Distribution of ferritin and hemosiderin in the liver, spleen and bone marrow of normal, phlebotomized and iron overloaded rats.

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Abstract

The distribution of ferritin has been studied in many tissues, but has not yet been established on the cellular level. We investigated the cellular distribution of ferritin in the liver, spleen and bone marrow using the immunoperoxidase method, and compared it with that of hemosiderin. We also examined changes in the distribution of these proteins after phlebotomy and iron overload. In normal rats, ferritin was seen in centrilobular hepatocytes, Kupffer cells, macrophages in the red and white pulp of the spleen and central macrophages in bone marrow. Hemosiderin was observed almost exclusively in the red pulp and partly in tangible body macrophages of the white pulp. After phlebotomy, neither ferritin nor hemosiderin were detectable in these cells except for ferritin-positive cells in the white pulp, which showed little change after either phlebotomy or iron overload. In iron overloaded rats, both ferritin and hemosiderin increased in hepatocytes and reticulo-endothelial (RE) cells. Ferritin-positive cells in the liver were mainly located in the periportal area. These results indicated that hepatocytes and RE cells except for those in the white pulp may play an important role in iron storage, and that ferritin-positive cells in the white pulp may have a function other than iron reserve. They also suggested that the zonal distribution of ferritin-positive hepatocytes may be due to microcirculation in the hepatic lobules.

KEYWORDS: immunoperoxidase method, ferritin, hemosiderin

DISTRIBUTION OF FERRITIN AND HEMOSIDERIN IN THE LIVER, SPLEEN AND BONE MARROW OF NORMAL, PHLEBOTOMIZED AND IRON OVERLOADED RATS

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Abstract. The distribution of ferritin has been studied in many tissues, but has not yet been established on the cellular level. We investigated the cellular distribution of ferritin in the liver, spleen and bone marrow using the immunoperoxidase method, and compared it with that of hemosiderin. We also examined changes in the distribution of these proteins after phlebotomy and iron overload. In normal rats, ferritin was seen in centrilobular hepatocytes, Kupffer cells, macrophages in the red and white pulp of the spleen and central macrophages in bone marrow. Hemosiderin was observed almost exclusively in the red pulp and partly in tingible body macrophages of the white pulp. After phlebotomy, neither ferritin nor hemosiderin were detectable in these cells except for ferritin-positive cells in the white pulp, which showed little change after either phlebotomy or iron overload. In iron overloaded rats, both ferritin and hemosiderin increased in hepatocytes and reticulo-endothelial (RE) cells. Ferritin-positive cells in the liver were mainly located in the periportal area. These results indicated that hepatocytes and RE cells except for those in the white pulp may play an important role in iron storage, and that ferritin-positive cells in the white pulp may have a function other than iron reserve. They also suggested that the zonal distribution of ferritin-positive hepatocytes may be due to microcirculation in the hepatic lobules.

Key words : immunoperoxidase method, ferritin, hemosiderin.

Iron is one of the metals essential to living organisms, and is stored in the form of ferritin or hemosiderin. Ferritin composes a major part of this storage iron and has been found in many tissues (1, 2). It is stored mainly in the liver, and in reticulo-endothelial cells (RE cells), particularly in the spleen and bone marrow (3, 4). Hemosiderin also exists in these sites under normal conditions. The amount of storage iron dynamically changes under iron depleted or iron overloaded conditions. In iron overload (such as hemochromatosis), hemosiderin granules are noted not only in RE cells but also in parenchymal cells of many organs, such as the liver, heart and pancreas (5-8). In these cells, hemosiderin is not distributed randomly but in a regular manner. Thus, in view of the close relationship between ferritin and hemosiderin (9, 10), the former is also thought to have a relatively definite distribution. However, it is difficult to demonstrate fer-

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ritin by hematoxylin-eosin or Prussian blue iron staining, whereas with hemosiderin, small brown pigments in hematoxylin-eosin sections and brilliant blue granules due to Prussian blue iron staining can be detected.   In recent years immunohisto-chemical methods have made it possible to demonstrate the distribution of ferritin on a cellular level.   There have been some reports (11-13) on the distribution of ferritin with these methods, but the results are not yet in agreement.   The changes in distribution of ferritin under iron deficiency and iron overload have not been fully investigated.

    In the present study, we investigated the cellular distribution of ferritin and hemosiderin in the liver, spleen and bone marrow, and examined the changes in their distribution after phlebotomy and iron overload using the immunoperoxidase method.

MATERIALS AND METHODS

Animals.   Wistar rats, weighing 250-300 g, were purchased from Shimizu Jikkenzairyo Co., Kyoto.   They were allowed food and water *ad libitum*.   The standard diet, purchased from Oriental Yeast Co., Tokyo, contains 0.016 % iron.

Reagents.   Chondroitin sulfate-iron colloid (Blutal) was donated by Dainippon Pharmaceutical Co., Osaka.   Carbonyl iron and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma Chemical Co., St. Louis.

Antibodies.   Rabbit antibody to rat liver ferritin purified by affinity chromatography (14) was kindly supplied by Dr. T. Ono (Shigei Medical Institute, Okayama).   Goat antiserum to rabbit IgG and peroxidase-antiperoxidase (PAP) complexes were obtained from Behringwerke AG, Marburg, and Dakopatts A/S, Copenhagen, respectively.   Optimal dilutions of the antibodies were determined in preliminary studies to give suitable sensitivity with a minimum of non-specific staining.

Experimental protocol.   Rats were divided into four groups: (1) normal control group (n= 5), (2) phlebotomy group (n=5) in which rats were bled at three day intervals, two to four times for a total of 5 to 10 ml from the orbital venous plexus, (3) iron overload group A (n=5) in which Blutal was injected once via the tail vein at 40 mg Fe/kg body weight, and (4) iron overload group B (n=6) in which rats were allowed a diet containing 2.5 % carbonyl iron for 2 weeks and then given the standard diet.

Tissue preparation.   One week after each treatment, rats were sacrificed by sectioning the abdominal aorta under ether anesthesia.   The liver, spleen and femur were removed quickly and cut into small pieces.   They were fixed in PLP (periodate-lysine-2 % paraformaldehyde) solution (15) overnight at 4 ℃, dehydrated and embedded in paraffin.   Serial sections, 4 μm in thickness, were prepared and dried overnight at 37℃.   They were dewaxed and rehydrated before histochemical procedures.

Immunohistochemical staining.   One serial section was stained using Perls' iron staining method (16).   Another was treated by the PAP method (17, 18) to determine the immunohistochemical localization of ferritin.   The prepared sections were washed in phosphate buffered saline (PBS, pH 7.2).   First, endogenous peroxidase activity of the sections was blocked using Streefkerk's method (19) or Isobe's method (20).   After pretreatment with normal goat serum to reduce non-specific staining, the sections were incubated with rabbit antibody to rat liver ferritin for 60 min.   Then the sections were incubated with goat antiserum to rabbit IgG for 30 min, fol-

lowed by incubation with PAP complexes for 30 min. The final reaction was achieved by incubating the sections with 0.02 % DAB and 0.005 % H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6, for 5 min. After nuclear counterstaining with hematoxylin, the sections were dehydrated and enclosed. All incubations were performed at room temperature, and after each step sections were rinsed three times in PBS. As negative controls, PBS, normal rabbit serum and anti-ferritin antibody absorbed with ferritin were employed in place of anti-ferritin antibody. Some of the sections were treated by the PAP method with anti-ferritin antibody, followed by Perls' iron staining, in order to determine whether the ferritin-positive cells had hemosiderin or not.

RESULTS

Sections incubated with PBS or normal rabbit serum in place of anti-ferritin antibody showed negative staining, and those incubated with absorbed anti-ferritin antibody showed a considerable decrease in staining. The sites positive for ferritin were easily distinguished from hemosiderin-positive sites by double staining with the PAP method and Perls' iron staining.

Normal Control Group

Liver. Hemosiderin granules were not detected in either hepatocytes or Kupffer cells (Fig. 1a). Immunohistochemical staining for ferritin revealed the central zones of the hepatic lobules around the central vein to be more intensely stained than the peripheral zones around the portal vein (Fig. 1b). However, Kupffer cells positive for ferritin were scattered throughout the hepatic lobules, and did not show zonal distribution such as seen in hepatocytes. On higher magnification, the hepatocytes and Kupffer cells showed fine granular brown pigments of DAB reaction products diffusely in their cytoplasm (Fig. 1c).

Spleen. In the red pulp, a large number of macrophages were stained for ferritin (Fig. 5b), and some of them also had hemosiderin (Fig. 5a). In the white pulp, the cells stained for ferritin were tingible body macrophages, marginal metalophils (21) and macrophages in the mantle zone, periarterial lymphatic sheath (PALS) and marginal zone (Fig. 5b). However, most of these cells were negative for iron staining, and only a few tingible body macrophages had hemosiderin (Fig. 5a).

Bone marrow. None of the bone marrow cells were positive for iron staining (Fig. 9a). Immunoreactive ferritin was detected only in central macrophages in the erythroblastic islands (Fig. 9b). On higher magnification, their dendritic cytoplasm surrounded erythroblasts in which ferritin was undetectable (Fig. 9c). Hematopoietic cells were rarely stained for ferritin.

Phlebotomy Group

Liver. Neither hepatocytes nor Kupffer cells had hemosiderin (Fig. 2a). Zonal distribution of ferritin in hepatic lobules became unclear with the decrease in staining of hepatocytes, and Kupffer cells became negative for ferritin.

Spleen. Both hemosiderin and ferritin disappeared in red pulp macrophages (Fig. 6a, 6b). Erythroid hyperplasia was seen in the red pulp (Fig. 6a). In the

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white pulp, the cells stained for ferritin in the normal control group remained stained (Fig. 6b) and a few tingible body macrophages in the germinal center also had hemosiderin (Fig. 6a).

Bone marrow.   Erythroid hyperplasia was observed.   Neither hemosiderin nor ferritin were detectable (Fig. 10a, 10b).

Iron Overload Group A (Intravenous Injection of Blutal)

Liver.   Kupffer cells and some endothelial cells in the peripheral and midzonal area of the hepatic lobules had hemosiderin (Fig. 3a), and these cells were also strongly stained for ferritin (Fig. 3b).   Thus, they were more dominant in peripheral than in central areas.   Hepatocytes showed little change.

Spleen.   A larger number of red pulp macrophages had hemosiderin (Fig. 7a), and they were also stained for ferritin (Fig. 7b).   In the white pulp, a few tingible body macrophages also had hemosiderin (Fig. 7a), and the cells stained for ferritin in the control group remained stained without an increase in their staining or number (Fig. 7b).

Bone marrow.   Two types of cells were positive for iron staining: central macrophages which were diffusely stained and endothelial cells which had some iron-positive granules (Fig. 11a).   Ferritin was detected in both central macrophages and some endothelial cells (Fig. 11b).

Iron Overload Group B (Oral Administration of Carbonyl Iron)

Liver.   The immunohistochemical localization of ferritin was almost the same as that of hemosiderin, that is, periportal hepatocytes were more intensely stained for ferritin than centrilobular hepatocytes (Fig. 4b).   The distribution of ferritin in this group was opposite to that in the control group.

Spleen.   The distribution of both hemosiderin and ferritin was similar to that in iron overload group A (Fig. 8a, 8b).   A larger number of red pulp macrophages were stained for both iron and ferritin.   In the white pulp, only a few tingible body macrophages in the germinal center had hemosiderin, and the cells stained for ferritin in the control group maintained staining for ferritin without an increase in their staining or number.

Bone marrow.   Central macrophages were positive for iron staining the same as in iron overload group A, but endothelial cells were negative in this group (Fig. 12a).   Central macrophages were more intensely stained for ferritin (Fig. 12b).

DISCUSSION

In this study we used the immunoperoxidase method with anti-ferritin antibody to demonstrate the distribution of ferritin.   The antibody mainly recognizes the protein moiety of holoferritin, but it also reacts with apoferritin or its subunits (22).   Strictly speaking, immunohistochemical methods using anti-ferritin antibody do not specifically demonstrate the localization of holoferritin, but actually demonstrate holoferritin, apoferritin and its subunits as a group.   According to a report (23)

that apoferritin with little iron makes up about 10 % of all ferritin molecules, with most of the remaining holoferritin molecules possessing approximately 2,000 iron atoms, positive sites mostly indicate the localization of holoferritin.

With this immunoperoxidase method, ferritin of erythroblasts (24) and lymphocytes (25), which have been reported to contain ferritin, was not clearly demonstrated. Thus, it was concluded that the sensitivity of the immunoperoxidase method was relatively low. In other words, cells stained for ferritin are considered to contain relatively large amounts of ferritin. This is also supported by the fact that our results are in good agreement with previous reports (3, 4) stating that hepatocytes and RE cells are abundant in ferritin.

In our study, ferritin-positive cells were distributed in central areas of the hepatic lobules. Such zonal distribution of ferritin has not been reported previously.

Lee *et al.* (12) demonstrated ferritin in normal and iron overloaded rats by immunofluorescence. They reported that livers without iron overload contained occasional Kupffer cells and hepatocytes that gave a weak positive immunofluorescent reaction, but did not refer to the zonal distribution which we observed. They also reported that hepatocytes, Kupffer cells and macrophages gave a positive immunofluorescent reaction after administration of iron dextran. However, they did not describe the exact distribution of ferritin-positive hepatocytes and Kupffer cells.

In view of the response after phlebotomy, the cells stained for ferritin in normal rats could be categorized into the following two groups: (A) hepatocytes, Kupffer cells, red pulp macrophages and central macrophages of bone marrow, and (B) the cells which were stained for ferritin in the white pulp, such as marginal metalophils, tingible body macrophages and other macrophages in the mantle zone, PALS and marginal zone.

In (A), ferritin became undetectable after phlebotomy. Storage iron in ferritin and hemosiderin in (A) might be transported to erythropoietic foci in the form of transferrin with an increase in erythropoiesis induced by blood depletion. Ferritin and hemosiderin might be catabolized after iron release, thus becoming histochemically undetectable.

In iron overloaded rats, ferritin and hemosiderin of these cells increased, but in a somewhat different manner between the two kinds of iron overload. After intravenous injection of colloidal iron, ferritin and hemosiderin increased in RE cells, such as Kupffer cells, red pulp macrophages, central macrophages and some endothelial cells, similar to previous reports (6) about the distribution of hemosiderin. Hepatocytes, however, showed little change. This distribution is similar to hemosiderosis in man (5, 6). After oral administration of carbonyl iron, which was thought to be absorbed in the small intestine and to be transported in the from of transferrin, ferritin and hemosiderin, increased chiefly in periportal hepatocytes, and both were detected in red pulp macrophages and in central macrophages of bone marrow. Kupffer cells and endothelial cells showed little change. The different response between hepatocytes and Kupffer cells was also mentioned by

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Bacon (26). Anyway, these ferritin-positive cells belonging to (A) may play a central role in iron reserve.

In both kinds of iron overload, most ferritin-positive cells also had hemosiderin. On the other hand, in the normal control group, the number of ferritin-positive cells was far greater than that of hemosiderin-containing cells. These histochemical results confirm previous findings (4, 27) that the hemosiderin/ferritin ratio becomes greater as the amount of storage iron increases.

On the contrary, ferritin and hemosiderin in (B) did not respond to phlebotomy or iron overload. Therefore, these cells may have some function other than iron reserve.

In the liver of patients with idiopathic hemochromatosis (5, 7) and experimentally iron overloaded animals, such as those administrated Fe-nitrilotriacetate (8), hemosiderin is seen predominantly in periportal hepatocytes, contrary to the liver of normal rats, where ferritin is seen in centrilobular hepatocytes. Hemosiderin and ferritin were also seen zonally in periportal hepatocytes after oral administration of carbonyl iron.

From this experimental evidence together with other reports (28, 29), the following mechanism was cosidered. The difference in distribution of ferritin in livers of normal and iron overloaded animals is more likely due to hepatic microcirculation than to differences in hepatocytes themselves, since periportal hepatocytes first encounter portal blood, which has transferrin more or less saturated with iron. If periportal hepatocytes were perfused with iron-saturated transferrin, they would incorporate iron from transferrin and synthesize ferritin. Centrilobular hepatocytes are perfused with less saturated transferrin, and, thus, they incorporate less or no iron. The gradient of ferritin distribution in hepatocytes from peripheral to central areas seems to be formed in this manner. In the same manner, if periportal hepatocytes were perfused with less saturated transferrin, they would release iron to transferrin and lose ferritin. Centrilobular hepatocytes were perfused with more saturated transferrin than periportal ones, thus releasing less or no iron and maintaining ferritin.

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LEGENDS FOR FIGURES

Figs. 1 to 4 show photomicrographs of liver sections, Figs. 5 to 8 show those of spleen sections, and Figs. 9 to 12 show those of bone marrow sections.   a: Perls' iron staining.   b: immunoperoxidase staining (PAP method) with anti-ferritin antibody.   All figures except for c, in which the magnification is 200 ×, are shown at 100 × magnification.

Fig. 1.   Normal control group.   a: Hemosiderin is not present in parenchymal cells, Kupffer cells or other types of cells.   b: Zonal distribution of immunoreactive ferritin in hepatic lobules. Parenchymal cells around the central vein (C) are more intensely stained than those around the portal vein (P).   c: Higher magnification of Fig. 1b.   Kupffer cells also stained for ferritin (arrows).

Fig. 2.   Phlebotomy group.   a: Hemosiderin is absent in parenchymal cells, Kupffer cells and other types of cells.   b: Ferritin is not detected in either hepatocytes or Kupffer cells.

Fig. 3.   Iron overload group A (intravenous injection of Blutal).   a: Hemosiderin is detected in Kupffer cells and some endothelial cells around the portal vein (P).   b: Ferritin is also strongly stained in Kupffer cells and some endothelial cells in the peripheral area, which are positive for iron staining. Hepatocytes show little change.

Fig. 4.   Iron overload group B (oral administration of carbonyl iron).   a: Hemosiderin is faintly positive in hepatocytes around the portal vein (P).   b: The distribution of ferritin is almost the same as that of hemosiderin.   Ferritin is strongly stained in periportal hepatocytes.   Note that the zonal distribution of ferritin in this group is opposite to that in the normal control group.

Fia 5.   Normal control group.   a: Some of the macrophages in the red pulp (R) have hemosiderin (arrows), and a few tingible body macrophages (arrow head) in the germinal center (G) have hemosiderin. b: Ferritin is stained in a large number of red pulp macrophages (R), macrophages in the white pulp and marginal metalophils (MM) which are arranged in a circle.   c: Higher magnification of the marginal sinus area of Fig. 5b.   Spindle shaped or polygonal cells arranged along the marginal sinus (arrows) are marginal metalophils (MM).   Tingible body macrophages are shown in the upper left of this figure (arrow heads).

Fig. 6.   Phlebotomy group.   a: Hemosiderin in red pulp macrophages is absent and is detected only in a few tingible body macrophages (arrow heads) in the germinal center.   Erythroid hyperplasia is shown in the red pulp (R).   b: Distribution of ferritin in the white pulp is similar to that in the normal control group.   On the other hand, ferritin is rarely detected in the red pulp.

Fig. 7.   Iron overload group A (intravenous injection of Blutal).   a: Numerous red pulp macrophages are positive after iron staining.   A few tingible body macrophages (arrow heads) are positive after iron staining.   b: Numerous red pulp macrophages are strongly stained for ferritin, and most of them are also stained due to iron staining.   The distribution of ferritin in the white pulp is similar to that in the normal control group.

Fig. 8.   Iron overload group B (oral administration of carbonyl iron).   Staining pattern of a

and b is similar to Fig. 7.   Arrow heads: iron positive tingible body macrophages.

Fig. 9.   Normal control group.   a: None of the bone marrow cells have hemosiderin.   b: Ferritin is detected only in central macrophages at the erythroblastic islands.   c: Higher magnification of Fig. 9b.   Ferritin-positive cells have clear, oval nuclei and dendritic cytoplasm which surround erythroblasts.

Fig. 10.   Phlebotomy group.   a and b: Erythroid hyperplasia is seen.   Ferritin and hemosiderin are undetectable in bone marrow cells, even in central macrophages.

Fig. 11.   Iron overload group A (intravenous injection of Blutal).   a: There are two types of hemosiderin-containing cells: central macrophages (arrows) which have abundant cytoplasm that is stained diffusely, and endotherial cells (arrow heads) which have some iron-positive granules.   b: Both types of cells are stained for ferritin.

Fig. 12.   Iron overload group B (oral administration of carbonyl iron).   a: Hemosiderin is shown only in central macrophages.   b: Central macrophages are strongly stained for ferritin.

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4b

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