concept of atherosclerosis being a (low-grade) chronic process. In this respect, it was important to avoid lytic infection, which could overrule the specific effects initiated by infection. We always carefully monitored cytotoxicity, and the severity of infection was calculated and monitored by immunofluorescence. Obviously, infections rates

were dependent on the nature and strain of the microorganism involved and on the susceptibility of cells to become infected. This was also illustrated in a study in which CMV infection resulted in a lytic infection in brain microvascular endothelial cells but not in aortic macrovascular cells. This could indicate that aortic macrovascular cells act as a reservoir of virus in the host[8]. Other experimental data show that RNA from influenza virus is present in atherosclerotic plaques but not in undamaged vessel[27]. This tropism may also explain how pathogens become internalized, especially into atherosclerotic plaques. It is difficult to develop an experimental model of persistent infection because, for example, while chemicals can be used to suppress the replication of intracellular organisms, they will also influence or stop cellular metabolism.

Detection of microorganisms involved in atherosclerosis and vascular disease

Given the nature of persistency, it is conceivable that the role of intracellular pathogens in atherogenesis is underestimated in clinical studies. A lack of standardized methods and inconsistent use of diagnostic procedures may give rise to discrepant results[28]. There is no "gold standard" for diagnostic methods to detect infection. Viral cultures, immunoassays, serologic and PCR methods may be insensitive, and performance and criteria are not always well defined. These considerations are important because inadequate detection may lead to ineffective treatment. Moreover, treatment may be inadequate if a single infection is targeted, especially because the effectiveness of treatment is probably determined by the pathogen burden. The importance of pathogen burden was clearly demonstrated in a study involving consecutive patients undergoing elective carotid endarterectomy. The *Cp* burden was associated with plaque expression of IL-6, which in turn was associated with serum levels of IL-6 and CRP[29]. Moreover, multifactorial disease processes, such as those involved in atherosclerosis, are unlikely to be activated by a single microbe or vascular risk factor. Several studies indicate that infections with multiple pathogens are common[30] and could contribute to coronary artery disease by precipitating inflammation and endothelial cell injury[31,32]. Increasing pathogen burden is also associated with an increased risk of myocardial infarction and death in patients with known coronary heart disease[33-35]. Therefore, the cumulative effects of simultaneous infections at various sites and/or repetitive infections may contribute to the pathogenesis and progression of atherosclerosis[36,37].

Evidence for a causal role of infections in atherogenesis ?

The role of infections in endothelial injury and vascular wall inflammation has been studied extensively but evidence for a causal relation is still lacking[2,4,38,39]. Several infectious agents have been investigated but none has proved to play a causative and specific role. *Cp* has been studied the most extensively, but the results of large clinical trials of antibiotics against this disease have on the whole been disappointing[40,41]. Nevertheless, certain infections are now considered to be a risk factor for atherosclerosis development, but current data do not allow us to determine whether infection is a cause or a co-factor in atherogenesis. Various criteria have been proposed to help discriminate between causal and non-causal associations. Atherosclerosis has been proposed as an infectious disease, but there is little evidence for this according to Koch's postulates[42] . Koch stated that the host infection must lead to disease and that the pathogen must be isolated from the infected host and should be present in all infected persons. However, it may not be correct to apply these criteria to chronic diseases. Apart from the fact that atherosclerosis is multifactorial disorder, the main characteristics of chronic infections are precisely that microorganisms are latent or persistent and may be hard to culture, if viable at all. It is possible that viral proteins are synthesized *de novo*, but that there is no or little productive infection. Therefore, some of Hill's criteria for causality may be more applicable for determining the causality of infection in atherosclerosis[43,44]. The results of the studies presented in this thesis meet most of these criteria, namely, strength of association, consistency, coherence, biologic plausibility, and dose-response relationship. An overview of the results from our studies shows that infection of various cell types consistently resulted in pro-inflammatory and procoagulant activity, hypofibrinolysis, and cytokine production, and that these effects were time and dose dependent. In experiments with *Cp*, treatment of hepatocytes (chapter 4) and endothelial cells (chapter 5) with macrolide antibiotics markedly decreased the inflammatory effects of the infection. As for biologic plausibility, the procoagulant effects of influenza infection *in vitro* are consistent with the clinical observation of coagulation disorders as complications of influenza. Causality between infections and occurrence of vascular diseases is difficult to establish in clinical studies. In the present thesis, several *in vitro* studies are presented showing that various infections are able to trigger cellular responses involved in atherogenesis. We therefore propose a causal role for intracellular pathogens in the etiology of vascular diseases.

Involvement of immune cells in atherogenesis

Macrophages are central mediators of cellular innate and adaptive immunity and are essential to the initiation and progression of vascular lesions. Once activated, they initiate the oxidation of LDL and rapidly take up oxidized LDL through specific scavenger receptors, leading to foam-cell formation. Activated macrophages also secrete a variety of pro-inflammatory products that affect lesion progression and plaque stability[45]. Although immune responses may rely mainly on tissue-resident macrophages, the infiltration of activated monocytes (and their differentiation into macrophages) into tissues is markedly increased under inflammatory conditions. Oxidized LDL and locally secreted chemokines affect the chemotactic migration of monocytes and T-cells, but not neutrophils, into the subendothelial space. In atherosclerosis, the recruitment of monocytes is essential for lesion formation. T- and B-cells may also have proatherogenic effects but are probably not obligatory for lesion initiation or progression. In the studies described in chapter 3 and chapter 9, we showed that monocytes and macrophages are susceptible to infection and can be infected directly or via transinfection. The observation that monocytes may also function as vehicles for delivering infectious agents to tissues (vessel wall, liver, abdominal fat) contributes to our understanding of the increased tissue inflammation seen in obesity. We and others have demonstrated that obesity is accompanied by inflammation, as evidenced by the overproduction of several adipokines[46]. Increased macrophage recruitment into various tissues may exacerbate the total inflammatory response to the infection and may have potent proatherogenic effects in the vasculature. Cross-talk between cells of the immune system, cells of other tissues (liver, fat), and the vessel wall, mediated by cytokines and other proinflammatory substances, is responsible for initiating a cascade of effects leading to vascular disorders. We conclude that monocyte and/or macrophage activation plays a central role in infection-induced inflammation, potentially contributing to atherogenesis.

Can *in vitro* experimental results be extrapolated to the *in vivo* situation?

While most suspected infectious agents have an atherogenic effect by initiating or aggravating a chronic vascular or systemic inflammatory process, influenza may have a rather different effect by triggering destabilization of already vulnerable plaques[47]. However, the current body of evidence is not conclusive about the causal relationship

between pathogens and cardiovascular disease. Animal and pathological studies probably provide the best evidence that infections have effects *in vivo* similar to those seen in atherosclerosis. Several studies have shown that *Cp* induces atherosclerosis in animals[48,49]. Moreover, inoculation of chicken with Ad 36 virus induces adiposity[50,51]. This may be associated with inflammation and subsequent vascular complications. Evidence that CMV causes atherosclerotic disease comes from studies of the development of atherosclerosis in transplant recipients[52-54]. CMV-negative recipients of CMV-positive donor hearts have an impaired epicardial endothelial function and an increased incidence of cardiovascular-related events and death during follow-up. CMV infection may contribute to allograft failure by accelerating coronary endothelial dysfunction[55].

In clinical studies, it is difficult or even impossible to distinguish between cause and effect. Even intervention trials do not confirm the role of pathogens in vascular disease. It is possible that pathogens are not associated with atherosclerosis at all, but the overall results of clinical and laboratory studies suggest that there is such a relation. The inconclusive results of intervention trials are probably due to the methodological shortcomings, such as underpowering, transient short-term effects, and low-dosage and low-duration regimens. Moreover, treatment of patients with cardiovascular diseases with conventional antibiotics may be ineffective because microorganisms become persistent or less susceptible to antibiotics.

The validity of using *in vitro* study results to explain *in vivo* events could be questioned. Cultured cells may not respond to infection in the same way as cells cultured from tissue explants or primary cells. Moreover, conclusions based on *in vitro* findings cannot necessarily be extrapolated directly to the *in vivo* situation, and clinical implications should be interpreted with caution. While the methods currently used for the culture and propagation of *C. pneumoniae* may not be analogous to the mechanisms by which the infection is propagated *in vivo*, the results of our *in vitro* studies suggest that antibiotic treatment of infection-induced atherosclerosis may improve endothelial function by restoring normal cell function. This may be the case not only *in vitro* but also *in vivo*. Another i*n vitro* study indicated that continuous *C. pneumoniae* infections may more closely resemble the events that occur *in vivo* and, therefore, may be a good model for the *in vitro* study of *C. pneumoniae* infection. In the same study macrolides did not completely eliminate the organism. This may be important because the failure of antibiotic therapy against *C. pneumoniae* infection in humans has been reported[56]. *In vitro* studies, such as those presented in this thesis, remain essential for providing

insight into the mechanisms by which microorganisms exert potential adverse effects on the vasculature.

Concluding remarks

In conclusion, the results of the studies presented in this thesis suggest that intracellular infections have a causal role in the etiology of vascular diseases. We suggest that intracellular microorganisms should be added to the list of factors that induce endothelial dysfunction and contribute to the development of atherosclerosis. We identified inflammation, procoagulant activity, and hypofibrinolysis to be major outcomes of intracellular infection of different types of cells. The *in vitro* studies presented in this thesis identified at least some of the mechanisms by which intracellular infections may contribute to the development of cardiovascular diseases.

Reference List

1 Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. Circulation 1999;100 (4):e20-8.

2 Libby P, Egan D, Skarlatos S. Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research. Circulation 1997;96 (11):4095-103.

3 Capron L. Role of infectious agents in the understanding of atherosclerosis. Rev Med Interne 2002 ;23 Suppl 1:8s -9s.

4 Kuo CC, Campbell LA, Rosenfeld ME. Chlamydia pneumoniae infection and atherosclerosis: methodological considerations. Circulation 2002;105(4):E34.

5 Higuchi ML, Sambiase N, Palomino S, Gutierrez P, Demarchi LM, Aiello VD, Ramires JA. Detection of Mycoplasma pneumoniae and Chlamydia pneumoniae in ruptured atherosclerotic plaques. Braz J Med Biol Res 2000;33(9):1023-6.

6 Kalayoglu MV, Libby P, Byrne GI. Chlamydia pneumoniae as an emerging risk factor in cardiovascular disease. Jama 2002 4;288(21):2724-31.

7 Bruggeman CA. Does Cytomegalovirus Play a Role in Atherosclerosis? Herpes 2000;7(2):51-54.

8 Jarvis MA, Nelson JA. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. Curr Opin Microbiol 2002;5(4):403-7.

9 Blankenberg S, Rupprecht HJ, Bickel C, Espinola-Klein C, Rippin G, Hafner G, Ossendorf M, Steinhagen K, Meyer J. Cytomegalovirus infection with interleukin-6 response predicts cardiac mortality in patients with coronary artery disease. Circulation 2001;103(24):2915-21.

10 Madjid M, Aboshady I, Awan I, Litovsky S, Casscells SW. Influenza and cardiovascular disease: is there a causal relationship? Tex Heart Inst J 2004;31(1):4-13.

11 Yudkin JS. Adipose tissue, insulin action and vascular disease: inflammatory signals. Int J Obes Relat Metab Disord 2003; Suppl 3:S25-8.

12 Calabro P, Yeh ET. Obesity, inflammation, and vascular disease: the role of the adipose tissue as an endocrine organ. Subcell Biochem 2007;42 :63-91.

13 Dhurandhar NV, Kulkarni PR, Ajinkya SM, Sherikar AA, Atkinson RL. Association of adenovirus infection with human obesity. Obes Res 1997;5(5):464-9.

14 Boomker JM, van Luyn MJ, The TH, de Leij LF, Harmsen MC. US28 actions in HCMV infection: lessons from a versatile hijacker. Rev Med Virol 2005;15(4):269-82.

15 Meier CR, Jick SS, Derby LE, Vasilakis C, Jick H. Acute respiratory-tract infections and risk of first-time acute myocardial infarction. Lancet 1998;351(9114):1467-71.

16 Harskamp RE, van Ginkel MW. Acute respiratory tract infections: a potential trigger for the acute coronary syndrome. Ann Med 2008 ;40 (2):121-8.

17 Simonsen L, Clarke MJ, Williamson GD, Stroup DF, Arden NH, Schonberger LB. The impact of influenza epidemics on mortality: introducing a severity index. Am J Public Health 1997;87(12):1944-50.

18 Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K. Mortality associated with influenza and respiratory syncytial virus in the United States. Jama 2003;289(2):179-86.

19 Glezen WP, Payne AA, Snyder DN, Downs TD. Mortality and influenza. J Infect Dis 1982;146(3):313-21.

20 Kaplan M, Yavuz SS, Cinar B, Koksal V, Kut MS, Yapici F, Gercekoglu H, Demirtas M. Detection of Chlamydia pneumoniae and Helicobacter pylori in atherosclerotic plaques of carotid artery by polymerase chain reaction. Int J Infect Dis 2006;10(2):116-23.

21 Ouchi K, Fujii B, Kudo S, Shirai M, Yamashita K, Gondo T, Ishihara T, Ito H, Nakazawa T. Chlamydia pneumoniae in atherosclerotic and nonatherosclerotic tissue. J Infect Dis 2000;181 Suppl 3:S441-3..

22 Yamashita K, Ouchi K, Shirai M, Gondo T, Nakazawa T, Ito H. Distribution of Chlamydia pneumoniae infection in the athersclerotic carotid artery. Stroke 1998;29(4):773-8.

23 Kwon TW, Kim DK, Ye JS, Lee WJ, Moon MS, Joo CH, Lee H, Kim YK. Detection of enterovirus, cytomegalovirus, and Chlamydia pneumoniae in atheromas. J Microbiol 2004 ;42(4):299-304.

24 Hu W, Liu J, Niu S, Liu M, Shi H, Wei L. Prevalence of CMV in arterial walls and leukocytes in patients with atherosclerosis. Chin Med J (Engl) 2001;114 (11):1208-10.

25 Gurevich VS, Pleskov VM, Levaia MV, Bannikov AI, Mitrofanova LB, Urazgil'deeva SA. [Influenza virus infection in progressing atherosclerosis]. Kardiologiia 2002;42(7):21-4.

26 Waldman WJ, Knight DA, Huang EH, Sedmak DD. Bidirectional transmission of infectious cytomegalovirus between monocytes and vascular endothelial cells: an in vitro model. J Infect Dis 1995;171(2):263-72.

27 Pleskov VM, Bannikov AI, Gurevich VS, Pleskova I. [Influenza viruses and atherosclerosis: the role of atherosclerotic plaques in prolonging the persistent form of influenza infection]. Vestn Ross Akad Med Nauk 2003;(4):10-3.

28 Boman J, Hammerschlag MR. Chlamydia pneumoniae and atherosclerosis: critical assessment of diagnostic methods and relevance to treatment studies. Clin Microbiol Rev 2002;15(1):1-20.

29 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(12):2648-53.

30 Qavi HB, Melnick JL, Adam E, Debakey ME. Frequency of coexistence of cytomegalovirus and Chlamydia pneumoniae in atherosclerotic plaques. Cent Eur J Public Health 2000;8(2):71-3.

31 Prasad A, Zhu J, Halcox JP, Waclawiw MA, Epstein SE, Quyyumi AA. Predisposition to atherosclerosis by infections: role of endothelial dysfunction. Circulation 2002;106(2):184-90.

32 High KP. Atherosclerosis and infection due to Chlamydia pneumoniae or cytomegalovirus: weighing the evidence. Clin Infect Dis 1999;28 (4):746-9.

33 Zhu J, Nieto FJ, Horne BD, Anderson JL, Muhlestein JB, Epstein SE. Prospective study of pathogen burden and risk of myocardial infarction or death. Circulation 2001;103(1):45-51.

34 Rupprecht HJ, Blankenberg S, Bickel C, Rippin G, Hafner G, Prellwitz W, Schlumberger W, Meyer J. Impact of viral and bacterial infectious burden on long-term prognosis in patients with coronary artery disease. Circulation 2001;104(1):25-31.

35 Smieja M, Gnarpe J, Lonn E, Gnarpe H, Olsson G, Yi Q, Dzavik V, McQueen M, Yusuf S. Multiple infections and subsequent cardiovascular events in the Heart Outcomes Prevention Evaluation (HOPE) Study. Circulation 2003;107(2):251-7.

36 Zhu J, Quyyumi AA, Norman JE, Csako G, Waclawiw MA, Shearer GM, Epstein SE. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. Am J Cardiol 2000;85(2):140-6.

37 Wanishsawad C, Zhou YF, Epstein SE. Chlamydia pneumoniae-induced transactivation of the major immediate early promoter of cytomegalovirus: potential synergy of infectious agents in the pathogenesis of atherosclerosis. J Infect Dis 2000;181(2):787-90.

38 Ieven MM, Hoymans VY. Involvement of Chlamydia pneumoniae in atherosclerosis: more evidence for lack of evidence. J Clin Microbiol 2005;43(1):19-24.

39 Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? Lancet 1997;350(9075):430-6.

40 Muhlestein JB, Anderson JL, Carlquist JF, Salunkhe K, Horne BD, Pearson RR, Bunch TJ, Allen A, Trehan S, Nielson C. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease: primary clinical results of the ACADEMIC study. Circulation 2000;102(15):1755-60.

41 O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S, Benner RJ, Fisher MR, Cook TD. Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. Jama 2003;290 (11):1459 -66.

42 Visseren FL, Erkelens DW. [Atherosclerosis as an infectious disease]. Ned Tijdschr Geneeskd 1999;143(6):291-5.

43 Hill AB. The environment and disease: Association or causation? Proc R Soc Med 1965;58:295-300.

44 Susser M. What is a cause and how do we know one? A grammar for pragmatic epidemiology. Am J Epidemiol 1991;133 (7):635-48.

45 Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired immunity in atherogenesis. Nat Med 2002;8(11):1218-26.

46 Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A, Brichard SM. Adipokines oversecreted by omental adipose tissue in human obesity. Am J Physiol Endocrinol Metab 2007;293(3):E656-65.

47 Madjid M, Naghavi M, Litovsky S, Casscells SW. Influenza and cardiovascular disease: a new opportunity for prevention and the need for further studies. Circulation 2003;108(22):2730-6.

48 Muhlestein JB, Anderson JL, Hammond EH, Zhao L, Trehan S, Schwobe EP, Carlquist JF. Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. Circulation 1998;97 (7):633-6.

49 de K, van Gorp EC, Keller TT, Ossewaarde JM, ten CH. Chlamydia pneumoniae infections in mouse models: relevance for atherosclerosis research. Cardiovasc Res 2005;65(2):317-27.

50 Dhurandhar NV, Israel BA, Kolesar JM, Mayhew GF, Cook ME, Atkinson RL. Increased adiposity in animals due to a human virus. Int J Obes Relat Metab Disord 2000;24(8):989-96.

51 Whigham LD, Israel BA, Atkinson RL. Adipogenic potential of multiple human adenoviruses in vivo and in vitro in animals. Am J Physiol Regul Integr Comp Physiol 2006;290(1):R190-4

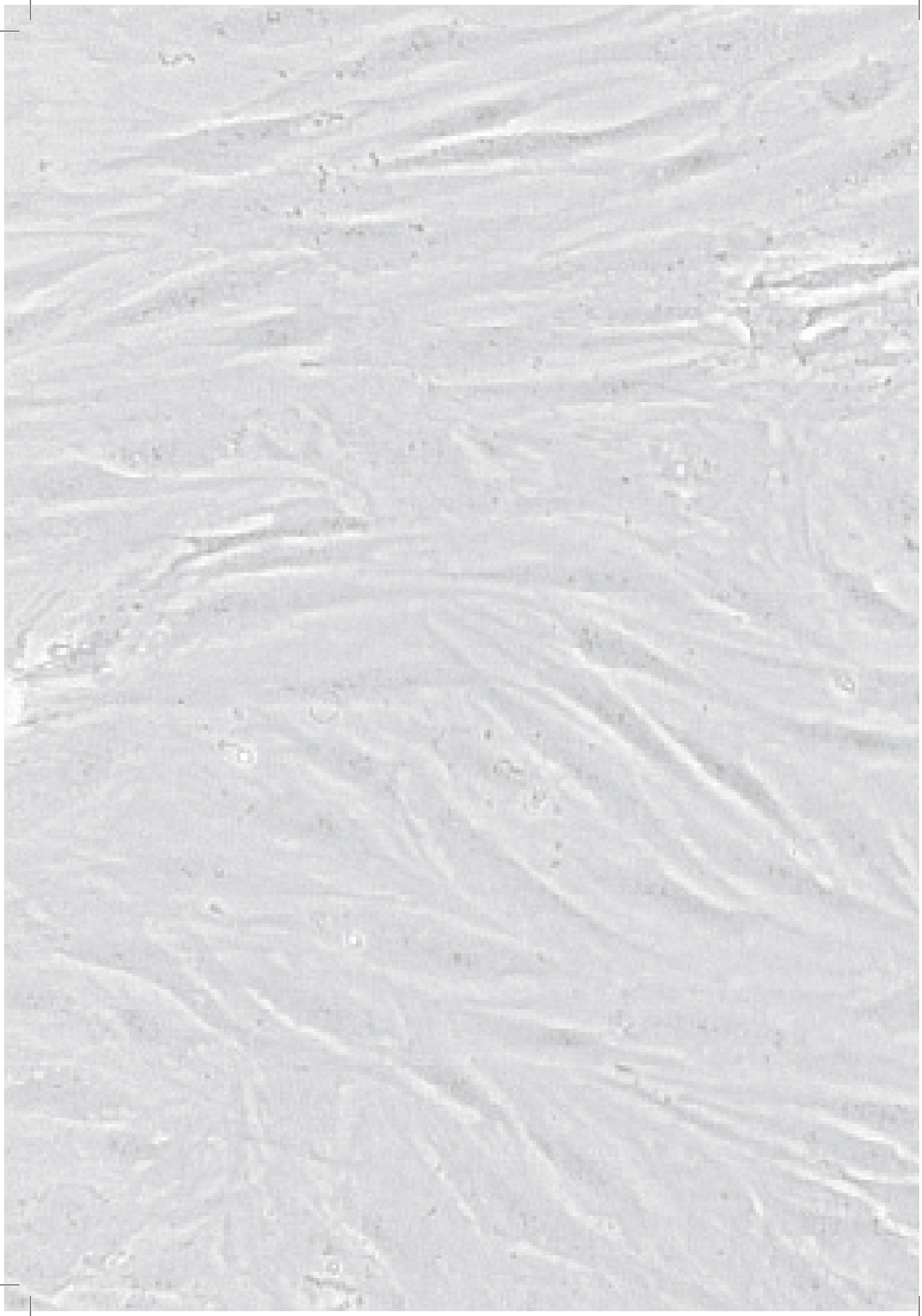
52 Grattan MT, Moreno-Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. Jama 1989;261(24):3561-6.

53 Loebe M, Schuler S, Zais O, Warnecke H, Fleck E, Hetzer R. Role of cytomegalovirus infection in the development of coronary artery disease in the transplanted heart. J Heart Transplant 1990;9(6):707-11.

54 McDonald K, Rector TS, Braulin EA, Kubo SH, Olivari MT. Association of coronary artery disease in cardiac transplant recipients with cytomegalovirus infection. Am J Cardiol 1989;64(5):359-62.

55 Petrakopoulou P, Kubrich M, Pehlivanli S, Meiser B, Reichart B, von SW, Weis M. Cytomegalovirus infection in heart transplant recipients is associated with impaired endothelial function. Circulation 2004;110(11 Suppl 1):II207 -12.

56 Kutlin A, Roblin PM, Hammerschlag MR. In vitro activities of azithromycin and ofloxacin against Chlamydia pneumoniae in a continuous-infection model. Antimicrob Agents Chemother 1999;43(9):2268-72.



9

Discussion

Summary

Samenvatting

Dankwoord

Curriculum vitae

Color figures

Chronic disorders, such as atherosclerosis, are currently considered to be associated with viral infections. Influenza virus, Cytomegalovirus (CMV), and *Chlamydia pneumoniae (Cp),* in particular, have been associated with cardiovascular disorders. However, it is unclear whether these infections cause these disorders or merely exacerbate existing processes. Severe complications can occur in acute infectious diseases such as influenza. For example, in the Netherlands about 2000 people die annually of mainly cardiovascular complications occurring secondary to influenza. Various factors, including viral infections, are thought to initiate or accelerate the process of atherosclerosis. When plaques rupture and their contents pass into the bloodstream, various systemic processes are evoked, such as enhanced coagulation and inflammatory responses. Chronic infections may also have a role in vessel wall damage and loss of the constitutionally protective properties of endothelial cells. Although the hypothesis that infections may underlie cardiovascular disorders is widely accepted, definite evidence is still lacking. In the studies described in this thesis, we performed several *in vitro* studies to investigate the hypothesis that cardiovascular disorders have an infectious etiology.

In the study described in **chapter 2,** we demonstrated that respiratory viruses (influenza A, influenza B, parainfluenza-1, respiratory syncytial virus, adenovirus, and CMV) infect lung fibroblasts and human umbilical vein endothelial cells *in vitro*. As determined in a one-stage clotting assay, all viral pathogens induced procoagulant activity in infected endothelial cells, decreasing the clotting time by a mean of 55%. When factor VII-deficient plasma was used, the clotting time was not reduced. These results indicated that the reduction in clotting time was factor VII dependent and therefore mediated by the extrinsic coagulation pathway. The induction of procoagulant activity was associated with a 4- to 5-fold increase in the expression of tissue factor (TF), as measured by the generation of factor Xa. Thus viral infection is accompanied by an increased expression of TF on endothelial cells. We concluded that both enveloped and non-enveloped respiratory viruses are capable of infecting cultured human endothelial cells, causing a shift from anticoagulant to procoagulant activity associated with the induction of TF expression.

The production of cytokines by monocytes may accelerate the process of atherosclerosis and may contribute to vascular disorders by eliciting plaque instability. Whether virus-infected monocytes initiate coagulation and produce (pro-) inflammatory cytokines

was investigated in the study described in **chapter 3**. For this study, we designed an improved method for isolating platelet-free monocytes. Freshly isolated human monocytes were incubated with CMV, *Cp,* and influenza A. The clotting time of CMV- and *Cp*-infected monocytes was reduced by 60%. Addition of factor VII-deficient plasma normalized the clotting time. As with endothelial cells, the clotting time of monocytes was factor VII dependent and therefore mediated by the extrinsic coagulation pathway. The initiation of coagulation by virus-infected monocytes was a result of the expression of TF. By using factor VII-deficient plasma and anti-TF antibodies, we showed that TF plays an important role in virus-induced monocytic procoagulant activity. This could be a direct effect of virus but could also be triggered by IL-6 produced by monocytes. By producing IL-6, infected monocytes could stimulate uninfected cells to express TF. Although infection with influenza A only marginally influenced clotting times, it strongly stimulated the production of IL-6 and IL-8. In contrast, the infection of monocytes with CMV and *Cp* only modestly stimulated the production of IL-6 and IL-8. During viral infections, monocytes predominantly produce the inflammatory cytokines IL-6 and IL-8. Interleukin-10 is detected predominantly in atherosclerotic lesions, but monocytes may produce this cytokine transiently. In this study, we did not find virus-infected monocytes to produce IL-10. The balance between proinflammatory and anti-inflammatory cytokines in the atherosclerotic plaque may be decisive for the progression of the lesion. Our results indicated that only small amounts of an infectious virus are needed to stimulate monocytes to exert considerable immunological effects. We concluded that the infection of monocytes by viruses triggers proatherosclerotic and prothrombotic processes *in vitro*.

The liver has multiple metabolic, endocrine, and secretory functions and is an important source of a variety of proteins, among which acute-phase proteins, coagulation factors, complement factors, cholesterol, and triglycerides. Inflammation caused by infection or tissue damage stimulates the secretion of inflammation-associated cytokines, including IL-1, IL-6 and tumor necrosis factor (TNF)-alpha. These cytokines stimulate hepatocytes to increase the synthesis and release of positive acute-phase proteins, including C-reactive protein (CRP). IL-6, also called hepatocyte-stimulating factor, is the major stimulus for CRP production. Fibrinogen is involved in hemostasis by stabilizing the fibrin matrix and is synthesized by hepatocytes under the control of inflammatory cytokines. In the study described in **chapter 4,** we reported that human HepG2 hepatocytes could be infected with the intracellular pathogens *Cp a*nd

CMV. We also investigated whether azithromycin was able to inhibit the infection of human hepatocytes by *Cp* and reduce the inflammatory response of hepatocytes. We showed that *in vitro* infection of hepatocytes with *Cp* led to time and dose-dependent production of IL-6 and that this inflammatory response was strongly and significantly inhibited by azithromycin, confirming the antibacterial properties of azithromycin against *Cp*. Fibrinogen production was increased by CMV and *Cp*, but azithromycin was not able to suppress this CMV- or *Cp*-induced production of fibrinogen. Apparently, the effects of azithromycin on *Cp*-infected hepatocytes are due to its antimicrobial properties and not to its anti-inflammatory properties.

Chronic infection could contribute to atherogenesis by a number of mechanisms, such as direct vascular injury and induction of a systemic inflammatory state, both of which lead to endothelial dysfunction. Nitric oxide (NO) is a key regulator of endothelial function. Under pathological conditions, uncoupling of endothelial nitric oxide synthase (eNOS) leads to vessel damage and endothelial dysfunction as a result of the production of oxygen radicals instead of NO. Excessive formation of reactive oxygen species (ROS) leads to inflammation, endothelial dysfunction, and increased adhesion of leukocytes. In the study described in **chapter 5,** we measured the *in vitro* production of eNOS, cyclic guanosine monophosphate (cGMP, as a surrogate for NO), and ROS by human umbilical vein endothelial cells (HUVEC) infected with *Cp* or CMV. We hypothesized that infection-induced atherosclerosis could be initiated by changes in NO metabolism and that this effect could be reversed by azithromycin treatment. eNOS, cGMP, and ROS production were attenuated in *Cp*-infected HUVEC, whereas infection with CMV did not change cGMP or ROS production and only marginally decreased eNOS production. Co-incubation with azithromycin restored eNOS, cGMP, and ROS production by *Cp*-infected HUVEC to levels comparable with those of uninfected HUVEC. These results suggested that antibiotic treatment for infection-induced atherosclerosis improves endothelial function.

Increased expression of endothelial adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and endothelial selectin (E-selectin), are markers of endothelial dysfunction. Given the role of iron in a wide range of infection processes, the presence of iron may complicate the infection-mediated activation of endothelial function. Iron has been found to accumulate in human atheroma, and several experimental studies have shown that iron chelation

or iron-deficient diets lead to a reduced formation of early atherosclerotic lesions. In the study described in **chapter 6,** we showed that *Cp* and CMV infection induced the expression of ICAM-1 and VCAM-1 and up-regulated the expression of E-selectin. The expression of adhesion molecules in HUVEC infected with *Cp* was increased by incubating the cells in iron-rich medium. Thus the endothelial response to chronic infections depends on the intracellular level of iron. However, the modulating effect of iron is not due to an increase in the infectivity or replication of the microorganism because the endothelial response was observed before *Cp* infection or replication was noticed. We also observed that both chelation and radical scavengers counteracted the effects of iron. These findings suggested that the main effect of iron is to prime endothelial cells by causing oxidative stress. Iron-primed endothelial cells may be more responsive to the paracrine effects of endothelial cell infection. In contrast, the level of intracellular oxygen-derived radicals in infected HUVEC was unchanged, indicating that the induction of ICAM-1 and VCAM-1 was not driven by infection-related radical formation. This suggested that infections exert their effects on endothelial activation through a pathway that is different from that for the formation of oxygen-derived radicals.

Abdominal adipose tissue not only absorbs and stores free fatty acids but also produces and secretes adipokines such as IL-6, TNF-alpha, PAI-1, and adiponectin. These factors may have effects on the arterial wall, on vascular risk factors, and on insulin resistance. By producing hormones and inflammatory cytokines, adipose tissue may have an important role in the development of diabetes and atherosclerosis. It has been suggested that obesity is a chronic inflammatory state in which adipocyte dysfunction has a crucial role. In the study described in **chapter 7,** we showed that several microorganisms were able to infect human adipocytes and pre-adipocytes *in vitro*. Infection of adipocytes with Adenovirus type 36, but not with Adenovirus type 2, led to increased production of IL-6. This may contribute to chronic low-grade inflammation, a process known to be involved in the development of cardiovascular diseases and type 2 diabetes. These findings support a potential role for microorganisms in inducing and maintaining adipocyte dysfunction. We also demonstrated that adiponectin was exclusively produced by adipocytes and not by pre-adipocytes and that IL-6 and PAI-1 were mainly produced by pre-adipocytes. Thus during their passage through the portal circulation, inflammatory substances are able to directly influence liver metabolism, potentially leading to an accelerated inflammatory response, e.g. CRP production,

and to changes in hemostasis as a result of the production of coagulant factors and proteins involved in fibrinolysis.

The inflammatory response of adipocytes to infection may also have paracrine effects by influencing the function of neighboring adipocytes and may attract macrophages into adipose tissue, increasing the capacity for the production of inflammatory mediators. In the study described in **chapter 8,** we tested this concept in an *in vitro* co-culture model of human adipocytes and macrophage-like differentiated human THP-1 cells. We first showed that transinfection occurred, namely, that infected adipocytes infected macrophages and vice versa. This phenomenon may explain how infectious agents can infect cells of various organ systems, such as vessel walls and adipose tissue, thus contributing to local and systemic inflammatory responses. Levels of IL-6 and PAI-1 were increased in co-cultures of human adipocytes and THP-1 macrophages infected with CMV or influenza A virus. The invasion of adipose tissue by microorganisms such as CMV and influenza A may initiate and perpetuate a chronic inflammatory response, attracting more macrophages into the already inflamed tissue, thereby exacerbating the total inflammatory response to the infection. Although adipocytes seem to be the major source of bioactive cytokines, the extent of macrophage recruitment may be decisive for the severity of inflammation. However, it is conceivable that infected monocytes invading adipose tissue trigger an inflammatory response. In addition, we showed that primarily macrophages were responsible for the increased levels of IL-6 measured in infected co-cultures. This response provides insight into how adipose tissue signals to remote tissues (endothelium, liver, muscle) and may provide a pathological link between obesity and its associated cardiovascular complications.

From the results presented in this thesis, we conclude:

- Intracellular infections with various pathogens, such as respiratory viruses, *Chlamydia pneumoniae* (*CP*), and cytomegalovirus (CMV), induce procoagulant activity in endothelial cells and monocytes *in vitro*. This procoagulant activity is TF dependent.

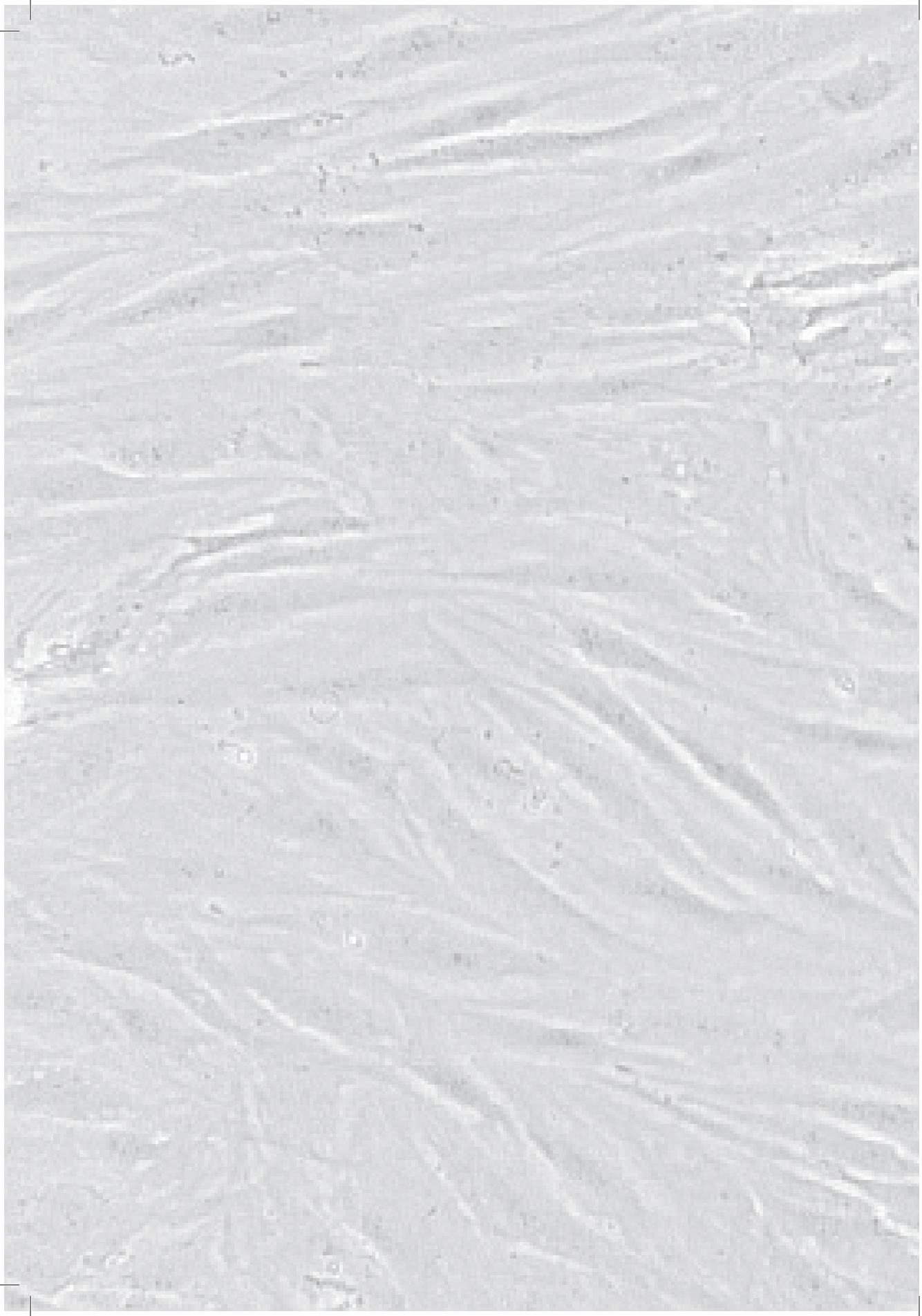
- Infection of endothelial cells with *Cp in vitro* attenuates eNOS, cGMP, and ROS production, and azithromycin reverses this attenuation.

- Infection of HepG2 human hepatocytes *in vitro* leads to an increased production of IL-6 and fibrinogen. Azithromycin dose dependently reduces *Cp*-induced IL-6, but not fibrinogen, production. We could not confirm that azithromycin has an anti-inflammatory effect.

- Infection of endothelial cells leads to increased expression of adhesion molecules. The endothelial response to chronic infections depends on the intracellular iron level.

- Adipocytes can be infected by several microorganisms *in vitro*. Infection of adipocytes with CMV, Ad 36, and influenza A leads to increased production of IL-6.

- Infection of co-cultures of human adipocytes and THP-1 macrophages with CMV and influenza A induces the overproduction of IL-6 and PAI-1.



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Er bestaan aanwijzingen dat infecties, met influenzavirus, Cytomegalovirus (CMV) en *Chlamydia pneumoniae (Cp)* in het bijzonder, het proces van atherosclerose ('aderverkalking') in gang kunnen zetten en/of versnellen. Dit kan gebeuren via directe infectie van de cellen in de binnenbekleding van de bloedvaten (endotheelcellen) en indirect door infectie van andere cellen en organen, waardoor een gegeneraliseerde ontstekingsreactie ontstaat. Hierdoor kunnen uiteindelijk aandoeningen aan bloedvaten ontstaan zoals een hartinfarct, herseninfarct of perifeer arterieel vaatlijden. In de laboratoriumstudies die deel uitmaken van dit proefschrift wordt onderzocht of bepaalde micro-organismen in staat zijn om verschillende cellen van verschillende organen te infecteren en wordt onderzocht wat het effect is op de functie van die cellen. Vooral wordt gekeken naar processen die een rol spelen bij het ontstaan van atherosclerose.

In **hoofdstuk 2** laten we zien dat luchtwegvirussen (influenza A en B, parainfluenza-1, respiratoir syncytieel virus (RSV), adenovirus en CMV) uit navelstrengen verkregen endotheelcellen kunnen infecteren. Als reactie op infectie ontstond verhoogde stollingsactiviteit op geïnfecteerde endotheelcellen. De factoren van het extrinsieke stollingssysteem, zoals tissue factor (weefselfactor, factor III) en factor VII, zijn eiwitten die op endotheelcellen aanwezig zijn. Tissue factor staat aan het begin van het stollingssysteem en wordt geactiveerd door beschadiging van de bloedvatwand. Uit een stollingsproef bleek dat de gemiddelde stoltijd met 55% verkort werd. Deze versnelde stolling was afhankelijk van stollingsfactor VII. Daarmee werd aangetoond dat de stolling via de tissue factor afhankelijke (extrinsieke) stollingsroute verliep. Naast de verkorte stollingstijden, nam ook de expressie van het weefselfactor eiwit op het oppervlak van geïnfecteerde endotheelcellen met een factor 4 tot 5 toe. De conclusie is dan ook dat respiratoire virussen in staat zijn om menselijke endotheelcellen te infecteren en dat de toegenomen expressie van tissue factor de endotheelcellen in een staat van verhoogde stollingsactiviteit brengt.

Monocyten behoren tot een fractie van witte bloedcellen die een rol spelen bij het opruimen van indringers zoals bacteriën. De productie van ontstekingseiwitten (cytokines) door monocyten kan het atherosclerotisch proces versnellen en bijdragen aan het ontstaan van vaatproblemen als gevolg van vorming van instabiele atherosclerotische plaques in de vaatwand. In **hoofdstuk 3** werd onderzocht of de met virus geïnfecteerde monocyten ook de bloedstolling in gang zetten en

ontstekingsbevorderende cytokines kunnen produceren. Door middel van een geoptimaliseerde isolatiemethode werden monocyten verkregen die geïnfecteerd werden met CMV, *Cp* en influenzavirus. De stollingstijden van CMV- en *Cp*-geinfecteerde monocyten bleken met ca. 60% verkort te zijn. Net als bij endotheelcellen bleek de stolling factor VII- afhankelijk te verlopen via de extrinsieke stollingsroute. De door geïnfecteerde monocyten op gang gebrachte stolling werd veroorzaakt door expressie van tissue factor. Tevens produceerden geïnfecteerde monocyten de ontstekingsbevorderende cytokines interleukine- 6 (IL-6) en interleukine-8 (IL-8). Onze resultaten geven aan dat slechts kleine hoeveelheden infectieus virus in staat zijn om monocyten aan te zetten tot een merkbare immunologische reactie. Geconcludeerd wordt dat infectie van monocyten leidt tot ontstekingsreacties en stollingsbevorderende processen die tot verergering van atherosclerose kunnen leiden.

De lever is een belangrijke producent van acute fase eiwitten, stollingsfactoren, cholesterol en triglyceriden. De aanmaak van acute fase eiwitten is een eerste normale reactie van het lichaam op ontstekingen. Ontsteking als gevolg van een infectie of weefselbeschadiging versterkt de productie van ontstekingsbevorderende cytokines als IL-1, IL-6 en tumor necrosis factor (TNF)-alfa. Deze cytokines stimuleren op hun beurt weer levercellen tot sterk verhoogde aanmaak van acute fase eiwitten zoals C-reactief proteïne (CRP). IL-6 is de belangrijkste cytokine die de lever aan kan zetten tot productie van CRP. De stollingsfactor fibrinogeen wordt aangemaakt door levercellen en is betrokken bij het stabiliseren van bloedstolsels. Onder abnormale omstandigheden, zoals chronische ontstekingsprocessen bij atherosclerose, kunnen plasmaconcentraties van acute fase eiwitten en stollingsfactoren langdurig verhoogd zijn. In **hoofdstuk 4** beschrijven wij dat menselijke levercellen in het laboratorium geïnfecteerd kunnen worden door de intracellulaire pathogenen *Cp a*nd CMV. Het bleek dat infectie van levercellen met *Cp* leidt tot een tijd- en dosisafhankelijke productie van IL-6. Ook hebben wij onderzocht of azitromycine in staat zou zijn om *Cp*-infectie en de ontstekingsreactie in levercellen af te remmen. Bepaalde antibiotica zoals azithromycine en roxithromycine (macroliden) zijn namelijk effectief voor de behandeling van intracellulaire infecties omdat langdurig hoge antibioticumconcentraties in de cellen bereikt kunnen worden. Toevoeging van azitromycine remde de ontstekingsreactie van geïnfecteerde levercellen en onderdrukte de infectie van *Cp in vitro*. Wij concluderen dat de waargenomen afname van de ontstekingsreacties na azithromycine gebruik het rechtstreeks gevolg is

van de antibacteriële werking van het antibioticum en niet een ontstekingsremmend effect van dit middel is.

Chronische infecties kunnen directe vaatschade maar ook systemische ontstekingsreacties tot gevolg hebben. In beide gevallen kan dit leiden tot een verstoorde functie van endotheelcellen (endotheeldisfunctie). Stikstofoxide (NO) is een belangrijke regulator van endotheelfunctie en zorgt o.a. voor vaatverwijding. Onder pathologische omstandigheden wordt minder NO gevormd en juist meer schadelijke reactieve zuurstofradicalen (reactive oxygen species, ROS), hetgeen leidt tot vaatbeschadiging en daardoor endotheeldisfunctie en ontstekingreacties. In **hoofdstuk 5** werd, na infectie van endotheelcellen met *Cp*- en CMV, de productie gemeten van NO, ROS en het enzym endotheliaal nitric oxide synthase (eNOS), wat zorgt voor aanmaak van NO. Omdat NO lastig te meten is werd in plaats daarvan cyclisch guanosine monofosfaat (cGMP) gemeten, als maat voor de beschikbaarheid van NO. Wij veronderstelden dat infecties atherosclerose op gang kunnen brengen door onderdrukking van NO-productie en dat toevoeging van azitromycine dit effect weer zou kunnen herstellen. *Cp*-geinfecteerde endotheelcellen bleken inderdaad minder eNOS, cGMP en ROS te produceren. Toevoeging van azitromycine herstelde de eNOS, cGMP en ROS-productie. De resultaten van deze studie suggereren dat behandeling met antibiotica de verstoorde endotheelfunctie ten gevolge van infecties mogelijk zou kunnen verbeteren.

Bij endotheeldisfunctie kan de expressie van de adhesiemoleculen vasculair cel adhesiemolecuul-1 (VCAM-1), intercellulair adhesiemolecuul-1 (ICAM-1) en endotheliaal selectine (E-selectin) verhoogd zijn. Infecties kunnen de aanleiding zijn voor de activatie van endotheel en de gelijktijdige aanwezigheid van ijzer kan dit proces versterken. Diverse studies hebben aangetoond dat vorming van atherosclerose afnam door ijzerchelatietherapie (wegvangen van ijzer) of ijzerarme diëten. In **hoofdstuk 6**, laten we zien dat *Cp*- en CMV-infecties de ICAM-1 en VCAM-1 expressie in endotheelcellen op gang brengen en de productie van E-selectin versterken. In *Cp*-geinfecteerde endotheelcellen werden deze effecten nog eens verder versterkt wanneer ijzerrijk medium werd toegevoegd. De aanwezigheid van ijzer beïnvloedt weliswaar de reactie van endotheelcellen op infectie, maar dit effect werd al gezien voordat infectie en vermenigvuldiging van *Cp* kon worden aangetoond. Omdat na infectie de concentratie van zuurstofradicalen in endotheelcellen niet veranderde, is

het ook niet waarschijnlijk dat de expressie van ICAM-1 en VCAM-1 werd aangestuurd door productie van radicalen. Kennelijk verloopt endotheelactivatie door infectie via een andere route. Dit geeft aan dat ijzer endotheelcellen eigenlijk vooral prikkelt door het opwekken van oxidatieve stress. Op deze wijze kunnen endotheelcellen echter wel gevoeliger worden voor de effecten van infecties en bijdragen aan het ontstaan van atherosclerose.

Buikvet zorgt niet alleen voor de opname en opslag van vrije vetzuren, maar is ook in staat tot productie en uitscheiding van adipokines (cytokines geproduceerd door vet). Vetzucht wordt ook wel voorgesteld als een staat van chronische ontsteking waarvan bekend is dat die betrokken is bij het ontstaan van hart- en vaatziekten en diabetes mellitus. Adipocytendisfunctie is daarbij cruciaal. Uit **hoofdstuk 7** blijkt dat ook adipocyten (vetcellen) en pre-adipocyten (voorloper vetcellen) geïnfecteerd kunnen worden met verschillende virussen. Infectie van adipocyten met adenovirus type 36, maar niet met adenovirus type 2, leidt tot verhoogde productie van IL-6 door adipocyten. Dit ondersteunt de hypothese dat micro-organismen mogelijk een rol spelen in het ontstaan en onderhouden van een chronisch laaggradig ontstekingsproces dat gepaard gaat met adipocytendisfunctie. Het feit dat ongeveer een derde deel van de totale IL-6 plasmaconcentratie afkomstig is van vetweefsel geeft aan dat ontstekingreacties in vetweefsel tot belangrijke biologische effecten kunnen leiden.

Micro-organismen die het vetweefsel infecteren kunnen een chronische ontstekingsreactie in gang zetten. De hierbij geproduceerde adipokines kunnen vervolgens de functie van naastgelegen cellen beïnvloeden (paracrien effect) en er voor zorgen dat meer macrofagen het vetweefsel binnendringen. Hierdoor kan de totale productiecapaciteit van ontstekingsbevorderende stoffen nog meer stijgen. Het is ook denkbaar dat toegenomen infiltratie van geïnfecteerde macrofagen in vetweefsel de aanzet geeft tot een ontstekingsreactie. In **hoofdstuk 8** werden menselijke vetcellen (adipocyten) en macrofagen samen gekweekt (co-cultuur) en geïnfecteerd. Wij konden laten zien dat infectie van adipocyten ook daadwerkelijk overgaat naar macrofagen en vice versa (transinfectie). CMV- en influenza A infectie in co-culturen van adipocyten en macrofagen leidt tot overproductie van IL-6 en plasminogeen activator inhibitor-1 (PAI-1). Directe communicatie tussen deze celtypen leidt kennelijk tot de aanzienlijk verhoogde staat van ontsteking die gezien wordt bij obesitas. Dit ondersteunt de

gedachte dat zowel adipocyten als macrofagen in vetweefsel betrokken zijn bij adipokine productie. Hoewel adipocyten de belangrijkste bron van cytokines lijken te zijn, is de mate van infiltratie door macrofagen waarschijnlijk bepalend voor de ernst van de ontstekingsreactie. Uiteindelijk bleek ook dat de stijging van IL-6 in geïnfecteerde co-culturen vooral aan macrofagen toegeschreven kon worden**.** Deze bevindingen verschaffen inzicht in de manier waarop infectie van vetweefsel op afstand betrokken kan zijn bij het activeren van andere weefsels (endotheel, lever, spieren) en zodoende een pathologische link zou kunnen zijn tussen obesitas en de verhoogde kans op het ontstaan van hart- en vaat aandoeningen.

Conclusies van dit proefschrift:

- Intracellulaire infecties met micro-organismen zoals respiratoire virussen, *Chlamydia pneumoniae* (*Cp*) en cytomegalovirus (CMV) leiden tot verhoogde stollings-activiteit van endotheelcellen en monocyten *in vitro* die geassocieerd is met verhoogde expressie van tissue factor.

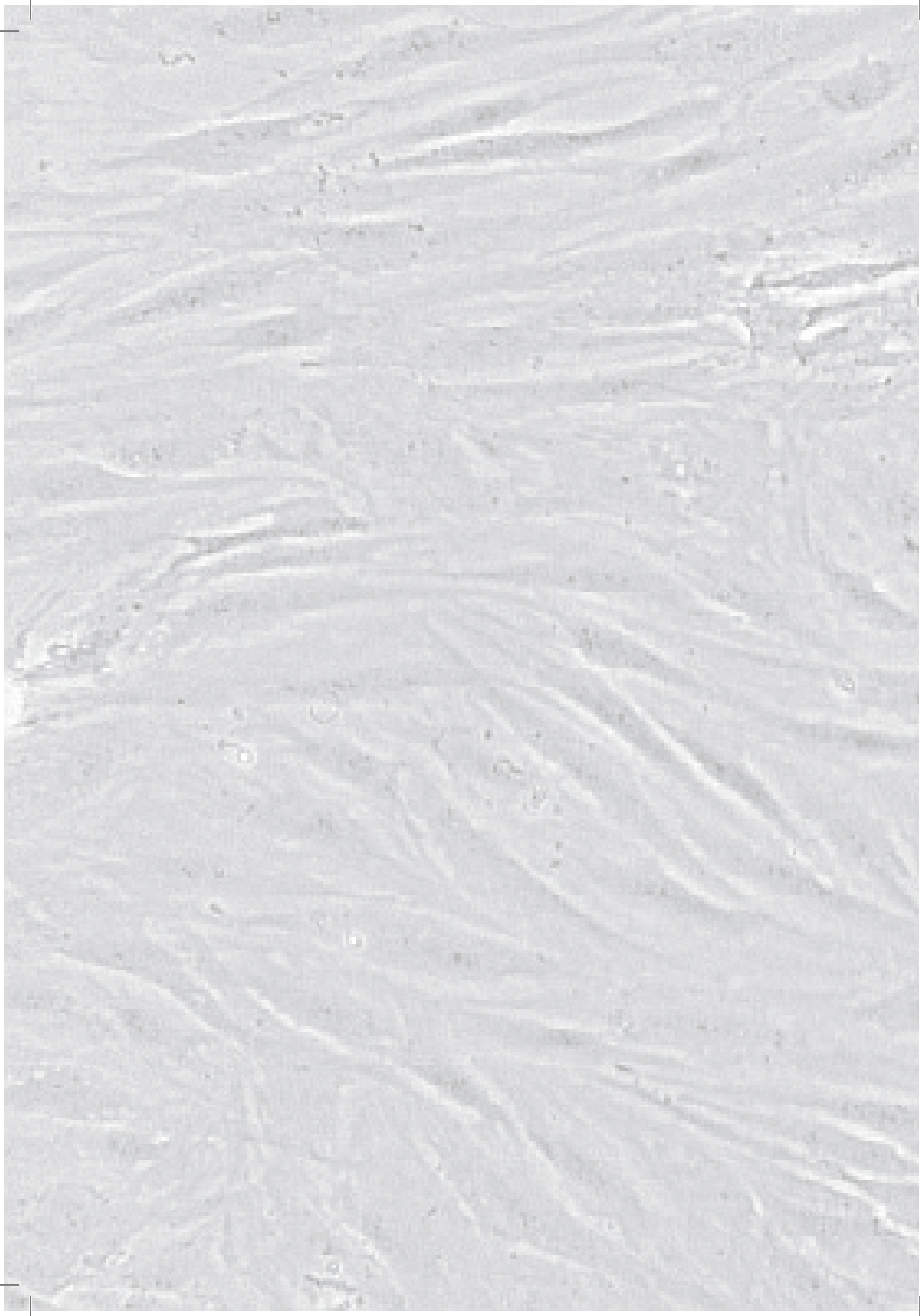
- *In vitro* infectie van endotheelcellen met *Cp* onderdrukt eNOS, cGMP en ROS productie. Incubatie met azitromycine voorkomt deze effecten.

- Infectie van levercellen *in vitro* leidt tot een toename van IL-6 en fibrinogeen productie. Toevoeging van azitromycine verlaagt weliswaar de door *Cp* geïnduceerde IL-6 productie, maar niet de fibrinogeen productie. Azithromycine heeft geen directe anti-inflammatoire effecten op levercellen.

- Infectie van endotheelcellen leidt tot toegenomen expressie van adhesiemoleculen op endotheelcellen. De mate waarin endotheelcellen op infecties reageren, hangt ook af van de ijzer concentratie in het kweekmedium van de cellen.

- Vetcellen zijn met verscheidene micro-organismen *in vitro* te infecteren. Infectie van vetcellen met CMV, Ad 36 and influenza A leidt tot verhoogde IL-6 productie.

- CMV- en influenza A infecties induceren toegenomen productie van IL-6 en PAI-1 in co-culturen van vetcellen en monocyten.



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Game, set en match: het boekje ligt er en ik ben trots op het resultaat!
Toen mij paar jaar geleden werd voorgesteld om te gaan promoveren moest ik daar eens rustig over nadenken. Later besefte ik dat ik er gewoon voor moest gaan. Uiteindelijk ben ik toch jarenlang met veel plezier aan het experimenteren geweest en het is mooi dat die inspanningen nu met dit proefschrift bekroond zijn.
Liters koffie, branderige ogen en een aantal doses hoofdpijn verder, kijk ik terug op een enerverende tijd. Achteraf gezien ook een vreemde periode waarin de studies uit dit proefschrift verweven waren met tal van andere bezigheden. Zo kon het gebeuren dat ik soms niet eens tijd had voor mijn zo geliefde celkweken. Vooral, nadat we in januari 2005 de TBC-exercitie in Zeist achter de rug hadden, raakte de toepassing van Elispot voor TB-diagnostiek in een stroomversnelling en ging veel van mijn tijd daaraan op.
Ik realiseer mij dat alle jaren van onderzoek geen solo actie geweest zijn. Hier aangekomen wil ik iedereen bedanken die een rol heeft gespeeld bij het tot stand komen van dit proefschrift. Ik ben er zeker van dat de bijdragen en steun van velen een wezenlijke bijdrage aan dit resultaat hebben geleverd. Toch wil ik een aantal mensen persoonlijk bedanken:

Dr. R.J.A. Diepersloot, beste Rob: Mijn eerste woorden van dank richt ik tot jou. Wat ik nu bereikt heb was niet mogelijk geweest zonder jouw onvoorwaardelijke steun en betrokkenheid. Ik dank je met name voor het vertrouwen en alle faciliteiten die je mij in al die jaren hebt geboden. Ik weet nog goed dat je begin jaren negentig naar het Diakonessenhuis kwam als tweede microbioloog. Vanaf dat moment ging het snel en in no time was de virologie in het Diak geboren. Voor we het wisten hadden we al een polio-epidemie te pakken. Daarna hebben we een en ander nog verder uitgebouwd tot wat nu state of the art units voor virologie en moleculaire diagnostiek zijn. In ben zeer content dat ik samen met jou aan de basis hiervan heb gestaan. Jouw onderzoeksmind heeft gelukkig altijd de overhand gehad, zelfs in de perioden dat je voorzitter was van de medische staf en later van het pensioenfonds. Ik heb daar altijd veel motivatie uit kunnen halen. Ik realiseer me ook als geen ander dat ik jarenlang in een unieke omgeving heb mogen werken en bouwen aan dit resultaat. Ik vind het een eer jou als co-promotor te mogen hebben en hoop dat dit proefschrift de kroon op ons werk van de afgelopen jaren mag zijn.

Prof. Dr. F.L.J. Visseren, beste Frank: Mijn onnoemelijke dank voor jouw enthousiasme en betrokkenheid bij mijn onderzoeksactiviteiten. Onvoorstelbaar dat ons eerste contact al dateert uit de vorige eeuw. Toen al was jouw enthousiasme aanstekelijk. Deze 'oude rot in het vak' (zoals jij mij zelf noemde) mocht jou op sleeptouw nemen tijdens jouw onderzoeksjaar in het Medisch Microbiologisch Laboratorium van het Diakonessenhuis Utrecht. Daarna hebben we nog vaak samen met Rob en Paul in de denktank gezeten. Nu zijn de zaken omgekeerd en ben jij al jaren mijn steun en toeverlaat op mijn weg naar de academie. Een altijd kritische, maar constructieve leermeester ben je voor mij geweest. Het schrijfwerk was voor mij altijd een hele bevalling en jouw commentaren op mijn hersenspinsels waren voor mij als eenvoudige analist meestal flink slikken, maar hebben mij wel gebracht waar ik nu ben. Geachte promotor: respect en dank!

Prof. I.M. Hoepelman, beste Andy: In maart 2008 hebben wij elkaar eigenlijk pas voor het eerst ontmoet. Op voorspraak van Frank en op basis van mijn al gepubliceerde werk gaf U mij het vertrouwen om dit promotietraject tot een goed einde te brengen. Ik ben U daar zeer erkentelijk voor.

Ik dank de hooggeleerde heren Prof. dr. J. Verhoef, Prof. dr. J.B.L. Hoekstra, Prof. dr. Ph. G. de Groot, Prof. dr. G. Pasterkamp en Prof. dr. J.J.M. Marx voor de bereidheid om zitting te nemen in de **beoordelingscommissie**

Artsen-microbioloog van het Diakonessenhuis: Beste Jan, Steven en Arend Jan. Dank dat mijn activiteiten zich in jullie warme belangstelling mochten verheugen. Ik heb jullie enthousiasme en betrokkenheid altijd zeer gewaardeerd.

Aan de **regionale Maatschap Medische Microbiologie** ben ik veel dank verschuldigd voor alle jaren dat zij het voor mij mogelijk hebben gemaakt mijn onderzoeks-activiteiten te kunnen ontplooien. Het bekostigen van de helft van mijn aanstelling is door de jaren heen de stabiele factor geweest om mijn onderzoekswerk te kunnen continueren. Beste Jan, Steven, Arend Jan, Thijs, Bartelt, Paul en Bart: dank voor jullie ondersteuning en belangstelling; ook zonder jullie was ik niet zo ver gekomen. Van de colloquia op woensdagmiddag heb ik veel geleerd.

Stichting Diabetes Research, Rob en Paul: Mijn grote dank dat jullie steeds kans gezien hebben om mijn onderzoek te blijven ondersteunen. Zelfs in tijden waarin het niet zo voorspoedig ging met de stichting wisten jullie altijd bronnen aan te boren. Ik besef heel goed dat ook deze steun onmisbaar is geweest om dit traject tot een goed einde te brengen. **Dr. K.P. Bouter, beste Paul**: daarnaast dank voor het denkwerk en jouw inspirerende adviezen.

Mijn lieve collega's van het MMI: Wat had ik zonder jullie gemoeten! Ik voel(de) me wel eens een einzelgänger en had soms het gevoel te verzuipen in de drukte en hectiek op het lab. Ik weet ook dat ik niet altijd de gemakkelijkste geweest ben, maar jullie hebben mij altijd in het juiste spoor weten te houden door jullie medeleven, belangstelling, gezelligheid en de bereidheid om soms gewoon eens een klein klusje voor mij te doen. Ik dank jullie allemaal voor de fijne samenwerking. Ik zal ook zeker een keertje vaker mee gaan stappen. Zonder iemand onbedoeld te vergeten, zijn er toch een paar mensen die ik speciaal wil bedanken. Hiske en Gwenny: op de virologie hebben wij heel wat zielenroerselen uitgewisseld en zijn een hoop meningen de revue gepasseerd. Soms ging het ook helemaal nergens over, maar ik heb er van genoten! Alle analisten van de viro: dicht bij elkaar in die kleine ruimte heeft toch ook wel wat en brengt vaak een hoop gezelligheid. Simone en Lene: Dank voor jullie zorgzaamheid: levenservaring, een luisterend oor en een half woord waren voor mij vaak genoeg. Medewerkers ELK: dank voor het bereiden van alle PBS en andere bijzondere maaksels. Steven Thijsen: dank voor jouw belangstelling en openheid. We hebben samen heel wat Elispots beoordeeld en onze ideeën over de TBC-diagnostiek uitgewisseld. TB is ook bij mij nog steeds latent aanwezig en ik verheug me nu al op onze verdere samenwerking. Ik waardeer het zeer dat je mij ook als paranimf bij hebt willen staan. Eva: dank voor de bewegingsvrijheid die je mij altijd gegund hebt. Tenslotte op dit moment bijna last, but not least: "SMASHES". Beste volleyballers: kwaliteit komt vanzelf bovendrijven en volgend seizoen zullen we iedereen verslaan met onze 'eigen slag'.

Mijn HLO-Stagiaires: Carla, Bianca, Renate, Petra, Marije, Fröken, Willemieke en Suzanne. Aan jullie ben ik heel veel dank verschuldigd. Ik heb jullie mogen begeleiden naar het HLO-diploma, maar al het praktische werk wat jullie voor mij hebben verricht is van onschatbare waarde.

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Roy Sanders Dank voor de fijne samenwerking en de fraaie vormgeving van dit proefschrift. Ik waardeer jouw geduld en creativiteit

Lieve moeders: Spijtig dat de mannen dit niet meer mee kunnen maken. Ook ik mis hen nog elke dag. Jullie stonden altijd voor ons klaar. Ik dank jullie vooral voor alle fijne jaren en de onvoorwaardelijke steun in alles wat ik deed. Ik heb vaak geprobeerd uit te leggen waar ik allemaal mee bezig was, want interesse lieten jullie altijd blijken. Jullie hebben me altijd mijn drukke leven 'gegund' en nooit beslag op me gelegd. Ik ben jullie daar heel dankbaar voor.

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幸運はライデン大学で楽しい時を過し、en 日本で会おう !!

Annelies, mijn lief: Dank voor je geduld als ik je weer eens probeerde uit te leggen wat er nou zo belangrijk was aan het knoeien met navelstrengen en hompen vet. Ik heb je vast vaak verveeld met mijn overdreven enthousiasme. Toch was ik thuis ook vaak afwezig en onbereikbaar. Desondanks heb je nooit op de rem getrapt en me altijd de vrijheid gegeven om te doen wat ik dacht dat goed was. Nu dit ei gelegd is beloof ik er wat vaker voor je te zijn. Want zeg nou zelf: het leven begint toch pas bij 50.. !?

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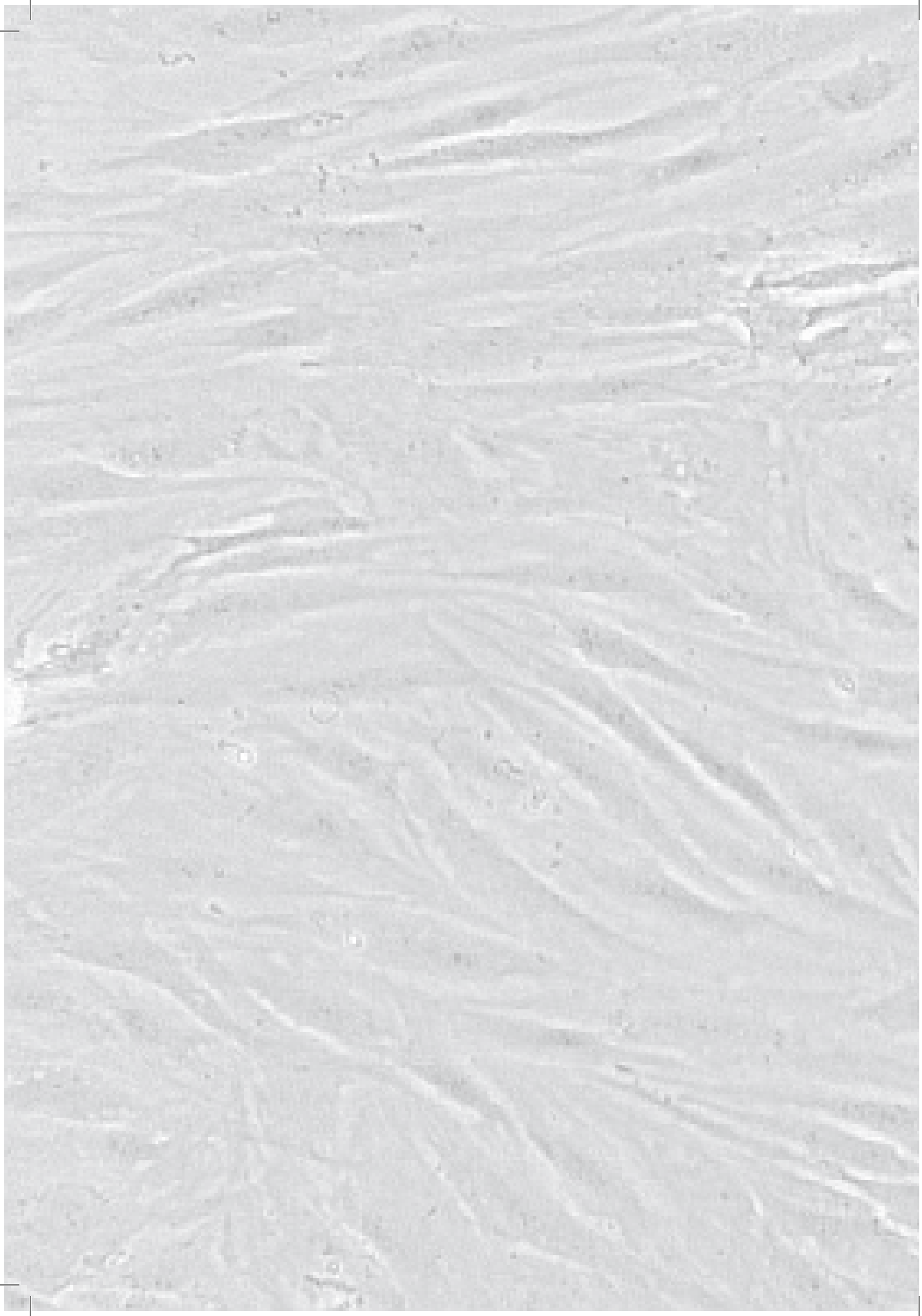
John

Juni 2009.



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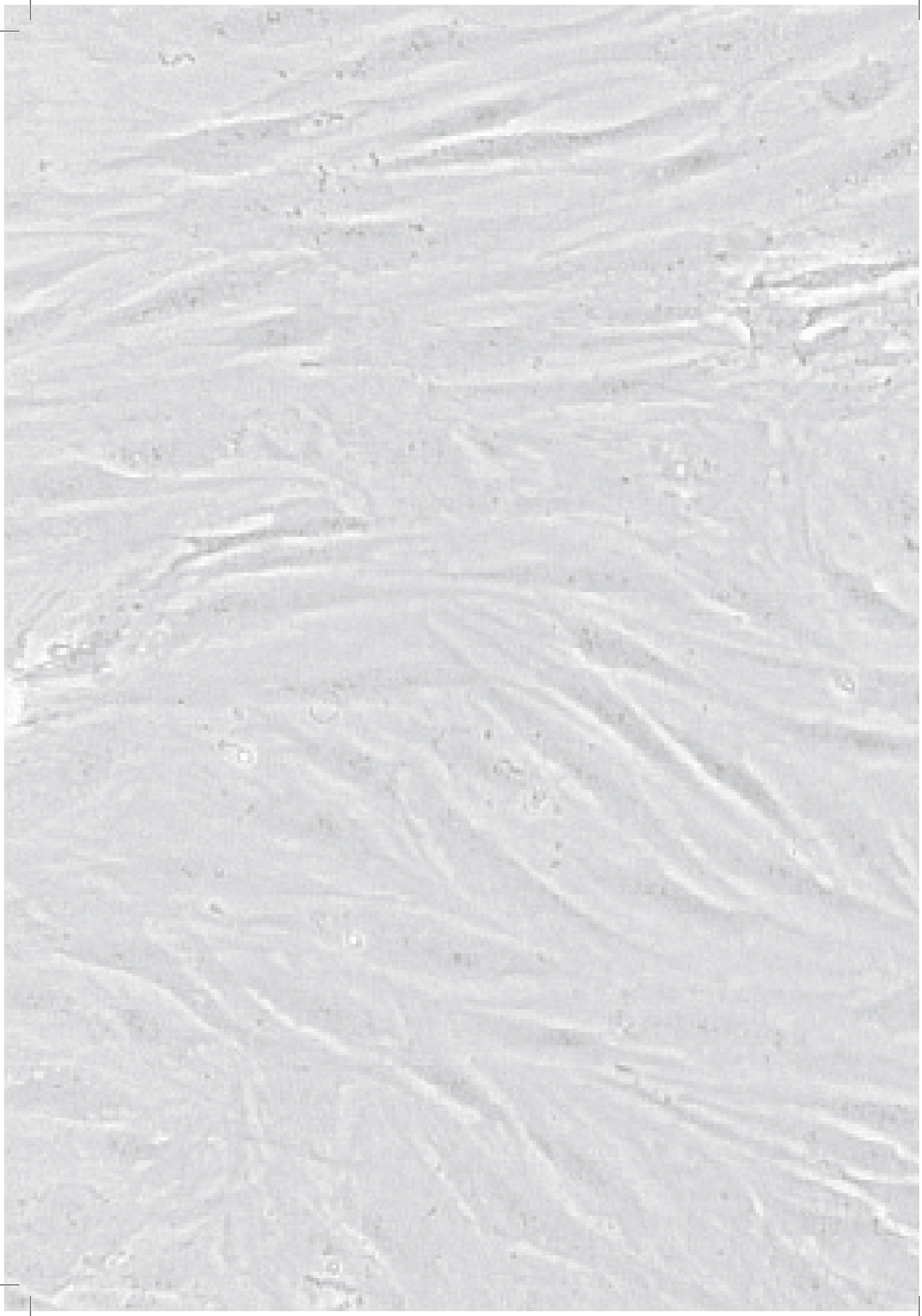
Color figures

Johannes Jacobus Maria Bouwman was born on April 28th 1959 in Utrecht, The Netherlands.

After graduation from secondary school (college De Klop, Utrecht) in 1976, he started his HBO-A education medical microbiology at the Ir. L.W. Gijsen Instituut in Utrecht. He fulfilled his training period at the CBSL Laboratories Hilversum and successfully completed his study in 1978. His career as a lab technician started at the Streeklaboratorium Deventer. In 1979 the Military Hospital Dr. A. Mathijsen in Utrecht became his next employer. From 1988 until 1990 he was a lab technician at the virology department of the GG&GD Amsterdam. At this department he acquired the knowledge and proficiency for the cell and viral culture techniques that eventually have been the basis for the fundamental research work presented in the studies of this thesis.

The Department of Medical Microbiology of the Diakonessen Hospital Utrecht has been the employer since December 1990. At first, all obligatory tasks as a lab technician in medical microbiology were performed. Starting from 1992 he was involved in development and implementation of a virology unit and started introducing molecular diagnostics in 1996. Next to these activities, in 1994 he became a laboratory attendant for HLO-trainees and medical students during their research- and graduation stage. In 2001 the research described in this thesis started, alternating part time and full time, under supervision of dr. RJ.A. Diepersloot (Department of Medical Microbiology; Diakonessen Hospital Utrecht) and dr. F.L.J. Visseren (Deptartment of Vascular Medicine; University Medical Center Utrecht). 2008 and 2009 were largely dedicated to research and the completion of this thesis.

John Bouwman married Annelies Hornstra in 1982. They live in Nieuwegein and have two daughters: Monique (1987) and Rianne (1990).



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Chapter 3

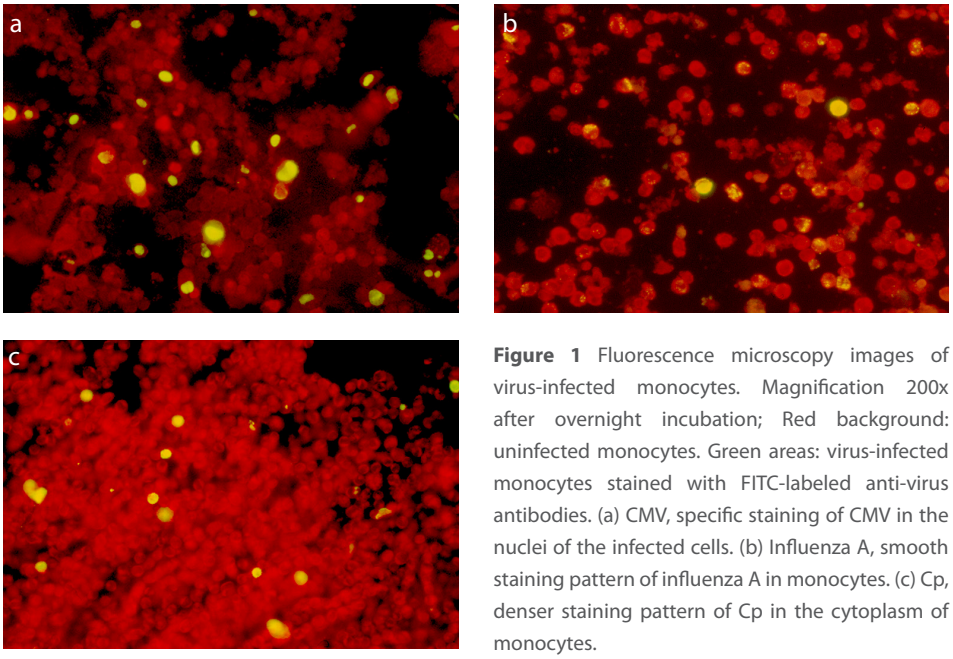

Figure 1 Fluorescence microscopy images of virus-infected monocytes. Magnification 200x after overnight incubation; Red background: uninfected monocytes. Green areas: virus-infected monocytes stained with FITC-labeled anti-virus antibodies. (a) CMV, specific staining of CMV in the nuclei of the infected cells. (b) Influenza A, smooth staining pattern of influenza A in monocytes. (c) Cp, denser staining pattern of Cp in the cytoplasm of monocytes.

Chapter 4

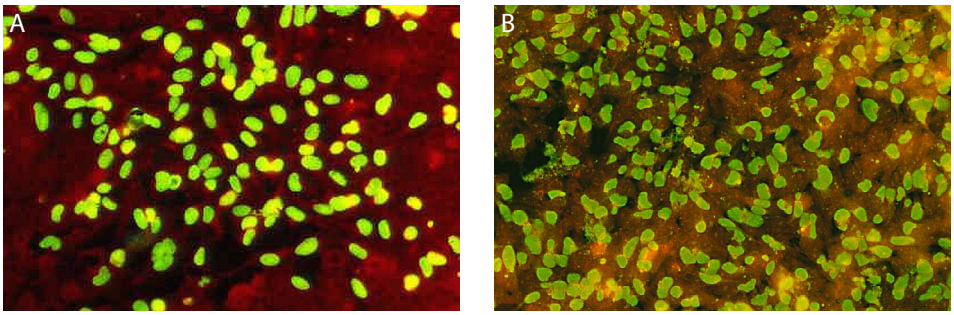

Figure 1 Infection of HepG2 cells. **A**: Fluorescence staining of CMV-infected HepG2 cells. Note apple-green fluorescence of CMV immediate early antigen in the nuclei of infected cells. **B**: Fluorescence staining of C. pneumoniae–infected HepG2 cells. Apple-green fluorescence (genus-specific) denotes Chlamydia antigens in the cytoplasm of infected cells. Background of each photo contains uninfected cells counterstained with Evans blue (red). Original magnification 100x

Chapter 5

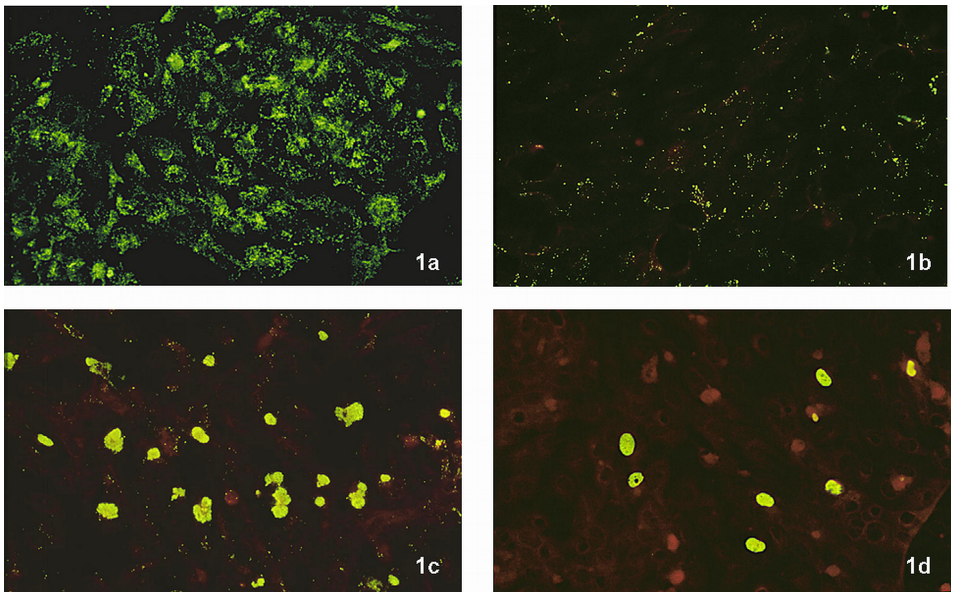

Figure 1. Fluorescence microscopy images (×200) of human umbilical vein endothelial cells (HUVECs) 48 h after inoculation. Indirect immunostaining with antihuman von Willebrand factor (a), anti-Cp (b,c) and anti-CMV (d) antibodies. Secondary antibody: FITC-labelled rabbit antimouse conjugate; counterstaining: Evans Blue. Red background: uninfected cells. Apple green fluorescence: positive identification of von Willebrand factor (a), inactivated *Cp* (b), *Cp* (MOI = 1) (c) and CMV (MOI = 1) (d).

Chapter 6

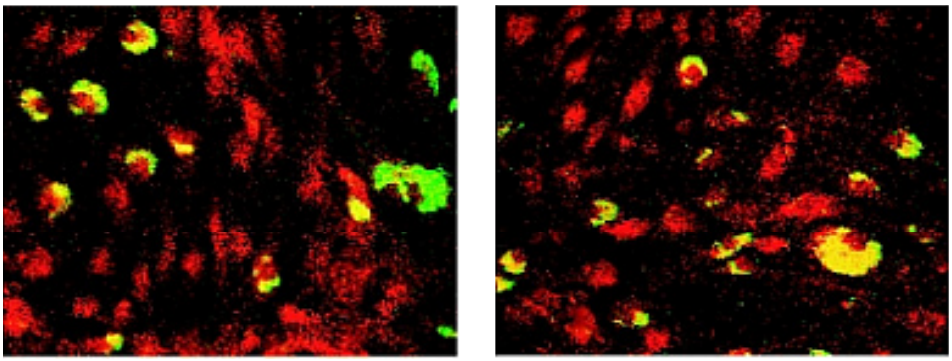

Figure 4d Confocal laser micrographs, representing four different slides, visualize the infectivity of Cp (green) on HUVECs (red) in the absence and presence of iron.

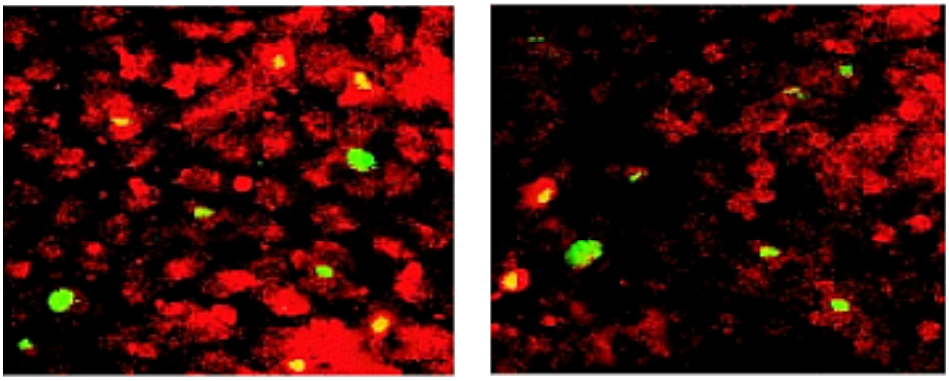

Figure 5d Confocal laser micrographs, representing four different slides, visualize the infectivity of CMV (green) on HUVECs (red) in the absence and presence of iron.

Chapter 7

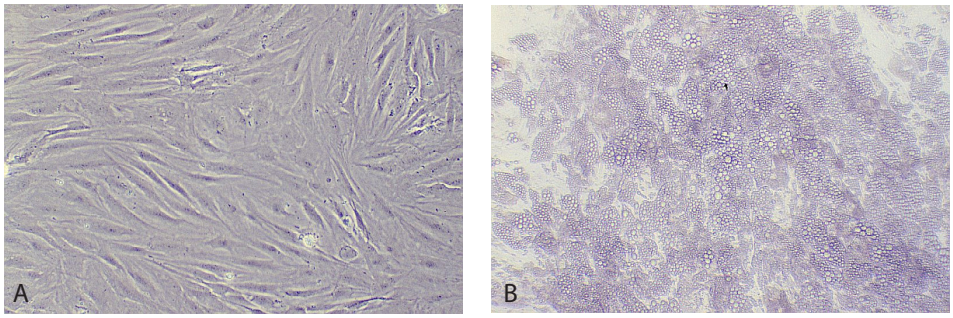

Figure 1 (Pre-) Adipocytes in culture (200x)

A Pre-adipocytes: spindle-shaped "fibroblast-like" cells grown to confluence.

B Adipocytes: differentiated cells 13 days after start of differentiation. Cells are rounded and have accumulated lipid droplets in their cytoplasm

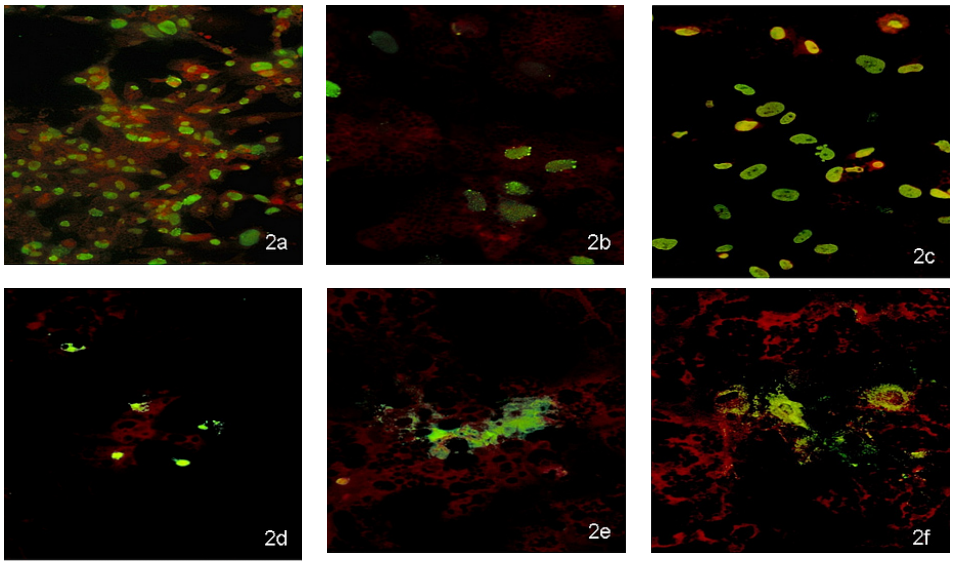

Figure 2. Fluorescent images of infected adipocytes (200x, 90% differentiated) after 48 hours infection with: Ad2, Ad 36, Cp, CMV, Inf, and RS-virus. Background (red): uninfected cells. Infected cells: apple green fluorescence either condensed in the nuclei (CMV;2c) or diffuse in the cytoplasm (Ad 2, Ad 36, Inf, RSV; 2a,b,d,e,f respectively). Cp-infected cells: compact fluorescent vacuoles in the cytoplasm (2d)

Chapter 8

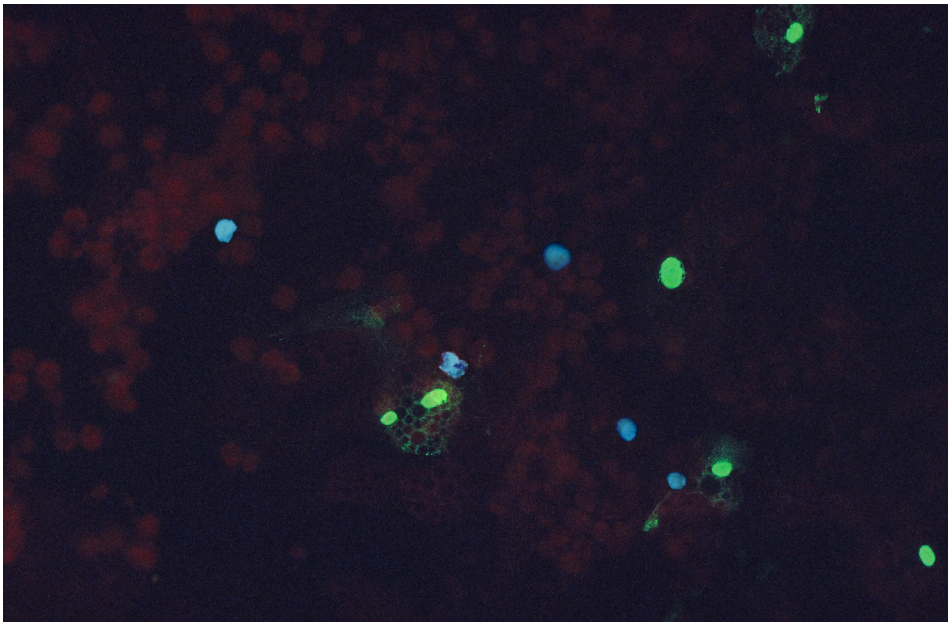

Figure 1 Dual color immunofluorescence image of transinfection of influenza A in a co-culture of adipocytes and THP-1 cells. Influenza infected THP-1 cells were co-cultured with uninfected adipocytes for 48 hours. THP-1 cells infected with influenza A demonstrate blue fluorescence (AMCA label). After 48 hours, influenza infection was transmitted from THP-1 cells to adipocytes, as indicated by apple green fluorescence (FITC label). Background: uninfected adipocytes and THP-1 cells (round) counterstained with Evans Blue (red). Lipid droplets in adipocytes are clearly visible as dark inclusions in the cytoplasm of adipocytes.