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H. 知的所有権の出願・取得状況 (予定を含む)

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

Ｉ Ｉ．　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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ＩＩＩ．　研究成果の刊行物　別刷

Detection of soluble HFE associated with soluble transferrin receptor in human serum

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Received September 30, 2010; Accepted November 25, 2010

DOI: 10.3892/ijmm.2010.584

Abstract. Hereditary hemochromatosis is an autosomal recessive disease, and 80-90% of patients exhibit Cys282Tyr or His63Asp mutations in the HFE gene. HFE, also known as major histocompatibility complex (MHC) class I-like molecule, binds to transferrin receptor 1 (TfR1) and β2-microglobulin at the cell surface, forming a complex. Some MHC class I molecules are known to be soluble, raising the possibility that HFE also has a soluble form. However, it is not known whether soluble HFE (sHFE) is present in human serum, and there has been no report on the possible binding between sHFE and soluble TfR (sTfR), which is the fragment of the extracellular domain of TfR1 released into the blood. In the present study, we purified an sTfR complex from pooled serum collected from healthy volunteers, showing that the main components of the complex are sTfR and transferrin. We also confirmed the existence of sHFE in this complex. This is the first report on the existence of sHFE in human serum.

Introduction

Transferrin receptor 1 (TfR1) is a type II membrane protein, which functions in iron uptake from transferrin by cells via a well-known recycling pathway (1-3). Two identical TfR1 subunits form a homodimer with a disulfide bond near the plasma membrane. In the recycling pathway, a portion of TfR1 is hydrolyzed at the point between Arg100 and Leu101 in the extracellular domain of TfR1. The extracellular domain of TfR1 is released from the cells into the blood and is detectable as soluble TfR (sTfR) (4-8). The concentration of sTfR is increased in iron deficiency anemia and autoimmune hemolytic anemia, and is decreased in aplastic anemia and the myelodysplastic syndrome. The concentration of circulating sTfR is therefore, a marker of erythropoiesis and iron storage (9-11).

TfR1 binds to HFE, the protein which when mutated is responsible for hereditary hemochromatosis, as well as to β2-microglobulin (β2m) at the cell surface, forming a complex (12-17). Hereditary hemochromatosis is an autosomal recessive disease, and 80-90% of patients exhibit Cys282Tyr or His63Asp mutations in the HFE gene. HFE binds to TfR1 in the intracellular endosome bearing the Tf-TfR1 complex, reducing the binding affinity between TfR1 and Tf (16,18-20). In addition, HFE has been reported to reduce the rate of TfR1 recycling and of iron uptake (21). HFE has a high similarity to the major histocompatibility complex (MHC) class I proteins. Some MHC class I molecules are known to be soluble (22-29), raising the possibility that HFE also has a soluble form. Recombinant soluble HFE (sHFE) protein produced by the transfection of the extracellular portion of HFE binds to sTfR, and sHFE can bind to the complex of Tf-TfR1 (13,30). However, it is not known whether sHFE is present in human serum, and there is no report concerned with possible binding between sHFE and sTfR in the circulation. Therefore, we investigated these possibilities.

Materials and methods

Cell cultures and liver tissues. Human hepatoma-derived HLF cells (Japanese Cancer Resources Bank, Tokyo) were cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate at 37°C in a 5% CO_2 incubator. Human liver samples were obtained from liver biopsies performed in patients with hepatitis, and used with written permission.

Antibodies. Anti-TfR1 monoclonal antibodies (clone Nos. TR101 and TR112, IgG1, Nissui, Tokyo), anti-Tf monoclonal

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Key words: transferrin, soluble transferrin receptor, soluble HFE, major histocompatibility complex class I molecule

antibody (Biodesign International, ME), and anti-CD5 antibody (B-B8, IgG1, Funakoshi) were used. Anti-HFE antiserum was obtained by vaccination of rabbits with a complex of a recombinant HFE sequence (171-182: RHKIRARQNRAYC) and keyhole limpet hemocyanin. Peroxidase-conjugated anti-mouse (Rockland, PA) or anti-rabbit (Chemicon, CA) antibodies were used as the secondary antibodies in Western blotting.

Purification of sTfR complex from pooled serum collected from healthy volunteers. Pooled serum (100 ml) collected from healthy volunteers was passed through an anti-TfR1 monoclonal antibody-bound Sepharose-4B column (column volume of 10 ml), and then washed with phosphate buffered saline (PBS). Elution was performed using ammonium peroxide solution (pH 11.0), and the fractions including sTfR were determined by enzyme-linked immunosorbent assay (ELISA) and collected. Next, collected fractions were equilibrated with 0.01 M Tris phosphate (pH 8.0), and further purified by passage through a monoQ column (linear gradient of NaCl, 0-0.5 M). Fractions including sTfR were analyzed by ELISA, for use in the present study. Protein concentrations were determined by the Lowry method (Bio-Rad, CA).

ELISA. Ninety-six-well plates were coated with 100 μg/ml of the anti-TfR1 antibody (TR112) for 3 h at room temperature, and then blocking was performed using PBS with 1% bovine serum albumin (BSA). Anti-TfR1 antibody clone TR101 was used for the detection. Color assays were performed using 4.5 mM disodium phenylphosphate and 2 mM 4-aminoantipyrine as the substrate. Absorbance was measured at 490 nm.

SDS-PAGE and silver staining. Cultured cells were harvested with a cell scraper, washed with PBS twice, and then incubated with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1.0% Nonidet P-40) for 30 min at 4°C. Human liver tissues were homogenized with lysis buffer. Both samples were centrifuged at 14,000 rpm for 10 min at 4°C and then the supernatant was collected. Collected samples were mixed with Laemmli sample buffer (Bio-Rad) for 5 min at 96°C and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 8 or 10% SDS-polyacrylamide gels. A silver stain reagent kit (Daiichi-Kagaku-Yakuhin) was used for silver staining.

Immunoprecipitation. Lysates obtained from cultured cells, human liver tissues, and purified sTfR fractions were incubated with protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) and centrifuged. Supernatants were collected, and primary antibody was added and incubated for 8 h at 4°C. Washing was performed with lysis buffer twice, then Laemmli sample buffer was added, followed by incubation for 5 min at 96°C. Finally, the samples were applied for SDS-PAGE.

Western blotting. After SDS-PAGE, proteins were transferred to nitrocellulose membranes. Nitrocellulose membranes were treated with blocking buffer (5% skim milk dissolved in 0.05% Tween-PBS) at 4°C overnight, washed with 0.05% Tween-PBS, and then incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing with 0.05% Tween-PBS, the ECL chemiluminescence system (Amersham, Buckinghamshire, UK) was used for detection. To determine the specificity of the produced anti-HFE antibody, Western blotting was performed as above using the anti-HFE antibody mixed with peptide (8 μg/ml) which was used as antigen for producing the anti-HFE antibody.

Transfection of HLF cells with the HFE gene. Total RNA was purified from human hepatoma-derived Chang cells using RNAgents total RNA isolation system (Promega). Complementary DNA was synthesized by reverse transcription with M-MLV Reverse Transcriptase (Promega) using an oligo dT primer (Invitrogen, CA). Nested polymerase chain reaction (PCR) was then performed using Taq DNA polymerase (Takara) and 3 primers (first sense: 5'-CTGAGCCTAGGCA ATAGCTG-3', second sense: 5'-TAGGGTGACTTCTGGAG CCA-3', first and second antisense: 5'-TCACGTTAGCTAAG ACGTA-3'). The entire coding region of the HFE gene without a stop codon (position -86 to 1043) was amplified by PCR. Forty thermal cycles of 94°C for 4 min, 55°C for 1 min, and 72°C for 2 min were performed. The PCR product was ligated in pT7 blue vector (Novagen, Madison, WI), and its sequences confirmed using the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Subcloning of the HFE gene to the mammalian expression pRc/CMV vector (Invitrogen) was then performed. Transfection of HLF cells with the pRc/CMV vector bearing the HFE gene was carried out by lipofection using the GenePorter kit (Gene Therapy Systems, CA). Cells were harvested 48 h after transfection.

Labeling of the sTfR fraction with ^{125}I. Purified sTfR fraction (20 μl, 50 μg/ml), IODO-Beads (Iodinating reagent, Pierce), 40 μCi of ^{125}I (Amersham), and 500 μl of 100 mM Tris-HCl were mixed and incubated for 15 min at room temperature. The mixture was passed through a PD-10 column (Bio-Rad), and collected fractions were assayed with a gamma counter. Fractions labeled with ^{125}I were then collected and used in the experiments.

Results

Purification of the sTfR complex from pooled sera of healthy volunteers. Due to the fact that the serum concentration of HFE was expected to be low, and a method for purifying only HFE was not available, we tried to purify sTfR that might include soluble HFE. Pooled serum collected from healthy volunteers was isolated by affinity chromatography using an anti-TfR1 monoclonal antibody-bound Sepharose-4B column. The sTfR complex was further purified by ion-exchange chromatography utilizing a monoQ column and a linear gradient of NaCl. The concentrations of eluted proteins as a function of NaCl concentration, as well as the absorbancies measured by ELISA for quantifying the concentration of sTfR in each fraction are shown in Fig. 1A. The concentrations of sTfR indicated by the absorbancies at OD 490 nm were relatively high in the fractions eluted in 0.2-0.3 M NaCl. These fractions were collected, and used for further experiments.

Characteristics of the purified sTfR fractions. To determine the characteristics of the purified sTfR fractions, 10 mg of purified protein were applied for SDS-PAGE electrophoresis

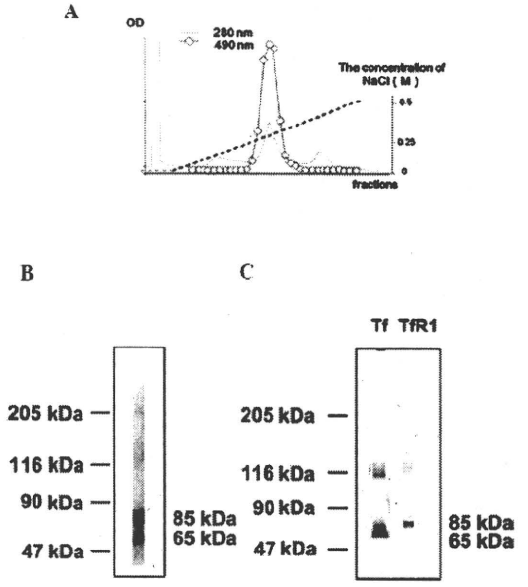

Figure 1. (A) Purification of sTfR by ion-exchange chromatography. Fractions including sTfR were concentrated from pooled serum collected from healthy volunteers using an anti-TfR1 antibody-bound Sepharose 4B column, and then passed through an ion-exchange monoQ column. Finally, fractions were eluted using the gradient concentrations of NaCl. The dotted line indicates the concentration of NaCl; the solid line indicates the concentrations of eluted proteins measured by the absorbancies at 280 nm; and the open circles indicate the concentration of sTfR measured by the absorbancies at 490 nm with the ELISA system. (B) Silver staining for the purified fraction of sTfR after SDS-PAGE. Two bands corresponding to 85 kDa and 65 kDa proteins were observed. (C) Western blot analysis for the purified fraction of sTfR after SDS-PAGE using an anti-Tf (Tf) and an anti-TfR1 antibody (TfR1). Two bands corresponding to 85 kDa and 65 kDa proteins were observed, indicating these proteins were sTfR and Tf, respectively.

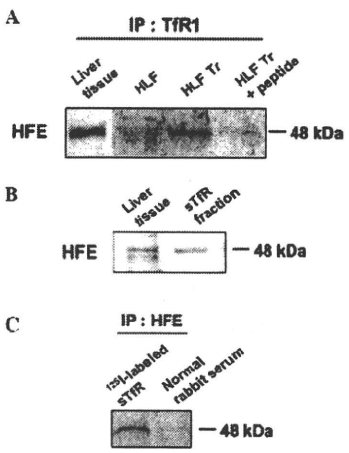

Figure 2. (A) Determination of the specificity of the anti-HFE antiserum. To determine the specificity of the anti-HFE antiserum produced by vaccination of a rabbit with the HFE peptide, lysates prepared as indicated were applied for immunoprecipitation by an anti-TfR1 antibody followed by Western blotting with the anti-HFE antiserum. Lysates were prepared from liver tissues, human hepatoma-derived HLF cells without the expression of HFE, transfected HLF cells with HFE expression vector (HLF-Tr), HLF-Tr lysate blocked by the peptide used as an antigen. (B) Western blotting for the purified sTfR fraction using the anti-HFE antiserum. The purified sTfR fraction was taken for SDS-PAGE followed by Western blotting using the anti-HFE antiserum. Bands corresponding to 48 kDa were observed in both samples. Lysates were prepared from liver tissues and the purified sTfR fraction. (C) Immmunoprecipitation from ^{125}I-labeled purified sTfR fraction using the anti-HFE antiserum. The purified sTfR fraction was labeled with ^{125}I and then subjected to immunoprecipitation using an anti-HFE antibody followed by SDS-PAGE. The 48 kDa protein was immunoprecipitated with the anti-HFE antibody. Normal rabbit serum was used as the negative control.

and silver staining. Although a broad non-specific band could be seen at the region of high molecular weight, silver staining showed clear bands with apparent molecular weights of 85 and 65 kDa (Fig. 1B). To identify these proteins, Western blotting was performed. The band at 85 kDa reacted with the anti-TfR1 antibody and the band at 65 kDa reacted with the anti-Tf antibody (Fig. 1C) confirming that the principal components of the sTfR fraction were sTfR and Tf. This result verifies a previous report concerning the main components of the sTfR fraction purified from pooled sera of healthy volunteers (31).

Determination of the specificity of the anti-HFE antibody against HFE. The specificity of the anti-HFE antibody made by immunization of a rabbit with HFE was determined. Lysates were prepared from liver tissues expressing HFE, human hepatoma-derived HLF cells that did not express any HFE, and the transiently transfected HLF cells (HLF-Tr) with HFE expression vector. SDS-PAGE was performed after immunoprecipitation with the anti-TfR1 antibody, and then Western blotting was performed using the anti-HFE antibody. The band at 48 kDa in the liver tissue was considered to be HFE, but no band was observed in HLF cells (Fig. 2A). HLF-Tr cells showed the band at the molecular weight of 48 kDa as the same as that in the liver tissue (Fig. 2A). The reactivity of the anti-HFE antibody was inhibited by the peptide which was used for vaccination of the rabbit, indicating that the produced antibody specifically recognized and reacted with HFE (Fig. 2A).

Detection of soluble HFE in the purified sTfR fractions. To determine if HFE was present in the purified sTfR fractions, lysates from liver tissues and purified protein were subjected to SDS-PAGE followed by Western blotting using the anti-HFE antibody. The band was observed in the purified sTfR fraction at 48 kDa similarly to the plasma membrane-bound HFE from liver tissues (Fig. 2B). The purified protein was then labeled with ^{125}I and immunoprecipitated with the anti-HFE antibody. The band observed at 48 kDa in the ^{125}I-labeled sTfR fraction was separated by SDS-PAGE after immunoprecipitation with the anti-HFE antibody (Fig. 2C). In contrast, no band was observed in the negative control when normal rabbit serum was used for immunoprecipitation. These results indicate that the size of sHFE was almost the same as that of the membrane-bound HFE. However, the concentrations of sHFE in healthy donors were considered to be very low compared to sTfR and Tf since no band was observed at 48 kDa with silver staining (Fig. 1B).

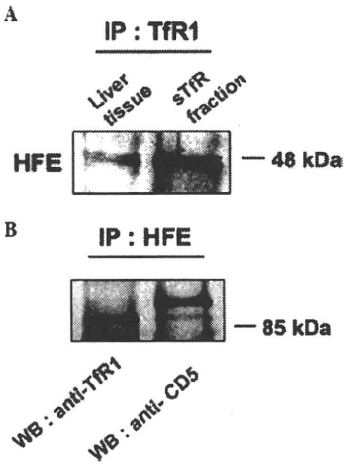

Figure 3. (A) Determination of sHFE bound with sTfR in the purified sTfR fraction by an anti-TfR1 antibody. Lysates prepared from liver tissues and the sTfR fraction were immunoprecipitated using an anti-TfR1 antibody. After immunoprecipitation, SDS-PAGE was performed, followed by Western blotting using the anti-HFE antibody. Bands corresponding to 48 kDa were observed in both lanes, indicating that both were precipitable with TfR1. (B) Detemination of soluble TfR bound with soluble HFE in the purified soluble TfR fraction by an anti-HFE antibody. Lysates prepared from the sTfR fraction were immunoprecipitated using an anti-HFE antibody. After immunoprecipitation, SDS-PAGE was performed, followed by Western blotting using an anti-TfR1 antibody or an anti-CD5 antibody (negative control). The band corresponding to the 85 kDa protein was observed in the left lane.

Determination of the complex formed between sTfR and sHFE. To determine whether sHFE forms a complex with TfR1, immunoprecipitation and Western blotting using an anti-TfR antibody and the anti-HFE antibody were performed. Western blotting and SDS-PAGE showed clear 48 kDa bands in the lysate prepared from liver tissues and the sTfR fraction (Fig. 3A). In contrast, Western blotting using the anti-TfR1 antibody after immunoprecipitation with the anti-HFE antibody and SDS-PAGE showed clear bands at 85 kDa in the sTfR fraction (Fig. 3B). No band at 85 kDa using the anti-CD5 antibody as a negative control was seen. We therefore, conclude that HFE on the cell surface is released into the serum as a soluble form with almost same molecular weight as the membrane-bound HFE, and binds to sTfR to form a complex.

Discussion

In the present study, sTfR fractions were purified from the serum of healthy volunteers using an affinity column with an anti-TfR1 antibody and a monoQ column. It was confirmed that the main contents of the purified fractions were sTfR and Tf. Soluble HFE was also found, although its amount was very small. This is the first report indicating the presence of HFE as a soluble form bound to sTfR in serum. There are reports that HFE forms lacking the α domain or a portion of it, apparently products of alternative splicing that alters the sequence with which HFE binds to TfR1 (32-34), resulting in sHFE that cannot form a complex with sTfR, may also circulate. Further investigation is warranted.

HFE belongs to MHC class Ib, and has constructive homology with MHC class Ia in which HLA-A, -B, and -C belong. They have the α chain as an extracellular domain (α1, α2, α3 domains), a transmembranous domain, and an intracellular domain and therefore, are transmembrane proteins. The existence of a soluble form has been reported in MHC class Ia, and it was also reported that the serum concentration and the molecular conformation are related to the disease activity and to complications in liver or kidney transplantations (22,26), to systemic lupus erythematosus, and to rheumatoid arthritis (25,28,29). MHC class Ib has also been reported to have a soluble form (35,36), and the serum concentration of HLA-G which belongs to this MHC class was useful for the diagnosis of placental abruption (37) and lymphoproliferative diseases (38). The clinical significance of the soluble HFE should be clarified by further studies.

Three conformational variants of the soluble forms of MHC class Ia and Ib have been identified (27-29,35,36). The first one has been digested just external to the cell membrane by metalloproteinase, resulting in a smaller molecular weight compared to the membrane-bound protein. The second type is produced by alternative splicing that leads to loss of the transmembrane portion of the protein. The third is released into the serum following shedding or destruction of cells and exhibits a molecular weight almost the same as that of the intact membrane-bound protein. The soluble HFE observed in the present study has a molecular weight of 48 kDa identical to that of membrane-bound HFE, implying that the soluble HFE possesses both transmembrane and intracellular portions and is released into the circulation by cell shedding or destruction.

We confirm that recombinant sHFE protein produced by CHO cells (13,30). Tf, and recombinant sTfR form a complex, with a stoichiometry resembling that observed at the cell surface where recombinant sTfR forms a dimer. It has also been reported that a Tf molecule and an sHFE molecule could bind to the dimer of sTfR, and that two sHFE molecules could bind to the dimer of sTfR when the concentration of sHFE is increased. However, the stoichiometry may change because sTfR in the serum may be a monomeric fragment from digestion at the region of disulfide bond formation. Complexes formed with one molecule of sTfR and one molecule of Tf, and with two molecules of sTfR and two molecules of Tf have been described (31), and a complex with two molecules of sTfR and one molecule of Tf has been found in decreased iron loads (39). We could find no data on whether a complex of sHFE and Tf exists. A complex of monomeric sTfR and sHFE and Tf is not expected because the binding site of sTfR and Tf partly overlaps with the binding site of sTfR and sHFE (30). However, the fact that two molecules of sTfR and two molecules of Tf could form a complex suggests the existence of a complex of two molecules of sTfR with one molecule of Tf and one molecule of sHFE.

We have shown that sHFE binds to sTfR. Whether the concentration of serum sHFE has some direct or indirect effect on iron absorption and iron sequestering of the cells remains unclear. Concentrations of sHFE might fluctuate with the volume of HFE-expressing cells or the catabolim of a growing cell. Further investigation is required to determine if there is an effect on iron metabolism, and a possible clinical relevance of the findings.

In conclusion, we purified an sTfR complex from pooled serum collected from healthy volunteers, showing that the main components of the complex were sTfR and Tf. We also confirmed the existence of sHFE in that complex. We believe this is the first report on the existence of sHFE in normal healthy people. HFE expressed on the cell surface was found to be released from the cells to the serum in a soluble form which complexes to sTfR. The clinical significance of our findings is not yet clear.

Acknowledgements

We thank Dr Philip Aisen for reviewing this manuscript.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Patients with Chronic Hepatitis C May be More Sensitive to Iron Hepatotoxicity than Patients with HFE-Hemochromatosis

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Abstract

Aim In chronic hepatitis C, iron might play an important role as a hepatotoxic co-factor. Therefore, venesection, a standard treatment for hemochromatosis, has been proposed as an alternative for patients who respond poorly to anti-viral therapy. To improve our understanding of iron-induced hepatotoxicity, we compared the responses to venesection between patients with chronic hepatitis C and those with HFE-hemochromatosis.

Methods Fourteen Japanese patients with chronic hepatitis C and eight Italian patients with HFE-hemochromatosis underwent repeated venesection with a serum ferritin endpoint of 20 and 50 ng/mL, respectively. Serum iron indices and liver function tests were measured in pre- and post treatment blood samples from each patient. Body iron stores were calculated using the removed blood volume.

Results In both patients with hepatitis and hemochromatosis, serum ferritin, aminotransferase and hepcidin 25 were reduced after venesection. The serum aminotransferase activity, but not the serum ferritin level, was predictive of effective iron removal treatment. Hepcidin regulation was set at an inappropriately low level in hemochromatosis patients (11.1 ± 9.2 ng/mL), but not so in hepatitis patients (30.7 ± 14.5 ng/mL). Inversely, the estimated body iron stores of hemochromatosis patients were 5,960 ± 2,750 mg, while those of hepatitis patients were 730 ± 560 mg. Judging from the liver enzyme reduction ratio, patients with hepatitis seemed to be more sensitive to iron hepatotoxicity than hemochromatosis patients.

Conclusion Even though the threshold of iron hepatotoxicity and benefit of its removal differ between patients with chronic hepatitis C and those with HFE-hemochromatosis, venesection is a valid choice of treatment to reduce liver disease activity in both diseases.

Key words: body iron store, hemochromatosis, HFE, hepatitis C, hepcidin, venesection

(Intern Med 49: 2371-2377, 2010)
(DOI: 10.2169/internalmedicine.49.4088)

Introduction

A door to genetic diagnosis of hereditary hemochromasis was opened in 1996 by Feder et al; C282Y homozygosity in *HFE* gene was found in 85% patients with hemochromatosis (1). Marked iron overload can induce organ and tissue damage by producing oxidative stress related to the presence

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Received for publication June 14, 2010; Accepted for publication August 20, 2010

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of "free iron" or "labile iron". Accordingly, iron depletion by venesection is the standard treatment for patients with HFE-hemochromatosis. Treatment at the pre-fibrosis stage is essential because hepatocellular carcinoma can develop in patients with advanced liver fibrosis even after iron removal (2). Iron-induced oxidative stress in the liver can also be induced by chronic infection with hepatitis C virus (HCV) and some studies suggest that in patients with chronic hepatitis C (CHC), the coexistence of even a slight amount of excess iron in the liver may exacerbate this oxidative stress and promote liver injury and fibrosis, and in some cases, promote carcinogenesis (3, 4). Over the last 20 years, the treatment of choice for CHC has been interferon (IFN) therapy (5). Even though the clinical administration of pegylated IFN and combination therapy with ribavirin have improved the viral clearance rate, these antiviral therapies are effective in eradicating the virus in only approximately 50% of patients (6). Therefore, venesection has been proposed as an alternative in CHC patients who either were contraindicated or responded poorly to IFN therapy, but the routine application of venesection for the treatment of CHC patients remains limited worldwide.

Hepcidin, a peptide synthesized in the liver, is the main regulator of iron homeostasis by inhibiting intestinal iron absorption and iron release by macrophages (7, 8). Functionally, hepcidin secreted into the circulation binds to ferroportin, the only known cellular iron exporter, inducing its internalization and degradation (9). Thus, the down-regulation of ferroportin controls iron efflux from enterocytes and reticuloendothelial cells into the circulation. Hemochromatosis proteins act as positive regulators of hepcidin. Thus, hepcidin synthesis is at a low level in patients with HFE-hemochromatosis, which is the primary explanation for the development of iron overload in these patients (10). Venesection further decreases serum and urinary hepcidin to very low levels in patients with HFE-hemochromatosis, indicating that they are still able to modulate, although inappropriately, hepcidin production in response to iron stores (11, 12). Investigation of hepcidin regulation has been limited due to the lack of reliable methods. Recently serum hepcidin levels of CHC patients were measured in 2 studies using different methods (13, 14). Both studies indicated that hepcidin regulation by iron stores is maintained in CHC and suggested that HCV infection can impair hepcidin production, which may be an important factor in hepatic iron accumulation.

Based on previous data on the possible beneficial effect of venesection in HFE-hemochromatosis and CHC (2-4), we investigated iron-induced hepatotoxicity and hepcidin regulation under these iron overload conditions.

Subjects and Methods

Fourteen Japanese patients with CHC (8 males and 6 females; aged 57 ± 8 years) and eight Italians with HFE-hemochromatosis (6 males and 2 females; aged 49 ± 17 years) were treated by venesection. Age-matched controls were selected from a database of healthy Japanese volunteers (8 males and 6 females; aged 56 ± 7). Inclusion criteria for CHC patients were: HCV-positive chronic hepatitis, alcohol intake <25 g/day, absence of coexisting hepatitis B virus (HBV) infections; absence of decompensated cirrhosis; absence of coexisting conditions that could influence iron parameters, such as acute and chronic inflammatory diseases and hematological disorders, venesection, iron supplementation or repeated transfusions. All CHC patients were either non-responders to IFN or had refused IFN therapy. Most patients were under long-term ursodeoxycholic acid treatment without interruption during venesection. Demographic information showing that the HFE mutant has an almost zero incidence among Japanese (15) permitted omission of HFE analysis in this population. Inclusion criteria for HFE-hemochromatosis were increased transferrin saturation and serum ferritin, and homozygosity for C282Y mutation in *HFE* (1). HBV, HCV or human immunodeficiency virus infections and high alcoholic intake were exclusion criteria for the hemochromatosis group. Patients with CHC received venesection with modified endpoints of serum ferritin of 20 ng/mL or hemoglobin of 12.0 g/dL based on a previous report (3) because iron deficiency anemia might decrease hepcidin production. A volume of 200 mL for female patients and 400 mL for male patients was removed every two weeks. Italian patients with HFE-hemochromatosis were treated by standard venesection with an endpoint of serum ferritin levels of less than 50 ng/mL. A volume of 350 mL for female patients and 400 mL for male patients was drawn each week.

Routine laboratory tests included hemoglobin, serum alanine aminotransferase (ALT) activities, serum iron, total iron binding capacity and ferritin concentration. Transferrin saturation (TS) was calculated according to the standard method. Serum hepcidin 25 was quantified by liquid chromatography tandem mass spectrometry in the laboratory of Kanazawa Medical University, and expressed as ng/mL as reported previously (16).

Because Hb did not change in venesection for hemochromatosis, body iron stores were simply estimated from the total iron removed using a modified version of the following formula reported previously (17) [mean Hb (g/dL)×0.034× total blood volume (mL)]. Body iron stores of hepatitis with post treatment anemia were adjusted by reduced hemoglobin (Hb) concentration during venesection: body iron stores (mg)=total iron removed (mg) - reduced blood iron (mg) [ΔHb (g/dL)×0.034×1/15×body weight (g)]. ΔHb (g/dL) was defined as the change in the concentration after venesection.

Reduction of ALT activity was calculated as [pre-treatment activity-post treatment activity]. Based on a hypothesis that sensitivity to iron-induced hepatotoxicity might be represented by a ratio of ALT reduction during venesection to body iron stores, the iron hepatotoxicity index (IHI) was calculated by [dividing reduction in ALT activity by body iron stores estimated from total volume of removed

Table 1. Laboratory Data of Controls and Patients Receiving Venesection

Subjects	Hb (g/dL)	Hepcidin (ng/mL)	Ferritin (ng/mL)	H/F ratio	TS (%)	ALT (U/L)
Japanese Control (n=14)						
Base line	14.0±1.4	33.7±17.9	110±81	0.48±0.47	35.8±9.3	20±7
Japanese CHC (n=14)						
Pre Treatment	14.6±1.1	30.7±14.5	250±132	0.14±0.07	45.3±12.9	90±27
Post Treatment	13.1±1.3	2.0±1.4	15±4	0.15±0.12	17.2±5.7	41±16
p1 (Pre vs. Cont)	ns	ns	<0.01	0.018	0.034	<0.01
p4 (Pre vs. Post)	<0.01	<0.01	<0.01	ns	<0.01	<0.01
Italian Hemochromatosis (n=8)						
Pre Treament	14.6±0.8	11.1±9.2	1347±620	0.01±0.01	83±14	40±21
Post Treatment	14.8±0.5	2.1±2.3	49±16	0.05±0.05	41±13	18±5
p1 (Pre vs. Control)	ns	<0.01	<0.01	<0.01	<0.01	ns
p2 (Pre vs. Pre CHC)	ns	<0.01	<0.01	<0.01	<0.01	<0.01
p3 (Post vs. Post CHC)	<0.01	ns	<0.01	0.012	<0.01	<0.01
p4 (Pre vs. Post)	ns	0.014	<0.01	ns	<0.01	0.016

H/F, hepcidn/ferritin, ALT; alanine aminotransferase, TS; transferrin saturation, CHC; chronic hepatitis C, *p1*; statistical analysis of pre-treatment values compared to control baseline, *p2*; statistical analysis of pre-treatment values compared to those of CHC, *p3*; statistical analysis of post treatment values compared to those of CHC, *p4*; statistical analysis of pre-treatment and post-treatment values.

There were sex differences in Hb and serum ferritin concentration, but not in serum levels of hepcidin and ALT in controls.

Pre-treatment patients with hemochromatosis were characterized by remarkably high levels of ferritin and transferrin saturation, and low levels of hepcidin and ALT compared to those with CHC. Baseline levels of hepcidin did not differ between controls and CHC, but the hepcidin/ferritin ratio, an iron regulatory hormone index adjusted by representative values of body iron stores, were low in CHC. Regardless of the different endpoints, both patients with CHC and those with hemochromatosis responded similarly to venesection treatment. Post treatment levels of hepcidin were quite low in all patients regardless of CHC or hemochromatosis.

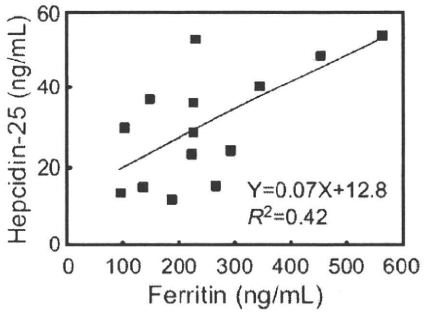

Figure 1. A correlation between serum levels of ferritin and hepcidin 25 in pre-treatment patients with chronic hepatitis C. Hepcidin might regulate iron homeostasis in chronic hepatitis C patients.

blood].

These procedures including venesection were performed in accordance with guidelines for Human Research at Nagoya University Hospital and Milano-Bicocca University. For statistical analysis, biochemical indices were expressed as mean ± SD, and differences between pre- and post-treatment values, and those among patient groups were analyzed using Student's t test. Gender differences were not considered in the study because of the small number of patients.

Results

Laboratory data of subjects at entry and post-venesection are summarized in Table 1. Pre-treatment levels of serum ferritin, transferrin saturation and ALT differed among groups. Serum ferritin levels and TS were markedly elevated in hemochromatosis, and slightly increased in CHC compared to those in controls. ALT levels were three times higher than the upper normal value in patients with CHC and only slightly increased in hemochromatosis patients. Pre-treatment hepcidin levels of hepatitis patients did not significantly differ compared with those of controls (33.7 ± 17.9 vs. 30.7 ± 14.5 ng/mL, p=0.64), but the hepcidin/ferritin ratio in hepatitis patients was significantly lower than that in controls (0.14 ± 0.07 vs. 0.48 ± 0.47, p=0.018). Serum hepcidin levels were significantly lower in patients with hemochromatosis. Markedly low levels of hepcidin and high levels of ferritin induced quite low values for the hepcidin index in hemochromatosis patients. A significant correlation was observed in the pre-treatment levels between serum hepcidin and ferritin in CHC patients (r=0.65, p=0.012) (Fig. 1), but not in controls and hemochromatosis patients (data not shown). The small number of controls and patients

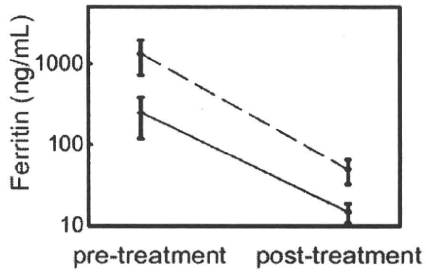

Figure 2. Reduction of serum ferritin levels in the 2 patient groups by venesection. Note that the endpoints were set differently: 20 ng/mL for chronic hepatitis C (a straight line), and 50 ng/mL for HFE-hemochromatosis (a dotted line). High starting points of 1347±620 ng/mL in hemochromatosis patients indicate severe iron overload in the genetic disorder as compared to those of 250±132 ng/mL in chronic hepatitis C patients. Vertical bars indicate mean ± SD.

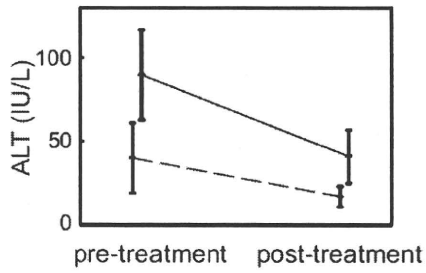

Figure 3. Reduction of serum ALT levels in the 2 patient groups by venesection. The parameter of biochemical liver disease was normalized in HFE-hemochromatosis (dotted line), but remained slightly elevated in chronic hepatitis C (straight line), suggesting that viral insult persisted after iron hepatotoxicity was removed. ALT; alanine aminotransferase. Vertical bars indicate mean ± SD.

with HFE-hemochromatosis might have caused a false negative result.

There were no side effects of venesection requiring discontinuation of therapy. Hemoglobin concentration remained normal during venesection in hemochromatosis patients but decreased slightly in CHC patients. The serum ferritin concentrations used for monitoring body iron stores decreased linearly from 250 ± 32 ng/mL to levels of <20 ng/mL in CHC, and from 1,347 ± 620 ng/mL to levels <50 ng/mL in hemochromatosis (Fig. 2). The treatment effectively reduced ALT to only slightly increased levels in CHC, and normalized these levels in hemochromatosis (Fig. 3). One exceptional patient with hemochromatosis showed an increase in the liver enzyme level from 15 to 23 U/L. Serum hepcidin was significantly decreased in both CHC and hemochromatosis reaching comparable levels after iron depletion (2.1 ± 2.3 ng/mL in hemochromatosis vs. 2.0 ± 1.4 ng/mL in

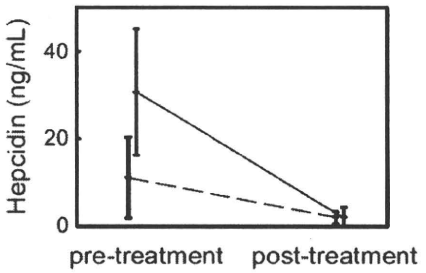

Figure 4. Reduction of serum hepcidin levels in the 2 patient groups by venesection. Serum hepcidin 25 levels of chronic hepatitis C (straight line) were similar to those of controls at the pre-treatment stage, while those of HFE-hemochromatosis (dotted line) were low at 11.1±9.2 ng/mL. In both patient groups, hepcidin levels were reduced to quite low levels after venesection. It is clear that the hepcidin system is set at lower levels in HFE-hemochromatosis than in chronic hepatitis C. Genetic setting with low hepcidin regulation caused a large amount of iron absorption in the intestine over 50 years. Vertical bars indicate mean ± SD.

CHC) (Fig. 4). One exceptional patient with hemochromatosis showed an increase in serum hepcidin from 3.5 to 7.7 ng/mL.

The regimens and effects of venesection for patients with CHC and hemochromatosis are summarized in Table 2. Estimated body iron stores of 730 ± 560 mg were removed from hepatitis patients over 7±3 months, while 5,960 ± 2,750 mg were removed from hemochromatosis patients over 15 ± 8 months. There were differences in the body iron stores and treatment periods between the 2 patient groups. In CHC patients, reduction of ALT activity by venesection was larger than that in hemochromatosis patients (49 ± 30 vs. 22 ± 20 U/L). There were correlations between the reduction in ALT activity and pre-treatment ALT activity in both patient groups (Fig. 5, 6). IHI was quite low in hemochromatosis with a significant difference between the 2 patient groups (0.097 ± 0.083 in CHC vs. 0.0032 ± 0.0046 U/L/mg in hemochromatosis).

Discussion

In the present paper, we compared the behavior of serum iron indices including hepcidin-25 and liver enzymes in CHC and HFE-hemochromatosis during the first stage of venesection to remove body iron stores. Since, in addition to genetic background, dietary customs between Italians and Japanese, and the venesection protocols used for CHC and hemochromatosis are different, this comparative study may involve confounding factors. However, most of the findings observed are potentially significant. Considering that both conditions responded to venesection, iron hepatotoxicity is involved in their pathogenesis, but these iron disorders substantially differ with regard to mechanisms and amounts of

Table 2. Regimens and Effects of Venesection Treatment in Chronic Hepatitis C and HFE-Hemochromatosis

	CHC	Hemochromatosis	Statistical Analysis
Treatment Periods (months)	7 ± 3	15 ± 8	0.03
Blood Volume Removed (mL)	2,010 ± 1,190	12,000 ± 5,560	<0.01
Iron Removed by Venesection. (mg)	940 ± 550	5,960 ± 2,750	<0.01
Iron in Reduced Hb (mg)	210 ± 150	negligible	
Body Iron Stores (mg)	730 ± 560	5,960 ± 2,750	<0.01
ALT Reduction (U/L)	49 ± 30	22 ± 20	0.02
Iron Hepatotoxicity Index	0.097 ± 0.083	0.0032 ± 0.0046	<0.01

CHC: chronic hepatitis C, ALT; alanine aminotransferase.

Treatment methods for CHC and hemochromatosis are different so that statistical analysis is not fully reliable. Body iron stores were calculated from the blood volume removed according to the formula described in the text. Iron hepatotoxicity index (IHI) was calculated by dividing reduction in ALT by body iron stores. Provided that iron-induced hepatotoxicity is represented by ALT reduction during iron removal treatment, IHI might be a sensitivity to iron hepatotoxicity. A high IHI in CHC suggests not only a high sensitivity to iron hepatotoxicity, but also a good benefit of iron removal treatment. In contrast, liver cells with HFE-mutation are highly tolerant of iron hepatotoxicity. Treatment regimens for hemochromatosis were stronger but effects were smaller than those for CHC.

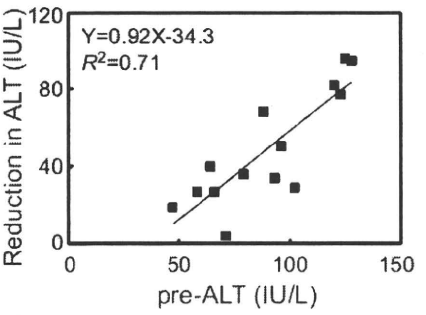

Figure 5. A good correlation between the reduction of ALT and pre-treatment ALT in chronic hepatitis C. Pre-treatment ALT level is predictive of efficacy by venesection. It may be important that other parameters including ferritin concentration are not predictive of treatment effects. In other words, the higher ALT is, the greater the treatment effect will be. ALT: alanine aminotransferase.

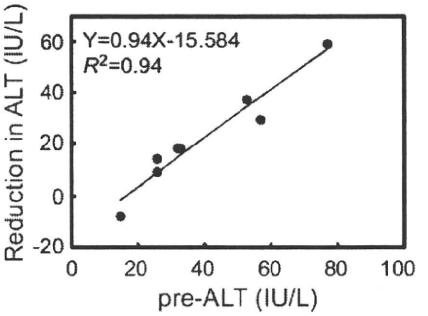

Figure 6. A good correlation between the reduction of ALT and pre-treatment ALT in HFE-hemochromatosis. Seven of 8 patients treated showed decreased serum ALT activities, while one of them had increased enzyme activity from 15 to 23 U/L after iron removal. It may be important that even though the levels are low, the effect of iron removal is ALT-dependent and not ferritin-dependent even in patients with HFE-hemochromatosis. ALT reduction may be the primary effect, and suppression of hepatic fibrosis might be a secondary event of venesection in both chronic hepatitis C and HFE-hemochromatosis. ALT: alanine aminotransferase.

iron overload. In HFE-hemochromatosis, iron accumulates due to HFE-dependent derangement of hepcidin production (10, 11). Serum hepcidin-25 levels in our Italian HFE-hemochromatosis patients were low at 11.1 ± 9.2 ng/mL. The current observations of circulating hepcidin 25 confirmed the findings previously observed in urine from patients with HFE-hemochromatosis (12). Low levels of serum hepcidin 25 were also found in Japanese patients with non-HFE hemochromatosis (18).

In CHC patients, the mechanism of hepatic iron accumulation appears to be more complex and is still not completely understood. Iron regulation by hepcidin might be partially impaired in CHC. Serum hepcidin-25 levels in our Japanese patients were relatively high at 30.7 ± 14.5 ng/mL compared to those in Italian HFE-hemochromatosis patients. Our findings, including the reduced hepcidin/ferritin ratio and correlation between hepcidin and ferritin concentrations fit well with reports showing that hepcidin induction was

relatively impaired in CHC patients, but also that its regulation by iron stores was maintained (13, 14). These findings are also in agreement with recent studies in animal and cellular models suggesting that HCV suppresses hepcidin production and may contribute to the development of iron overload in CHC (19, 20).

Previous study reported that none of the iron indices were predictive of the venesection effect, but rather the pretreatment ALT activities were predictive in CHC (21). The present study confirmed that this is also likely in HFE-hemochromatosis. The pretreatment ALT was higher, the reduction in ALT was larger in both CHC and HFE-hemochromatosis. Active liver cell damage could be removed by venesection regardless of the amount of stored iron. HCV co-infection in hemochromatosis patients markedly increases the risk of cirrhosis in the presence of a relatively low amount of iron (22). Therefore, with or without HCV infection, venesection should be recommended for HFE-hemochromatosis patients not only to remove excess iron, but also to suppress biochemical liver damage as in CHC patients. Provided that iron-induced hepatotoxicity is represented by ALT reduction during venesection, IHI obtained by [dividing ALT reduction by body iron stores] might be representative of sensitivity to iron hepatotoxicity under disease conditions. The IHI suggests not only sensitivity to iron hepatotoxicity, but also beneficial effects of venesection. CHC patients with a high IHI may be more sensitive to iron-induced liver damage than patients with HFE-hemochromatosis with low IHI. Therefore, liver cells with HFE-mutation are more tolerant to iron hepatotoxicity than liver cells infected by HCV.

In HFE-hemochromatosis patients, iron accumulates slowly and redox active iron emerges only when the storage capacity for ferritin and hemosiderin is overwhelmed. In other words, because of the high tolerance to iron hepatotoxicity, clinical manifestation might be delayed in HFE-hemochromatosis. The mean amount of iron removed from HFE hemochromatosis was reported to be 4.98 g (3.9-6.1 g in the range) (12), while that of CHC was 0.61 g (0-1.6 g in the range) (17). The results of the current study did not differ from those in the literature. It is also important that the amount of body iron in CHC patients is considered a non-toxic level in healthy subjects because sensitivity to iron hepatotoxicity disappeared in complete responders to IFN without iron removal (13). However, the combination of even a slightly increased iron level with HCV-related insult may act synergistically to increase iron hepatotoxicity and the risk of progressive liver damage so that iron removal can be beneficial in this setting (3, 4).

Patients with HFE-hemochromatosis showed normalized serum ALT activity after venesection, while all CHC patients showed significantly reduced but not normalized activity on biochemical liver function test. Iron hepatotoxicity was totally removed from HFE-hemochromatosis at 50 ng/mL of serum ferritin, while it remained in CHC around 20 ng/mL serum ferritin. The modified endpoint of 20 from 10 ng/mL for CHC still induced a reduction of Hb concentration in the posttreatment period (1.6 ± 1.1 g/dL). This incomplete effect of venesection in CHC indicates at least 2 compartments of hetatotoxicity of iron-induced oxidative stress and HCV-dependent insult. Hb concentration did not change in HFE-hemochromatosis with a high serum ferritin endpoint of 50 ng/mL. The high endpoint for HFE-hemochromatosis may account for the good tolerance to venesection to remove large amounts of body iron stores. Thus, threshold and sensitivity for iron hepatotoxicity were apparently different under the 2 conditions.

The remarkable reduction in serum hepcidin levels after venesection for iron overload conditions indicates suppressed internalization of ferroportin to enhance iron absorption in the gut. Thus, patients will rapidly recover iron stores if receiving a normal diet. Based on those findings, low-iron diets may be recommended to reduce active iron absorption in the intestine during the second stage of venesection in order to maintain free from iron-induced hepatotoxicity in Japanese patients with CHC (4, 23). In HFE-hemochromatosis with good tolerance to venesection, the effect of dietary iron restriction might be negligible.

To summarize an iron-induced hepatotoxicity under these 2 disease conditions, CHC patients were loaded with a minimum iron burden, but were more sensitive to iron-induced hepatotoxicity and less tolerant of venesection. In contrast, HFE-hemochromatosis patients were loaded with excessive iron due to genetic dysregulation of the hepcidin system and were more tolerant to both iron-induced hepatotoxicity and venesection to remove large amounts of body iron stores.

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VIII 遺伝と代謝

ヘモクロマトーシス

Hemochromatosis

Key words：HFE, transferrin receptor 2, hepcidin, hemojuvelin, ferroportin

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1. 概念・定義

ヘモクロマトーシスは肝硬変，糖尿病，心筋症などの全身性の多臓器障害を伴う鉄過剰症である．鉄過剰症は大きく原発性と二次性に分けられる．我が国においては原発性の遺伝性ヘモクロマトーシスはほとんど存在しないので，まず，二次性を考慮する．遺伝性ヘモクロマトーシスは遺伝子診断の時代を迎えて，より疾患概念が鮮明になってきた．複数の原因遺伝子が発見されているが，それらの変異によって同じような鉄代謝異常を呈する．ヘモクロマトーシス型は鉄代謝調節の遺伝的欠損によって消化管からの持続的な鉄吸収過剰状態となり，循環プールに流入する鉄を制御できず，結果として，様々な実質臓器，特に肝臓，膵臓，心臓に鉄が過剰蓄積し，多臓器障害の症候を呈する．

2. 疫　　学

遺伝性ヘモクロマトーシスはコーカシアンに最も多い遺伝性肝疾患である．しかし，もともとヘモクロマトーシスの臨床像が非特異的で緩徐に進行することから，診断に至らない無治療例が少なくない．古典的所見である肝硬変，皮膚色素沈着，糖尿病，関節痛を呈する典型例は非常に少なく，米国においても入院患者20,000人あたり1人の低頻度である．しかし，剖検の検討では1,000人あたり1-2人の頻度であり，更に最近の西欧の一般人口を対象にしたスクリーニング調査ではヘモクロマトーシス原因遺伝子*HFE*のホモ接合型遺伝子変異は200人に1人で，しかも明らかな鉄過剰症を発症しているのは300人に1人の高頻度であることがわかった．*HFE*のヘテロ接合型遺伝子変異は一般人口の10人に1人と報告されている．一方，日本，韓国では非HFE型の報告は散見されるが，HFEの主要変異であるC282Yは存在せず，わずかにH63Dのヘテロ接合型が報告されているだけでHFE関連の鉄過剰症はない．

3. 病　　因

1世紀以上も前に，ヘモクロマトーシスは肝内鉄過剰に関連する糖尿病，ブロンズ様皮膚，肝硬変の三徴を呈する疾患として初めて認識された．1889年に初めてhemochromatosisと命名されるようになり，1935年には遺伝性疾患と認識された．Davidらは責任遺伝子が第6染色体短腕上にあるHLA-A locusに近接していることを報告し，*HFE*遺伝子がクローニングされた[1]．そしてヘモクロマトーシスの60-100％がHFEのC282Y変異をもつことが報告され，その後第二の変異であるH63Dが1-10％の頻度で認められることが報告された．

a. ヘモクロマトーシス型とferroportin病

HFEのC282YまたはH63Dのホモ接合型変異または各ヘテロ接合型の複合でヘモクロマトーシスの表現型を呈する．まれなタイプとしてtransferrin receptor 2(TfR2，*TFR2*遺伝子)変異，若年発症の重症型としてhepcidin(HEPC，*HAMP*遺伝子)変異，hemojuvelin(HJV，*HFE2*遺伝子)変異がHFE変異以外のヘモクロマトーシス型として報告されている．表現型が異なる

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表1　鉄過剰症のカテゴリー

遺伝性ヘモクロマトーシス(常染色体劣性遺伝)
　ヘモクロマトーシス型(食餌鉄吸収，鉄取り込み亢進)
　　HFE 関連ヘモクロマトーシス
　　非 HFE 関連ヘモクロマトーシス
　　　成人型ヘモクロマトーシス(*TFR2*)
　　　若年性ヘモクロマトーシス(*HAMP*，*HFE2*)
　ferroportin 病(細胞鉄放出低下)

二次性鉄過剰症
　鉄輸送障害
　　遺伝性疾患
　　　サラセミア
　　　無(低)トランスフェリン血症
　　　無セルロプラスミン血症
　　後天性疾患
　　　鉄芽球性貧血
　　　骨髄過形成性疾患を合併した無効造血
　　　慢性炎症性貧血

食餌鉄増加：アフリカン鉄過剰症
鉄過剰投与：大量輸血，鉄剤投与
その他
　慢性肝疾患関連鉄過剰症(アルコール性，ウイルス性)
　代謝疾患関連鉄過剰症
　晩発性皮膚ポルフィリン症
　新生児ヘモクロマトーシス
　遺伝性高フェリチン血症

ferroportin 病の原因として，ferroportin(FPN，*SLC40A1* 遺伝子)変異がある(表1)．

4. 病　　態

a. 上部小腸からの鉄吸収亢進

我が国における一般の食事中の鉄量は8-10 mg/日で，欧米においても10-20 mg/日である．このうち1-2 mg/日の鉄が上部小腸から吸収される．脱落する消化管粘膜などによる鉄の喪失量は1-2 mg/日で鉄の出納のバランスが取れている．しかし，ヘモクロマトーシスでは消化管の鉄輸送の亢進があるため，成人では3-4 mg/日の鉄が吸収され，1年間で500-1,000 mg の鉄が蓄積すると考えられる．最初の表現型は血清トランスフェリン飽和度の上昇である．組織に鉄が蓄積するに従って総体内貯蔵鉄量に比例して鉄血清フェリチンの上昇が認められるようになる．

b. 遺伝子変異と鉄代謝異常

鉄代謝ホルモン HEPC は肝臓で産生され血中に分泌される鉄代謝調節分子で，腸管細胞，マクロファージに発現している鉄放出トランスポーター FPN の発現量を調節している．HEPC が FPN に結合すると FPN の細胞内分解が促進され，細胞外循環プールへの鉄放出が抑制される[2]．ヘモクロマトーシス型遺伝子変異の共通した特徴は，HEPC の遺伝子レベルの発現低下または活性低下によって，腸管細胞の基底膜側に分布する FPN の発現が増加し，循環プールへの鉄吸収の制御機能が欠損する hepcidin-ferroportin 調節系の破綻である(図1)．HEPC 活性が低下する HEPC 遺伝子変異以外のヘモクロマトーシス型である HFE，TfR2[3]，HJV[4] の遺伝子変異では肝細胞における鉄感受性を障害されるため HEPC の発現誘導シグナルが障害されている．原因となる鉄関連分子の変異によって遅発性軽症型から若年性重症型まで様々であるが，