Histological Study of Iron Deposits in Selenium-deficient Rats†

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Recent reports from this and other laboratories have indicated that selenium (Se) deficiency causes hematological abnormalities that are characterized by an increased sensitivity to hemolysis, 1–3) formation of methemoglobin in vitro, 4,5) an increase in the population of abnormal erythrocytes that contain Heinz bodies, 3,6–7) and a decrease in the proportion of aged erythrocytes. 8) Previous studies in our laboratory have shown that Se deficiency is associated with marked increases in the levels of iron in the serum and in various organs. 9) Increases in serum iron concentrations are accompanied by decreased serum levels of transferrin, with resultant higher transferrin saturation in Se-deficient [Se(−)] rats. A higher proportion of serum transferrin is, therefore, saturated with iron in Se(−) rats and, as a consequence, there appears to be a considerable increase in the amount of non-transferrin-bound iron. These findings suggest that an excess of iron promoted by Se deficiency might play a role in pathogenesis because excess iron in various tissues can cause injury to cells. 10,11)

Earlier studies did not include histological analyses that allowed evaluation of the relative size and location of iron deposits. In this paper, we report the detection of iron deposits by light microscopy in sections of liver and kidney after staining with Prussian blue and the identification of sites of iron deposits by X-ray microanalysis in conjunction with electron microscopy. The sites of iron deposits are an important factor in the development of tissue abnormalities due to excess iron.

Materials and Methods

Animals and diets. All animals were handled in accordance with institutional guidelines of Kyoto University, as outlined in the Guide for the Care and Use of Laboratory Animals. Weanling male Wistar rats (Shimizu Laboratory Supplies Co., Kyoto), weighing 40–50 g were housed individually in hanging, stainless-steel, wire-bottomed cages in a temperature- and humidity-controlled room on a 12-h light-dark cycle. The rats were randomly assigned to two groups so that distributions of body weight were similar. They were given free access to distilled water and Torula yeast-based selenium-deficient [Se(−)] or Se-adequate [Se(+)] diet; containing 0.1 mg Se/kg as sodium selenite) diet for 8 or 82 weeks. Excised tissues were embedded in either paraffin or epoxy resin. A dramatic increase was observed in iron deposition in the liver and kidneys of rats on the Se(−) diet. Prussian blue-stained sections under the light microscope showed iron deposits in the parenchymal cells and Kupffer cells of liver and in the proximal tubules of kidneys. The liver and kidneys of Se(−) rats had considerably altered morphology: lysosomes were enlarged and contained electron-dense areas. X-Ray microanalysis showed that the areas that corresponded to the lysosomes contained iron. No iron deposits were observed in sections of kidney and liver from rats fed the Se(+) diet. Thus, these studies identified subcellular sites of iron deposition in the liver and kidneys of Se(−) rats. These iron deposits may be an important factor in the pathogenesis of Se deficiency.

Analysis. Iron was measured after wet digestion with a mixture of perchloric acid and nitric acid by inductively coupled argon plasma emission spectrometry, as described previously. 9) Glutathione peroxidase activity was assayed by a modified version of the method of Paglia and Valentine, as previously described. 11)

Light microscopy. Portions of the liver and kidney were perfused with saline and fixed in neutral buffer solution that contained 10% formaldehyde (Nacalai Tesque, Kyoto) for 1 week. Small blocks were excised from the fixed samples, dehydrated in the routine manner with a graded ethanol series (from 70% to 100% ethanol), and then ethanol was replaced with xylene. The dehydrated small blocks were embedded in paraffin (60°C for 30 min; Histosec, Merck, Darmstadt, Germany). The paraffin blocks were sectioned on a microtome at 5–10 µm. The sections were laid out on warm water and transferred to glass slides. For staining with Prussian blue, the slides were incuated in a mixture of 2% potassium ferrocyanide and 1% hydrochloric acid for 15 min. The stained preparations were examined with a light microscope (XF21; Nikon Optics Co., Tokyo).

Electron microscopy. Portions of liver and kidney were perfused with saline and then with 2.5% glutaraldehyde (Nacalai Tesque) in 0.05 mol/liter phosphate buffer (pH 7.4) at 22°C. The perfused samples were cut into 1-mm cubes, fixed in 2.5% glutaraldehyde in 0.05 mol/liter phosphate buffer solution (pH 7.4), kept for 2 h at room temperature, and then rinsed...
Fig. 1. Staining with Prussian Blue of Liver Tissues from Se(+) (A) or Se(−) (B) Rats after 8 Weeks of Feeding. Note the large numbers of blue granules (dark blue dots) in parenchymal cells (arrowheads), Kupffer cells (long arrows), and microsiderotic nodules of phagocytic cells (m) between cords or groups of hepatocytes.

Fig. 2. Staining with Prussian Blue of Kidney Tissues from Se(+) (A) or Se(−) (B) Rats after 82 Weeks of Feeding. A: Glomerulus and convoluted tubules from a Se(+) rat. B: Glomerulus and convoluted tubules from a Se(−) rat. Iron is stained as dark granules within epithelial cells (arrowheads) of proximal convoluted tubules (P).
three times in 0.05 mol/liter phosphate buffer (pH 7.4). After rinsing, all samples were postfixed in 1% osmium tetroxide (Nacalai Tesque) in 0.05 mol/liter phosphate buffer (pH 7.4) for 1 h, dehydrated in a graded ethanol series (from 70 to 100% ethanol), transferred to propylene oxide for 30 min, embeded in low-viscosity Epon 812 (Nacalai Tesque), placed in an oven at 60°C for 48 h until the resin had hardened, and then removed from the molds as blocks. Blocks containing portions of liver and kidney were trimmed and sectioned on an ultramicrotome (Reichert-Jung OmU2, A. G. Herrmaler, Vienna, Austria). To identify the orientation of samples, thick sections (0.02–0.1 μm) were first sampled with glass knives and viewed under a light microscope. Ultrathin sections were made with a diamond knife (Diatome, Berne, Switzerland), and sections were collected on 100-mesh copper grids. The sections were counterstained with 2% uranyl acetate for 10 min to increase the staining of membranes and examined with an electron microscope (H-700; Hitachi Ltd., Tokyo) at an accelerating voltage of 100 kV.

X-Ray microanalysis. X-Ray microanalysis in combination with electron microscopy was done using sections mounted on 100-mesh copper grids for liver and on 100-mesh nickel grids for kidney and an X-ray energy spectroscopy system (model 7000; Kevex Corp., Foster City, CA, U.S.A.) interfaced with the electron microscope (H-700). The technique involves focusing an electron beam on the subcellular area of interest to generate spectra characteristic of the metal present in that area. Microanalysis was done at an accelerating voltage of 75 kV, an electron beam intensity of 2.5 × 10^-4 A, and a counting time of 100 s. X-Ray mapping for iron was obtained by processing the Fe Kα, X-ray peaks.

Results

Body weight, organ weight, and iron content

After 8 weeks, body weights, and organ weights were similar in all subgroups of rats, irrespective of whether they had eaten a Se(−) or a Se(+) diet. The architecture of the liver and kidneys appeared normal in routine histological analyses of both Se(−) and Se(+) rats after staining with hematoxylin and eosin (data not shown). Measurement of the mineral content of the tissues showed that Se deficiency caused a significant increase in the iron content of livers (Table).

After 24 weeks or more, Se(−) rats displayed a progressive decrease in body weight gain as compared with Se(+) rats (data not shown). After 82 weeks, the liver, kidney, spleen, and heart of Se(−) animals were larger than those of Se(+) animals. In these organs from Se(−) animals, iron contents were higher than in those from Se(+) animals (Table). The accumulation of iron in these various organs, when expressed in terms of relative body weight, was, 13, 9, 10, and 6 times greater, respectively, in Se(−) rats than in Se(+) rats. However, there was no gross evidence of cirrhosis or fatty infiltration in the excised liver, nor of necrosis in the kidney, although some kidneys showed sporadic signs of lymphocytic infiltration and fibrous hyperplasia in the interstitium.

Light microscopy

In the livers of Se(−) rats after 8 weeks, staining by Prussian blue was observed in the parenchymal cells and Kupffer cells, and staining was stronger in phagocytic cells situated in the sinusoids (Fig. 1B). Such mesocidoerotic nodules are also observed in livers of iron-overloaded rats.12 By contrast, none of the sections from livers of control rats were stained with Prussian blue (Fig. 1A).

No staining was observed in the kidneys of either Se(−) or Se(+) rats after 8 weeks of feeding on the specific diets. This finding corroborated the insignificant increase in the iron content of the kidneys in Se(−) rats (Table). However, after rats had spent 82 weeks on an Se(−) diet, their kidneys stained heavily for iron. The staining was confined primarily to the epithelial cells of the proximal tubules in Se(−) rats (Fig. 2B). No staining was seen in the analogous tissues of Se(+) rats (Fig. 2A).

Electron microscopy

Electron microscopy confirmed the deposition of iron in the liver and kidneys of rats fed a Se(−) diet for 8 and 82 weeks, respectively. In the livers of Se(−) rats after 8 weeks, lysosomes were observed to be enlarged while the majority of mitochondria had normal morphology. Heavy electron-dense deposits were found in large, irregularly shaped lysosomes in both hepatocytes (Fig. 3B) and Kupffer cells (data not shown). The hepatocytes (Fig. 3A) and Kupffer cells (data not shown) of control rats had lysosomes with normal morphology, with moderate electron-dense deposits, and mitochondria with no appreciable electron-dense deposits. The kidneys from Se(−) rats after 8 weeks showed

<table>
<thead>
<tr>
<th>8 wk</th>
<th>Se(−)</th>
<th>Se(+)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>221 ± 9 (6)</td>
<td>230 ± 3 (6)</td>
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<tr>
<td>Organ fresh weight (g/100 g body wt)</td>
<td></td>
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<tr>
<td>Liver</td>
<td>3.3 ± 0.1 (6)</td>
<td>3.3 ± 0.1 (6)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.37 ± 0.02 (6)</td>
<td>0.36 ± 0.01 (6)</td>
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<tr>
<td>Spleen</td>
<td>0.19 ± 0.01 (6)</td>
<td>0.18 ± 0.01 (6)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.33 ± 0.02 (6)</td>
<td>0.32 ± 0.01 (6)</td>
</tr>
<tr>
<td>Iron content, μg/g fresh wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>3.0 ± 0.11* (6)</td>
<td>1.37 ± 0.07 (6)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.76 ± 0.03 (6)</td>
<td>0.76 ± 0.04 (6)</td>
</tr>
<tr>
<td>Spleen</td>
<td>31 ± 2* (6)</td>
<td>12 ± 1 (6)</td>
</tr>
<tr>
<td>Heart</td>
<td>1.17 ± 0.06 (6)</td>
<td>1.13 ± 0.07 (6)</td>
</tr>
<tr>
<td>Glutathione peroxidase activity in erythrocytes, unit/g hemoglobin</td>
<td>13.1 ± 1.6* (6)</td>
<td>159 ± 11 (6)</td>
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* Values are means ± SEM, with n in parenthesis. All means for the Se(−) group are significantly different at p < 0.001 from those of Se(+) group, as measured by Student’s t-test.

The averages for right and left kidneys.
Fig. 3. Electron Microscopy of Liver Tissues from Rats Fed a Se (+) (A) or a Se (−) (B) Diet for 8 Weeks. These electron micrographs show electron-dense deposits (arrowheads) within hepatocytes and Kupffer cells of a Se (−) rat.

Fig. 4. Electron Microscopy of Kidney Tissues from Rats Fed a Se (+) (A) or a Se (−) (B) Diet for 82 Weeks. These electron micrographs show electron-dense deposits (arrowheads) within the secondary lysosomes of a Se (−) rat.
no obvious increases in the intensity of staining of electron-dense deposits (data not shown). After 82 weeks, however, secondary lysosomes were enlarged and contained electron-dense areas in Se(−) rats (Fig. 4B), unlike those in Se(+) rats (Fig. 4A).

*X-Ray microanalysis*

Results of X-ray mapping of iron on the sections of livers from rats fed the experimental diets for 8 weeks are shown in Fig. 5. Panels B and D show the X-ray maps for iron, and panels A and C show the superimposition of the iron

![Image](image.png)

**Fig. 5.** X-Ray Microanalysis Energy Imaging and Mapping for Liver Tissues of Rats Fed a Se(+) (A, B) or a Se(−) (C, D) Diet for 8 Weeks.

X-Ray map of iron in an Se(−) rat (Fig. 5D, arrowheads) reflects the same pattern as the lysosomes in the ultrastructural image in Fig. 5C. The X-ray map of iron in the Se(+) rat (Fig. 5B) does not show any organelle-specific pattern when it is compared with the ultrastructural image in Fig. 5A.
maps on the electron micrographs. The superimposed images indicate that, in the livers from Se(−) rats, most of the iron was localized in the areas that corresponded to lysosomes. In contrast to those in Se(−) rats, the X-ray maps of iron in Se(+) rats did not reveal any organelle-specific pattern (Figs. 5A and 5B) nor did they show any appreciable deposition of iron in lysosomes (Fig. 6A).

The localization of the excess iron in lysosomes was confirmed by X-ray microanalysis energy emission spectroscopy. Peaks at 6.4 and 7.1 keV are characteristic of iron. Spectra were recorded from eight different areas for each of three rats in the respective subgroups. A large iron peak was detected in the spectra from lysosomal areas of the livers of rats fed an Se(−) diet for 8 weeks (Fig. 6B), but no appreciable iron peak was recorded from nonlysosomal areas, such as mitochondria, the nucleus, and the cytosol (data not shown). The spectra showed a large peak specific for iron in lysosomal areas in Se(−) rats (Figs. 5C and 5D), as compared to the background spectra obtained with Se(+) rats (Figs. 5A and 5B). As with the livers from Se(−) rats, sections of kidneys from rats fed the Se(−) diet for 82 weeks gave a large peak that corresponded to iron in lysosomes (Fig. 7). No other organelles were sites of the prominent deposition of iron.

**Discussion**

Previous studies have shown that Se deficiency increases levels of iron in serum and in various tissues as a result of the liberation of iron by enhanced hemolysis. In this study,
activity, and the level of substrate heme is increased sufficiently to induce the enzyme that liberates free iron from heme. This iron may be taken up by the liver and, in severe cases, by the kidney after endocytosis or autophagocytosis and degradation within the lysosomal storage vesicles of increased amounts of iron. The localization of iron in the liver and kidneys is often seen in patients with intravascular hemolysis, as, for example, in case of paroxysmal nocturnal hemoglobinuria or of hemolytic transfusion reactions. Selenium as sodium selenite has been also noted to stabilize erythrocyte membranes and to reduce their high susceptibility to complement-mediated lysis in paroxysmal nocturnal hemoglobinuria. Therefore, these results suggest that the rats in our study were probably suffering from long-term exposure to Se deficiency. The effects could be due to a decrease in total-body Se, namely, in whole blood, erythrocyte, plasma, and tissue Se.

Reticuloendothelial deposition of iron tends not to produce clinically significant organ dysfunction, but excess iron in parenchymal cells is believed to be toxic. Iron overload in parenchymal cells over a long period damages the tissue, with damage being apparent first in the liver but later in the pancreas, skin, joints, endocrine organs, heart, and other tissues. After 82 weeks of feeding, a progressive decrease in body weight gain and enlarged liver, kidney, spleen, and heart were observed in our Se(-) rats, with corresponding doubling of the concentration of iron (Table). Although gross evidence of tissue damage, namely, fibrosis or cirrhosis of the liver was not evident in these animals, some kidneys showed evidence of lymphocytic infiltration and fibrous hyperplasia in the interstitium (data not shown). The absence of liver damage in the animals studied here does not exclude the possibility that excess iron is involved in Se deficiency-induced liver necrosis in rats. Iron causes an increase in lipid peroxidation in vitro in subcellular organelles, but the onset of cellular disruption in isolated hepatocytes exposed to iron is delayed.

In addition to iron deposits, Se deficiency caused the enlargement of lysosomes, which may play a role in the pathogenesis of Se deficiency. Overloading of lysosomes with iron could result in Fenton reactions, peroxidation of lysosomal membranes, and leakage of lytic enzymes into the cell sap with ensuing cell damage; this phenomenon has been observed in liver biopsy tissues from patients with hereditary and secondary hemochromatosis. Therefore, an increase in lysosomal staining may reflect pathologic changes in Se deficiency. Further study is required to find whether Se deficiency-promoted, excess iron can amplify and perpetuate cell damage, aiding in the production of oxygen free radicals and the release of lysosomal enzymes.

We propose that cellular damage produced by Se deficiency in various tissues is mostly, if not completely, the consequence of an excess of iron in tissues, which leads to peroxidation of cell membranes and oxidation of intracellular protein and, ultimately, to damage to cells. These studies have identified the subcellular sites of iron in the liver and kidneys of Se(-) rats and shown that these deposits may be important in the pathogenesis of selenium deficiency.
References