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Iron loading increases cholesterol accumulation and macrophage scavenger receptor I expression in THP-1 mononuclear phagocytes

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Abstract

Epidemiological studies have established that a high level of iron body stores is associated with increased risk of acute coronary heart disease. To explain this association, it has been proposed that iron catalyzes the production of highly reactive forms of free oxygen species, and thus, promotes low-density lipoprotein (LDL) oxidation, a lipoprotein that plays a critical role in atherogenesis. However, few studies have provided evidence to support this hypothesis. In the present study, we determined the effect of iron loading of THP-1 mononuclear phagocytes on LDL metabolism. We demonstrated that iron loading of THP-1 cells stimulated conjugated diene formation in LDL in the culture medium. In addition, iron loading of THP-1 cells significantly increased cholesteryl ester accumulation in cells exposed to native LDL, suggesting that during the incubation of the cells with native LDL, the LDL became oxidized and was taken up by the cells. We further demonstrated that the degradation of ^{125}I-oxidized LDL was significantly increased in iron-loaded THP-1 cells. Lastly, we demonstrated that iron loading of THP-1 cells stimulated scavenger receptor expression in these cells. In conclusion, this study demonstrates that loading of mononuclear phagocytes with iron leads to oxidization of LDL, increased cellular cholesterol accumulation and scavenger receptor expression, and supports the hypothesis that increased macrophage iron levels promote atherogenesis.

1. Introduction

In 1981, Sullivan [1] first reported that high levels of iron body stores were correlated with coronary heart disease (CHD) and he hypothesized that high iron stores are a risk factor for CHD. After this report, several epidemiological studies have been published supporting this hypothesis [2-7]. Furthermore, a study conducted by Salonen et al [8] in randomly selected eastern Finnish men further demonstrated that the association between high iron level and CHD was even stronger in men with high serum low-density lipoprotein cholesterol (LDL-C) than those with normal LDL, suggesting a synergistic role of high iron stores and high LDL-C. Because it is known that iron is a potent catalyst in lipoprotein oxidation, and the oxidized LDL (oxLDL) has been shown to be involved in atherogenesis [9,10], these studies led to the hypothesis that high levels of body iron stores may promote atherosclerotic coronary disease by catalyzing LDL oxidation. However, few laboratory studies have provided evidence to verify this hypothesis.

Oxidized LDL incubated with macrophages in culture stimulates cholesteryl ester accumulation in the cell [9,10] and leads to foam cell formation. The cholesteryl ester-laden foam cell is the hallmark of the early atherosclerotic lesion [11]. It has been demonstrated that oxLDL has the potential to promote the transformation of macrophages into foam cells [10,12,13]. Thus, it is conceivable that the enhancement of LDL oxidation by iron-loaded cells would lead to increased uptake of oxLDL by mononuclear phagocytes. In the present study, we have investigated the effect of iron loading of THP-1 monocytes on the LDL oxidation and

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subsequent uptake of oxLDL by the cells. We have also investigated the effect of cell iron loading on the expression of scavenger receptors. Our results show that iron loading not only increases LDL oxidation and LDL uptake, but also stimulates scavenger receptor expression.

2. Materials and methods

2.1. Culture of THP-1 monocytes

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Manassas, Va). The cells were cultured in a 5% CO_2 atmosphere in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum and penicillin, streptomycin, and amphotericin B (Sigma Corp, St Louis, Mo). The medium was changed every 3 days. The cell density in the culture dishes was always kept between 2×10^5 and 2×10^6 cells/mL. Cell viability was determined using trypan blue exclusion. Cells were not treated with phorbol esters before use in an experiment.

2.2. Iron loading with transferrin and measurement of intracellular ferritin

THP-1 cells were resuspended at 1×10^6/mL in phenol red–free IMDM supplemented with 100 μg/mL human holotransferrin (Sigma) and incubated at 37°C for 24 hours. After the incubation, the cells were sedimented by centrifugation at 1000 rpm for 5 minutes, the medium was discarded, and the cells were washed with ice-cold phosphate-buffered saline (PBS) containing 0.1% (wt/vol) bovine serum albumin. The cells were disrupted by adding 250 μL M-PER, lysis buffer (Pierce Biotechnology, Inc, Rockford, Ill) to the cell pellet, and the suspension was vortexed for 30 minutes and sonicated for 30 seconds at 4°C. The cell lysates were stored at −70°C until ferritin levels were determined. The ferritin concentration in the lysates was determined using Fer-Iron II immunoradiometric assay (Ramco Laboratories, Inc, Stafford, Tex); the polyclonal antibody used in this assay was raised against human spleen transferrin and recognizes both holo- and apoferritin. Transferrin addition to the media at 100 μg/mL led to an approximate 1.5-fold increase in the intracellular ferritin level after 24 hours of incubation. When higher transferrin concentration (200 μg/mL) was used for the iron loading, there was a significant decrease in the cell number and in trypan blue exclusion, suggesting a toxic effect of iron at high concentrations.

2.3. LDL isolation

Low-density lipoprotein was isolated from venous plasma of healthy volunteers using a single spin density gradient ultracentrifugation procedure as described in Ref. [14]. Briefly, the density of plasma was increased to 1.21 g/mL by the addition of solid potassium bromide, and an aliquot was layered under a nitrogen-saturated salt solution at d = 1.019 g/mL in a Beckman VTi50 polyallomer tube (Beckman Instruments, Palo Alto, Calif). The samples were centrifuged at 60 000 rpm at 7°C for 18 hours. The LDL band was aspirated, filter sterilized, and stored under a nitrogen atmosphere at 4°C in foil-covered glass vials until used.

2.4. LDL oxidation procedure

Oxidized LDL was prepared as described previously [14]. Briefly, the aqueous antioxidant EDTA used in the preparation of LDL was first removed by gel filtration. Immediately after chromatography, the in vitro oxidation reaction was initiated by the addition of $CuCl_2$ (40 μmol/L, final concentration). The absorbance at 234 nm was continuously monitored in a Beckman DU 640 spectrophotometer (Beckman Instruments, La Jolla, Calif) with the sample compartment heated to 25°C. The maximal rate of oxidation was calculated from the slope of the absorbance curve during the propagation phase and was expressed as absorbance units per minute. The extent of oxidation was measured as the difference between the initial and the maximal absorbance at 234 nm.

2.5. LDL metabolism in THP-1 cells

Macrophages (1×10^6) previously incubated for 24 hours with or without transferrin were incubated for an additional 24 hours with native LDL (250 μg protein/mL) in IMDM supplemented with 10% fetal bovine serum. After incubation with LDL, the cells were washed 5 times with ice-cold PBS. The lipids were extracted from the pellet with hexane/isopropanol (3:2, vol/vol) solution, each time for 45 minutes as described previously [15]. Free and total cholesterol levels in cellular lipid extracts were determined using gas chromatography as described previously [14]. Cholesteryl ester levels in the extracts were calculated as the difference between total and free cholesterol values. After lipid extraction, the cell pellet was solubilized with buffer containing NaOH (0.2 mol/L) and 1% sodium dodecyl sulfate, and protein content was determined by a modified Lowry procedure [16]. Cell cholesterol content was normalized by cell protein content.

2.6. Measurement of LDL oxidation

Cells previously loaded with iron as detailed above and control cells without iron-loading were incubated at a density of 1.5×10^6/mL with IMDM containing 250 μg/mL of native LDL protein. Native LDL, at the same concentration, also was incubated in cell-free medium and used as control. After incubation at 37°C for 24 hours, the cells were centrifuged at 1000 rpm for 5 minutes. The supernatants were removed from the pelleted cells and the density was raised to d = 1.063 g/mL. Low-density lipoprotein was isolated from the cell culture supernatants by ultracentrifugation as described above under LDL isolation. Conjugated dienes were measured using spectrophotometry at 234 nm as previously described [14].

2.7. Degradation and accumulation of radiolabeled oxLDL

Oxidized LDL was labeled with radioactive iodine (^{125}I) as described previously [17]. THP-1 cells (1×10^6) were incubated with ^{125}I-oxLDL (10 μg protein/mL medium) at 37°C for 24 hours. After the incubation, the cells were washed with PBS as described above. The medium was used to determine the rates of total, nonspecific, and receptor-mediated degradation of ^{125}I-oxLDL by THP-1 cells as previously described [18]. The above levels were corrected by subtracting spontaneous oxLDL degradation from the calculated values (small amounts of ^{125}I-labeled acid soluble material that was found in parallel incubations without cells). The intracellular accumulation (binding + internalization) of ^{125}I-oxLDL was determined in cells washed as described above and after lysing the cell pellets in 0.8 mL of NaOH (0.2 mol/L) containing 1% sodium dodecyl sulfate and measuring the amount of ^{125}I radioactivity associated with the cells. The same aliquot of the solubilized cells was used to determine cell protein. Values were corrected by cell protein.

2.8. Scavenger receptor expression

After the cells were loaded with iron, the medium was discarded and the cell pellet was washed twice with ice-cold PBS. The cells were then lysed in buffer containing HEPES (100 mmol/L, pH 7.9), NaCl (2 mol/L), $CaCl_2$ (20 mmol/L), $MgCl_2$ (25 mmol/L), 1.5% Triton X-100, phenylmethylsulfonyl fluoride (PMSF) (10 μL/mL), and leupeptin (10 μL/mL). Cell protein concentration was determined and 25 μg of cell lysate was electrophoresed in a 10% polyacrylamide gel. After the electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer, Boston, Mass) and the membrane was blotted with anti-scavenger receptor antibodies including rabbit anti-CD36, rabbit anti–macrophage scavenger receptor I (MSR-I), goat anti–lectin-like oxidized lipoprotein recepter-1 (LOX-I), goat anti–class B scavenger receptor-1 (SR-BI), and rabbit anti–MSR-II. All the antibodies except rabbit anti–MSR-II were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Rabbit anti–MSR-II was prepared in our laboratory. After washing, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin G (IgG) (Calbiochem, San Diego, Calif) or horseradish peroxidase-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology) [13]. The receptors were visualized by incubating the membrane for 1 minute with Western Lightning chemiluminescence reagents and exposure to x-ray film for 15 seconds to 1 minute (Perkin Elmer).

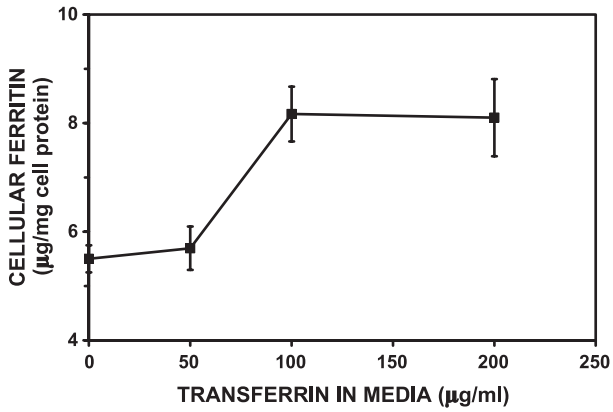

Fig. 1. Iron loading of THP-1 cells. THP-1 cells were incubated with 0 to 200 μg/mL human holotransferrin at 37°C for 24 hours. After the incubation, the cells were lysed and cellular ferritin concentration was determined as described in "Materials and methods" section. Results shown are mean ± SD of 3 experiments with each incubation conducted in quadruplicate.

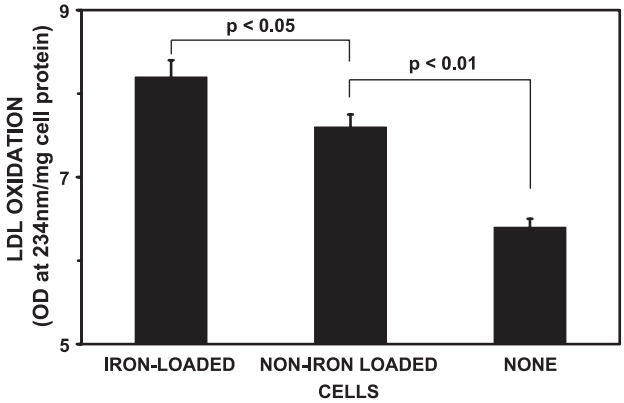

Fig. 2. Oxidation of LDL by iron-loaded THP-1 cells. Iron-loaded and non–iron-loaded cells at density 1.5×10^6/mL were incubated with IMDM containing 250 μg LDL protein per milliliter of native LDL. Native LDL at the same concentration in IMDM without any cells served as a control. After incubation at 37°C for 24 hours, the LDL in the supernatants was isolated by raising the density of the media to d = 1.063 g/mL and centrifugation as described in "Materials and methods" section. Conjugated dienes were measured at 234 nm using spectrophotometry. The data presented are mean ± SD of 3 experiments conducted in triplicate. Low-density lipoprotein protein recovery in the ultracentrifuge supernatant averaged 94%.

2.9. Statistical analyses

To compare the cholesterol amount between iron-loaded and control cells, Duncan test for several independent samples was used. In studies of LDL accumulation and degradation, the groups were compared using the 2-tailed unpaired Student t test. Differences were considered significant when $P \leq .05$.

3. Results

3.1. Increased iron loading in THP-1 cells

To determine the optimal concentration of transferrin for adequately promoting iron loading of THP-1 cells, the cells were incubated with increasing concentrations (0-200 μg/mL) of transferrin for 24 hours. Fig. 1 shows a concentration-dependent increase of intracellular ferritin after incubation of THP-1 cells with transferring for 24 hours. The increase in the intracellular ferritin concentration reached a plateau with transferrin concentrations between 100 and 200 μg/mL. Transferrin at 200 μg/mL resulted in decreased cell protein concentration and trypan blue exclusion, and therefore, was considered toxic to the

cells. Thus, 100 μg/mL was used as the optimal transferrin concentration to promote iron loading in THP-1 cells.

3.2. Iron loading of THP-1 cells increases LDL oxidation

To determine the effect of iron loading of THP-1 cells on cell-mediated LDL oxidation, native LDL was incubated with iron-loaded THP-1 cells for 24 hours, or with control cells not previously loaded with iron. The LDL was then recovered from the conditioned medium for analysis of conjugated dienes. Fig. 2 shows that LDLs exposed to iron-loaded cells have significantly higher concentrations of conjugated dienes than those exposed to control cells that

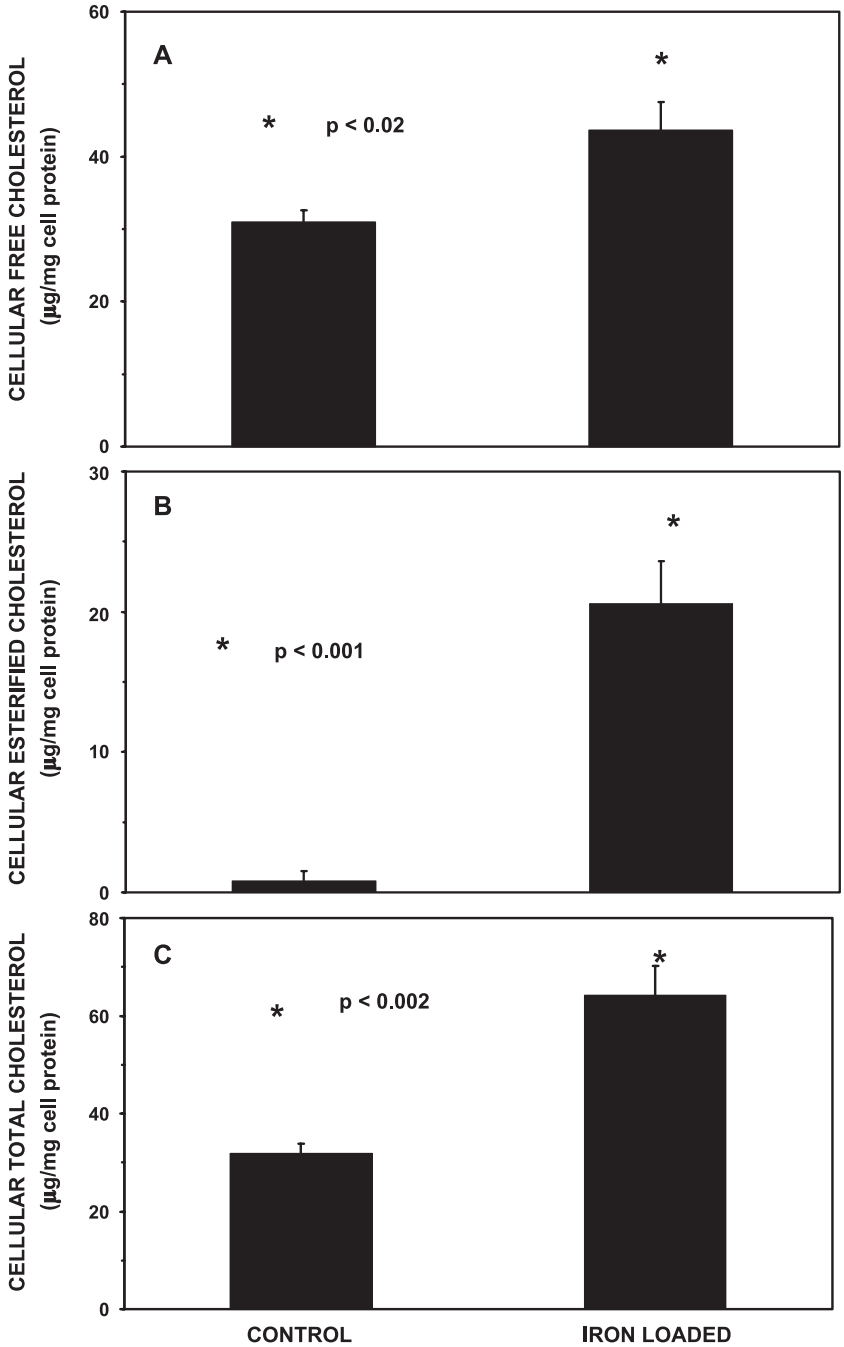

Fig. 3. Cholesterol mass accumulation in iron-loaded THP-1 cells and in control THP-1 cells not loaded with iron. Native LDL was added to cell culture media at 250μg LDL protein/mL and the cells were incubated for 24 hours as described in the "Materials and methods" section. After the incubation period, cellular lipids were extracted and cellular-free (panel A), esterified (panel B), and total cholesterol (panel C) levels were determined using gas chromatography. Low-density lipoprotein added to the medium at the indicated level was not cytotoxic as indicated by trypan blue exclusion from more than 98% of the cells. Levels shown are the mean ± SD of duplicate experiments with each incubation conducted in quadruplicate.

were not previously loaded with iron ($P < .01$) or to cell-free incubations.

3.3. Iron loading increases cholesterol accumulation in THP-1 cells incubated with native LDL

We demonstrated above that the conjugated diene concentration of native LDL incubated with iron-loaded THP-1 increased significantly during incubation with iron-loaded THP-1 cells. Previous studies have shown that oxLDL is metabolized by mononuclear phagocytes via scavenger receptors, leading to cholesteryl ester accumulation [9,10]. Therefore, we investigated if LDL incubated with iron-loaded THP-1 cells would stimulate cholesterol accumulation in the cells. We incubated iron-loaded THP-1 cells with native LDL and measured cholesterol accumulation by the cells. As shown in Fig. 3, the metabolism of native LDL by iron-loading of THP-1 cells resulted in the accumulation of significantly more cellular-free (panel A, $P < .02$), esterified (panel B, $P < .001$), and total cholesterol (panel C, $P < .002$) compared to non–iron-loaded control cells incubated with native LDL.

3.4. Iron loading also increases uptake of oxLDL

To further investigate the observed increase in cholesterol accumulation in iron-loaded THP-1 cells incubated with LDL, we determined the rates of accumulation and degradation of ^{125}I-oxLDL by iron-loaded THP-1 cells compared to control cells. We anticipated that iron loading would have no effect on the uptake of oxLDL. Unexpectedly, we found that the rates of receptor-mediated accumulation and degradation of oxLDL were significantly increased in iron-loaded cells (Table 1). These results suggest that iron loading in THP-1 cells also increased the expression of receptors for oxLDL.

3.5. Iron loading increases scavenger receptor expression

Because iron loading of THP-1 increased the uptake of oxLDL, we determined the effect of iron loading on the expression of scavenger receptor A (MSR I and II), scavenger receptor SR-BI, CD36, and LOX-I by immunoblotting. Results showed that iron loading specifically increased MSR-I by 100%, but had no effect on CD36 (Fig. 4). Iron loading also had no effect on SR-BI and LOX-I expression (data not shown).

Table 1
Effect of iron loading of THP-1 cells on the catabolism of ^{125}I-oxLDL

^{125}I-oxLDL catabolism	THP-1 status	Receptor-mediated catabolism (ng/mg cell protein)	P
Accumulation	Iron loaded	42 ± 22	.08
	Control	3 ± 27	
Degradation	Iron loaded	204 ± 63	.01
	Control	86 ± 21	

Results shown are the mean ± SD of duplicate experiments with cells incubated in quintuplicate.
Specific activity of the ^{125}I-oxLDL preparations uses averaged 332 ± 67 cpm/ng LDL protein.

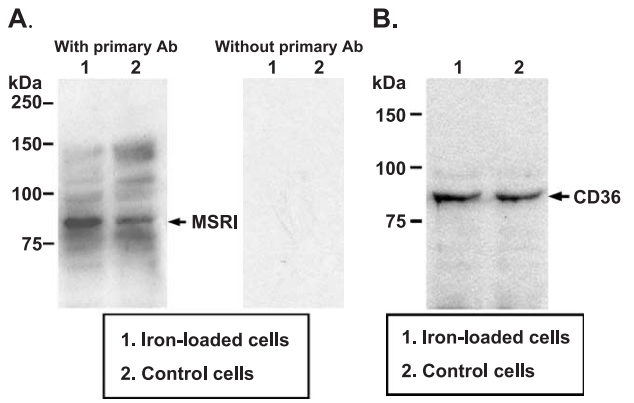

Fig. 4. Western blot of scavenger receptors. After iron loading, cells were then lysed and 25 μg of cell protein was electrophoresed in a 10% polyacrylamide gel. After the electrophoresis, proteins were transferred to a PVDF membrane and the membrane was blotted with antibodies against MSR I and II, and CD36. After washing, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG. The receptors were visualized by incubating the membrane for 1 minute with chemiluminescence reagents and exposure to x-ray film for 15 seconds to 1 minute. Immunoblots shown are representative of those resulting from 3 studies.

4. Discussion

Iron is hypothesized to function as a transition metal that catalyzes the conversion of oxygen free radicals to their highly reactive forms [2]. These reactive oxygen species modify lipoproteins and contribute to the formation of atherogenic lipoproteins such as oxLDL [2,9,10]. Oxidized LDL leads to cholesterol accumulation in the macrophages and results in foam cell formation. Thus, the oxidative modification of LDL within the vessel wall has been implicated in the pathogenesis of atherosclerosis. Our present study demonstrates that iron loading of THP-1 cells increases LDL oxidation. Previously, Abdalla et al [19] reported that iron promoted phorbol myristate acetate–stimulated LDL oxidation by neutrophils and increased uptake of oxLDL by human monocyte-derived macrophages. Yuan et al also demonstrated that preexposure of human macrophages to $FeCl_3$, Fe-ADP, Fe-EDTA, or hemoglobin caused an increased LDL oxidation and intracellular LDL accumulation by these cells [20]. All these studies strongly suggest that elevation of intracellular iron levels may play an important role in atherogenesis by oxidizing LDL.

Our study shows that iron loading of THP-1 cells leads to a significantly increased cholesteryl ester accumulation when cells are exposed to native LDL. Because expression of the native LDL receptor is tightly regulated by a negative feedback mechanism, native LDL is unable to promote cholesteryl ester accumulation in mononuclear phagocytes [20]. Thus, we put forward the hypothesis that the increased content of cholesteryl ester in THP-1 cells loaded with iron

after incubation with normal LDL was due to the ability of these cells to catalyze oxidation of LDL during the incubation. This postulate was confirmed by our results showing that the levels of conjugated dienes in the LDL isolated from the medium of iron-loaded cells were considerably higher than those obtained in the LDL isolated from the medium of control cells not loaded with iron. The oxidation of LDL by iron-loaded cells and the subsequent uptake of the oxLDL by the cells via scavenger receptors may contribute to the observed increase in cholesteryl ester accumulation in iron-loaded THP-1 cells. Interestingly, native LDL that undergoes cell-mediated oxidation seems to lead to levels of intracellular cholesteryl ester accumulation higher than expected. This may be the result of a higher affinity of this cell-mediated oxLDL to scavenger receptors and therefore increased uptake by macrophages. Alternatively, cell-mediated oxLDL may lead to greater inhibition of lysosomal proteases than copper-oxLDL [21], and thus favor accumulation of cholesterol esters.

In contrast to previous studies [22,23], we did not use either organic ferrous/ferric salts or erythrocytes to increase the intracellular ferritin level. We loaded the macrophages with iron by a physiological mechanism that is believed to be involved in body iron overload, namely through the transferrin receptor pathway. Iron acquired from transferrin is released in the early endosome from which it is transported to the cytoplasm and then to the mitochondria for metabolic use. In contrast, iron taken up by macrophages through phagocytosis of erythrocytes seems to follow a different pathway within the cell. In general, intracellular iron may be found in the lysosomes. Ferritin represents the storage form of iron, and in addition, in the so-called labile iron pool. It is believed that a steady state exists among these 3 intracellular iron compartments, and that increase in ferritin level results in elevation of the free iron pool containing the reactive form of iron that is responsible for hydroxide anion and hydroxide radical production and further prooxidative reactions.

The increase in cholesteryl ester content observed in iron-loaded cells seemed difficult to explain solely on the basis of LDL oxidation and uptake because the level of scavenger receptor expression in non–cholesterol-loaded cells is low. Thus, we decided to determine, using a short incubation period to minimize oxidation of native LDL, if the catabolism of both native and oxLDL were modified in iron-loaded cells. We found no difference in the catabolism of native LDL between iron-loaded and unloaded cells, indicating that native LDL receptor expression is not affected by iron loading (data not shown). In contrast, we observed a significant increase in the receptor-mediated uptake of oxLDL in iron-loaded cells compared to that in control cells, suggesting that the expression of scavenger receptors in iron-loaded cells is up-regulated.

To determine which of the known scavenger receptors were up-regulated in iron-loaded cells, we performed Western blot analysis for MSR-I, MSR-II, CD36, LOX-I, and SR B-I. We demonstrated a significantly higher expression of MSR-I in iron-loaded cells when compared to unloaded cells. To our knowledge, this is the first time that up-regulation of a specific type of scavenger receptor has been demonstrated in iron-loaded cells.

Iron seems to play a direct role in monocyte-macrophage differentiation. It has been demonstrated [24] that iron induces a cyclin-dependent kinase inhibitor p21(WAF1/CIP1) causing the cell cycle blocking at the G(1)S interface and preventing premature apoptosis. This differentiation stimulus may thus be accompanied by increased expression of scavenger receptors. It is well known that expression of scavenger receptors is relatively low in circulating monocytes, but it is substantially increased during their activation and differentiation. Free oxygen radicals may up-regulate the MSR-I (but not MSR-II) activity by stabilizing the MSR-I messenger RNA through a posttranscriptional mechanism that is independent of oxidative stress-induced nuclear factor κ B pathway [25]. In our studies, the approximate 50% increase in intracellular ferritin achieved with iron-saturated transferrin presumably led to a sufficient increase of oxygen free radicals to result in higher MSR-I activity. In vivo, plasma ferritin reflects the iron stores in tissues. Nutritional studies demonstrated that diet supplemented with iron led to an increase in plasma ferritin concentrations of 30% to 60% [26,27]. Thus, the present studies reflect physiologically relevant levels of iron loading.

In summary, we have demonstrated that THP-1 monocytes, after iron loading using a physiological medium, show an increased expression of MSR-I scavenger receptor on their surface. This lead to a significantly increased uptake of oxLDL and to cholesteryl ester accumulation within these cells. On the basis of these results, we hypothesize that iron plays a significant role in foam cell formation, and that the elevation of body iron stores may contribute to the pathogenesis of atherosclerosis.

Acknowledgments

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