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CAN IRON CHELATORS INFLUENCE THE PROGRESSION OF ATHEROSCLEROSIS?

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□ *Epidemiological studies and experimental data suggest iron involvement in atherosclerosis. The relation between iron and atherosclerosis is complex and remains contradictory. In thalassemia patients, non transferrin bound iron (NTBI) and free hemoglobin (Hb) are present in plasma and may accelerate atherogenesis, but its progression may be inhibited by iron chelators.*

The mechanism whereby iron may stimulate atherogenesis has been intensively investigated. Non transferrin bound iron and sera from subjects with hemochromatosis induced endothelial activation with expression of vascular adhesion molecules and endothelial inflammatory chemokines. Such events could be inhibited by iron chelators and oxygen radical scavengers with intracellular activity. Iron chelators may be effective in preventing vascular damage in patients with high concentrations of NTBI as found in thalassemia.

Keywords Atherosclerosis, Hemochromatosis, Thalassemia, Non transferrin bound iron (NTBI), Hemoglobin (Hb), Endothelium, Monocytes, Iron chelators, Deferoxamine (DFO), Deferiprone (L1)

IRON NEEDS AND HAZARDS

Worldwide iron deficiency is the most important cause of anemia (1). A normal subject needs to absorb between 1 and 2 mg elementary iron from the diet, which should contain 10 to 15 mg iron per day to avoid iron deficiency. The diet is iron-deficient in many third world countries. As a

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consequence, food fortification with iron is a common practice in many countries, and food additives like multivitamins usually contain iron. These apparently beneficial measures may have a deleterious effect in patients with hereditary hemochromatosis (HH), and in anemias that are associated with iron overload (e.g., thalassemias), as well as in infectious diseases such as malaria and tuberculosis (2). Normally, iron in plasma is safely attached to transferrin. In patients with HH and iron overload anemias non transferrin bound iron (NTBI) can be detected in plasma. *In vitro* experiments have demonstrated that NTBI is able to generate hydroxyl radicals. The exact chemical structure of NTBI is, however, unknown, and several molecular species occur (3). It has been shown that patients with HH and thalassemia, who seemed to be effectively treated, had NTBI in their plasma (4,5). In patients with severe hemolysis, including thalassemias and malaria, free hemoglobin (Hb) that exceeds the binding capacity of haptoglobin could have similar or even worse effects compared to NTBI.

ATHEROSCLEROSIS

Atherosclerosis is an inflammatory condition (6) and the primary cause of heart disease and stroke, claiming the lives of millions of people each year in developed countries, and rapidly increasing in prevalence in developing countries (7). The atherosclerotic lesion is preceded by adhesion of monocytes (MN) to the vascular endothelium and their infiltration into the subendothelial space. Expression of adhesion molecules on both cell types causes a rolling interaction by selectins, followed by a firm attachment by means of integrins. Adherent MN migrate into the subendothelial space under the influence of chemoattractant molecules, and differentiate into foam cells, macrophages loaded with oxidized low-density lipoproteins (oxLDL), forming fatty streaks and lesions of the vessel wall. The classical risk factors for atherosclerosis, such as hypercholesterolemia, smoking, male gender, hypertension, diabetes and age, have been suggested by epidemiological observations. However, other risk factors such as the individual immune system and iron may be critical in the development of atherosclerosis and its clinical outcome (8). In Table 1, molecular mechanisms and gene products are listed that are involved in atherogenesis (9–11). Many molecules are part of a very complex network (12), and iron may be able to interfere with this network in many ways.

IRON AND ARTERIAL DISEASE: EVIDENCE FROM EPIDEMIOLOGY

In 1981, Sullivan (13) postulated that iron depletion can protect against ischemic heart disease. This hypothesis was an attempt to explain the male

TABLE 1 Processes and Molecules Involved in Atherogenesis

LDL accumulation in subendothelial matrix:

- ApoB (interaction of LDL with matrix proteoglycans)
- ApoA (accumulation of lipoprotein A)
- 15-LO, 5-LO, iNOS, MPO, Lipases (LDL oxidation)

Antioxidant properties of HDL:

- PON1, PAF-AH, ApoA1 (degrade oxidized phospholipids)

Monocyte adhesion to endothelium:

- E-Selectin, P-Selectin, VCAM-1, ICAM-1, CCRI (heme, iron, oxidized LDL, homocysteine, estrogen, infection and diabetes formed AGEs may activate endothelial cells with expression of adhesion proteins)
- CS-1 (splice variant of fibronectin)
- VLA-4, MCP1 (adhesion proteins on MN)

Lymphocyte adhesion to endothelium:

- VCAM-1, CXCR3, IP10, ITAC, Mig (on endothelial cells)
- CS-1 (splice variant of fibronectin)
- VLA-4 (on lymphocytes)
- IFN- γ (induced chemokine production)

Foam cell formation:

- M-CSF (macrophage differentiation)
- CRP, SR-A, CD36 (uptake of oxidized LDL)
- ABCA1, ApoE, ACAT (cholesterol efflux from macrophage)
- FAS, p53 (apoptosis of cholesterol-loaded macrophage)

Smooth muscle cell proliferation:

- CD40, OncostatinM (MN and macrophages)
- CD40L, IFN- γ (proinflammatory), IL-4, IL-10, IL-13 (anti inflammatory), (modulation of atherosclerosis by T cells: IFN- γ reduces 15-LO, while IL-4 and IL-13 induce)
- Estrogen (reduce lipoprotein levels, stimulate prostacyclins, NO production)
- PDGF, Angiotensin II (from hypertension)
- Homocysteine, CRP (induced by infection)

Instability of atherosclerotic plaques:

- IFN- γ (inhibits the production of matrix by SMC)
- MMPs (from macrophages, degrade extracellular matrix)
- Oxysterols, BMPs (calcification of matrix-scaffold secreted by pericyte-like cells)
- CD40-CD40L (coagulation cascade, thrombosis)

LDL: low-density lipoprotein; ApoB: apolipoprotein B; ApoA: apolipoprotein A; 15LO: 15-lipoxygenase; 5-LO: 5-lipoxygenase; iNOS: inducible nitric oxide synthase; MPO: myeloperoxidase; HDL: high-density lipoprotein; PON1: serum paraoxonase-1; PAF-AH: Platelet activating factor acetyl hydrolase; ApoA1: apolipoprotein A-1; E-selectin: endothelial selectin; P-selectin: platelet selectin; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1; CCRI: chemokine receptor 1; CS-1: splice variant of fibronectin; VLA-4: very late antigen-4; MCP1: monocyte chemoattractant protein-1; CXCR3: chemokine (C-X-C motif) receptor 3; IP10: Interferon- γ -inducible 10 kD protein; ITAC: interferon-inducible T cell- α chemoattractant; Mig: monokine induced by interferon- γ ; IFN- γ : interferon γ ; M-CSF: macrophage colony-stimulating factor; CRP: C-reactive protein; SR-A: class A scavenger receptors; CD36: multifunctional membrane protein, acts as a scavenger receptor on macrophages; ABCA1: ATP-binding cassette, sub-family A (ABC1), member 1; ApoE: apolipoprotein-E; ACAT: acylcholesterol acyl transferase; FAS: TNF receptor superfamily, member 6, mediator of the mitochondrial caspase-dependent pathway; P53: tumor suppressor protein p53; CD40: integral membrane protein, member of the tumor necrosis factor superfamily; CD40L: ligand for CD40 (CD154); IL: interleukin; PDGF: platelet-derived growth factor; SMC: smooth muscle cells; MMPs: matrix metalloproteinases; BMPs: bone morphogen proteins.

gender risk, and the subsequent loss of the protective effect of the female gender menopause, in developing coronary heart disease. The results of epidemiological studies were conflicting regarding the association between dietary iron intake and cardiovascular disease (14–19). Such investigations have studied the risk of dietary iron, blood donations, indicators of iron stores, plasma iron, NTBI or mutations of the hereditary hemochromatosis gene (HFE) with disease parameters or death resulting from cardiovascular events or stroke. Studies were not always performed in a sufficiently old population or during a sufficiently long observation period. Obviously this often generated inconclusive results for iron involvement in slowly developing vascular pathology. In our group, we found that in a postmenopausal population, high serum ferritin values increased the risk for stroke (20), and high amounts of dietary heme iron (but not inorganic iron) gave an increased risk for myocardial infarction (21).

The original hypothesis of Sullivan (13) focused on the protective effects of iron depletion on ischemic heart disease, whereas later it gained a broader interpretation suggesting a deleterious role for excess iron in cardiovascular disease. A different approach of the “iron hypothesis” was chosen by our group, relating atherosclerotic disease (both cardiovascular and cerebrovascular) to a *genetic* non variable parameter, the C282Y mutation of HFE, the gene related to HH. It was detected that C282Y +/- *heterozygosity* alone was harmless (22), but it was a strong and independent risk factor for atherosclerosis-related early mortality, only if complicating factors such as smoking or hypertension existed (23). Both conditions relate to production of oxygen radicals that may have detrimental effects on vascular cells through the oxidation of biomolecules such as lipids and proteins. In a normal postmenopausal population aged 49 to 70 years at enrollment, NTBI was not a risk factor for atherosclerotic disease during a median follow-up of 4.3 years (24), but its values were in a much lower range than observed in iron overload disorders. In a large population study no increased frequency of cardiovascular disease was found for HH homozygotes (25). Such patients, however, have iron-poor macrophages compared to normal subjects (26) due to a higher release of Fe(II) by the ferroportin transporter, as a result of low hepcidin production in HH hepatocytes. Paradoxically, HH homozygotes might be protected against atherogenesis as their foam cells also should not be able to accumulate iron.

Atherosclerosis-related vascular complications in β -thalassemia (thal)/Hb E [β 26(B8)Glu→Lys, GAG→AAG] patients may result from iron induced oxidation of lipoproteins (27). Epidemiological studies on the impact of consistently high NTBI and free Hb in plasma of thalassemia patients on atherosclerotic disease do not yet exist, but this may become possible in the growing population of aging patients who were effectively treated with iron chelators.

IRON AND ATHEROSCLEROSIS: EXPERIMENTAL DATA

The cholesterol accumulating in foam cells is derived from oxidatively modified oxLDL in plasma that can be generated by iron- and copper-catalyzed free radical production (28). 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 (29). Ferritin is highly expressed in atherosclerotic lesions (30). Iron is co-localized with ceroid, an insoluble complex of oxidized-lipid and protein, in foam cells and smooth muscle cells (31). In hypercholesterolemic rabbits, iron overload stimulated the formation of atherosclerotic lesions (32). Dietary iron restriction also protected apoE-deficient mice from developing such lesions (33). Finally, iron chelation in experimental rabbits showed an anti atherosclerotic effect by reducing plaque formation (34).

Vascular endothelial dysfunction is able to initiate the development of atherosclerosis and promotes thrombosis that leads to vessel occlusion and acute cardiovascular events (6). Endothelial activation induces the expression of vascular adhesion molecules and inflammatory chemokines from endothelial cells. This in turn stimulates blood cells like MN and T-lymphocytes to attach and migrate into the subendothelial space. Several studies have shown the importance of iron to induce early functional and structural vascular abnormalities due to endothelial dysfunction (35) that is associated with induction of oxidative stress. Oxygen radicals may be involved in the regulation of transcription factors, such as nuclear-factor κ B (NF- κ B) (36), important for the transcription of a large number of genes like endothelial adhesion molecules and inflammatory cytokines. It was recently shown that deferoxamine (DFO) inhibits low-density lipoprotein (LPS)-induced, reduced nicotinamide adenine dinucleotide (NADPH) oxidase-mediated oxidative stress and NF- κ B activation, and adhesion molecule expression in a murine model of local inflammation by inhibiting p22^{phox} protein expression (37). Iron *in vitro* up regulates interleukin-6 (IL-6) production by endothelial cells (38), while iron chelators inhibit tumor necrosis factor- α (TNF- α)-mediated up regulation of endothelial adhesion molecule expression (39,40). Moreover, radical species may also impair nitrogen monoxide (NO) production, leading to the condition of arterial stiffness (41). Nitrogen monoxide has been shown to mediate anti atherosclerotic 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 (42), or by iron depletion (43). Erythrophagocytosis by macrophages was observed in atherosclerotic lesions (44), mediating the accumulation of heme iron, and possibly activation of MN. Moreover, iron deposits also stimulate more macrophage infiltration to the atherosclerotic lesions (45).

EXPERIMENTAL STUDIES ON THE EFFECTS OF IRON AND IRON CHELATORS ON MONOCYTE-ENDOTHELIUM INTERACTION

We have studied effects of different chemical forms of NTBI on the interaction of MN with human umbilical vein endothelial cells (HUVEC) that are functional arteries (46). The HUVECs were isolated and cultured as described by Jaffe *et al.* (47), with minor modifications. Peripheral blood mononuclear cells (PBMC) were isolated from donor blood (Sanquin Blood Bank, Utrecht, The Netherlands) by Ficollpaque density gradient centrifugation. Two different methods of MN isolation were used depending on the amount of cells needed. For cytoadherence assay, MN were isolated by counter current centrifugal elutriation (48), while for detection of the integrins, the negative immunoselection MN isolation kit (MiltenyiBiotec, CLB Sanquin, Amsterdam, The Netherlands) was used. Further details on all methods used can be found elsewhere (46,49).

An increase in the level of intracellular labile iron, due to incubation with low molecular weight iron, induced adhesion of MN to endothelium, which is a crucial event in atherosclerotic plaque formation. A concordant increase in the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and endothelial selectin (E-selectin) on endothelial cells, as well as very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) on MN was observed. Furthermore, inclusion of membrane-permeable iron chelators and radical scavengers resulted in a complete inhibition of these effects of iron. These studies support that intracellular labile iron may thereby contribute to the pathogenesis of atherosclerosis, by promoting MN adherence to endothelial cells through the production of oxygen-derived radicals.

The effects of NTBI were studied using a wide range of concentrations of Fe(II) and Fe(III), mostly bound to citrate. However, the behavior of NTBI may be different in plasma. Therefore, we have performed experiments on endothelial activation and induction of MN adherence to endothelium using human sera (49). Non transferrin bound iron has been detected in iron overload diseases (50,51). This form of iron may exert pro-oxidant effects and modulate cellular function and inflammatory response. Measured by a recently developed high-throughput fluorescence-based assay (52), serum NTBI was found to be higher in both homozygotes of HFE C282Y mutation of HH (7.9 ± 0.6 mol/L, $p < 0.001$) and heterozygotes (4.0 ± 0.5 mol/L, $p < 0.001$), compared to controls (1.6 ± 0.2 mol/L). The effects of these sera on MN adhesion and endothelial activation were examined. Adhesion of normal human MN to C282Y homozygote- and heterozygote-serum-treated HUVEC was higher (25.0 ± 0.9 and $22.1 \pm 0.7\%$, respectively) compared to controls ($17.6 \pm 0.5\%$, 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 oxygen 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 MN-endothelial interactions that may also contribute to the development of atherosclerosis and neurodegenerative diseases.

CAN IRON CHELATORS INFLUENCE PROGRESSION OF ATHEROSCLEROSIS IN THALASSEMIA?

This question cannot yet be answered. In severe forms of thalassemia, NTBI is higher and more toxic than in HH. However, it should be realized that in normal subjects some Hb is always present in plasma that is safely bound to haptoglobin, and rapidly removed from plasma by the liver (53,54). We could demonstrate *in vitro* that release of Hb by macrophages is part of physiological iron kinetics (26). During hemolysis there is excess release of Hb from circulating red blood cells, or after erythrophagocytosis by macrophages, exceeding the binding capacity of haptoglobin. In addition to NTBI, free Hb occurs that is very toxic for the endothelium (55). Following hemolysis, Hb is rapidly oxidized to metHb that easily releases heme. Free hydrophobic heme can readily enter endothelial cells and iron will be liberated by heme oxygenase (56). Endothelial cells up regulate heme oxygenase-1, releasing Fe(II) from heme together with carbon monoxide (CO) and the antioxidant biliverdin, as a defense against this very toxic molecule (57,58). Toxic Fe(II) can be sequestered by ferritin that is up regulated as well. This labile Fe(II), however, is also accessible for iron chelators, in particular for low-molecular-weight chelators such as deferoxamine (DFO). Such iron chelators may effectively prevent iron catalyzed oxygen radical mediated events like molecular damage and activation of the inflammatory cascade, including up regulation of endothelial adhesion proteins. Effects in chronic and acute hemolysis may differ considerably. In chronic hemolysis atherogenesis may prevail. Acute hemolysis, which can even occur against the background of iron deficiency, will generate an abundance of toxic heme and Fe(II), and may stimulate thrombotic events. This situation is relevant for regions with a high frequency of malaria and co-morbidity of severe iron deficiency and hereditary hemolytic anemias. In most studies, iron supplements that may increase NTBI, cause a higher risk of malaria mortality in children (59), probably by promoting cerebral vascular occlusion. In such situations iron chelators may be beneficial (60,61). Results of clinical studies, however, are contradictory and may depend on study design and the relatively slow penetration into cells of DFO compared

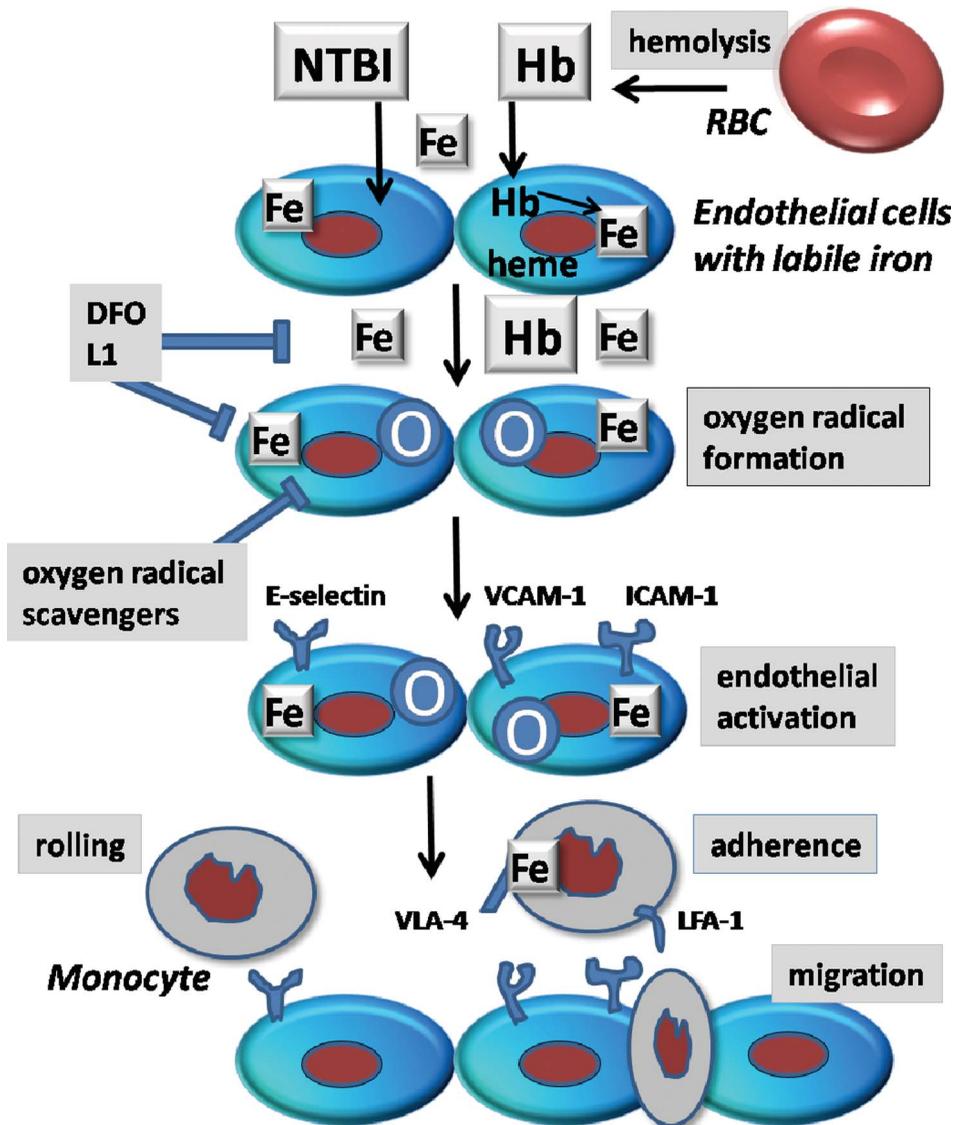


FIGURE 1 Hypothetical sequence of events initiated by NTBI and Hb leading to adherence of MN to endothelial cells. Non transferrin bound iron and Hb (after enzymatic release of iron from heme by hemoxygenase) increase the level of intracellular labile iron, and oxygen-derived free radicals are generated as a result. The radicals activate the intracellular machinery of endothelial cells leading to the expression of endothelial surface adhesion molecules. These adhesion molecules promote MN adherence to the endothelium. NTBI: non transferrin-bound iron; Hb: hemoglobin; RBC: red blood cell; Fe: iron; DFO: deferoxamine; L1: deferiprone; E-selectin: endothelial selectin; VCAM-1: vascular cell endothelial molecule-1; ICAM-1: intercellular adhesion molecule-1; VLA-4: very late antigen-4; LFA-1: lymphocyte function-associated antigen-1.

to L1 (62,63). In investigations on a possible beneficial effect of iron chelators on malaria outcome, the aim should not only be their interaction with iron in parasitized cells, but also on blocking the adherence of blood cells to endothelium. The impact of the use of iron chelators in hemolytic disorders goes beyond the aim of only prevention of atherosclerosis.

CONCLUSIONS

Toxic forms and quantities of NTBI and heme stimulate early atherogenic and probably late thrombotic events promoting cardiovascular morbidity and mortality. The sequence of events leading to increased expression of adherence proteins on endothelium is summarized in Figure 1. The atherosclerotic changes developing in aging thalassemia patients due to chronic exposure of vessel walls to increased amounts of NTBI and heme are not yet predictable. Patients with thalassemia major, receiving effective iron chelator treatment, may be better protected than less severe patients with thalassemia intermedia. Extensive laboratory, clinical and epidemiological studies are needed to investigate potential benefits of iron chelators for prevention of atherosclerotic damage in patients with acute and chronic hemolysis.

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