Iron modulates phagocyte-endothelial cell interactions

Implications for atherosclerosis

Apriliana E. R. Kartikasari

Iron modulates phagocyte-endothelial cell interactions
Implications for atherosclerosis

Thesis, Faculty of Medicine, Utrecht University, The Netherlands

Electronically published by the University Library, Utrecht University, The Netherlands

ISBN	90-393-41818
Author	Apriliana E. R. Kartikasari
Cover	The secret nature for quality life
Cover design	Maarten de Geest
Printing	Febodruk B.V., The Netherlands
Correspondence	april.kartikasari@gmail.com

Iron modulates phagocyte-endothelial cell interactions

Implications for atherosclerosis

IJzer moduleert fagocyt-endotheliale cel interacties

Implicaties voor atherosclerose

(met een samenvatting in het Nederlands)

Zat besi memodulasi interaksi fagosit-sel endotel

Implikasi untuk aterosklerosis

(dengan ringkasan dalam bahasa Indonesia)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op
gezag van de Rector Magnificus, Prof. Dr. W. H. Gispen, ingevolge het besluit van
het College voor Promoties in het openbaar te verdedigen op maandag
13 februari 2006 des middags te 16.15 uur

door

Apriliana Ellya Ratna Kartikasari

geboren op 24 april 1975

te Demak, Midden Java, Indonesië

Promotor

Prof. Joannes J. M. Marx, MD, PhD

Eijkman-Winkler Centre,
University Medical Centre Utrecht, Utrecht, The Netherlands

Co-promotores

Niki A. Georgiou, PhD

Eijkman-Winkler Centre,
University Medical Centre Utrecht, Utrecht, The Netherlands

Frank L. J. Visseren, MD, PhD
Department of Vascular Medicine
University Medical Centre Utrecht, Utrecht, The Netherlands

The work described in this thesis was funded by a grant from the European Commission, QLK1-CT-2002-00444, awarded to Prof. J. J. M. Marx, MD, PhD.

Financial support by the Netherlands Heart Foundation and Alzheimer Nederland for the publication of this thesis is gratefully acknowledged.

The printing of the thesis was also financially supported by:

Akzo-Nobel Chemicals Pte. Ltd., Singapore
Pfizer B.V., The Netherlands
J. E. Jurriaanse Stichting, The Netherlands
Vifor International Inc., Switzerland
Merck Sharp & Dohme B.V., The Netherlands
Dr. Ir. van de Laar Stichting, The Netherlands

To my parents, my family,
and everyone involved in this endevour

Paranymphs:

Ružena Stránská, PhD

J. Henny van Kats-Renaud

Assessment committee:

Prof. I. M. Hoepelman, MD, PhD (University Medical Centre Utrecht, The Netherlands) - **chairman**

Prof. L. Koenderman, MD, PhD (University Medical Centre Utrecht, The Netherlands)

Prof. H. A. Koomans, MD, PhD (University Medical Centre Utrecht, The Netherlands)

Prof. R. C. Hider, PhD (King's College London, United Kingdom)

CONTENTS

CHAPTER 1

Iron metabolism and coronary artery disease: an introduction

Based on:

Apriliana E. R. Kartikasari, Niki A. Georgiou, Joannes J. M. Marx

Iron intake and cardiovascular disease

Functional foods, cardiovascular disease and diabetes

CRC Press LLC, USA, ISBN 0-8493-2559-5 and Woodhead Publishing Ltd, UK,

ISBN 1-85573-735-3, 2004, Chapter 6: 99-125

Iron is an essential dietary component, necessary for a number of cellular functions, like respiration and immune response. Excess or deficiency of iron can lead to impaired cellular functions. The importance of iron resides in its capacity to participate in electron exchange reactions. However, this same property makes it capable of catalysing free radical generation that can be deleterious to cells. Oxidative damage caused by free radicals is believed to be related to the pathogenesis of numerous diseases, such as atherosclerosis, diabetes, cancer and neurodegenerative diseases. Therefore, these seemingly paradoxical properties of iron require a balanced regulation of cellular iron levels. Since there is no physiological means of iron excretion from the human body, iron homeostasis is achieved by its regulated intestinal absorption as well as its efficient conservation. Several genetic predispositions, inflammatory disorders and infections can lead to disturbance of iron homeostasis. This chapter describes the metabolism of iron and its regulation. In particular, this chapter discusses potential roles of iron in the cellular processes that may lead to the development of atherosclerotic artery disease.

1. METABOLISM OF IRON

Since there is no specific means of iron excretion, iron is reutilised in the body, and only a small fraction is gained or lost each day. The daily iron losses are mostly from desquamation of epithelia. Greater iron losses may occur during growth in childhood, hemorrhages, menstruation and pregnancy in women.

A well-balanced diet containing sufficient iron is therefore needed to balance the 1 or 2 mg of daily losses. The efficiency of iron absorption is regulated by body requirements to maintain iron homeostasis. In the body, iron is mainly needed to form the porphyrin complex of hemoglobin (30 mg/kg), myoglobin in muscle cells (4 - 8 mg/kg) as well as other iron-containing enzymes. Up to 30% of body iron (12 mg/kg) can be stored as ferritin and hemosiderin in the bone marrow, the spleen and the liver.

1.1. CHEMICAL PROPERTIES OF IRON

Iron (Fe) belongs to the transition metal sub-family. The two main oxidative states are the divalent, Fe(II), and the trivalent, Fe(III). In aqueous media, the maximal solubility of Fe(III) is very low at 10^{-17} mol/L, whereas Fe(II) solubility is much greater at 10^{-1} mol/L. However, in the presence of oxygen, Fe(II) is rapidly oxidised to Fe(III). Because of the low solubility of iron, organisms have been forced to evolve proteins that are able to bind Fe(III) and keep it thermodynamically stable, and proteins that are able to catalyse the reduction of Fe(III) to make it kinetically available for biological processes. Through the Fenton reaction,[1] under biologically-relevant conditions, Fe(II) is capable of transforming molecular oxygen to the superoxide radical (O_2^-), and weak oxidant hydrogen peroxide (H_2O_2) into hydroxyl radical

($OH^{\cdot}$), the major damaging reactive species in nature.[2] In the reductive environment of the cell, Fe(III) may non-enzymatically be reduced back to Fe(II) giving rise to a vicious cycle of radical production.

1.2. Iron absorption

The extent of iron absorption is mainly affected by the level of body iron, the degree of erythropoesis, the amount of iron in the diet, and the composition of the diet itself. Other conditions, such as hypoxia, pregnancy, and inflammation, may also alter the absorption. Iron absorption can also be increased in conditions like primary hemochromatosis.

Both heme-iron and soluble complexes of iron are mainly absorbed in the duodenum and the upper jejunum of the small intestine. Heme iron derived from digested hemoproteins is taken up by the enterocytes through heme carrier protein-1 (HCP-1).[3] Inside the enterocytes, the heme is broken down by heme oxygenase-1. The released iron then enters the intracellular iron pool (LIP).

Non-heme iron complexes mainly exist in two oxidation states, the soluble Fe(II) and the almost insoluble Fe(III). The acidic environment of the stomach and some naturally occurring reductants reduce Fe(III), increase the solubility and hence the uptake of iron. The remaining insoluble Fe(III) can be taken up by the enterocytes after a reduction step catalysed by duodenal cytochrome-b (Dcytb).[4] The soluble Fe(II) is taken up by the enterocytes through a divalent metal transporter called Nramp-2, divalent cation transporter-1 (DCT-1) or divalent metal transporter-1 (DMT-1).[5]

The transport of iron from the enterocytes across the basolateral membrane to the circulation is mediated by an iron-regulated protein called Ireg-1 or Ferroportin-1.[4] The expression of the protein is localised in the duodenum and also in several other organs, such as the macrophages and the placenta where iron transfers between maternal and fetal circulations.

1.3. Cellular iron uptake and utilisation

1.3.1 Iron trafficking and uptake into cells

The intestinal mucosal cells and macrophages can donate iron to the plasma as Fe(II). The majority of this iron is rapidly oxidised by hephaestin in the basolateral membrane of the mature villus enterocytes, or ceruloplasmin secreted by the liver to the plasma. Transferrin-bound iron is offered to cells expressing the transferrin receptor, with the majority going to the erythroblasts in the bone marrow for hemoglobin synthesis.

Transferrin is produced by the liver and secreted to the plasma. It is also produced locally in the testes and the central nervous system. The protein contains two structurally similar subunits, each with one Fe(III) binding site. Both monoferric and diferric transferrins are internalised by receptor-mediated endocytosis. This endosome undergoes acidification to pH 5.5, weakening the association between iron

and transferrin. Iron is then reduced to Fe(II) and transported to the cytosol by DMT-1.[4] Once in the cytosol, Fe(II) becomes part of the LIP. The intact receptor-apotransferrin then recycles to the cell surface, where neutral pH promotes detachment of apotransferrin into the circulation. The average transferrin molecule with a half-life of eight days may be used up to one hundred times for iron delivery.

Only around 20 to 45% of the transferrin binding sites is occupied in the circulation, so that most of available transferrin molecules are not occupied by iron. Nevertheless, non-transferrin-bound iron (NTBI) can be detected in some conditions, such as in the serum of hemochromatosis patients[6] and end-stage renal disease patients receiving intravenous iron supplemetation.[7-13] NTBI is attached to a variety of ligands such as citrate, albumin, amino acids or sugars.[14,15] Non-hematopoetic tissues, mainly the liver, and also endocrine organs, kidneys, heart and the endothelium lining the blood vessels, preferentially take up NTBI utilising the DMT-1 transporter, of which expression can be downregulated by high cellular iron content.

1.3.2 CELLULAR IRON UTILISATION

Inside the cell, Fe(II) enters the LIP, which is a pool of weakly bound iron. Because of the reductive environment of the cell, iron in LIP is, on average, in the divalent state, although transient Fe(III) is expected as a result of cellular oxidations. Normally, the LIP represents only 3–5% of the total cellular iron, but this proportion changes with the iron status of the cell. Moreover, cells with higher LIP invariably exhibit higher levels of reactive oxygen species. Thus, the LIP is a marker not only of total cell iron content but could also determine the redox state of the cell.[16] Iron in LIP is able to enter various intracellular locations, mostly mitochondria (especially for heme biosynthesis), and ferritin (for storage).[17]

Heme biosynthesis occurs in all tissues, although the principal sites of synthesis are erythroid cells (~85%) and hepatocytes (accounting for nearly all the rest of heme synthesis). In hepatocytes, heme is incorporated into cytochromes, in particular the P_{450} class which is important for detoxification. In erythroid cells, almost all of the heme is synthesised for hemoglobin. Normally after 120 days, senescent red blood cells are engulfed by macrophages. In macrophages, the globin is converted into amino acids to be recycled. The heme is catabolised by heme oxygenase to biliverdin, iron and carbon monoxide. Most of the carbon monoxide is excreted through the lungs, while the iron is then either stored as ferritin or released into the plasma via Ireg-1. The released iron is oxidised to Fe(III) by ceruloplasmin and is again bound to circulating transferrin.

.

1.3.3 CONTROL MECHANISM FOR IRON HOMEOSTASIS IN CELLS

In vertebrates, cellular iron levels are post-transcriptionally controlled by the activity of iron regulatory proteins (IRPs), cytosolic proteins that bind to structural elements called iron-responsive

elements (IREs). The activities of IRPs are regulated by distinct posttranslational mechanisms in response to cellular iron levels. IRPs also respond, albeit differentially, to iron-independent signals, such as hydrogen peroxide, hypoxia, or nitric oxide.[18] IREs are found in the untranslated region of the mRNAs of the major proteins that regulate cellular iron homeostasis, such as DMT-1, Ireg-1, ferritin, transferrin and TfR-1.

In the case of ferritin production, for example, low intracellular iron conditions allow the binding of IRP to IRE, stopping protein synthesis. High intracellular iron level, on the other hand, prevents IRP-IRE binding, which results in increased ferritin synthesis. This IRP-IRE interaction provides control mechanism for intracellular iron homeostasis.[19]

1.4. Regulation of systemic iron homeostasis

Because iron is important for many biological processes, yet is highly reactive, its availability must be controlled not only at a cellular level but also systemically (Figure 1). In general, situations that require decreased iron availability are associated with decreased intestinal absorption and retention of iron by recycling macrophages. In contrast, situations requiring increased iron availability are associated with increased intestinal absorption and enhanced macrophage iron release.[20]

The circulating peptide hepcidin was recently discovered and its presence explains how iron release from absorptive enterocytes and macrophages is regulated in concert. Hepcidin, structurally similar to the defensin antimicrobial peptides,[21] is capable of negatively regulating intestinal iron absorption and macrophage iron release. It is primarily produced by the hepatocytes, secreted into the plasma, and cleared by the kidneys. Increased levels of hepcidin are produced in non-genetic iron overload and inflammation, while decreased hepcidin expression is seen in iron deficiency, accelerated erythropoesis and hypoxia.[22,23]

The proinflammatory cytokine interleukin-6 (IL-6) is able to stimulate hepcidin production.[23,24] Hepcidin production is currently hypothesised to be regulated in response to transferrin saturation, through a signal cascade triggered by the HFE protein.[25] This may explain why in hereditary hemochromatosis with mutated HFE, there is a drop in hepcidin synthesis despite high body iron stores.

The mechanism of action of hepcidin involves binding to ferroportin and promoting ferroportin internalisation and degradation. Loss of ferroportin from cell membrane ablates cellular iron export.[26] This mechanism explains the regulation of iron absorption from enterocytes as well as how macrophage recycling of iron is regulated. In addition to direct effects on ferroportin and iron export, hepcidin is expected to have secondary effects on cellular iron intake. A block of iron export by hepcidin would result in a rise in intracellular iron and the suppression of synthesis of the DMT1, thus reducing iron uptake.[27]

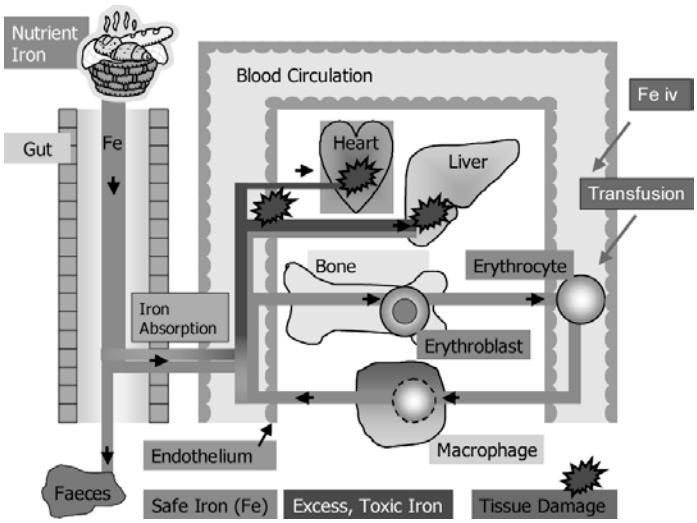

Figure 1. Illustration of body iron metabolism. Conditions that lead to excess iron and tissue damages are indicated. Adapted from Marx and Hider.[28]

1.5. DISORDERS OF IRON HOMEOSTASIS

Disorders in iron homeostasis may lead to conditions of iron deficiency or overload. Iron deficiency is a condition where the iron intake does not meet body's demands. Iron overload, on the other hand, is characterised by a progressive increase in the total body iron content followed by an abnormal iron deposition in multiple organs.

1.5.1. HEREDITARY HEMOCHROMATOSIS

Mutations in several genes described are known to result in pathological iron overload. The excess iron accumulates over time, leading to tissue damage and organ failure (Figure 1). Clinical consequences include hepatic failure, liver carcinoma, arthritis, diabetes, endocrine abnormalities and cardiac failure. The most common form of hemochromatosis results from homozygous mutations in an HLA class I-like gene, HFE.[29] The majority of the patients carry a missense mutation (C282Y) in HFE. Other mutations and polymorphisms (H63D, S65C, I105T, G93R) have been identified, but their contributions to hemochromatosis are not clearly understood. Most individuals with this missense C282Y mutation absorb excessive amount of dietary iron irrespective of the level of body iron. The

molecular function of HFE has remained obscure, however some observations have implicated HFE in the regulation of hepcidin expression.[30]

A less common type of hemochromatosis is juvenile hemochromatosis,[31] due to mutations either in the gene encoding hepcidin,[32] or in a novel gene, hemojuvelin (*HJV*).[33] This type of hemochromatosis is more severe than HFE hemochromatosis and it is characterised by rapid iron loading and clinical manifestations within the second decade of life. The striking similarity between the two genetic disorders suggests that hepcidin and HJV are components of the same regulatory system.

Another type of recessively inherited hemochromatosis is associated with mutations in the gene encoding transferrin receptor-2 (TfR-2).[34] This disease is phenotypically similar to HFE hemochromatosis. TfR-2 is expressed exclusively in hepatocytes, however its role in regulating iron homeostasis is unknown.

Mutations that lead to dominantly inherited iron-overload have been discovered, including mutations in the ferroportin gene and the ferritin gene. The ferroportin-associated disease shows macrophage-predominant iron loading,[35,36] while the defective ferritin molecule causes a disorder in the iron-storing process.[37]

While each of these hemochromatosis disease genes was initially discovered through the study of patients homozygous for mutations in a single gene, it is now clear that some patients with severe hemochromatosis carry mutations in more than one gene.

1.5.2. ACQUIRED IRON–OVERLOAD

Acquired hemochromatosis can be caused by chronic hemolysis and frequent blood transfusions, such as in aplastic anemia, sickle cell anemia, and thalassemia. Phlebotomy is mostly impossible in these cases. The end-organ manifestations of iron overload, like cirrhosis, cardiac failure, hepatocellular carcinoma, diabetes mellitus and hypopituitarism resemble the manifestations in hereditary hemochromatosis patients. The treatment most commonly used is a continuous administration of an iron-chelating agent. Deferoxamine is a widely used iron chelator, which needs to be administered intravenously. Several oral iron chelators have been developed in recent years, including deferiprone that has been registered in several countries, as well as CP502 (Apotex, Canada) and ICL670A (Novartis, Switzerland) that have entered clinical trials.

.

1.5.3. ACQUIRED ANEMIA

Several conditions, such as malnutrition, severe hemorrhages and pregnancy may lead to anemia. Several infections and chronic inflammation conditions could also lead to the state of so-called anemia of inflammation. Intravenous iron supplementation for these patients using iron dextran or iron sucrose is commonly practiced.

Anemia of inflammation is a common consequence of chronic infections, including HIV and tuberculosis, and can develop within days during sepsis.[38] Anemia of inflammation is also seen in non-infectious inflammatory disorders, including chronic kidney diseases, rheumatologic diseases, inflammatory bowel disease, multiple myeloma and other malignancies.[39] These anemias are characterised by decreased plasma iron and the presence of iron in bone-marrow macrophages, indicating impaired mobilisation of iron from the stores.

IL-6 production in inflammatory disorders or IL-6 supplementation like in cancer patients causes hypoferremia through an increase in hepcidin production.[24,27] The iron imbalance probably has a role in host defense by limiting the availability of iron to invading microorganisms. Because most of iron in the transferrin compartment is destined for the bone marrow, hypoferremia diminishes the amount of iron available for hemoglobin synthesis and erythrocyte production. Indeed, clinical and experimental situations in which hepcidin is overproduced are commonly associated with anemia.[23]

2. Iron and atherosclerosis

2.1. Atherosclerosis: a combination of hypercholesterolemia and inflammation

Atherosclerosis is the primary cause of heart disease and stroke (Figure 2). Death arising as a complication of atherosclerosis claims the lives of millions of people each year in the Western world, and also rapidly increases in prevalence in developing countries. The treatment of hypercholesterolemia has been shown to profoundly reduce morbidity and mortality from coronary artery disease (CAD).[40,41] Research efforts the last 20 years have been increasingly focused on the pathobiology of hypercholesterolemia in initiating atherosclerotic lesion in the artery wall. The findings have brought the general conclusion that atherosclerosis is a chronic inflammatory disease initiated and enhanced by the presence of hypercholesterolemia.[42]

The atherosclerotic lesion is preceded by the adhesion and infiltration of monocytes through the vascular endothelium into the subendothelial space (Figure 3). Monocytes differentiate to macrophages that are capable of taking up phospholipids, leading to an accumulation of lipid-foam cells, forming a fatty streak. Fatty streaks, however, may progress to lesions or may eventually disappear. It is now evident that the rupture-prone plaque, rather than the progressive vessel narrowing from continued growth of the plaque, participates in myocardial infarction and ischemia.

There is diversity in expression of disease even in cases of extreme hypercholesterolemia. What accounts for these differences in the rate of lesion formation and clinical presentation? Since atherosclerosis is a complex disease with multifactorial etiology, it is likely that different factors play a role in different individuals. The classical risk factors, such as hypercholesterolemia, smoking, male gender, hypertension, diabetes, and age have been suggested by epidemiological observations.

However, other risk factor, like the status of individual's immune system, is also critical in the development of atherosclerosis and its clinical outcome.

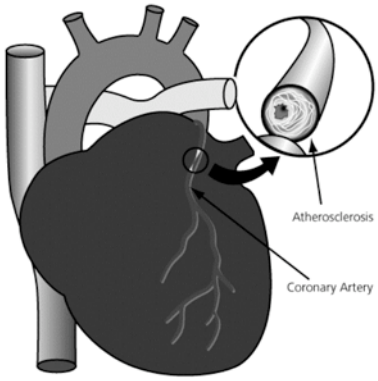

Figure 2. Illustration of atherosclerotic lesion in coronary artery showing narrowing of the artery

2.2. IRON AND CARDIOVASCULAR DISEASE: EVIDENCE FROM EPIDEMIOLOGY?

In significant parts of the Western world, iron overload is found in the population more often than iron deficiency. Consequently, the potential hazards of iron excess are and should be gaining more attention. A hypothesis has been postulated by Sullivan in 1981.[43] This hypothesis proposed the possible benefits of iron depletion against ischemic heart disease. Following Sullivan's proposal, the involvement of iron in atherosclerosis and coronary heart disease has been extensively investigated. A review of epidemiological studies and experimental data (Figure 3) suggested evidence for iron involvement in atherosclerosis.[44] However, an early meta-analysis of prospective studies could not yet confirm this evidence.[45]

2.3. IRON AND ATHEROSCLEROSIS: *IN VITRO* AND *IN VIVO* STUDIES

2.3.1. OXIDATIVE MODIFICATION OF CHOLESTEROL

The cholesterol accumulating in foam cells is derived from circulating plasma low-density lipoproteins (LDL). However, only oxidatively modified LDL (oxLDL) is taken up by macrophages massively in the subendothelial space, through a distinct receptor than for native LDL, without regulated feedback mechanism. OxLDL is directly chemotactic for monocytes and T cells. Moreover, oxLDL induces changes in the endothelial cells ranging from activation to loss of endothelial integrity.

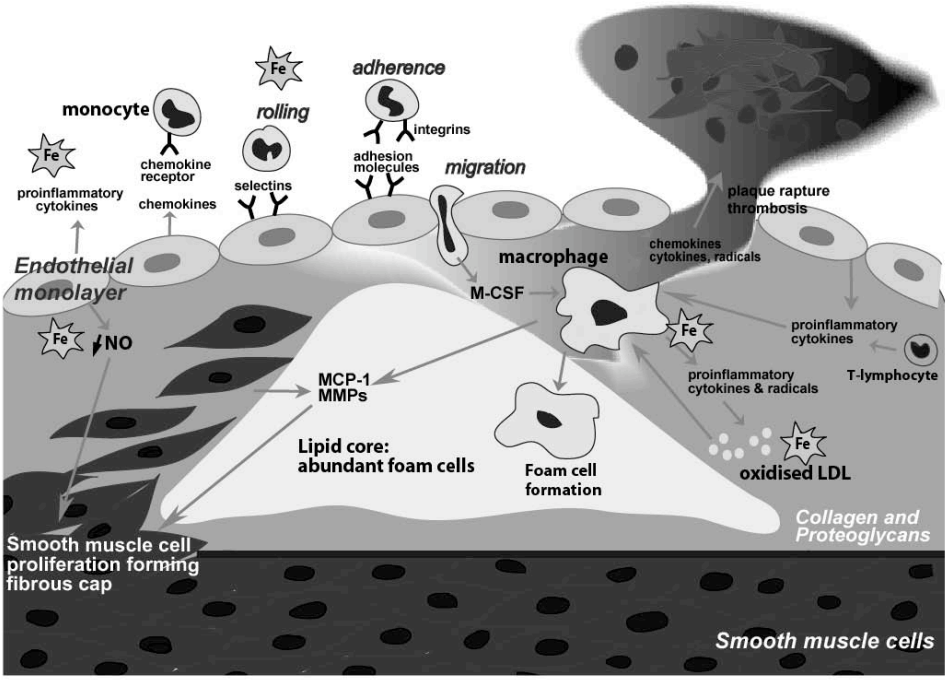

Figure 3. Illustration of the events of atherogenesis leading to plaque rapture. Leukocytes from the blood stream respond to the chemotactic stimuli along the vessel wall, secreted by activated endothelium. The selectins on endothelial cells trigger tethering of the leukocytes to the endothelium. Arrest and firm adhesion are mediated by the binding of leukocyte integrins, like lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) to the adhesion receptors on endothelial cells, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), respectively. The adhesion step is followed by leukocyte transendothelial migration, mediated by the junctional molecules, such as platelet endothelial cell adhesion molecule-1 (PECAM-1). In atherosclerotic lesions, monocytes differentiate to macrophages, which are capable of taking up oxLDL without a feedback-regulated process, leading to the formation of foam cells. Thrombosis is followed when the atherosclerotic plaque is destabilised, and this may lead to myocardial infarction and ischemia. The plausible involvement of iron in the progression of this disease is indicated.

The mechanism by which iron may stimulate atherogenesis is unclear. *In vitro* it has been shown that iron-catalysed free radicals result in oxidation of native LDL.[46] Moreover, the interior of

advanced human atherosclerotic lesions is a highly prooxidant environment containing reduction-oxidation active iron and copper ions that induce lipid peroxidation.[47] Ferritin was found to be highly expressed in the atherosclerotic lesions.[48] Furthermore, iron is co-localised with ceroid, an insoluble complex of oxidised-lipid and protein, extracellularly and also intracellularly in the foam cells and smooth muscle cells.[49]

In hypercholesterolemic rabbits, iron overload stimulated the formation of atherosclerotic lesions.[50] Dietary iron restriction also protected apoE-deficient mice from developing such lesions.[51] Finally, iron chelation in experimental rabbits showed an anti-atherosclerotic effect by reducing plaque formation.[52]

2.3.1. ENDOTHELIAL ACTIVATION AND VASCULAR DYSFUNCTION

Vascular endothelial dysfunction plays an important role both in initiating the development of atherosclerosis and promoting thrombosis that leads to vessel occlusion and acute cardiovascular events.[42] Endothelial activation induces the expression of adhesion molecules and many of inflammatory chemokines. This in turn stimulates blood cells like monocytes and T-lymphocytes to attach and migrate into the subendothelial space (Figure 3).

Several studies have shown the importance of iron for the induction of early functional and structural vascular abnormalities due to endothelial dysfunction[53] which is associated with the subsequent induction of oxidative stress. The oxygen-derived radicals may be involved in the regulation of transcription factors, such as nuclear-factor κB (NF-κB),[54] important for the transcription of a large number of genes, like endothelial adhesion molecules and inflammatory cytokines. Iron *in vitro* upregulates IL-6 production by endothelial cells,[55] while iron chelators inhibit tumor necrosis factor-α–(TNF-α))-mediated upregulation of endothelial adhesion molecule expression.[56,57] Moreover, the radical species may also impair the nitrogen monoxide (NO) production, leading to the condition of arterial stiffness.[58] NO has been shown to mediate antiatherosclerotic properties of the endothelium by inhibition of platelet aggregation, adhesion molecule expression, and vascular smooth muscle cell proliferation. The vascular condition could be improved after administration of an iron chelator,[59] or by iron depletion.[60]

2.3.3. MONOCYTES AND MACROPHAGES

Monocytes are initially attached to lesion-prone sites by cell adhesion molecules expressed on activated endothelial cells. The initial adhesion involves the selectins that mediate a rolling interaction. This event is followed by a firm attachment by means of the integrins. Adherent monocytes migrate into the subendothelial space under the influence of chemoattractant molecules. These monocytes then differentiate into macrophages. Several endogenous and microbial molecules can induce activation of

macrophages leading to the release of inflammatory molecules and other atherogenic proteins. Activated macrophages also express myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and NADPH oxidases that produce antimicrobial radicals promoting activation of other inflammatory cells as well as oxidation of LDL. Moreover, the scevenger receptors on macrophages mediate the engorgement of the available oxLDL.

The involvement of iron in this inflammatory process is shown by its capability to induce secretion of TNF-α[61] and IL-1[62] by monocytes. The cytokines promote iron sequestration in macrophages.[63] Erythrophagocytosis by macrophages also occurred in the lesions,[51] mediating the accumulation of iron, and possibly activation of monocytes. Moreover, iron deposits also stimulate more macrophage infiltration to the atherosclerotic lesions.[64]

2.3.4. LYMPHOCYTES, NEUTROPHILS, AND SMOOTH MUSCLE CELLS

T-cell infiltrates are always present in atherosclerotic lesions. Such infiltrates are predominantly $CD4^+$T cells, which are reactive to disease-related antigens and oxLDL. A minor T-cell subpopulation, natural killer T cells, is also prevalent in early lesions, which recognise lipid antigens. $CD8^+$T cells are also present in atherosclerotic lesions, which recognise foreign antigens in the lesions. When T cells recognise an antigen, an activation cascade occurs, resulting in the expression of a set of cytokines, cell-surface molecules, and enzymes, which in turn activate macrophages and may accelerate atherosclerosis. T-cell cytokines induce the production of large amounts of molecules downstream in the cytokine cascade. As a result, elevated levels of IL-6 and C-reactive protein (CRP) may be detected in the peripheral circulation.[42]

Neutrophils or polymorphonuclear leukocytes (PMN) are the most common type of leukocytes found in the circulation and a major component of the innate immune response. These short-lived phagocytic cells are primarily involved in acute inflammation by engulfing microbes, releasing anti-microbial peptides, and killing invading microbes through the respiratory burst. Although neutrophils generally are not detected in stable atherosclerotic plaques, they are prevalent in ruptured plaques in patients with acute coronary syndromes. Myeloperoxidases and NADPH oxidases induced after neutrophil and monocyte activation may contribute to atherosclerosis by oxidising LDL, leading to uncontrollable uptake by macrophages. Although, myeloperoxidase was found in atherosclerotic plaques,[65] and myeloperoxidase levels were significantly higher in patients with coronary heart disease than in normal controls,[66] this does not necessarily show the involvement of neutrophils in atherogenesis. This is because monocytes are also able to produce myeloperoxidases and NADPH oxidases, although in much lesser extent than neutrophils. It has been proposed that atherosclerosis is in part a response towards infectious agents detected in the atherosclerotic lesions like *Chlamydia pneumoniae* (Cp) and cytomegalovirus (CMV).[67-70] Myeloperoxidase, therefore, may be brought to the lesions by phagocytes responding to an infectious agent. In addition, naturally occurring antimicrobial peptides stored in the azurophilic granules of neutrophils, defensins and cathelicidins, have also been

found in high concentrations in atherosclerotic plaques in humans.[71] Both proteins exhibit chemotactic activities towards many immune cells, interfere with endothelial and vascular smooth muscle cell functions, and exhibit prothrombotic activity. Taken together, neutrophils may be involved in atherogenesis, however further investigation is needed to determine the mechanisms of their possible contributions.

Another key process of atherosclerosis involves the proliferation of vascular smooth muscle cells (VSMCs). In early atherosclerosis, VSMCs may contribute to the development of the atheroma through the production of pro-inflammatory mediators like chemotactic proteins, like monocyte chemotactic protein-1 (MCP-1) and matrix metaloproteinases (MMPs) required for the alteration of vascular matrix and the retention of lipoproteins. VSMCs may also be important in maintaining the stability of the plaque through the formation of a firm fibrous cap. In advanced lesions, fibroblasts and VSMCs with extracellular calcification form a fibrocalcific plaque.

2.3.5. PLAQUE RAPTURE

Activated macrophages, T cells, and mast cells in atherosclerotic plaques produce several types of molecules, such as inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules that can destabilise lesions. They inhibit the formation of stable fibrous caps, attack collagen in the cap, and initiate thrombus formation. All these reactions can induce the activation and rupture of plaque, thrombosis, and ischemia.

The involvement of iron in the process of plaque rapture has been investigated. Iron deposits have been shown to stimulate plaque rupture.[64] Iron overload increases the susceptibility of rat hearts to oxygen reperfusion damage.[72,73] Several studies showed a protective effect of iron chelators in the post-ischemic cardiac injury period in animals, indicating that iron plays a role in reperfusion injury in tissues after ischemic insult.[73-78] Moreover, dietary iron restriction not only protected apoE-deficient mice from developing the lesions, but also from having plaque rupture.[79]

2.3.6. ROLE OF INFECTIONS

Infections have been linked to atherosclerosis and CAD. Elevated titers of antibodies against Cp were found in patients with CAD, while chlamydial protein products have been found in the lesions.[67,68] It was then speculated that this microbe might cause atherosclerosis. Cp can modulate immune-cell as well as vascular-cell activity. However, infection does not cause atherosclerosis in animals, although it may stimulate disease progression and plaque activation.

CMV, a herpes virus, has also been linked to CAD.[69,70] Similar to Cp, CMV increases experimental atherosclerosis. Clinical data imply an important role for CMV in transplantation-related arteriosclerosis causing graft rejection. However, more studies will be needed to determine whether the virus is involved in CAD.

Since several types of pathogens may contribute to CAD, it is unlikely that a single microbe causes atherosclerosis. Instead, the total burden of infection at various sites may affect the progression of atherosclerosis and elicit clinical manifestations. Moreover, body iron metabolism may also complicate the outcome of infections in modifying atherogenesis, as iron could modulate the growth of microorganisms.[42]

3. SCOPE OF THIS THESIS

Given the growing evidence of iron involvement in CAD, and also the higher frequency of CAD as well as iron overload particularly in the Western world, due to habitual changes such as nutrient supplementation and sedentary lifestyle, elucidating the mechanisms of which iron involved in differential events of atherosclerotic development has been the primary aim of the research described in this thesis. Both *in vitro* and *ex vivo* approaches were used to answer the following questions:

Chapter 2 Does iron modulate the course of monocyte adhesion to endothelium, the primary event of atherogenic inflammation process? If it does, what is the mechanism? Is it intracellular or extracellular? Does this process involve the production of oxygen-derived radicals? Would iron chelation or radical scavenging affect this process?

Chapter 3 Is non-transferrin bound iron (NTBI) present in the sera of hemochromatosis HFE C282Y carriers as well as normal individuals? Is this naturally occurring NTBI capable of promoting endothelial activation and subsequent monocyte adherence? Would C-reactive protein, the inflammation marker and potent risk predictor of CAD correlate to NTBI level and endothelial activation?

Chapter 4 Could Cp and CMV, the two potentially atherogenic microorganisms, promote endothelial activation? Would iron and iron chelation affect this process? If they do, what is the mechanism?

Chapter 5 Is iron involved in the multi-step recruitment of cytokine-mediated monocyte rolling and adhesion under physiological flow conditions? Does it also play a role in cytokine-dependent monocyte infiltration through endothelium? If it does, what is the mechanism? Would iron chelation or radical scavenging affect this process?

Chapter 6 Would EDTA chelation therapy with high-doses ascorbic acid supplementation, an alternative treatment for coronary artery disease practiced worldwide, be beneficial or detrimental for endothelial function? What is the mechanism?

Chapter 7 Do low molecular weight iron and several pharmacological iron preparations affect the course of neutrophil adherence and transmigration through endothelial cells as a crucial event in acute innate immunity that may potentially be involved in atherogenesis? If they do, what is the mechanism?

Chapter 8 Do iron and iron chelators modulate the phagocyte-derived-myeloperoxidase- as well as copper-dependent low density lipoprotein oxidations? Do they influence the activity of this enzyme?

In the last chapter of this thesis, the overall findings and conclusions of the research will be discussed and placed in broader perspectives. This thesis is concluded with a summary as a brief overview of the findings.

4. REFERENCES

1. Fenton, H.J.H. oxidation of tartaric acid in the presence of iron. *J Chem Soc* **65**, 899-910 (1894).
2. Halliwell, B. & Gutteridge, J.M. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett* **307**, 108-112 (1992).
3. Shayeghi, M. *et al.* Identification of an intestinal heme transporter. *Cell* **122**, 789-801 (2005).
4. McKie, A.T. *et al.* An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* **291**, 1755-1759 (2001).
5. Gunshin, H. *et al.* Cloning and characterisation of a mammalian proton-coupled metal-ion transporter. *Nature* **388**, 482-488 (1997).
6. de Valk, B. *et al.* Non-transferrin-bound iron is present in serum of hereditary haemochromatosis heterozygotes. *Eur J Clin Invest* **30**, 248-251 (2000).
7. Banyai, S., Rainer, V., Druml, W., Horl, W.H., & Sunder-Plassmann, G. Bleomycin-detectable free iron (BDI) is present in patients on intravenous (IV) iron therapy. *J Am Soc Nephrol* **9**, 199 (1998).
8. Boogaerts, M.A., Yamada, O., Jacob, H.S., & Moldow, C.F. Enhancement of granulocyte-endothelial cell adherence and granulocyte-induced cytotoxicity by platelet release products. *Proc Natl Acad Sci U S A* **79**, 7019-7023 (1982).
9. Breuer, W., Hershko, C., & Cabantchik, Z.I. The importance of non-transferrin-bound iron in disorders of iron metabolism. *Transfus Sci* **23**, 185-192 (2000).
10. Breuer, W. *et al.* The assessment of serum nontransferrin-bound-iron in chelation therapy and iron supplementation. *Blood* **95**, 2975-2982 (2000).
11. Esposito, B.P., Breuer, W., Slotki, I., & Cabantchik, Z.I. Labile iron in parenteral iron formulations and its potential for generating plasma non-transferrin-bound iron in dialysis patients. *Eur J Clin Invest* **32 Suppl 1**, 42-49 (2002).
12. Parkkinen, J., von, B.L., Peltonen, S., Gronhagen-Riska, C., & Rosenlof, K. Catalytically active iron and bacterial growth in serum of haemodialysis patients after i.v. iron-saccharate administration. *Nephrol Dial Transplant* **15**, 1827-1834 (2000).
13. Scheiber-Mojdehkar, B., Lutzky, B., Schaufler, R., Sturm, B., & Goldenberg, H. Non-transferrin-bound iron in the serum of hemodialysis patients who receive ferric saccharate: no correlation to peroxide generation. *J Am Soc Nephrol* **15**, 1648-1655 (2004).
14. Loreal, O. *et al.* Determination of non-transferrin-bound iron in genetic hemochromatosis using a new HPLC-based method. *J Hepatol* **32**, 727-733 (2000).
15. Grootveld, M. *et al.* Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterisation by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *J Biol Chem* **264**, 4417-4422 (1989).
16. Kruszewski, M. Labile iron pool: the main determinant of cellular response to oxidative stress. *Muta. Res* **531**, 81-92 (2003).
17. Breuer, W., Epsztejn, S., & Cabantchik, Z.I. Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). *J Biol Chem* **270**, 24209-24215 (1995).
18. Pantopoulos, K. Iron metabolism and the IRE/IRP regulatory system: an update. *Ann N Y Acad Sci* **1012**, 1-13 (2004).

19. Hentze, M.W. *et al.* Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. *Science* **238**, 1570-1573 (1987).

20. Andrews, N.C. Molecular control of iron metabolism. *Best Pract Res Clin Haematol* **18**, 159-169 (2005).

21. Park, C.H., Valore, E.V., Waring, A.J., & Ganz, T. Hepcidin, a urinary antimicrobial peptide synthesised in the liver. *J Biol Chem* **276**, 7806-7810 (2001).

22. Nicolas, G. *et al.* The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest* **110**, 1037-1044 (2002).

23. Nemeth, E. *et al.* Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* **101**, 2461-2463 (2003).

24. Nemeth, E. *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* **113**, 1271-1276 (2004).

25. Frazer, D.M. & Anderson, G.J. The orchestration of body iron intake: how and where do enterocytes receive their cues? *Blood Cells Mol Dis* **30**, 288-297 (2003).

26. Nemeth, E. *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalisation. *Science* **306**, 2090-2093 (2004).

27. Ganz, T. Hepcidin - a regulator of intestinal iron absorption and iron recycling by macrophages. *Best. Pract. Res Clin Haematol* **18**, 171-182 (2005).

28. Marx, J.J.M. & Hider, R.C. Iron: an essential but potentially harmful nutrient. *Eur J Clin Invest* **32 Suppl 1**, 1-2 (2002).

29. Feder, J.N. *et al.* A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nat Genet* **13**, 399-408 (1996).

30. Bridle, K.R. *et al.* Disrupted hepcidin regulation in HFE-associated hemochromatosis and the liver as a regulator of body iron homeostasis. *Lancet* **361**, 669-673 (2003).

31. Perkins, K.W., Mcinnes, I.W., Blackburn, C.R., & Beal, R.W. Idiopathic hemochromatosis in children; report of a family. *Am J Med* **39**, 118-126 (1965).

32. Roetto, A. *et al.* Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nat Genet* **33**, 21-22 (2003).

33. Papanikolaou, G. *et al.* Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. *Nat Genet* **36**, 77-82 (2004).

34. Camaschella, C. *et al.* The gene TFR2 is mutated in a new type of hemochromatosis mapping to 7q22. *Nat Genet* **25**, 14-15 (2000).

35. Montosi, G. *et al.* Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. *J Clin Invest* **108**, 619-623 (2001).

36. Njajou, O.T. *et al.* A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. *Nat Genet* **28**, 213-214 (2001).

37. Kato, J. *et al.* A mutation, in the iron-responsive element of H ferritin mRNA, causing autosomal dominant iron overload. *Am J Hum Genet* **69**, 191-197 (2001).

38. van Iperen, C.E., van de, W.A., & Marx, J.J.M. Acute event-related anemia. *Br J Haematol* **115**, 739-743 (2001).

39. Jurado, R.L. Iron, infections, and anemia of inflammation. *Clin Infect Dis* **25**, 888-895 (1997).

40. Sacks, F.M. *et al.* The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and recurrent events trial investigators. *N Engl J Med* **335**, 1001-1009 (1996).

41. Shepherd, J. *et al.* Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland coronary prevention study group. *N Engl J Med* **333**, 1301-1307 (1995).

42. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).

43. Sullivan, J.L. Iron and the sex difference in heart disease risk. *Lancet* **1**, 1293-1294 (1981).

44. de Valk, B. & Marx, J.J.M. Iron, atherosclerosis, and ischemic heart disease. *Arch Intern Med* **159**, 1542-1548 (1999).

45. Danesh, J. & Appleby, P. Coronary heart disease and iron status: meta-analyses of prospective studies. *Circulation* **99**, 852-854 (1999).

46. Heinecke, J.W., Rosen, H., & Chait, A. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest* **74**, 1890-1894 (1984).

47. Smith, C., Mitchinson, M.J., Aruoma, O.I., & Halliwell, B. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem J* **286 (Pt 3)**, 901-905 (1992).

48. Pang, J.H. *et al.* Increased ferritin gene expression in atherosclerotic lesions. *J Clin Invest* **97**, 2204-2212 (1996).

49. Lee, F.Y., Lee, T.S., Pan, C.C., Huang, A.L., & Chau, L.Y. Colocalisation of iron and ceroid in human atherosclerotic lesions. *Atherosclerosis* **138**, 281-288 (1998).

50. Araujo, J.A. *et al.* Iron overload augments the development of atherosclerotic lesions in rabbits. *Arterioscler Thromb Vasc Biol* **15**, 1172-1180 (1995).

51. Lee, T.S., Shiao, M.S., Pan, C.C., & Chau, L.Y. Iron-deficient diet reduces atherosclerotic lesions in apoE-deficient mice. *Circulation* **99**, 1222-1229 (1999).

52. Minqin, R., Watt, F., Huat, B.T., & Halliwell, B. Correlation of iron and zinc levels with lesion depth in newly formed atherosclerotic lesions. *Free Radic Biol Med* **34**, 746-752 (2003).

53. Rooyakkers, T.M. *et al.* Ferric saccharate induces oxygen radical stress and endothelial dysfunction *in vivo*. *Eur J Clin Invest* **32 Suppl 1**, 9-16 (2002).

54. Baldwin, A.S., Jr. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* **107**, 3-6 (2001).

55. Visseren, F.L.J. *et al.* 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* **32 Suppl 1**, 84-90 (2002).

56. Zhang, W.J. & Frei, B. Intracellular metal ion chelators inhibit TNF-alpha-induced SP-1 activation and adhesion molecule expression in human aortic endothelial cells. *Free Radic Biol Med* **34**, 674-682 (2003).

57. Koo, S.W., Casper, K.A., Otto, K.B., Gira, A.K., & Swerlick, R.A. Iron chelators inhibit VCAM-1 expression in human dermal microvascular endothelial cells. *J Invest Dermatol* **120**, 871-879 (2003).

58. Cheung, Y.F., Chan, G.C., & Ha, S.Y. Arterial stiffness and endothelial function in patients with beta-thalassemia major. *Circulation* **106**, 2561-2566 (2002).

59. Duffy, S.J. *et al.* Iron chelation improves endothelial function in patients with coronary artery disease. *Circulation* **103**, 2799-2804 (2001).

60. Failla, M. *et al.* Radial artery wall alterations in genetic hemochromatosis before and after iron depletion therapy. *Hepatology* **32**, 569-573 (2000).

61. Lopez, M. *et al.* Tumour necrosis factor-alpha transcription in transferrin-stimulated human blood mononuclear cells: is transferrin receptor involved in the signalling mechanism? *Br J Haematol* **120**, 829-835 (2003).

62. Szkaradkiewicz, A. Interleukin-1 production by human monocytes induced in culture with K562 cells. *Res Exp Med (Berl)* **191**, 201-208 (1991).

63. Ludwiczek, S., Aigner, E., Theurl, I., & Weiss, G. Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood* **101**, 4148-4154 (2003).

64. Kolodgie, F.D. *et al.* Intraplaque hemorrhage and progression of coronary atheroma. *N Engl J Med* **349**, 2316-2325 (2003).

65. Daugherty, A., Dunn, J.L., Rateri, D.L., & Heinecke, J.W. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J Clin Invest* **94**, 437-444 (1994).

66. Zhang, R. *et al.* Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* **286**, 2136-2142 (2001).

67. Campbell,L.A. & Kuo,C.C. *Chlamydia pneumoniae*--an infectious risk factor for atherosclerosis? *Nat Rev Microbiol* **2**, 23-32 (2004).

68. Belland, R.J., Ouellette, S.P., Gieffers, J., & Byrne, G.I. *Chlamydia pneumoniae* and atherosclerosis. *Cell Microbiol* **6**, 117-127 (2004).

69. Danesh, J., Collins, R., & Peto, R. Chronic infections and coronary heart disease: is there a link? *Lancet* **350**, 430-436 (1997).

70. 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* **96**, 4095-4103 (1997).

71. Kougias, P. *et al.* Defensins and cathelicidins: neutrophil peptides with roles in inflammation, hyperlipidemia and atherosclerosis. *J Cell Mol Med* **9**, 3-10 (2005).

72. Voogd, A., Sluiter, W., van Eijk, H.G., & Koster, J.F. Low molecular weight iron and the oxygen paradox in isolated rat hearts. *J Clin Invest* **90**, 2050-2055 (1992).

73. van der Kraaij, A.M., Mostert, L.J., van Eijk, H.G., & Koster, J.F. Iron-load increases the susceptibility of rat hearts to oxygen reperfusion damage. Protection by the antioxidant (+)-cyanidanol-3 and deferoxamine. *Circulation* **78**, 442-449 (1988).

74. Bolli, R. *et al.* The iron chelator desferrioxamine attenuates postischemic ventricular dysfunction. *Am J Physiol* **253**, H1372-H1380 (1987).

75. Williams, R.E., Zweier, J.L., & Flaherty, J.T. Treatment with deferoxamine during ischemia improves functional and metabolic recovery and reduces reperfusion-induced oxygen radical generation in rabbit hearts. *Circulation* **83**, 1006-1014 (1991).

76. Badylak, S.F., Simmons, A., Turek, J., & Babbs, C.F. Protection from reperfusion injury in the isolated rat heart by postischaemic deferoxamine and oxypurinol administration. *Cardiovasc Res* **21**, 500-506 (1987).

77. Reddy, B.R., Kloner, R.A., & Przyklenk, K. Early treatment with deferoxamine limits myocardial ischemic/reperfusion injury. *Free Radic Biol Med* **7**, 45-52 (1989).

78. van der Kraaij, A.M., van Eijk, H.G., & Koster, J.F. Prevention of postischemic cardiac injury by the orally active iron chelator 1,2-dimethyl-3-hydroxy-4-pyridone (L1) and the antioxidant (+)-cyanidanol-3. *Circulation* **80**, 158-164 (1989).

79. Lee, H.T., Chiu, L.L., Lee, T.S., Tsai, H.L., & Chau, L.Y. Dietary iron restriction increases plaque stability in apolipoprotein-e-deficient mice. *J Biomed Sci* **10**, 510-517 (2003).

CHAPTER 2

Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells

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

Arteriosclerosis, Thrombosis and Vascular Biology
2004, 24(12):2257-62

Intracellular Labile Iron Modulates Adhesion of Human Monocytes to Human Endothelial Cells

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

Objective—Elevated iron stores and high plasma iron concentration have been linked to an increased risk of atherosclerosis. Iron may thereby affect the interaction of monocytes to endothelium, an initial event in the formation of atherosclerotic plaques.

Methods and Results—Addition of 10 μmol/L non–transferrin-bound iron to the incubation medium caused a 2-fold increase in monocyte adhesion to human umbilical vein endothelial cells (HUVECs). A concordant increase in the expression of the following adhesion molecules was observed: vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and endothelial selectin on HUVECs as well as very late antigen-4, and lymphocyte function–associated antigen-1 on monocytes. The inclusion of either deferiprone or salicylaldehyde isonicotinoylhydrazone counteracted these effects. Intracellular iron chelation by deferoxamine was completed only after 10 hours of incubation, shown by reversal of iron-quenched intracellular calcein signal, and concurrently the effects of iron were blunted. The membrane-impermeable chelator, diethylenetriamine pentaaceticacid, failed to negate iron effects, even after 48 hours of treatment. Furthermore, only membrane-permeable superoxide or hydroxyl radical scavengers were capable of preventing HUVEC activation by iron.

Conclusions—Non–transferrin-bound iron increases the level of intracellular labile iron, which promotes monocyte recruitment to endothelium and may thereby contribute to the pathogenesis of atherosclerosis. Iron-induced adhesion molecule expression was observed, and this event may involve the production of oxygen radicals. (***Arterioscler Thromb Vasc Biol.*** **2004;24:2257-2262.**)

Key Words: iron ■ atherosclerosis ■ monocytes ■ endothelium ■ adhesion molecules

Atherosclerosis has been associated with several important environmental and genetic risk factors. It is characterized by inflammatory changes leading to plaque formation and, furthermore, to plaque rupture and arterial thrombosis. Transendothelial migration of leukocytes is a fundamental inflammatory mechanism in atherogenesis.[1] This process is partly mediated by the interaction between endothelial adhesion molecules and their ligands on monocytes.

Elevated concentrations of adhesion molecules have been observed in human atherosclerotic plaques, including 2 members of the immunoglobulin superfamily of adhesion receptors, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as a member of the selectin family, endothelial selectin (E-Selectin). Moreover, a significant correlation has been found between the degree of macrophage infiltration and endothelial ICAM-1, VCAM-1, and E-selectin expression in atherosclerotic lesions.[2]

The infiltration of leukocytes consists of consecutive adhesion-mediated events. The first step of adhesion involves binding of selectins to carbohydrate ligands that triggers tethering of the leukocytes to the activated endothelium along the vessel wall. Arrest and firm adhesion of the leukocytes on activated endothelial cells occur depending on the activation of the integrins very late antigen-4 (VLA-4) and lymphocyte function–associated antigen-1 (LFA-1).[3]

In 1981, Sullivan[4] suggested that iron depletion may protect against ischemic heart disease. Many epidemiological studies have tested this iron hypothesis. Some showed that the level of body iron stores, as measured by serum ferritin concentration, is positively correlated to the incidence of cardiovascular diseases.[5–11] However, others have yielded conflicting results.[12–18]

Recent studies have demonstrated that carriers of the HFE mutant gene C282Y, responsible for autosomal recessive hereditary hemochromatosis, have a significantly higher plasma concentration of non–transferrin-bound iron (NTBI)

Original received June 14, 2004; final version accepted September 9, 2004.

From the Eijkman-Winkler Center for Medical Microbiology (A.E.R.K., N.A.G., F.L.J.V., H.v.K.-R., B.S.v.A., J.J.M.M.), Infectious Diseases, and Inflammation and Eijkman Graduate School for Immunology and Infectious Diseases, and the Departments of Vascular Medicine (F.L.J.V.) and Internal Medicine (B.S.v.A.), University Medical Center Utrecht, The Netherlands.

Correspondence Prof Joannes J.M. Marx, MD, PhD, Eijkman-Winkler Center for Medical Microbiology, Infectious Diseases, and Inflammation, University Medical Center Utrecht, 100 Heidelberglaan G04.614, 3584CX Utrecht, The Netherlands. Email jmarx@azu.nl

than the normal population[19] and are at an increased risk of cardiovascular-related death or myocardial infarction.[20–22] However, some other studies failed to reveal an increased risk of atherosclerosis in population carrying HFE mutant gene.[23–27] A positive association between frequent blood donations and reduced risk of myocardial infarction has been reported,[28–30] presumably caused by iron depletion. However, in one study, iron depletion caused by blood donation did not appear to reduce the risk of cardiovascular diseases.[31] Additionally, a community-based prospective cohort study showed that heme iron intake was significantly correlated with total iron stores and the risk of cardiovascular diseases.[32–35]

Given the potential role of iron in atherosclerosis, we investigated the effects of iron-rich and iron-withholding conditions in the course of human monocyte adhesion to human umbilical vein endothelial cells (HUVECs) as one of the earliest events of atherosclerotic plaque formation. Several iron chelators of different cell-membrane permeability were used to investigate the involvement of extracellular and intracellular labile iron in this process. The involvement of toxic oxygen species formed through iron-catalyzed Fenton reaction was also investigated.

Methods

HUVEC Isolation and Culture

HUVECs were isolated and cultured as described by Jaffe et al[36] with minor modifications (for details, please see the online Methods, available at http://atvb.ahajournals.org).

Monocyte Isolation

Two different methods of monocyte isolation were used depending on the amount of cells needed (see online Methods): the countercurrent centrifugal elutriation[37] and the negative immunoselection monocyte isolation kit (Miltenyi Biotec).

Preparation of Iron Solutions and Chelator-Bound Iron

Two different iron compounds were used in this study. A 10-mmol/L Fe(II)ammoniumsulfate (Merck) solution was made by dissolving the iron crystals in distilled water at room temperature. 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°C for 30 minutes. Iron solutions were always freshly prepared and filter-sterilized before use. Fe(III)citrate was used, because the majority of NTBI is found in a complex form of Fe(III) to citrate, as shown by NMR spectroscopy of serum from patients with iron overload.[38]

Chelator stocks were prepared as follows: 10 mmol/L deferoxamine (DF; Novartis) in PBS, 30 mmol/L deferiprone (L1; Duchefa Biochemie) in PBS, 10 mmol/L diethylenetriamine pentaaceticacid (DTPA; Sigma) in PBS, and 20 mmol/L salicylaldehyde isonicotinoylhydrazone (SIH)[39] in dimethylsulfoxide. Preparation of fully-saturated chelator-bound iron solutions was done a few minutes before such solutions were added to the incubation medium (see online Methods).

The detectable iron from the endothelial basal medium-2, which is the medium used to perform all of the experiments involving HUVECs, was measured by Vitros 950 Chemistry System (Ortho-Clinical Diagnostics; see online Methods). With no external iron addition, the baseline iron level was counted to be ≈0.36 μmol/L. In all experiments, plastic materials with low affinity for iron were used. The contaminant iron level in the working containers and the basal cell culture growth medium is relatively low compared with the iron concentrations used in this study.

In Vitro Cytoadherence Assay

Monocytes and HUVECs were pretreated with different combinations of compounds of interest before the assay. After treatment, monocytes were labeled with 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM, Molecular Probes). The cytoadherence of monocytes (25×10^4 cells per well) to HUVEC monolayer (5×10^4 cells per well) was performed in a 96-well plate for 30 minutes at 37°C with gentle agitation. Fluorescence was read using a filter pair for excitation at 485 nm and emission at 530 nm in a CytoFluor II fluorescence microplate reader (Perseptive Biosystems). This value represented total fluorescing monocytes added to each well. After thorough washing, fluorescence was again measured, and the value represented the remaining monocytes firmly attached to the HUVEC monolayer. The percentage of adhesion was defined as the value of remaining fluorescence divided by the value of total fluorescence multiplied by 100.

Fluorescence-Activated Cell Sorting

After treatment with various compounds of interest, HUVECs or monocytes were incubated with fluorescence-labeled monoclonal antibodies against the adhesion molecules of interest. The level of the adhesion molecule expression on these cells was then analyzed by the FACS analysis using a Becton Dickinson FACScan (see online Methods).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Viability Assay for HUVECs and Trypan Blue Exclusion Test for Monocytes

Cellular viability of HUVECs was monitored by the MTT (Sigma) method,[40] whereas cellular viability of monocytes after the various compound incubations was monitored using the trypan blue exclusion test (see online Methods).

Calcein-AM Assay

The membrane permeability of iron chelators was followed by the calcein-AM assay[41] (see online Methods). One hundred μmol/L of Fe(III)citrate was loaded to confluent HUVECs for 1 hour before the addition of 0.125 μmol/L calcein-AM (30 minutes at 37°C). After the signal was stabilized, the chelator of interest was added. This fluorescence signal (excitation=485 nm; emission=530 nm) was followed for a specified time period. Reversal of the quenched calcein signal by addition of a chelator gives an indication as to the membrane permeability of the chelator at a specific time point.

Data Analysis

Results are expressed as means+SEM. Differences in quantitative measures were tested for significance using the unpaired 2-tailed Student *t* test.

Results

Cell Viability

After 48 hours of incubation with up to 300 μmol/L Fe(II)ammoniumsulfate or Fe(III)citrate, the viability of HUVECs was >95% as determined by the MTT assay (not shown). TC_{50} of DF, L1, SIH, and DTPA were also determined. TC_{50} increased when chelators were bound to iron (Table I, available online at http://atvb.ahajournals.org) indicating their specificity for iron. The MTT assay was also performed to assure that the concentrations of compounds of interest used in all experiments retained 95% cell viability. Monocytes were >95% viable after treatment with <100 μmol/L Fe(II)ammoniumsulfate or Fe(III)citrate for 3 hours shown by the trypan blue exclusion test (not shown).

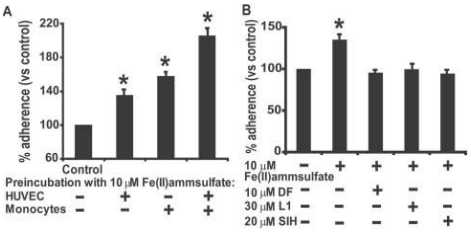

Figure 1. Effects of iron (A) and chelator-bound iron (B) on monocyte adhesion to endothelium (mean±SEM; n=6; *different from control, $P<0.05$). Source of iron was Fe(II)ammoniumsulfate with incubation time of 5 hours on HUVECs (A and B) and 1 hour on monocytes (A).

Iron Modulates Human Monocyte Adhesion to Human Vascular Endothelium

The extent of monocyte adhesion to the endothelium was determined by an in vitro cytoadherence assay. Confluent HUVECs were incubated for 5 hours, whereas monocytes were incubated for 1 hour with Fe(II)ammoniumsulfate. Compared with untreated cells, more iron-treated monocytes adhered to HUVECs and, similarly, more monocytes adhered to iron-treated HUVECs (Figure 1A). A 2-fold increase in adhesion was observed when both endothelial cells and monocytes were pretreated with iron. When HUVECs were incubated with either DF-, L1-, or SIH-saturated iron, this phenomenon was no longer observed (Figure 1B), indicating that the increase in monocyte adhesion to HUVECs was attributed to the presence of iron, the level of which was modulated by external addition of NTBI.

Effects of Iron on VCAM-1, ICAM-1, and E-Selectin Expression on HUVECs and the Counter Receptors: VLA-4 and LFA-1 on Monocytes

Incubation of endothelial cells for 48 hours with increasing concentrations of Fe(II)ammoniumsulfate or Fe(III)citrate increased VCAM-1, ICAM-1, and E-selectin expression on HUVECs as observed by the FACS analysis (Figure 2A through 2D). No difference was seen between Fe(II)ammoniumsulfate and Fe(III)citrate, which may be because of the

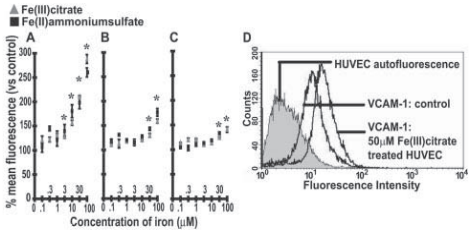

Figure 2. Effects of iron on the expression of endothelial adhesion molecules: VCAM-1 (A), ICAM-1 (B), and E-selectin (C) after 48 hours incubation (mean±SEM; n=6; *different from control, $P<0.001$). The mean fluorescence from monoclonal antibody against adhesion molecules, analyzed by FACS, correlates to the expression of adhesion molecules on the cell surface (illustrated in D).

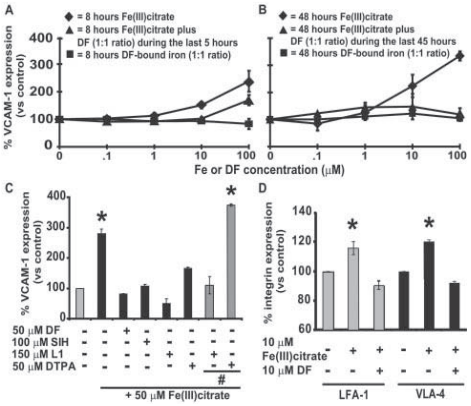

Figure 3. The expression of adhesion molecules on HUVECs or monocytes after treatment with iron or chelator-bound iron. VCAM-1 expression (mean±SEM; n=3) was lower in 8 hours (A) compared to 48 hours (B) 100 μmol/L Fe(III)citrate-treated HUVEC ($P<0.001$; n=6). Forty-five hours (B) but not 5 hours (A) of DF treatment was enough to negate iron activation on HUVECs. C, VCAM-1 expression on HUVECs after 48 hours of treatment with iron or chelator-bound iron freshly prepared before being added to the incubation medium (mean±SEM; n=3; *different from control, $P<0.001$). #Iron was preloaded to HUVECs for 3 hours before a chelator was added for 45 hours. Monocyte surface integrin expression was altered (D) after 1-hour incubation with iron but remained the same as control after 1-hour incubation with DF-bound iron freshly prepared before being added to the incubation medium (mean±SEM; n=3; *different from control, $P<0.05$).

long incubation time under conditions that promote oxidation of Fe(II). The enhancement of VCAM-1 expression is much more pronounced than ICAM-1 or E-selectin with lower dosage of iron (ie, 1 to 20 μmol/L; Figure 2A). The upregulation of all 3 adhesion molecule expressions was more marked as higher dosage of iron (Figure 2A through 2C) or longer incubation with iron (Figure 3A and 3B) was applied. These suggest an iron-induced process as dose- and time-dependent, causing an augmentation of HUVEC activation as more iron accumulates intracellularly. Saturating iron with either DF, L1, or SIH completely blunted the effects of iron in VCAM-1 expression (Figure 3C) as well as ICAM-1 and E-selectin (not shown), confirming the involvement of iron in this process.

Subsequent increase in the expression of VLA-4 and LFA-1 was observed on monocytes after 1 hour of 10 μmol/L Fe(III)citrate treatment, suggesting that monocyte activation was attributed to the presence of iron. Incubation with DF-bound iron resulted only in the basal level of expression of these 2 integrins, confirming the involvement of iron in monocyte activation (Figure 3D).

Intracellular Iron Involved in Iron-Induced Monocyte Adhesion to Endothelium

Using the calcein-AM quenching test, the cell-membrane permeability of 4 different chelators was assayed (Figure 4A). L1 and SIH were highly permeant, as after a 2-hour incubation period these 2 chelators could considerably restore the

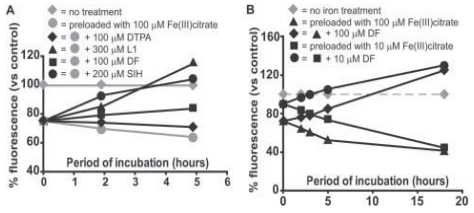

Figure 4. The calcein-AM assay for assessing the membrane permeability of iron chelators. One hundred percent of fluorescence means no calcein quenching by iron; <100% shows calcein quenching. The time needed for the chelators to enter HUVECs is illustrated as the time to dequench calcein signal after iron treatment (A; mean; n=3). B, Completion of intracellular iron chelation by DF after 10 hours of incubation (mean; n=3).

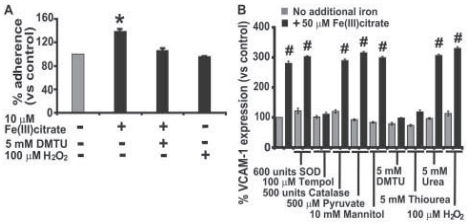

Figure 5. Effects of radical scavengers on iron-activated HUVECs, shown by the cytoadherence assay of monocytes to HUVECs (A; mean±SEM; n=5; *different from control, $P<0.05$) and VCAM-1 expression on HUVECs (B; mean±SEM; n=6; #different from control, $P<0.001$).

quenched calcein signal. In contrast, DTPA was ineffective at restoring the signal, confirming its membrane impermeability. DF, on the other hand, slowly dequenched calcein signal. Ten hours were needed for 100 μmol/L DF to completely restore the signal previously quenched by 100 μmol/L Fe(III)citrate (Figure 4B). However, <5 hours were required for 10 μmol/L DF to restore the signal quenched by 10 μmol/L Fe(III)citrate.

Treatment with DF for 5 hours on HUVECs pretreated with 100 μmol/L Fe(III)citrate did not fully counteract iron-induced VCAM-1 expression (Figure 3A). After 45 hours of DF treatment (Figure 3B), HUVECs expressed the same amount of VCAM-1 as the non–iron-treated cells. The results suggest that chelation of intracellular iron was necessary to counteract the effects of iron.

A similar experiment was also done using L1 and DTPA (Figure 3C). L1 easily penetrated the endothelial cells and was also capable of counteracting the effects of iron on HUVECs. DTPA, on the other hand, was ineffective. These results confirm the need of chelating intracellular iron to prevent HUVEC activation by iron.

Involvement of Oxygen Radicals in Iron-Promoted Cytoadherence

Promotion of monocyte adhesion to endothelium by iron was no longer observed when HUVECs were coincubated with 5 mmol/L dimethylthiourea (DMTU), a scavenger for both H_2O_2 and OH. Incubation of confluent HUVECs with H_2O_2 alone did not induce further monocyte adhesion to endothelium (Figure 5A). These results suggest that iron may produce its effects through the production of reactive oxygen species other than H_2O_2.

Furthermore, a range of both membrane-permeable and -impermeable scavengers with specific scavenging activity for either O_2^-, H_2O_2, or $OH^\cdot$ was tested to reveal their involvement in iron-promoted endothelial adhesion molecule expression (Figure 5B). Besides DMTU (H_2O_2 and $OH^\cdot$ scavenger), only thiourea ($OH^\cdot$ scavenger) and tempol (O_2^- scavenger) could counteract the effects of iron on VCAM-1 upregulation. Neither membrane-impermeable scavengers, superoxide dismutase (O_2^- scavenger), catalase (H_2O_2 scavenger), and mannitol ($OH^\cdot$ scavenger), a membrane-permeable H_2O_2 scavenger (pyruvate), nor urea (a compound control for DMTU and thiourea) could neutralize the effects of iron. H_2O_2 alone could not increase the basal expression of VCAM-1. H_2O_2 in combination with iron gave the same level of VCAM-1 expression as iron alone. Similar results from this set of experiments were observed in endothelial ICAM-1 and E-selectin expression (not shown). These results suggest that iron activates HUVECs through the production of either O_2^- or $OH^\cdot$ or both within the cells.

Discussion

Monocyte migration across the endothelium is an initial pathogenic event of atherosclerosis.[1] Iron in vitro has been shown to upregulate interleukin-6 production by HUVECs,[42] whereas iron chelators inhibit the tumor necrosis factor-α–mediated upregulation of endothelial adhesion molecules.[43,44]

In iron overload diseases with 100% transferrin saturation, such as hemochromatosis, a labile form of iron may exist. Furthermore, it is believed that labile iron may also be present in response to other triggers disturbing iron homeostasis. NTBI can be detected in plasma and is bound to ligands with substantially less affinity than transferrin, such as citrate[38] and albumin. NTBI is capable of freely entering the cell with no feedback-regulated process.[45] Intracellular iron may only be bound to low-molecular weight ligands, catalytically active and capable of initiating free radical reactions.[46] The expansion of intracellular labile iron, which may parallel that of NTBI, may lead to cell toxicity.

In this study, we used Fe(II)ammoniumsulfate and Fe(III)citrate as the source of free iron in the incubation medium to investigate the effects of intracellular catalytically-active iron in monocyte interaction to endothelium. Ten μmol/L of iron could directly activate and stimulate the expression of adhesion molecules on both HUVECs and monocytes as well as promote monocyte adhesion to endothelial cells. No enhancement in the adhesion molecule expression and the cytoadherence was observed when chelator-bound iron was added, confirming the involvement of iron in this process.

Proinflammatory cytokines have been shown to be involved in the development of atherosclerosis.[1] These cytokines induce iron uptake by monocytes and macrophages.[47] Subsequently, our results show an upregulation of monocyte integrin expression due to the presence of labile iron, suggesting that iron may enhance monocyte activation.

L1 and SIH nullified the effect of iron on HUVECs, most probably by chelating intracellular iron. In contrast, DTPA gave no counteracting effect once iron had accumulated. The effectiveness of DF to negate iron effects was time- and concentration-dependent: the higher the level of intracellular iron, the longer the time needed for DF to counteract the effect of iron. This may be because of the route through which DF enters the cells.[48] DF enters intact cells by endocytosis. It slowly accumulates in the endosome-lysosome complex, and after longer periods of exposure it is capable of removing intracellular iron. This suggests chelation of intracellular labile iron as an effective way to prevent iron-induced cell activation.

In HUVECs, iron-induced adhesion molecule expression was in a concentration- (0 to 100 μmol/L) and time-dependent manner. Resting levels of iron in the cultured HUVECs might be 10-fold less than in freshly isolated HUVECs,[49,50] and $\approx$1/3 of the amount of NTBI in the loading medium may enter the cells.[49] Because NTBI is generally in the range of 1 to 20 μmol/L,[51] the results in this study may not only be significant for various conditions of iron overload but also meaningful for normal physiological situations, as an increase in intracellular labile iron in vivo may incline adhesion. This suggests that any condition that disturbs iron homeostasis, even in normal population, may increase the risk for developing atherosclerosis. Furthermore, this study implies that stimulation of adhesion in vivo may reach its lowest point in a state of iron deficiency, which may be protective against atherosclerosis but may also lead to a defective immune function.

Antioxidants have been shown to protect against endothelial dysfunction associated with atherosclerosis,[52] whereas iron chelators were capable of preventing hydroxyl radical damage.[53] Reactive oxygen species, possibly formed through iron-catalyzed Fenton reaction, may be involved in atherogenesis. In our study, DMTU inhibited iron-induced monocyte adherence to HUVECs. Furthermore, only DMTU and 2 other membrane-permeable scavengers, tempol and thiourea, were able to inhibit iron-induced endothelial adhesion molecule expression, suggesting that O_2^- and OH^- are involved in the process of iron-induced monocyte recruitment to HUVECs.

In conclusion, additional NTBI increases the level of intracellular labile iron. This labile iron may play a role as an independent risk factor in atherosclerosis by promoting monocyte recruitment to endothelium. It modulates the expression of cell adhesion molecules on HUVECs as well as the integrins on monocytes. Iron chelators commonly used for the treatment of secondary iron overload can effectively prevent these iron-mediated events. Furthermore, intracellular labile iron may promote its effect through the production of oxygen radicals, particularly superoxide and hydroxyl radicals.

Acknowledgments

This work was supported by European Commission, QLRT-2001-0044 (http://www.nutrientirontoxicity.nl). We thank J.J.M. Bouwman (Diakonessen Hospital, Utrecht, the Netherlands) for providing the umbilical cords and Prof P. Ponka (McGill University, Montreal, Canada) for providing the iron chelator SIH.

References

1. Lusis AJ. Atherosclerosis. *Nature*. 2000;407:233–241.
2. 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;93:672–682.
3. Ronald JA, Ionescu CV, Rogers KA, Sandig M. Differential regulation of transendothelial migration of THP-1 cells by ICAM-1/LFA-1 and VCAM-1/VLA-4. *J Leukoc Biol*. 2001;70:601–609.
4. Sullivan JL. Iron and the sex difference in heart disease risk. *Lancet*. 1981;1:1293–1294.
5. Salonen JT, Nyyssonen K, Korpela H, Tuomilehto J, Seppanen R, Salonen R. High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. *Circulation*. 1992;86: 803–811.
6. Kiechl S, Aichner F, Gerstenbrand F, Egger G, Mair A, Rungger G, Spogler F, Jarosch E, Oberhollenzer F, Willeit J. Body iron stores and presence of carotid atherosclerosis. Results from the Bruneck Study. *Arterioscler Thromb*. 1994;14:1625–1630.
7. Kiechl S, Willeit J, Egger G, Poewe W, Oberhollenzer F. Body iron stores and the risk of carotid atherosclerosis: prospective results from the Bruneck study. *Circulation*. 1997;96:3300–3307.
8. Tuomainen TP, Punnonen K, Nyyssonen K, Salonen JT. Association between body iron stores and the risk of acute myocardial infarction in men. *Circulation*. 1998;97:1461–1466.
9. Klipstein-Grobusch K, Koster JF, Grobbee DE, Lindemans J, Boeing H, Hofman A, Witteman JC. Serum ferritin and risk of myocardial infarction in the elderly: the Rotterdam Study. *Am J Clin Nutr*. 1999;69:1231–1236.
10. Haidari M, Javadi E, Sanati A, Hajilooi M, Ghanbili J. Association of increased ferritin with premature coronary stenosis in men. *Clin Chem*. 2001;47:1666–1672.
11. Wolff B, Volzke H, Ludemann J, Robinson D, Vogelgesang D, Staudt A, Kessler C, Dahm JB, John U, Felix SB. Association between high serum ferritin levels and carotid atherosclerosis in the study of health in Pomerania (SHIP). *Stroke*. 2004;35:453–457
12. Manttari M, Manninen V, Huttunen JK, Palosuo T, Ehnholm C, Heinonen OP, Frick MH. Serum ferritin and ceruloplasmin as coronary risk factors. *Eur Heart J*. 1994;15:1599–1603.
13. Frey GH, Krider DW. Serum ferritin and myocardial infarct. *W V Med J*. 1994;90:13–15.
14. Solymoss BC, Marcil M, Gilfix BM, Gelinas F, Poitras AM, Campeau L. The place of ferritin among risk factors associated with coronary artery disease. *Coron Artery Dis*. 1994;5:231–235.
15. Moore M, Folsom AR, Barnes RW, Eckfeldt JH. No association between serum ferritin and asymptomatic carotid atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. *Am J Epidemiol*. 1995;141: 719–723.
16. Eichner JE, Qi H, Moore WE, Schechter E. Iron measures in coronary angiography patients. *Atherosclerosis*. 1998;136:241–245.
17. Sempos CT, Looker AC, Gillum RE, Mcgee DL, Vuong CV, Johnson CL. Serum ferritin and death from all causes and cardiovascular disease: the NHANES II Mortality Study. National Health and Nutrition Examination Study. *Ann Epidemiol*. 2000;10:441–448.
18. Knuiman MW, Divitini ML, Olynyk JK, Cullen DJ, Bartholomew HC. Serum ferritin and cardiovascular disease: a 17-year follow-up study in Busselton, Western Australia. *Am J Epidemiol*. 2003;158:144–149.
19. de Valk B, Addicks MA, Gosriwatana I, Lu S, Hider RC, Marx JJ. Non–transferrin-bound iron is present in serum of hereditary haemochromatosis heterozygotes. *Eur J Clin Invest*. 2000;30:248–251.
20. Roest M, van der Schouw YT, de Valk B, Marx JJ, Tempelman MJ, de Groot PG, Sixma JJ, Banga JD. Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. *Circulation*. 1999;100:1268–1273.
21. Tuomainen TP, Kontula K, Nyyssonen K, Lakka TA, Helio T, Salonen JT. Increased risk of acute myocardial infarction in carriers of the hemochromatosis gene Cys282Tyr mutation: a prospective cohort study in men in eastern Finland. *Circulation*. 1999;100:1274–1279.
22. Rasmussen ML, Folsom AR, Catellier DJ, Tsai MY, Garg U, Eckfeldt JH. A prospective study of coronary heart disease and the hemochromatosis gene (HFE) C282Y mutation: the Atherosclerosis Risk in Communities (ARIC) Study. *Atherosclerosis*. 2001;154:739–746.
23. Hetet G, Elbaz A, Gariepy J, Nicaud V, Arveiler D, Morrison C, Kee F, Evans A, Simon A, Amarenco P, Cambien F, Grandchamp B. Association studies between haemochromatosis gene mutations and the risk of cardiovascular diseases. *Eur J Clin Invest*. 2001;31:382–388.

24. Candore G, Balistreri CR, Lio D, Mantovani V, Colonna-Romano G, Chiappelli M, Tampieri C, Licastro F, Branzi A, Averna M. Association between HFE mutations and acute myocardial infarction: a study in patients from Northern and Southern Italy. *Blood Cells Mol Dis*. 2003; 31:57–62.
25. Fox CJ, Cullen DJ, Knuiman MW, Cumpston GN, Divitini ML, Rossi E, Gochee PA, Powell LW, Olynyk JK. Effects of body iron stores and haemochromatosis genotypes on coronary heart disease outcomes in the Busselton health study. *J Cardiovasc Risk*. 2002;9:287–293.
26. Franco RF, Zago MA, Trip MD, ten Cate H, van den EA, Prins MH, Kastelein JJ, Reitsma PH. Prevalence of hereditary haemochromatosis in premature atherosclerotic vascular disease. *Br J Haematol*. 1998;102:1172–1175.
27. Battiloro E, Ombres D, Pascale E, D'Ambrosio E, Verna R, Arca M. Haemochromatosis gene mutations and risk of coronary artery disease. *Eur J Hum Genet*. 2000;8:389–392.
28. Tuomainen TP, Salonen R, Nyyssonen K, Salonen JT. Cohort study of relation between donating blood and risk of myocardial infarction in 2682 men in eastern Finland. *BMJ*. 1997;314:793–794.
29. Salonen JT, Tuomainen TP, Salonen R, Lakka TA, Nyyssonen K. Donation of blood is associated with reduced risk of myocardial infarction. The Kuopio Ischaemic Heart Disease Risk Factor Study. *Am J Epidemiol*. 1998;148:445–451.
30. Meyers DG, Jensen KC, Menitove JE. A historical cohort study of the effect of lowering body iron through blood donation on incident cardiac events. *Transfusion*. 2002;42:1135–1139.
31. Ascherio A, Rimm EB, Giovannucci E, Willett WC, Stampfer MJ. Blood donations and risk of coronary heart disease in men. *Circulation*. 2001; 103:52–57.
32. Klipstein-Grobusch K, Grobbee DE, den Breeijen JH, Boeing H, Hofman A, Witteman JC. Dietary iron and risk of myocardial infarction in the Rotterdam Study. *Am J Epidemiol*. 1999;149:421–428.
33. Ascherio A, Willett WC, Rimm EB, Giovannucci EL, Stampfer MJ. Dietary iron intake and risk of coronary disease among men. *Circulation*. 1994;89:969–974.
34. Tzonou A, Lagiou P, Trichopoulou A, Tsoutsos V, Trichopoulos D. Dietary iron and coronary heart disease risk: a study from Greece. *Am J Epidemiol*. 1998;147:161–166.
35. Snowdon DA, Phillips RL, Fraser GE. Meat consumption and fatal ischemic heart disease. *Prev Med*. 1984;13:490–500.
36. 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;52:2745–2756.
37. Wahl LM, Katona IM, Wilder RL, Winter CC, Haraoui B, Scher I, Wahl SM. Isolation of human mononuclear cell subsets by counterflow centrifugal elutriation (CCE). I. Characterization of B-lymphocyte-, T-lymphocyte-, and monocyte-enriched fractions by flow cytometric analysis. *Cell Immunol*. 1984;85:373–383.
38. Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A, Sadler PJ. Non–transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *J Biol Chem*. 1989;264:4417–4422.
39. Ponka P, Borova J, Neuwirt J, Fuchs O, Necas E. A study of intracellular iron metabolism using pyridoxal isonicotinoyl hydrazone and other synthetic chelating agents. *Biochim Biophys Acta*. 1979;586:278–297.
40. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55–63.
41. Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik ZI. Fluorescence analysis of the labile iron pool of mammalian cells. *Analytical Biochemistry*. 1997;248:31–40.
42. 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.
43. Zhang WJ, Frei B. Intracellular metal ion chelators inhibit TNFα-induced SP-1 activation and adhesion molecule expression in human aortic endothelial cells. *Free Radic Biol Med*. 2003;34:674–682.
44. 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;120:871–879.
45. Kaplan J, Jordan I, Sturrock A. Regulation of the transferrin-independent iron transport system in cultured cells. *J Biol Chem*. 1991;266: 2997–3004.
46. Konijn AM, Glickstein H, Vaisman B, Meyron-Holtz EG, Slotki IN, Cabantchik ZI. The cellular labile iron pool and intracellular ferritin in K562 cells. *Blood*. 1999;94:2128–2134.
47. Ludwiczek S, Aigner E, Theurl I, Weiss G. Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood*. 2003;101:4148–4154.
48. Cable H, Lloyd JB. Cellular uptake and release of two contrasting iron chelators. *J Pharm Pharmacol*. 1999;51:131–134.
49. 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;289:664–669.
50. Varani J, Dame MK, Gibbs DF, Taylor CG, Weinberg JM, Shayevitz J, Ward PA. 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;66:708–714.
51. Esposito BP, Breuer W, Sirankapracha P, Pootrakul P, Hershko C, Cabantchik ZI. Labile plasma iron in iron overload: redox activity and susceptibility to chelation. *Blood*. 2003;102:2670–2677.
52. Diaz MN, Frei B, Vita JA, Keaney JF Jr. Antioxidants and atherosclerotic heart disease. *N Engl J Med*. 1997;337:408–416.
53. Marx JJ, van Asbeck BS. Use of iron chelators in preventing hydroxyl radical damage: adult respiratory distress syndrome as an experimental model for the pathophysiology and treatment of oxygen-radical-mediated tissue damage. *Acta Haematol*. 1996;95:49–62.

CHAPTER 3

Endothelial activation and induction of monocyte adherence to endothelium by non-transferrin-bound iron present in human sera

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

The FASEB Journal
2005, Epub 10.1096/fj.05-4700fje

ABSTRACT

Non-transferrin-bound iron (NTBI) has been detected in iron overload diseases. This form of iron may exert pro-oxidant effects and modulate cellular function and inflammatory response. The present study has aimed to investigate the effects of serum NTBI on monocyte adherence to endothelium. Measured by a recently developed high-throughput fluorescence-based assay, serum NTBI was found to be higher in both homozygotes of HFE C282Y mutation of hereditary hemochromatosis (7.9±0.6 μmol/L, P<0.001) and heterozygotes (4.0±0.5 μmol/L, P<0.001), compared to controls (1.6±0.2 μmol/L, n=21). The effects of these sera on monocyte adhesion and endothelial activation were examined. Adhesion of normal human monocytes to C282Y homozygote- and heterozygote-serum-treated human umbilical vein endothelial cells was higher (25.0±0.9% and 22.1±0.7% respectively) compared to controls (17.6±0.5%, both P<0.001). For the three groups combined, the expression of adhesion molecules, ICAM-1, VCAM-1, and E-selectin, was positively correlated to NTBI levels but not to the inflammatory marker, C-reactive protein. Furthermore, accumulation of intracellular labile iron and oxidative radicals within the cells due to NTBI was evidenced. Finally, counteraction of NTBI-induced endothelial activation was observed using iron chelators. These findings therefore identify a physiological function of NTBI in monocyte-endothelial interactions that may also contribute to the development of atherosclerosis and neurodegenerative diseases.

Key words: Iron • monocytes • endothelium • inflammation • adhesion molecules

INTRODUCTION

Non-transferrin-bound iron (NTBI) has been detected in patients with primary and secondary hemochromatosis, particularly in patients with transferrin saturation above 45%.[1] In subjects with the HFE C282Y mutation of hereditary hemochromatosis, NTBI has been detected not only in the sera of homozygotes, but also of carriers, using and HPLC-based method.[2] The autosomal recessive mutation of the HFE gene is common among the Caucasian population with prevalence of 10% and is responsible for 80-90% of hemochromatosis cases.[3] In the plasma, NTBI is believed to bind to ligands with substantially less affinity than transferrin, such as citrate, citrate-carbonate,[4] albumin, or other serum proteins.[5] In contrast to ferritin or transferrin-bound iron, NTBI, is hypothesised to be more readily available for catalysing free radical formation, which are capable of causing cellular damage through various mechanisms.[6] Because the chemical nature of NTBI in the plasma is largely unknown, in order to explore the biological function of serum NTBI, it is crucial to use sera with a sufficiently wide range of NTBI in experimental set-up.

Oxygen-derived free radicals have been implicated as key mediators of the signaling pathways that underlie inflammation. Immune cells use these radical species in order to support their functions

and therefore need adequate levels of antioxidant defenses to avoid any harmful effects of intracellular reduction-oxidation state imbalance.[7] An essential inflammatory process is the binding and transmigration of leukocytes through endothelium to gain access to the inflamed sites. This inflammatory event has been implicated in the development of many diseases, such as atherosclerosis and neurodegenerative diseases. Oxygen-derived free radicals that could be generated by NTBI through the Fenton reaction may augment this particular inflammatory process. The pro-oxidant iron has been shown to generate free radicals in endothelial cells[8] and induce monocyte adhesion to these cells.[9] Moreover, iron has been shown to promote interleukin-6 production by endothelial cells[10] as well as induce cell toxicity.[11]

These findings prompt the question whether NTBI present in human sera plays a role in immune function involving monocyte-endothelial interactions. To that purpose, we used the sera from control subjects and HFE C282Y carriers, which provide wide range levels of serum NTBI and investigated the effects of these sera on endothelial activation and subsequent monocyte adherence to the endothelium. NTBI was measured using a recently developed fluorescence-based assay.[12] Changes in cellular reduction-oxidation status due to NTBI, which could bring about alteration in cellular function and downstream protein expression, were examined. Furthermore, the potential counteracting effects of iron chelation were also investigated.

EXPERIMENTAL PROCEDURES

Baseline iron level

The iron content of the EBM-2 medium 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.

Serum samples

Serum samples of nine C282Y homozygous hemochromatosis patients, all being treated with phlebotomies (most of them on maintenance treatment) and eight C282Y heterozygous subjects were from a previous study[2] and approved by the institutional review board of the University Medical Centre Utrecht (Utrecht, The Netherlands). All of these subjects gave informed consent. Twenty-one control sera were obtained from healthy donors provided and approved by Sanquin blood bank to be used for research purposes. These healthy donors were not screened for HFE mutations. None of the subjects studied had inflammatory diseases, liver diseases or history of alcohol abuse.

Serum samples were isolated by centrifuging blood samples at 2500 g for 20 minutes, followed by heat inactivation (56^0C, 60 minutes), and continued with an additional centrifugation at 2500 g for 20 minutes. The obtained sera were stored at -20^0C until experiments were carried out.

Serum iron parameter measurements

Serum ferritin, serum iron, serum transferrin, and transferrin saturation were measured (Central Diagnostic Laboratory, University Medical Centre Utrecht, The Netherlands) using routine laboratory methods.

Fluorescence-based one-step NTBI measurement

The assay was carried out as described by Breuer and Cabantchik.[12] Briefly, the serum sample was mixed with reagent A (hepes-buffered saline (HBS) containing 10 mmol/L sodium oxalate (BDH Chemicals, Ontario, Canada), 0.1 mmol/L gallium chloride (Sigma-Aldrich, Zwijndrecht, The Netherlands), and 0.6 μmol/L 5-(4,6-dichlorotriazinyl)-aminofluorescein (DCTAF, Molecular Probes, Eugene, USA) -apotransferrin) or reagent B (same as reagent A, but containing 25 μM apotransferrin (Kamada, Haifa, Israel)). In reagent A, the accessible iron binds to the fluorescein-apotransferrin and quenches its fluorescence, whereas in reagent B the iron binds to the excess nonfluorescent apotransferrin rather than to fluorescein-apotransferrin, resulting in higher fluorescence. After incubation of one hour, fluorescence was read using a filter pair for excitation at 485 nm and emission at 530 nm using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA). The ratio of the fluorescence readings (A/B) was inversely proportional to the concentration of NTBI in the original sample, in which the amount was determined from a Fe(III)nitrilotriacetate (Sigma-Aldrich) calibration curve, in a range of 0.4-25 μmol/L. The arbitrary zero value[12] of the calibration curve was set to the highest value of A/B.

High-sensitivity C-reactive protein test (hs-CRP)

The measurement of hs-CRP in each serum was performed by a commercial high-sensitivity assay on a Behring Nephelometer Analyser II (BNII, Dade-Behring, Germany).

HUVEC isolation and culture

HUVECs were isolated and cultured as described by Jaffe et al.[13] Confluent cells from passages two to three were used for all experiments. HUVECs were always used during and maintained at a cobblestone confluent density for all conducted experiments.

Monocyte isolation

Peripheral blood mononuclear cells (PBMC) were isolated from donor blood (Sanquin blood bank, Utrecht, the Netherlands) by Ficollpaque density gradient centrifugation. Monocytes were isolated using the negative immuno-selection monocyte isolation kit (MiltenyiBiotec, CLB Sanquin, Amsterdam, The Netherlands) according to supplier's instructions. This method resulted in purity of >90% as analysed by flow cytometry. Purified monocytes were suspended at a concentration of $5x10^6$ cells/ml in RPMI1640 (BioWhittaker) supplemented with 2 mM L-glutamine and 0.2% human serum albumin (CLB Sanquin) prior to use.

Preparation of iron and chelators

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°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 was maintained 7.8 in incubation medium.

Viability assay

Cellular viability of HUVECs was monitored by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT,Sigma-Aldrich) method.[14] Briefly, cells were seeded into a 96-well microtitre plate, and grown to confluence. Individual sera with iron chelator over a range of concentrations (0-1000 μmol/L) were added to the plate. Incubation was for a period of 48 hours before the MTT assay was performed. Compound cytotoxicity was expressed as a TC_{50} denoting the concentration resulting in 50% loss of cell viability, as calculated by Calcusyn.[15] After 48 hours of serum treatment, HUVEC viability was >95%.

The amount of viable freshly isolated monocytes was monitored by trypan blue (Sigma-Aldrich) exclusion. Briefly, 10 μL cell suspension was mixed with 90 μL trypan blue isotonic solution (0.2% w/v). The amount of viable cells was >95%, determined microscopically using a hemocytometer.

***In vitro* cytoadherence assay**

Confluent HUVECs were pretreated with 50% individual sera in EBM-2 medium for 48 hours prior to the assay. Monocytes were labeled with 2 μmol/L 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM, Molecular Probes). Cytoadherence of monocytes ($25x10^4$ cells/well) to HUVEC monolayer ($5x10^4$ cells/well) was performed in a 96-well plate for 30 minutes at 37^{0}C with gentle agitation. After addition of monocytes to HUVECs at monocytes:HUVECs ratio of 5:1, fluorescence was read using a filter pair for excitation at 485 nm and emission at 530 nm using the Cytofluor II microplate reader. This value represented total fluorescing monocytes added to each well. After thorough washing with RPMI1640, fluorescence was again measured and the value represented the remaining monocytes firmly attached to the HUVEC monolayer. The percentage of adhesion was defined as the value of remaining fluorescence divided by the value of total fluorescence multiplied by 100.

Fluorescence-activated cell sorting (FACS)

After treatment with 50% individual sera for 48 hours with or without iron chelator for the last 24 hours of treatment, 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 4^0C. 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.[16] After incubation with 50% serum in EBM-2 medium for 48 hours or otherwise indicated, HUVEC were harvested by incubating with 0.2% trypsin-EDTA at 37^0C 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, confluent HUVECs were pretreated with 50% serum in EBM-2 medium for 48 hours or otherwise indicated prior to the assay.[17] HUVECs were then washed one time and followed by incubation with 0.125 µmol/L calcein-AM (30 minutes at 37^oC). The cells were washed twice to remove the remaining extracellular calcein-AM before fluorescence signal of calcein (excitation = 485 nm; emission = 530 nm) was read in the Cytofluor II microplate reader. After a stable basal fluorescence signal was observed, deferiprone (100 µmol/L) or Fe(III)citrate (50 µmol/L) was added to the incubation medium. Addition of this permeant membrane iron chelator, led to its competitive binding of intracellular labile iron, subsequent release of calcein-bound iron, and an increase in fluorescence intensity by dequenching of calcein signal. This fractional increase in fluorescence intensity after addition of a chelator correlates with the amount of labile iron within HUVEC.

Calcein-fluorescing cells were fixed in PBS containing 3% paraformaldehyde (Polysciences, Warrington, USA) and 0.02% glutaraldehyde (Merck, Darmstadt, Germany) and visualised using Leica TCS SP2 confocal scanning laser microscope and Leica confocal software (Leica Microsystems, GmbH, Heidelberg, Germany).

Data analysis

Results are expressed as means $\pm$ standard errors of the means (SEM). Differences in quantitative measures were tested for significance using one-way analysis of variance with Bonferroni post-test for further analysis of the data. Between NTBI and other variables, two-tailed Pearson regression coeficient (R^2) was computed. Significance was established when $P<0.050$. All statistics were performed using the GraphPad Prism 4 statistical program (San Diego, USA).

RESULTS

The baseline iron level

With no external iron addition, the baseline iron level in the basal cell culture growth medium, EBM-2, was 0.36 μmol/L. To avoid any external iron contaminant, in all experiments, plastic materials with low affinity for iron were used.

Serum ferritin, serum transferrin, serum iron, transferrin saturation and NTBI levels

Mean values of serum ferritin, serum iron, transferrin saturation and NTBI were higher in HFE C282Y homozygotes compared to the HFE unscreened controls (all $P<0.050$). Serum iron and transferrin saturation were higher in the sera of HFE C282Y homozygotes compared to heterozygotes and heterozygotes compared to controls (all $P<0.050$, Table 1). In this study, the HFE unscreened control subjects included some blood donors with serum ferritin levels indicating iron depletion. The fluorescence-based one-step NTBI assay measured higher levels of NTBI in HFE C282Y homozygotes (7.9 ± 0.6 μmol/L, n=9) compared to heterozygotes (4.0 ± 0.6 μmol/L, n=8, $P<0.001$) and controls (1.6 ± 0.2 μmol/L, n=21, $P<0.001$, Figure 1a). The difference in NTBI levels between heterozygotes and controls was also significant ($P<0.001$). These NTBI values were calculated after adjusting for the arbitrary zero value (see experimental procedures), with within sample variation of 0.24 μmol/L as the average of the errors of the means. The result is in agreement with the previous study,[2] using an HPLC-based method, which unlike the assay used in this present study, measures NTBI with a separation step from other serum proteins. Furthermore, NTBI values were positively correlated to the standardised measurement of iron parameters, including transferrin saturation ($R^2=0.909$, $P<0.001$, n=38) and serum iron ($R^2=0.872$, $P<0.001$, n=38) in all the three groups combined (Figure 2a-b), which is in agreement with another study.[18] Moreover, serum ferritin was correlated to NTBI with a positive exponential relationship (log-linear $R^2=0.635$, $P<0.001$, n=38, Figure 2c). Considering the value of arbitrary zero that falls between 0 to 1.5 μmol/L of iron concentrations,[12] the plot of serum ferritin versus NTBI indicates that NTBI was detectable from the sera with serum ferritin of as low as 10 μg/L. Additionally, similar significant correlations found within the control group (NTBI vs. transferrin saturation, $R^2=0.605$, $P<0.001$, n=21; NTBI vs. serum iron $R^2=0.555$, $P<0.001$, n=21; and NTBI vs. serum ferritin, log-linear $R^2=0.321$, $P=0.008$, n=21) also identify the presence of NTBI in these healthy subjects.

Serum hs-CRP levels of HFE C282Y homozygotes and heterozygotes

Mean value of serum inflammatory marker, hs-CRP, were 1.2 mg/L (HFE C282Y homozygotes, n=9), 1.4 mg/L (C282Y heterozygotes, n=8) and 0.6 mg/L (controls, n=21, Figure 1b). hs-CRP from all sera ranged from 0.2 to 3.9 mg/L. There was no significant difference in the mean values between the groups, indicating that the difference in NTBI levels were not due to a difference in inflammation status between the groups.

HFE C282Y homozygote and heterozygote sera modulate human monocyte adhesion to human endothelial cells

The adherence of monocytes to endothelial cells was investigated, where HUVECs were conditioned with EBM-2 containing 50% individual sample sera for 48 hours. The range of adhered monocytes to HUVECs was 13.4% to 21.8% in the control group (n=21) with a mean of 17.6±0.5% (Figure 1c). The adherence of monocytes was significantly higher on HUVECs treated with 50% individual heterozygous sera, ranging from 18.9% to 25.0% (mean=22.1±0.7%, P<0.001, n=8) compared to controls. Moreover, monocyte adhesion ranging from 21.4% to 28.4% (mean=25.0±0.9%, n=9) was observed when homozygote sera were used, and was also significantly higher than both heterozygous sera (P<0.050) and controls (P<0.001). Collectively, the results suggest that constituents present in both of the sera of HFE C282Y homozygotes and heterozygotes are capable of modifying the level of monocyte adherence to endothelial cells.

Table 1. Iron parameters and NTBI levels in the sera of HFE C282Y homozygotes, heterozygotes and normal controls.

	Normal controls (n=21)	HFE C282Y heterozygotes (n=8)	HFE C282Y homozygotes (n=9)
Serum Ferritin (μg/L)	25.5 (3.0-88.0)	343.0 (35.0-1045.0)	928.3 (43.0-4229.0)*
Serum Iron (μmol/L)	15.8 (6.0-28.0)	24.9 (16.0-49.0)*	40.0 (31.0-45.0) *†
Transferrin (g/L)	2.5 (1.5-3.6)	2.2 (1.3-2.5)	1.9 (1.5-2.5)*
Transferrin saturation (%)	24.1 (9.1-40.7)	44.5 (27.7-92.8)*	84.7 (56.2-99.6) *†
NTBI (μmol/L)	1.6 (0.2-3.1)	4.0 (2.5-8.1)*	7.9 (5.1-10.0)*†

Values represent the mean, ranges of values are shown in parentheses. NTBI: non-transferrin-bound iron.

* P<0.050 for differences between heterozygotes or homozygotes and normal controls

† P<0.050 for differences between homozygotes and heterozygotes

Serum NTBI, hs-CRP, and the expression of VCAM-1, ICAM-1 and E-selectin on HUVECs

To investigate how sera of C282Y carriers promote monocyte adherence, the expression of the adhesion molecules on treated HUVECs was analysed. The expression of VCAM-1, ICAM-1 and E-selectin, the adhesion molecules involved in monocyte recruitment to endothelium,[19] was measured by FACS analysis. The sera were heat-inactivated before use to avoid the influence of complement systems as well as other heat-labile proteins. The complement systems have been shown to promote leukocyte-endothelial interactions,[20] and endothelial adhesion molecule expression.[21] The conventional method of heat-inactivation largely abrogates the activity of heat-labile enzymes, such as superoxide dismutase,[22] and cytokines,[23] as well as lipopolysaccharide-potentiating activity of the serum,[24] without affecting the iron-bound proteins, such as transferrin[25] and albumin.[26] The heat-inactivation procedure did not affect our NTBI, serum ferritin, serum iron, serum transferrin, transferrin saturation (data not shown) or hs-CRP measurements.[27]

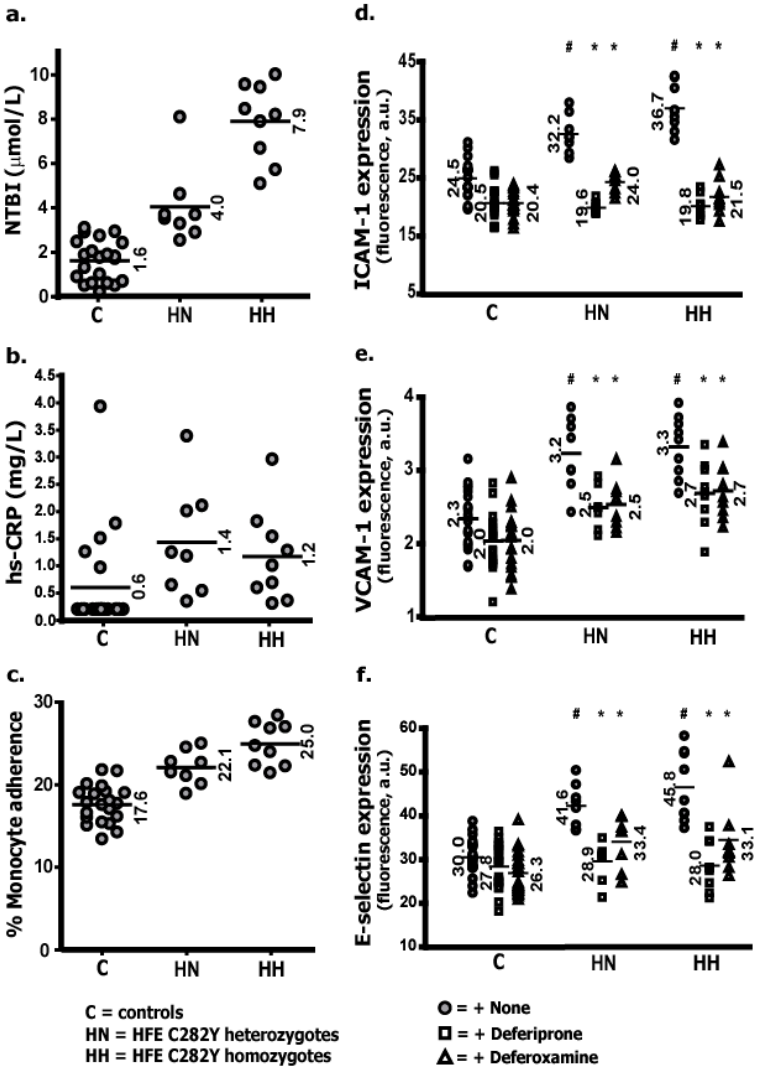

Figure 1. Serum NTBI levels and hs-CRP of HFE C282Y homozygotes, heterozygotes and controls, and their effects on endothelial activation and the adhesiveness of monocytes to endothelium. Scatter plots with the means of the three measured groups, including controls (C, n=21), HFE C282Y heterozygotes (HN, n=8) and homozygotes (HH, n=9), of (a) NTBI, (b) hs-CRP, and (c) percentage of monocyte adherence to HUVECs treated for 48 hours with 50% individual sera (each value is the mean of 4 independent experiments in triplicate). (d) ICAM-1, (e) VCAM-1 and (f) E-selectin expression of respective HUVECs from indicated groups, with lines indicating means of each group. HUVECs were treated with 50% individual sera either of controls, C282Y heterozygotes or C282Y homozygotes for 48 hours, +/- 10 μmol/L deferoxamine or 30 μmol/L deferiprone for the last 24 hours of incubation with sera, as indicated. Each value of point is the mean of 4 to 9 independent experiments. # P<0.001, higher than the control group. * P<0.010, lower than the respective heterozygote or homozygote group with no chelation. a.u.= arbitrary unit.

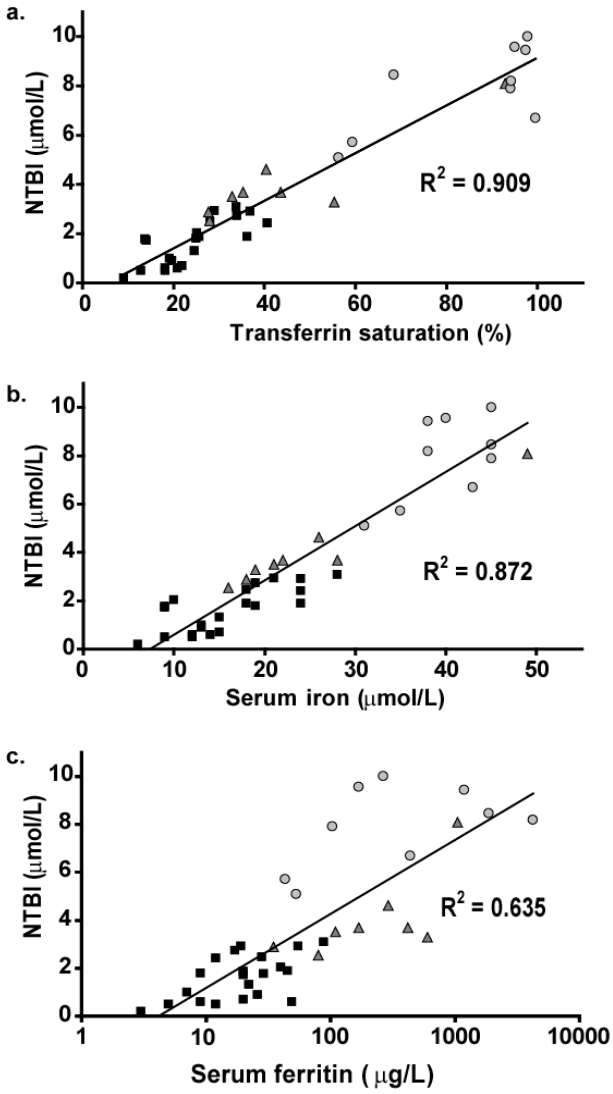

Figure 1. Positive correlation between NTBI and transferrin saturation or serum iron. Linear regression plots of NTBI (Y-axis) and (a) transferrin saturation, (b) serum iron, or (c) serum ferritin (X-axis). Black squares = controls (n=21), Grey triangles = HFE C282Y heterozygotes (n=8), Grey circles = HFE C282Y homozygotes (n=9)

Incubation of endothelial cells for 48 hours with 50% individual sera resulted in various levels of adhesion molecule expression. The mean values of those three adhesion molecules were found to be higher in both C282Y-homozygote- and heterozygote-serum-incubated HUVECs compared to controls (both P<0.001, Figure 1d-f, circles). The levels of expression were then tested for association with the values of NTBI or hs-CRP of the respective sera, using Pearson regression analysis. All of the above-mentioned molecules were positively correlated to the NTBI levels of the sera (ICAM-1, R^2=0.574, P<0.001; VCAM-1, R^2=0.399, P<0.001; E-selectin, R^2=0.453, P<0.001, n=38) depicted as linear regression on Figure 3). hs-CRP, on the other hand, did not correlate to the adhesion molecule expression (ICAM-1, R^2=0.054, P=0.161; VCAM-1, R^2=0.028, P=0.313; E-selectin, R^2=0.120, P=0.123, n=38). This finding suggests that NTBI, under the current experimental conditions, modulated the levels of adhesion molecule expression, and consequently the extent of monocyte adhesion to the endothelial cells.

Intracellular radical formation and accumulation of labile iron within HUVECs

NTBI-induced adhesion molecule expression on endothelial cells may possibly be mediated by alteration in cellular reduction-oxidation status. The levels of cellular oxidative stress in HUVECs were therefore examined, using the oxygen radical sensitive DCF fluorescence probe, where the fluorescence intensifies with increased level of oxidative stress. HUVECs incubated for 48 hours with 50% serum containing 9.4 μmol/L NTBI produced a two-fold higher fluorescence compared to those incubated with serum containing 0.6 μmol/L NTBI (Figure 4a). When HUVECs were incubated with serum containing 0.6 μmol/L NTBI, which had been premixed with 30 μmol/L Fe(III)citrate, a three-fold increase in free radicals formation was observed (Figure 4a). Here, Fe(III)citrate was added to supplement NTBI to the incubating serum, since the majority of NTBI is found in complex forms to citrate. This finding indicates that NTBI increased cellular oxidative stress and that NTBI-induced adhesion molecule expression on HUVECs was mediated by oxygen-derived free radicals.

The pro-oxidant condition in cells can be promoted by an increase of intracellular labile iron.[28] Influx of iron from the various iron species in the serum may take place during the 48 hours incubation of HUVECs. Some may accumulate in the cytoplasmic labile iron pool. HUVECs incubated for 48 hours with 50% serum containing 9.4 μmol/L NTBI has lower basal calcein fluorescence compared to those incubated with serum containing 0.6 μmol/L NTBI (Figure 5a-b), due to quenching of the calcein signal. This result demonstrates increased level of intracellular labile iron due to NTBI. Further quenching of calcein signal was observed when HUVECs were incubated for 48 hours with 50% serum containing 0.6 μmol/L NTBI, premixed with 50 μmol/L Fe(III)citrate (Figure 5a-b). Additionally, when Fe(III)citrate at a final concentration of 15 μmol/L was added to the incubation medium, after calcein was loaded to the cells, quenching of the basal calcein signal was also observed (Figure 5a). On the other hand, dequenching of basal calcein signal was observed when 45 μmol/L deferiprone was added to the incubation medium. Collectively, these results indicate that serum NTBI augmented the level of cytoplasmic

HUVECs incubated for 48 hours with 50% serum containing 9.4 μmol/L NTBI premixed with 90 μmol/L deferiprone or 50% serum containing 0.6 μmol/L NTBI premixed with 30 μmol/L Fe(III)citrate and 90 μmol/L deferiprone were subjected to DCF and Calcein assays. The amount of oxygen-derived free radicals (Figure 4b) and the basal calcein fluorescence (Figure 5a and not shown) in these HUVECs were at the same level as HUVECs treated with 50% serum containing 0.6 μmol/L NTBI. This result confirms that iron chelation prevented while NTBI induced the formation of oxygen-derived free radical formation and labile iron accumulation.

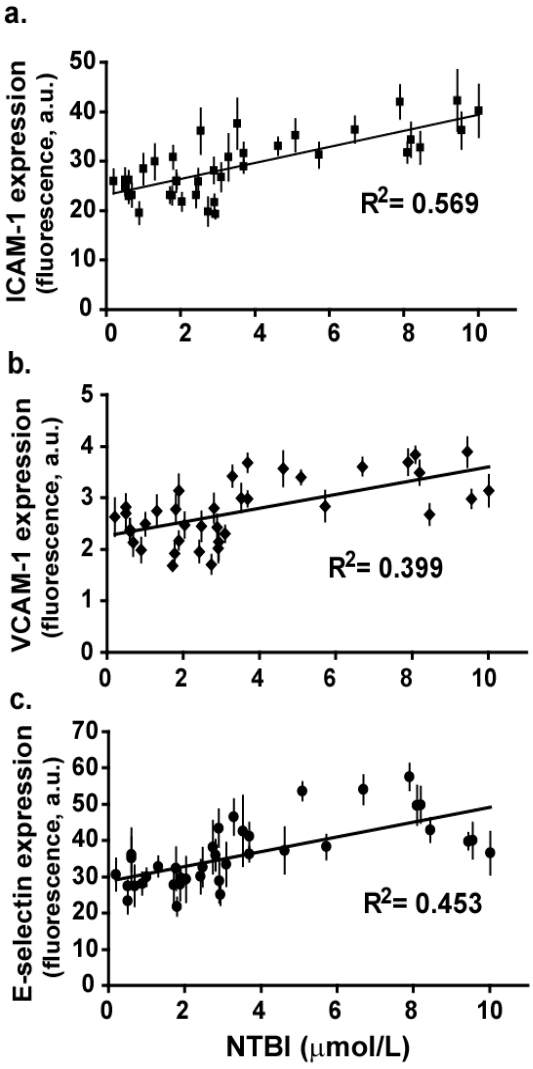

Figure 3. Positive correlation between NTBI and expression of endothelial adhesion molecules, ICAM-1, VCAM-1 and E-selectin. Linear regression plots of NTBI (X-axis) and endothelial adhesion molecule expression (Y-axis) on HUVECs treated for 48 hours with 50% individual sera containing various level of NTBI. Each value of adhesion molecule expression is expressed as mean ± SEM of 7-9 independent experiments. a.u.= arbitrary unit.

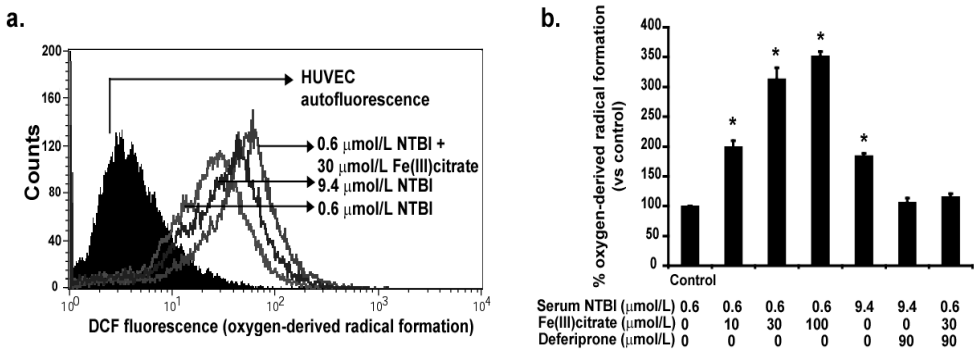

Figure 4. Oxygen-derived radical formation within HUVECs. (a) represents the FACS analysis on DCF Fluorescence in HUVECs after indicated treatments for 48 hours. (b) shows the percentage of oxygen-derived radicals formed in HUVECs after indicated treatments for 48 hours compared to control. * = higher than control ($P<0.001$, n=3). All indicated concentrations are half of the final concentrations in the incubation medium (dilution 1:1).

Iron chelation significantly reduced the induction of endothelial adhesion molecule expression

Since there is a positive correlation between adhesion molecule expression and NTBI, addition of a chelator with high specificity for iron would then be expected to lower adhesion molecule expression. To test this, deferiprone and deferoxamine, were included in this experimental set-up. HUVECs were treated in total for 48 hours with 50% individual sera. After 24 hours of serum incubation, 30 μmol/L deferiprone or 10 μmol/L deferoxamine was added to the incubation solution. The concentration of 10 μmol/L for deferoxamine was chosen to cover the highest NTBI level in this experiment. This concentration is below the TC_{50} (15 $\pm$ 2.3 μmol/L) of the compound on HUVECs.[9] Deferoxamine is a hexadentate chelator, in which one molecule can bind all six ligands of one iron atom. Three molecules of a bidentate iron chelator, like deferiprone, are required to fully chelate one iron atom. Therefore, in this experiment 30 μmol/L concentration was chosen for deferiprone, which is also below its TC_{50} value (100 $\pm$ 11.3 μmol/L).[9]

Treated HUVECs were grouped based on C282Y genetic variation of the incubating sera and the chelator added. The expression of adhesion molecules was then analysed. The mean value of each group and significance of difference between groups are shown on Figure 1d-f. NTBI-induced VCAM-1, ICAM-1 and E-selectin expression was significantly lowered by the addition of either deferiprone or deferoxamine. Both chelators most effectively reduced ICAM-1 expression. This finding confirms the involvement of NTBI in the induction of adhesion molecule expression. Deferiprone lowered the expression of ICAM-1 and E-selectin more effectively than deferoxamine. The different effectiveness of chelation, shown in this study, might be due to the nature of the two chelators, as deferoxamine is slowly entering the cells and therefore, needs longer time to chelate intracellular labile iron.[9] Chelation

of intracellular labile iron is necessary to reduce the upregulation of adhesion molecule expression.[9,29]

DISCUSSION

We investigated the relation between serum NTBI, and adhesion of monocytes to endothelium. This process plays a significant role in host-defense mechanism against microbial infections, and homing of monocytes to the tissues. However, in many age-associated diseases, like atherosclerosis and neurodegenerative diseases, this inflammatory event has been implicated as an initiating process towards the development of the disease.

The involvement of iron in atherosclerosis and coronary heart disease has recently been extensively investigated. A review of epidemiological studies and experimental data suggested evidence for iron involvement in atherosclerosis.[30] However, an early meta-analysis of prospective studies could not yet confirm this evidence.[31] In this study, we focused on NTBI, the non-sequestered form of iron, which unlike ferritin and transferrin-bound iron, is potentially capable of catalysing free radical formation. Recently, it has been shown that the redox active component of NTBI, so-called labile plasma iron (LPI), is measurable in the sera of HFE C282Y hemochromatosis patients.[32] In this study, sera of HFE C282Y homozygotes, heterozygotes and normal subjects with wide-range levels of serum NTBI were used. Our results showed for the first time that NTBI present in human sera were involved in the process of monocyte adherence to endothelium. This finding suggests the involvement of iron in the process of inflammation, and furthermore in the development of diseases like atherosclerosis. In experimental animal models of atherosclerosis, the pro-oxidant iron has been shown to promote plaque formation.[33-35]

Iron chelation markedly improved vascular-endothelial function in patients of coronary artery disease,[36] HFE C282Y hemochromatosis,[37,38] and in subjects with homocysteine-induced endothelial dysfunction.[39] Blood donation with substantial depletion of storage iron proves to acutely improve vascular function without pharmacological interventions.[40,41] Serum ferritin is known to be a measure for body iron storage. This parameter however, can be highly influenced by inflammation. Considering the lack of relationship to hsCRP in our study, serum ferritin could therefore describe the level of iron storage in our population. For those blood donors with ferritin levels indicating iron depletion, NTBI was found to be very low or lacking. The very low NTBI level was associated and may have lead to the measured least state of endothelial activation. Our findings may therefore explain the results from the aforementioned studies on how low iron status *in vivo* could benefit vascular endothelial function.

Although the evidence from epidemiological studies linking HFE C282Y mutation to the development of cardiovascular disease is inconclusive, a large scale study involving 12.239 postmonopausal women revealed an 18.85 fold increased risk of cardiovascular death in subjects who were heterozygous for HFE C282Y and additionally were smokers and hypertensive, while 2.26 fold increased for non-carriers who were smokers and hypertensive, both compared to non-smoking and non-hypertensive controls.[42] NTBI in these C282Y carriers may have modulated the reduction-oxidation status while interaction with environmental stressors like smoking and hypertension leads to

overexposure of radicals and triggers their sensitivity towards cardiovascular disease. Furthermore, in our study, a correlation analysis between endothelial adhesion molecule expression and serum NTBI levels of the three groups combined revealed a strong positive association. This suggests that the NTBI levels could influence the levels of adhesion molecule expression, regardless of the HFE genotypes. This also implies that populations with increased NTBI levels may have an inclination towards enhanced monocyte adhesion to endothelium.

In neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's diseases, accumulation of iron has been characterised, which catalyses metal-induced oxidative stress in the disease process.[43] The chronic inflammation has been associated with a broad spectrum of neurodegenerative diseases of aging.[44] Neuroinflammation, characterised by the accumulation of reactive microglia, is present in the degenerating areas. The intensity of the activation of these microglial cells, as the brain representatives of the monocyte phagocytic system, is related to a spectrum of inflammatory mediators like adhesion molecules and free radicals generated by a variety of local cells, including the vascular endothelium of the blood-brain barrier.[44,45] Evidence for the role of inflammation and infiltration of inflammatory cells through the brain endothelium in neurodegenerative diseases has recently emerged.[46-48] Moreover, leukocyte-mediated breakdown of blood–brain barrier followed by recruitment into the central nervous system is indeed a process characteristic of several neurodegenerative diseases.[46,48] Our findings on the role of NTBI in endothelial activation as well as monocyte-endothelial interactions identify a potential pathophysiological nature of NTBI in the development of neurodegenerative diseases.

Monitoring the levels of oxygen-derived free radical formation and labile iron inside the cells unraveled the mechanism underlying NTBI-induced endothelial activation. Influx of iron initially joins the labile iron pool in cytoplasm, which is soon stored safely in ferritin or being used for the synthesis of iron-containing proteins. Iron in this transit cytoplasmic pool is metabolically and catalytically reactive[17, 49] therefore the level is tightly regulated in order to minimise any potentially toxic reactions. Prolonged exposure to high serum NTBI on HUVECs in this study had augmented the level of intracellular labile iron. This coincided with induction of cellular reduction-oxidation state imbalance, which had lead to the phenotype of primed endothelial cells.

Both deferiprone and deferoxamine were capable of reducing the induction of endothelial adhesion molecule expression by C282Y sera. *In vivo*, the two iron chelators have been shown to prevent hydroxyl radical damage.[50] In this study, deferiprone and deferoxamine may have chelated NTBI in those sera, reduced the formation of free radicals, and thereby inhibited endothelial activation. These results not only confirm the involvement of naturally occurring NTBI present in the sera in enhancing the process of monocyte adhesion to endothelium, but also demonstrate the possible beneficial effects of iron chelation or induced iron depletion to lessen monocyte-endothelial interactions in individuals with increased serum NTBI levels.

Recently, the adhesion molecule ICAM-1 has been shown to have a predictive value of carotid atherosclerosis progression independently of traditional risk factors and hs-CRP.[51] Concordantly, in our experimental conditions, NTBI induced the expression of endothelial adhesion molecules regardless the

levels of hs-CRP. Additionally, many factors other than NTBI in the sera could also be involved in the process of endothelial activation.

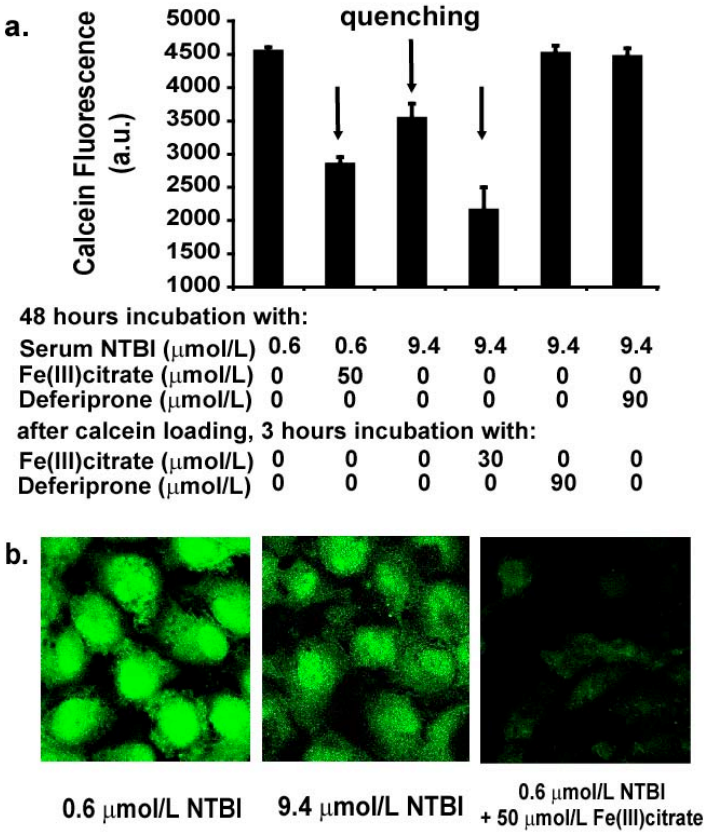

Figure 5. Accumulation of labile iron intracellularly. (a) shows the fluorescence of intracellular calcein signal of HUVECs (n=3), which is quenched by iron, after indicated treatments. (b) visualises the calcein signal from HUVECs after indicated treatments for 48 hours, using a confocal laser microscope. All indicated concentrations are half of the final concentrations in the incubation medium (dilution 1:1). a.u.= arbitrary unit.

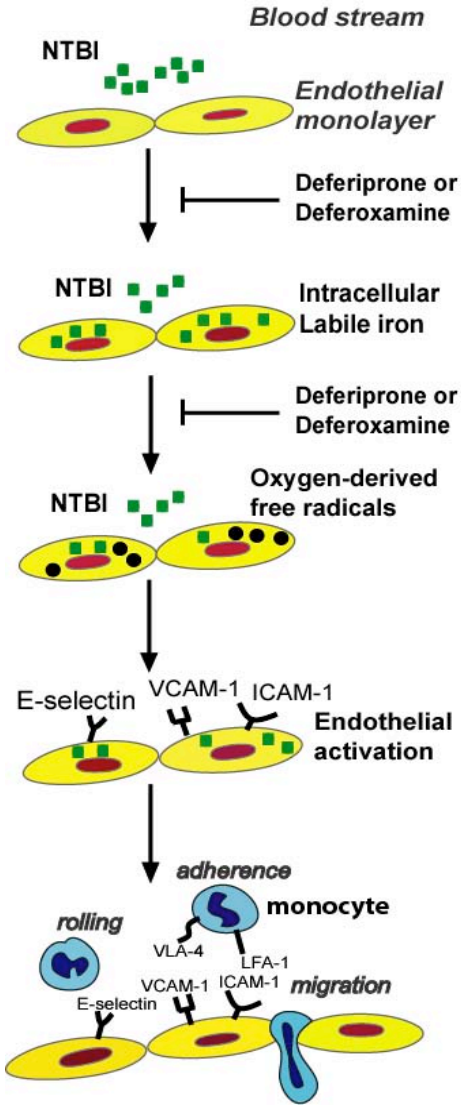

Figure 6. Illustration of the reported findings with current knowledge showing a hypothetical sequence of events initiated by NTBI leading to inflammation. NTBI increases the level of intracellular labile iron, and oxygen-derived free radicals were generated as a result. The radicals activate the cellular machinery of endothelial cells leading to the expression of endothelial surface adhesion molecules. These adhesion molecules promote the inflammatory event of monocyte adherence to the endothelium.

In conclusion, NTBI from human sera promotes monocyte adhesion to endothelium, by upregulating the expression of endothelial adhesion molecules (Figure 6). The findings support the involvement of NTBI in physiological process of monocyte homing to the tissues, as well as in host-defense mechanism against microbial infections. This study also suggests for a role of NTBI in diseases involving monocyte-endothelial interactions, such as artherosclerosis or neurodegenerative diseases.

Acknowledgements: This work was supported by a grant from the European Commission, QLK1-CT-2002-00444. We thank Mr. J.J.M. Bouwman from the Diakonessen Hospital, Utrecht, the Netherlands, for providing the umbilical cords

References

1. Loreal, O. *et al.* Determination of non-transferrin-bound iron in genetic hemochromatosis using a new HPLC-based method. *J Hepatol* **32**, 727-733 (2000).
2. de Valk, B. *et al.* Non-transferrin-bound iron is present in serum of hereditary hemochromatosis heterozygotes. *Eur J Clin Invest* **30**, 248-251 (2000).
3. Feder, J.N. *et al.* A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis. *Nat. Genet.* **13**, 399-408 (1996).
4. Grootveld, M. *et al.* Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterisation by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *J Biol Chem* **264**, 4417-4422 (1989).
5. Batey, R.G., Lai Chung, F.P., Shamir, S., & Sherlock, S. A non-transferrin-bound serum iron in idiopathic hemochromatosis. *Dig Dis Sci* **25**, 340-346 (1980).
6. Gutteridge, J.M., Rowley, D.A., Griffiths, E., & Halliwell, B. Low-molecular-weight iron complexes and oxygen radical reactions in idiopathic hemochromatosis. *Clin Sci (Lond)* **68**, 463-467 (1985).
7. Cuzzocrea, S., Riley, D.P., Caputi, A.P., & Salvemini, D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol Rev* **53**, 135-159 (2001).
8. Zweier, J.L., Broderick, R., Kuppusamy, P., Thompson-Gorman, S., & Lutty, G.A. Determination of the mechanism of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. *J Biol Chem* **269**, 24156-24162 (1994).
9. Kartikasari, A.E.R. *et al.* Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. *Arterioscler Thromb Vasc Biol* **24**, 2257-2262 (2004).
10. Visseren, F.L.J. *et al.* 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* **32 Suppl 1**, 84-90 (2002).
11. Pietrangelo, A. Iron, friend or foe? "Freedom" makes the difference. *J Hepatol* **32**, 862-864 (2000).
12. Breuer, W. & Cabantchik, Z.I. A fluorescence-based one-step assay for serum non-transferrin-bound iron. *Anal Biochem* **299**, 194-202 (2001).
13. Jaffe, E.A., Nachman, R.L., Becker, C.G., & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* **52**, 2745-2756 (1973).
14. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63 (1983).
15. Chou, T.C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**, 27-55 (1984).
16. Vanden Hoek, T.L., Li, C., Shao, Z., Schumacker, P.T., & Becker, L.B. Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J Mol Cell Cardiol* **29**, 2571-2583 (1997).
17. Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W., & Cabantchik, Z.I. Fluorescence Analysis of the labile iron pool of mammalian cells. *Anal Biochem* **248**, 31-40 (1997).
18. Jacobs, E.M. *et al.* Results of an international round robin for the quantification of serum non-transferrin-bound iron: Need for defining standardization and a clinically relevant isoform. *Anal Biochem* **341**, 241-250 (2005).
19. O'Brien, K.D., McDonald, T.O., Chait, A., Allen, M.D., & Alpers, C.E. 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* **93**, 672-682 (1996).
20. Morigi, M. *et al.* Xenogeneic serum promotes leukocyte-endothelium interaction under flow through two temporally distinct pathways: role of complement and nuclear factor-kappaB. *J Am Soc Nephrol* **10**, 2197-2207 (1999).

21. Lozada, C. *et al.* Identification of C1q as the heat-labile serum cofactor required for immune complexes to stimulate endothelial expression of the adhesion molecules E-selectin and intercellular and vascular cell adhesion molecules-1. *Proc Natl Acad Sci U S A* **92**, 8378-8382 (1995).

22. Vouldoukis, I. *et al.* Antioxidant and anti-inflammatory properties of a Cucumis melo LC. extract rich in superoxide dismutase activity. *J Ethnopharmacol* **94**, 67-75 (2004).

23. Mahanty, S., Kalwar, R., & Rollin, P.E. Cytokine measurement in biological samples after physicochemical treatment for inactivation of biosafety level 4 viral agents. *J Med Virol* **59**, 341-345 (1999).

24. Meszaros, K., Aberle, S., White, M., & Parent, J.B. Immunoreactivity and bioactivity of lipopolysaccharide-binding protein in normal and heat-inactivated sera. *Infect Immun* **63**, 363-365 (1995).

25. Ikemoto, H. & Ventura, M.M. Differential scanning calorimetry of the thermal denaturation of human serotransferrin. *An Acad Bras Cienc* **51**, 165-171 (1979).

26. Farruggia, B. & Pico, G.A. Thermodynamic features of the chemical and thermal denaturations of human serum albumin. *Int J Biol Macromol* **26**, 317-323 (1999).

27. McCarthy, P.L., Jekel, J.F., & Dolan, T.F., Jr. Comparison of acute-phase reactants in pediatric patients with fever. *Pediatrics* **62**, 716-720 (1978).

28. Halliwell, B. & Gutteridge, J.M. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett* **307**, 108-112 (1992).

29. Zhang, W.J. & 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* **34**, 674-682 (2003).

30. de Valk, B. & Marx, J.J.M. Iron, atherosclerosis, and ischemic heart disease. *Arch Intern Med* **159**, 1542-1548 (1999).

31. Danesh, J. & Appleby, P. Coronary heart disease and iron status: meta-analyses of prospective studies. *Circulation* **99**, 852-854 (1999).

32. Le Lan, C. *et al.* Redox active plasma iron in C282Y/C282Y hemochromatosis. *Blood*(2005).

33. Day, S.M. *et al.* Chronic iron administration increases vascular oxidative stress and accelerates arterial thrombosis. *Circulation* **107**, 2601-2606 (2003).

34. Araujo, J.A. *et al.* Iron overload augments the development of atherosclerotic lesions in rabbits. *Arterioscler Thromb Vasc Biol* **15**, 1172-1180 (1995).

35. Lee, T.S., Shiao, M.S., Pan, C.C., & Chau, L.Y. Iron-deficient diet reduces atherosclerotic lesions in apoE-deficient mice. *Circulation* **99**, 1222-1229 (1999).

36. Duffy, S.J. *et al.* Iron chelation improves endothelial function in patients with coronary artery disease. *Circulation* **103**, 2799-2804 (2001).

37. Failla, M. *et al.* Radial artery wall alterations in genetic hemochromatosis before and after iron depletion therapy. *Hepatology* **32**, 569-573 (2000).

38. Gaenzer, H. *et al.* Association between increased iron stores and impaired endothelial function in patients with hereditary hemochromatosis. *J Am Coll Cardiol* **40**, 2189-2194 (2002).

39. Zheng, H., Dimayuga, C., Hudaihed, A., & Katz, S.D. Effect of dexrazoxane on homocysteine-induced endothelial dysfunction in normal subjects. *Arterioscler Thromb Vasc Biol* **22**, E15-E18 (2002).

40. Sullivan, J.L. Stored iron and vascular reactivity. *Arterioscler Thromb Vasc Biol* **25**, 1532-1535 (2005).

41. Zheng, H., Cable, R., Spencer, B., Votto, N., & Katz, S.D. Iron stores and vascular function in voluntary blood donors. *Arterioscler Thromb Vasc Biol* **25**, 1577-1583 (2005).

42. Roest, M. *et al.* Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. *Circulation* **100**, 1268-1273 (1999).

43. Ross, C.A. & Poirier, M.A. Protein aggregation and neurodegenerative disease. *Nat Med* **10 Suppl**, S10-S17 (2004).

44. Marchetti, B. & Abbracchio, M.P. To be or not to be (inflamed) - is that the question in anti-inflammatory drug therapy of neurodegenerative disorders? *Trends Pharmacol Sci* **26**, 517-525 (2005).

45. McGeer, P.L. & McGeer, E.G. Inflammation and the degenerative diseases of aging. *Ann N Y Acad Sci* **1035**, 104-116 (2004).

46. Lossinsky, A.S. & Shivers, R.R. Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review. *Histol Histopathol* **19**, 535-564 (2004).

47. Pachter, J.S., de Vries, H.E., & Fabry, Z. The blood-brain barrier and its role in immune privilege in the central nervous system. *J Neuropathol Exp Neurol* **62**, 593-604 (2003).

48. Dietrich, J.B. The adhesion molecule ICAM-1 and its regulation in relation with the blood-brain barrier. *J Neuroimmunol* **128**, 58-68 (2002).

49. Breuer, W., Epsztejn, S., & Cabantchik, Z.I. Dynamics of the cytosolic chelatable iron pool of K562 cells. *FEBS Lett* **382**, 304-308 (1996).

50. Marx, J.J.M. & van Asbeck, B.S. Use of iron chelators in preventing hydroxyl radical damage: adult respiratory distress syndrome as an experimental model for the pathophysiology and treatment of oxygen-radical-mediated tissue damage. *Acta Haematol* **95**, 49-62 (1996).

51. Kondo, K. *et al*. Associations of soluble intercellular adhesion molecule-1 with carotid atherosclerosis progression. *Atherosclerosis* **179**, 155-160 (2005).

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.

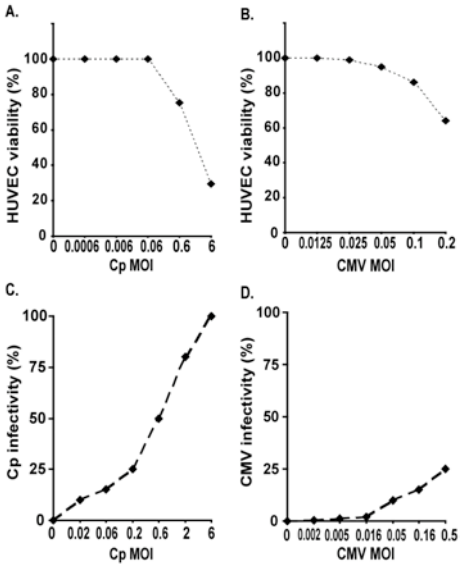

Figure 1. Endothelial viability and microorganism infectivity. (A) shows the percentage of HUVEC viability determined by MTT cytotoxicity assay 2 days after Cp-infection at indicated MOI, while (B) shows HUVEC viability 4 days after CMV infection. (C) shows the percentage of Cp infectivity towards HUVECs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

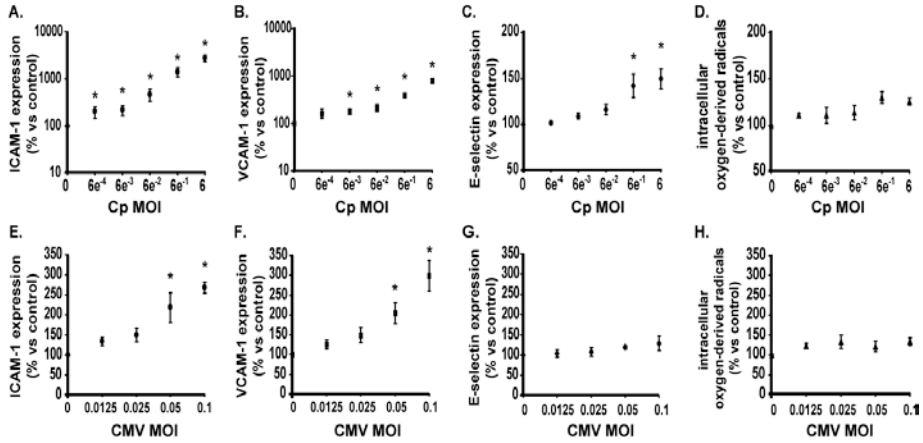

Figure 2. Induction of adhesion molecule expression by Cp or CMV infection. The expression of ICAM-1 (A), VCAM-1 (B), E-selectin (C) on, and the levels of intracellular oxygen-derived radicals (D) in HUVECs after Cp infection at indicated MOI (mean±SEM; n=4; *P<0.05). The expression of ICAM-1 (E), VCAM-1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

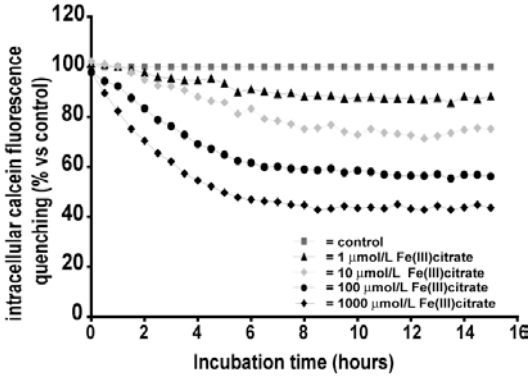

Figure 3. Endothelial intracellular iron level is modulated by addition of low molecular weight iron. Data represent the normalised mean fluorescence of intracellular calcein in HUVECs in the presence of specified concentrations of Fe(III)citrate, monitored up to 15 hours (n=3). Quenching indicates the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

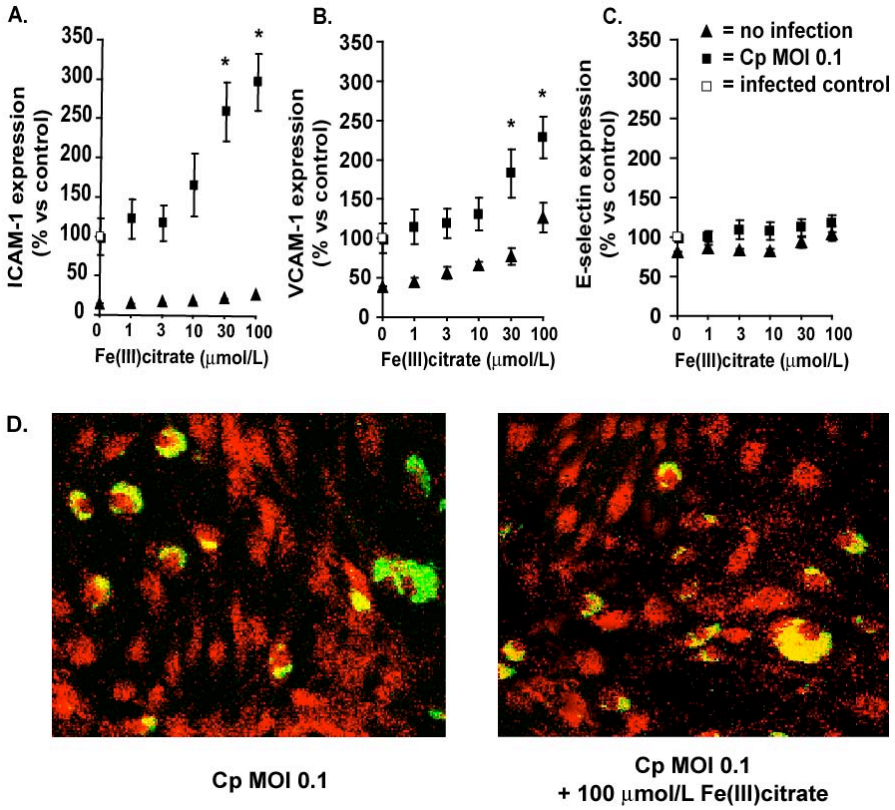

Figure 4. Iron modulates Cp-induced adhesion molecule expression. The expression of ICAM-1 (A), VCAM-1 (B), E-selectin (C) on HUVECs 2 days after Cp infection in the presence of increasing iron concentrations. (mean±SEM; n=4; *P<0.05). (D) confocal laser micrographs, representing 4 different slides,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

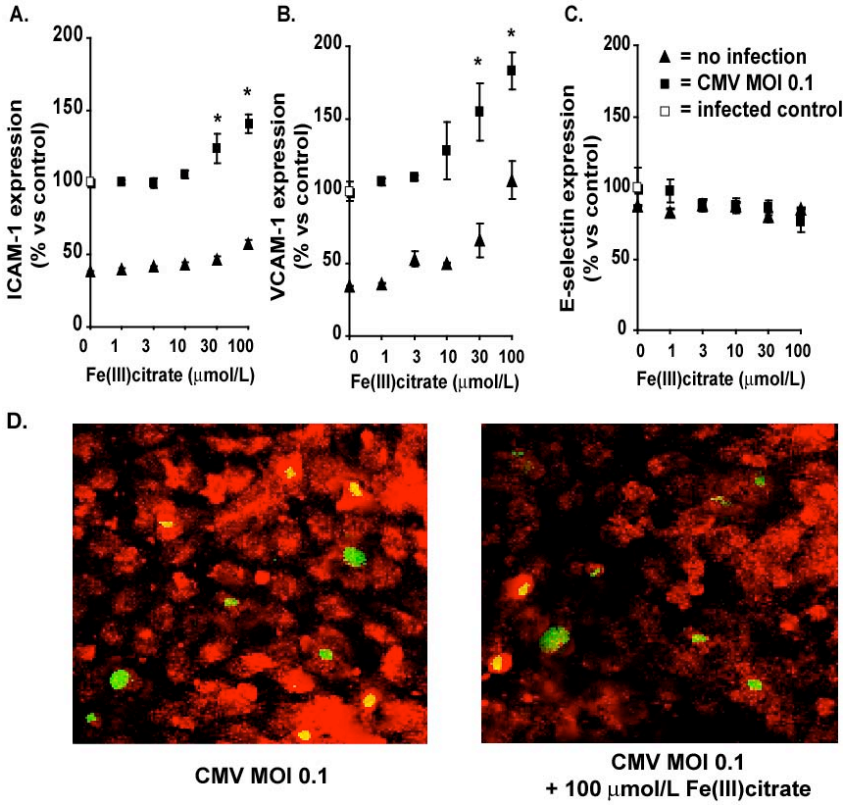

Figure 5. Iron modulates CMV-induced adhesion molecule expression. The expression of ICAM-1 (A), VCAM-1 (B), E-selectin (C) on HUVECs 4 days after CMV infection in the presence of increasing iron concentrations. (mean±SEM; n=4; *P<0.05). (D) confocal laser micrographs, representing 4 different slides,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

.

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.

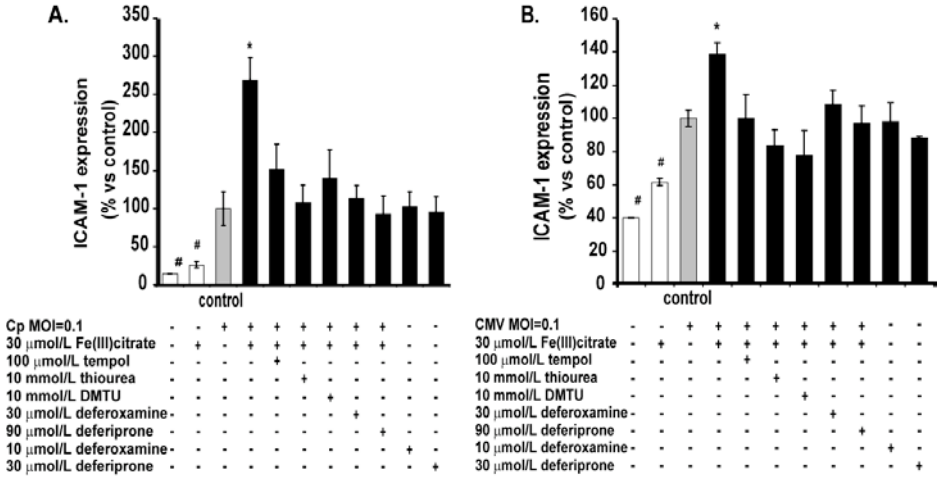

Figure 6. Effects of iron chelation and radical scavenging on infections. The expression of ICAM-1 on Cp (A) or CMV (B) infected HUVECs in the presence of indicated compounds. (mean±SEM; n=3; *higher than control P<0.05; #lower than control P<0.05).

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.

Acknowledgements: This work was supported by a grant from the European Commission, QLK1-CT-2002-00444. We thank A. Huisman, PhD from the University Medical Centre Utrecht, Utrecht, The Netherlands, for the basal iron measurement using Vitros®.

References

1. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).
2. Libby, P., Ridker, P.M., & Maseri, A. Inflammation and atherosclerosis. *Circulation* **105**, 1135-1143 (2002).
3. Campbell, L.A. & Kuo, C.C. *Chlamydia pneumoniae*--an infectious risk factor for atherosclerosis? *Nat Rev Microbiol* **2**, 23-32 (2004).
4. Belland, R.J., Ouellette, S.P., Gieffers, J., & Byrne, G.I. *Chlamydia pneumoniae* and atherosclerosis. *Cell Microbiol* **6**, 117-127 (2004).
5. Danesh, J., Collins, R., & Peto, R. Chronic infections and coronary heart disease: is there a link? *Lancet* **350**, 430-436 (1997).
6. 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* **96**, 4095-4103 (1997).
7. Ieven, M.M. & Hoymans, V.Y. Involvement of *Chlamydia pneumoniae* in atherosclerosis: more evidence for lack of evidence. *J Clin Microbiol* **43**, 19-24 (2005).
8. Printseva, O.Y., Peclo, M.M., & Gown, A.M. Various cell types in human atherosclerotic lesions express ICAM-1. Further immunocytochemical and immunochemical studies employing monoclonal antibody 10F3. *Am J Pathol* **140**, 889-896 (1992).
9. van der Wal, A.C., Das, P.K., Tigges, A.J., & Becker, A.E. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am J Pathol* **141**, 1427-1433 (1992).
10. O'Brien, K.D., McDonald, T.O., Chait, A., Allen, M.D., & Alpers, C.E. 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* **93**, 672-682 (1996).
11. O'Brien, K.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* **92**, 945-951 (1993).
12. Gaydos, C.A., Summersgill, J.T., Sahney, N.N., Ramirez, J.A., & Quinn, T.C. Replication of *Chlamydia pneumoniae in vitro* in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun* **64**, 1614-1620 (1996).
13. Kaukoranta-Tolvanen, S.S., Laitinen, K., Saikku, P., & Leinonen, M. *Chlamydia pneumoniae* multiplies in human endothelial cells *in vitro. Microb Pathog* **16**, 313-319 (1994).
14. Godzik, K.L., O'Brien, E.R., Wang, S.K., & Kuo, C.C. *In vitro* susceptibility of human vascular wall cells to infection with *Chlamydia pneumoniae*. *J Clin Microbiol* **33**, 2411-2414 (1995).
15. Bouwman, J.J.M. *et al.* Azithromycin reduces *Chlamydia pneumoniae*-induced attenuation of eNOS and cGMP production by endothelial cells. *Eur J Clin Invest* **35**, 573-582 (2005).
16. Krull, M. *et al.* Signal transduction pathways activated in endothelial cells following infection with *Chlamydia pneumoniae*. *J Immunol* **162**, 4834-4841 (1999).
17. Kaukoranta-Tolvanen, S.S., Ronni, T., Leinonen, M., Saikku, P., & Laitinen, K. Expression of adhesion molecules on endothelial cells stimulated by *Chlamydia pneumoniae*. *Microb Pathog* **21**, 407-411 (1996).
18. Schumacher, A. *et al.* Does infection with *Chlamydia pneumoniae* and/or *Helicobacter pylori* increase the expression of endothelial cell adhesion molecules in humans? *Clin Microbiol Infect* **8**, 654-661 (2002).
19. Shahgasempour, S., Woodroffe, S.B., & Garnett, H.M. 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* **41**, 121-129 (1997).
20. Guetta, E., Scarpati, E.M., & DiCorleto, P.E. Effect of cytomegalovirus immediate early gene products on endothelial cell gene activity. *Cardiovasc Res* **50**, 538-546 (2001).
21. Sedmak, D.D., Knight, D.A., Vook, N.C., & Waldman, J.W. Divergent patterns of ELAM-1, ICAM-1, and VCAM-1 expression on cytomegalovirus-infected endothelial cells. *Transplantation* **58**, 1379-1385 (1994).
22. Burns, L.J. *et al.* Intercellular adhesion molecule-1 expression in endothelial cells is activated by cytomegalovirus immediate early proteins. *Transplantation* **67**, 137-144 (1999).
23. Knight, D.A., Waldman, W.J., & Sedmak, D.D. Cytomegalovirus-mediated modulation of adhesion molecule expression by human arterial and microvascular endothelial cells. *Transplantation* **68**, 1814-1818 (1999).
24. Ewig, S. & Torres, A. Is *Chlamydia pneumoniae* an important pathogen in patients with community-acquired pneumonia? *Eur Respir J* **21**, 741-742 (2003).
25. Forbes, B.A. Acquisition of cytomegalovirus infection: an update. *Clin Microbiol Rev* **2**, 204-216 (1989).

26. Kartikasari, A.E.R. *et al.* Iron and atherosclerosis: Iron chelators decrease adhesion of monocytes to vascular endothelium. *Biomarkers Environment* **6 Suppl 1**, 34-38 (2003).

27. Kartikasari, A.E.R. *et al.* Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. *Arterioscler Thromb Vasc Biol* **24**, 2257-2262 (2004).

28. Visseren, F.L.J.*et al.* 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* **32 Suppl 1**, 84-90 (2002).

29. Varani, 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* **66**, 708-714 (1992).

30. Ryter, S.W., Si, M., Lai, C.C., & Su, C.Y. Regulation of endothelial heme oxygenase activity during hypoxia is dependent on chelatable iron. *Am J Physiol Heart Circ Physiol* **279**, H2889-H2897 (2000).

31. 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* **289**, 664-669 (2001).

32. Satoh, T. & Tokunaga, O. Intracellular oxidative modification of low density lipoprotein by endothelial cells. *Virchows Arch* **440**, 410-417 (2002).

33. Zhang, W.J. & Frei, B. Intracellular metal ion chelators inhibit TNF-alpha-induced SP-1 activation and adhesion molecule expression in human aortic endothelial cells. *Free Radic Biol Med* **34**, 674-682 (2003).

34. Koo, S.W., Casper, K.A., Otto, K.B., Gira, A.K., & Swerlick, R.A. Iron chelators inhibit VCAM-1 expression in human dermal microvascular endothelial cells. *J Invest Dermatol* **120**, 871-879 (2003).

35. Ramachandran, A., Ceaser, E., & rley-Usmar, V.M. 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 U S A* **101**, 384-389 (2004).

36. Dhanasekaran, A. *et al.* Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis. *J Biol Chem* **279**, 37575-37587 (2004).

37. Soares, M.P. *et al.* Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation. *J Immunol* **172**, 3553-3563 (2004).

38. Kartikasari, A.E.R. *et al.* Endothelial activation and induction of monocyte adhesion by nontransferrin-bound iron present in human sera. *FASEB J*(2005). DOI: 10.1096/fj.05-4700fje

39. Kaplan, J., Jordan, I., & Sturrock, A. Regulation of the transferrin-independent iron transport system in cultured cells. *J Biol Chem* **266**, 2997-3004 (1991).

40. Marx, J.J.M. Primary and secondary haemochromatosis. *Transfus Sci* **23**, 183-184 (2000).

41. Sullivan, J.L. Iron and the sex difference in heart disease risk. *Lancet* **1**, 1293-1294 (1981).

42. Kartikasari, A.E.R., Georgiou N.A., & Marx J.J.M. Iron intake and cardiovascular disease in *Functional foods, cardiovascular disease and diabetes* (ed. Arnoldi A) 99-125 (Woodhead Publishing Ltd, Abington, Cambridge, UK and CRC Press LLC, Boca Raton, Florida, USA, 2004).

43. Stadler, N., Lindner, R.A., & Davies, M.J. 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* **24**, 949-954 (2004).

44. Lee, T.S., Shiao, M.S., Pan, C.C., & Chau, L.Y. Iron-deficient diet reduces atherosclerotic lesions in apoE-deficient mice. *Circulation* **99**, 1222-1229 (1999).

45. Matthews, A.J. *et al.* Iron and atherosclerosis: inhibition by the iron chelator deferiprone (L1). *J Surg Res* **73**, 35-40 (1997).

46. Minqin, R. *et al.* The iron chelator desferrioxamine inhibits atherosclerotic lesion development and decreases lesion iron concentrations in the cholesterol-fed rabbit. *Free Radic Biol Med* **38**, 1206-1211 (2005).

47. Minqin, R., Watt, F., Huat, B.T., & Halliwell, B. Correlation of iron and zinc levels with lesion depth in newly formed atherosclerotic lesions. *Free Radic Biol Med* **34**, 746-752 (2003).

48. Jaffe, E.A., Nachman, R.L., Becker, C.G., & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* **52**, 2745-2756 (1973).

49. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63 (1983).

50. Chou, T.C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**, 27-55 (1984).

51. Vanden Hoek, T.L., Li, C., Shao, Z., Schumacker, P.T., & Becker, L.B. Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J Mol Cell Cardiol* **29**, 2571-2583 (1997).

52. Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W., & Cabantchik, Z.I. Fluorescence analysis of the labile iron pool of mammalian cells. *Analytical Biochemistry* **248**, 31-40 (1997).

53. Beatty, W.L., Morrison, R.P., & Byrne, G.I. Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol Rev* **58**, 686-699 (1994).

54. Halwachs-Baumann, G. *et al.* Human trophoblast cells are permissive to the complete replicative cycle of human cytomegalovirus. *J Virol* **72**, 7598-7602 (1998).

55. Visseren,F.L.J., Verkerk,M.S., Bouter,K.P., Diepersloot,R.J., & Erkelens,D.W. Interleukin-6 production by endothelial cells after infection with influenza virus and cytomegalovirus. *J Lab Clin Med* **134**, 623-630 (1999).

56. Freidank, H.M., Billing, H., & Wiedmann-Al-Ahmad, M. Influence of iron restriction on *Chlamydia pneumoniae* and *C. trachomatis*. *J Med Microbiol* **50**, 223-227 (2001).

57. Al-Younes, H.M., Rudel, T., Brinkmann, V., Szczepek, A.J., & Meyer, T.F. Low iron availability modulates the course of *Chlamydia pneumoniae* infection. *Cell Microbiol* **3**, 427-437 (2001).

CHAPTER 5

Intracellular iron enhances monocyte adhesion to TNF-α-activated endothelial cells under physiological flow conditions and MCP-1-dependent transendothelial migration

Apriliana E. R. Kartikasari, Frank L. J. Visseren, Joannes J. M. Marx,
Sanne van Mullekom, Laurien H. Ulfman, J. Henny van Kats-Renaud,
B. Sweder van Asbeck, Niki A. Georgiou

Submitted

ABSTRACT

Infiltration of monocytes across the endothelial barrier is not only considered to contribute to the initiation but also to the progression of atherosclerosis. Given the plausible roles of iron in inflammatory processes, we have investigated the effects of iron on monocyte rolling and firm adhesion to TNF-α-activated endothelial cells in a physiological flow model, as well as their diapedesis through the endothelial monolayer. Iron enhanced TNF-α-induced endothelial activation, resulting in a rise on monocyte adhesion. Moreover, an increase in the number of firmly adhered monocytes was observed using iron-loaded monocytes compared to controls. Blockade of the integrins, α4 and β2, restored more than 60% of monocyte rolling. However, in this condition, the number of firmly adhered cells was higher for iron-loaded monocytes than controls, while no difference in the rolling velocity was observed, suggesting that iron could have affected receptors other than the blocked integrins. Iron loading indeed upregulated CCR-2 and CXCR-2 but not PECAM-1 expression on monocytes, and resulted in increased MCP-1-dependent transendothelial migration. Furthermore, both iron-induced adhesion and transmigration could be mitigated by iron chelation. Additionally, accumulation of oxygen-derived radicals due to iron loading was evidenced. We conclude that intracellular iron is an important enhancer of cytokine-mediated consecutive processes of monocyte-endothelial interactions. This finding reveals an immunomodulatory function of iron in inflammation.

Key words: inflammation • cytokines • atherosclerosis • monocytes • endothelium

INTRODUCTION

Recruitment of inflammatory monocytes from the blood stream to the sites of injury or infection is a fundamental mechanism in many inflammatory diseases, like atherosclerosis.[1] The process consists of consecutive adhesion-mediated events. The first event involves the selectins that trigger tethering of the leukocytes to the activated endothelium along the vessel wall.[2] This rolling event is followed by arrest and firm adhesion of monocytes. The rolling step occurs with the binding of the integrins, very late antigen-4 (VLA-4, α4β1) and lymphocyte function-associated antigen-1 (LFA-1, αLβ2), to their counter receptors on endothelial cells, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1).[2] Firm adhesion is followed by transendothelial migration of monocytes, with one of the mediators being platelet endothelial cell adhesion molecule-1 (PECAM-1).[3]

Chemokines, the family of chemotactic cytokines, are capable of activating the integrins as well as inducing directed migration of leukocytes.[4] Endothelial cells synthesise chemokines upon stimulation with cytokines. Monocyte chemotactic protein-1 (MCP-1, a CC chemokine) and Interleukin-8 (IL-8, a CXC chemokine), presented by endothelial cells, and their receptors on monocytes, i.e. CCR-2 for MCP-1, and CXCR-1 and CXCR-2 for IL-8, trigger adhesion as well as infiltration of monocytes.[5] The

endothelial surface-bound melanoma growth stimulatory activity-α (GRO-α, a CXC chemokine)) together with its receptor on monocytes, CXCR-2,[6,7] mediates the abrupt arrest of monocytes possibly through an instantaneous conformation change of the integrins.[8]

Infiltrating monocytes may have an important impact in the progression of atherosclerotic lesions.[1] This hypothesis is supported by the findings that mice lacking MCP-1, IL-8, or the receptors are resistant to atherosclerosis.[9-13] Furthermore, MCP-1 has been found in human atheroma.[14,15] The degree of macrophage infiltration is also significantly increased in human atheroma,[16] with concentrations of ICAM-1, VCAM-1, and endothelial selectin (E-Selectin) being elevated in the plaques.[16]

There is growing evidence that iron plays a role in inflammatory processes involved in atherogenesis.[17,18] We and others have shown that iron influences the activation state of endothelial cells,[19-24] as well as leukocyte.[19,25-27] Coincidently, the transition metal iron as well as copper have been found accumulating in human atheroma.[28] Moreover, several other studies show reduced formation of early atherosclerotic lesions by means of iron chelation or iron-deficient diets in experimental animals.[29-32]

There are many abnormal conditions that can lead to disturbances of iron homeostasis. In hereditary hemochromatosis and β-thalassemia where patients receive frequent blood transfusions the increased body iron stores may lead to the formation of labile non-transferrin-bound iron (NTBI).[33] The labile NTBI is freely entering cells with no feedback-regulated process,[34] and is capable of initiating free radical reactions.[35] In the setting of infections, iron homeostasis could also be altered, resulting in a pathogenic condition, the so-called anemia of inflammation, with a feature of low serum iron resembling iron deficiency anemia.

In this study, we have investigated the biological effects of iron and iron chelation in the inflammatory process of monocyte-endothelial interactions under physiological flow conditions. This flow model provides a system mimicking the multi-step recruitment cascade of leukocyte infiltration in the vasculature.

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, which have lower affinity for iron compared to glass were used.

Endotoxin-free conditions

Iron solutions were tested for the presence of LPS using a limulus amebocyte lysate assay (Cambrex Bioscience, Walkersville, USA). To ensure LPS-free conditions, all the materials were kept under pyrogen-free conditions.

HUVECs isolation and culture

HUVECs were isolated and cultured as described by Jaffe et al.[36] Confluent cells from passages two to three were used for all experiments. To minimise donor-to-donor variability, HUVECs were pooled from three to four donors for each experiment. Moreover the cells were always used during and maintained at a cobblestone confluent density for all conducted experiments.

Monocyte isolation

Peripheral blood mononuclear cells (PBMC) were isolated from donor blood (Sanquin blood bank, Utrecht, the Netherlands) by Ficollpaque density gradient centrifugation. An extensive washing step was performed by centrifugation at 200g for 8 minutes at 4^0C without deceleration for at least six times, in order to eliminate most of the platelets. Monocytes were then isolated using the negative immuno-selection monocyte isolation kit (MiltenyiBiotec, CLB Sanquin, Amsterdam, The Netherlands) according to supplier's instructions. This method resulted in purity of >90% as analysed by flow cytometry. Purified monocytes were suspended at a concentration of $5x10^6$ cells/mL in RPMI1640 (BioWhittaker) supplemented with 2 mmol/L L-glutamine and 0.2% human serum albumin (CLB Sanquin) prior to use.

Viability assay

Cellular viability of HUVECs was monitored by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich) method.[37] Compound cytotoxicity was expressed as a TC_{50} denoting the concentration resulting in 50% loss of cell viability, as calculated by Calcusyn.[38] Cellular viability of monocytes after the various compound incubations was monitored using the trypan blue exclusion test. In all experiments, both HUVECs and monocytes were >95% viable after indicated treatments

Preparation of iron solutions and chelator-bound iron

The majority of NTBI is found in the complex form of Fe(III) to citrate.[39] A 10 mmol/L Fe(III)citrate (Sigma, St.Louis, USA, 1:6 iron-citrate molar ratio) stock solution was made by dissolving the iron crystals in distilled water at 56^oC for 30 minutes. Iron solutions were always freshly prepared and filter-sterilised prior to use.

Chelator stocks were 10 mmol/L deferoxamine (Novartis, Arnhem, The Netherlands) in PBS and 30 mmol/L deferiprone (Duchefa Biochemie, Haarlem, The Netherlands) in PBS and stored at -20^0C prior to use. Six ligands can bind to an iron atom. One deferoxamine molecule being a hexadantate chelator, or three molecules of a bidentate chelator, like deferiprone, are required to fully chelate one iron atom. Chelator-bound iron solutions were always freshly prepared prior to experiments.

Calcein assay for measurement of intracellular labile iron

The accumulation of labile iron inside cells due to incubation with NTBI was tested by the calcein-AM assay.[40] Calcein-AM (Molecular Probes) is a fluorescent probe with a lipophilic acetoxymethylester (AM) moiety that makes it permeable through cell membranes. Once inside the cell, the AM group will be cleaved by non-specific esterases, resulting in a charged form that hardly leaks out of cells. As a weak iron chelator, once inside the cells, the fluorescence signal of calcein can be quenched by iron.

In this assay, cells were incubated with 0.125 μmol/L calcein-AM (30 minutes at 37°C), then washed twice to remove the remaining extracellular calcein-AM before the fluorescence signal of calcein (excitation = 485 nm; emission = 530 nm) was followed using a Flexstation (Molecular Devices, Workingham, UK) at 37°C. After a stable basal fluorescence signal was observed, iron was added to the incubation medium. 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.

Dichlorofluorescein assay for measurement of intracellular oxygen-derived radicals

Carboxydichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, USA) is a nonpolar compound that is converted into a membrane-impermeable non-fluorescent polar derivative (DCFH) by cellular esterases after incorporation into cells. The trapped DCFH is rapidly oxidised to fluorescent 2,7-dichlorofluorescein (DCF) by intracellular hydrogen peroxide and hydroxyl radicals [41]. Cells were incubated in DCFH-DA at a final concentration of 5 μmol/L for 30 minutes and washed. The emission of the trapped, oxidised DCF in 10.000 cells was analysed by flow cytometry.

Monocyte perfusion and evaluation

Perfusions under steady flow were performed in a modified form of transparent parallel-plate perfusion chamber as previously described.[42] HUVECs were subcultured to confluent monolayers on a coverslip (18 mm X 18 mm) precoated with glutaraldehyde-linked gelatin. The micro-chamber has a slit height of 0.2 mm and width of 2 mm when the coverslip with HUVECs were mounted on a circular plug of the chamber. HUVECs were activated using 2 ng/mL TNF-α (Boehringer Mannheim, Germany) for 4 hours prior to each perfusion.

Monocytes were aspirated from a reservoir through the perfusion chamber. The perfusions were performed as individual runs at 37°C. During perfusions, the flow chamber was placed on a microscope stage (DM RXE; Leica, Weitzlar, Germany), which was equipped with a B/W CCD video camera (Sanyo, Osaka, Japan). The entire period of perfusion was recorded on tape using a VHS video recorder connected to the camera. The monocytes in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. Video images were evaluated for the number of rolling and adherent cells using the image analysis software Optimas 6.1 (Media Cybernetics systems, Silver Springs, MD).

The monocyte suspension of $2x10^6$ cells/mL after 2 minutes preincubation at 37^0C, was perfused during 5 minutes at a shear stress of 2.0 dynes/cm^2, using a perfussion buffer containing 20

mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L $MgSO_4$, 1.2 mmol/L KH_2 PO_4, supplemented with 5 mmol/L glucose, 1.0 mmol/L $CaCl_2$, and 0.5% HSA. The mean number of surface-adherent monocytes were measured after the 5 minutes perfusion at a minimum of 50 randomised high power fields (total surface of at least 2 mm^2).

For rolling experiments, monocytes were incubated with the blocking monoclonal antibodies, HP2/1 (anti–α4-integrin, Immunotech, Marseille, France) and IB4 (anti–β2-integrin, isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection, Rockville, MD). The incubation was for 15 minutes at 4^0C. Monocytes were then diluted with perfusion medium to $2x10^6$ cells/mL. Rolling was measured by the capture of a sequence of 50 frames representing an adjustable time interval (Δt, with a minimal interval of 80ms). At each frame, the position of every cell was detected, and for all subsequent frames the distance moved by each cell and the number of images in which a cell appears in focus was measured. The velocity of a cell (v) in μm/s was calculated from the equation: $v = L/\Delta t(x - 1)$, in which L is the covered distance (μm) and x is the number of images in which a cell appears. The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/s.

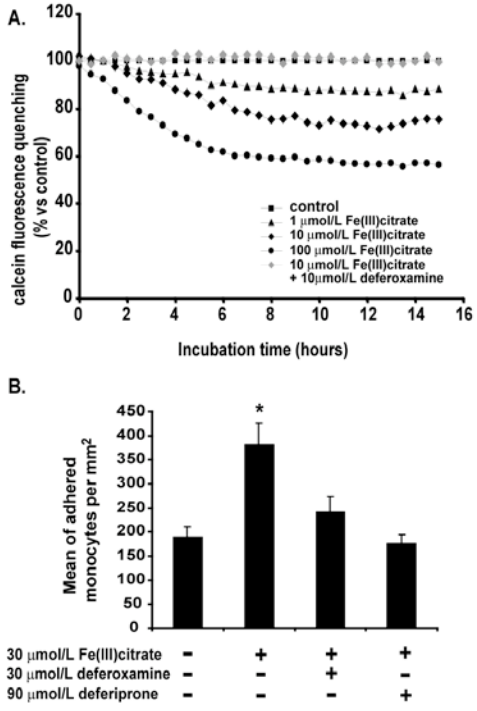

Figure 1. **Endothelial intracellular iron induced monocyte arrest under flow.** (A) Data represent the normalised fluorescence of intracellular calcein of HUVECs in the presence of specified concentrations of Fe(III)citrate, monitored for 15 hours (n=3). Quenching indicates the presence of intracellular labile iron. (B) Firm adhesion of non-treated monocytes on HUVECs under shear stress of 2 dynes/cm^2. HUVECs were incubated with indicated compound(s) for 48 hours, before 4 hours treatment with 2 ng/mL TNF-α. (n=4, *P<0.01).

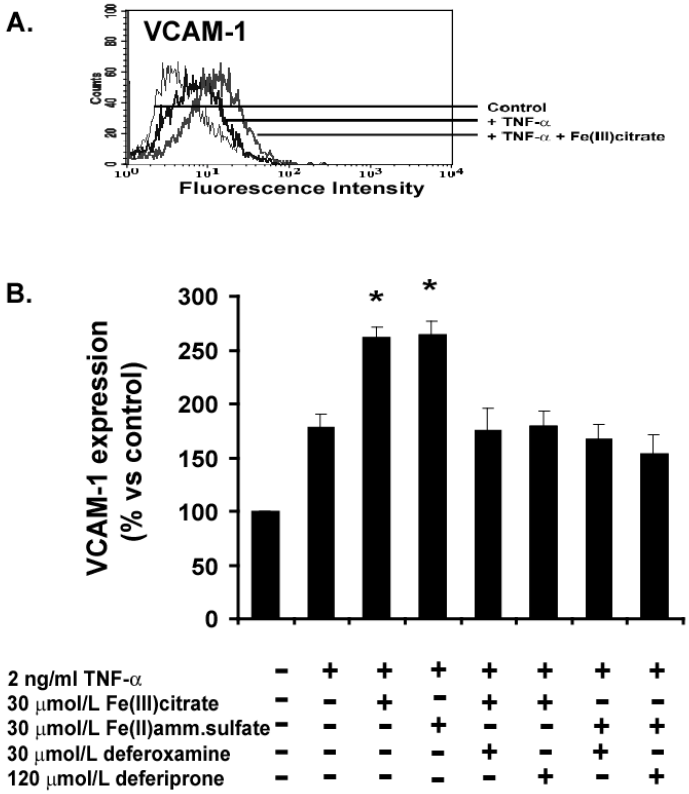

Figure 2. **Enhanced TNF-α activation of endothelial cells by iron.** (A) An illustration of FACS analysis of VCAM-1 expression on HUVECs. The shift to the right indicates more expression of the protein. (B) shows the expression level of VCAM-1 after indicated treatments and 4 hours coincubation with 2 ng/mL TNF-α at the end of each treatment (n=3, *P<0.01, higher compared to controls).

Measurement of cell adhesion molecules by fluorescence-activated cell sorting (FACS)

Analysis of proteins expressed on the surface of either monocytes or HUVECs was performed according to standard techniques. Briefly, the cells were 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), Cychrome-conjugated E-selectin antibody (BDBiosciences), FITC-conjugated PECAM-1 antibody (Sigma), PE-conjugated CCR-2 antibody (BDBiosciences), PE-conjugated CXCR-2 antibody (BDBiosciences), for 30 minutes at 4^0C, then washed prior to FACS analysis. Each flow cytometric measurement was performed using a Becton Dickinson FACScan and 10.000 events were analysed.

Transendothelial migration assay

Migration of monocytes through an endothelial monolayer was evaluated using 24-well Costar Transwell chambers (3 μm pore size, Corning, New York, USA). HUVECs were subcultured to confluent monolayers on Transwell inserts which had been precoated with fibronectin. Monocytes were labeled with 2 μmol/L 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM, Molecular Probes). 100 μL monocyte suspension (1-2 x 10^6 cells/mL) was seeded in the upper chamber and 600 μL of medium containing 10 nmol/L MCP-1 was added to the lower well. The chamber was incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2 for 3 hours. Fluorescence of monocytes was read using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA) at 485 nm excitation and 530 nm emission. The percentage of transmigration was defined as the value of fluorescence of migrated monocytes divided by the value of total fluorescence, multiplied by 100.

Data analysis

Results are expressed as means ± standard error of 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

Endothelial iron loading enhanced firm adhesion of monocytes under flow

With no external iron addition, the baseline iron level in the basal cell culture medium was 0.36 μmol/L. LPS concentrations in different iron solutions were less than 1 pg/mL. Incubation of HUVECs with iron of as low as 1 μmol/L, already increased intracellular labile iron level shown by the quenched calcein signal (Figure 1a). The level of intracellular iron was stabilised after 6 to 8 hours of iron incubation.(Figure 1a). Incubation of HUVECs with fully chelated iron did not quench the calcein signal, indicating that iron chelation prevented iron loading to cells (Figure 1a).

Both non-iron-loaded and iron-loaded HUVECs could not support firm adhesion of monocytes at 2 dynes/cm^2 (not shown). After HUVECs were activated with 2 ng/mL TNF-α for 4 hours, a two-fold increase in the number of adherent monocytes was observed after perfusion on iron-loaded HUVECs compared to controls (Figure 1b). Preincubation of HUVECs with either deferoxamine or

deferiprone–chelated iron did not increase adhesion (Figure 1b). This suggests that the level of monocyte adhesion under flow was alterable by endothelial iron, the level of which was modulated by external addition of iron. In all of conditions mentioned above, rolling interactions were negligible (not shown).

The endothelial iron loading increased TNF-α-induced VCAM-1 expression to 60%, as observed by FACS analysis (Figure 2a-b). However, this treatment did not significantly alter ICAM-1, E-selectin and PECAM-1 expression (not shown). Chelation of iron with either deferoxamine or deferiprone counteracted this effect of iron (Figure 2b), confirming the involvement of iron in this observed sequel.

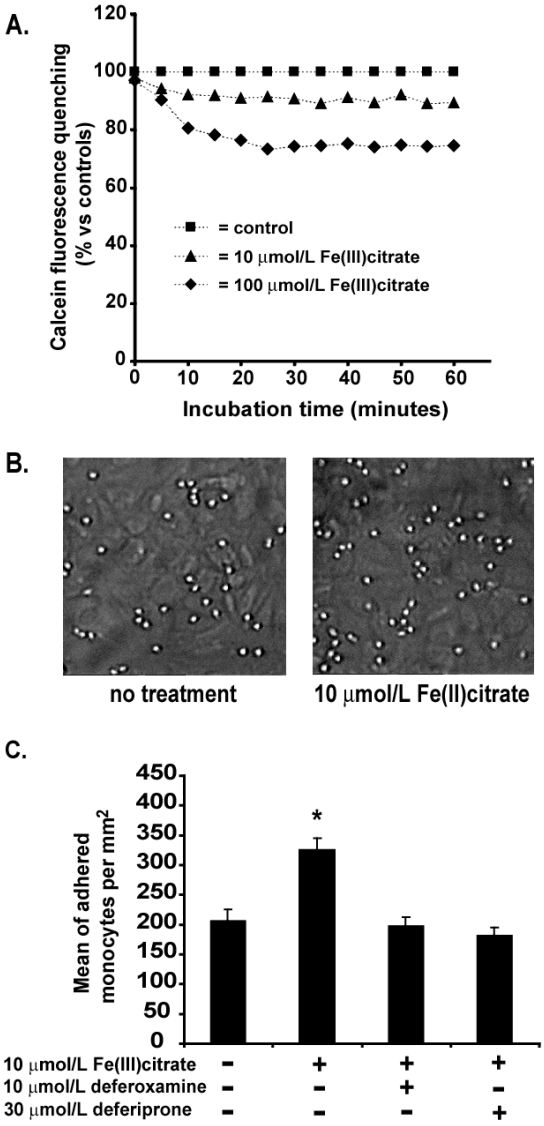

Figure 3. Increased intracellular iron enhanced monocyte arrest under flow. (A) Data represent the normalised mean fluorescence of intracellular calcein of monocytes in the presence of specified concentration of Fe(III)citrate or Fe(II)ammoniumsulfate, monitored for 2 hours (n=3). Quenching indicates the presence of intracellular labile iron. (B) Illustration of firm adhesion under flow after monocytes were subjected to indicated treatments. (C) shows the amount of arrested monocytes on HUVECs under shear stess of 2 dynes/cm^2 after monocytes were incubated with the indicated compound(s) for 1 hour. (n=5, *P<0.01).

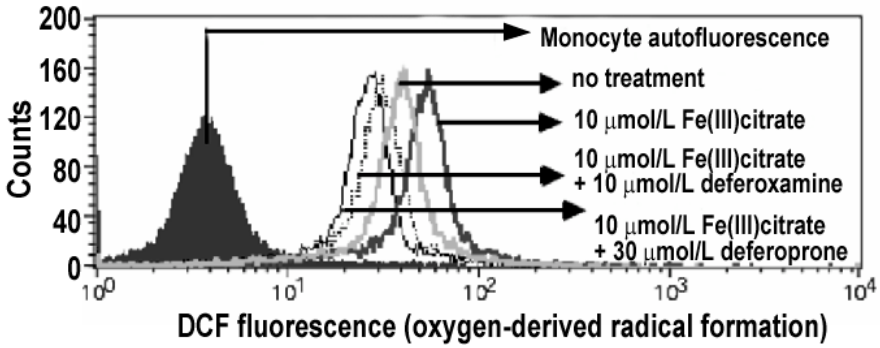

Figure 4. Iron induces generation of oxygen-derived radicals. IIlustration of FACS analysis on DCF-fluorescence probe for oxygen-derived radicals. The shift to the right indicates an increase in intracellular radical production.

Monocyte iron loading augmented adhesion to endothelium under flow

Accumulation of intracellular labile iron inside the monocytes was already detected and stabilised after 10 minutes of incubation with Fe(III)citrate as shown by the Calcein assay (Figure 3a). Incubation of monocytes with 100 μmol/L iron for 1 hour at 37^0C did not affect cellular viability as evaluated by trypan blue staining, nor the total cell counts of monocytes (not shown). A significant increase in adhesion was observed when monocytes preincubated with 10 μmol/L for 1 hour were perfused compared to non-iron-loaded controls (Figure 3b-c). Preincubation of monocytes with either deferoxamine or deferiprone–bound iron did not enhance adhesion (Figure 3c). This suggests that increased level of intracellular iron in monocytes triggered adhesion to vascular endothelium.

Iron induced oxygen-derived radical formation in monocytes

The increased level of intracellular iron in monocytes may promote the production of oxygen-derived radicals leading to monocyte activation. Using the DCF-fluorescence probe, an increased intracellular radical generation due to iron loading was observed (Figure 4). This effect could be counteracted using either of the iron chelators, deferoxamine or deferiprone, confirming that the intracellular redox status is alterable by the level of labile iron.

The intracellular labile iron did not alter the rolling of monocytes

TNF-α-activated HUVECs are known to support mostly firm adhesion of monocytes.[5] In this study, we have indeed observed this phenomenon. However, when the monocytes were incubated with

the monoclonal antibodies directed against α4 and β2 integrins, we were able to restore more than 60% of rolling interactions. Using this condition, the effects of an increased intracellular iron level on monocyte rolling were investigated. Rolling velocity of iron-loaded monocytes was similar to controls (18.09 ± 0.67 vs. 18.36 ± 0.22 μm/sec, P>0.05). The numbers of rolling and adherent cells reported in this study were the average of the amount of cells along the perfusion chamber. This was because in both iron and non-iron treated conditions, a tendency of having decreasing amounts of cells rolling along the perfusion chamber was observed. The number of rolling monocytes did not differ after incubation with or without iron (141 ± 17 vs. 128 ± 15 monocytes/mm^2, P>0.05, Figure 5). However, the level of firm adhesion of monocytes preincubated with 10 μmol/L Fe(III)citrate for 1 hour was significantly higher than that of controls (125 ± 23 vs. 44 ± 10 monocytes/mm^2, P<0.05, Figure 5). This finding suggests that intracellular labile iron may promote firm adhesion of monocytes mediated by receptors other than the integrins.

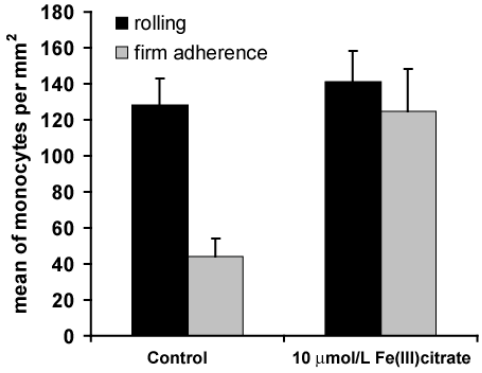

Figure 5. Effects of α4 and β2 blocking on rolling and arrest of monocytes. The figure shows the mean numbers of rolling and adhered monocytes, after α4 and β2 blocking. Monocytes were treated for 1 hour with or without 10 mmol/L Fe(III)citrate (n=3, *P<0.01).

Iron induced CCR-2, CXCR-2, but not PECAM-1 expression

Besides the expression of adhesion molecules, monocyte infiltration through the endothelial barrier is critically regulated by chemokines. We analysed the effects of iron on the expression level of CCR-2 and CXCR-2, the main chemokine receptors on monocytes, regulating the rolling and firm adhesion events. Figure 6 shows the expression levels of CCR-2, CXCR-2 and PECAM-1 on monocytes after incubation with various concentrations of iron. Concentration of 10 μmol/L already

upregulated the expression of CCR-2 as well as CXCR-2, indicating that intracellular iron also modulated the chemokine pathway. Induction of these two receptors could lead subsequently to enhanced transendothelial migration of the monocytes. In this study, however, PECAM-1 expression remained unaffected by iron loading.

MCP-1-dependent transendothelial migration of monocytes was enhanced by intracellular labile iron

Since intracellular iron upregulated the expression of the chemokine receptors, we investigated the effects of iron loading on transendothelial migration of monocytes towards the chemoattractant MCP-1. The level of MCP-1-dependent transendothelial migration of monocytes was increased when either monocytes or HUVECs were iron-loaded (Figure 7). The transmigration level increased even more when both type of cells were iron-loaded, resulting in a 1.6 fold increase of transmigrating monocytes compared to non-iron-loaded controls. Furthermore, iron chelation could counteract the effect of iron (Figure 7), confirming the role of iron in transmigration.

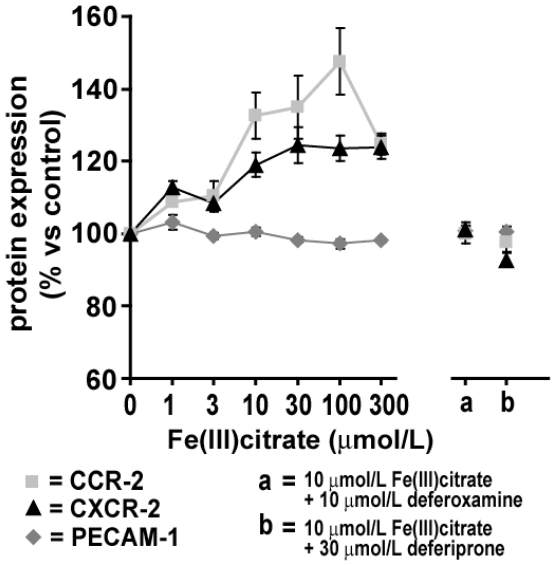

Figure 6. Monocyte surface protein expression and oxygen-derived radical generation. The graph shows the expression level of CCR-2, CXCR-2 and PECAM-1 on monocytes after 1 hour of the indicated treatment. (n=3 to 10).

DISCUSSION

To investigate the effects of iron in the consecutive process of monocyte infiltration through endothelium, mimicking the inflammatory event of the vasculature, human monocytes were perfused

over a monolayer of TNF-α-activated HUVECs. Without TNF-α activation, the iron-loaded HUVECs could not support monocyte adherence under flow. These HUVECs possessed a higher level of intracellular labile iron compared to untreated controls. It has been shown previously that iron induced most effectively VCAM-1 expression.[19,21,22] VCAM-1 alone however, is not sufficient[5] to promote monocyte firm adhesion at shear of 2 dynes/cm^2 indicating that other signals expressed by activated endothelial cells are necessary for firm adhesion. Upon stimulation with TNF-α, iron-loaded HUVECs promoted more monocyte firm adhesion compared to non-iron-loaded controls, and expressed higher levels of VCAM-1. This finding suggests that intracellular labile iron is capable of modulating cytokine activation signals on endothelial cells, which in turn augment monocyte recruitment to sites of inflammation. In this study, we also observed that iron loading on monocytes had lead to a significant induction of monocyte firm adhesion to TNF-α-activated HUVECs under flow. This finding suggests that an increase in iron status may enhance the process of inflammation. In addition, this study may imply that in a state of iron deficiency, the response towards cytokine activation *in vivo* may be inefficient and this could lead to an impaired immune response.

In this study, blockade of α4 and β2 integrins restored more than 60% of the rolling interactions, with an average rolling velocity of 18.36±0.22 μm/sec. In the presence of these blocking antibodies, a significantly higher number of iron-activated monocytes was firmly adhering compared to controls, although there was no change in the rolling velocity nor the number of rolling cells. This result suggests that iron may have prevented detachment of the readily adhering cells, since there is no increase in the number of cells leaving the rolling stage. This finding also suggests that iron could have affected a set of receptors other than integrins that are involved in the adhesion process. Flow cytometry indeed showed upregulation of surface proteins, CCR-2 and CXCR-2 by iron. These chemokine receptors can bind to chemokines, like MCP-1, IL-8 and GRO-α, to promote monocyte extravasation.[5-7] Collectively, these findings suggest that iron may enhance monocyte activation and arrest through both the adhesion molecules as well as the chemokine pathways.

In this study, without changes in PECAM-1 expression level, iron-loaded monocytes could more effectively transmigrate towards an MCP-1 gradient, compared to controls. Iron-induced expression of CCR-2 on monocytes could have potentiated the effects of MCP-1, independent of the level of PECAM-1 expression. It has been shown that MCP-1-mediated monocyte infiltration is dependent on the amount of surface expression of CCR-2 on the monocytes.[43] Additionally, CCR-2-deficient mice exhibit a pronounced defect in the process of leukocyte extravasation.[13] In atherosclerosis-prone mice, disruption of CCR-2 had markedly decreased atherosclerotic lesion formation.[10] Our current study demonstrates enhancement of CCR-2 expression as well as monocyte transmigration by increased intracellular iron level, suggesting the role of iron in this inflammatory event.

Iron is capable of catalysing the formation of oxygen-derived radicals,[35] promoting the expression of adhesion molecules[19,21-23,27] chemokines[44,45], and also their receptors.[46] In agreement with these studies, we have observed increased oxygen-derived radical formation in iron-loaded cells. This finding suggests that enhancement of monocyte-endothelial interactions by intracellular iron is mediated by alteration of the reduction-oxidation status of the cells. Moreover, our finding may be particularly important in artherosclerotic and ischemic disease where an inflammatory infiltrate is a constituent of

the pathogenesis, while oxygen-derived radicals could aggravate the cytokine-induced inflammatory events. Prolonged presence of oxygen-derived radicals, possibly occurring in iron-overload conditions, may lead to the loss of control of the inflammatory response, which in turn could result in the development of persistent inflammatory disorders.

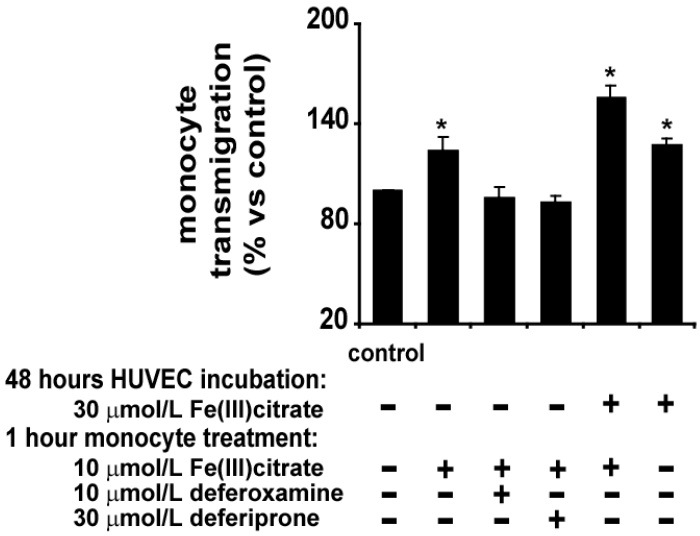

Figure 7. Transendothelial migration of monocyte induced by intracellular iron. The graph shows the level of transendothelial migration of monocytes compared to controls, after the indicated treatment (n=3 to 7, *P<0.05).

In summary, using a flow model mimicking *in vivo* conditions, we have demonstrated the role of intracellular labile iron in enhancing the cytokine-induced consecutive processes of monocyte-endothelial interactions. Intracellular iron alters both adhesion molecule and chemokine receptor expression that is possibly initiated by the formation of intracellular oxygen-derived radicals. Furthermore, the iron-triggered monocyte-endothelial interactions can effectively be prevented by iron chelation. Taken together, our study identifies an immunomodulatory function of iron in the complex process of monocyte recruitment and transmigration during inflammation. The identification of cellular conditions responsible for monocyte-endothelial interactions could provide insight into biological

mechanisms of inflammatory diseases, like atherosclerosis, and could offer tools for therapy and prevention of disease.

***Acknowledgements*:** This work was supported by a grant from the European Commission, QLK1-CT-2002-00444. We thank Mr. J.J.M. Bouwman from the Diakonessen Hospital, Utrecht, the Netherlands, for providing the umbilical cords.

References

1. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).
2. Imhof, B.A. & urrand-Lions, M. Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol* **4**, 432-444 (2004).
3. Liao, F., Ali, J., Greene, T., & Muller, W.A. Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration *in vitro* and *in vivo*. *J Exp Med* **185**, 1349-1357 (1997).
4. Johnston, B. & Butcher, E.C. Chemokines in rapid leukocyte adhesion triggering and migration. *Semin Immunol* **14**, 83-92 (2002).
5. Gerszten, R.E. *et al.* MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* **398**, 718-723 (1999).
6. Weber, K.S., von, H.P., Clark-Lewis, I., Weber, P.C., & Weber, C. Differential immobilization and hierarchical involvement of chemokines in monocyte arrest and transmigration on inflamed endothelium in shear flow. *Eur J Immunol* **29**, 700-712 (1999).
7. Huo, Y. *et al.* The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J Clin Invest* **108**, 1307-1314 (2001).
8. Shamri, R. *et al.* Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat Immunol* **6**, 497-506 (2005).
9. Gu, L. *et al.* Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* **2**, 275-281 (1998).
10. Boring, L., Gosling, J., Cleary, M., & Charo, I.F. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394**, 894-897 (1998).
11. Smith, J.D. *et al.* Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc Natl Acad Sci U S A* **92**, 8264-8268 (1995).
12. Boisvert, W.A., Santiago, R., Curtiss, L.K., & Terkeltaub, R.A. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest* **101**, 353-363 (1998).
13. Kuziel, W.A. *et al.* Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc Natl Acad Sci U S A* **94**, 12053-12058 (1997).
14. Nelken, N.A., Coughlin, S.R., Gordon, D., & Wilcox, J.N. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest* **88**, 1121-1127 (1991).
15. Yla-Herttuala, S. *et al.* Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc Natl Acad Sci U S A* **88**, 5252-5256 (1991).
16. O'Brien, K.D., McDonald, T.O., Chait, A., Allen, M.D., & Alpers, C.E. 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* **93**, 672-682 (1996).
17. Kartikasari, A.E.R., Georgiou, N.A. & Marx, J.J.M. Iron intake and cardiovascular disease in *Functional foods, cardiovascular disease and diabetes* (ed. Arnoldi A) 99-125 (Woodhead Publishing Ltd, Abington, Cambridge, UK and CRC Press LLC, Boca Raton, Florida, USA, 2004).
18. Sullivan, J.L. Stored iron and vascular reactivity. *Arterioscler Thromb Vasc Biol* **25**, 1532-1535 (2005).

19. Kartikasari, A.E. *et al.* Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. *Arterioscler Thromb Vasc Biol* **24**, 2257-2262 (2004).

20. Visseren, F.L. *et al.* 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* **32 Suppl 1**, 84-90 (2002).

21. Zhang, W.J. & 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* **34**, 674-682 (2003).

22. Koo, S.W., Casper, K.A., Otto, K.B., Gira, A.K., & Swerlick, R.A. Iron chelators inhibit VCAM-1 expression in human dermal microvascular endothelial cells. *J Invest Dermatol* **120**, 871-879 (2003).

23. Soares, M.P. *et al.* Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation. *J Immunol* **172**, 3553-3563 (2004).

24. Kartikasari, A.E.R. *et al.* Endothelial activation and induction of monocyte adhesion by non-transferrin-bound iron present in human sera. *FASEB J* (2005). DOI: 10.1096/fj.05-4700fje

25. Kuvibidila, S.R., Kitchens, D., & Baliga, B.S. *In vivo* and *in vitro* iron deficiency reduces protein kinase C activity and translocation in murine splenic and purified T cells. *J Cell Biochem* **74**, 468-478 (1999).

26. Leung, S. *et al.* Differential inhibition of inducible T cell cytokine secretion by potent iron chelators. *J Biomol Screen* **10**, 157-167 (2005).

27. Whitley, W.D., Hancock, W.W., Kupiec-Weglinski, J.W., DeSousa, M., & Tilney, N.L. Iron chelation suppresses mononuclear cell activation, modifies lymphocyte migration patterns, and prolongs rat cardiac allograft survival in rats. *Transplantation* **56**, 1182-1188 (1993).

28. Stadler, N., Lindner, R.A., & Davies, M.J. 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* **24**, 949-954 (2004).

29. Lee, T.S., Shiao, M.S., Pan, C.C., & Chau, L.Y. Iron-deficient diet reduces atherosclerotic lesions in apoE-deficient mice. *Circulation* **99**, 1222-1229 (1999).

30. Matthews, A.J. *et al.* Iron and atherosclerosis: inhibition by the iron chelator deferiprone (L1). *J Surg Res* **73**, 35-40 (1997).

31. Minqin, R. *et al.* The iron chelator desferrioxamine inhibits atherosclerotic lesion development and decreases lesion iron concentrations in the cholesterol-fed rabbit. *Free Radic Biol Med* **38**, 1206-1211 (2005).

32. Minqin, R., Watt, F., Huat, B.T., & Halliwell, B. Correlation of iron and zinc levels with lesion depth in newly formed atherosclerotic lesions. *Free Radic Biol Med* **34**, 746-752 (2003).

33. Marx, J.J. & Hider, R.C. Iron: an essential but potentially harmful nutrient. *Eur J Clin Invest* **32 Suppl 1**, 1-2 (2002).

34. Kaplan, J., Jordan, I., & Sturrock, A. Regulation of the transferrin-independent iron transport system in cultured cells. *J Biol Chem* **266**, 2997-3004 (1991).

35. Marx, J.J. & van Asbeck, B.S. Use of iron chelators in preventing hydroxyl radical damage: adult respiratory distress syndrome as an experimental model for the pathophysiology and treatment of oxygen-radical-mediated tissue damage. *Acta Haematol* **95**, 49-62 (1996).

36. Jaffe, E.A., Nachman, R.L., Becker, C.G., & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* **52**, 2745-2756 (1973).

37. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63 (1983).

38. Chou, T.C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**, 27-55 (1984).

39. Grootveld, M. *et al.* Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *J Biol Chem* **264**, 4417-4422 (1989).

40. Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W., & Cabantchik, Z.I. Fluorescence Analysis of the Labile Iron Pool of Mammalian Cells. *Anal.Biochem.***248**, 31-40 (1997).

41. Vanden Hoek, T.L., Li, C., Shao, Z., Schumacker, P.T., & Becker, L.B. Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J Mol Cell Cardiol* **29**, 2571-2583 (1997).

42. Sakariassen, K.S., Aarts, P.A., de Groot, P.G., Houdijk, W.P., & Sixma, J.J. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. *J Lab Clin Med* **102**, 522-535 (1983).

43. Kaufmann, A., Salentin, R., Gemsa, D., & Sprenger, H. Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1 alpha during differentiation of human monocytes to macrophages. *J Leukoc Biol* **69**, 248-252 (2001).

44. Chen, X.L., Zhang, Q., Zhao, R., & Medford, R.M. Superoxide, H2O2, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase. *Am J Physiol Heart Circ Physiol* **286**, H1001-H1007 (2004).

45. Satriano, J.A. *et al.* Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-alpha and immunoglobulin G. Evidence for involvement of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase. *J Clin Invest* **92**, 1564-1571 (1993).

46. Saccani, A. *et al.* Redox regulation of chemokine receptor expression. *Proc Natl Acad Sci U S A* **97**, 2761-2766 (2000).

CHAPTER 6

Endothelial cell activation by EDTA chelation therapy

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

Submitted

ABSTRACT

Ethylenediamine-tetraacetic acid (EDTA) chelation therapy is practiced as an alternative treatment for coronary artery disease, believing that removal of metal ions like copper and iron by EDTA is able to prevent atherogenic oxidative stress. A large-scale clinical trial has been launched recently evaluating benefits and hazards of EDTA chelation therapy. Little is known, however, on its biological effects on vascular endothelium. In this study, we examined the effects of metal chelation with EDTA on the expression of endothelial ICAM-1, VCAM-1 and E-selectin as well as the adhesion of monocytes to the endothelium, being a crucial event in atherogenesis. In a serum free condition, addition of EDTA enhanced, rather than reversed, iron-induced endothelial adhesion molecule expression. In the presence of copper, EDTA moderately induced ICAM-1 expression. In 50% human pooled serum, EDTA alone, without added iron or copper, enhanced the expression of all three proteins. EDTA appeared to promote accumulation of intracellular labile iron, inducing oxygen radical mediated endothelial cell activation. This activation could be reversed by deferoxamine or deferiprone, two iron chelators used for treatment of secondary iron overload. High doses vitamin C, routinely added to EDTA therapy, augmented rather than reversed EDTA effects, as well as inducing monocyte adherence to vascular endothelium. Endothelial activation by EDTA-vitamin-C treatment, following interaction with transition metal iron, therefore, may cause harmful effects when being used for the treatment of coronary artery disease. In particular subjects with iron overload could be at risk after EDTA chelation therapy.

Key words: EDTA • vitamin C • adhesion molecules • coronary artery disease

INTRODUCTION

Ethylenediamine-tetraacetic acid (EDTA) chelation is practiced worldwide as an alternative therapy for coronary artery disease.[1] It is administered as an intravenous infusion of 250-500ml solution containing 50mg of disodium-EDTA per kilogram of lean body weight, and is routinely supplemented with high doses vitamin C (4-20grams), minerals and iron. This infusion is commonly given once or twice a week in a series of thirty or more sessions. Follow-up treatments are believed to be beneficial for sustaining improvement and preventing recurrence of symptoms.[1]

Although some non-controlled case reports have suggested beneficial effects of EDTA chelation therapy, its efficacy on coronary artery disease has not been confirmed in clinical trials.[2] Due to the high number of patients receiving this treatment, the United States National Institutes of Health (NIH) have recently started the first large-scale five-year randomised trial on the safety and efficacy of EDTA chelation therapy for post-myocardial infarction patients.[3-5] This would determine its efficacy and/or adverse effects for the treatment of coronary artery disease.

To understand the true effects of EDTA chelation therapy biologically relevant mechanisms of interaction with the vascular system have yet to be examined.[6,7] It has been proposed that EDTA therapy reduces oxidative stress by removing transition metals,[1] like iron and copper, which accumulate in atherosclerotic lesions.[8] This might result in reduced arterial inflammation,[9] being effective against atherogenesis.[1] However, in a cell-free system, EDTA has been shown to potentiate the iron-driven Fenton reaction, resulting in formation of radical species, in a very complex fashion, depending on the metal/chelator ratio and competing molecules.[10] Vitamin C can provide antioxidant effects, supporting the therapy's efficacy,[1] but a high concentration of vitamin C can exert pro-oxidant properties, especially when it interacts with transition metal ions.[11-13] Oxidative stress has been shown to promote atherogenesis by catalysing LDL-oxidation, nitrogen-monoxide preservation, cellular oxidative injury, platelet activation, smooth muscle cell proliferation, inflammatory gene activation and transendothelial migration of leukocytes.[14]

Leukocyte migration through vascular endothelium is the initial process in atherogenesis[15] and is facilitated by endothelial adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial selectin (E-Selectin). In human atherosclerotic lesions, expression of ICAM-1, VCAM-1 and E-selectin is elevated.[16] The expression of these adhesion molecules are inducible by oxidative stress[14] and iron.[17,18] In the present study, we assessed the effects of EDTA chelation and vitamin C on the expression of adhesion molecules by human endothelial cells and on the adherence of human monocytes to endothelium.

EXPERIMENTAL PROCEDURES

Expression of endothelial adhesion molecules

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords as described by Jaffe et al.[19] Cells were cultured in tissue culture flasks precoated with fibronectin, using EBM-2 medium (Clonetics®). Culturing was carried on in a humidified 37°C incubator with 5% CO_2 and cells from passages 1-2 were used in all experiments.

Cellular viability of HUVECs was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay.[20] Adhesion molecule expression was measured using fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson FACScan). 20.000 events were analysed for each FACS measurement.

***In vitro* cytoadherence assay**

Peripheral blood mononuclear cells (PBMC) were isolated from donor blood (Sanquin blood bank, Utrecht, the Netherlands) by Ficollpaque density gradient centrifugation. Monocytes were isolated using the negative immuno-selection monocyte isolation kit (Miltenyi Biotec) according to supplier's instructions. This method resulted in purity of >90% as analysed by flow cytometry.

HUVECs were pretreated with compounds of interest prior to the assay. Cytoadherence of 2'7'-bis-(2-carboxyethyl)-5.6-carboxyfluorescein (BCECF-AM)-labelled monocytes ($25x10^4$ cells/well) to HUVEC monolayer ($5x10^4$ cells/well) was performed in 96-well plates for 30 minutes at 37^0C with gentle agitation. Fluorescence was read using a filter pair for excitation at 485nm and emission at 530 nm in a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems). This value represented total fluorescing monocytes added to each well. After thorough washing, fluorescence was again measured and the value represented the remaining monocytes firmly attached to the HUVEC monolayer. The percentage of adhesion was defined as the value of remaining fluorescence divided by the value of total fluorescence multiplied by 100.

Calcein-AM assay

Calcein-AM (Molecular Probes), a permeant fluorescent probe for cytosolic iron was used to monitor intracellular labile iron level.[21] Calcein-AM has a lipophilic acetoxymethylester (AM) moiety that makes it permeable through cell membranes. Once inside the cell, the AM group will be cleaved by non-specific esterases, resulting in a charged form that hardly leaks out of cells. As a weak iron chelator, once inside the cells, the fluorescence signal of calcein can be quenched by iron. This property is used in this study to detect intracellular labile iron.

After treatment with compounds of interest, confluent HUVECs were incubated with 0.125 μmol/L calcein-AM (30 minutes at 37^oC). Following the washing step, the fluorescence signal of calcein (excitation = 485 nm; emission = 530 nm) was monitored for a specified time period with or without the presence of compounds of interest, quenching of calcein signal relative to the control indicating the presence of labile iron intracellularly.

Statistical analysis

Data are expressed as means $\pm$ standard error of the mean. 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

EDTA promoted adhesion molecule expression

In this study, we investigated the effects of metal chelation with EDTA on endothelial adhesion molecule expression. Confluent HUVECs, incubated with 10 μmol/L Fe(III)citrate or 10 μmol/L Cu(II)chloride for 48 hours, were treated with 10 μmol/L EDTA during the last 24 hours of incubation. Iron chelation with EDTA resulted in enhancement, rather than the expected reversal, of iron-induced VCAM-1 (Figure 1A), ICAM-1 and E-selectin expression (not shown). EDTA also moderately induced ICAM-1 expression with copper incubation (not shown). Furthermore, co-incubation with 10 μmol/L deferoxamine or 30 μmol/L deferiprone lowered iron-EDTA-induced adhesion molecule expression

(Figure 1A), confirming that interaction between EDTA and the metal ions was involved in endothelial cell activation.

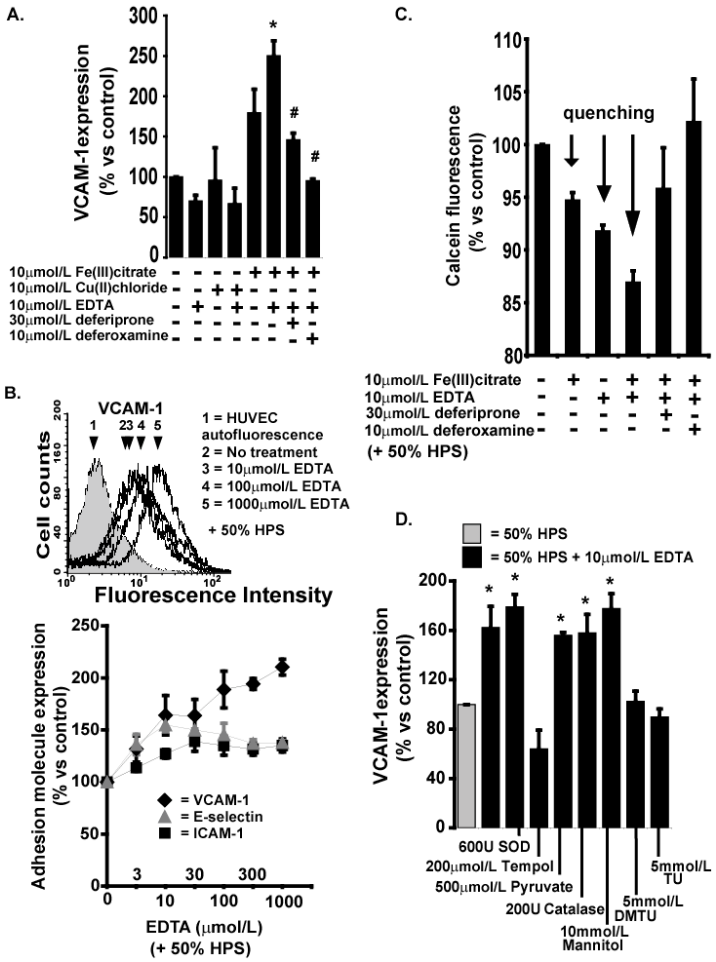

Figure 1. EDTA induced endothelial adhesion molecule expression. Confluent HUVECs were incubated for 48 hours with 50% HPS, Fe(III)citrate or Cu(II)chloride, and treated with EDTA or radical scavengers during the last 24 hours of incubation, as indicated. (A) In serum free condition, EDTA-iron-containing medium induced VCAM-1 expression (mean±SEM; n=6, **P*<0.05 vs controls), deferiprone and deferoxamine counteracted the effects (#*P*<0.05 vs *). (B) Adhesion molecule expression was altered with increasing EDTA concentration (0-1000 μmol/L) (mean, n=6), the top panel illustrates FACS analysis of VCAM-1 expression on HUVECs. (C) EDTA increased the level of intracellular labile iron (mean, n=3), shown by the quenching of calcein signal. (D) Effects of radical scavengers on VCAM-1 expression (mean±SEM; n=6, **P*<0.05 vs control).

In the circulation EDTA may interact with other metal ions, and compete for iron with other ligands present in plasma. To investigate the consequence of any interaction, HUVECs were incubated with EBM-2 medium containing 50% human pooled serum (HPS) obtained from 30 healthy individuals (University Medical Centre Utrecht, The Netherlands) for 48 hours, and increasing concentrations of EDTA (0-1000 μmol/L, pH=7.4) at the last 24 hours of incubation. The range of EDTA concentrations covered calculated concentrations in the circulation after an intravenous infusion. HUVEC viability was >95% in all conditions monitored by MTT assay (not shown). Increasing concentrations of EDTA resulted in enhanced endothelial adhesion molecule expression, with VCAM-1 being most pronounced (Figure 1B). The modest concentration of 3 μmol/L EDTA already caused a 40% increased VCAM-1 expression.

The effect of EDTA chelation at the level of intracellular labile iron was investigated by monitoring intracellular calcein fluorescence. Quenching of intracellular calcein signal was observed when iron was added to the incubation medium containing 50% HPS (Figure 1C). This quenching was also observed when EDTA alone was added to the incubation medium, indicating that EDTA also modulated the level of intracellular labile iron. Further quenching of calcein signal in the presence of iron and EDTA, as well as dequenching by deferoxamine or deferiprone, suggests that EDTA may promote low-molecular-weight iron uptake by endothelial cells.

Several radical scavengers were used to investigate the role of oxidative stress in the process of EDTA-induced adhesion molecule expression. Only the membrane-permeable radical scavengers, tempol, thiourea, and DMTU, but not the membrane-impermeable scavengers, superoxide dismutase, catalase and mannitol, nor the hydrogen peroxide scavenger, pyruvate, were able to counteract EDTA-induced VCAM-1 expression (Figure 1D), suggesting that intracellular radicals mediate EDTA-induced endothelial activation.

Vitamin C enhanced EDTA-induced endothelial activation

EDTA therapy routinely includes a supplement of high doses vitamin C to function as an antioxidant. Here, we tested the effects of vitamin C, in combination with EDTA, on adhesion molecule expression. Increasing concentrations of sodium-ascorbate (0-10 mmol/L, pH=7.4) were included for 24 hours of incubation with or without addition of EDTA. Vitamin C concentrations were based on calculated concentrations after an intravenous infusion. High doses ascorbate (>100 μmol/L) upregulated VCAM-1 and E-selectin moderately, and these were enhanced by coincubation with EDTA (Figure 2A). Low doses (<100 μmol/L) of sodium-ascorbate neither affected nor reversed EDTA-induced adhesion molecule expression (not shown). Furthermore, monocyte adherence to HUVECs treated with EDTA in combination with ascorbate was significantly enhanced (Figure 2B).

Since ascorbate has a reducing capacity and the addition of high doses ascorbate may have caused iron release from protein complexes, the effects of ascorbate on intracellular iron level (Figure 2C) were investigated. 10 mmol/L sodium-ascorbate following preincubation with 50% HPS caused a tremendous drop in calcein signal, suggesting a raise in the intracellular labile iron level. Addition of deferoxamine or deferiprone dequenched the calcein signal, confirming increased availability of free intracellular iron by ascorbate.

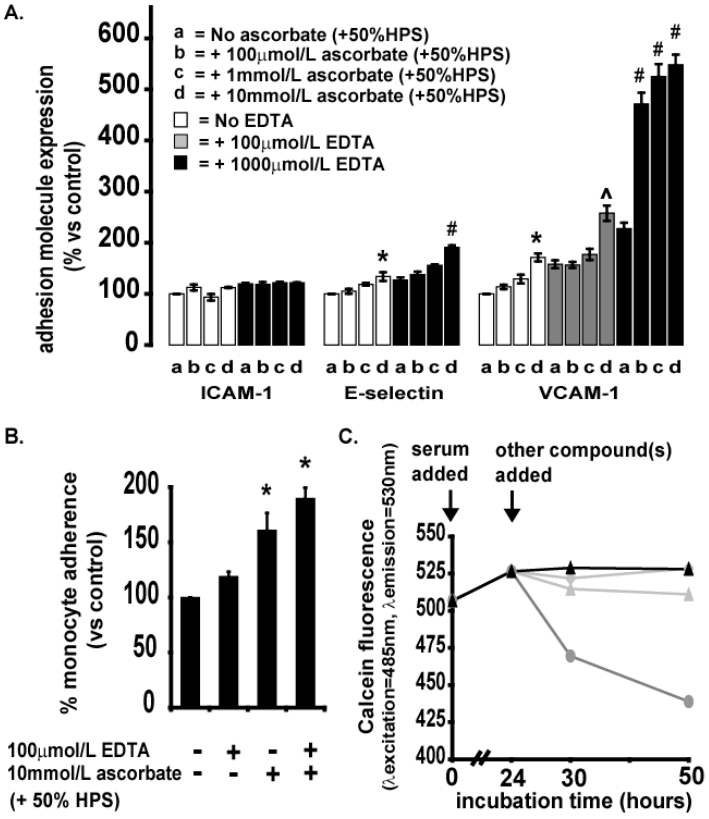

Figure 2. Vitamin C aggravated iron-EDTA-induced adhesion molecule expression. Confluent HUVECs were incubated for 48 hours with 50% HPS and treated with EDTA and ascorbate during the last 24 hours of incubation, as indicated. (A) 10 mmol/L ascorbate enhanced both EDTA-induced VCAM-1 and E-selectin expression (mean±SEM; n=6, **P*<0.05 vs the respective a □, ^P<0.05 vs the respective a ▩, #P<0.05 vs the respective a ■). (B) Monocyte adherence to HUVECs after indicated treatments (mean±SEM; n=3, **P*<0.05 vs controls) (C) The calcein fluorescence was followed as indicated. HUVECs were incubated in 50% HPS (black triangles), with 10mmol/L ascorbate (grey circles), and 100 μmol/L deferoxamine (grey diamonds) or 300 μmol/L deferiprone (grey triangles). Ascorbate raised intracellular labile iron level, shown by the quenching of calcein signal (mean, n=5), while chelators could reverse this effect.

DISCUSSION

Several beneficial effects of EDTA therapy for the treatment of atherosclerosis have been suggested,[1] with antioxidant effects being most plausible.[7] However, these potential benefits have not been scientifically explored.[6,7] Moreover, definitive evidence for the efficacy of EDTA therapy has been lacking in randomised trials.[2] The present study demonstrates that metal chelation, particularly iron, with EDTA promotes the expression of atherogenic adhesion molecules. Paradoxically, the process involves free radical production and leads to endothelial activation, as opposed to the proposed antioxidant benefits of chelation therapy. This study, using biologically relevant media, shows for the first time that interaction of EDTA with iron may, in contrast to the suggested chelation benefits, initiate the process of vasculature damage.

EDTA-induced adhesion molecule expression was mediated by accumulation of intracellular labile iron. This could be due to mobilisation of iron by EDTA from transferrin[22] or other ligands,[23] making it more available as low molecular weight iron, and thus capable of participating in oxidation-reduction reactions.

Low doses of ascorbate neither affected nor reversed EDTA-induced endothelial activation, suggesting the absence of antioxidant properties of ascorbate in the endothelial activation process. High doses vitamin C even aggravated EDTA-induced endothelial activation, in contrast to the expected therapy's benefits.[1] Possible explanations could be that vitamin C as a strong reducing agent mobilises iron from its ligands and facilitates iron uptake by cells.[24] Ascorbate can also be taken up by HUVECs[25] and may enlarge the intracellular iron pool by mobilising iron from ferritin.[26] Furthermore, the prooxidant nature of ascorbic acid has been described when it interacts with transition metals.[11-13]

Collectively, our results implicate altered expression of endothelial adhesion molecules by EDTA therapy, which are partly due to interaction with metal iron and free-radical generation. This finding therefore suggests possible deleterious effects, and does not support the proposed benefits of EDTA therapy in combination with high doses vitamin C, when being used for the treatment of coronary artery disease. Subjects with increased plasma iron levels, like dialysis patients and those with hereditary or secondary hemochromatosis, could even be at risk after EDTA chelation therapy.

Acknowledgements: This study was supported by the European Commission, project QLK1-CT-2002-00444. We thank Mr. J.J.M. Bouwman from the Diakonessen Hospital, Utrecht, The Netherlands, for providing the umbilical cords.

References

1. Rozema, T.C. The protocol for the safe and effective administration of EDTA and other chelating agents for vascular disease, degenerative disease, and metal toxicity. *J Adv Med* **10**, 5-100 (1997).
2. Ernst, E. Chelation therapy for coronary heart disease: An overview of all clinical investigations. *Am Heart J* **140**, 139-141 (2000).
3. Holden, C. Alternative medicine. NIH trial to test chelation therapy. *Science* **297**, 1109 (2002).
4. Bonetta, L. Trial tests chelation's effect on heart disease. *Nat Med* **8**, 906-907 (2002).
5. http://nccam.nih.gov/chelation/chelationstudy.htm. National Intitute of Health (2004).
6. Villarruz, M.V., Dans, A., & Tan, F. Chelation therapy for atherosclerotic cardiovascular disease. *Cochrane Database Syst Rev* CD002785 (2002).
7. Lamas, G.A. & Ackermann, A. Clinical evaluation of chelation therapy: is there any wheat amidst the chaff? *Am Heart J* **140**, 4-5 (2000).
8. Stadler, N., Lindner, R.A., & Davies, M.J. 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* **24**, 949-954 (2004).
9. Hornig, B. & Drexler, H. Reversal of endothelial dysfunction in humans. *Coron Artery Dis* **12**, 463-473 (2001).
10. Engelmann, M.D., Bobier, R.T., Hiatt, T., & Cheng, I.F. Variability of the Fenton reaction characteristics of the EDTA, DTPA, and citrate complexes of iron. *Biometals* **16**, 519-527 (2003).
11. Sardi, B. High-dose vitamin C and iron overload. *Ann Intern Med* **140**, 846-847 (2004).
12. Podmore, I.D. *et al.* Vitamin C exhibits pro-oxidant properties. *Nature* **392**, 559 (1998).
13. Buettner, G.R. & Jurkiewicz, B.A. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat Res* **145**, 532-541 (1996).
14. Diaz, M.N., Frei, B., Vita, J.A., & Keaney, J.F., Jr. Antioxidants and atherosclerotic heart disease. *N Engl J Med* **337**, 408-416 (1997).
15. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).
16. O'Brien, K.D., McDonald, T.O., Chait, A., Allen, M.D., & Alpers, C.E. 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* **93**, 672-682 (1996).
17. Kartikasari, A.E.R. *et al.* Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. *Arterioscler Thromb Vasc Biol* **24**, 2257-2262 (2004).
18. Kartikasari AER *et al.* Endothelial activation and induction of monocyte adhesion by non-transferrin-bound iron present in human sera. *FASEB J* DOI: 10.1096/fj.05-4700fje (2005).
19. Jaffe, E.A., Nachman, R.L., Becker, C.G., & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* **52**, 2745-2756 (1973).
20. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63 (1983).
21. Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W., & Cabantchik, Z.I. Fluorescence Analysis of the Labile Iron Pool of Mammalian Cells. *Anal Biochem* **248**, 31-40 (1997).
22. Baldwin, D.A. The kinetics of iron release from human transferrin by EDTA. Effect of salts and detergents. *Biochim Biophys Acta* **623**, 183-198 (1980).
23. MacPhail, A.P., Patel, R.C., Bothwell, T.H., & Lamparelli, R.D. EDTA and the absorption of iron from food. *Am J Clin Nutr* **59**, 644-648 (1994).
24. May, J.M., Qu, Z.C., & Mendiratta, S. Role of ascorbic acid in transferrin-independent reduction and uptake of iron by U-937 cells. *Biochem Pharmacol* **57**, 1275-1282 (1999).
25. Ek, A., Strom, K., & Cotgreave, I.A. The uptake of ascorbic acid into human umbilical vein endothelial cells and its effect on oxidant insult. *Biochem Pharmacol* **50**, 1339-1346 (1995).
26. Bienfait, H.F. & van den Briel, M.L. Rapid mobilisation of ferritin iron by ascorbate in the presence of oxygen. *Biochim Biophys Acta* **631**, 507-510 (1980).

CHAPTER 7

Neutrophil adherence and transmigration through endothelial cells are enhanced by physiological and therapeutic iron complexes

Niki A. Georgiou, Apriliana E. R. Kartikasari, J. Henny van Kats-Renaud,
B. Sweder van Asbeck, Joannes J. M. Marx

Submitted

ABSTRACT

The role of iron in polymorphonuclear cell (PMN, neutrophil) – endothelial cell interactions has scarcely been studied. In innate immunity this is one of the key interactions in fighting and clearing out an invading pathogen. As non-transferrin bound iron (NTBI) has been detected in various patient groups, including end-stage renal disease patients, and even in healthy humans, we questioned whether NTBI could be contributing to neutrophil recruitment to the endothelium. 10 μmol/L Fe(III)citrate incubation on endothelial cells for 18 hours, increased the adherence of both treated and non-treated PMN. The increased neutrophil recruitment did not proceed via the classic CD11b/CD18 and ICAM-1 interactions, instead we noted enhancement of the pro-adhesive P-selectin on HUVECs after iron incubation. Furthermore, increased amount of lactoferrin on the phagocyte membrane was noted on iron-treated PMN. Iron dextran incubation of both PMN and endothelial cells significantly enhanced transendothelial migration at concentrations of as low as 25 μg/mL, without significant enhancement of PECAM-1 expression. However, treatment of endothelial cells alone with iron dextran at concentrations of up to 100 μg/mL did not enhance PMN transmigration. In addition, iron sacharate did not enhance neutrophil transmigration, in the same way as iron dextran, although from both iron preparations, NTBI concentrations of up to 6 μmol/L were measured. In conclusion, this study reports enhanced adhesion and transmigration of PMN trough an endothelial monolayer by iron. Pharmacological forms of iron given in end-stage renal disease patients on dialysis could modulate the degree of PMN recruitment in inflammation. This finding implicates that iron may play a role as a modulator of innate immune activation.

Key words: NTBI• neutrophils • endothelium• innate immunity • atherosclerosis

INTRODUCTION

Neutrophil and vascular endothelium interactions constitute an important initiation event in the response to an inflammatory stimulus, being the first step in the acute innate immunity. A bacterial infection will result in rapid recruitment of polymorphonuclear granulocytes (PMN, neutrophils) from the circulation to sites of microbial invasion. This recruitment is achieved by various host stimuli (C3a, C5a, IL-8) and pathogen-derived stimuli (LPS, fMLP).[1] Neutrophils express selectins (PSGL-1 or CD162) and integrins (CD11b/CD18) that bind to countereceptors on endothelial cells, respectively P-selectin and ICAM-1. After transmigration, neutrophils move to a site of infection under a chemotactic gradient (chemotaxis). Moreover, the activated PMN produce superoxide through the NADPH-oxidase system. Products of this superoxide in the presence of either chloride ion or iron are hypochlorous acid and the hydroxyl radical, respectively, which are used to efficiently kill ingested bacteria.[2] Neutrophils are also

equipped with microbicidal enzymes, like elastase, myeloperoxidase and peptides to efficiently carry out microbial killing.

In acute innate immunity, neutrophil-endothelial interactions are of crucial importance. There are however clinical situations whereby aggregation of neutrophils and adherence to endothelium can lead to endothelial injury.[3,4] Endothelial injury by reactive oxygen species or neutrophil elastase have been linked to acute lung injury and may play a role in the pathophysiology of the adult respiratory distress syndrome (ARDS).[5-7]

In end stage renal disease (ESRD) patients, intravenous iron supplements are administered to complement recombinant human erythropoetin administraton in order to treat anemia in these patients, which is mainly the result of insufficient renal production of erythropoetin. Intravenous iron supplementation can result in the formation of labile non-transferrin bound iron (NTBI) capable of facilitating redox reactions.[8] It was estimated that the biologically available or labile iron fraction can be 2.5-5.8% of the total iron in various iron preparations, including iron sacharate and dextran.[9] The nature of NTBI is believed to be a heterogeneous fraction of iron not bound to transferrin or the storage protein ferritin, but bound to albumin, citrate, and other unidentified negatively charged ligands.[10,11] NTBI can be detected in plasma of patients with hereditary hemochromatosis, in synovial fluid of patients with rheumatoid arthritis and in transfusion-dependent thalassemic patients. The occurence of NTBI in plama of dialysis patients has also been reported.[5,8,12-16]

The biological effects of this potentially toxic form of iron in PMN-endothelial cell interactions has been, thus far, poorly studied. Given the large group of patients who could harbour this form of iron, we have studied the effects of physiological forms of NTBI as well as the commercially used iron complexes Venofer® (iron sacharate) and Cosmofer® (iron dextran) on human neutrophil adherence and transmigration through human umbilical vein endothelial cells (HUVEC). We have also investigated the interactions that facilitate adherence and transmigration of neutrophils under these conditions.

EXPERIMENTAL PROCEDURES

Preparation of iron solutions and other materials

Fe(III)citrate with metal-to-chelate ratios of 1:20 was prepared at pH 1.0 using Fe(III)chloride (Fluka, Buchs SG, Switzerland) and sodium citrate (J.T. Baker, Deventer, the Netherlands). After 10 min at low pH, the pH was increased slowly to pH 7.4 under continuous stirring with NaOH. Fe(II) was prepared freshly under hypoxic conditions by dissolving Fe(II)ammoniumsulphate (Merck, Darmstadt, Germany) in milliQ which has been bubbled with nitrogen gas for at least 15 minutes. Venofer® (ferrioxidesacharate, 20mg/mL) was from Vifor, St, Gallen, Switzerland, and Cosmofer® (iron dextran, 50mg/mL) was from Nebo a/s, Holsbaek, Denmark. Ten-fold concentrations were prepared for each dilution in Hank's balanced buffered solution (HBBS, Cambrex Biosciences, Verviers, Belgium) supplemented with 0.4% human serum albumin (HSA, CLB, The Netherlands) and stocks were kept at 4°C. All solutions had a pH of between 6.9 - 7.2. Concentrations used in these experiments include the

maximum plasma concentration of each agent after intravenous push injection (C_{max}) of 100 mg of iron sucrose (30 μg/mL) or 100 mg iron dextran (30-34 μg/mL). Other materials used were PMA (Sigma), fMLP (Sigma) and recombinant TNF-α (Sigma).

PMN isolation

Blood obtained from healthy human volunteers was collected into tubes containing sodium heparin (Vacuette, Greiner, Alphen a/d Rijn, The Netherlands) as anticoagulant. Neutrophils were isolated as previously described.[17] For the isolation, heparinized blood was diluted 1:1 (vol/vol) with pyrogen-free PBS and layered onto a gradient of Ficoll (Pharmacia) and Histopaque (density, 1.119 g/mL, Sigma). After centrifugation for 20 min at 320 g, the neutrophils were collected from the Histopaque phase. Cells were subjected to a brief hypotonic shock with water, washed, and suspended at 5 _ 10^6 cells/mL in RPMI medium containing 0.05% HSA.

HUVEC isolation and culture

HUVEC were isolated and cultured as described by Jaffe et al.[18] Culturing was in a humidified 37°C incubator with 5% CO_2, in endothelial growth medium-2 (EGM-2, Clonetics®). Cells from passages 2-3 were used for all experiments.

PMN adhesion to HUVEC

Freshly isolated PMN were labelled with 3 μmol/L 2'7'-bis-(2-carboxyethyl)-5.6-carboxyfluorescein (BCECF)-AM (Molecular Probes/Invitrogen, Carlsbad, California, USA) at room temperature for 20 minutes and after washing were incubated with 10 μmol/L iron solution for 1 hour at 37°C. Control PMN were left untreated at 37°C. 2.5 x 10^5 PMN were added on each confluent monolayer of HUVEC in 96-well plate well (ratio PMN:HUVEC 10:1) and allowed to adhere under gentle agitation for 20 minutes. Total fluorescence was measured in a CytoFluor II fluorescence microplate reader (Perseptive Biosystems) using 485 nm excitation and 530 nm emmission. After carefully washing the unattached PMN four times with warm HBBS containing 0.4% HSA, fluorescence from attached PMN was measured. The percentage adhesion is resting fluorescence after washing x 100 divided by total fluorescence before washing.

Flow cytometry

After treatment with various compounds of interest the medium containing the compounds was removed and cells washed. HUVEC were harvested by incubating with 0.2% trypsin-EDTA at 37°C for 1 minute. Analysis of proteins expressed on the surface of either PMN or HUVEC was performed according to standard techniques. Briefly, the cells were incubated with fluorescence-labeled monoclonal antibodies against the surface proteins: FITC-conjugated ICAM-1 antibody (R&Dsystem, Minneapolis, USA), PE-labelled CD162 (PSGL-1) antibody (Becton Dickinson, NJ, USA), FITC-conjugated P-selectin (CD62P) antibody (R&D Systems), rabbit anti-human Lactoferrin (Sigma) with FITC-labelled secondary goat anti-rabbit IgG (Sigma), FITC-labelled CD18 antibody (Becton Dickinson), and 44a monoclonal antibody (anti-CD11b, from secreting hybridoma cells obtained from ATTC) with

FITC-labelled secondary goat anti-mouse IgG (DAKOa/s, Denmark). All samples were analyzed on a FACSscan (Becton Dickinson) using FACScalibur software. The photomultipliers for side scatter, FL1 (530) and FL2 (580 nm) were operated using logarithmic amplification. Instrument calibration was performed daily using Calibrite beads (BDIS) according to manufacturer's recommendations. PMN and HUVEC were identified by forward and side scatter analysis. 10,000 events were counted.

Fluorescence-based one-step NTBI measurement

The assay was carried out as described by Breuer and Cabantchik.[19]

Neutrophil transendothelial migration

6,5 mm transwell plates (Corning Incorporated, NY, USA) with 3 μm pore polyester membrane inserts were coated for at least one hour with fibronectin, 600 μL EGM-2 medium was added in the well and in the insert 100 μL EGM-2 medium containing between 8 x 10^4 and 1 x10^5 HUVEC. After overnight incubation at 37 ºC confluency was reached. A mixed HUVEC preparation from two or three donors from passages 1-3 was used to perform transmigration experiments. PMN were treated with each compound concentration for 1 hour at 37 ºC in HBBS containing 0.08% HSA, while HUVEC for 1 hour in EGM-2 medium. Transmigration was allowed to proceed without washing under a 10 nmol/L fMLP chemotactic gradient for 2 hours. Fluorescence of transmigrated BCECF-AM labelled PMN was measured in a CytoFluor II fluorescence microplate reader (Perseptive Biosystems) using 485nm excitation and 530nm emmission. We define 100% transmigration as that achieved when both PMN and HUVEC were untreated.

RESULTS

PMN adherence to endothelium

A dose dependent increase in PMN adherence to endothelium was noted either after 4 hours (Figure 1A) or 18 hours (Figure 2B) HUVEC incubations with Fe(III)citrate (1:20). Incubating PMN for one hour resulted in an increase in adherence, however this increase was not significant. Only after overnight HUVEC incubation with iron was the increase in adherence was significant already at 3μmol/L Fe(III)citrate, the concentration that is easily reached in hemochromatosis patients as well as patients undergoing dialysis with human recombinant erythropoetin (rh-EPO) and iron supplementation.[8, 16]

Expression of CD18/CD11b and PSGL-1 on PMN and counter-receptors ICAM-1 and P-selectin on HUVEC under iron-rich conditions

The main players in leukocyte rolling and attachment to endothelium are CD18/CD11b and P-selectin glycoprotein ligand-1 (PSGL-1 or CD162). Their counter-receptors on endothelium are ICAM-1 and P-selectin (CD62), respectively. These molecules are essential for innate immunity and inflammation. The interaction of PSGL-1 with P-selectin mediates tethering, rolling and weak adhesion

of leukocytes[20,21] during which they become sufficiently activated *in situ* by cytokines and chemoattractants for integrin-mediated firm adhesion.[22,23]

We looked for the expression of the aforementioned molecules in the presence of iron after noting increased adherence of PMN on endothelium. Freshly prepared Fe(II)ammonium sulphate as well as Fe(III)citrate in the ratio 1:20 were used in the concentrations of 1, 3, 10 and 30 μmol/L. PMN were treated for 1 hour and HUVEC for either 1 hour or 18 hours. On PMN, CD18 and CD11b expression was not upregulated by iron (Figure 2A and B). PSGL-1 was also not affected (not shown). ICAM-1 expression after 1 hour or 18 hours compound incubations was not affected by either Fe(II)ammoniumsulfate or Fe(III)citrate (not shown). We conclude that enhancement of PMN adherence by iron proceeds via an ICAM-1 independent pathway.

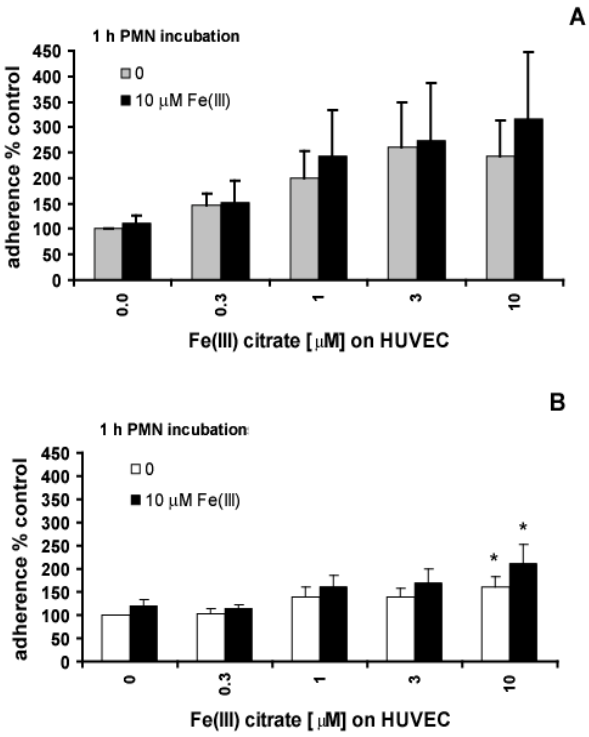

Figure 1. Neutrophil adherence to endothelium 4 hours (A) and 18 hours (B) after Fe (III) citrate incubation of HUVEC. White bars indicate adherence of untreated PMN on HUVEC and black bars adherence of PMN treated for 1 hour with 10 μmol/L Fe(III)citrate. The range of 0-10 μmol/L Fe(III)citrate was used to treat HUVEC. Results are the mean ± SEM of 4 experiments (A) and 7 experiments (B) performed four-fold, statistical analysis using ANOVA and Student Newman Keuls test, $p<0,05$. 100% adherence is set at the value obtained with untreated cell types.

Expression of membrane lactoferrin on PMN under iron-rich conditions

The iron-binding glycoprotein lactoferrin is able to cause PMN aggregation and facilitate adherence.[24] Lactoferrin is stored in granules and is secreted to the surface upon stimulation. We looked for the expression of membrane lactoferrin under iron-rich conditions and found a dose dependent increase of membrane lactoferrin expression by both Fe(II)ammoniumsulfate and Fe(III)citrate (Figure 3). The increased lactoferrin expression was counteracted by the iron chelator deferoxamine pointing to the involvement of iron in this process.

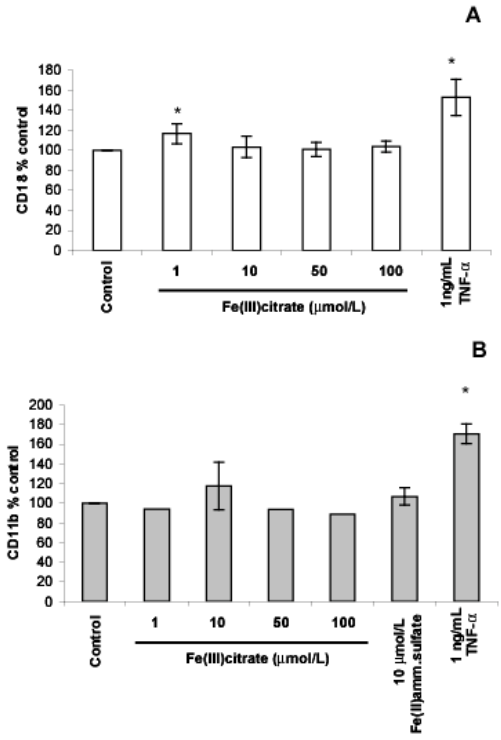

Figure 2. CD18 (A) and and CD11b (B) expression on neutrophils treated with either Fe(II)ammoniumsulfate or Fe(III)citrate for one hour. Results are the mean ± SEM of 3-6 experiments (A) and 1-3 experimens (B) performed in duplicate, statistical analysis using Student's t-test, $p<0,05$.

PMN transmigration after iron sacharate and iron dextran incubations

Experiments performed with 10 μmol/L Fe(III)citrate incubated with either PMN or HUVEC for one hour resulted in a 2.5-fold increase in transmigration when both cell types were incubated with iron (not shown). Incubation of either cell type failed to result in any change in transmigration (not shown). Given the above observation and after having established the increased adherence of PMN to HUVEC by Fe(II) and Fe(III), we wanted to look at the process of transmigration in the presence of two iron

preparations used to supplement treatment with rh-EPO in hemodialysis patients. Concentrations of iron sacharate and iron dextran of 1, 25, 50 and 100 μg/mL were used to study PMN tansmigration through HUVEC monolayers. The equivalent range of iron concentrations that these dilutions cover were calculated to be between 0,02 – 2 μmol/L for iron sacharate and between 0,01 – 1 μmol/L for iron dextran.

Table 1. PMN transmigration.

Cell types preincubated with iron complexes	Iron dextran concentration (μg/mL)	Transmigration after iron dextran incubations (% control)	Significance (P) Iron dextran vs control
HUVEC+/PMN+	1	117 ± 8	0,06
	25	121 ± 5	0,002
	50	147 ± 15	0,011
HUVEC-/PMN+	1	142 ± 12	0,006
	25	145 ± 9	0,000
	50	128 ± 10	0,019
HUVEC+/PMN-	1	110 ± 9	0,293
	25	102 ± 9	0,829
	50	89 ± 6	0,097
	100	91 ± 7	0,228

Cell types preincubated with iron complexes	Iron sacharate concentration (μg/mL)	Transmigration after Iron sacharate incubations (% control)	Significance (P) Iron sacharate vs control
HUVEC+/PMN+	1	132 ± 24	0,219
	25	105 ± 16	0,763
	50	105 ± 11	0,662
HUVEC-/PMN+	1	149 ± 30	0,14
	25	106 ± 20	0,772
	50	101 ± 12	0,936
HUVEC+/PMN-	1	104 ± 14	0,782
	25	96 ± 14	0,782
	50	107 ± 7	0,347
	100	107 ± 5	0,199

Control transmigration is untreated HUVEC and PMN and is set at 100%. % transmigration at each compound incubation is calculated compared to the 100% control. HUVEC+/PMN+ represents both cells types treated with iron preparations, HUVEC+/PMN- represents only HUVEC treated with iron preparations etc. Statistical analysis using Student's t-test, $p<0,05$, n=5 for iron sacharate and n=6 for iron dextran.

Non-treated PMN did not result in significant changes when HUVEC were either treated for one hour or not treated with either iron sacharate or iron dextran (Table 1). PMN treatment with iron dextran resulted in increased transmigration at concentrations of > 1 μg/mL when HUVEC were either pre-treated or not treated with the same compound concentration (Table 1). Non-treated PMN did not act

significantly different on iron dextran treated HUVEC with transmigration staying within 110 and 89% of control. A 45% increase in transmigration was noted with 25 μg/mL iron dextran treated PMN on non-treated HUVEC. Moreoever, treatment of HUVEC did not enhance this effect (Table 1).

Iron sacharate did not share the effect of transmigration enhancement observed with iron dextran. The values at 1 μg/mL concentrations for the HUVEC-/PMN+ combination were higher than the HUVEC+/PMN+ combination but the differences were not significant (Table 1).

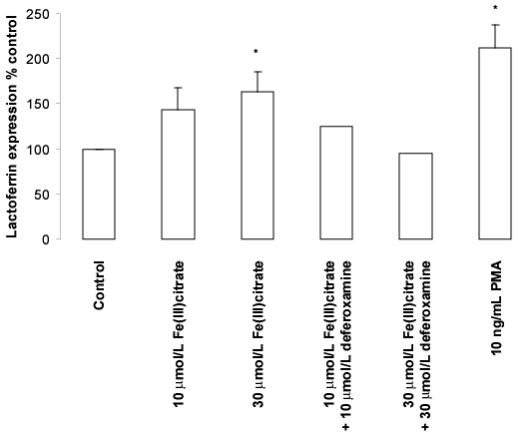

Figure 3. Membrane lactoferrin expression on human PMN after 1 hour compound incubations. Control represents cells incubated with no iron. In the Fe(III)citrate/deferoxamine combinations, these two compounds were added together at least 30 minutes before incubation with cells. Statistical analysis using Student's t-test, $p<0,05$, n=2-7.

Adhesion molecule expression on HUVEC after 1 h iron sacharate/iron dextran incubations

We have examined the expression of various key adhesion molecules on HUVEC after incubating HUVEC for 1 h with the concentations iron sacharate and iron dextran, which had been used for the transmigration experiments. ICAM-1 expression remained unchanged in all compound incubations examined (not shown). Expression of PECAM-1, which is one of the receptors involved in transmigration, also remained unaffected by both iron sacharate and iron dextran (not shown). The proadhesive receptor P-selectin however, was up regulated by 1 and 25 μg/mL iron dextran (Figure 4).

DISCUSSION

The immediate effect of labile iron on PMN recruitment to endothelium has been scarcely studied. In this study we have looked into the effect of mononuclear forms of iron including Fe(III)citrate and Fe(II)ammoniumsulphate, as well as the iron preparations routinely used in combination with rh-

EPO in hemodialysis patients, iron sacharate and iron dextran on PMN recruitment and transmigration through HUVEC. We have found that levels of iron as low as 3 μmol/L result in enhanced adherence of PMN on HUVEC (Figure 1B). These concentrations of NTBI have even been reported in healthy subjects,[25] implying that even at physiological concentrations of iron, enhanced recruitment of phagocytes to the endothelium during bacterial invasion is possible. This could make iron a modulator of innate immunity.

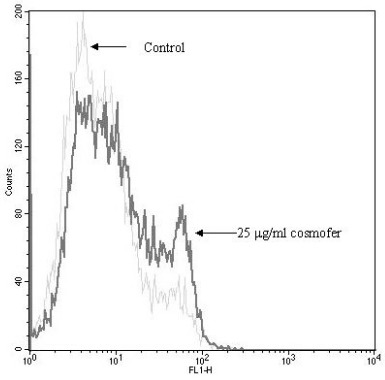

Figure 4. Representative experiment showing displacement of P-selectin FL1-fluorescence in the presence of 1 μg/mL iron dextran. A shift to the right indicated increased expression of P-selectin.

CD11a/CD18 and CD11b/CD18 are expressed on resting PMN isolated from blood.[26] In this study, no significant changes have been observed in the expression of CD18/CD11b on PMN and the counter-receptor ICAM-1 on HUVEC after iron treatment. Enhancement of PMN adherence has been described in hypoxanthine-xantine oxidase treated HUVEC,[27] without upregulation of ICAM-1. They describe the involvement of a carbohydrate ligand on HUVEC interacting with L-selectin on PMN. ICAM-1, unlike P- and E-selectin is expressed constitutively on the endothelial cell surface, although it can also be markedly induced by cytokines, including TNF-α and IL-1β.[28] In our experiments we similarly failed to see upregulation of ICAM-1 expression on HUVEC one hour after either iron sacharate or iron dextran incubations (not shown). This does not exclude the possibility that adhesion is still via ICAM-1 - CD11b/CD18 interactions without noted increase of any of the two receptors.

In this present study we observed upregulation of the proadhesive P-selectin on HUVEC after 1h iron dextran incubations as low as 1 μg/mL (Figure 4). Interestingly, iron sacharate did not show the same stimulatory effect as might be expected, and this could explain the difference in the results of transmigration experiments. Iron dextran enhanced PMN transmigration whereas iron sacharate had no effect (Table 1). The iron in both iron sacharate and iron dextran is in complexes, which make the iron unavailable until processing by the reticuloendothelial system. However, a labile iron form may exist on the surface of these complexes, which could be available to the cells before processing. This labile iron could take part in oxidation-reduction reactions and facilitate an altered oxidative status in the cells.

Indeed, we found NTBI of up to 6 μmol/L in both iron dextran and iron sacharate preparations used in our experiments, however the nature of NTBI from the two iron preparations may differ that could be responsible for the observed results. In addition, the production of oxygen-derived radicals by HUVEC in the presence of iron has been shown, and it is possible that the radicals promotes adherence of PMN to endothelium [29].

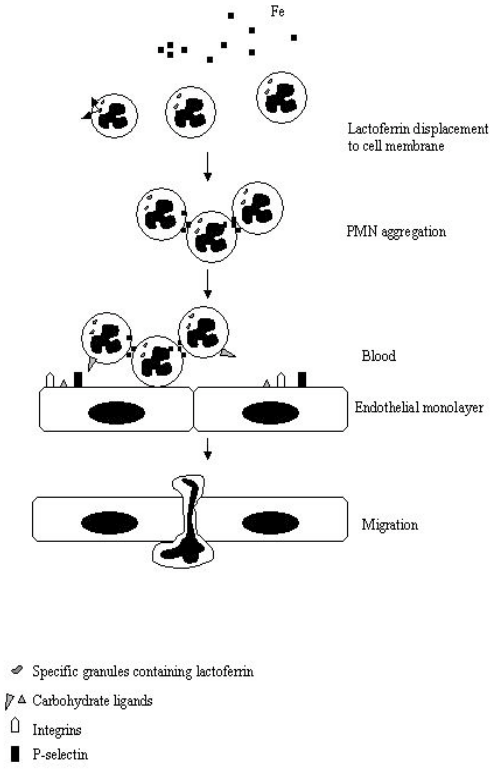

Figure 5. Proposed model for enhancement of PMN recruitment to endothelium under iron-rich conditions. Lactoferrin secretion to the cellular membrane is induced. PMN aggregation will stimulate adherence to endothelium without notable increased in ICAM-1 - CD11b/CD18 interactions. Proadhesive P-selectin on HUVEC is induced. PMN transmigration proceeds without notable upregulation of PECAM-1 (see also

Lactoferrin is an iron-binding protein from glandular secretions and is stored in granules of neutrophils, which express the protein on the membrane upon activation. It is not expressed constitutively on the membrane, but within 15 min of stimulation with either 1 μg/ml PMA or 2 ng/ml TNF-α it appears on the surface.[30] Iron rich conditions could stimulate the release of lactoferrin to the cell surface. Iron has been previously shown to induce PMN aggregation with iron complexes concentrating at the interface of two PMN (Hoepelman, I. M., personal communication). In addition, iron complexes seemed to be taken up by the PMN probably by pinocytosis (Hoepelman, I. M., personal

communication). The uptake of iron could facilitate lactoferrin secretion from specific granules and have resulted in increased PMN aggregation and subsequent adherence to HUVEC. In our experimental set-up, we have also observed enhancement of the pro-adhesive P-selectin by iron without notable changes in ICAM-1 expression. Studying the signaling pathways that are switched on after engagement of selectins and adhesion molecules by counter-receptors on PMN, and also other receptors like growth factor receptors, should provide more insight into the actual mechanism of PMN adherence to endothelium under iron rich conditions (Figure 5).

A previous study has reported an impairment of transendothelial leukocyte migration by iron sucrose and iron gluconate.[31] In opposite to the findings in that previous study,[31] we report enhanced leukocyte migration by iron treatment. This is most probably due to our choice of fluorescent label in carrying out the transmigration experiments, namely BCECF-DA, instead of the weak iron-chelator, calcein used in that study.[31] Our findings on enhancement of adherence and transmigration of PMN through HUVEC by iron provides a role of iron in innate immune activation. Furthermore, our group has recently published the enhanced adherence of human monocytes on HUVEC under artificial iron-rich conditions[29] as well as by using serum of hemochromatosis patients with raised NTBI levels.[25] The overall implication of these findings *in vivo* are yet to be studied in a controlled setting. Moreover, the true benefits of this enhanced adhesion and transmigration by iron complexes still have to be assessed in relevant groups of patients.

Acknowledgements: This study was supported by the European Commission, project QLK1-CT-2002-00444. We thank Mr. J.J.M. Bouwman from the Diakonessen Hospital, Utrecht, The Netherlands, for providing the umbilicalcords.

References

1. Schiffmann, E., Corcoran, B.A., & Wahl, S.M. N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc. Natl Acad Sci U S A* **72**, 1059-1062 (1975).
2. Roos, D., van Bruggen, R., & Meischl, C. Oxidative killing of microbes by neutrophils. *Microbes Infect* **5**, 1307-1315 (2003).
3. Varani, J. *et al.* Pulmonary endothelial cell killing by human neutrophils. Possible involvement of hydroxyl radical. *Lab Invest* **53**, 656-663 (1985).
4. Ward, P.A., Till, G.O., Kunkel, R., & Beauchamp, C. Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. *J Clin Invest* **72**, 789-801 (1983).
5. Boogaerts, M.A., Yamada, O., Jacob, H.S., & Moldow, C.F. Enhancement of granulocyte-endothelial cell adherence and granulocyte-induced cytotoxicity by platelet release products. *Proc Natl Acad Sci U S A* **79**, 7019-7023 (1982).
6. Sacks, T., Moldow, C.F., Craddock, P.R., Bowers, T.K., & Jacob, H.S. Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. An *in vitro* model of immune vascular damage. *J Clin Invest* **61**, 1161-1167 (1978).
7. Zeiher, B.G., Matsuoka, S., Kawabata, K., & Repine, J.E. Neutrophil elastase and acute lung injury: prospects for sivelestat and other neutrophil elastase inhibitors as therapeutics. *Crit Care Med* **30**, S281-S287 (2002).
8. Kooistra, M.P. *et al.* Nontransferrin-bound iron in the plasma of haemodialysis patients after intravenous iron saccharate infusion. *Eur J Clin Invest* **32 Suppl 1**, 36-41 (2002).

9. Van Wyck, D., Anderson, J., & Johnson, K. Labile iron in parenteral iron formulations: a quantitative and comparative study. *Nephrol Dial Transplant* **19**, 561-565 (2004).

10. Hershko, C., Graham, G., Bates, G.W., & Rachmilewitz, E.A. Non-specific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. *Br J Haematol* **40**, 255-263 (1978).

11. Hider, R.C. Nature of nontransferrin-bound iron. *Eur J Clin Invest* **32 Suppl 1**, 50-54 (2002).

12. Banyai, S., Rainer, V., Druml, W., Horl, W.H., & Sunder-Plassmann, G. Bleomycin-detectable free iron (BDI) is present in patients on intravenous (IV) iron therapy. *J Am Soc Nephrol* **9**, 199. (1998).

13. Breuer, W. *et al.* The assessment of serum nontransferrin-bound iron in chelation therapy and iron supplementation. *Blood* **95**, 2975-2982 (2000).

14. Esposito, B.P., Breuer, ., Slotki, I., & Cabantchik, Z.I. Labile iron in parenteral iron formulations and its potential for generating plasma nontransferrin-bound iron in dialysis patients. *Eur J Clin Invest* **32 Suppl 1**, 42-49 (2002).

15. Parkkinen, J., von Bonsdorff, L., Peltonen, S., Gronhagen-Riska, C., & Rosenlof, K. Catalytically active iron and bacterial growth in serum of haemodialysis patients after i.v. iron-saccharate administration. *Nephrol Dial Transplant* **15**, 1827-1834 (2000).

16. Scheiber-Mojdehkar, B., Lutzky, B., Schaufler, R., Sturm, B., & Goldenberg, H. Non-transferrin-bound iron in the serum of hemodialysis patients who receive ferric saccharate: no correlation to peroxide generation. *J Am Soc Nephrol* **15**, 1648-1655 (2004).

17. Troelstra, A. *et al.* Dual effects of soluble CD14 on LPS priming of neutrophils. *J Leukoc Biol* **61**, 173-178 (1997).

18. Jaffe, E.A., Nachman, R.L., Becker, C.G., & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* **52**, 2745-2756 (1973).

19. Breuer, W. & Cabantchik, Z.I. A fluorescence-based one-step assay for serum non-transferrin-bound iron. *Anal Biochem* **299**, 194-202 (2001).

20. Lawrence, M.B. & Springer, T.A. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* **65**, 859-873 (1991).

21. Rosen, S.D. Cell surface lectins in the immune system. *Semin Immunol* **5**, 237-247 (1993).

22. Laudanna, C., Kim, J.Y., Constantin, G., & Butcher, E. Rapid leukocyte integrin activation by chemokines. *Immunol Rev* **186**, 37-46 (2002).

23. Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-314 (1994).

24. Oseas, R., Yang, H.H., Baehner, R.L., & Boxer, L.A. Lactoferrin: a promoter of polymorphonuclear leukocyte adhesiveness. *Blood* **57**, 939-945 (1981).

25. Kartikasari, A.E.R. *et al.* Endothelial activation and induction of monocyte adhesion by nontransferrin-bound iron present in human sera. *FASEB J* DOI: 10.1096/fj.05-4700fje (2005).

26. Kuijpers, T.W. *et al.* Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* **78**, 1105-1111 (1991).

27. Sellak, H., Franzini, E., Hakim, J., & Pasquier, C. Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood* **83**, 2669-2677 (1994).

28. Luscinskas, F.W. *et al.* Cytokine-activated human endothelial monolayers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1. *J Immunol* **146**, 1617-1625 (1991).

29. Kartikasari, A.E.R. *et al.* Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. *Arterioscler Thromb Vasc Biol* **24**, 2257-2262 (2004).

30. Afeltra, A. *et al.* Expression of lactoferrin on human granulocytes: analysis with polyclonal and monoclonal antibodies. *Clin Exp Immunol* **109**, 279-285 (1997).

31. Sengoelge, G. *et al.* Impairment of transendothelial leukocyte migration by iron complexes. *J Am Soc Nephrol* **14**, 2639-2644 (2003).

CHAPTER 8

Iron chelators interfere with phagocyte-derived myeloperoxidase-mediated oxidation of native low density lipoprotein

Niki A. Georgiou, Apriliana E. R. Kartikasari, Suzanne J. Korporaal, Frank L. J. Visseren, J. Henny van Kats-Renaud, B. Sweder van Asbeck, Joannes J. M. Marx

In preparation

ABSTRACT

Lipid peroxidation is a major contributor in the pathogenesis of atherosclerosis. Oxidised low density lipoprotein (oxLDL) is recognised by scavenger receptors on monocyte derived macrophages which attach and migrate to the intima under various chemotactic gradients. The uncontrollable uptake of oxLDL results in the formation of foam cells, which will eventually contribute to atherogenic plaque formation. Among the various pathways whereby low-density lipoprotein is oxidised is by products from the reaction between neutrophil myeloperoxidase (MPO) and H_2O_2. Iron and copper have also been implicated in oxLDL formation. The aim of this study was to investigate the involvement of iron and iron chelators in the *in vitro* oxidation of human, native LDL. The iron chelators deferoxamine, deferiprone and the experimental iron chelator CP502 were used. Their contribution in the MPO/H_2O_2 and Copper(II)-catalysed oxidation of LDL was examined. The activity of the heme containing MPO enzyme was also monitored in the presence of these compounds. 10 μmol/L deferoxamine and 30 μmol/L of each deferiprone and CP502 inhibited the rate of diene formation in both catalysed reactions. An increase in lag times of oxidation indicate that all three chelators act as antioxidants in the process of LDL oxidation. The enzymatic activity of MPO remained unchanged. Iron chelation can significantly inhibit LDL oxidation without affecting MPO enzymatic activity. LDL oxidation is a pathway whereby iron could participate in the pathogenesis of atherosclerosis.

Key words: iron • iron chelator • LDL • myeloperoxidase • atherosclerosis

INTRODUCTION

Atherosclerosis is a chronic inflammatory disorder[1,2] with the oxidation of LDL, the major carrier of cholesterol in the blood, thought to contribute significantly to atherogenesis.[3,4] The LDL molecule carries positively charged proteins, ApoB100s, on its surface, which bind to the negatively charged LDL receptor. Masking of the positive charge of ApoB100 by oxidation makes oxLDL a ligand for scavenger receptors instead of the LDL receptor. Reactive oxygen species have been shown to modify and even fragment apolipoprotein B100.[5]

The proatherogenic properties of oxLDL are not limited to their uncontrollable uptake by scavenger receptors on macrophages resulting in foam cells formation. OxLDL also inhibits macrophage motility, trapping them and allowing the lesion to develop. It is also a monocyte chemoattractant resulting in recruitment of monocytes to the artery wall. Furthermore, oxLDL prevents vascular endothelium from releasing NO. Under normal conditions NO is secreted to prevent accumulation of leukocytes and platelets. Moreover, OxLDL is cytotoxic, inducing the release of lysosomal enzymes and lipids that are capable of enhancing the development of an atherosclerostic lesion. Proteolytic enzymes secreted from necrotic macrophages are thought to promote rupture of fatty

plaques by destabilising the fibrous cap around the atherosclerotic lesion. In addition, OxLDL has been visualised in plaques, plasma and arteries in humans and experimental animals.

Various pathways leading to LDL oxidation have been described, one of them is facilitated by oxidants generated by the heme protein myeloperoxidase (MPO). Enzymatically active MPO is secreted by activated white blood cells (WBC) including monocytes, macrophages, and polymorphonuclear granulocytes (neutrophils, PMN). MPO has been found in atherosclerotic tissue, colocalising with white blood cells. WBC secrete MPO which will oxidise Cl^- to hypochlorous acid (HOCl) in the presence of H_2O_2. In addition to this, it will convert L-tyrosine to 3-chlorotyrosine. The two radical products of MPO, HOCl and 3-chlorotyrosine can oxidise LDL. MPO has also been reported to quench NO and thus contribute to endothelial dysfunction.[6]

Another pathway of LDL oxidation proceeds via reactive metal ions. The two metal ions standardly used *in vitro* to generate oxLDL are copper and iron. *In vivo* they are tightly bound to proteins (e.g. copper in ceruloplasmin and iron on transferrin and in ferritin). However, when there is tissue damage these metals can be released, forming labile complexes and allowing catalytic activity resulting in the formation of hydroxyl radicals. Under such conditions, also hemin or hemoglobin can promote LDL oxidation. Ion-dependent LDL oxidation is expected to be of significance in advanced lesions.

Non-transferrin bound iron (NTBI) is currently considered to be one of the toxic forms of iron, partly via its potential to form the hydroxyl radical, which has been implicated in the pathogenesis of various conditions, including atherosclerosis.[7,8] Increased malondialdehyde levels as an indication of oxLDL levels has been reported in plasma of β-thalassemia intermedia patients with secondary iron overload.[9] In another study, a group of 15 β-thalassemia intermedia patients with high malondialdehyde (MDA) levels and low antioxidant status were followed. After 9 months of oral treatment with 600 mg/day Vitamin E, their parameters were the same as of controls treated for 6 months.[10] Superoxide ion generation in β-thalassemia red blood cells is 8-fold higher than that of normal erythrocytes.[11] Superoxide anion could participate in the formation of peroxynitrite, which has also been reported to result in LDL oxidation. Increased MDA plasma levels and significantly decreased plasma superoxide dismutase activity have also been observed following intravenous administration of 100 mg iron sucrose to hemodialysis patients.[12]

As MPO is a heme protein we have looked into the involvement of iron in the process of LDL oxidation by this enzyme. We hypothesise that the activity of MPO, being an iron-dependent enzyme, could be modulated by iron levels. In the current study we have also investigated the involvement of iron and iron chelators in the MPO/H_2O_2 and copper(II) dependent oxidation of LDL. The iron chelators tested included CP502, in addition to the establishe deferoxamine and deferiprone. CP502 has been targeted as a new iron-chelating candidate for further development in early pre-clinical testing.[13,14]

EXPERIMENTAL PROCEDURES

PMN isolation

Blood obtained from healthy human volunteers was collected into tubes containing sodium heparin (Vacuette, Greiner, Alphen a/d Rijn, The Netherlands) as anticoagulant. Neutrophils were isolated as previously described.[15] For the isolation, heparinised blood was diluted 1:1 (vol/vol) with pyrogen-free PBS and layered onto a gradient of Ficoll (Pharmacia) and Histopaque (density, 1.119 g/ml; Sigma, St. Louis, USA). After centrifugation for 20 min at 320 x *g*, the neutrophils were collected from the Histopaque phase. Cells were subjected to a brief hypotonic shock with water, washed, and suspended at 5 _ 10^6 cells/ml in RPMI medium containing 0.05% human serum albumin (HSA) (Sanquin, Amsterdam, The Netherlands).

Myeloperoxidase activity measurements

Freshly isolated PMN were incubated for 4 hours with either 100 μmol/L deferoxamine or 300 μmol/L deferiprone. 2 x 10^5 cells were used for each compound incubation. The cells were lysed in hexadecyltrimethylammonium bromide (HTAB) and 0.53 mmol/L O-dianisidine dihydrochloride (Sigma) was used as substrate and 166 μmol/L H_2O_2 (Merck, Darmstadt, Germany) was used to initiate the reaction. Absorbance at 460 nm was measured with a 15 second interval for 5 minutes in a Genesis™ 10 series spectrophotometer (Spectronic Unicam, Rochester, NY, USA).

Human lipoprotein Isolation

Lipoproteins were isolated as described before.[16] In short, fresh, non-frozen plasma from 3 healthy subjects (Sanquin Blood bank, Amsterdam), each containing less than 100 mg of lipoprotein(a)/liter, was pooled, and LDL (density range 1.019–1.063 kg/liter) was isolated by sequential flotation in a Beckman L-80 ultracentrifuge.[17] Centrifugations (175, 000 x *g*, 20 h, 10 °C) were carried out in the presence of NaN_3 and EDTA. Lipoproteins were stored at 4 °C under nitrogen for not longer than 14 days and before each experiment dialysed overnight against 10^4 volumes 150 mmol/L NaCl. ApoB100 and lipoprotein(a) concentrations were measured using the Behring Nephelometer 100. The concentration of LDL was expressed as grams of apoB100 protein/L.

Lipoprotein oxidation

LDL was dialysed overnight in PBS before each oxidation. Between 50-100 μg protein/mL was used for each oxidation. Oxidation proceeded by two different routes: either by 5 μmol/L Cu(II)chloride (Sigma-Aldrich, WI, USA) or by the combination 150 mU/mL MPO (Calbiochem Darmstadt, Germany)/166 μmol/L H_2O_2. Oxidation was followed for 6 hours measuring at 3 minutes interval, at 37 °C. Oxidative modification of LDL was followed by the formation of conjugated dienes measured at 234nm and expressed as % oxidation compared to native LDL. The lag time of oxidation which gives an indication of the compound's ability to delay the oxidation of LDL (antioxidant properties) was also expressed as % control. Absorbance was measured by a Biochrom-4060 variable length scanning

spectrophotometer (Pharmacia, Pfizer, Ontario, Canada) and measurement of diene rate formation and lag times were calculated using the Biochrom-4060 software (Version 2.0, 1993 by L. Bunnage, copyright Pharmacia 1992).

Initiation of LDL oxidation

LDL was added in a glass cuvette at total volume of 1 mL PBS and as soon as all compounds were added OD measurements were initiated. In the reactions whereby H_2O_2 is present this was added lastly and measurements initiated directly. For the Cu(II) oxidation route, a 0.5 mmol/L stock Cu(II) chloride (Sigma) is freshly prepared each time in distilled water. 5 μmol/L Cu(II) was added lastly before initiation of measurements commenced. Further dilutions were in PBS. Iron (III) citrate with metal-to-chelate ratios of 1:20 (mononuclear) was prepared at pH 1 using Fe(III)chloride (Fluka, Buchs SG, Switzerland) and sodium citrate (J.T. Baker, Deventer, The Netherlands). After 10 min at low pH the pH was increased slowly to pH 7.4 under continuous stirring with NaOH. Chelator stocks were 10 mmol/L deferoxamine (Novartis, Arnhem, The Netherlands), 30 mmol/L deferiprone (Duchefa Biochemie, Haarlem, The Netherlands) and 30 mmol/L CP502 (Apotex Inc., Toronto, Ontario, Canada), all in PBS. Myeloperoxidase was stored in 1000 mU/mL aliquots in potassium phosphate buffer at pH 6. All compounds were diluted in PBS and stocks were stored at –20 °C unless otherwise stated, until use.

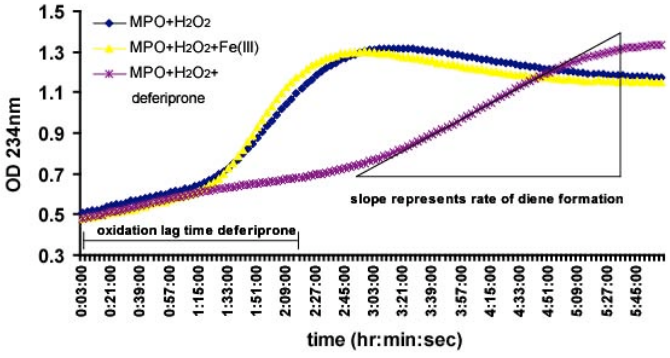

Figure 1. **Representative experiment showing reaction kinetics of LDL oxidation.** OD at 234 nm was followed for 6 hours at 3 minutes intervals at 37 °C. The reaction was initiated by the addition of 166 μmol/L H_2O_2. In this experiment lag time of oxidation by deferiprone is 134 min and rate of diene formation is 2.5 nmol/mg apoB/min (derived from the steepest ascending part of the slope of OD234 measurement). In this experiment lag time of oxidation by 150 mU MPO/mL/166 μmol/L H_2O_2 and 150 mU MPO/mL/166 μmol/L H_2O_2 in the presence of 10 μmol/L Fe(III)citrate were 72 and 87 min, respectively, and the rate of diene formation similar at 5.5 and 4.9 nmol/mg apoB/min, respectively. In this graph the antioxidant nature of deferiprone in the MPO/H_2O_2-mediated LDL oxidation is demonstrated.

RESULTS

Myeloperoxidase enzyme activity

MPO activity measured from non-treated and iron chelator treated PMN was unchanged. 0.110 OD change/min was noted for the untreated cells and 0.103 and 0.100 OD change /min for the 100 μmol/L deferoxamine and 300 μmol/L deferiprone incubations, respectively.

Kinetics of LDL oxidation and experimental readout

A representative experiment showing the reaction kinetics of the oxidation of native LDL is shown in Figure 1. In this example the MPO/ H_2O_2-catalysed LDL oxidation is followed in the presence of either 10 μmol/L Fe(III) or 30 μmol/L deferiprone and compared to control (only MPO/H_2O_2). A shift of the curve to the right represents an increase in lag time and thus and increase in the time needed for initiation of the oxidation reaction (Figure 1). The slope (derived from the steepest ascending part of the slope of OD234 measurement) gives an indication of the rate of diene formation. In this particular experiment, lag time of oxidation by deferiprone is 134 min and rate of diene formation is 2.5 nmol/mg apoB/min. Lag time of oxidation by 150 mU MPO/mL/166 μmol/L H_2O_2 and 150 mU MPO/ml/166 μmol/L H_2O_2 / 10 μmol/L FeIII were 72 and 87 min, respectively, and the rate of diene formation similar at 5.5 and 4.9 nmol/mg apoB/min, respectively. No effect of Fe(III) on MPO/H_2O_2 catalysed LDL oxidation was noted. In this graph the antioxidant nature of deferiprone in the MPO/H_2O_2 mediated LDL oxidation is demonstrated.

Effect of iron and chelation in the *in vitro* MPO/H_2O_2-catalysed LDL oxidation

In this set of experiments 150 mU MPO/mL/166 μmol/L H_2O_2 was used to initiate native LDL oxidation, with H_2O_2 being added lastly before initiation of measurements. The presence of Fe(III) did not influence the progression of LDL oxidation in the MPO/H_2O_2-calalysed reaction (Figures 2A and 2B). Deferiprone at a concentration of 30 μmol/L lowered the rate of diene formation by 60% (Figure 2A), from 5.7± 1.3 to 1.8 ± 0.3 nmol/ mg B100/min. The lag time of LDL oxidation by deferiprone was doubled (Figure 2B) from 65 ± 7.7 to 126 ± 9.8 minutes, showing the antioxidant nature of deferiprone. 10 μmol/L deferoxamine showed similar properties to 30 μmol/L deferiprone although less pronounced. At deferoxamine concentration of 10 μmol/L, rate of diene formation was significantly decreased by 37% (figure 2A), from 5.7± 1.3 to 3.6 ± 1.2 nmol/ mg B100/min. The lag time of LDL oxidation by deferoxamine was almost doubled (Figure 2B) from 65 ± 7.7 to 100 ± 21.8 minutes, showing the antioxidant nature of deferoxamine. Similarly, 30 μmol/L CP502 lowered the rate of diene formation by 57 % although not significantly (Figure 2A) from 5.7± 1.3 to 2.5 ± 0.3 nmol/ mg B100/min. The lag time of LDL oxidation by CP502 was similar to deferiprone, doubled (Figure 2B) from 65 ± 7.7 to 126 ± 13.9 minutes, showing the antioxidant nature of CP502 (Figure 2B).

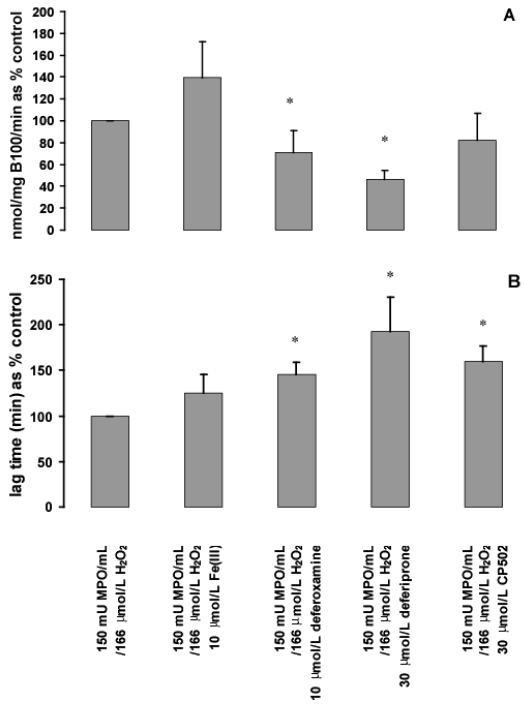

Figure 2: 150 mU MPO/ml /166 μmol/L H_2O_2 catalysed LDL oxidation. (A) represents rate of diene formation (derived from the steepest ascending part of the slope of OD234 measurements, see Figure 1) and (B) is lag time of oxidation (time needed for initiation of oxidation reaction, indicated by the point of increasing OD234 slope, see Figure 1), both expressed as % control (n=3-9)

Effect of iron chelation in the *in vitro* Cu(II)-catalysed LDL oxidation

In this set of experiments 5 μmol/L Cu(II) was used to initiate the oxidation reaction of native LDL, by being added lastly in the glass cuvette. The presence of the iron chelators deferoxamine, deferiprone and CP502 in Cu(II)-catalysed LDL oxidation was investigated. At deferoxamine concentration of 10 μmol/L, the rate of diene formation was significantly decreased by 70% (Figure 3A), from 11 ± 1.2 to 3.8 ± 1.1 nmol/ mg B100/min. The lag time of LDL oxidation by deferoxamine was more than doubled (Figure 3B) from 39.0 ± 8.3 to 90.7 ± 23.3 minutes, showing the antioxidant nature of deferoxamine. Deferiprone at a concentration of 30 μmol/L lowered the rate of diene formation by 80% (figure 3A), from 11 ± 1.2 to 2.4 ± 1.0 nmol/ mg B100/min. The lag time of LDL oxidation by deferiprone was almost tripled (Figure 3B) from 39.0 ± 8.3 to 114.8 ± 13.8 minutes, showing the antioxidant nature of deferiprone. Similar to deferoxamine, 30 μmol/L CP502 lowered the rate of diene formation by 70 % (Figure 3A) from 11 ± 1.2 to 4.1 ± 0.25 nmol/ mg B100/min. The lag time of LDL oxidation by CP502 was similar to deferiprone, tripled (Figure 3B) from 39.0 ± 8.3 to 135 ± 14 minutes, showing the antioxidant nature of CP502 (Figure 3B). The effects of all three deferoxamine, deferiprone and CP502 were significant in both diene formation and lag time measurements.

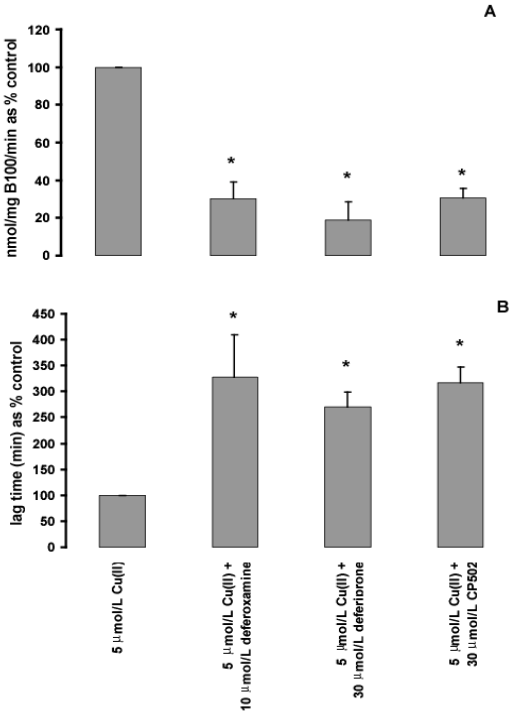

Figure 3: 5 μmol/L Cu(II)-catalysed LDL oxidation: (A) represents diene formation (derived from the steepest ascending part of the slope of OD234 measurements, see fig. 1) and (B) lag time of oxidation (time needed for initiation of oxidation reaction, indicated by the point of increasing OD234 slope, see fig. 1), both expressed as % control (n=2-6)

DISCUSSION

Recent clinical studies in humans strongly support a role for MPO in atherogenesis. In a study comparing 100 totally or subtotally MPO-deficient individuals with 118 normal controls randomly selected, a protective effect against cardiovascular damage was noted in the first group.[18] In addition, the MPO-deficient group had a higher occurrence of severe infections and chronic inflammation processes.[18] MPO polymorphisms have also been implicated in coronary artery disease.[19] One polymorphism described for the MPO gene is located at the promoter region and involves a G to A substitution at position 463 bp upstream of the SP1 transcription start site.[20] The G allele is associated with a higher level of MPO expression than the A allele, of which was less frequent in patients with coronary artery disease.[20] Other studies have shown that subjects with two copies of the G allele have an increased risk of developing a cardiovascular event in normal subjects,[21] as well as in end-stage renal disease patients.[22]

In the present study, we show that iron chelation can interfere with various pathways of LDL oxidation, namely the MPO/H_2O_2 and Cu(II)-catalysed reactions. In these two pathways studied, both

Fe(III) and Cu(II) were in their oxidised form. No chelator is exclusively specific for one metal, and in the same way, all three chelators used in this study are able to chelate other metals. Both deferoxamine and deferiprone can chelate zinc, deferiprone with a higher affinity than deferoxamine.[23] The chelation of zinc by iron chelators was shown to result in murine thymocyte apoptosis and it was suggested that deferiprone due to its small size can access zinc pools not available to the larger deferoxamine molecule.[24] 14% of patients receiving long-term treatment with deferiprone had zinc deficiency, whereas this did not occur in patients receiving deferoxamine.[23] This chelator has been shown to have appreciable affinity for copper and with *in vitro* systems it was shown to be able to mobilise copper from albumin and to faciliate its movement across a cell membrane model [25]. The reduction of LDL oxidation observed in this study could be the result of Cu(II) chelation by deferoxamine, deferiprone and CP502.

Iron chelation did not affect the activity of the MPO enzyme, even though the MPO/H_2O_2 catalysed LDL oxidation was affected. MPO contains heme iron, however this heme iron appears to be firmly bound and unavailable for chelation.[26, 27] Deferoxamine, however, was shown to affect the oxidation reaction facilitated by MPO, without affecting the enzymatic activity. In this case, deferoxamine probably affects products formed by MPO.[27]

LDL has been shown to be oxidised by endothelial cells when pretreated with ferritin.[28] The oxidation of LDL was inhibited by apoferritin (100%), deferoxamine (60% inhibition) and by the cell permeable antioxidants DMSO (80%) and butylated hydroxytoluene (BHT) (99%).[28] In that study, iron mobilised from ferritin appears to be essential for the formation of free radicals, which in turn facilitate LDL oxidation. Since NTBI has been positively correlated to ferritin levels,[8] NTBI may also involve in MPO driven reactions with an effect on levels of LDL oxidation. Moreover, the involvement of iron in the toxicity of oxLDL to cultured vascular cells has been demonstrated with deferoxamine pre-incubations preventing the lysis of cells by oxLDL.[29] In addition, heme molecule that carries iron ion has been shown to mediate the oxidative modification of LDL.[30-32] The results from aforementioned studies, together with our findings in this current study suggest that iron could enhance the rate of native LDL oxidation and thereby, may contribute to the progression of cardiovascular damage. Catalytically active NTBI may interact with phagocyte derived oxygen metabolites, resulting in formation of the proatherogenic oxLDL. Our findings also suggest that iron chelators could interfere with the course of LDL oxidation.

Acknowledgement: This study was supported by the European Commission, project QLRT-2002-00444

References

1. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809 (1993).
2. Libby, P. Molecular bases of the acute coronary syndromes. *Circulation* **91**, 2844-2850 (1995).
3. Berliner, J.A. *et al.* Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation* **91**, 2488-2496 (1995).
4. Podrez, E.A., Abu-Soud, H.M., & Hazen, S.L. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med* **28**, 1717-1725 (2000).
5. Napoli, C. *et al.* Glycosylation enhances oxygen radical-induced modifications and decreases acetylhydrolase activity of human low density lipoprotein. *Basic Res Cardiol* **92**, 96-105 (1997).
6. Brennan, M.L. & Hazen, S.L. Amino acid and protein oxidation in cardiovascular disease. *Amino. Acids* **25**, 365-374 (2003).
7. Kartikasari, A.E.R. *et al.* Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. *Arterioscler Thromb Vasc Biol* **24**, 2257-2262 (2004).
8. Kartikasari, A.E.R. *et al.* Endothelial activation and induction of monocyte adhesion by non-transferrin-bound iron present in human sera. *FASEB J* DOI: 10.1096/fj.05-4700fje (2005).
9. Livrea, M.A. *et al.* Oxidative modification of low-density lipoprotein and atherogenetic risk in beta-thalassemia. *Blood* **92**, 3936-3942 (1998).
10. Tesoriere, L. *et al.* Oral supplements of vitamin E improve measures of oxidative stress in plasma and reduce oxidative damage to LDL and erythrocytes in beta-thalassemia intermedia patients. *Free Radic Res* **34**, 529-540 (2001).
11. Scott, M.D. *et al.* Effect of excess alpha-hemoglobin chains on cellular and membrane oxidation in model beta-thalassemic erythrocytes. *J Clin Invest* **91**, 1706-1712 (1993).
12. Lim, P.S., Wei, Y.H., Yu, Y.L., & Kho, B. Enhanced oxidative stress in haemodialysis patients receiving intravenous iron therapy. *Nephrol. Dial. Transplant.* **14**, 2680-2687 (1999).
13. Novakovic, J., Tesoro, A., Thiessen, J.J., & Spino, M. Metabolic and pharmacokinetic evaluation of a Novel 3-hydroxypyridinone iron chelator, CP502, in the rat. *Eur. J Drug Metab Pharmacokinet* **29**, 221-224 (2004).
14. Tam, T.F. *et al.* Iron chelator research: past, present, and future. *Curr Med Chem* **10**, 983-995 (2003).
15. Troelstra, A. *et al.* Dual effects of soluble CD14 on LPS priming of neutrophils. *J Leukoc Biol* **61**, 173-178 (1997).
16. Hackeng, C.M. *et al.* Low-density lipoprotein enhances platelet secretion via integrin-alphaIIbbeta3-mediated signaling. *Arterioscler Thromb Vasc Biol* **19**, 239-247 (1999).
17. Havel, R.J., Eder, H.A. & Bragdon, J.H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* **34**, 1345-1353 (1955).
18. Kutter, D. *et al.* Consequences of total and subtotal myeloperoxidase deficiency: risk or benefit ? *Acta Haematol* **104**, 10-15 (2000).
19. Nikpoor, B., Turecki, G., Fournier, C., Theroux, P., & Rouleau, G.A. A functional myeloperoxidase polymorphic variant is associated with coronary artery disease in French-Canadians. *Am Heart J* **142**, 336-339 (2001).
20. Piedrafita, F.J. *et al.* An Alu element in the myeloperoxidase promoter contains a composite SP1-thyroid hormone-retinoic acid response element. *J Biol Chem* **271**, 14412-14420 (1996).
21. Asselbergs, .W., Reynolds, W.F., Cohen-Tervaert, J.W., Jessurun, G.A., & Tio, R.A. Myeloperoxidase polymorphism related to cardiovascular events in coronary artery disease. *Am J Med* **116**, 429-430 (2004).
22. Pecoits-Filho, R. *et al.* A functional variant of the myeloperoxidase gene is associated with cardiovascular disease in end-stage renal disease patients. *Kidney Int Suppl* S172-S176 (2003).
23. al-Refaie, F.N. *et al.* Zinc concentration in patients with iron overload receiving oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one or desferrioxamine. *J Clin Pathol* **47**, 657-660 (1994).
24. Maclean, K.H., Cleveland, J.L., & Porter, J.B. Cellular zinc content is a major determinant of iron chelator-induced apoptosis of thymocytes. *Blood* **98**, 3831-3839 (2001).
25. Pall, H. *et al.* Ocular toxicity of desferrioxamine--an example of copper promoted auto-oxidative damage? *Br J Ophthalmol* **73**, 42-47 (1989).
26. Klebanoff, S.J. The iron-H2O2-iodide cytotoxic system. *J Exp Med* **156**, 1262-1267 (1982).
27. Klebanoff, S.J. & Waltersdorph, A.M. Inhibition of peroxidase-catalysed reactions by deferoxamine. *Arch Biochem Biophys* **264**, 600-606 (1988).

28. Satoh, T. & Tokunaga, O. Intracellular oxidative modification of low density lipoprotein by endothelial cells. *Virchows Arch* **440**, 410-417 (2002).

29. Kuzuya, M. *et al.* Involvement of intracellular iron in the toxicity of oxidised low density lipoprotein to cultured endothelial cells. *Biochem Int* **22**, 567-573 (1990).

30. Balla, G., Jacob, H.S., Eaton, J.W., Belcher, J.D., & Vercellotti, G.M. Hemin: a possible physiological mediator of low density lipoprotein oxidation and endothelial injury. *Arterioscler Thromb* **11**, 1700-1711 (1991).

31. Klouche, K. *et al.* Mechanism of *in vitro* heme-induced LDL oxidation: effects of antioxidants. *Eur J Clin Invest* **34**, 619-625 (2004).

32. Camejo, G. *et al.* Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages. *J Lipid Res* **39**, 755-766 (1998).

CHAPTER 9

General Discussion

Main conclusions and current perspectives

Coronary artery disease (CAD) is currently the major cause of death in the Western world, and is also rapidly emerging in other countries. The disease results from lipidemia, coupled with a chronic inflammatory condition of the vascular wall, that leads to vessel occlusion and organ damage. To this date, despite intense efforts to determine the pathogenesis of atherosclerosis, this process remains poorly understood.

In 1981, Sullivan[1] suggested possible benefits of iron depletion against ischemic heart disease. This hypothesis was an attempt to explain the male gender risk, and the subsequent loss of the protective effect of female gender after menopause, in developing CAD. Following Sullivan's proposal, basic and clinical data have begun to provide plausible explanations for a link between iron and atherosclerosis.[2] In this thesis, the involvement of iron in several inflammatory processes of atherosclerosis has been investigated.

BODY IRON STORES

Several measures of body iron content have been widely used in clinical laboratories, including serum iron, serum ferritin and transferrin-bound iron. A different form of iron, the so-called non-transferrin-bound iron (NTBI), has been detected in HFE C282Y hemochromatosis patients with transferrin saturation above 45%.[3] NTBI is the non-sequestered form of iron, which unlike ferritin and transferrin-bound iron, is potentially capable of catalysing free radical formation.

Using a newly developed fluorescence-based one-step NTBI assay,[4] one study described in this thesis has found significantly positive correlations between NTBI and other measures of body iron content, both in HFE C282Y carriers as well as in healthy subjects (Chapter 3).[5] This finding, reported for the first time, indicates the presence of NTBI in normal subjects. NTBI, previously found to be pathogenic, may thus also participate, as a normal physiological form of iron, in body iron metabolism.

IRON AND ENDOTHELIAL ACTIVATION

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.[6] Several studies have shown the importance of iron status for vascular endothelial function. For instance, iron chelation was shown to be capable of markedly improving vascular endothelial function in patients of CAD,[7] of HFE C282Y hemochromatosis,[8,9] and in subjects with homocysteine-induced endothelial dysfunction.[10] In experimental rat, iron chelation improves endothelial dysfunction[11] while iron overload promotes vascular atherosclerotic growth.[12] Furthermore, blood donation with substantial depletion of storage iron proves to acutely improve vascular function without pharmacological interventions.[13,14] In addition, iron induces early functional and structural vascular abnormalities due to endothelial dysfunction[15] while in genetic hemochromatosis, iron induces

hypertrophy of the radial artery. This iron effects are associated with subsequent induction of oxidative stress. The radical species may also impair the nitrogen monoxide (NO) production, leading to the condition of arterial stiffness,[16] that can be improved by iron chelation,[7] or by iron depletion.[8]

One well-characterised phenotype of endothelial dysfunction is increased expression of the endothelial adhesion molecules, E-selectin, ICAM-1 and VCAM-1.[17] In this thesis, incubation of endothelial cells with low molecular weight iron mimicking NTBI (Chapter 2)[18] or with the naturally occurring NTBI in sera of both normal and HFEC282Y carriers (Chapter 3)[5] promoted accumulation of iron in the intracellular labile iron pool (LIP). Iron in this transit cytoplasmic LIP is metabolically and catalytically reactive [19, 20] therefore the level is tightly regulated. In this study, prolonged exposure to NTBI on endothelial cells, nevertheless had augmented the level of LIP. Increased LIP levels had promoted the induction of cellular reduction-oxidation state imbalance, which had lead to increased expression of endothelial adhesion molecules with the phenotype of primed endothelial cells. As expected, intracellular iron chelation could counteract the effects of iron. These findings indicate that chelation of intracellular labile iron is an effective way to prevent iron-induced cell activation. These results support the view that low iron status *in vivo* could benefit vascular endothelial function. Furthermore, since NTBI is capable of modulating the reduction-oxidation status of the cells, interaction with environmental stressors, like smoking and hypertension,[21] may thus lead to overexposure of radicals and triggers sensitivity to develop atherosclerosis and cardiovascular diseases.

Despite the number of risk factors known to induce atherosclerosis, many cardiovascular events occur in individuals without these current known risk factors. Attempts in the last decade to predict cardiovascular risk more accurately have led to the emergence of a novel risk factor, C-reactive protein (CRP), which has proved to be as good a risk predictor as LDL cholesterol, and furthermore independently predicts recurrent events in patients with known CAD. CRP is a chronic inflammatory marker, and is thought to promote directly all stages of atherosclerosis. An additional study in this thesis (Chapter 3) found no significant correlation between CRP levels and endothelial activation. This result indicates that CRP *per se* does not modulate endothelial function, as has also been suggested by one study.[22] This finding suggests that although CRP could predict CAD events, it is not necessarily the main culprit of the disease.

IRON AND POTENTIALLY ATHEROGENIC MICROORGANISMS

Chronic inflammation plays a crucial role in CAD and other manifestations of atherosclerosis.[6] Several intracellular pathogens, including *Chlamydia pneumoniae* (Cp) and cytomegalovirus (CMV), may promote the chronic inflammation. Concurrently, the two microorganisms have been found in atherosclerotic plaques.[23-26] However, the evidence for the role of Cp and CMV in atherogenesis is still conflicting.[25] In this thesis (Chapter 4), both low-grade chronic Cp and CMV infections had readily upregulated the expression of endothelial adhesion molecules, possibly mediated by paracrine

interactions between endothelial cells to generate a response towards infections.[27] Our results, therefore, support the hypothesis that Cp and CMV infections are likely to contribute to atherosclerosis.

Cp requires proliferating cells to stay viable. This is because it cannot synthesise its own dNTPs. The conversion of NTPs to dNTPs, the crucial building blocks of DNA, is catalysed by the enzyme ribonucleotide reductase that is iron dependent. Since intracellular pathogens require iron to remain viable and to proliferate, while the host immune system require iron for proper functioning, the iron status would then be an important modifying factor in host-microbe interactions. In this study (Chapter 4), the expression of adhesion molecules on endothelial cells infected with either Cp or CMV was further enhanced when iron-rich medium was used during the infection. The infectivity and the growth of microorganisms, at the point of adhesion molecule assessment, were not enhanced by iron nor inhibited by iron chelation. This finding indicates that iron had already modulated endothelial response towards chronic low-grade infections before any significant changes were observed in the infectivity or the growth of the microorganisms. The iron-primed endothelial cells may have been more responsive towards the paracrine effects of infection. These findings may therefore imply that Cp or CMV infections in a population with increased body iron levels could render the patients to be more prone to the risk of developing endothelial dysfunction and furthermore atherosclerotic vascular disease.

IRON AND MONOCYTE INFILTRATION

Recruitment of monocytes to sites of inflammation is a fundamental event in many inflammatory diseases, such as atherosclerosis.[6] In this study, short incubations of freshly isolated monocytes with iron increased the level of intracellular labile iron with subsequent increased oxygen-derived radical formation. Under both static (Chapter 2 and Chapter 3) and flow conditions (Chapter-5) iron-loaded monocytes adhered more efficiently than non-iron-loaded monocytes on endothelial monolayer, possibly due to upregulation of the integrins. Under flow conditions, after blockade of the integrins, more iron-loaded monocytes could still adhere on TNF-α-activated endothelial cells, compared to non-iron-loaded monocytes, indicating that iron may have also modulated other receptors than the integrins. Flow cytometry indeed showed upregulation of chemokine receptors, CCR-2 and CXCR-2 by iron. Compared to non-iron-loaded cells, these iron-loaded monocytes could also more effectively transmigrate through HUVEC monolayer against an MCP-1 gradient, the potent chemoattractant for monocytes to transmigrate towards injured or infected tissues.[28] In summary, these findings indicate that intracellular iron enhanced monocyte-endothelial cell interactions under inflammatory conditions by altering the reduction-oxidation status of the cells. This is particularly important in artherosclerotic-ischemic disease where an inflammatory infiltrate is a constituent of the pathogenesis, while oxygen-derived radicals could aggravate the induction of inflammation. Prolonged presence of oxygen-derived radicals, possibly occurring in iron-overload conditions, may thus lead to a loss of antiinflammatory control, leading to the development of persistent inflammatory disorders, like atherosclerosis. On the other hand, in the case of anemia of inflammation, the feature of having low iron in the circulation may not only be due to the

effects of the host defense mechanism in limiting the availability of iron to invading microorganisms,[29] but could also be the result of an active effort of the immune system to avoid a prolonged inappropriate inflammatory response. In addition, the whole pattern of body iron content from various follow-up time points rather than one time point measurement may possibly give a better picture on the true effects of iron in modulating the risk for CAD.

Monocyte infiltration also plays a role in neurodegenerative diseases. Neuroinflammation, characterised by the accumulation of reactive microglia, is present in the degenerating areas. The intensity of the activation of these microglial cells, as the brain representatives of the monocyte phagocytic system, is related to a spectrum of inflammatory mediators like adhesion molecules and free radicals generated by a variety of local cells, including the vascular endothelium of the blood-brain barrier.[30,31] Leukocyte-mediated breakdown of blood–brain barrier followed by recruitment into the central nervous system is indeed a process characteristic of several neurodegenerative diseases.[32,33] Moreover, in the lesions, accumulation of iron has been characterised, which catalyses metal-induced oxidative stress in the disease process.[34] The findings in this book on the role of iron in monocyte-endothelial cell interactions, therefore, identify a potential pathophysiological nature of iron in the development of neurodegenerative diseases.

EDTA CHELATION THERAPY

EDTA chelation therapy in combination with high doses of ascorbic acid has been practiced widely as an alternative medication for atherosclerotic heart disease and stroke. Several beneficial effects of EDTA therapy for the treatment of atherosclerosis have been suggested,[35] with antioxidant effects being most plausible.[36] However, this thesis (Chapter 6) demonstrated that metal chelation by EDTA, particularly in combination with high doses of ascorbic acid promotes, rather than inhibits, endothelial activation. Paradoxically, the process involves free radical production, in contrast to the proposed antioxidant benefits of chelation therapy.[35] These findings lead to the suggestion that EDTA chelation therapy may even promote the process of vasculature damage.

Due to the high number of patients receiving this treatment, the United States National Institutes of Health (NIH) have started the first large-scale randomised trial on the safety and efficacy of EDTA chelation therapy for post-myocardial infarction patients.[37-39] The findings in this study provide a valuable mechanistic knowledge on the true effects of this treatment.

IRON AND NEUTROPHIL INFILTRATION

The neutrophil is a major component of the innate immune response, involved primarily in acute inflammation by engulfing damaged tissue and bacteria, releasing antimicrobial peptides, and killing invading microbes through the respiratory burst. In chapter 7, the levels of neutrophil adherence and

transendothelial migration were investigated after both neutrophils and/or endothelial cells were treated with low molecular weight iron, or the widely used intravenous iron supplements, iron saccharate or iron dextran. Iron dextran and not iron saccharate, increased neutrophil transendothelial migration, without significant changes in the expression of CD18/CD11b on neutrophils as well as the counter-receptor ICAM-1 on endothelial cells, the main interaction facilitating firm adhesion of neutrophils on endothelium after inflammation. In this study, however, iron-loaded neutrophils were shown to express more lactoferrin on the cell surface, compared to non-iron-loaded cells. The increased surface expression of lactoferrin may have also promoted neutrophil aggregation and subsequently adherence to endothelial cells. Moreover, the proadhesive P-selectin on endothelial cells was significantly upregulated after iron dextran treatment, but not after iron saccharate treatment, which may explain the differences in the transmigration experiments. *In vivo*[40] both iron saccharate and iron dextran maintain to be large iron-containing complexes, which makes the iron unavailable until processing by the reticuloendothelial system. However, there has been ongoing debate on the existence of labile iron on the surface of these complexes, which could be available to circulating cells before processing. In the study presented in this thesis, NTBI was detected in the medium as well as serum, supplemented with either preparation, indicating that both iron preparations contained labile iron. However, the nature of labile iron presented by iron saccharate may differ from that of iron dextran, which may lead to different activation state of cells, and the extent of neutrophil transendothelial migration.

The fact that iron enhances adherence and transendothelial migration of neutrophils through endothelial cells makes iron an important modulator in innate immune response. The true benefits and drawbacks of this enhanced adhesion and transmigration by iron complexes still have to be assessed in groups of patients receiving iron saccharate and iron dextran supplements, such as in end-stage renal disease patients. Moreover, although neutrophils generally are not detected in stable atherosclerotic plaques, their involvement in some stages of atherosclerotic development is plausible. For instance, myeloperoxidase secreted by neutrophils, is capable of catalysing LDL oxidation and has been abundantly found in atherosclerotic lesions.[41,42]

In addition, with detected pathogens like Cp and CMV in atherosclerotic plaques, the presence of neutrophils, as a major component of the innate immune response against intruding microorganisms could be expected. Therefore, the findings in this study may imply a role of iron in accelerating atherosclerotic lesion formation through neutrophil activation.

IRON AND PHAGOCYTE–MEDIATED LDL OXIDATION

LDL oxidation is a main event in atherogenesis. Copper and iron-catalysed free radicals are able to oxidise native LDL.[43] Concurrently, the two transition metals accumulate in atherosclerotic lesions.[44] Myeloperoxidase, a heme-containing enzyme produced primarily by neutrophils, and in a lesser extent by monocytes, is capable of catalysing LDL oxidation in the presence of hydrogen

peroxide. In this study (Chapter 8), the involvement of iron and iron chelators in the process of LDL oxidation were investigated. The iron chelators include deferoxamine, deferiprone and CP502. The latter has recently entered preclinical trials. Iron alone did not further enhance MPO/H_2O_2-driven LDL oxidation. Iron chelation did not interfere with MPO activity. However, all iron chelators could reduce the rate as well as the time of initiation of oxidation in both MPO/H_2O_2-driven and copper(II)-driven LDL oxidation reactions. These results suggest that iron may modulate the extent of LDL oxidation possibly by enhancing radical formation. These findings therefore imply that catalytically active iron in *in vivo* situations, may interact with the phagocyte-derived oxygen metabolites, enhancing the formation of atherogenic oxidised LDL, but may also enhance the phagocytic activity of neutrophils during bacterial infections. The study also demonstrated counteracting effects of iron chelators in the process of LDL oxidation.

CONCLUDING REMARKS

The inflammatory events leading to atherosclerosis and its associated clinical manifestations are influenced by various risk factors. *In vitro* studies described in this thesis show that several inflammatory responses are alterable by iron. Increased intracellular labile iron promotes intracellular oxidation-reduction imbalance that results in cell activation. The studies presented in this thesis point towards an immunomodulatory role of iron in inflammation. The beneficial function of iron for immune response is shown by enhanced phagocyte-endothelial cell interactions, leading to increased recruitment and transendothelial migration of phagocytes. On the other hand, together with its ability to promote oxidative modification of cholesterol, iron may have a significant contribution in aggravating atherogenesis, especially in the presence of other stressors, such as infections. In addition, this study has identified harmful effects of EDTA chelation therapy in combination with Vitamin C when being used for the treatment of coronary artery disease. On the basis of the findings in this thesis, iron depletion would be beneficial to reverse undesired inflammation conditions. The identification of the role of iron in inflammation suggests an additional factor to consider for prevention and therapy of inflammatory diseases, like atherosclerotic vascular disease as well as neurodegenerative diseases.

References

1. Sullivan, J.L. Iron and the sex difference in heart disease risk. *Lancet* **1**, 1293-1294 (1981).
2. Kartikasari, A.E.R., Georgiou N.A. & Marx J.J.M. Iron intake and cardiovascular disease in *Functional foods, cardiovascular disease and diabetes* (ed. Arnoldi A) 99-125 (Woodhead Publishing Ltd, Abington, Cambridge, UK and CRC Press LLC, Boca Raton, Florida, USA, 2004).
3. Loreal, O. *et al.* Determination of non-transferrin-bound iron in genetic hemochromatosis using a new HPLC-based method. *J Hepatol* **32**, 727-733 (2000).
4. Breuer, W. & Cabantchik, Z.I. A fluorescence-based one-step assay for serum non-transferrin-bound iron. *Anal Biochem* **299**, 194-202 (2001).

5. Kartikasari, A.E.R. *et al.* Endothelial activation and induction of monocyte adhesion by non-transferrin-bound iron present in human sera. *FASEB J.* DOI: 10.1096/fj.05-4700fje (2005).

6. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* **352**, 1685-1695 (2005).

7. Duffy, S.J. *et al.* Iron chelation improves endothelial function in patients with coronary artery disease. *Circulation* **103**, 2799-2804 (2001).

8. Failla, M. *et al.* Radial artery wall alterations in genetic hemochromatosis before and after iron depletion therapy. *Hepatology* **32**, 569-573 (2000).

9. Gaenzer, H. *et al.* Association between increased iron stores and impaired endothelial function in patients with hereditary hemochromatosis. *J Am Coll Cardiol* **40**, 2189-2194 (2002).

10. Zheng, H., Dimayuga, C., Hudaihed, A., & Katz, S.D. Effect of dexrazoxane on homocysteine-induced endothelial dysfunction in normal subjects. *Arterioscler Thromb Vasc Biol* **22**, E15-E18 (2002).

11. Ishizaka, N. *et al.* Iron chelation suppresses ferritin upregulation and attenuates vascular dysfunction in the aorta of angiotensin II-infused rats. *Arterioscler Thromb Vasc Biol* **25**, 2282-2288 (2005).

12. Ishizaka, N. *et al.* Iron overload augments angiotensin II-induced cardiac fibrosis and promotes neointima formation. *Circulation* **106**, 1840-1846 (2002).

13. Sullivan, J.L. Stored iron and vascular reactivity. *Arterioscler Thromb Vasc Biol* **25**, 1532-1535 (2005).

14. Zheng, H., Cable, R., Spencer, B., Votto, N., & Katz, S.D. Iron stores and vascular function in voluntary blood donors. *Arterioscler Thromb Vasc Biol.***25**, 1577-1583 (2005).

15. Rooyakkers, T.M. *et al.* Ferric saccharate induces oxygen radical stress and endothelial dysfunction *in vivo*. *Eur J Clin Invest* **32 Suppl 1**, 9-16 (2002).

16. Cheung, Y.F., Chan, G.C., & Ha, S.Y. Arterial stiffness and endothelial function in patients with beta-thalassemia major. *Circulation* **106**, 2561-2566 (2002).

17. Libby, P., Ridker, P.M., & Maseri, A. Inflammation and atherosclerosis. *Circulation* **105**, 1135-1143 (2002).

18. Kartikasari, A.E. *et al.* Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. *Arterioscler Thromb Vasc Biol* **24**, 2257-2262 (2004).

19. Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W., & Cabantchik, Z.I. Fluorescence Analysis of the Labile Iron Pool of Mammalian Cells. *Anal Biochem* **248**, 31-40 (1997).

20. Breuer, W., Epsztejn, S., & Cabantchik, Z.I. Dynamics of the cytosolic chelatable iron pool of K562 cells. *FEBS Lett* **382**, 304-308 (1996).

21. Roest, M. *et al.* Heterozygosity for a hereditary hemochromatosis gene is associated with cardiovascular death in women. *Circulation* **100**, 1268-1273 (1999).

22. Taylor, K.E., Giddings, J.C., & van den Berg, C.W. C-reactive protein-induced *in vitro* endothelial cell activation is an artefact caused by azide and lipopolysaccharide. *Arterioscler Thromb Vasc Biol* **25**, 1225-1230 (2005).

23. Campbell, L.A. & Kuo, C.C. *Chlamydia pneumoniae*--an infectious risk factor for atherosclerosis? *Nat Rev Microbiol* **2**, 23-32 (2004).

24. Belland, R.J., Ouellette, S.P., Gieffers, J., & Byrne, G.I. *Chlamydia pneumoniae* and atherosclerosis. *Cell Microbiol* **6**, 117-127 (2004).

25. Danesh, J., Collins, R., & Peto, R. Chronic infections and coronary heart disease: is there a link? *Lancet* **350**, 430-436 (1997).

26. 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* **96**, 4095-4103 (1997).

27. Visseren, F.L.J., Verkerk, M.S., Bouter, K.P., Diepersloot, R.J., & Erkelens, D.W. Interleukin-6 production by endothelial cells after infection with influenza virus and cytomegalovirus. *J Lab Clin. Med* **134**, 623-630 (1999).

28. Imhof, B.A. & urrand-Lions, M. Adhesion mechanisms regulating the migration of monocytes. *Nat. Rev Immunol* **4**, 432-444 (2004).

29. Jurado, R.L. Iron, infections, and anemia of inflammation. *Clin Infect Dis* **25**, 888-895 (1997).

30. McGeer, P.L. & McGeer, E.G. Inflammation and the degenerative diseases of aging. *Ann N Y Acad Sci* **1035**, 104-116 (2004).

31. Marchetti, B. & Abbracchio, M.P. To be or not to be (inflamed) - is that the question in anti-inflammatory drug therapy of neurodegenerative disorders? *Trends Pharmacol Sci* **26**, 517-525 (2005).

32. Lossinsky, A.S. & Shivers, R.R. Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review. *Histol Histopathol* **19**, 535-564 (2004).

33. Dietrich, J.B. The adhesion molecule ICAM-1 and its regulation in relation with the blood-brain barrier. *J Neuroimmunol* **128**, 58-68 (2002).

34. Ross,C.A. & Poirier,M.A. Protein aggregation and neurodegenerative disease. *Nat Med* **10 Suppl**, S10-S17 (2004).

35. Rozema, T.C. The protocol for the safe and effective administration of EDTA and other chelating agents for vascular disease, degenerative disease, and metal toxicity. *J Adv Med* **10**, 5-100 (1997).

36. Lamas, G.A. & Ackermann, A. Clinical evaluation of chelation therapy: is there any wheat amidst the chaff? *Am Heart J* **140**, 4-5 (2000).

37. Holden, C. Alternative medicine. NIH trial to test chelation therapy. *Science* **297**, 1109 (2002).

38. Bonetta, L. Trial tests chelation's effect on heart disease. *Nat Med* **8**, 906-907 (2002).

39. http://nccam.nih.gov/chelation/chelationstudy.htm. National Intitute of Health (2004).

40. Kooistra, M.P. *et al.* Nontransferrin-bound iron in the plasma of hemodialysis patients after intravenous iron saccharate infusion. *Eur J Clin Invest* **32 Suppl 1**, 36-41 (2002).

41. Zhang, R. *et al.* Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* **286**, 2136-2142 (2001).

42. Kougias, P. *et al.* Defensins and cathelicidins: neutrophil peptides with roles in inflammation, hyperlipidemia and atherosclerosis. *J Cell Mol Med* **9**, 3-10 (2005).

43. Heinecke, J.W., Rosen, H., & Chait, A. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest* **74**, 1890-1894 (1984).

44. Stadler, N., Lindner, R.A., & Davies, M.J. 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* **24**, 949-954 (2004).

SUMMARY

Atherosclerotic heart disease has claimed the lives of millions of people each year in the Western world, and rapidly increases in prevalence in many other places. The disease results from cholesterolemia, coupled with a chronic inflammatory condition of the vascular wall that lead to vessel occlusion and various clinical manifestations.

Elevated iron stores and high plasma iron concentrations have been linked to an increased risk of atherosclerosis. To this day, basic and clinical data continue to provide explanations for a link between iron and atherosclerosis (Chapter 1). This thesis has aimed to investigate the role of iron in the inflammatory events crucial in atherogenesis, especially in the course of phagocyte-endothelial cell interactions.

In vitro studies described in this thesis reveals an immunomodulatory function of iron in inflammation. Chapter 2 and 3 show that NTBI promotes accumulation of intracellular labile iron and production of oxygen-derived free radicals, leading to cell activation. Activation of endothelial cells is a well-characterised phenotype that leads to endothelial dysfunction *in vivo*, which not only will initiate the development of atherosclerosis, but also plays a role at a critical late step of thrombosis, promoting vessel occlusion and acute cardiovascular events. In combination with chronic infections, iron enhances infection-induced endothelial activation (Chapter 4), implying that in the presence of other stressors, iron may contribute significantly in aggravating atherogenesis. In addition, in Chapter 6, the drawbacks of EDTA chelation therapy particularly by inducing endothelial activation are described. The use of this alternative therapy for atherosclerosis therefore needs to be critically reconsidered, especially in regards to its effectiveness and safety. Furthermore, iron also promotes monocyte (Chapter 2 and 5) and neutrophil (Chapter 7) activation. An increased number of phagocyte infiltrates may thus complicate the progression of aterosclerotic vascular disease. Infiltration of monocytes in the blood-brain barrier also plays a role in neuroinflammation, mediating the development of neurodegenerative diseases. Finally, Chapter 8 demonstrates the involvement of iron in enhancing the rate and the production of oxidised LDL cholesterol, especially through interactions with phagocyte-derived oxygen metabolites.

Based on the findings in this thesis, iron depletion and iron chelation could be beneficial for such unfavourable conditions generated by iron. The identified modifying role of iron in inflammation described in this study (concluded in Chapter 9) offers an additional strategy for prevention and therapy of inflammatory diseases, like atherosclerotic vascular disease and neurodegenerative diseases.

SAMENVATTING

Atherosclerose eist ieder jaar vele miljoenen levens in de westerse wereld, en neemt hand over hand toe in vele andere gebieden. De ziekte wordt veroorzaakt door cholesterolemia gekoppeld aan een chronische staat van ontsteking van de bloedvat-wand, en leidt tot stremming van de bloedsomloop in bepaalde delen van het lichaam en verscheidene andere klinische manifestaties.

Verhoogde ijzer-voorraden in het lichaam en hoge ijzer concentraties in het plasma zijn in verband gebracht met een verhoogd risico op atherosclerose. Dit verband wordt ook nu nog ondersteund door fundamenteel en klinisch onderzoek (Hoofdstuk 1). Dit proefschrift heeft tot doel de rol van ijzer tijdens de voor atherosclerose cruciale ontstekingsfase te onderzoeken, in het bijzonder tijdens fagocyt-endotheliale cel interacties.

De *in vitro* studies beschreven in dit proefschrift onthullen een modulerende rol van ijzer in het afweersysteem tijdens ontsteking. In Hoofdstuk 2 en 3 is het aangetoond dat NTBI de ophoping van intracellulair onstabiel ijzer en de productie van zuurstof-afgeleide vrije radicalen bevordert, wat leidt tot activatie van cellen. Deze activatie is een goed gekarakteriseerd fenotype dat leidt tot disfunctie van de betrokken endotheliale cellen *in vivo*. Het initieert niet alleen de ontwikkeling van atherosclerose, maar speelt ook het een rol in een critische late stap in thrombose-vorming, waardoor vaatafsluiting en acute cardiovasculaire incidenten worden bevorderd. In combinatie met chronische infecties (Hoofdstuk 4) versterkt ijzer infectie-geinduceerde endotheliale activatie, wat impliceert dat ijzer, in aanwezigheid van andere stressoren, significant bij zou kunnen dragen aan de verergering van atherosclerose. Verder worden in Hoofdstuk 3 de nadelen van de EDTA chelatie therapie besproken, in het bijzonder in relatie tot endotheliale activatie. Het al dan niet gebruiken van deze alternatieve therapie bij het tegengaan van atherosclerose dient kritisch te worden heroverwogen vanwege twijfels over effectiviteit en veiligheid. Voorts bevordert ijzer ook monocyt (Hoofdstuk 2 en 5) en neutrofiel (Hoofstuk 7) activatie. Verhoogde aantallen fagocyt-infiltranten gedurende ontsteking zouden de voortgang van de atherosclerotische vaatziekten kunnen compliceren. De infiltratie van monocyten door de bloed-brein barriere speelt ook een rol in zenuwontsteking, en stimuleert de ontwikkeling van neurodegeneratieve ziekten. Ten slotte wordt in Hoofdstuk 8 gedemonstreerd dat ijzer de productie van geoxideerd LDL cholesterol verhoogt door interacties met uit fagocyten afkomstige zuurstof metaboliten.

Gebaseerd op de bevindingen gepresenteerd in dit proefschrift wordt gesteld dat ijzer depletie en ijzer chelatie voordelig kunnen zijn in condities waar ijzer ongewenste effecten als atherosclerose teweegbrengt. De gevonden modificerende rol van ijzer tijdens ontstekingen (beschreven in Hoofdstuk 9) draagt een nieuwe strategie aan voor de preventie en behandeling van ontstekingsziekten zoals atherosclerotische vaatziekten en neurodegeneratieve ziekten.

RINGKASAN

Penyakit jantung aterosklerosis mengancam hidup jutaan orang tiap tahun. Prevalensinya meningkat secara pesat, tidak hanya di negara-negara barat tetapi juga di berbagai negara lainnya. Penyakit ini disebabkan oleh penimbunan kolesterol dan selaras dengan suatu kondisi peradangan kronis pada dinding pembuluh darah, yang dapat menyebabkan penyumbatan pembuluh dan bermacam perwujudan klinis.

Berdasarkan observasi/pengamatan akan adanya tendensi terkena jantung koroner karena kandungan zat besi yang tinggi dalam tubuh, hingga saat ini banyak penelitian dasar dan penelitian klinis/kedokteran dilakukan untuk menjelaskan hubungan antara zat besi dan aterosklerosis (Bab 1). Tujuan penelitian pada buku ini adalah untuk mempelajari pengaruh zat besi pada proses peradangan kronis yang menyebabkan aterosklerosis, khususnya dalam proses interaksi antara sel fagosit dan sel endotel.

In vitro studi dalam thesis ini menggambarkan peranan zat besi pada peradangan kronis. Bab 2 dan 3 mendemonstrasikan bahwa NTBI dapat mencetuskan akumulasi zat besi di dalam sel dan produksi radikal bebas, yang menyebabkan pengaktifan sel. Aktifasi sel endotel adalah fenotipe yang menggambarkan kelumpuhan dari fungsi endotel pada pembuluh darah. Aktifasi sel endotel tidak hanya menstimulasi tumbuhnya aterosklerosis, tetapi juga dapat mencetuskan penyumbatan pembuluh darah yang mengakibatkan terjadinya serangan jantung. Dikombinasi dengan infeksi kronis (Bab 4), zat besi dapat meningkatkan pengaruh infeksi pada proses pelumpuhan dari fungsi endotel pada pembuluh darah. Disini diperlihatkan bahwa zat besi dapat memberikan kontribusi yang signifikan dalam mempercepat pertumbuhan aterosklerosis ketika berinteraksi dengan factor-faktor lain penyebab aterosklerosis. Sebagai tambahan, Bab 6 menjelaskan kemungkinan efek samping dari terapi EDTA khususnya dalam proses terjadinya aktifasi sel endotel. Dengan adanya penemuan ini, maka penggunaan terapi EDTA untuk aterosklerosis perlu dipertimbangkan lagi, terutama dari segi efektifitas dan keselamatannya. Selain sel endotel, zat besi juga dapat mengaktifasi sel fagosit (Bab 2, 5 dan 7). Aktifasi ini menyebabkan peradangan yang dapat mengkomplikasi/mempercepat pertumbuhan aterosklerosis. Pada pembuluh darah otak, peradangan ini juga dapat mencetuskan pertumbuhan penyakit neurodegenerasi. Bab 8 pada thesis ini menggambarkan pengaruh zat besi dalam meningkatkan laju dan produksi dari pengoksidasian kolesterol LDL/jahat, dengan cara membentuk radikal bebas dari produk metabolisme yang dihasilkan fagosit sel.

Berdasarkan penemuan-penemuan tersebut diatas, maka disarankan bahwa pengurangan zat besi dalam darah dapat bermanfaat untuk menghindari pengaruh buruk dari zat besi. Identifikasi atas pengaruh zat besi dalam proses peradangan kronis yang digambarkan dalam thesis ini (dikonklusikan di Bab 9) menawarkan strategi tambahan untuk pencegahan dan terapi penyakit-penyakit peradangan kronis, seperti penyakit aterosklerosis pada pembuluh darah dan juga penyakit neurodegenerasi.

ABBREVIATIONS

BSA	Bovine serum albumin
C282Y	The substitution of tyrosine for cysteine at amino acid position 282
CAD	Coronary artery disease
CMV	Cytomegalovirus
Cp	*Chlamydia pneumoniae*
CRP	C-reactive protein
CVD	Cardiovascular disease
DCF	2,7-dichlorofluorescein
E-selectin	Endothelial selectin
EDTA	Ethylenediamine tetraacetic acid
EGM-2	Endothelial growth medium-2
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GRO-α	Endothelial surface-bound melanoma growth stimulatory activity-α
HBBS	Hank's balanced buffer solution
HSA	Human serum albumin
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL-6	Interleukin-6
LFA-1	Lymphocyte function-associated antigen-1
LIP	Labile iron pool
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotctic protein-1
MOI	Multiplicity of infection
MPO	Myeloperoxidase
NTBI	Non-transferrin-bound iron
OxLDL	Oxidised low density lipoprotein
P-selectin	Platelet selectin
PBS	Phosphate buffered saline
PECAM-1	Platelet endothelial cell adhesion molecule-1
PMN	Polymorphonuclear leukocytes, neutrophils
SD	Standard deviation
SEM	Standard error of the mean
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell endothelial molecule-1
VLA-4	Very late antigen-4

CONTRIBUTORS

Eijkman-Winkler Centre

Apriliana E. R. Kartikasari
Niki A. Georgiou
J. Henny van Kats-Renaud
Sanne van Mullekom
Maarten de Geest
Joannes J. M. Marx

Department of Vascular Medicine

Frank L. J. Visseren

Department of Internal Medicine

B. Sweder van Asbeck

Department of Pulmonary Diseases

Laurien H. Ulfman

and Department of Haematology,
Thrombosis, and Haemostasis Laboratory
University Medical Centre Utrecht
Heidelberglaan 100, 3584CX, Utrecht
The Netherlands

Suzanne J. Korporaal

Department of Medical Microbiology,
and Immunology,
Diakonessen Hospital
Bosboomstraat 1, 3582 KE, Utrecht
The Netherlands

John J. M. Bouwman

ACKNOWLEDGEMENTS

After three years and a couple of months living in Holland, mostly for searching, learning, experimenting, thinking, back to experimenting, writing and finally a lot of writing… the work for this thesis is finally accomplished. I realise that what I have experienced here is truly through a lot of help from many people. Therefore I would like to acknowledge many people for their support and help.

I deeply thank my promoter, Prof. Jo Marx who has offered me the PhD position and provided me with full guidance as well as motivation. Even when he worked for 30%, he was always 100% there for me. Jo, you have inspired me to become scientifically sincere and critical. Thank you for all the enjoyable and exciting time we worked together. Those conferences have made me part of the 'international family' of the field. The Eijkman graduate school was also an unforgettable experience, the courses and especially the annual retreats. The day after my promotion will be your lecture, 'ironing the vessel wall', an excellent title Jo! Although the farewell is on the way, it is great that you continue collaborating in many projects with Eijkman Jakarta.

I also wish to thank Prof. Jan Verhoef for a wonderful opportunity to join the EWI. Thank you for your interest and support especially during my final year.

My deep gratitude goes to Dr. Niki Georgiou, who has guided me throughout my PhD days. Niki, thank you for being my co-promoter, for the guidance and help you have given me, and also for the sincere friendship and care. I really admire your attitude and intelligence. You were involved in many important aspects described in this thesis, and put a lot of effort to make this thesis a better form. I don't know if I could ever thank you enough, especially for those 'hang in there' spirits you have given me during the 'tough' last few months of my PhD. Niki, thank you! We have made it!!!

My gratitude also goes to Dr. Frank Visseren, for being my co-promoter, for his ideas and critical opinions on vascular knowledge, and also for getting collaborations going. Frank, thank you for all those positive thinking and support throughout these years.

I also thank Dr. Sweder van Asbeck for the 'radicals' spirit. Sweder, thank you for your ideas and support, lets find the 'python' there!

Henny, thank you for those experiments you have done for me, especially during the last year. I enjoyed our good time together, also with Jan. I am grateful to both of you for the care all this time. Moreover, Henny, thank you for being my paranymph, checking up my thesis and arranging the party!

Ružena, (used to be) my 'kamergenoot', my best friend, (and now) my paranymph, I really enjoyed the time we spent together, from staying late in the lab, travelling to Czech Republic, actually I wouldn't forget that 'spanking season' and your family, Jan Primus and the 'chasing' nights, cooking with Lucie, swimming with Maki, and a lot more. You have inspired me with your passion in this science world. Ruza, thank you for those nice times also for flying over all the way from the US to my promotion.

Sanne, my student, thank you for your hard work and effort doing the rolling! Maarten, thanks for the infection work, hope you graduate soon. Btw, do those orang-utans have names?

My 'oude kamergenoten', Annemarie, Gunnar, Dirk, Miguel, Bart, Ben, David, Peter-Jan, thank you for the warm welcome to the 'AIO kamer', and my deepest-best wishes for the recent groom Ewout and the bride Floor! My 'kamergenoten', Thuy-Nga, Annette, Jovanka, Maya, Ilse, and Anthony, I enjoyed the lively and fresh atmosphere, it is fun and inspiring! Best wishes and success for your PhDs.

Wil and Theo, thank you so much for your help and support during my stay in Holland. Both of you are like my parents here. You have helped me through these years!! The 'speedy' Piet, the man of 602, thank you for having me in the lab! More UMC estafette's to go? Machiel, really great photographs. Jos and Kok, thank you for the critical comments, and those monoclonal antibodies… Harm, Pauline, Frank, Jelle, Karla, Erik, Maartje, Suzan, Carla, Noortje, Marleen, Candida, Dimitri, Marcel, Sylvain, Bertie, Willem, Hans, Miriam, Mabel, Ellen, Karin, Irma and many more, thank you for the spirits, the lessons, the parties, BBQs, koepels, I had a real fun time in EWI…! Risma, actually I prefer to call you Ika… makasih Ka buat dinnersnya, gosip dan cerita-ceritanya, belanjanya, juga makasih buat Buyung, Anton dan Hendri, di jaman-jamannya Rijnlaan…. Henk and Loek, the computer masters, thank you for helping me with those impossible things. Jos and Roel, thanks for organising my 'orders'. Yfke, thank you for your help during my stay in Holland. Btw, you really are the best cook. Lenny and Rowena, thanks for making all those bureaucracy easy for me. Zwaniek, Daphne, Asli and Edhiyana, I enjoyed the time we spent during those conferences. Also my gratitudes for John, Laurien, Albert and more colleagues with whom I have enjoyed spending time in both working discussion and social way.

My special friend Leo. I enjoyed those amsterdam's nights, the cookings, all of our fights and laughs! The lovely 'family', Sana, Elsa, Elena, Giorgos, Vicky, Lorenzo, Fernando, Meindert, Duart, Luca, Timo, Gianluca, Aart, Eyk, Leo, Pipit, Maarten, Jeroen, and others. I have lots of real fun times with you all, partying, travelling, sporting, teasing each other, actually also learning from each other. I hope that our friendship stay for good. Nyoman, I had a great time in England, thanks for coming over from Australia. My regards to everyone in Brisbane.

My gratitude also goes to the group from the Eijkman Institute Jakarta, especially to Prof. Sangkot Marzuki, who has guided me from the very beginning of my scientific career, and convinced me to learn biochemistry. Prof, thank you for your patience, I don't think I would have gone this far without your guidance. My special gratitude to Dr Alida and Dr. Ning, I have really learned so much from both of you in thalassemia laboratory. Saya juga ingin berterimakasih untuk Dr Hera, mbak Emi, dan semua teman-teman di Eijkman atas pertolongannya.

Finally, I would like to thank my parents, who has supported me all these years, although I was always far away. My sister, mbak Ita, she has been an angel, supporting and helping me for a lot of things. Also my gratitudes to mbak Ida, mas Heri, mbak Lisna, and little Nafa. I enjoyed all the time spent with you all. To Ms. and Mr. van Boxtel, and all of the family, thank you for the warm welcome in the family and also for all of the support. I feel grateful to get to know how sincere dutch families are.

Last but not least, I would like to thank the person who is very special and always nearby to me. Jeroen, thank you for your patience and support. You have given me a tremendous amount of help in preparing this book, from reading most of my drafts critically, translations, staying up late, until checking up the final version. Thank you.

PUBLICATIONS

Kartikasari AER, Georgiou NA, Visseren FLJ, van Kats-Renaud JH, van Asbeck BS, Marx JJM. Endothelial activation and induction of monocyte adhesion by non-transferrin-bound iron present in human sera. The FASEB Journal. Express article. DOI 10.1096/fj.05-4700fje. 2005.

Kartikasari AER, Georgiou NA, Visseren FLJ, van Kats-Renaud JH, van Asbeck BS, Marx JJM. Intracellular labile iron modulates adhesion of human monocytes to human endothelial cells. Arteriosclerosis, Thrombosis and Vascular Biology. 24(12): 2257-62. 2004.

Kartikasari AER, Georgiou NA, Marx JJM. Iron intake and cardiovascular disease. In Functional foods, cardiovascular disease and diabetes, Arnoldi A (edt.) CRC Press, Boca Raton FL USA, ISBN 0-8493-2559-5, and Woodhead Publishing Ltd, Cambridge UK, ISBN 1-85573-735-3. Chapter 6: 99-125. 2004.

Kartikasari AER, Georgiou NA, Visseren FLJ, van Kats-Renaud JH, van Asbeck BS, Marx JJM. Iron and atherosclerosis. Biomarkers and Environment. 6 Suppl 1:34-38. 2003.

Keenihan SH, Gramzinksi R, Ratiwayanto S, Hadiputranto H, Riberu W, Soebianto S, Rusjdy F, Syafruddin D, Kartikasari AER, Djojosubroto M, Setianingsih I, Harahap A, Krisin, Fryauff D, Richie T, Charoenvit Y, Marwoto HA, Kumar S, Hoffman S, Marzuki S, Baird K. Plasmodium falciparum. Mechanisms of innate and acquired protection against Plasmodium falciparum in Javanese transmigrant adults and children newly resident in malaria-endemic Northwest Papua. Advances in Experimental and Medical Biology. 531:83-102. 2003.

Duggleby RG, Kartikasari AER, Wunsch RM, Lee YT, Kil MW, Shin JY, Chang SI. Expression in Escherichia coli of a putative human acetolactate synthase. Journal of Biochemistry and Molecular Biology. 33(3):195-201. 2000.

ABSTRACTS

Kartikasari AER, Georgiou NA , van Mullekom S, Visseren FLJ, van Kats-Renaud JH, van Asbeck BS, Marx JJM. Non-transferrin bound iron alters adhesion of human monocytes to human vascular endothelium under shear stress. European Journal of Clinical Investigation, 35 Suppl 2:33. 2004.

Georgiou NA, Kartikasari AER, de Geest MC, Bouwman JJM, Visseren FLJ, van Asbeck BS, Marx JJM. Effect of iron on monocyte attachment to microorganism-infected human endothelial cells. European Journal of Clinical Investigation. 35 Suppl 2:33. 2004.

Kartikasari AER, Georgiou NA, Visseren FLJ, van Kats-Renaud JH, van Asbeck BS, Marx JJM. EDTA therapy modulates the expression of adhesion molecules essential in atherogenesis. European Journal of Clinical Investigation. 35 Suppl 2:48. 2005.

Georgiou NA, Kartikasari AER, Visseren FLJ, van Kats-Renaud JH, van Asbeck BS, Marx JJM. Involvement of iron chelators in low density lipoprotein oxidation. European Journal of Clinical Investigation. 35 Suppl 2. 2005.

CURRICULUM VITAE

Apriliana Ellya Ratna Kartikasari was born on April 24th 1975 in Demak, Central Java, Indonesia. She grew up in Depok, West Java, a little but busy town near Jakarta, which she always regards as home. To be noted by the readers, the last name of the author is not a family name. It is not the custom of where the author came from to pass on a family name. There, people are more recognised based on their first or shorten name. In this case, the author is best known as April. After junior high school, the author started to study physics and life sciences at the Santa Ursula high school in Central Jakarta. In 1993, she studied chemical engineering at the Institute Technology of Bandung, Indonesia. April continued to study biochemistry in 1994, at the University of Queensland, Brisbane, Australia. She was awarded the degree of bachelor of science with an upper class honours on a final year project aiming to express and characterise a putative human acetolactate synthase, supervised by Dr. R. G. Duggelby, with the help of the head of the biochemistry department, Prof. Dr. J. de Jersey.

In 1998, she started working at CSIRO, Brisbane, Australia as a junior scientist on a project aiming to generate transgenic sugarcanes lacking polyphenol oxidase using antisense technology, under the supervision of Dr. J. E. Vickers and Dr. C. P. Grof. April returned to Indonesia in 2000 and joined the thalassemia research group, at Eijkman Institute for Molecular Biology, Jakarta, supervised by Dr. I. Setianingsih, Dr. A. Harahap, and Prof. Dr. S. Marzuki. In June 2002, she started the project as described in this thesis at Eijkman-Winkler Centre, University Medical Centre Utrecht, Utrecht, The Netherlands, under the supervision of Dr. N. A. Georgiou, Dr. F. L. J. Visseren, Dr B. S. van Asbeck and Prof. Dr. J. J. M. Marx