Iron is an essential metal involved in a wide spectrum of physiological functions in the body, such as oxygen transport and enzymatic reactions. Nevertheless, excess iron can be dangerous because it promotes the generation of free radicals, which subsequently lead to tissue damage. The association between body iron status and the risk of cardiovascular disease was first postulated by Sullivan1 in the early 1980s. Subsequently, experiments with animal models have supported the role of iron in myocardial reperfusion injury.2–7 Furthermore, evidence has accumulated to suggest the role of iron in promoting the oxidation of LDL.8–10 which is one of the early events occurring within the subendothelial space of arteries prone to the formation of atherosclerotic plaques.11 Nevertheless, epidemiological studies conducted by a number of investigators to establish a link between body iron stores and cardiovascular disease in human populations have yielded inconclusive results.12–21 Therefore, whether iron is a risk factor for atherosclerosis and ischemic heart disease remains to be clarified.

A recent study from our laboratory demonstrated that ferritin, the iron storage protein, is highly expressed in human atherosclerotic lesions.24 Perls’ iron staining further revealed the colocalization of ferritin and iron deposition in advanced lesions, which provides the pathological evidence to support the implication of iron in the development of vascular disease. In an attempt to further characterize the role of iron in atherosclerosis, in the present study, apolipoprotein E (apoE)-deficient mice, which have been shown to spontaneously develop atherosclerotic plaques with features similar to those seen in humans,25,26 were used as the animal model to assess the association between iron deposition and the progression of the disease. Additional experiments were designed to treat the apoE-deficient mice with a low-iron diet for 3 months, and the effect on lesion formation was further examined. The results clearly show that restriction of dietary iron significantly attenuates the progression of atherosclerotic lesions in these animals.
Methods

Animals

Homozygous apoE-deficient mice of C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, Me), bred, and maintained under conventional housing conditions in our animal facility. C57BL/6 mice were from the National Animal Center of Taiwan. For dietary experiments, apoE-deficient mice 3 months old were divided into 2 groups and matched for sex, body weight, and plasma cholesterol. One group received a regular AIN-76 semipurified diet (ICN) supplemented with 34 to 36 mg iron/kg in the form of ferric citrate, and the other group was fed a low-iron diet (AIN-76 without iron supplementation, ICN). Our iron determination showed that the AIN-76 regular diet contained 50 mg iron/kg, and the low-iron diet contained 11.5 mg iron/kg. Both groups were housed in plastic cages with stainless steel grid tops. Food and distilled water were given ad libitum. The average amount of diet eaten by each mouse was ~2.5 g/d in both groups.

Figure 1. Iron deposition in aorta (A through C), heart (E through G), and liver (I through K) of apoE-deficient mice at various ages. A, E, and I are tissue sections prepared from a 3-month-old apoE-deficient mouse. B, F, and J are tissue sections from a 5-month-old mouse. C, G, and K are sections from a 6-month-old mouse. D, H, and L are sections of aorta, heart, and liver, respectively, prepared from a 6-month-old C57BL/6 mouse. Iron deposits are revealed as brown color stains. Magnification: A through H, ×400; I through L, ×200.
Iron in Atherosclerosis

TABLE 1. Effect of Low-Iron Diet on Body Weight, Hematocrit, Serum Cholesterol, Iron Content, Ferritin, and Alkaline Phosphatase in Sera of ApoE-Deficient Mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Diet</th>
<th>Weight, g</th>
<th>Hematocrit, %</th>
<th>Serum hemoglobin, g/dL</th>
<th>Serum cholesterol, mg/dL</th>
<th>Total serum iron, μg/dL</th>
<th>TIBC, μg/dL</th>
<th>Serum ferritin, μg/L</th>
<th>Serum alkaline phosphatase, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Chow Diet</td>
<td>28.9±4.8 (12)</td>
<td>47.5±3.1 (9)</td>
<td>15.1±1.8 (9)</td>
<td>459.8±170.5 (10)</td>
<td>148.3±40.6 (11)</td>
<td>351.6±63.0 (11)</td>
<td>308.2±58.0 (10)</td>
<td>106.3±23.6 (12)</td>
</tr>
<tr>
<td>Females</td>
<td>Chow Diet</td>
<td>23.5±3.5 (12)</td>
<td>52.0±2.8 (11)</td>
<td>15.0±1.4 (9)</td>
<td>528.0±150.0 (11)</td>
<td>136.3±45.8 (12)</td>
<td>320.8±83.5 (12)</td>
<td>321.4±56.8 (10)</td>
<td>100.2±33.4 (12)</td>
</tr>
<tr>
<td></td>
<td>Low-Iron Diet</td>
<td>30.2±6.0 (12)</td>
<td>47.8±3.6 (11)</td>
<td>15.2±1.6 (9)</td>
<td>432.9±190.3 (10)</td>
<td>133.2±36.2 (12)</td>
<td>310.6±60.0 (12)</td>
<td>227.4±59.2 (10)</td>
<td>61.5±22.2 (12)</td>
</tr>
<tr>
<td></td>
<td>Low-Iron Diet</td>
<td>23.9±4.0 (12)</td>
<td>52.5±3.3 (10)</td>
<td>14.7±1.5 (9)</td>
<td>472.3±191.0 (12)</td>
<td>137.6±35.5 (12)</td>
<td>315.9±115.9 (12)</td>
<td>224.3±62.5 (10)</td>
<td>95.5±25.5 (12)</td>
</tr>
</tbody>
</table>

TIBC indicates total iron-binding capacity. Numbers in parentheses are numbers of animals examined in each group. Data are mean±SD.

Significant difference vs control group placed on chow diet: *P<0.01, †P<0.005.

The handling of animals was in accordance with institutional guidelines.

Quantification of Aortic Atherosclerosis
After 3 months of dietary intervention, animals were killed by exsanguination under carbon dioxide anesthesia. The thorax and abdomen were opened, and blood samples were collected from the vena cava. After vascular perfusion with ice-cold PBS, the heart with the attached aorta and other organs were removed, fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned at 5 μm for histological staining or other experiments as described in the following sections. For the quantification of the atherosclerotic lesions of apoE-deficient mice, 45 serial sections from the aortic sinus or arch of each mouse were collected. A total of 10 to 12 sections sampled from every 4 consecutive sections were stained with hematoxylin and eosin, and photomicrographs were taken. The cross-sectional area of a given photomicrograph was calculated from the average of the area quantified from the 10 to 12 sections.

Determination of Serum Cholesterol, Iron, Hemoglobin, Ferritin, and Alkaline Phosphatase
The serum level of total cholesterol was determined by an enzymatic method using an assay kit (Sigma Chemical Co). Serum iron and total iron binding capacity were determined by use of Ferrozine as chromogen. Serum total hemoglobin was measured with Drabkin’s reagent (Sigma). Serum ferritin was measured with an immunosassay kit (Boehringer Mannheim), in which the polyclonal anti-human ferritin antibody exhibited high cross-reactivity to mouse liver ferritin. Alkaline phosphatase activity was determined with p-nitrophenyl phosphate as substrate.

Determination of Tissue Iron Concentration and Iron Histochemistry
Tissue samples were dried overnight at 106°C and weighed. Samples were then solubilized in 6 mol/L HNO3 by heating at 100°C to release protein-associated iron. The iron was reduced by ascobic acid and then quantified after being complexed with Ferrozine. Iron deposits on tissue sections were examined by Perl’s Prussian blue reaction with 3,3’-diaminobenzidine (DAB) intensification as described previously.

Susceptibility of Lipoproteins to Oxidation
Plasma samples of 3 mice were pooled and subjected to density-gradient ultracentrifugation. The lipoprotein fraction collected from the density range of 1.019 to 1.063 was dialyzed extensively against PBS at 4°C, and the final concentration was adjusted to 100 μg/mL before assay. In vitro lipoprotein oxidation was conducted at room temperature by addition of CuSO4 to a final concentration of 10 μmol/L. Absorption at 234 nm was monitored continuously as an index of conjugated diene formation. The lag phase was defined as the intercept of the tangent drawn to the steepest segment of the propagation phase to the horizontal axis.

Measurement of Anti–Oxidized LDL Antibodies
Human LDL was isolated as described previously. Oxidized LDL was prepared by incubating LDL (1 mg/mL) with 5 μmol/L CuCl2 in PBS overnight at 37°C. Before each assay, microtiter plates were freshly coated with native LDL or oxidized LDL at a concentration of 5 μg/mL in PBS overnight at 4°C, followed by blocking with 1% BSA for 2 hours at room temperature. Diluted sera (1:40) from animals were incubated for 2 hours at room temperature in duplicate wells. After 3 washes with PBS containing 0.1% Tween-20, goat anti-mouse IgM conjugated with alkaline phosphatase (Sigma) (1:1000 dilution) was added to each well, and incubation continued for 2 hours at room temperature. Plates were washed again, and the alkaline phosphatase activity was determined with p-nitrophenyl phosphate as substrate and read at 405 nm. The titer of anti–oxidized LDL antibody was defined as the difference between the optical density readings obtained from the binding to oxidized LDL and to native LDL.

Immunohistochemistry
The antisera against malondialdehyde (MDA)-modified LDL were generated by immunization of rabbits with homologous MDA-modified LDL. Tissue sections were pretreated with 3% H2O2 in methanol for 10 minutes at room temperature to
exhaust endogenous peroxidase activities. After incubation in PBS containing 1% BSA and 1% goat serum at room temperature for 30 minutes, sections were treated with rabbit anti–MDA-modified LDL antiserum (1:50 dilution) for another 30 minutes followed by three 5-minute washes in PBS. Sections were then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (Gibco BRL) for 30 minutes at room temperature. After washing, color was developed with 0.1% DAB. As negative control, the tissue section was incubated with preimmunized rabbit serum.

Statistical Analysis
Data were expressed as mean±SD. Group data were analyzed by unpaired Student’s t test. A value of P<0.05 was considered statistically significant.

Results
To examine whether the iron deposit was present in the arterial lesions of various stages from the apoE-deficient mice, Perls’ staining was performed. As illustrated in Figure 1A, there was virtually no positive staining in early lesions taken from a 3-month-old mouse. However, when an intermediate lesion taken from a 5-month-old animal was examined, the iron stain clearly appeared in the endothelium (Figure 1B). Increased iron deposits were detected in advanced lesions from a 6-month-old mouse in which the staining was present in intima enriched in foam cells as well as in smooth muscle cells of the media layer.

Figure 2. Effect of low-iron diet on atherosclerotic lesions developed in apoE-deficient mice. ApoE-deficient mice of either sex were fed a regular chow diet (open bars) or low-iron diet (hatched bars) for 3 months, and extents of atherosclerosis in aortic root (A) and aortic arch (B) were assessed. Data are expressed as mean±SD. Number of animals examined in each group was 12. Significant difference vs control group placed on chow diet: *P<0.025, **P<0.01, ***P<0.005.

Figure 3. Iron deposition in aortic lesions of apoE-deficient mice fed a regular diet (A and C) or low-iron diet (B and D). Lesions of aortic roots of male mice (A and B) or female mice (C and D) were subjected to iron staining and counterstained with hematoxylin. Figures shown are representatives observed from each group of animals. Magnification ×100.
Iron in Atherosclerosis

Table 3. Effect of Low-Iron Diet on the Iron Contents of Aorta, Heart, and Liver of ApoE-Deficient Mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Diet</th>
<th>Iron Concentration, μg/g dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Male</td>
<td>96.3±24.5 (8)</td>
</tr>
<tr>
<td></td>
<td>Chow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iron-restricted</td>
<td>45.2±4.7 (8)†</td>
</tr>
<tr>
<td>Female</td>
<td>Chow</td>
<td>66.8±21.0 (8)</td>
</tr>
<tr>
<td></td>
<td>Iron-restricted</td>
<td>48.7±20.2 (8)*</td>
</tr>
<tr>
<td>Heart</td>
<td>Male</td>
<td>357.0±21.9 (8)</td>
</tr>
<tr>
<td></td>
<td>Chow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iron-restricted</td>
<td>285.9±43.2 (8)‡</td>
</tr>
<tr>
<td>Female</td>
<td>Chow</td>
<td>305.3±23.8 (8)</td>
</tr>
<tr>
<td></td>
<td>Iron-restricted</td>
<td>244.2±30.4 (8)‡</td>
</tr>
<tr>
<td>Liver</td>
<td>Male</td>
<td>377.0±111.6 (15)</td>
</tr>
<tr>
<td></td>
<td>Chow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iron-restricted</td>
<td>264.6±114.2 (15)†</td>
</tr>
<tr>
<td>Female</td>
<td>Chow</td>
<td>502.9±148.2 (15)</td>
</tr>
<tr>
<td></td>
<td>Iron-restricted</td>
<td>453.0±101.4 (15)‡</td>
</tr>
</tbody>
</table>

Numbers in parentheses are numbers of animals in each group. Data are mean±SD. Significant difference vs control group placed on chow diet: *P<0.05, †P<0.01, ‡P<0.005.

Additional experiments were conducted to elucidate whether restriction of dietary iron intake would affect the progression of atherosclerosis in these animals. As shown in Table 1, receiving a low-iron diet for 3 months did not significantly affect the concentrations of serum iron or lag time for the lipoprotein preparations isolated from iron-restricted mice appeared to be longer than that from control group littermates of either sex (105.5±16.1 versus 81.7±14.6 minutes, P<0.025, for males, and 140.1±21.2 versus 97.1±27.6 minutes, P<0.005, for females) (Table 2).

The effect of low iron intake on the extent of atherosclerosis in the aortic root and arch of these animals was also assessed. Morphometric quantification of the area of lesions revealed that a low-iron diet reduced atherosclerosis in the aortic root significantly, by 24% and 37% for female (P<0.025) and male (P<0.005) mice, respectively (Figure 2A). The reduction in lesion area of the aortic arch was 30% and 44% for female (P<0.01) and male (P<0.005) animals, respectively (Figure 2B). Perl’s staining of the sectioned aortas revealed that the iron deposition was less evident in iron-restricted animals (Figure 3). Direct quantification of iron concentrations in different tissues again revealed that low iron intake resulted in significant reduction in iron contents of aortic tissue and heart in both sexes (Table 3), whereas the effect on liver iron content was evident only in male but not female mice.

When the sections of atherosclerotic lesions were immunostained for the presence of epitopes of MDA-modified LDL, the results demonstrated that the stains were much stronger in the sections from control animals (Figure 4), supporting the idea that iron-catalyzed free radical reaction promotes the lipid/LDL oxidation in vascular walls. Furthermore, in parallel with the extent of oxidative modification of LDL in lesions, the titer of autoantibodies to oxidized LDL in sera of the control mice was relatively higher than that of littermates in the iron-restricted group in either sex (P<0.05 for females; P<0.025 for males) (Figure 5).

Discussion

In the present study, apoE-deficient mice were used as an animal model to characterize the role of iron in atherosclerosis. As illustrated in Figure 1, iron deposition was visible in the endothelium of intermediate lesions from these mice. When the lesion progressed to a more advanced plaque, the distribution of iron deposits was extended to the area within the intima and some in the media as well. These observations reveal a close association between the severity of the atherosclerosis and the extent of iron deposition in apoE-deficient mice. The iron deposits were also present in tissue sections of liver and heart in an age-dependent manner. Because no iron stain was detected in the sections from the normal C57BL/6 mice of the same age, it is unlikely that the iron deposition in the liver and heart of apoE-deficient mice resulted from the aging process. Nevertheless, it is not clear whether the iron overload in both tissues results from the complication of atherosclerosis or is an independent pathological process.

Additional experiments were conducted to elucidate whether restriction of dietary iron intake would affect the progression of atherosclerosis in these animals. As shown in Table 1, receiving a low-iron diet for 3 months did not significantly affect the concentrations of serum iron or...
hemoglobin or the hematocrit level in apoE-deficient mice, indicating that the dietary intervention did not affect the minimal iron requirement essential for normal hematopoiesis in these mice. Similar results were reported previously in a study showing that iron depletion by treatment with desferrioxamine and iron-deficient diet in C57BL/6 mice for 50 days did not provoke significant changes in hematocrit and serum iron level. These data may suggest that the amount of iron present in a chow diet is in excess of that required by these animals. It was shown that the apoE-deficient mice have high serum ferritin levels (\( > 200 \mu g/L \)), which may be relevant to the iron depositions and high tissue iron contents observed in these animals. Nevertheless, the low iron intake appeared to significantly reduce the level of body iron stores, as shown by the lower serum ferritin in littermates of the iron-restricted group. To evaluate whether the low-iron diet benefited the animals by decreasing oxidative stress, the susceptibility of lipoproteins to in vitro–induced oxidation was assessed. The result clearly demonstrates that the lag time for conjugated diene formation was significantly prolonged in lipoproteins isolated from the iron-restricted group, indicating that dietary intervention affected the antioxidant status and increased the oxidation resistance in these animals. Apparently, the present observation is consistent with a recent study by Salonen et al., who reported that lowering body iron stores by phlebotomy could lengthen the lag time of serum lipoprotein oxidation in male smokers. Likewise, when serum alkaline phosphatase activity, a marker of
liver damage, was measured, it was noted that the enzyme activity was markedly reduced by the low-iron diet in male littermates. However, the enzyme activity of female mice was not significantly affected by the dietary intervention. The biochemical mechanism(s) underlying the sexual difference is not clear. It has been shown that female inbred mice exhibit higher susceptibility to diet-induced atherosclerosis.\(^3\)\(^3\)\(^3\) We also found that female apoE-deficient mice fed a chow diet developed more severe atherosclerotic lesions than male littermates (Figure 2). Nevertheless, the apoE-deficient mice receiving a low-iron diet developed smaller lesions in aortic roots and arches than control mice fed a chow diet in both sexes. Along with the plaque formation, the iron deposition and the epoxides of MDA-modified LDL were more evident in lesions of control mice of either sex. Quantitative assessment of tissue iron further demonstrated that the reduction of iron content was evident not only in aortas but also in hearts of animals placed on a low-iron diet. These data clearly indicate that low iron intake reduces the iron concentrations in vascular tissues and inhibits lesion progression in these animals.

It is believed that the effect of iron on atherosclerosis is largely dependent on the state of hypercholesterolemia in experimental animals. Consistent with the present study is a report by Araujo et al.,\(^4\) who demonstrated that an iron overload by intravenous injection of iron dextran in rabbits does not initiate atherosclerosis but augments lesion formation in rabbits fed a high-cholesterol diet. Furthermore, a recent study by Van Lenten et al.\(^5\) showed that an atherogenic high-cholesterol diet can influence iron homeostasis in mice. It appears that the interplay between lipid and iron is important in determining the extent of tissue damage and atherosclerosis developed in these animals. A previous study demonstrated that the iron chelator desferrioxamine is effective in inhibiting the proliferation of vascular smooth muscle cells in culture and preventing myointimal proliferative lesions in animals,\(^6\) suggesting that iron may affect lesion formation by influencing the proliferative activity of vascular smooth muscle cells. In any event, these observations support the detrimental role of iron in promoting the development of vascular disease. Our data provide an important pathological basis supporting the possible link between the body iron stores and the development of atherosclerotic cardiovascular disease. Although iron alone may not be sufficient in the initiation of the plaque formation, an increase in iron content may exacerbate the oxidative insults in local tissues and accelerate the progression of the disease.

Acknowledgments

This work was supported by grants from the Institute of Biomedical Sciences, Academia Sinica, and the National Science Council of Taiwan, ROC. The authors would like to thank Dr. J.L. Sullivan for critical review and valuable suggestions on this manuscript.

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Circulation. 1999;99:1222-1229
doi: 10.1161/01.CIR.99.9.1222

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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