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# Secretion of Ferritin by Iron-laden Macrophages and Influence of Lipoproteins

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Increasing evidence supports a role of cellular iron in the initiation and development of atherosclerosis. We and others reported earlier that iron-laden macrophages are associated with LDL oxidation, angiogenesis, nitric oxide production and apoptosis in atherosclerotic processes. Here we have further studied perturbed iron metabolism in macrophages, their interaction with lipoproteins and the origin of iron accumulation in human atheroma. In both early and advanced human atheroma lesions, hemoglobin and ferritin accumulation correlated with the macrophage-rich areas. Iron uptake into macrophages, via transferrin receptors or scavenger receptor-mediated erythrophagocytosis, increased cellular iron and accelerated ferritin synthesis at both mRNA and protein levels. The binding activity of iron regulatory proteins was enhanced by desferrioxamine (DFO) and decreased by hemin and iron compounds. Iron-laden macrophages exocytosed both iron and ferritin into the culture medium. Exposure to oxidized low-density lipoprotein (oxLDL, $\geq 50\,\mu g/mL$) resulted in $<20\%$ apoptosis of iron-laden human macrophages, but cells remained impermeable after a 24 h period and an increased excretion of ferritin could be observed by immunostaining techniques. Exposure to high-density lipoprotein (HDL) significantly decreased ferritin excretion from these cells. We conclude: (i) erythrophagocytosis and hemoglobin catabolism by macrophages contribute to ferritin accumulation in human atherosclerotic lesions and; (ii) iron uptake into macrophages leads to increased synthesis and secretion of ferritin; (iii) oxidized LDL and HDL have different effects on these processes.

*Keywords*: Atherosclerosis; Ferritin; Erythrophagocytosis; Macrophages; Lipoproteins; Lysosomal iron

## INTRODUCTION

Epidemiological and experimental studies suggest that development of atherosclerosis may be associated with the amount of iron storage in the human body.[1–2] Even though epidemiological evidence is still inconsistent, results from experimental studies clearly suggest that redox-active iron is pro-atherogenic. As we recently pointed out,[2] misconstruction of study populations and lack of good methods to assay tissue iron may be the major reasons. Further testing of the iron hypothesis also requires a better understanding of cellular iron metabolism in atherogenesis, particularly in macrophages.

Generally, iron found in serum accounts for only 0.004% of iron stored in the body and is readily bound by serum macromolecules such as transferrin, ferritin, haptoglobin, hemopexin and albumin. The majority of the iron in mammals is located intracellularly as erythrocyte hemoglobin and ferritin (68% and 27%, respectively, in a 70 kg man), which may be more important in atherogenesis.[3] Indeed, in apolipoprotein E (apoE)-deficient mice, iron accumulation occurs in atherosclerotic lesions, heart and liver in an age-dependent manner.[4] Hemoglobin deposits in atherosclerotic plaques are significantly associated with iron and lipid accumulation as well as

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lesion instability.[5] Hemeoxygenase-1 (HO-1) overexpression in vascular tissues reduces the iron overload and atheroma formation in the mice, thereby suggesting a role for vascular iron in atherogenesis.[6]

*In vitro* studies have shown that transition metals, particularly iron, are needed for the induction of low-density lipoprotein (LDL) oxidation by monocytes/macrophages.[7–9] Iron compounds, such as hemin and hemoglobin, alone or in the presence of low concentrations of $H_2O_2$, can induce LDL oxidation *in vitro*.[10–13] We and others earlier reported that macrophages, one of the major cellular components of the atheroma, are capable of endo- and exocytosing iron compounds, causing LDL oxidation.[14,15] Similarly, exposure to the iron chelator DFO suppressed ferritin-induced LDL oxidation by more than 60% in endothelial cell cultures.[16] Furthermore, interaction between iron--laden macrophages and LDL results in enhanced ceroid accumulation and foam cell formation, suggesting that LDL oxidized by iron-mediated reactions is poorly degraded in macrophage-derived foam cells.[17–19]

In atherosclerotic lesions and normal arterial tissues, the presence of both copper and iron in a catalytically active form has been reported.[20–22] Recently, a significant elevation of iron levels was demonstrated in both early and advanced human carotid atheroma using electron paramagnetic resonance. Iron accumulation in atherosclerotic lesions also positively correlated with cholesterol levels.[23] The evident iron and ferritin accumulation in atherosclerotic lesions occurs primarily in macrophage-rich areas.[14,18,23]

Other observations implying a role for iron in the atherosclerotic process include endothelial dysfunction,[24] early lesion formation,[25] iron-dependent human platelet activation and hydroxyl radical formation,[26] proliferation of smooth muscle cells,[27] impaired myocardial perfusion and functions[28] and apoptotic cell death in atheroma plaques.[29]

Although histopathological studies have demonstrated that accumulation of iron and iron-laden macrophages in atherosclerotic lesions is associated with atherogenesis,[6,18,20–22] abnormal iron metabolism of human macrophages and its atherogenic implications remain to be elucidated. It is well known that oxidized or aged red blood cells (RBCs) are removed from the circulation and atherosclerotic tissues by macrophages (erythrophagocytosis). Iron released by this process comprises most of the iron that is circulated in humans.[30]

In the present study, we investigated:

(i) erythrophagocytosis and its relation to ferritin accumulation in human atherosclerotic lesions;
(ii) the regulation of ferritin gene expression in iron-laden macrophages following erythrophagocytosis; and
(iii) the distribution of iron and ferritin in iron-laden human macrophages and the interaction between such iron-laden macrophages and lipoproteins.

## METHODS

### Cells

Human monocyte-derived macrophages (HMDMs) were isolated from healthy blood donors and grown in RPMI 1640 complete culture medium, as described previously.[19] J-774 cells, a murine macrophage cell line, were cultured in F10 complete medium. Macrophages were exposed to $Fe^{3+}$ (ferric chloride 0–100 μM or ferric ammonium citrate 0–100 μg/ml), hemin (0–100 μM) or oxidized red blood cells (20%, 0–80 μl/ml). Cell culture media and serum were from Invitrogen, Karlsruhe, Germany.

### Cell Viability and Apoptosis

In order to assess cell viability and characterize the cell death after various treatments, we used several assays that discriminate between apoptosis and necrosis. Necrotic cells were detected by their uptake of membrane-impermeable dyes propidium iodide (0.5 μg/ml) and trypan blue dye (0.1%).

HMDMs were cultured in complete medium as stated above in the presence of various concentrations of iron complexes and lipoproteins. Giemsa-stained cultures were prepared to analyze nuclear morphology by light microscopy. Apoptosis was also evaluated by DAPI staining with DAPI-containing mounting medium. A total of at least 200 cells from each sample were assessed according to typical morphological criteria of apoptosis.

### Immunocyto- and Histochemistry

J-774 cells and human macrophages were exposed to ferric iron for different time periods. Ferritin immunocytochemistry was performed as previously described.[30]

For immunohistochemistry, normal human arterial segments from mammary arteries ($n = 4$) and non-diseased parts of carotid arteries were used for experimental controls. Following carotid endarterectomy, segments of human carotid aorta were collected from patients ($n = 35$) with different degrees of carotid atherosclerosis. The samples were fixed in 10% phosphate-buffered formalin and 2–3 arterial segments of each sample were

embedded in paraffin. Routine hematoxylin and eosin staining were performed in all cases.

In order to localize ferritin and hemoglobin in macrophages of the vessel specimens, monoclonal anti-human CD 68 (a macrophage marker), polyclonal rabbit anti-human hemoglobin and rabbit anti-human ferritin antibodies were used for immunohistochemistry assays as described before.[29] In most experiments a peroxidase-conjugated secondary antibody was applied and peroxidase activity detected using diaminobenzidine (DAB). In some experiments an alkaline phosphatase-conjugated secondary antibody was employed and alkaline phosphatase activity visualized by 4-benzoylamino-2,5-diethoxybenzenediazonium chloride (Fast Blue BB).

Iron deposits on arterial tissue sections were examined by Perls' Prussian blue reaction as described previously.[14]

### Preparation of Oxidized Erythrocytes

It is known that patients with atherosclerosis often have elevated levels of lipid peroxidation in their erythrocytes, which may facilitate erythrophagocytosis.[2] The latter is the main way for senescent red blood cells to be removed from circulation and the process is closely associated with turnover of hemoglobin-derived iron and oxidative stress. The exposure of erythrocytes to UV light induces a low level of peroxidation of the erythrocyte membrane and by using such oxidized erythrocytes, erythrophagocytosis can be imitated in cultured macrophages.[31] In the present study, UV-oxidized erythrocytes (UVRBC) were prepared by UV irradiation (λ 254 nm) for 2 h, as previously described.[31]

### Preparation of Lipoproteins

LDL ($1.025 < d < 1.050$ g/ml, Apo-B 3.20 g/L, Apo-A-1 $< 0.28$ g/l) and HDL ($1.0\ 63 < d < 1.21$ g/ml, Apo-A-1 2.12 g/l, Apo-B $< 0.34$ g/l) were freshly isolated from human plasma by sequential ultracentrifugation.[32] The concentrations of apolipoproteins were measured with the Behring TurbiTimeSystem (Dade Behring Marburg GmbH, Marburg, Germany). To inhibit lipid peroxidation LDL and HDL were prepared in the presence of EDTA (1.4 mg/ml). LDL samples were photo-oxidized by UV irradiation (λ 254 nm) for 3 h at room temperature (UV-oxLDL).[14]

### RNA Isolation and Analysis

HMDMs and J-774 cells were treated as indicated, snap-frozen in liquid nitrogen, and stored at −80°C. TRIzol (GIBCO-BRL, Paisley, UK) was used for RNA isolation as specified by the manufacturer. Total RNA (10 μg) of control cells, or cells exposed to iron compounds or UVRBC, was electrophoresed through a denaturing 1.2% agarose gel with 2.2 mol/L formaldehyde and then transferred onto Hybond-N nylon membranes (Boehringer Mannheim, Indianapolis, IN, USA) in 10 × SSC. The membranes were hybridized with 10 pmol ferritin H-chain antisense oligonucleotide (5′-GGG GGT CAT TTT TGT CAG TGG CCA GTT TGT-3′),[33] which was labeled with digoxigenin nucleotides tailed with the DIG Oligonucleotide Tailing Kit. Signals were analyzed using the DIG luminescence detection kit (Boehringer Mannheim) according to the manufacturer's protocol.

### Electrophoresis Mobility Shift Assay (EMSA)

Following different treatments, J-774 cells were washed with cold PBS. Cytosolic extracts were prepared by resuspending cells in cold lysis buffer.[34] To get an IRE-probe, plasmid pSPT-fer was transcribed *in vitro* in the presence of [$^{32}$P]-UTP as described.[35] RNA–protein complexes were formed and resolved on native polyacrylamide gels as previously described and visualized by phosphor imaging.[34]

### Metabolic Labeling and Immunoprecipitation

After exposure to different substances as indicated, metabolic labeling and immunoprecipitation were done as described previously.[36]

### Fe Assays

After culturing for different time period in complete culture media with either iron compounds or erythrocytes, excreted iron and the iron content of macrophages were measured by atomic absorption spectrophotometry as described previously.[31] A modified Timm sulfide-silver method was used to localize cellular iron by light microscopy and electron microscopy, as described previously.[14,29]

### Ferritin ELISA

Excreted and cellular ferritin protein levels of human macrophages were assayed after incubation with iron, UVRBC and lipoproteins, as described before.[14,31]

### Statistics

All results are expressed as means ± SEM. Comparisons among groups were made by one-way ANOVA. When significance was detected, a *post hoc* Bonferroni multiple-comparison test was performed.

A probability value of less than 5% was considered to be significant (*$p < 0.05$, **$p < 0.01$).

## RESULTS

### Hemoglobin and Ferritin Accumulation Correlate with the Macrophage-rich Areas in Early and Advanced Human Atheroma

In order to examine the origin of iron depositions in atheroma, we studied hemoglobin and ferritin expression in normal and atherosclerotic arterial vessels from humans. In normal arterial tissues from mammary and non-diseased parts of carotid arteries there was no detectable ferritin immunoreactivity, but there were some weak hemoglobin positive signals. However, in early atherosclerotic tissues, there was evident immunoreactivity to hemoglobin (Fig. 1A) and ferritin (Fig. 1B) mainly in CD68-positive cells (macrophages), suggesting an ongoing erythrophagocytosis even in early lesions. This indicates that hemoglobin deposits are not necessarily a result of micro-haemorrhage in advanced atheroma. With atheroma progression, pronounced hemoglobin and ferritin accumulations were often seen in advanced lesions (Fig. 1C and D). Compared to intact early lesions ($n = 8$), advanced and ruptured atheroma plaques ($n = 18$) show more pronounced ferritin accumulation (ferritin positive areas estimated by image analysis are $16.48 \pm 5.69$ vs. $35.14 \pm 4.82$, respectively). Furthermore, hemoglobin deposit areas (Fig. 1E) are often positive to Perls' Prussian blue reaction (Fig. 1F), indicating that hemoglobin-derived iron contributes to iron accumulation in atheroma and consequently causes ferritin accumulation in the process of atheroma progression.

### Iron Compounds and Erythrophagocytosis Increase Ferritin mRNA and Protein Levels and Decrease IRP Binding Activity in Macrophages

The above results led us to the reason that hemoglobin catabolism and iron-uptake by macrophages might cause alterations in ferritin expression and localization. To examine this link in more detail we studied human macrophages which were iron-loaded by exposure to $Fe^{3+}$ or UVRBC, and analysed the expression of ferritin mRNA in these cells. Total RNA from macrophages, which had been loaded with $Fe^{3+}$ or UVRBC for 12 h, was subjected to Northern blot analysis using a ferritin H chain probe. Compared to controls (a), both $Fe^{3+}$ (b) and UVRBC (c) clearly increased ferritin mRNA in human macrophages (Fig. 2A).

We next examined the synthesis of ferritin by simultaneous metabolic labelling with [$^{35}$S]-methionine followed by immunoprecipitation (Fig. 2B). The iron chelator DFO dramatically decreased ferritin synthesis, implying that the synthesis is largely dependent on the size of the cellular pool of low molecular weight iron. Hemin had a stronger effect on ferritin synthesis compared to the same amount of iron loading (Fig. 2B, 100 μM for 4 h), suggesting that heme released during degradation of erythrocytes may serve as a potential inducer following erythrophagocytosis.

Regulation of ferritin synthesis by cellular iron levels largely occurs at the post-transcriptional level via binding of iron regulatory proteins (IRP-1 and IRP-2) to the iron responsive elements (IREs) of the ferritin mRNAs.[37] Electromobility shift assays (EMSA) using extracts from treated J-774 cells and radioactive IRE probes confirmed that $Fe^{3+}$ decreased and DFO increased IRP binding activity. Addition of β-mercaptoethanol artificially achieves

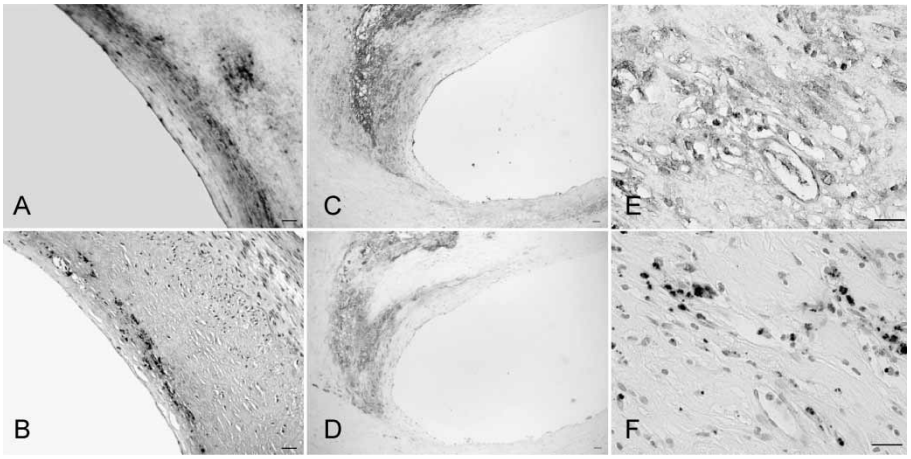


FIGURE 1 Hemoglobin (A, C, E) and ferritin immunohistochemistry (B, D) in serial sections of early (A, B) and advanced (C, D) human carotid atherosclerotic lesions. Note that hemoglobin and ferritin accumulation often correspond in atherosclerotic lesions, particularly in the advanced ones (C, D). Deposited hemoglobin in the lesions (E) led to iron accumulation in the same region (F). Alkaline phosphatase activity was visualized with Fast Blue in A (blue) and peroxidase activity was demonstrated with DAB in B, C, D, E (brown). Iron deposits are revealed as blue color stains by Perls' Prussian blue reaction (F). Bars = 20 μm in A, B, E and F and 40 μm in C and D.

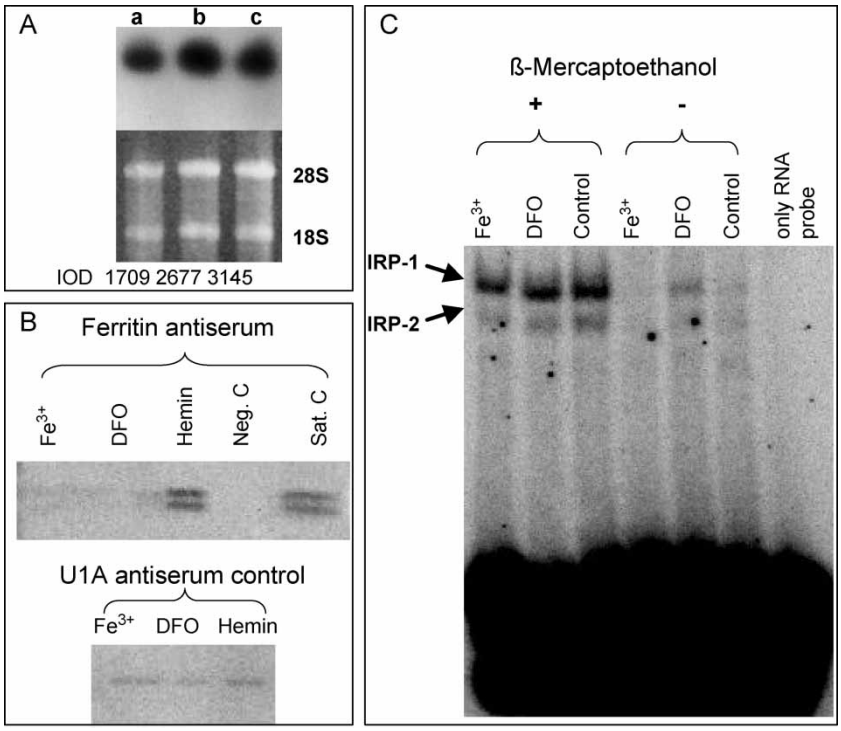


FIGURE 2 Upregulation of ferritin mRNA in human macrophages by $Fe^{3+}$ or UVRBC. For Northern blotting (A panel), 10 μg of total RNA was loaded in each lane. Ethidium bromide staining demonstrated equal amounts of 28S and 18S ribosomal RNA from the samples. Data from densitometry are tabulated below each figure. IOD stands for Integrated Optical Density. The (a), (b) and (c) stand for control cells, or cells treated for 12 h with 50 μM $Fe^{3+}$ or with 20 μl UVRBC, respectively. (B panel) shows effect of iron compounds on ferritin synthesis. Cells were treated with 100 μM of $Fe^{3+}$, Hemin or DFO for 4 h and ferritin synthesis was then measured by metabolic $^{35}S$-methionine labeling followed by immunoprecipitation. Equal amounts of samples were incubated with ferritin antibodies and U1A antiserum (non-regulated control). The deletion or 3-fold higher concentrations of ferritin antiserum were used as negative or saturated controls, respectively. In C panel the effect of iron compounds and DFO on IRP binding activity is shown by EMSA. Cells were treated with 100 μM of $Fe^{3+}$ or DFO for 4 h. Extracts were prepared from the treated and untreated cells. β-Mercaptoethanol (2%) was used to fully activate IRP binding and visualize total levels of IRPs.



full binding activity, and was used as a control to demonstrate that iron affected IRP at the binding activity level (Fig. 2C). These findings suggest that macrophage ferritin synthesis can be regulated at both the mRNA and translational levels by the DFO-binding iron pool.

### Exposure of Macrophages to Iron Compounds and Erythrocytes Increases Intravacuolar Iron and Results in Iron and Ferritin Excretion

To trigger iron uptake via different routes, human macrophages were first loaded for 12 h with varying amounts of iron complexes, followed by measurement of total cellular iron by atomic absorption spectrophotometry. Exposure to $Fe^{3+}$ or UVRBC increased the intracellular iron as expected. By ELISA analysis we observed a 2- to 4-fold increase in *de novo* synthesis of ferritin in the UVRBC or iron-laden human macrophages. However, we noticed simultaneous iron excretion from these cells, which may also affect the intracellular iron levels.

To directly visualize sub-cellular iron distribution and excretion in macrophages, these cells were assayed using the sulfide-silver method at both light- (Fig. 3A–C) and electro-microscopical levels (Fig. 3D–F) after exposure to iron compounds and UVRBC. Compared to control cells (Fig. 3A), a dramatic accumulation of lysosomal and cytosolic iron was detected (Fig. 3B). After careful washing and incubation of the iron-loaded macrophages in fresh culture medium for another 24 h, we observed a clear reduction of iron particles in these cells (Fig. 3C), suggesting an ongoing iron excretion. Electron microscopy demonstrated more iron particles in many dilated vacuoles and secondary lysosomes in UVRBC-exposed cells (Fig. 3E) compared to $Fe^{3+}$-treated cells (Fig. 3F), suggesting degradation of hemoglobin in the vacuolar compartments.

We next questioned whether or not iron excreted by the macrophages, after exposure to iron complex, was in the form of ferritin. ELISA analysis of supernatants for ferritin (Fig. 4A) clearly showed that macrophages excreted higher amounts of ferritin into the extracellular environment after pre-treatment with $Fe^{3+}$ or UVRBC. Exposure to $Fe^{3+}$ for different time periods revealed a gradual increase in exocytosed ferritin levels (Fig. 4A, lower panel). Loading of different amount of UVRBC for a fixed

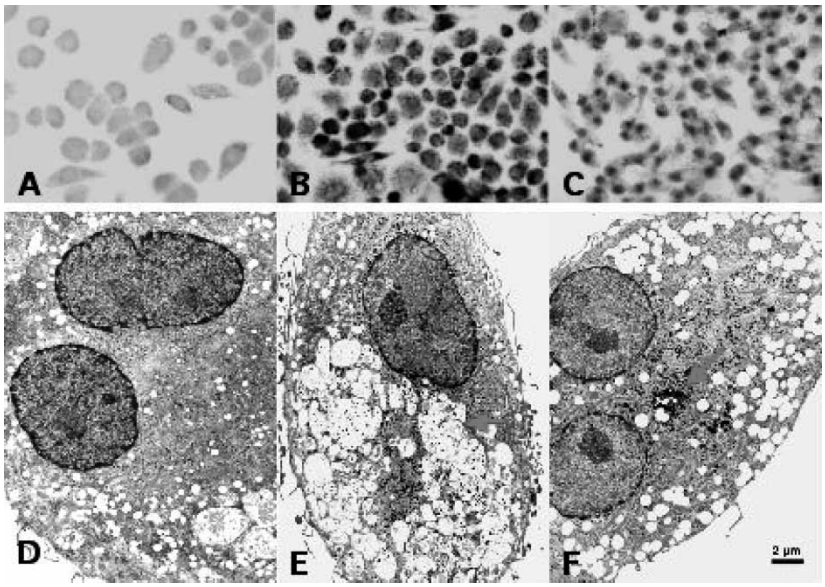


FIGURE 3 Cytochemical demonstrations of iron sequestration and excretion by macrophages at light microscopic (A–C) and electron microscopic levels (D–F). Precipitated silver particles indicate the location and amount of reactive-low-molecular-weight iron in the cells. Control cells only show occasional granular precipitates. Compared to control cells (A), cells after 12 h of exposure to 50 μM $Fe^{3+}$ show enhanced granular iron precipitates with a lysosomal-type distribution (B). After culturing in normal medium for another 24 h, the iron-loaded cells show much less silver precipitation of iron in their cytosol, although some larger positive particles remain, suggesting ongoing iron excretion by the cells (C). At the electron microscopic level, a control HMDM shows occasional precipitated silver granules in the vacuole compartment and cytosol (black spots seen in D). After exposure for 12 h to UVRBC (E) and $Fe^{3+}$ compound (F), cells show large amounts of vacuole and cytosolic silver granules, indicating iron sequestration in the cytosol and lysosomal vacuoles in HMDMs. Arrows indicate lysosomes (F) and lysosomal vacuoles (E). Note the UVRBC-exposed cell (E) contains many dilated vacuoles and lysosomal compartments that are filled with iron due to erythrocyte degradation (arrows).

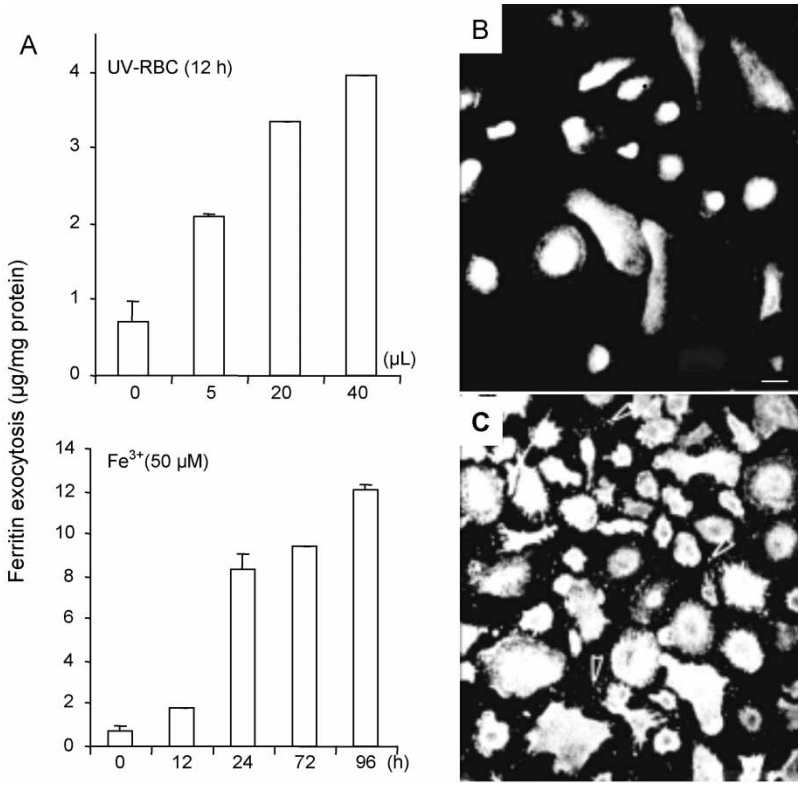


FIGURE 4 Ferritin excretion after $Fe^{3+}$ or UVRBC exposure. (A) Cells were exposed to different amounts of UVRBC for 12 h or to 50 μM $Fe^{3+}$ for different time periods. After exposure cells were rinsed and returned to standard culture conditions for another 24 h. Subsequently, conditioned medium was collected and ferritin content assayed using ELISA (normalized to total protein), and given as means ± SEM ($n = 2–4$). (B and C) Ferritin immunocytochemistry of human macrophages was visualized by confocal microscopy. B: control cells and C: cells exposed to 50 μM $Fe^{3+}$ for 48 h. Note the presence of ferritin particles intracellularly under both conditions. Ferritin was also found extracellularly in cells exposed to iron, as indicated by the arrowheads. Bar = 20 μm.

period of time (12 h) also gradually increased levels of secreted ferritin (Fig. 4A, upper panel). Compared to control cells (Fig. 4B), ferritin excreted (pointed by arrowheads) from iron-laden macrophages can be also visualized by ferritin immunocytochemistry (Fig. 4C).

### Effects of Lipoproteins on the Excretion of Iron and Ferritin by Iron-laden Macrophages

To study whether lipoproteins influence the above homeostasis of iron and ferritin, macrophages were first pre-incubated with $Fe^{3+}$ for 12 h and then treated with different forms of lipoproteins for another 24 h. As reported earlier,[19,31] the addition of iron (<100 μM $Fe^3$), hemoglobin (<25 μg/ml) or oxidized erythrocytes to human macrophages resulted in a minor loss of cell viability (>90% viable cells) as estimated by lactic dehydrogenase retention, propidium iodide assay and the trypan blue exclusion test. The iron loading treatments did not significantly affect cellular protein levels (data not shown here).

Subsequent exposure to UV-oxLDL (50 μg/ml for 24 h) caused apoptosis of some iron-laden human macrophages (<20%) without further loss of cell membrane permeability, as estimated by Giemsa staining, DAPI staining and the above-mentioned cell viability tests (Figs. 5 and 6).

Intriguingly, simultaneous immunocytochemistry demonstrated ferritin excretion in these cells parallel to the occurrence of apoptosis. Two categories of cells could be observed: one in which the macrophages have normal nuclei but excrete secretory granules with ferritin positivity (defined as type I cells, Fig. 6A–D), and the other in which the macrophages contain condensed/fragmented nuclei, are more shrunken and exhibit strong ferritin immunofluorescence in the absence of secretory structures (defined as type II cells, Fig. 6C and D). Neither of these cell types could be observed in cells not exposed to oxLDL. The result suggests that although oxLDL causes <20% apoptosis in iron-laden macrophages (Fig. 5), the observed ferritin secretion is the result of an active secretion rather than cell lysis, since all the iron- and lipoprotein-treated cells remain impermeable to the cell vital dyes, trypan blue and propidium iodide (>95% cells are viable in the group).

Changes in intra- and extracellular iron in human macrophages were assayed after exposure to different forms of lipoproteins. Using iron atom absorption spectrophotometry, a general decrease in intracellular iron and an increase of excreted iron was detected. Native LDL that had been incubated with iron-loaded macrophages for 24 h decreases intracellular iron excretion to the same extent as oxLDL (Fig. 7A). This is not surprising as native

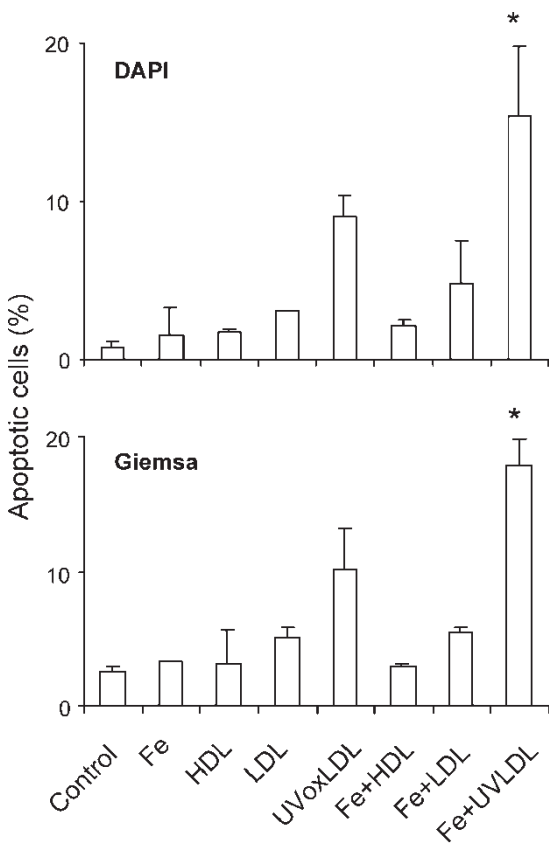


FIGURE 5 Morphological assessments of human macrophage apoptosis after exposure to iron compounds and lipoproteins. Human macrophages were cultured in complete culture medium in the presence or absence of 50 μM $Fe^{3+}$ for 12 h, rinsed and then exposed to 50 μg/ml LDL, 50 μg/ml oxLDL or 50 μg/ml HDL for another 24 h. The cells were stained with Giemsa or DAPI, respectively, as described in the "Methods" section. The figure shows the mean percentage of apoptotic cells ± standard deviation (SD). *$p < 0.05$ vs. Control, Fe, HDL, LDL, Fe + HDL, or Fe + LDL-treated cells.

lipoproteins may become oxidized after exposure to iron-laden macrophages for 12–24 h as demonstrated earlier.[14,19] Interestingly, HDL significantly decreased the intracellular iron content in iron-laden cells (Fig. 7A, *$p < 0.05$). This result suggests that HDL may play a role in binding and transportation of iron under atherogenic conditions, which is consistent with an earlier observation.[39]

We then studied the effects of different lipoproteins on ferritin excretion from iron-laden human macrophages. Incubation with oxLDL, but not native LDL, caused a significant increase in ferritin excretion from iron-laden cells when compared to control, Fe-, or Fe and native LDL-treated cells, whereas incubation with HDL resulted in a significant decrease of the excretion (Fig. 7B). A similar effect of HDL was observed in UVRBC-treated macrophages. The ferritin content in cell pellets remained at the same levels among the lipoprotein-treated cells. There was no significant difference in ferritin excretion between control cells and LDL-, oxLDL- or HDL-treated cells when iron

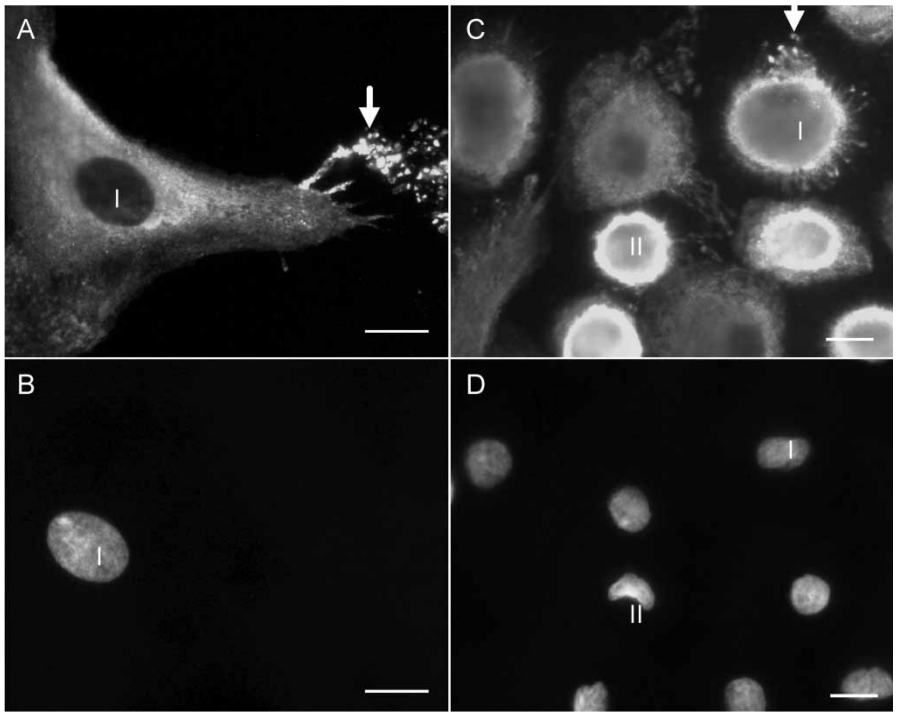


FIGURE 6 Ferritin immunocytochemistry and morphological assessment of apoptosis. Human macrophages were treated with 50 μM $Fe^{3+}$ for 12 h, rinsed and then exposed to 50 μg/ml oxLDL for another 24 h. The cells were then immunostained with ferritin antibody (A and C). Alterations in apoptotic nuclei, exhibiting highly fluorescent, condensed chromatin, were also evaluated by DAPI staining (B and D). Fluorescence microscopy showed that oxLDL treatment resulted in enhanced ferritin secretion from protrusion(s) of iron-laden macrophages (A and C, shown by arrows). Ferritin secretion particles appear mainly in normal cells (type I). Apoptotic cells (II) show increased ferritin immuno-fluorescence and condensed or fragmented nuclei as marked. Bars = 20 μm.



had not been added. Although native LDL may be oxidized by exposure to iron-laden macrophages it does not significantly affect ferritin secretion from iron-laden macrophages. These results suggest that oxidized LDL, but not native LDL, may perturb iron metabolism in iron-laden macrophages during development of atherosclerotic lesions.

## DISCUSSION

Monocytes/macrophages of the reticuloendothelial system recycle large amounts of iron from senescent erythrocytes and phagocytose damaged cells through several pathways. In this study we demonstrated, for the first time, that iron-laden human macrophages excrete ferritin following erythrophagocytosis or endocytotic uptake of iron compounds. Oxidized LDL and HDL have different effects on such excretion. The iron and ferritin exocytosed by iron-laden macrophages *in vivo* may be responsible for the oxidation of LDL and other oxidative damage to arterial cells observed in human atherosclerotic lesions.

Uptake of iron and erythrocytes clearly enhances ferritin expression at both mRNA and protein levels. Ferritin induction is believed to occur primarily at the translational level via inhibition of IRPs' binding to IREs within the ferritin mRNAs, but also occurs at the transcriptional level. In particular, heme released from degraded erythrocytes may initiate a strong induction of ferritin expression in arterial cells, as heme has been shown to be a potent regulator of IRP activity.

Accumulation of ferritin and redox-active iron has been observed in atheroma, mainly in macrophages and macrophage-derived foam cells.[13,28] This tissue iron may contribute to oxidative stress in atherogenesis in several ways, as suggested in previous studies.[13,39,40] In the present study, we demonstrate that induction of ferritin, at both mRNA and protein levels by iron and erythrophagocytosis, may lead to increased excretion of ferritin from human macrophages.

Furthermore, lipoproteins do influence this process. HDL causes a decrease in ferritin excretion from iron-laden macrophages. The mechanisms behind this are unclear, but may relate to the ability of HDL to bind metals. Previous work has demonstrated that apolipoprotein A-I-containing lipoproteins bind iron and copper tightly. Moreover, the apolipoprotein A-I in HDL fractions contains both transferrin and ceruloplasmin, which may contribute to inhibition of the *in vitro* oxidation of LDL by HDL.[38]

In contrast, oxLDL increases ferritin excretion from iron-laden human macrophages. In one earlier report, LDL was shown to influence cellular

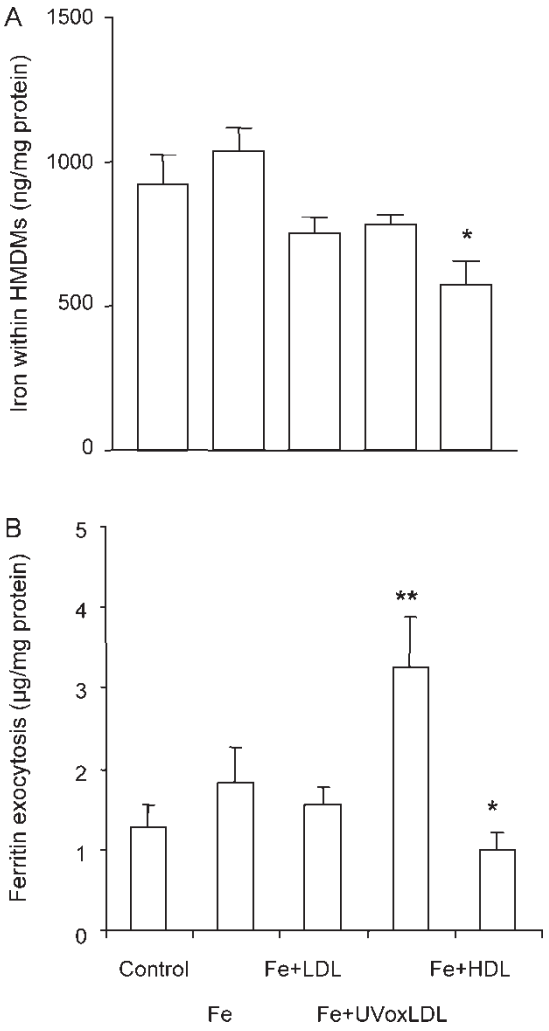


FIGURE 7 Effects of lipoproteins on intracellular iron content and ferritin excretion of human macrophages. Cells were exposed to 50 μM $Fe^{3+}$ for 12 h, rinsed and then exposed to 50 μg/ml LDL, 50 μg/ml oxLDL, or 50 μg/ml HDL for 24 h. Total cellular iron (A) was measured by atomic absorption as described in the Methods ($n = 4$). $^{*}p < 0.05$ vs. Fe-treated cells. Ferritin (B) in supernatant was assayed by ELISA ($n = 4$–6). $^{*}p < 0.05$ vs. Fe-treated cells. $^{**}p < 0.01$ vs. Control, Fe, or Fe + LDL-treated cells.

ferritin in a co-culture of non-iron-laden human endothelial and smooth muscle cells.[41] Our results further indicate that interaction of oxLDL and macrophages not only leads to foam cell formation but may also affect cellular iron homeostasis. These findings may shed new light on iron and ferritin accumulation in macrophage foam cells of atherosclerotic lesions and its implications in atherogenesis.

Hemoglobin, heme-compounds and worn-out erythrocytes may be linked to atherogenesis in several different ways. Others earlier demonstrated that lipid peroxidation of erythrocyte membranes is significantly higher in patients with coronary heart disease, whereas antioxidant enzyme activities, including those of superoxide dismutase and glutathione peroxidase, are lower than in controls.[42] The macrophage hemoglobin scavenger receptor (HbSR/CD163) is a key molecule in the process of removing hemoglobin released from ruptured erythrocytes. Released hemoglobin is bound to haptoglobin, which can be recognized by HbSR/CD163 and further endocytosed by monocytes and macrophages.[43] Kolodgie *et al.*[44] recently demonstrated an association between intraplaque hemorrhage, an increase in the size of the necrotic core and lesion instability in human coronary plaques. They also showed that an injection of autologous rabbit erythrocytes into existing lesions produced plaques with more crystallized cholesterol, lipid, iron accumulation and foam cells than controls. These findings, together with the results of the present study, strongly support our earlier working hypothesis that erythrophagocytosis and iron accumulation in macrophages contribute to the initiation and development of atheroma lesions via several mechanisms including hemoglobin catabolism-related iron metabolism, lipid peroxidation and foam cell formation.[13,30]

In conclusion, erythrophagocytosis and hemoglobin catabolism by macrophages are related to ferritin accumulation in human atherosclerotic lesions. Exposure of macrophages to iron compounds or erythrophagocytosis leads to increased synthesis and secretion of ferritin by human macrophages, and oxidized LDL and HDL have different effects on these processes.

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## *References*

[1] Sullivan, J.L. (1981) "Iron and the sex difference in heart disease risk", *Lancet* **1**, 1293–1294.
[2] Yuan, X.M. and Li, W. (2003) "The iron hypothesis of atherosclerosis and its clinical impact", *Ann. Med.* **35**, 1–15.
[3] Devlin, T. (1986) *Textbook of Biochemistry with Clinical Correlations*, 2nd Ed. (A Wiley Medical Publication, New York).
[4] Lee, T.S., Shiao, M.S., Pan, C.C. and Chau, L.Y. (1999) "Iron-deficient diet reduces atherosclerotic lesions in apoE-deficient mice", *Circulation* **99**, 1222–1229.
[5] Kolodgie, F.D., Gold, H.K., Burke, A.P., Fowler, D.R., Kruth, H.S., Weber, D.K., Farb, A., Guerrero, L.J., Hayase, M., Kutys, R., Narula, J., Finn, A.V. and Virmani, R. (2003) "Intraplaque hemorrhage and progression of coronary atheroma", *N. Engl. J. Med.* **349**, 2316–2325.
[6] Juan, S.H., Lee, T.S., Tseng, K., Liou, W.J.Y., Shyue, S.K., Wu, K.K. and Chau, L.Y. (2001) "Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development

of atherosclerosis in apolipoprotein E-deficient mice", *Circulation* **104**, 1519–1525.
[7] Witztum, J.L. (1994) "The oxidation hypothesis of atherosclerosis", *Lancet* **344**, 793–795.
[8] Xing, X., Baffic, J. and Sparrow, C.P. (1998) "LDL oxidation by activated monocytes: characterization of the oxidized LDL and requirement for transition metal ions", *J. Lipid Res.* **39**, 2201–2208.
[9] Paolicchi, A., Minotti, G., Tonarelli, P., Tongiani, R., De Cesare, D., Mezzetti, A., Dominici, S., Comporti, M. and Pompella, A. (1999) "Gamma-glutamyl transpeptidase-dependent iron reduction and LDL oxidation—a potential mechanism in atherosclerosis", *J. Investig. Med.* **47**, 151–160.
[10] Lachance, P.A. (1998) "Overview of key nutrients: micronutrient aspects", *Nutr. Rev.* **56**, S34–S39.
[11] Camejo, G., Halberg, C., Manschik-Lundin, A., Hurt-Camejo, E., Rosengren, B., Olsson, H., Hansson, G.I., Forsberg, G.B. and Ylhen, B. (1998) "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.
[12] Grinshtein, N., Bamm, V.V., Tsemakhovich, V.A. and Shaklai, N. (2003) "Mechanism of low-density lipoprotein oxidation by hemoglobin-derived iron", *Biochemistry.* **17**, 6977–6985.
[13] Miller, Y.I., Altamentova, S.M. and Shaklai, N. (1997) "Oxidation of low-density lipoprotein by hemoglobin stems from a heme-initiated globin radical: antioxidant role of haptoglobin", *Biochemistry.* **36**, 12189–12198.
[14] Yuan, X.M., Li, W., Olsson, A.G. and Brunk, U.T. (1996) "Iron in human atheroma and LDL oxidation by macrophages following erythrophagocytosis", *Atherosclerosis* **124**, 61–73.
[15] Fuhrman, B., Oiknine, J. and Aviram, M. (1994) "Iron induces lipid peroxidation in cultured macrophages, increases their ability to oxidatively modify LDL, and affects their secretory properties", *Atherosclerosis* **111**, 65–78.
[16] Satoh, T. and Tokunaga, O. (2002) "Intracellular oxidative modification of low density lipoprotein by endothelial cells", *Virchows Arch.* **440**, 410–417.
[17] Cromheeke, K.M., Kockx, M.M., De Meyer, G.R., Bosmans, J.M., Bult, H., Beelaerts, W.J., Vrints, C.J. and Herman, A.G. (1999) "Inducible nitric oxide synthase colocalizes with signs of lipid oxidation/peroxidation in human atherosclerotic plaques", *Cardiovasc. Res.* **43**, 744–754.
[18] Lee, F.Y., Lee, T.S., Pan, C.C., Huang, A.L. and Chau, L.Y. (1998) "Colocalization of iron and ceroid in human atherosclerotic lesions", *Atherosclerosis* **138**, 281–288.
[19] Yuan, X.M., Brunk, U.T. and Olsson, A.G. (1995) "Effects of iron- and hemoglobin-loaded human monocyte-derived macrophages on oxidation and uptake of LDL", *Arterioscler. Thromb. Vasc. Biol.* **15**, 1345–1351.
[20] Sorenson, E., Skiles, E.H., Xu, B., Aleryani, S. and Kostka, P. (2000) "Role of redox-active iron ions in the decomposition of S-nitrosocysteine in subcellular fractions of porcine aorta", *Eur. J. Biochem.* **267**, 4593–4599.
[21] Smith, C., Mitchinson, M.J., Aruoma, O.I. and Halliwell, B. (1992) "Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions", *Biochem. J.* **286**, 901–905.
[22] Evans, P.J., Smith, C., Mitchinson, M.J. and Halliwell, B. (1995) "Metal ion release from mechanically-disrupted human arterial wall. Implications for the development of atherosclerosis", *Free Radic. Res.* **23**, 465–469.
[23] Stadler, N., Lindner, R.A. and Davies, M.J. (2004) "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**, 1–7.
[24] Duffy, S.J., Biegelsen, E.S., Holbrook, M., Russell, J.D., Gokce, N., Keaney, J.F. and Vita, J.A., Jr. (2001) "Iron chelation improves endothelial function in patients with coronary artery disease", *Circulation* **103**, 2799–2804.
[25] Minqin, R., Watt, F., Huat, B.T. and Halliwell, B. (2003) "Correlation of iron and zinc levels with lesion depth in newly formed atherosclerotic lesions", *Free Radic. Biol. Med.* **15**, 746–752.
[26] Pratico, D., Pasin, M., Barry, O.P., Ghiselli, A., Sabatino, G., Iuliano, L., FitzGerald, G.A. and Violi, F. (1999) "Iron-dependent human platelet activation and hydroxyl radical formation: involvement of protein kinase C", *Circulation* **99**, 3118–3124.
[27] Porreca, E., Ucchino, S., Di Febbo, C., Di Bartolomeo, N., Angelucci, D., Napolitano, A.M., Mezzetti, A. and Cuccurullo, F. (1994) "Antiproliferative effect of desferrioxamine on vascular smooth muscle cells *in vitro* and *in vivo*", *Arterioscler. Thromb.* **14**, 299–304.
[28] Say, A.E., Gursurer, M., Yazicioglu, M.V. and Ersek, B. (2002) "Impact of body iron status on myocardial perfusion, left ventricular function, and angiographic morphologic features in patients with hypercholesterolemia", *Am. Heart J.* **143**, 257–264.
[29] Yuan, X.M. (1999) "Apoptotic macrophage-derived foam cells of human atheromas are rich in iron and ferritin, suggesting iron-catalysed reactions to be involved in apoptosis", *Free Radic. Res.* **30**, 221–231.
[30] Moura, E., Noordermeer, M.A., Verhoeven, N., Verheul, A.F. and Marx, J.J. (1998) "Iron release from human monocytes after erythrophagocytosis *in vitro*: an investigation in normal subjects and hereditary hemochromatosis patients", *Blood* **92**, 2511–2519.
[31] Yuan, X.M., Olsson, A.G. and Brunk, U.T. (1996) "Macrophage erythrophagocytosis and iron exocytosis", *Redox Rep.* **2**, 9–17.
[32] Havel, R.J., Eder, H.A. and Bragdons, J.H. (1955) "The distribution and chemical composition of ultracentrifugall separated lipoproteins in human serum", *J. Clin. Investig.* **34**, 1345–1353.
[33] Dhar, M., Chauthaiwale, V. and Joshi, J.G. (1993) "Sequence of a cDNA encoding the ferritin H-chain from an 11-week-old human fetal brain", *Gene* **126**, 275–278.
[34] Melefors, O., Goossen, B., Johansson, H.E., Stripecke, R., Gray, N.K. and Hentze, M.W. (1993) "Translational control of 5-aminolevulinate synthase mRNA by iron-responsive elements in erythroid cells", *J. Biol. Chem.* **268**, 5974–5978.
[35] Mullner, E.W., Neupert, B. and Kuhn, L.C. (1989) "A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA", *Cell* **58**, 373–382.
[36] Melefors, O. (1996) "Translational regulation *in vivo* of the *Drosophila melanogaster* mRNA encoding succinate dehydrogenase iron protein via iron responsive elements", *Biochem. Biophys. Res. Commun.* **221**, 437–441.
[37] Eisenstein, R.S. (2000) "Iron regulatory proteins and the molecular control of mammalian iron metabolism", *Annu. Rev. Nutr.* **20**, 627–662.
[38] Francis, G.A. (2000) "High density lipoprotein oxidation: *in vitro* susceptibility and potential *in vivo* consequences", *Biochim. Biophys. Acta* **1483**, 217–235.
[39] Kunitake, S.T., Jarvis, M.R., Hamilton, R.L. and Kane, J.P. (1992) "Binding of transition metals by apolipoprotein A-I-containing plasma lipoproteins: inhibition of oxidation of low density lipoproteins", *Proc. Natl Acad. Sci. USA* **89**, 6993–6997.
[40] Itakura, K., Oya-Ito, T., Osawa, T., Yamada, S., Toyokuni, S., Shibata, N., Kobayashi, M. and Uchida, K. (2000) "Detection of lipofuscin-like fluorophore in oxidized human low-density lipoprotein. 4-Hydroxy-2-nonenal as a potential source of fluorescent chromophore", *FEBS Lett.* **473**, 249–253.
[41] Brasen, J.H., Kivela, A., Roser, K., Rissanen, T.T., Niemi, M., Luft, F.C., Donath, K. and Yla-Herttuala, S. (2001) "Angiogenesis, vascular endothelial growth factor and platelet-derived growth factor-BB expression, iron deposition, and oxidation-specific epitopes in stented human coronary arteries", *Arterioscler. Thromb. Vasc. Biol.* **21**, 1720–1726.
[42] Van Lenten, B.J., Prieve, J., Navab, M., Hama, S., Lusis, A.J. and Fogelman, A.M. (1995) "Lipid-induced changes in intracellular iron homeostasis *in vitro* and *in vivo*", *J. Clin. Investig.* **95**, 2104–2110.
[43] Akkus, I., Saglam, N.I., Caglayan, O., Vural, H., Kalak, S. and Saglam, M. (1996) "Investigation of erythrocyte membrane lipid peroxidation and antioxidant defense systems of patients with coronary artery disease (CAD) documented by angiography", *Clin. Chim. Acta* **244**, 173–180.
[44] Madsen, M., Graversea, J.H. and Moestrup, S.K. (2001) "Haptoglobin and CD163: captor and receptor gating hemoglobin to macrophage lysosomes", *Redox Rep.* **6**, 386–388.