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

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Abstract

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 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.

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.
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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

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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 a 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-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 haemochromatosis 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.

Materials and 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 during 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^{-1} l-glutamine (Gibco), 5 mL non-essential amino acids (Gibco), 10 mg L^{-1} vancomycin (Faulding Pharmaceuticals, Brussels, Belgium), 4 mg L^{-1} amphotherycin B (Fungizone; Bristol-Meyers Squibb, Woerden, the Netherlands) and 10 mg L^{-1} 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^{-1} 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^{-1} sucrose-phosphate-glutamic acid (SPG) medium (2·088 g L^{-1} K_2HPO_4, 1·088 g L^{-1} KH_2PO_4, 68·46 g L^{-1} saccharose, 7·16 g L^{-1} l-glutamine, 10% FBS, 2·5 mg L^{-1} amphotherycin B, 23 mg L^{-1} vancomycin and 18 mg L^{-1} 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 (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, 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^{-1} 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 of 10 mmol L^{-1} deferoxamine (Novartis, Arnhem, the Netherlands) and 30 mmol L^{-1} 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 & Dsystem, 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 analysed.

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 DCFH-DA at a final concentration of 5 μm^{-1}, 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^{-1} 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 $P < 0{\cdot}05$.

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 titres of 6×10^6 Cp mL^{-1} and 2×10^5 CMV mL^{-1}. As HUVEC density was 10^5 cells cm^{-2} at a cobblestone confluency, 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 (Fig. 1a) or CMV infection (Fig. 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 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 (Fig. 1c). Additionally, CMV at an MOI of 0·1, gave rise to < 10% infection (Fig. 1d).

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

Chlamydia pneumoniae (Cp) infection markedly induced ICAM-1 (Fig. 2a) and VCAM-1 expression (Fig. 2b). A

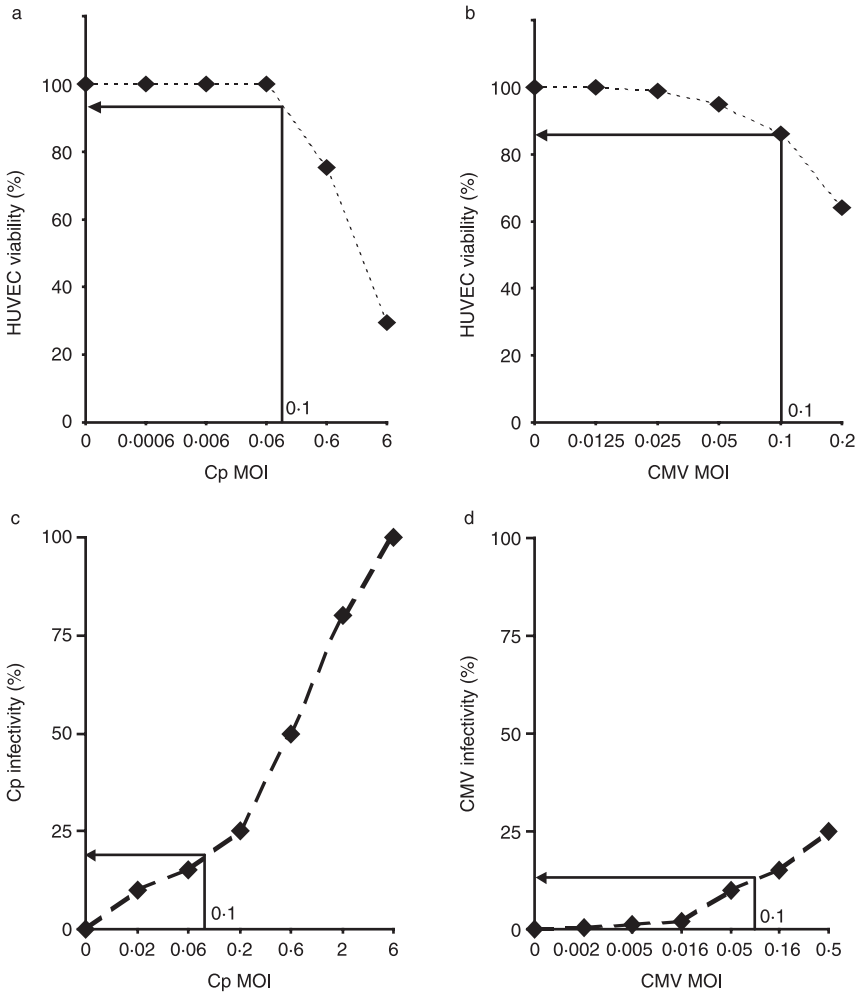


Figure 1 Endothelial viability and microorganism infectivity. (a) Shows the percentage of human umbilical vein endothelial cell (HUVEC) viability determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cytotoxicity assay, 2 days after *Chlamydia pneumoniae* (Cp) infection 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, $n = 3$).

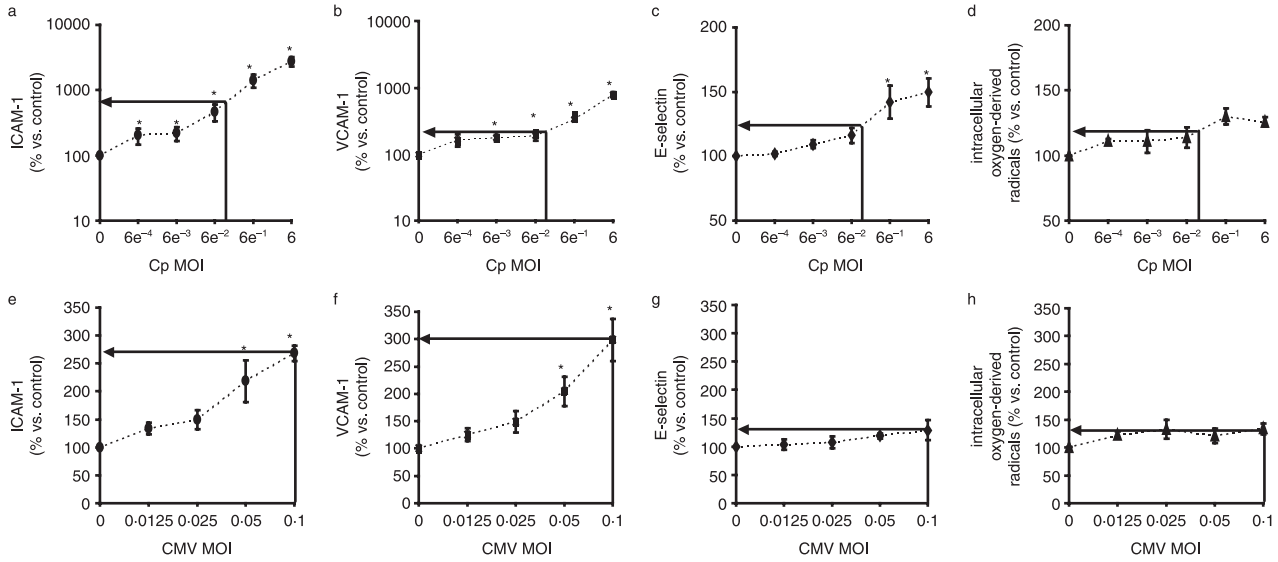


Figure 2 Induction of adhesion molecule expression by Cp or CMV infection. The expression of (a) intercellular adhesion molecule-1 (ICAM-1); (b) vascular cell adhesion molecule-1 (VCAM-1); (c) endothelial selectin (E-selectin); and (d) the levels of intracellular oxygen-derived radicals in human umbilical vein endothelial cells (HUVECs) after *Chlamydia pneumoniae* (Cp) infection at an indicated multiplicity of infection (MOI) (mean ± SEM, $n = 4$, $^{*}P < 0{\cdot}05$). 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, $n = 4$, $^{*}P < 0{\cdot}05$).

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 (Fig. 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 (Fig. 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- (Fig. 2d) and CMV-infected HUVECs (Fig. 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^{-1}. Accumulation of intracellular labile iron within HUVECs was monitored by following the fluorescence of calcein for up to 15 h (Fig. 3). An iron concentration of as low as 1 μmol L^{-1} 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 cytoplasmic labile iron.

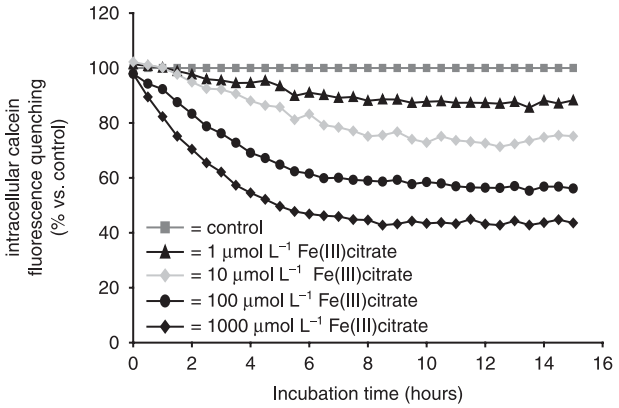


Figure 3 Endothelial intracellular iron level is modulated by addition of low molecular weight iron. Data represent the normalized mean fluorescence of intracellular calcein in human umbilical vein endothelial cells (HUVECs) in the presence of specified concentrations of Fe(III)citrate, monitored up 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 (Fig. 4a–c). Iron of 30 μmol L^{-1} significantly enhanced Cp-induced VCAM-1expression two-fold and ICAM-1 expression almost three-fold. Infectivity and the size of inclusions of Cp in HUVECs

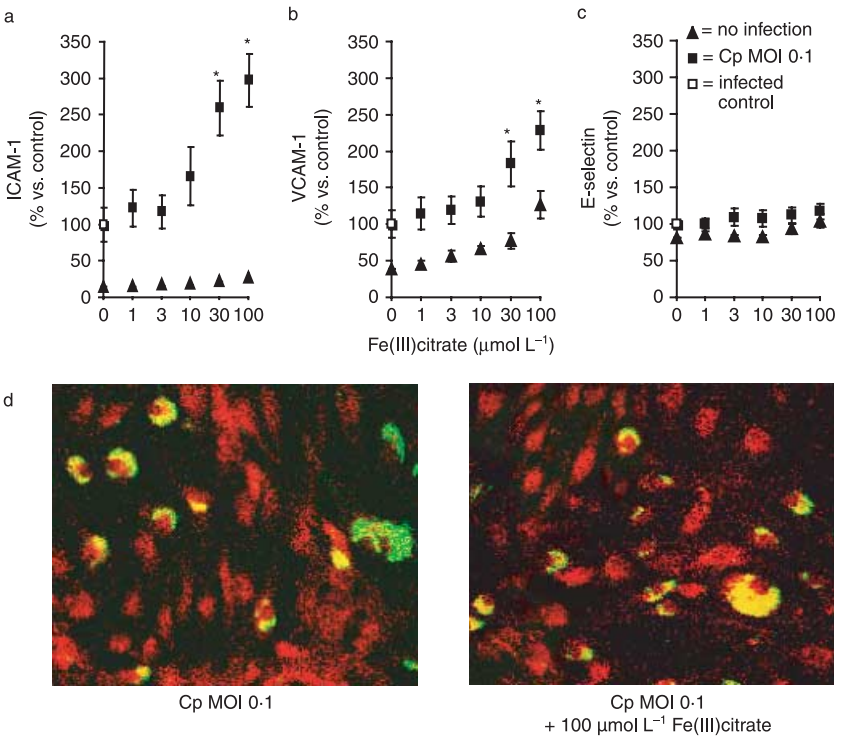


Figure 4 Iron modulates Cp-induced adhesion molecule expression. The expression of (a) intercellular adhesion molecule-1 (ICAM-1); (b) vascular cell adhesion molecule-1 (VCAM-1); and (c) endothelial selectin (E-selectin) on human umbilical vein endothelial cells (HUVECs) 2 days after *Chlamydia pneumoniae* (Cp) infection in the presence of increasing iron concentrations (mean ± SEM, $n = 4$, $^{*}P < 0{\cdot}05$). (d) Confocal laser micrographs, representing four different slides, visualize the infectivity of Cp (green) on HUVECs (red) in the absence and presence of iron.

were not affected by various concentrations of iron, ranging between 0 and 1000 μmol L^{-1}. This result was obtained by examination using fluorescence microscopy after immunostaining with monoclonal antibody against Cp (Fig. 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 up-regulated by iron (Fig. 5a–b), whereas E-selectin was not affected (Fig. 5c). Iron of 30 μmol L^{-1} 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^{-1} (Fig. 5d). This result indicates that iron could also modulate endothelial response towards chronic CMV infection, without affecting initial infectivity and the growth of CMV.

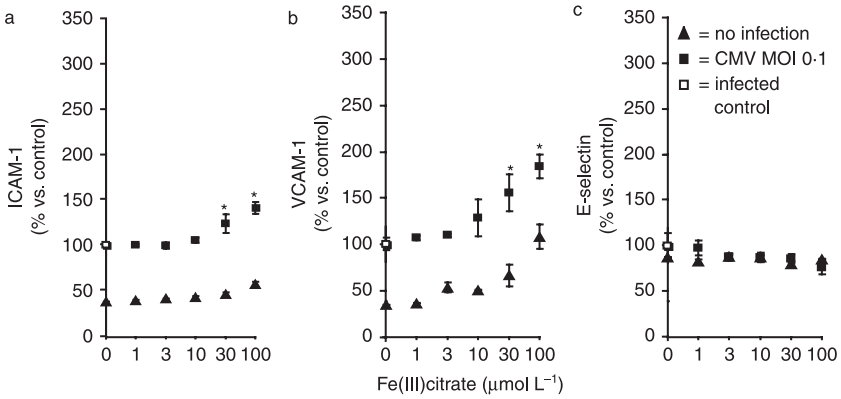
Iron chelation and radical scavenging could counteract potentiating effects of iron on infections

Addition of chelator-bound iron no longer modulated Cp or CMV-induced endothelial adhesion molecule expression (Fig. 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 (Fig. 6a–b).

Furthermore, addition of iron chelator alone did not significantly down-regulate Cp or CMV-induced adhesion molecule expression (Fig. 6a–b), suggesting that infections may induce endothelial activation through a distinct pathway than iron. Concentrations of 10 μmol L^{-1} for deferoxamine and 30 μmol L^{-1} 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^{-1}, deferiprone = 100 ± 11·3 μmol L^{-1}) [24].

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



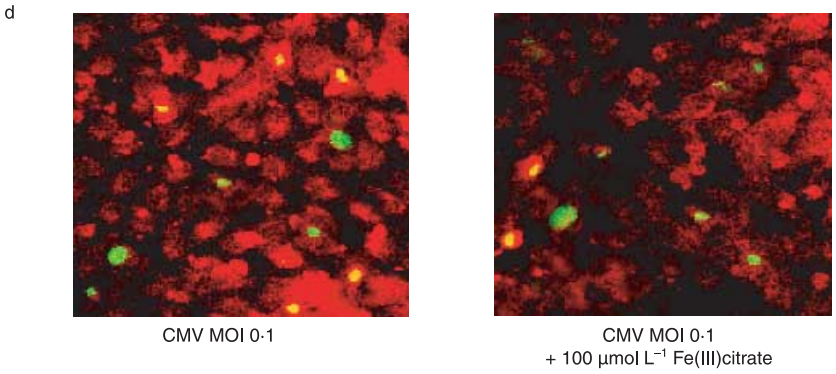


Figure 5 Iron modulates CMV-induced adhesion molecule expression. The expression of (a) intercellular adhesion molecule-1 (ICAM-1); (b) vascular cell adhesion molecule-1 (VCAM-1); and (c) endothelial selectin (E-selectin) on human umbilical vein endothelial cells (HUVECs) 4 days after cytomegalovirus (CMV) infection in the presence of increasing iron concentrations. (mean ± SEM, $n = 4$, $^{*}P < 0{\cdot}05$). (d) Confocal laser micrographs, representing four different slides, visualize the infectivity of CMV (green) on HUVECs (red) in the absence and presence of iron.

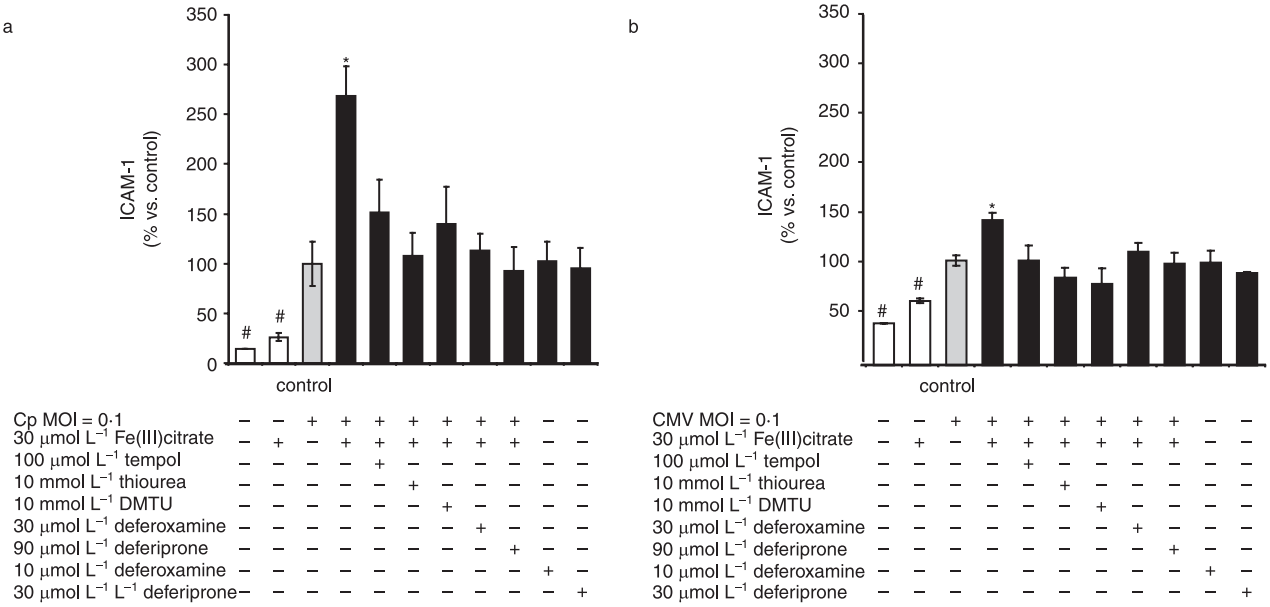


Figure 6 Effects of iron chelation and radical scavenging on infections. The expression of intercellular adhesion molecule-1 (ICAM-1) on (a) *Chlamydia pneumoniae* (Cp) or (b) cytomegalovirus (CMV) infected human umbilical vein endothelial cells (HUVECs) in the presence of indicated compounds. (mean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

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] 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 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.

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