CHAPTER 4

Iron mediates differential activation of human endothelial cells in response towards *Chlamydia pneumoniae* or Cytomegalovirus infection

Apriliana E. R. Kartikasari, Niki A. Georgiou, Maarten de Geest,
J. Henny van Kats-Renaud, John J. M. Bouwman, B. Sweder van Asbeck,
Joannes J. M. Marx, Frank L. J. Visseren

Submitted

ABSTRACT

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 artherogenic. 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. In this study, we measure endothelial ICAM-1, VCAM-1 and E-selection expression using flow cytometry, as an indication of endothelial activation. An increased number of infected endothelial cells in a monolayer population lead to a raised expression of adhesion molecules of the whole cell population, suggesting paracrine interactions. Iron additively upregulated Cp-induced VCAM-1 expression, while synergistically potentiated Cp-induced ICAM-1 expression. Together with CMV, iron also stimulated ICAM-1 and VCAM-1 expression. 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 through an increased cellular oxidative stress. We conclude that endothelial response towards chronic infections is alterable by intracellular iron levels. This finding implies that iron status in populations positive for Cp or CMV infections could be an important determinant in having increased risk of developing atherosclerosis.

Key words: infection • atherosclerosis • adhesion molecules

INTRODUCTION

Chronic inflammation plays a crucial role in coronary artery disease (CAD) and other manifestations of atherosclerosis.[1] Immune cells and their effector molecules participate in and are also capable of accelerating the progression of the lesions. The pathogenic inflammatory event of atherosclerosis is characterised by over recruitment of leukocytes to the sites of inflammation. Leukocyte infiltration is mediated by activated vascular endothelial cells, which are expressing their surface selective adhesion molecules to attract and bind various types of leukocytes, such as monocytes and T-lymphocytes.[1] The activation of endothelial cells therefore is a key initial step of the atherogenic inflammatory cascade.

Several stimuli like oxidised lipoproteins, oxidative stress as well as infectious agents are capable of triggering endothelial cell activation that leads to local inflammation, endothelial dysfunction, and finally atherosclerotic lesion formation.[2] A large number of studies have linked CAD risk to certain persistent microorganism infections, like *Chlamydia pneumoniae* (Cp) and cytomegalovirus (CMV)[3-6] Lack of correlations between Cp serology and atherosclerotic lesion, however, has been observed in several studies.[7] Moreover, the evidence for the role of CMV in atherogenesis is still conflicting.[5] Further studies are therefore warranted especially to unravel the pathological mechanisms of infections in arterial disease.[5,6]

Endothelial cell activation, the key mechanism of atherosclerotic inflammation, is characterised by upregulation 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.[8-11] Cp, the gram-negative obligate intracellular bacterium, is capable of infecting endothelial cells[12-15] as well as inducing the expression of adhesion molecules on these cells.[16,17] Cp infection also leads to increased soluble adhesion molecules in human.[18] Differential induction of adhesion molecule expression by CMV has also been demonstrated.[19-23] The upregulation 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 a high infection prevalence in the community [24,25] Cp has been established as a respiratory pathogen and contributed to 10 – 20 % of community-acquired pneumonia,[24] while 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.[25]

Recently, we and others have shown that iron status influences the endothelial activation state.[26-38] There are many abnormal conditions that may cause increased body iron stores, and formation of low molecular weight labile forms of iron that are capable of freely entering cells with no feedback-regulated process.[39] These conditions include hereditary hemochromatosis and secondary iron overload like in thalassemia with frequent blood transfusions.[40] These forms of iron may play an important role in the development of cardiovascular disease.[41,42] Coincidently, iron has been found accumulating in human atheroma.[43] Several other studies show reduced formation of early atherosclerotic lesions by means of iron chelation or iron-deficient diets in experimental animals.[44-47] 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-catalysed 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 hemochromatosis and secondary iron overload, this study provides important new insights and advances to the knowledge of the pathological mechanism of infections in arterosclerotic artery disease.

EXPERIMENTAL PROCEDURES

Baseline iron level

The iron content of the endothelial growth medium-2 (EGM-2, Clonetics, Wallkersville, 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). In this measurement, iron was first freed from any complexes by lowering the pH of the solution. The released iron was then complexed with a coloring agent for detection at a wavelength of 600 nm. To avoid any external iron contamination, in all experiments, plastic materials with lower affinity for iron compared to glass were used.

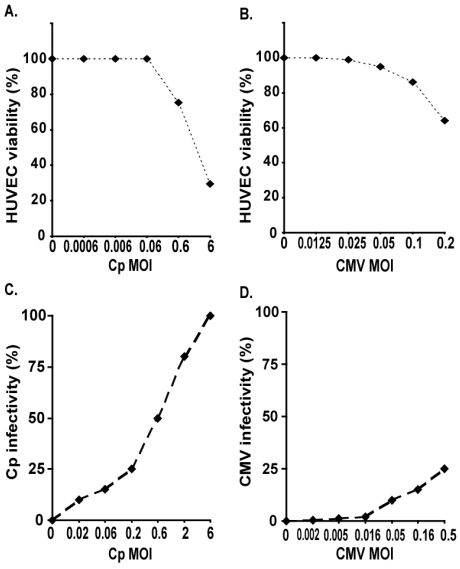

Figure 1. Endothelial viability and microorganism infectivity. (A) shows the percentage of HUVEC viability determined by MTT cytotoxicity assay 2 days after Cp-infection at indicated MOI, while (B) shows HUVEC viability 4 days after CMV infection. (C) shows the percentage of Cp infectivity towards HUVECs determined by immunostaining, and (D) shows CMV infectivity. (all data, n=3)

HUVEC isolation and culture

HUVECs were isolated and cultured as described by Jaffe et al.[48] To minimise 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 during and maintained at a cobblestone confluent density for all conducted experiments.

Propagation of microorganisms

Human embryonic lung (HEL) and buffalo green monkey cells (BGM) were cultured at 37^0C and 5% CO_2 in minimal essential medium Eagle with Earle's salts (EMEM, Gibco) containing 10% FBS. 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 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 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 resuspended in the same medium containing 2% FBS, 10% DMSO (Sigma-Aldrich, Zwijndrecht, The Netherlands) and supplements.

Cp-strain AR39 was propagated in BGM cells at 37^0C/5% CO_2 in EMEM containing 10% FBS and 0.1% cycloheximide (Sigma-Aldrich) and supplements. After 72 hours 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 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 amphotherycin 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 -80^0C until further use. The $TCID_{50}$ of CMV was determined by daily examination of the infected HEL cells for cytopathologic effects during 1 week, while the tissue culture infective dose ($TCID_{50}$) 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).[15]

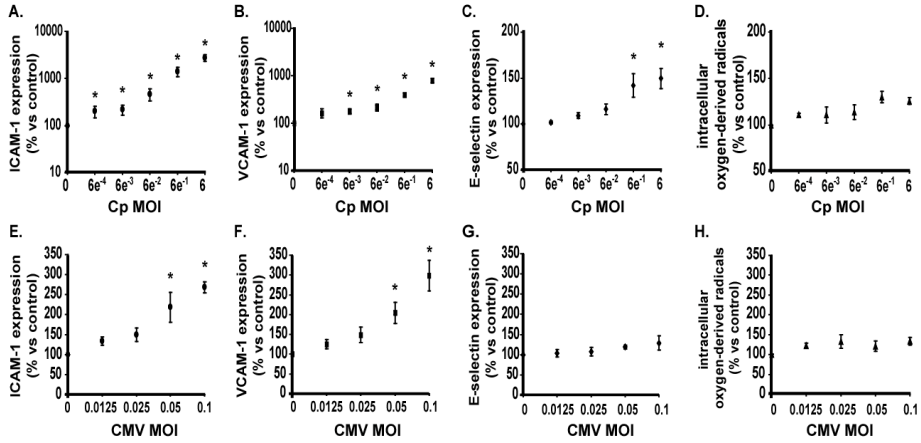

Figure 2. Induction of adhesion molecule expression by Cp or CMV infection. The expression of ICAM-1 (A), VCAM-1 (B), E-selectin (C) on, and the levels of intracellular oxygen-derived radicals (D) in HUVECs after Cp infection at indicated MOI (mean±SEM; n=4; *P<0.05). The expression of ICAM-1 (E), VCAM-1 (F), E-selectin (G) on, and the levels of intracellular oxygen-derived radicals (H) in HUVECs after CMV infection at indicated MOI (mean±SEM; n=4; *P<0.05).

Inoculation and immunostaining of endothelial cells

Cp and CMV were prediluted in endothelial EGM-2 medium and added at multiplicity of infection (MOI) of 0.1 for both Cp and CMV. Uninfected cells and filtrate of microorganisms through a 100 kD Microcon filter (Millipor, 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, BioRad, Redmond, WA, USA) and CMV (anti-CMV immediate early antigen clone E13, no.12-003, Argene, Varilhes, France).

Confocal scanning laser microscopy

For visualisation purpose, infected immunostained cells were fixed in PBS containing 3% paraformaldehyde (Polysciences, Warrington, USA) and 0.02% glutaraldehyde (Merck, Darmstadt, Germany) and visualised 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 (Sigma, 1:6 iron-citrate molar ratio) solution was made by dissolving the iron crystals in distilled water at 56^{o}C for 30 minutes. Iron solutions were always freshly prepared and filter-sterilised prior to use. The iron chelators were prepared as stocks in PBS of 10 mmol/L deferoxamine (Novartis, Arnhem, The Netherlands) and 30 mmol/L deferiprone (Duchefa Biochemie, Haarlem, The Netherlands), and stored at –20^{0}C prior to use. Final pH in incubation medium was maintained at 7.8. Several radical scavengers were used in some experiments, including tempol (Sigma), thiourea (OPG, Utrecht, The Netherlands) and 1,3 -dimethyl-2-thiourea (DMTU, Aldrich Chemical).

Viability assay

Cellular viability of HUVECs was monitored by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich) method.[49] Compound cytotoxicity was expressed as a TC_{50} denoting the concentration resulting in 50% loss of cell viability, as calculated by Calcusyn.[50]

Fluorescence-activated cell sorting (FACS)

HUVECs were harvested by incubating with 0.2% trypsin-EDTA at 37^{0}C for 3 minutes. The cells were then incubated with fluorescence-labeled monoclonal antibodies against the surface proteins, FITC-conjugated ICAM-1 antibody (R&Dsystem, Minneapolis, USA), PE-conjugated VCAM-1 antibody (BDBiosciences, San Diego, USA), or Cychrome-conjugated E-selectin antibody (BDBiosciences), for 30 minutes at 4^{0}C. Each flow cytometric measurement was performed using a Becton Dickinson (San Jose, USA) FACScan and 10.000 events were analysed.

2,7-dichlorofluorescein (DCF) assay

Carboxydichlorofluorescein diacetate (DCFH-DA, Molecular Probes) is a nonpolar compound that is converted into a membrane-impermeable non-fluorescent polar derivative (DCFH) by cellular esterase after incorporation into cells. The trapped DCFH is rapidly oxidised to fluorescent 2,7-dichlorofluorescein (DCF) by intracellular hydrogen peroxide and hydroxyl radicals.[51] HUVEC were harvested by incubating with 0.2% trypsin-EDTA at 37^{0}C for 3 minutes. Cells were then resuspended in DCFH-DA at a final concentration of 5 µmol/L, incubated for 30 minutes at room temperature and washed. The emission of the trapped, oxidised DCF in 10.000 cells was analysed on a FACScan.

Calcein assay

In this assay,[52] cells were incubated with 0.125 µmol/L calcein-AM (30 minutes 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.

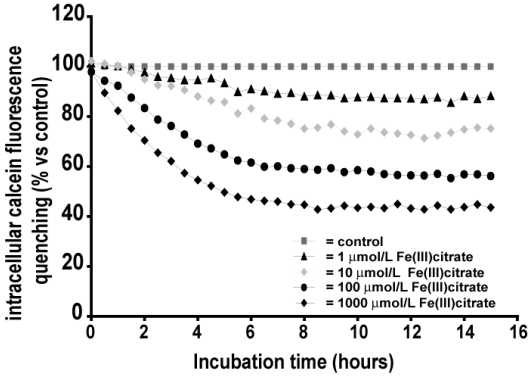

Figure 3. Endothelial intracellular iron level is modulated by addition of low molecular weight iron. Data represent the normalised mean fluorescence of intracellular calcein in HUVECs in the presence of specified concentrations of Fe(III)citrate, monitored up to 15 hours (n=3). Quenching indicates the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

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 $TCID_{50}$ values of the stock Cp and CMV were calculated as 5.5 and 4, respectively, indicating titers of 6 x 10^6 Cp/mL and 2 x 10^5 CMV/mL. Since HUVEC density was 10^5 cells/cm^2 at a cobblestone confluency, to obtain an MOI of 0.1 for Cp and CMV, Cp was diluted 300 times while CMV was diluted 10 times before being used for HUVEC inoculation.

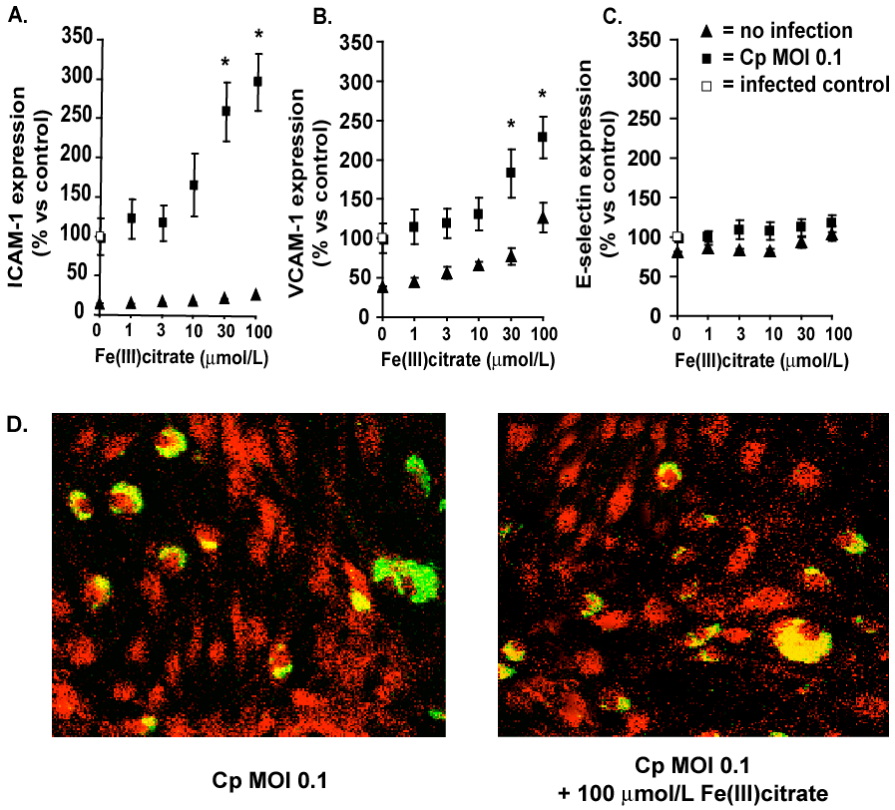

Figure 4. Iron modulates Cp-induced adhesion molecule expression. The expression of ICAM-1 (A), VCAM-1 (B), E-selectin (C) on HUVECs 2 days after Cp infection in the presence of increasing iron concentrations. (mean±SEM; n=4; *P<0.05). (D) confocal laser micrographs, representing 4 different slides, visualise the infectivity of Cp (green) on HUVECs (red) in the absence and presence of iron.

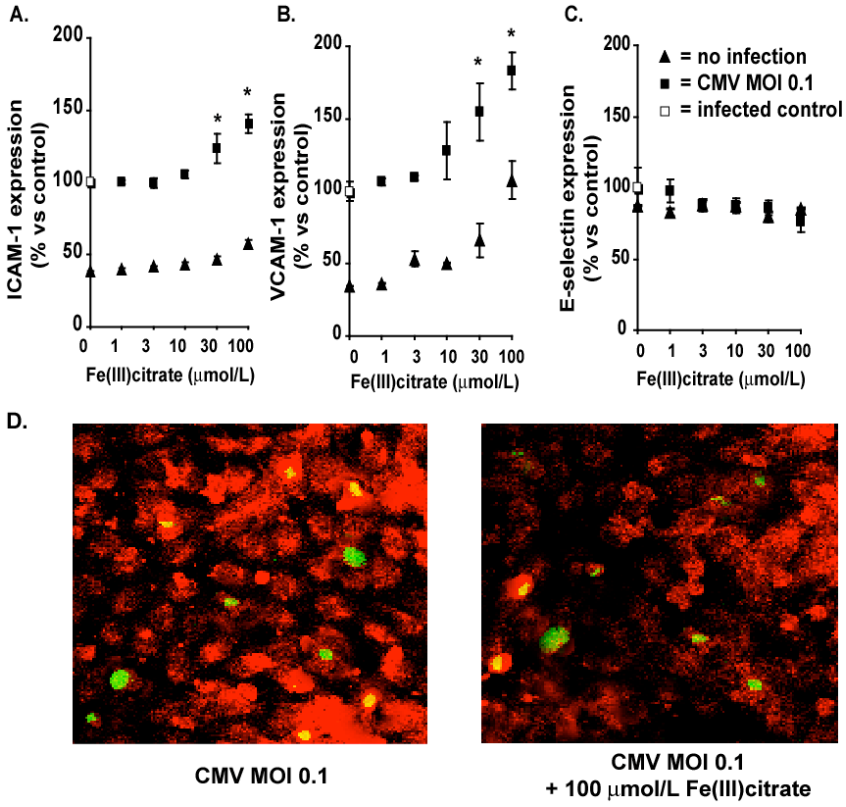

Figure 5. Iron modulates CMV-induced adhesion molecule expression. The expression of ICAM-1 (A), VCAM-1 (B), E-selectin (C) on HUVECs 4 days after CMV infection in the presence of increasing iron concentrations. (mean±SEM; n=4; *P<0.05). (D) confocal laser micrographs, representing 4 different slides, visualise the infectivity of CMV (green) on HUVECs (red) in the absence and presence of iron.

Cytotoxicity testing

The viability of HUVECs, after Cp infection, at an MOI of 0.1 was >85% (Figure 1a). Similarly CMV infection at an MOI of 0.1 (Figure 1b) resulted in HUVEC viability of >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 to 72 hours,[53] while the slow-replicating CMV enters the early stage of infection at 72 to 96 hours post-infection, and reaching the late stage of infection at 7 days post-infection.[54] 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

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 MOI of as low as 0.0006. E-selectin was significantly upregulated by Cp starting at MOI of 0.6 (Figure 2c).

CMV at MOI of as low as 0.05 induced more than two-fold increase in both ICAM-1 and VCAM-1 expression, while E-selectin upregulation was negligibly noticed (Figure 2e-g). The filtrate of microorganisms through a 100 kD Microcon filter did not result in upregulation 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.

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 hours (Figure 3). An iron concentration of as low as 1 μmol/L was able to quench 5% of calcein signal after 2 hours and 10% after 5 hours, 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 cytoplasmic 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, while ICAM-1 expression was synergistically upregulated by iron (Figure 4a-c). Iron of 30 μmol/L significantly enhanced Cp-induced VCAM-1expression by two folds and ICAM-1 expression by almost three folds. Infectivity and the size of inclusions of Cp in HUVECs were not affected by various concentrations of iron, ranging between 0-1000 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.

CMV-induced endothelial adhesion molecule expression was enhanced by iron

Both CMV-induced VCAM-1 and ICAM-1 expression were additively upregulated by iron (Figure 5a-b), while E-selectin was not affected (Figure 5c). Iron of 30 μmol/L significantly enhanced CMV-induced VCAM-1expression by 1.5 folds, and CMV-induced ICAM-1 expression by 1.3 folds. 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 0-1000 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.

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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 downregulate 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 $\pm$ 2.3 μmol/L, deferiprone = 100 $\pm$ 11.3 μmol/L).[27]

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] Since serological associations were found between Cp or CMV infections and acute myocardial infarctions as well as chronic coronary heart disease,[3-6] there has been much effort on determining how endothelial infection by Cp or CMV causes endothelial dysfunction. One well-characterised 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 upregulated 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,[55] are crucial to generate response towards infections. 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.

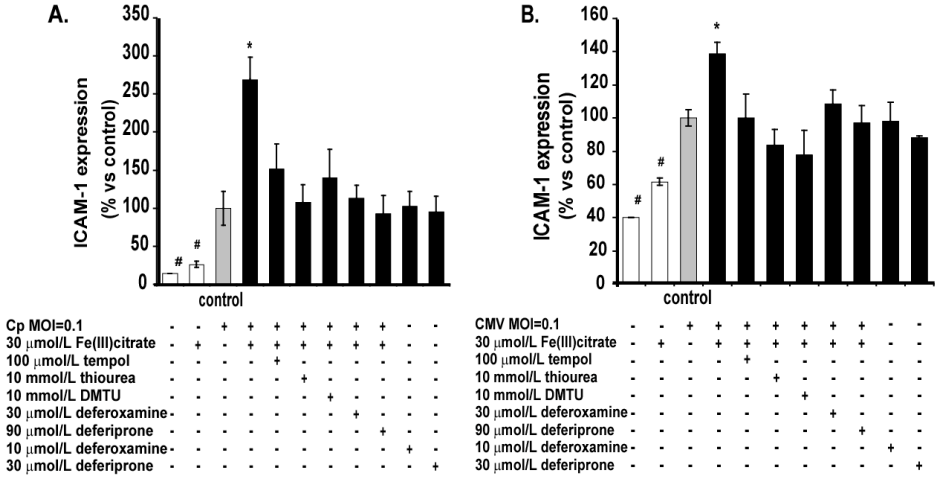

Figure 6. Effects of iron chelation and radical scavenging on infections. The expression of ICAM-1 on Cp (A) or CMV (B) infected HUVECs in the presence of indicated compounds. (mean±SEM; n=3; *higher than control P<0.05; #lower than control P<0.05).

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.[56,57] 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.[16] 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[16,17] that in turn was potentiated by iron.[33]

Iron chelators alone, however, did not downregulate Cp or CMV-induced adhesion molecule expression, since there are 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, i.e. in the order of 10 folds less than in freshly isolated cells [29,31] that further chelation probably 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 different pathway than the formation of oxygen-derived radicals.

CMV infection has a relatively slow development cycle.[54] The early stage of infection is started at 72 to 96 hours post-infection[54] During this time CMV has produced the immediate early gene products that are capable of inducing endothelial activation [20,22]. In this study, we harvested HUVECs 4 day post-infection, to allow CMV-induced endothelial activation to take place, without propagation of the microorganism. Upregulation of both ICAM-1 and VCAM-1 was observed, while E-selectin was not affected significantly. This result confirms previous studies[19-21] showing differential induction of endothelial adhesion molecule expression by CMV, based on the stage of infection. Furthermore, the iron-primed endothelial cells generated higher levels of CMV-induced ICAM-1 and VCAM-1 expression, compared to controls, 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,[3-6] although some studies showed lack of evidence.[5,7] Our study demonstrated modulating effects of iron on the endothelial response towards chronic infections of Cp and CMV. This finding may imply that Cp or CMV infections in a population with increased body iron levels or disturbed iron homeostasis could aggravate the susceptibility to endothelial dysfunction and furthermore atherosclerotic vascular disease.

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