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Abnormal iron delivery to the bone marrow in neonatal hypotransferrinemic mice

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Abstract

Hypotransferrinemic (HP) mice have a splicing defect in the transferrin gene, resulting in $< 1\%$ of the normal plasma levels of transferrin. They have severe anemia, suggesting that transferrin is essential for iron uptake by erythroid cells in the bone barrow. To clarify the significance of transferrin on iron delivery to the bone marrow, iron concentration and ^{59}Fe distribution were determined in 7-day-old HP mice. Iron concentration in the femur, bone containing the bone marrow, of HP mice was approximately twice higher than in wild type mice. Twenty-four h after injection of $^{59}FeCl_3$, ^{59}Fe concentration in the bone and bone marrow of HP mice was also twice higher than in wild type mice. The present findings indicate that iron is abnormally delivered to the bone marrow of HP mice. However, the iron seems to be unavailable for the production of hemoglobin. These results suggest that transferrin-dependent iron uptake by erythroid cells in the bone marrow is essential for the development of erythrocytes.

Introduction

Iron is an essential transition metal for the development of human and animals (Wrigglesworth & Baum 1980). Iron bound to transferrin is transported into cells via the receptor-mediated endocytosis (Crichton & Charloteaux-Wauters 1987; Huebers & Finch 1987). Transferrin, a serum glycoprotein, is primarily synthesized in the liver and has long been considered as an important molecule for the transport of iron.

The hypotransferrinemic (HP) mouse, a naturally occurring mouse mutant, has a point mutation or small deletion in the transferrin gene that results in defective splicing of transferrin precursor mRNA (Bernstein 1987). As a consequence of this mutation, the HP mouse produces $< 1\%$ of the normal circulating level of plasma transferrin. The affected animals are small, pale and severely anemic at birth and require weekly injections of serum or purified transferrin for survival. Gastrointestinal iron absorption is increased in HP mice, and iron is abnormally deposited in the liver and pancreas (Craven *et al.* 1987; Buys *et al.* 1991).

Hereditary hemochromatosis is characterized by the triad of increased iron absorption by gastrointestinal cells, high or total iron saturation of plasma transferrin, and abnormal iron deposition in the tissues, especially in the liver (Dadone *et al.* 1982; Edwards *et al.* 1982). The similar pattern of liver iron deposition in the two disorders of hypotransferrinemia and hereditary hemochromatosis may be due to saturation or lack of apotransferrin.

On the other hand, apparent difference in the distribution of transferrin receptor is not observed between HP and wild type mice (Dickinson & Connor 1998). The receptor-mediated endocytosis is considered to be important for iron uptake by erythroid cells in the bone marrow (Huebers & Finch 1987). Because of abnormal iron deposition in the liver via non-transferrin-

mediated transport mechanism, there is the possibility that iron is inadequately delivered to the bone marrow of HP mice. Thus, it is important to study iron distribution in the bone marrow of HP mice.

In the present study, to examine the significance of transferrin on the production of hemoglobin, iron concentration and ^{59}Fe distribution were determined in 7-day-old HP mice, which were not injected with transferrin.

Materials and methods

Chemicals

$^{59}FeCl_3$ [110-925 MBq (3–25 mCi)/mg Fe] was purchased from Amersham Pharmacia Biotech plc., Buckinghamshire, UK.

Animals

HP mice are originally BALBcj strain (Bernstein 1987). Because heterozygotes have 50% of the normal circulating level of transferrin, heterozygotes for the HP mutation were determined by the immunoblot analysis of serum levels of transferrin as described previously (Takeda *et al.* 1998). HP mice were obtained by matings of heterozygote mice for the HP mutation. HP mice were identified at birth because they were small and pale compared to unaffected wild type and non-mutant heterozygote littermates. Mice were housed under standard laboratory conditions (23 $\pm$ 1 °C, 55 $\pm$ 5% humidity). Mice had access to tap water and were fed a conventional mouse chow diet (Oriental Yeast Co., Ltd., Yokohama, Japan) *ad libitum*. The lights were automatically turned on at 8:00 and off at 20:00. All experiments were carried out in accordance with the 'Principles of laboratory animal care' of the National Institute of Health and the University of Shizuoka.

Tissue iron concentration

The tissues excised from five HP and wild type mice (7-day-old) were ashed for 2 h at 70 °C in 0.5 ml of nitric acid in glass centrifuge tubes. The samples were diluted with distilled deionized water before analysis. Analysis of the samples (20 μl), which was done in duplicate, was conducted using a flameless atomic absorption spectrophotometer (Nippon Jarrel-Ash AA-880 Mark-II, Kyoto, Japan) with a HU21 graphite cuvette and fitted with an iron detection lamp. The accuracy was tested by analyzing the samples added a standard iron solution.

Autoradiography

$^{59}FeCl_3$ [7.4 kBq (0.2 μCi)/67 ng Fe/50 μl/g body weight] diluted with 0.9% NaCl was subcutaneously injected into four HP mutant (1.71 $\pm$ 0.31 g body weight) and wild type (8.57 $\pm$ 0.33 g body weight) mice at 7 days of age. The mice were housed with their dams. The blood was collected from the common carotid arteries of the mice under deep diethyl ether anesthesia 24 h after injection. The mice were decapitated, frozen immediately with dry ice, fixed quickly with ice-cold 8% sodium carboxymethyl cellulose on each specimen stage, and sliced at 300 μm thickness at -20 °C with a microtome (Cryostat HM505E, Microm Laborgerate GmbH, Heidelberg, Germany). The serial sagittal slices were dried in a Cryostat at -20 °C. The distribution of radioactivity in each area of the slices was determined by autoradiography (Bio-imaging Analyzer BAS 2000, Fuji Photo Film Co. Ltd., Tokyo, Japan) after exposure to the imaging plates (Fuji imaging plate, 20 $\times$ 40 cm, Fuji Photo Film Co. Ltd.) for approximately 7 days. The exact time of exposure was determined by taking account of the physical decay. Radioactivity [photo-stimulated luminescence (PSL)/mm^2] in each area from the autoradiograms of the selected slices was measured quantitatively with a bio-imaging analyzer, and corrected according to PSL/mm^2 of internal standards in each autoradiogram.

γ ray counting

The blood obtained from HP and wild type mice were weighed and counted for the radioactivity in a γ-counter (Packard 5530, Packard Instrument Co., Inc, Meriden, CT).

Results

To examine the influence of transferrin deficiency on iron distribution of neonatal mice, iron concentration in HP and wild type mice was measured by a flameless atomic absorption spectrophotometer. Iron concentration in the liver was the highest of the tissues tested in both HP and wild type mice (Figure 1). Iron concentration in the liver of HP mice was higher than in wild type mice, although it was not significant. Iron concentration in the kidney and femur, bone containing

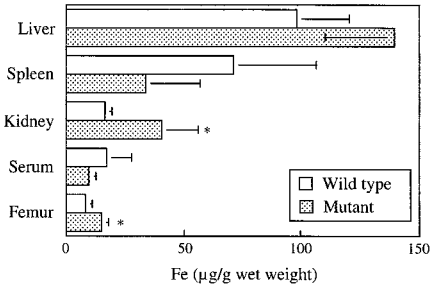

Fig. 1. Iron concentration in HP mice. Each value (mean ± SD) represents iron concentration determined by a flameless atomic absorption spectrophotometer ($n = 5$). Asterisks indicate significant difference (*, $P < 0.05$: t-test) from wild type (control).

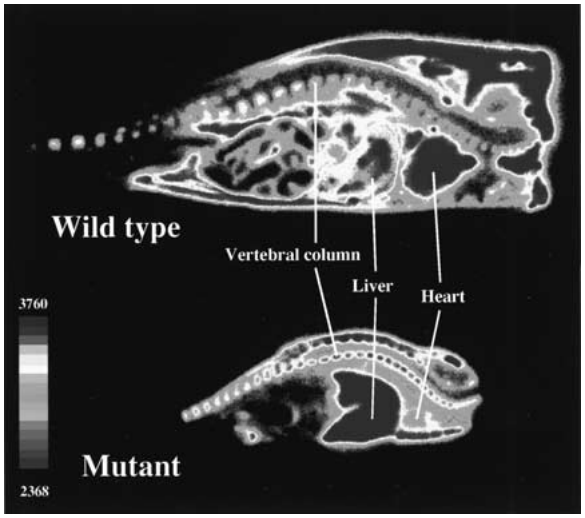

Fig. 2. ^{59}Fe imaging of HP mice. The radioimaging 24 h after injection of $^{59}FeCl_3$ was performed on selected sagittal slices of HP and wild type mice. The experiment was done in quadruplicate and the 4 images in each group were almost identical.

the bone marrow, of HP mice was also higher than in wild type mice. On the other hand, iron concentration in the spleen and serum of HP mice was approximately a half of that of wild type mice.

Because iron concentrations in tissues were changed by transferrin deficiency, ^{59}Fe distribution in HP mice was compared with that in wild type mice. Twenty-four h after injection of $^{59}FeCl_3$, ^{59}Fe distribution was remarkably different between HP and wild type mice (Figure 2). ^{59}Fe was highly concentrated in the liver in HP mice. ^{59}Fe concentration in the bone and bone marrow of the vertebral column of HP mice was higher than in wild type mice. While, ^{59}Fe was high level in the heart in the wild type mice. Regarding the blood, ^{59}Fe level (5.8% injected dose/g wet weight) in HP mice was approximately one-fifth of

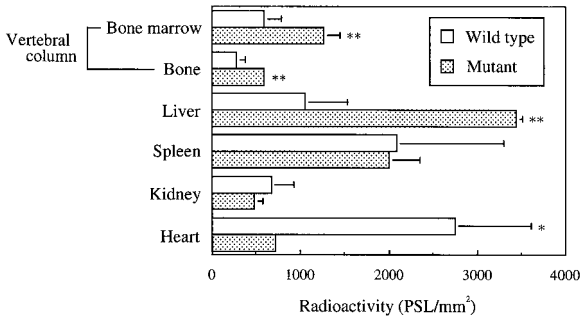

Fig. 3. ^{59}Fe distribution in HP mice. Each value (mean ± SD), which was measured with a bio-imaging analyzer, represents the radioactivity (PSL/mm^2) in the autoradiograms obtained in Figure 2 ($n = 4$). Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$: t-test) from wild type (control).

that (25.2% injected dose/g wet weight) in wild type mice.

When ^{59}Fe distribution was measured quantitatively by a bio-imaging analyzer, ^{59}Fe concentration in the liver was the highest of the tissues tested in HP mice and was approximately three times higher than in wild type mice (Figure 3). ^{59}Fe concentration in the bone and bone marrow of the vertebral column in HP mice was approximately twice higher than in wild type mice.

Discussion

HP mice are known to be severely hypochromic (Bernstein 1987). Smears of their blood revealed extensive polychromatophilia, anisocytosis, poikilocytosis, numerous target cells, stippled erythrocytes, microreticulocytes and occasional microspherocytes. Scanning electron microscopy revealed that a large portion of the red blood cell is spherocytic. Cellulose acetate electrophoresis of the hemoglobins of HP mice revealed no abnormalities. HP mice resemble human atransferrinemia (Cap *et al.* 1968; Hayashi *et al.* 1993). Both are early onset lethal diseases in which homozygotes have refractory iron-deficient hypochromic anemia.

Because transferrin-bound iron is considered to be involved in the development of erythrocytes (Huebers & Finch 1987), it is important to examine whether iron is delivered to the bone marrow in HP mice. However, HP mice usually died within a few days after birth when they were not injected with serum or purified transferrin for survival. In the present study, 7-day-old HP mice, which were not injected with

transferrin, could be obtained fortunately. Iron concentration and ^{59}Fe distribution in the 7-day-old HP mice were determined by flameless atomic absorption spectrophotometry and autoradiography, respectively.

Iron concentration in the femur of HP mice was approximately twice higher than in wild type mice, suggesting that iron is abnormally delivered to the bone and/or bone marrow. On the other hand, Craven *et al.* (1987) reported that ^{59}Fe is scarcely taken up in the femur in adult HP mice. Thus, ^{59}Fe distribution in the tissues, especially in the bone containing the bone marrow, was determined in HP mice by means of autoradiography. Twenty-four h after injection of $^{59}FeCl_3$, ^{59}Fe distribution was remarkably different between HP and wild type mice. ^{59}Fe concentration in the bone and bone marrow of the vertebral column in HP mice was approximately twice higher than in wild type mice. It is likely that iron is abnormally delivered to the bone marrow and is deposited there. The iron seems to be unavailable for the production of hemoglobin. ^{59}Fe level in the blood of HP mice was remarkably lower than in wild type mice and ^{59}Fe concentration in the liver of the former was much higher than in the latter. The present findings suggest that iron is delivered to the bone marrow, as well as the liver, via non-transferrin-mediated transport mechanism.

Craven *et al.* (1987) demonstrated that ^{59}Fe is scarcely detected in the erythrocytes of adult HP mice 3 days after oral administration. In rat reticulocytes, iron bound to transferrin is taken up by the receptor-mediated endocytosis (Thorstensen 1988). On the other hand, in rabbit and mouse reticulocytes, a portion of iron released from transferrin can be transported across the plasma membrane (Nunez *et al.* 1983). In human erythroleukemia K562, iron is taken up by transferrin-independent transport mechanism (Inman & Wessling-Resnick 1993) and/or trivalent cation-specific transport mechanism (Attieh 1999). An interesting subject is whether iron is actually taken up by erythroid cells in the bone marrow in the case of saturation or lack of apotransferrin.

Levy *et al.* (1999) demonstrated that transferrin receptor is necessary for the development of erythrocytes. The present findings suggest that transferrin-dependent iron uptake by erythroid cells in the bone marrow is essential for the development of erythrocytes in neonatal mice.

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