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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)

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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).

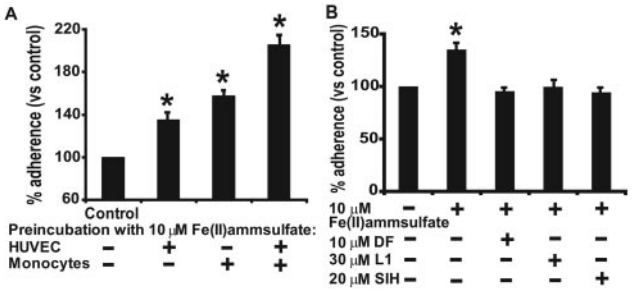

Figure 1. Effects of iron (A) and chelator-bound iron (B) on monocyte adhesion to endothelium (mean±SEM; n=6; *different from control, $P<0.05$). Source of iron was Fe(II)ammoniumsulfate with incubation time of 5 hours on HUVECs (A and B) and 1 hour on monocytes (A).

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

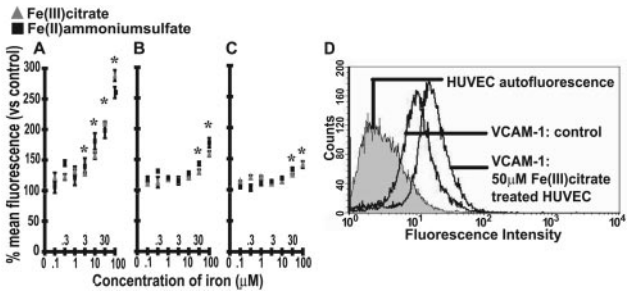

Figure 2. Effects of iron on the expression of endothelial adhesion molecules: VCAM-1 (A), ICAM-1 (B), and E-selectin (C) after 48 hours incubation (mean±SEM; n=6; *different from control, $P<0.001$). The mean fluorescence from monoclonal antibody against adhesion molecules, analyzed by FACS, correlates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

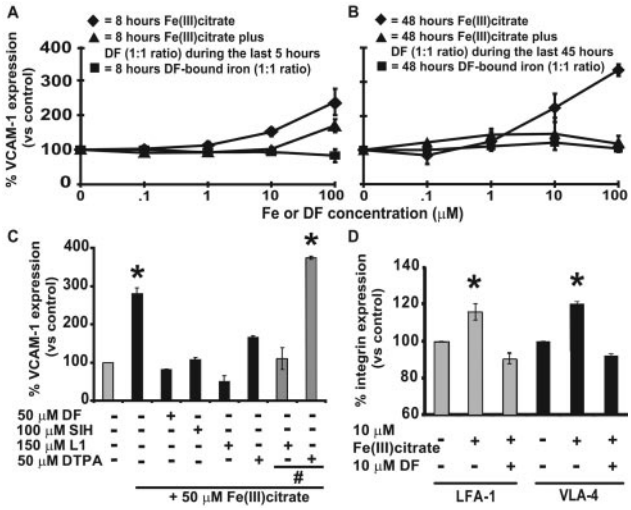

Figure 3. The expression of adhesion molecules on HUVECs or monocytes after treatment with iron or chelator-bound iron. VCAM-1 expression (mean±SEM; n=3) was lower in 8 hours (A) compared to 48 hours (B) 100 μmol/L Fe(III)citrate-treated HUVEC ($P<0.001$; n=6). Forty-five hours (B) but not 5 hours (A) 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

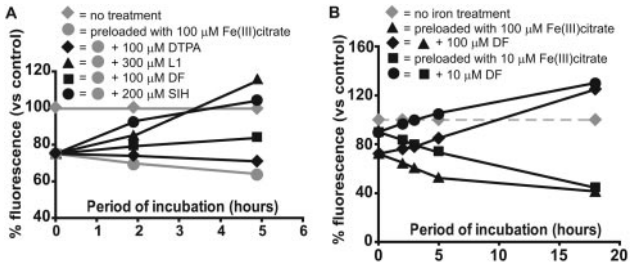

Figure 4. The calcein-AM assay for assessing the membrane permeability of iron chelators. One hundred percent of fluorescence means no calcein quenching by iron; <100% shows calcein quenching. The time needed for the chelators to enter HUVECs is illustrated as the time to dequench calcein signal after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

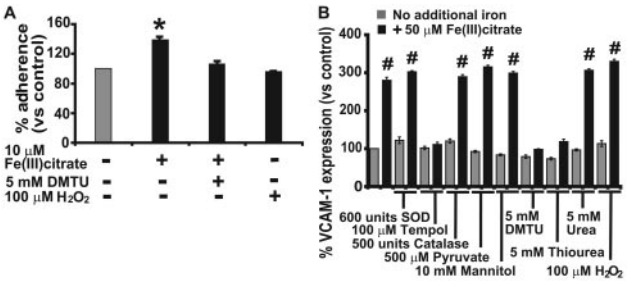

Figure 5. Effects of radical scavengers on iron-activated HUVECs, shown by the cytoadherence assay of monocytes to HUVECs (A; mean±SEM; n=5; *different from control, $P<0.05$) and VCAM-1 expression on HUVECs (B; mean±SEM; n=6; #different from control, $P<0.001$).

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 $OH^\cdot$ 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.

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INTRACELLULAR LABILE IRON MODULATES ADHESION OF HUMAN MONOCYTES TO HUMAN ENDOTHELIAL CELLS

(Online-Supplement)

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METHODS

HUVEC isolation and culture

HUVEC were isolated and cultured as described by Jaffe et al.[36] with minor modifications. Briefly, the umbilical vein was canulated at each end and washed gently with phosphate-buffered saline (PBS). 0.05% trypsin containing 0.02% ethylenediamine tetraaceticacid (Life Technologies) was injected into the vein, and left at 37^{o}C for 20 minutes. The cells were collected by flushing the vein with PBS, continued with centrifuging the suspension at 250g for 10 minutes. The pellet was resuspended in endothelial cell basal medium (EBM-2, Clonetics®) supplemented with 2% fetal bovine serum. Cells were cultured in tissue culture flasks (Nunc), precoated with fibronectin (Roche). Culturing was carried on in a humidified 37^{o}C incubator with 5% CO_2, and confluent cells from passages 2-3 were used for all experiments.

Monocyte isolation

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 monocyte isolation were used depending on the amount of cells needed. For cytoadherence assay, monocytes were isolated by the counter current centrifugal elutriation[37] while for detection of the integrins, the negative immunoselection monocyte isolation kit (MiltenyiBiotec) was used according to supplier's instructions. Both method resulted in a purity of >90% as analyzed by flow cytometry. Purified monocytes were suspended at a concentration of 10^6 cells/ml in RPMI1640 (BioWhittaker) supplemented with 2mM L-glutamine and 0.2% human serum albumin (CLB Sanquin) prior to use.

Preparation of iron solutions and chelator-bound iron

Two different iron compounds were used in this study. A 10mM Fe(II)ammoniumsulfate (Merck) solution was made by dissolving the iron crystals in distilled water at room temperature. A 10mM 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-sterilized prior to use. Fe(III)citrate was used since 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: 10mM deferoxamine (DF) (Novartis) in PBS, 30mM deferiprone (L1) (Duchefa Biochemie) in PBS, 10mM diethylenetriamine pentaaceticacid (DTPA) (Sigma) in PBS, and 20mM 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. The ratio of iron to chelator in which chelator is fully-saturated by iron depends on the number of ligands provided by the chelator. Six ligands can bind to an iron atom. Three molecules of a bidentate iron chelator, like L1, therefore are required to fully chelate one iron atom, while two SIH molecules are necessary to saturate one iron atom as it is a tridentate chelator. Both hexadentate chelators, DF and DTPA, chelate iron fully in a 1:1 ratio.

The detectable iron from the EBM-2, which is the medium used to perform all of the experiments involving HUVEC, was measured by Vitros®, which is a technique routinely used in the clinical diagnostic laboratory of the University Medical Center Utrecht for measuring iron concentration in serum samples. 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 600nm. With no external iron addition, the baseline iron level was counted to be around 0.36μM. 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 to the iron concentrations used in this study.

In vitro cytoadherence assay

Monocytes and HUVEC were pretreated with different combinations of compounds of interest prior to 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 ($25x10^4$ 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. Fluorescence was read using a filter pair for excitation at 485nm and emission at 530nm in a cytofluor fluorescence microplate reader. 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 (FACS)

After treatment with various compounds of interest as mentioned earlier, or H_2O_2 (GmbH), urea (USB)), or either one of the scavengers: 1,3-dimethyl-2-thiourea (DMTU) (Aldrich Chemical), superoxide dismutase (SOD, Sigma), tempol (Sigma), catalase (Sigma), pyruvate (Fluka), mannitol (Merck), thiourea (OPG), HUVEC were first harvested by incubating with 0.2% trypsin-EDTA at 37^{0}C for 3 minutes. Analysis of proteins expressed on the surface of either monocytes 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), PE-conjugated VCAM-1 antibody (BDBiosciences), Cychrome-conjugated E-selectin antibody (BDBiosciences), FITC-conjugated LFA-1 antibody (Becton Dickinson), PE-conjugated VLA-4 antibody (BDBiosciences), for 30 minutes at 4^{0}C prior to the FACS analysis. Each flow cytometric measurement was performed using a Becton Dickinson FACScan and 10.000 events were analyzed.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) viability assay for HUVEC and trypan blue exclusion test for monocytes

Cellular viability of HUVEC was monitored by the MTT (Sigma) method.[40] Briefly, cells were seeded into a 96-well microtitre plate, and grown to confluence. The compound of interest was added to the plate over a range of concentrations (0-1000mM). 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.

Cellular viability of monocytes after various compound incubations was monitored using the trypan blue exclusion test. Briefly, 10µl cell suspension was mixed with 90µl trypan blue isotonic solution (0.2% w/v). Cell viability was determined using a haemocytometer under a microscope.

Calcein-AM assay

The permeability of iron chelators was followed by the calcein-AM assay.[41] 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. This property is useful to detect any intracellular labile iron.

In this assay, 100µM of Fe(III)citrate was loaded to confluent HUVEC for 1 hour before the addition of 0.125µM calcein-AM (30 minutes at 37^{o}C). After the signal was stabilized, the chelator of interest was added. This fluorescence signal (excitation =

485nm; emission = 530nm) 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 + standard error of mean (SEM). Differences in quantitative measures were tested for significance using the unpaired two-tailed student's t-test

TABLE I

Table I. HUVEC viability as measured by the MTT assay after 48 hours incubation with chelator or iron-saturated chelator

Iron chelator	TC_{50} (μM)		Iron : chelator ratio
	without iron	bound to iron	
DF	15 ± 2.3	>500	1 : 1
L1	100 ± 11.3	>500	1 : 3
SIH	50 + 4.6	200 + 12.3	1 : 2
DTPA	70 ± 5.7	400 ± 43.5	1 : 1

TC_{50}: concentration reducing HUVEC viability to 50%; DF: deferoxamine; L1: deferiprone; SIH: salicylaldehyde isonicotinoylhydrazone; DTPA: diethylenetriamine pentaaceticacid; MTT: 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; HUVEC: Human umbilical vein endothelial cells. The results are the mean from triplicates of three separate experiments.