polymyxin B-immobilized fiber column is therefore considered to be an effective option to save intractable HLH patients, particularly when HLH persists after treatment for an underlying condition. However, further studies are necessary to confirm the usefulness of this strategy.

**The authors state that they have no Conflict of Interest (COI).**

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ORIGINAL ARTICLE

# The bone marrow hematopoietic microenvironment is impaired in iron-overloaded mice

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## Abstract

*Objectives:* Increasing numbers of reports have described hematopoietic improvement after iron chelation therapy in iron-overloaded patients. These observations indicate that excess iron could affect hematopoiesis unfavorably. To investigate how excess iron affects hematopoiesis *in vivo*, we generated iron-overloaded mice and examined hematopoietic parameters in these mice. *Methods:* We generated iron-overloaded mice by injecting 200 mg of iron dextran into C57BL/6J mice, and immature hematopoietic cells in the bone marrow were evaluated by flow cytometric analyses, colony-forming assays, and bone marrow transplantation analyses. We also examined changes in molecular profiles of the hematopoietic microenvironment. *Results and Conclusions:* Iron-overloaded (IO) mice did not show significant defects in the hematopoietic data of the peripheral blood. Myeloid progenitor cells in the bone marrow were increased in IO mice, but the number and function of the erythroid progenitors and hematopoietic stem cells were not significantly affected. However, bone marrow transplantation from normal donors to IO recipients showed delayed hematopoietic reconstitution, which indicates that excess iron impacts the hematopoietic microenvironment negatively. Microarray and quantitative RT-PCR analyses on the bone marrow stromal cells demonstrated remarkably reduced expression of CXCL12, VCAM-1, Kit-ligand, and IGF-1 in the iron-overloaded mice. In addition, erythropoietin and thrombopoietin levels were significantly suppressed, and increased oxidative stress was observed in the IO bone marrow and liver. Consequently, our findings indicate that excess iron can damage bone marrow stromal cells and other vital organs, disrupting hematopoiesis presumably by increased oxidative stress.

**Key words** iron overload; hematopoiesis; bone marrow microenvironment; hematopoietic stem cell; erythropoietin; thrombopoietin; reactive oxygen species

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Iron is an essential metal for hemoglobin synthesis and some vital enzymatic functions to maintain cellular homeostasis; therefore, iron deficiency leads to anemia and sometimes organ dysfunction. However, it is also known that excess iron increases reactive oxygen species (ROS) and negatively affects vital organs such as the liver, heart, and endocrine glands (1). Iron generates hydroxyl radicals by interacting with hydrogen peroxide (Fenton reaction). The hydroxyl radical is known as the most potent ROS, which can damage vital cellular components including lipid membranes, proteins, and nucleic acids, leading to derangement of cellular functions and eventually cell death and organ failure (2, 3).

Clinically, an increasing number of data have shown that organ dysfunction induced by iron overload can lead to high mortality (4). Sufficient iron chelation therapy (ICT) can remove excessive iron and ameliorate organ dysfunction in iron-overloaded patients, and accumulating data indicate that efficient ICT improves survival of transfusion-dependent patients with lower-risk MDS (5, 6).

Recently, we experienced a case of MDS with transfusional iron overload, in which hematopoietic data improved unexpectedly after administration of deferasirox without any other specific treatments (7), and increasing numbers of similar cases are being reported (8–12). These clinical

observations indicate that iron overload could affect hematopoiesis unfavorably, albeit through unknown mechanisms. Therefore, by generating iron-overloaded mice, we investigated how excess iron affects the hematopoietic system.

## Materials and methods

### Iron-overloaded mice

To generate iron-overloaded mice, we injected 25 mg of iron dextran (Sigma-Aldrich, St. Louis, MI, USA) in 1 mL phosphate-buffered saline intraperitoneally into six-week-old C57BL/6J mice (CLEA Japan, Tokyo, Japan), eight times over 4 wk – 200 mg in total (13). These mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Jichi Medical University according to institutional guidelines under an approved protocol.

Serum iron concentration was measured by the Nitroso-PSAP (2-Nitroso-5-[N-n-propyl-N-(3-sulfopropyl) amino] phenol) method. Sections of the liver and spleen were fixed in formaldehyde, and tissue iron concentrations were measured by atomic absorption spectrometry (SRL, Tokyo, Japan).

### Flow cytometric analysis

Bone marrow cells were collected from femoral and tibial bones and treated with RBC lysis buffer (QIAGEN, Dusseldorf, Germany) to remove erythrocytes. Mature lineage-positive cells were labeled with biotin-conjugated antibodies against CD5, B220, CD11b, Gr-1, and Ter119 (Lineage Depletion Kit mouse; Miltenyi Biotec, Bergisch Gladbach, Germany) and magnetically depleted by the autoMACS system (Miltenyi Biotec). Collected lineage-negative cells were stained with fluorescent conjugated antibodies: streptavidin-PerCP, CD34-FITC, CD48-APC, CD117-PE-Cy7, CD150-PE, Sca1-APC-Cy7 (BD Bioscience, Franklin Lakes, NJ, USA), and CD16/32-eFluor450 (eBioscience, San Diego, CA, USA); thereafter, specifically differentiated fractions were analyzed. Data acquisition was performed with BD FACSAria flow cytometer equipped with FACS Diva software (BD Biosciences), and data were analyzed by FlowJo cytometry software (Tree Star Inc., Ashland, OR, USA).

### Colony-forming cell assay

To investigate immature erythroid cells, lineage-negative bone marrow cells ($2 \times 10^4$) were cultured in methylcellulose culture medium (Stem Cell Technologies, Vancouver, Canada) for 10 d, and erythroid burst-forming units (BFU-E) were counted.

### Bone marrow transplantation

To analyze hematopoietic reconstitution capacity of the bone marrow cells, Ly45.1 mice were sublethally irradiated with 11 Gy of $\gamma$ rays (GammaCell 40, Nordion, Ottawa, ON, Canada), and 2 d after irradiation, bone marrow cells from Ly45.2 donor mice were transplanted intravenously. In the transplantation experiment from iron-overloaded donors to normal recipients, $1.0 \times 10^6$ Ly45.2 cells were injected to recipients along with the same number of Ly45.1 normal competitor cells. In the experiment from normal donors to iron-overloaded recipients, $1.0 \times 10^6$ Ly45.1 normal donor cells were transplanted to Ly45.2 iron-overloaded recipients. After transplantation, chimerism of the donor cells in the peripheral blood was analyzed every 2 wk. To distinguish between the two congenic cells, anti-mouse CD45.1-PE and CD45.2-FITC antibodies (BD Biosciences) were used.

### Microarray analysis and quantitative real-time polymerase chain reaction (qRT-PCR)

Bone marrow non-hematopoietic stromal cells were prepared by depleting $CD45^+$ and $Ter119^+$ cells. A cDNA library was prepared from these non-hematopoietic cells using RNAeasy Kit (QIAGEN) and SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). To prepare a sufficient amount of cDNA, a pool of eight normal and nine iron-overloaded mice were used for the experiment.

Comprehensive gene expression analyses were performed by Agilent Expression Array (SurePrint G3 Mouse GE 8 × 60K Microarray, Takara Bio, Shiga, Japan).

Quantitative RT-PCR was performed using TaqMan Gene Expression Assay probes and ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Results were quantified using the Delta C (T) method.

To perform qRT-PCR, the following TaqMan probes were used: VCAM1 (Assay ID: Mm01320970_m1), CXCL12 (Assay ID: Mm00445553_m1), Kit-ligand (Assay ID: Mm00442972_m1), IGF1 (Assay ID: Mm00439560_m1), thrombopoietin (Assay ID: Mm00437040_m1), beta-actin (Assay ID:Mm00607939_s1).

### Enzyme-linked immunosorbent assay

Mouse serum erythropoietin (EPO) concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using the Quantikine Mouse/Rat Epo Immunoassay kit (R&D Systems, Minneapolis, MN, USA) and Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA).

### Measurement of oxidative stress

Oxidative stress levels in the bone marrow samples were examined by flow cytometric analysis using 2, 7-dichlorodihydrofluorescein diacetate (DCFDA) (Invitrogen). Bone marrow cells were incubated with 5 μM DCFDA for 30 min at 37°C, and then, samples were suspended in cold FACS buffer and immediately analyzed by BD FACSAria.

Liver samples were analyzed by Western blotting for malondialdehyde (MDA), an organic compound naturally generated by lipid peroxidation. Frozen samples were homogenized in an ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (Sigma-Aldrich), 5 mM EDTA, and 1 mM PMSF (phenyl methyl sulfonyl fluoride). The lysate was incubated on ice for 15 min, and the centrifuged supernatant was analyzed by SDS-PAGE and immunoblot with rabbit polyclonal antibody for MDA (ab27642; Abcam, Cambridge, UK).

### Statistical analysis

The results are shown as the mean ± standard deviation (SD) and compared by the unpaired Student's *t*-test for differences in means.

## Results

### Iron-overloaded mice showed significant iron accumulation in vital organs

Macroscopically, iron-overloaded mice showed pigmented skin indicating excess iron deposition in the body (Fig. 1A).

Iron-overloaded (IO) mice vs. normal controls showed significantly higher iron levels in serum iron (2292.5 ± 1025.2 vs. 176.3 ± 62.4 μg/dL; $P < 0.01$), liver (79 153.1 ± 16 960.7 vs. 371.4 ± 139.0 μg/dry gram; $P < 0.01$), and spleen (78 840.6 ± 20 711.0 vs. 1383.0 ± 897.8 μg/dry gram; $P < 0.01$) (Fig. 1B).

Histological examination confirmed excessive iron deposition in the bone marrow, liver, spleen, and heart, as is often the case in iron-overloaded patients (14, 15) (Fig. 1C).

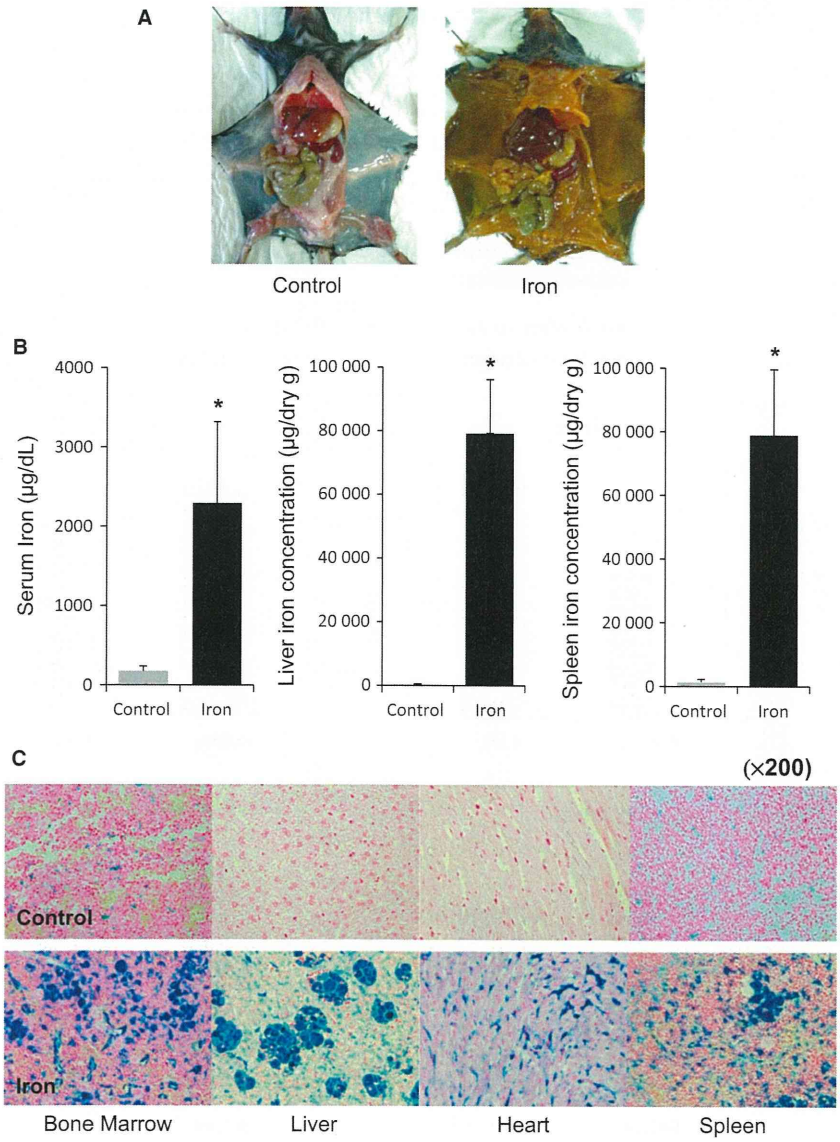


**Figure 1** Iron accumulated in vital organs of iron-overloaded mice. (A) Macroscopic findings of iron-overloaded mice at 4 wk, intraperitoneally injected with 200 mg of iron in total. Control mice were injected with phosphate-buffered saline. (B) Serum, liver, and spleen iron concentration at 4 wk. Serum samples were collected from 4 control and 4 iron-injected mice (*$P < 0.01$). Iron content in the liver and spleen was calculated as the ratio of ash weight to dry weight. Samples were extracted from 3 control and 3 iron-injected mice (*$P < 0.01$). (C) Histological examinations (iron stain, ×200) of vital organs. In iron-injected mice, depositions of excess iron (stained in blue) are distributed in the bone marrow, liver, heart, and spleen.

These results demonstrate that this experimental murine model reflects an iron-overloaded pathogenic condition.

### Peripheral blood counts were not remarkably influenced in iron-overloaded mice

To investigate how excess iron affects the hematopoietic system, we examined blood counts of the peripheral blood. On the completion of 4-wk iron injections, platelet counts in iron-overloaded mice were lower than in controls (104 ± 23 vs. 134 ± 9 × $10^4$/μL; $P = 0.003$), but they were nearly within the normal range. The number of white blood cells (WBCs) and the hemoglobin (Hb) levels did not show significant difference between IO and control mice, although in both groups, WBCs were increased compared with those of the baseline (Fig. 2).

### Myeloid progenitors were increased but erythroid progenitors and very immature hematopoietic cells were unaffected in the bone marrow of iron-overloaded mice

To investigate whether excess iron affects the bone marrow hematopoiesis, we analyzed the proportion of immature hematopoietic cells in the bone marrow. On completion of iron injection, bone marrow cells were collected and flow cytometric analyses were performed. In the iron-overloaded bone marrow, the proportion of immature hematopoietic cells defined by Lineage$^-$/Sca1$^+$/c-Kit$^+$ (LSK) was significantly higher than that of the control bone marrow (0.20% ± 0.07 vs. 0.12% ± 0.04; $P = 0.048$) (Fig. 3 A, C). But, in more immature hematopoietic stem cell (HSC) populations defined by CD34$^-$/LSK, CD150$^+$/CD48$^-$ (SLAM-HSC), and CD150$^+$/CD48$^-$/LSK (SLAM/LSK), no significant difference was observed between the IO and control groups (Fig. 3 B, C). In the population of hematopoietic progenitor cells, the proportions of common myeloid progenitors (CMP), CD34$^{high}$ and CD16/32$^{low}$, and granulocyte monocyte progenitors (GMP), CD34$^{high}$ and CD16/32$^{high}$, showed significant increase in the iron-overloaded bone marrow, while megakaryocyte erythroid progenitors (MEP), CD34$^{low}$ and CD16/32$^{low}$, did not (Fig. 3 A, D).

A recent study has reported impaired proliferation of erythroid progenitors from iron-overloaded MDS patients (16). Therefore, to confirm the number of erythroid progenitors more precisely, we performed an *in vitro* colony assay. We sorted and cultured lineage-negative immature hematopoietic cells from iron-overloaded and control mice, but we found no significant difference in the number of erythroid colonies (Fig. 3E).

These results indicate that a portion of myeloid progenitors increase in iron-overloaded mice, but the numbers of immature erythroid and hematopoietic stem cells are unaffected by excess iron in our experimental system.

### Immature hematopoietic cell function is unaffected in iron-overloaded mice

To further examine whether functional defects may exist in the hematopoietic stem/progenitor cells in iron-overloaded

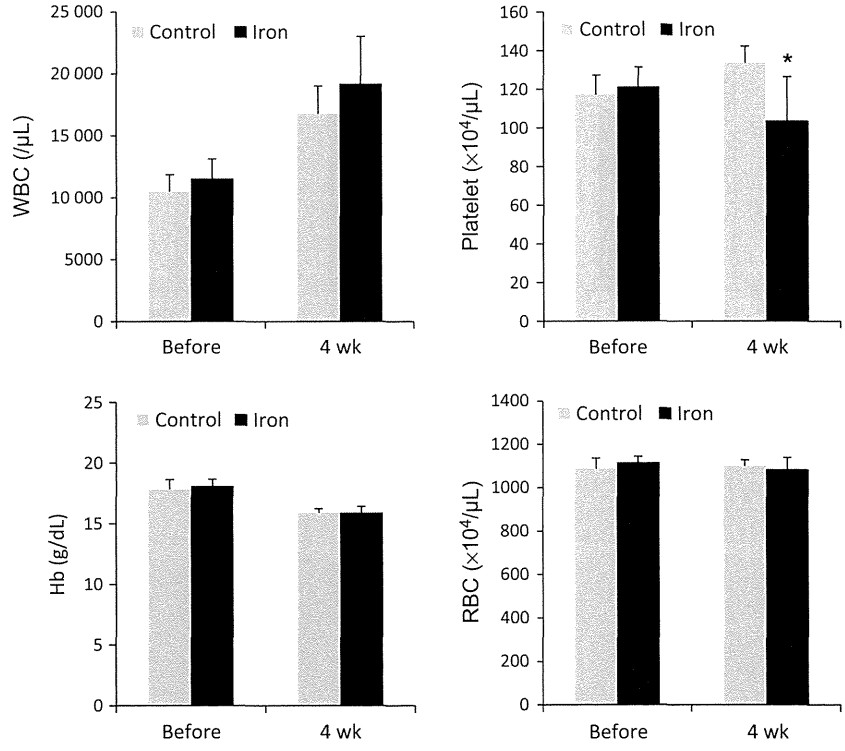


**Figure 2** Complete blood count in the peripheral blood of control and iron-overloaded mice before and after 4 wk of injection (200 mg of iron). Values are mean ± SD calculated in 8 control and 9 iron-overloaded mice. *$P = 0.003$.

mice, we performed bone marrow transplantation (BMT) assays.

We collected bone marrow cells from iron-overloaded or normal Ly45.2 mice and transplanted them to sublethally irradiated normal Ly45.1 recipients along with the same number of Ly45.1 normal competitor cells (Fig. 4A). As a result, we found no significant difference in the chimerism of the donor cells in the recipients' peripheral blood between the iron-overloaded and normal donor cells at 4 wk after BMT (49.7% ± 7.9 vs. 54.1% ± 3.1; $P = 0.35$) and at 8 wk after BMT (59.6% ± 3.7 vs. 59.8% ± 7.2; $P = 0.97$), suggesting that the hematopoietic reconstitution capacity of hematopoietic stem/progenitor cells in iron-overloaded mice is not significantly affected by excess iron (Fig. 4B).

## Hematopoietic recovery was delayed in iron-overloaded recipients after BMT

Next, to examine whether the excess iron affects the bone marrow microenvironment, we transplanted normal donor cells to iron-overloaded and normal recipients and compared hematopoietic recovery after BMT (Fig. 4C).

When we transplanted $1 \times 10^6$ normal bone marrow cells, hematopoietic reconstitution was significantly delayed in iron-overloaded mice. At 2 wk after BMT, significant impairment was observed in iron-overloaded mice compared to controls in WBC (2000 ± 1333 vs. 4330 ± 741/μL; $P < 0.001$), Hb (11.7 ± 1.3 vs. 14.6 ± 0.6 g/dL; $P < 0.001$), and platelets (17 ± 3 vs. 60 ± 12 × $10^4$/μL; $P < 0.001$). The delay in platelet recovery remained significant even at 4 wk after BMT for WBC (8160 ± 1927/μL vs. 12 440 ± 2 585/μL;

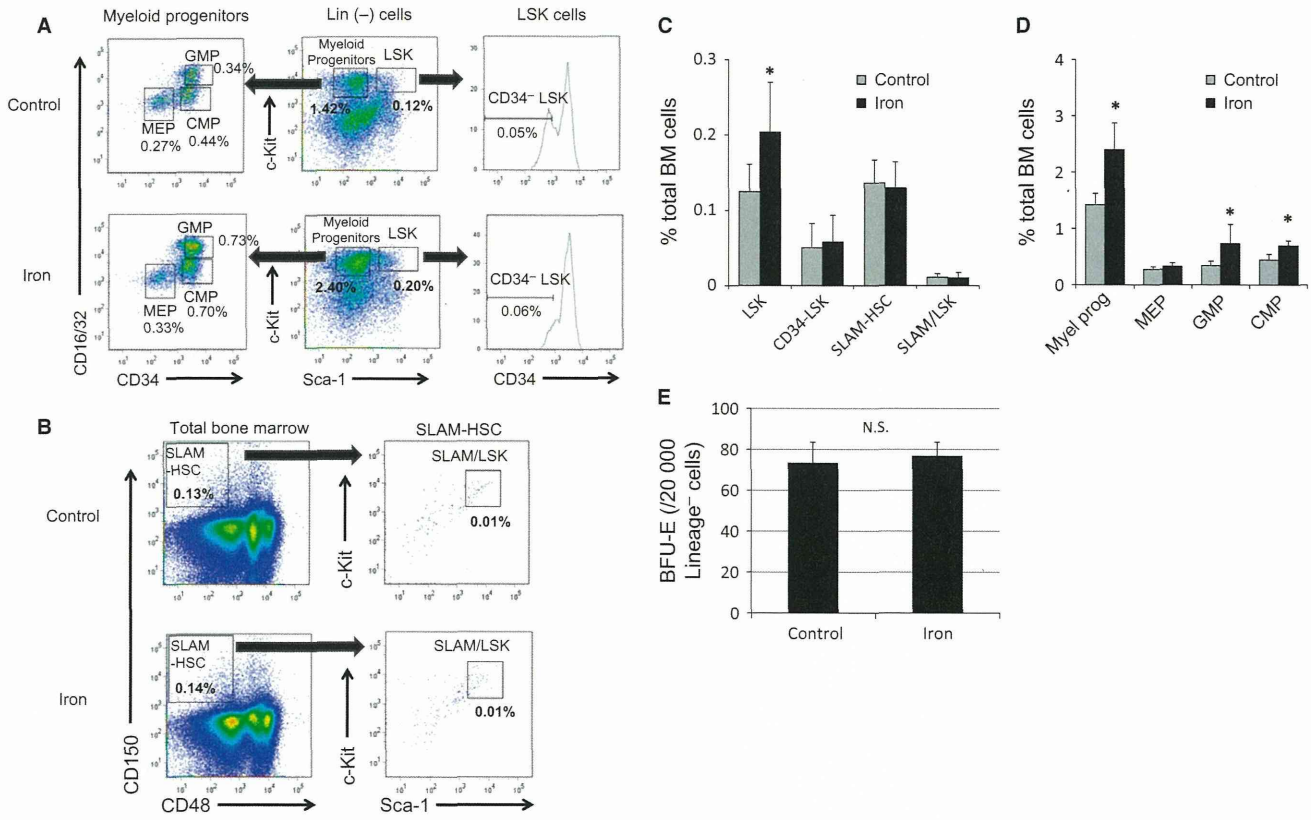


**Figure 3** Some fractions of hematopoietic progenitors were increased in iron-overloaded mice, but there was no significant difference in the fraction of immature erythroid cells and most immature hematopoietic cells. (A) Analysis on LSK and other hematopoietic progenitors (CMP, GMP, MEP). The proportion of LSK and myeloid progenitors (Sca-$1^{low}$/c-$Kit^{high}$) is shown in the central panels. Right and left panels show the fraction of $CD34^-$LSK cells and specific hematopoietic progenitors (CMP, GMP, MEP), respectively. (B) Analysis on SLAM-HSCs. Right panels show the proportion of the most immature hematopoietic cells, SLAM/LSK. Values described at each gating square indicate mean percentage in total viable cells. (C, D) Mean ± SD percentage of specific hematopoietic stem/progenitor fractions (C) and progenitors (D) in total viable cells. In iron-overloaded mice, LSK cells were significantly increased, but there was no difference in the most immature hematopoietic fraction, such as $CD34^-$KSL and SLAM-HSC (C). In iron-overloaded mice, myeloid progenitor cells were significantly increased (*$P < 0.05$), but there was no significant difference in the MEP fraction (D). (E) The *in vitro* colony assay also showed no significant difference in the number of immature erythroid cells (BFU-E) ($P > 0.05$). LSK, $Lineage^-$/Sca-$1^+$/c-$Kit^+$; CMP, common myeloid progenitor; GMP, granulocyte monocyte progenitor; MEP, megakaryocyte erythroid progenitor; BFU-E, burst-forming units erythroid; N.S., not significant.

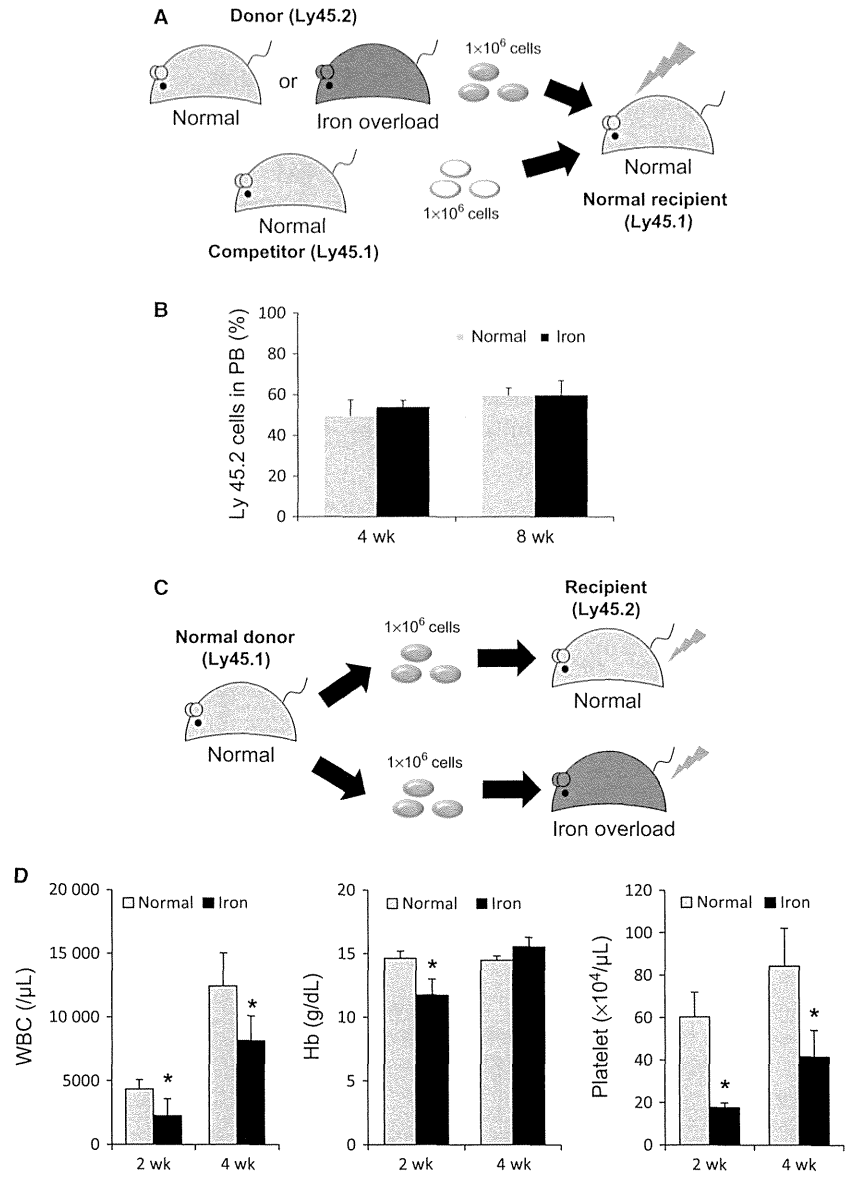


**Figure 4** Bone marrow transplantation assays with iron-overloaded donors or recipients. (A) Transplantation of bone marrow cells ($1 \times 10^6$ cells) from iron-overloaded mice or normal mice (Ly45.2) into sublethally irradiated normal recipient mice (Ly45.1) along with the same number of normal competitor cells (Ly45.1). (B) Chimerism of donor cells (Ly45.2) in the peripheral blood 4 and 8 wk after transplantation. There was no significant difference between iron-overloaded and control donors. (C) Transplantation of $1 \times 10^6$ normal donor cells to normal or iron-overloaded recipient mice. (D) Blood cell counts in recipients 2 and 4 wk after transplantation. In iron-overloaded mice, recovery of WBC, hemoglobin, and platelets was significantly delayed (*$P < 0.05$).

$P < 0.001$) and platelets ($41 \pm 13 \times 10^4/\mu L$ vs. $84 \pm 18 \times 10^4/\mu L$; $P < 0.001$) (Fig. 4D). These results indicate that excess iron disturbs the bone marrow microenvironment, which supports hematopoietic reconstitution.

### Expression of some molecules essential for hematopoiesis was reduced in the bone marrow stromal cells of iron-overloaded mice

Bone marrow transplantation assays showed susceptibility of the bone marrow microenvironment to excess iron. Therefore, to investigate the molecular basis for these bone marrow defects, we collected non-hematopoietic bone marrow stromal cells ($CD45^-/Ter119^-$) from the iron-overloaded mice and analyzed gene expression profiles by microarray analyses.

As a result, notable changes in the expression of common hematopoietic cytokines, chemokines, and adhesion genes were identified in the iron-overloaded stromal cells. Interestingly, expression of most genes was reduced. These included CXCL12, VCAM-1, IGF-1 (insulin-like growth factor-1), and Kit-ligand also known as stem cell factor (SCF), all of which are key molecules for hematopoiesis (Fig. 5A).

Quantitative RT-PCR (qRT-PCR) analyses confirmed reduced expression of the genes; the mRNA levels of CXCL12, VCAM-1, Kit-ligand, and IGF-1 in the iron-overloaded stromal cells were less than one twentieth of normal controls (Fig. 5B). As these gene products are essential for efficient hematopoiesis in the bone marrow, reduced expression of these genes can negatively affect hematopoietic

**A**

| Gene | Log 2 ratio | Gene | Log 2 ratio | Gene | Log 2 ratio |
|---|---|---|---|---|---|
| Bmp6 | −4.92 | Bmp7 | −3.13 | Tnfsf9 | 2.09 |
| Mmp2 | −4.69 | Cldn5 | −3.05 | Mmp9 | 2.13 |
| Nrcam | −4.36 | Notch3 | −2.96 | Ccr7 | 2.14 |
| Cxcl12 | −4.25 | Vcam1 | −2.96 | Cd34 | 2.52 |
| Cxcl14 | −3.86 | Sdc3 | −2.92 | Ltb | 2.73 |
| Vegfc | −3.70 | Pdgfc | −2.91 | Cxcr5 | 3.23 |
| Cdh2 | −3.64 | Bmp4 | −2.86 | | |
| Cdh5 | −3.61 | Neo1 | −2.78 | | |
| Jag1 | −3.48 | Ccl19 | −2.72 | | |
| Tnfsf11 | −3.44 | Sdc2 | −2.40 | | |
| Cx3cl1 | −3.39 | Bmp2 | −2.32 | | |
| Csf1 | −3.32 | Hspg2 | −2.22 | | |
| Bmp5 | −3.28 | Igf1 | −2.21 | | |
| Kitl | −3.21 | Jam3 | −2.04 | | |
| Cadm1 | −3.19 | | | | |

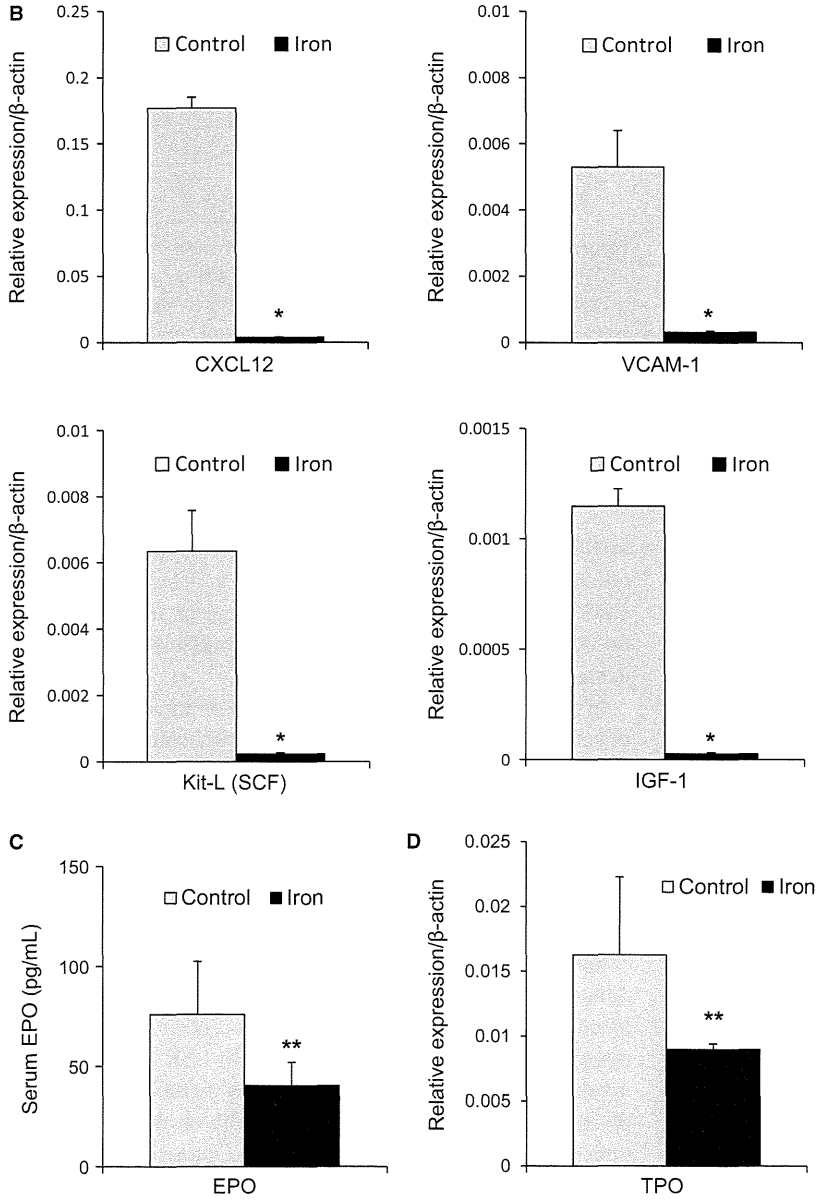


**Figure 5** Expression of hematopoietic chemokines and adhesion molecules in non-hematopoietic cells in the bone marrow, and serum EPO concentration and expression of TPO mRNA in the liver. (A) Microarray analyses on the non-hematopoietic bone marrow cells ($CD45^{-}/Ter119^{-}$) collected from normal or iron-overloaded mice. Genes related to hematopoiesis (chemokines, cytokines, and adhesion molecules) that were significantly suppressed (less than one-fourth) or significantly increased (more than four times) in iron-overloaded mice are shown. The values are shown as logarithm base 2. (B) Reduction in gene expression was confirmed by qRT-PCR. In the iron-overloaded bone marrow, expression of CXCL12, VCAM-1, Kit-ligand, and IGF-1 were significantly reduced ($*P < 0.01$). (C) Serum EPO concentration was measured by ELISA, and (D) expression of TPO mRNA in the liver was analyzed by qRT-PCR. In iron-overloaded mice, EPO levels in the serum and expression of TPO mRNA were significantly reduced ($**P < 0.05$). EPO, erythropoietin; TPO, thrombopoietin.

recovery after bone marrow transplantation in iron-overloaded recipients.

### Impaired production of erythropoietin and thrombopoietin in iron-overloaded mice

Hematopoietic recovery after bone marrow transplantation can be affected by hematopoietic cytokines, such as erythropoietin (EPO) and thrombopoietin (TPO), both of which are essential for RBC and platelet production, respectively. Interestingly, it has been reported that iron chelation therapy can increase production of EPO (17), and therefore, we examined production of these cytokines in iron-overloaded mice.

First, we analyzed serum EPO levels by ELISA. The result showed that the level of serum EPO was significantly reduced in iron-overloaded mice compared to controls (40.4 ± 11.6 pg/mL vs. 75.8 ± 26.5 pg/mL; $P < 0.001$), which indicates that production of EPO in the kidney may be impaired by excess iron (Fig. 5C). Moreover, qRT-PCR analysis of TPO in the liver demonstrated that the mRNA level of TPO in the iron-overloaded liver was also reduced to less than half of normal controls (Fig. 5D). As TPO is generated primarily in the liver, this result indicates that impaired production of TPO induced by excessive iron in the liver may affect hematopoietic recovery.

### Oxidative stress is increased in the iron-overloaded bone marrow and liver

It is known that excess iron can negatively affect vital organs through ROS production, and impaired stromal functions observed in iron-overloaded mice may be a consequence of increased oxidative stress in the bone marrow.

Therefore, to measure the oxidative stress in the bone marrow, we performed flow cytometric analyses with DCFDA (18), a fluorescent reagent used to detect reactive oxygen intermediates, on immature bone marrow cells (lineage-negative cells). The results showed significant aggravation of oxidative stress in iron-overloaded mice—over twice as high as in normal mice (FITC mean; 20 019 ± 6864 vs. 8721 ± 623; $P = 0.047$) (Fig. 6A).

In the liver, Western blot analyses for MDA showed a significantly increased signal in the iron-overloaded samples, which indicates higher oxidative levels (Fig. 6B).

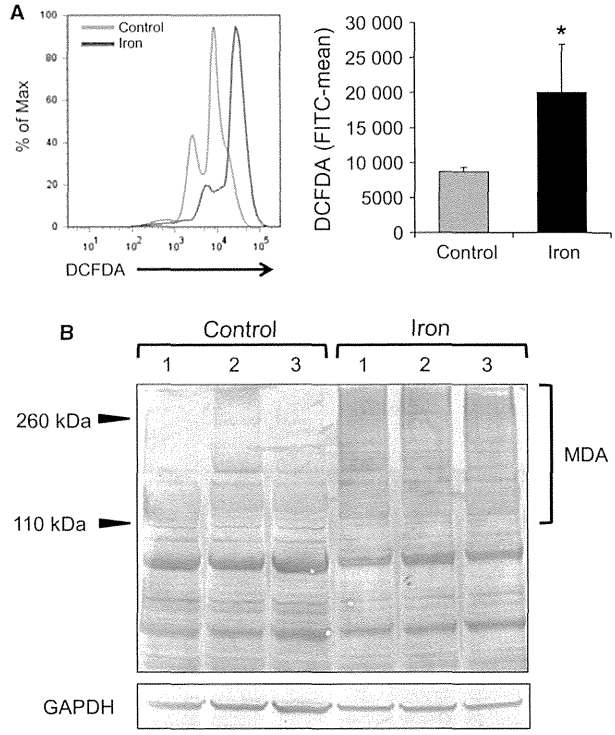


**Figure 6** Oxidative stress in iron-overloaded bone marrow and liver. (A) Flow cytometric analysis of oxidative stress on bone marrow lineage-negative cells. In iron-overloaded mice, signals of DCFDA were significantly higher than those in control mice (*$P < 0.05$). (B) Western blotting on MDA, an indicator of oxidative stress, in liver tissue. The amount of MDA was larger in the iron-overloaded liver than in control. At the bottom of each lane, blotting on GAPDH is shown as a control. DCFDA, 2,7-dichlorodihydrofluorescein diacetate; MDA, malondialdehyde.

## Discussion

Recently, numerous reports have described hematopoietic improvement after ICT (7–11). Although several hypothetical explanations have been proposed (17, 19–22), the mechanism underling this interesting phenomenon still remains unclear. One possible explanation is the negative effect of excess iron on HSCs. It is known that HSCs are susceptible to ROS, and ROS can damage their hematopoietic reconstitution capacity (23). Because iron is a potent generator of ROS and iron overload can be pathogenic for HSC functions, we investigated how excess iron affects hematopoiesis using iron-overloaded mice.

In this study, we generated iron-overloaded mice by injecting iron dextran solution intraperitoneally, as in a previous study concerning muscle dysfunction in iron-overloaded mice (13). We confirmed that our mice were actually iron-overloaded; macroscopic and microscopic analyses of the tissues showed deposition of excess iron, and quantitation of liver iron concentrations revealed higher levels.

Hematopoietic examination showed no pathological cytopenia in the peripheral blood of iron-overloaded mice. Although the number of platelets was slightly smaller than in control mice, it was still within the normal range. Bone marrow analyses showed an increase in LSK cells and other myeloid progenitors, but there was no significant change in the number of immature erythroid cells or the most immature hematopoietic stem cell fractions, namely $CD34^-$ LSK and SLAM-LSK. As bone marrow transplantation assays did

not show any significant defects in hematopoietic reconstitution capacity of HSCs in iron-overloaded mice, we conclude that HSCs are not affected significantly by excess iron in this experimental system.

The actual mechanism for the increase in LSK and myeloid progenitor populations in the bone marrow of iron-overloaded mice remains unclear. Because iron itself has some inflammatory characteristics, repeated intraperitoneal injection may have caused some biologic stress, although we confirmed that serum IL-6 and TNFα levels were not significantly increased in the iron-overloaded mice (data not shown).

In contrast, when iron-overloaded recipients were transplanted with normal donor cells, hematopoietic recovery was significantly delayed. This result suggests that excess iron could hamper the hematopoietic microenvironment. Interestingly, in the iron-overloaded bone marrow stromal cells, we found remarkable reductions in several vital molecules for hematopoiesis. Among these, by qRT-PCR methods, we confirmed reduced expression of CXCL12, VCAM-1, IGF-1, and SCF, all of which are key molecules for efficient hematopoiesis. Reduced expression of these molecules in the bone marrow can be an important clue to the defects in hematopoietic recovery observed in iron-overloaded conditions.

In addition to the pathological change in the bone marrow, we also found lower serum concentration of EPO and reduced expression of TPO in the liver in iron-overloaded mice. As EPO is an essential cytokine for red blood cell production, impaired production of EPO in iron overload could be an important factor to delay erythropoietic recovery. TPO is also an essential cytokine for the proliferation of HSCs and platelet production, and it is mainly produced by hepatocytes (24–26). To our knowledge, this is the first report that describes insufficient TPO production in the iron-overloaded condition, and insufficient TPO could have contributed to the delayed hematopoietic recovery after bone marrow transplantation. To confirm our results, we are planning a clinical study to investigate actual TPO levels in iron-overloaded patients.

Hematopoietic recovery after ICT indicates negative effects of excess iron on hematopoietic cells. But, in this study, we did not observe significant cytopenia in iron-overloaded mice, nor did we find qualitative or quantitative defects in erythroid progenitors or HSCs in these mice. Therefore, we have not found any results that can explain why ICT improves hematopoiesis in some iron-overloaded patients. This may partly be due to our iron-overloaded mice being generated by injecting iron into normal mice. In actual cases, hematopoietic recovery after ICT is observed in patients with hematopoietic disease; and in these patients, iron has accumulated gradually by repeated red blood cell transfusions. On this point, our animal models may not reflect the precise pathological condition of iron-overloaded patients. If abnormal hematopoietic cells in patients react differently to the biologic stress induced by excess iron, experiments using other relevant model mice may be necessary to understand how excess iron affects hematopoiesis. Otherwise, our animal model may possibly reflect pathological conditions of hereditary hemochromatosis. Patients with hereditary hemochromatosis usually do not show hematopoietic impairment, and our results indicate subclinical impairment in the bone marrow microenvironment. Hematopoietic supportive functions of these patients have not been elucidated enough, and it will be an important issue to examine these functions in actual patients.

Recently, Lu *et al.* (27) reported that *in vitro* culture of healthy human bone marrow cells in media with excess iron decreased the number of $CD34^+$ immature hematopoietic cells, which contradicts our observations. However, excess iron in the *in vitro* culture media can interfere with cellular functions more directly than *in vivo* conditions, and in that respect, iron can be more toxic. Consequently, this discrepancy may be due to the difference in the experimental systems. They also reported that immature erythroid cells (BFU-E) in iron-overloaded MDS patients were significantly reduced and that after ICT, colony-forming capacity was partially recovered. This indicates that excess iron can reduce immature erythroid cells *in vivo*. As we mentioned

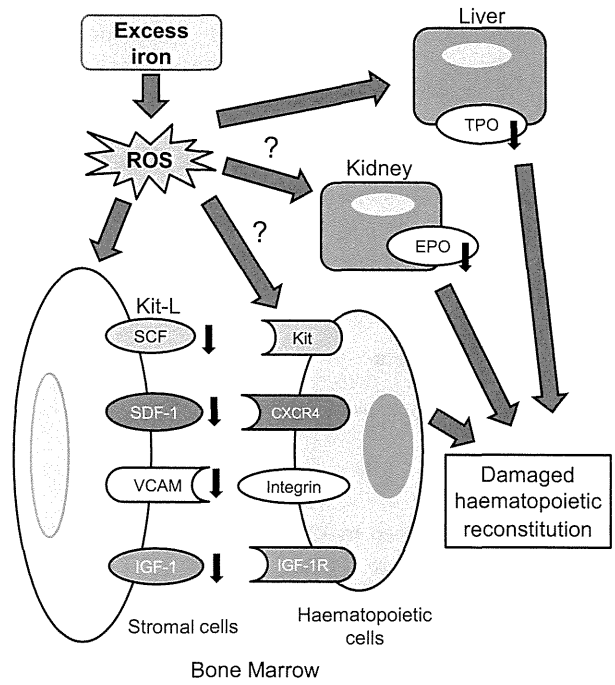


**Figure 7** Proposed schematic diagram of adverse effects of excess iron on the hematopoietic system. Excess iron increases ROS in vital organs, such as bone marrow and liver. ROS can damage hematopoietic stromal cells and reduce some important molecules for hematopoiesis. ROS may also reduce production of TPO in the liver and EPO in the kidney. The disturbance of hematopoietic supporting machinery can be one of the negative effects induced by iron overload. ROS, reactive oxygen species; TPO, thrombopoietin; EPO, erythropoietin.

previously, abnormal erythroid cells in MDS patients may react differently to excess iron, and therefore, to resolve this issue, other relevant murine models will be needed.

We found that iron overload can significantly delay hematopoietic recovery after bone marrow transplantation. Thus far, few reports have documented delayed engraftment after hematopoietic stem cell transplantation in iron-overloaded patients, but this can be because sufficient numbers of HSCs are transplanted in the usual clinical practice, and impaired recovery may be masked by the sheer volume of the transplanted cells. Actually, we have observed that the delay in recovery of white blood cells and red blood cells became indistinct when $2.0 \times 10^6$ cells were transplanted (data not shown). Although we have not investigated amelioration of post-transplant hematopoietic recovery after ICT in these model mice, our results suggest an advantage of vigorous ICT for pretransplant iron-overloaded patients to reduce the risk of delayed engraftment.

Recently, CXCL12-abundant reticular cells (CAR cells), which specifically produce CXCL12, were found in the bone marrow niche, and their critical function for hematopoiesis has been discovered. So, it is of great interest to investigate how iron affects these cells (28).

Development of novel iron chelators has enabled us to treat iron overload very efficiently, and the biologic relationship between iron overload and hematopoiesis is attracting attention. However, very little is known about how iron overload impacts immature hematopoiesis and the hematopoietic microenvironment in the bone marrow. To the best of our knowledge, this is the first report that reveals the negative effects of excess iron on the hematopoietic microenvironment (Fig. 7). Our study intimates important clues for future investigation and clinical practice in the treatment of iron overload.

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## Conflict-of-interest

The authors report no potential conflict of interest.

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ORIGINAL ARTICLE

# Distribution of serum erythropoietin levels in Japanese patients with myelodysplastic syndromes

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**Abstract** Erythropoiesis-stimulating agents (ESAs) are used to ameliorate anemia in lower-risk myelodysplastic syndromes (MDS). Serum erythropoietin (EPO) level <500 IU/L is widely accepted as a major predictive factor for response to ESAs. However, few data about EPO levels in the Japanese population are available. We therefore evaluated distribution of serum EPO levels in Japanese patients with MDS. Forty-three cases were analyzed; 30 were classified as lower-risk MDS (low or intermediate-1 by the international prognostic scoring system). Twenty-two cases were transfusion dependent. The overall median hemoglobin level was 7.7 g/dL. The median value of serum EPO was 254 IU/L (range: 16.4–23,000). Serum EPO levels had a strong inverse correlation with hemoglobin levels, and a significantly larger proportion of patients showed high EPO levels (>500 IU/L) in the transfusion-dependent group. In the higher-risk group, no significant correlation between EPO and hemoglobin was observed. Regression analyses showed that serum EPO of 500 IU/L corresponds to 8.29 g/dL of hemoglobin in lower-risk MDS. The results indicate that patients with hemoglobin levels of 8.0 g/dL or more, who are still transfusion independent, may be good candidates for ESA treatment.

**Keywords** Myelodysplastic syndromes (MDS) · Anemia · Erythropoietin · Erythropoiesis-stimulating agents (ESAs)

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## Introduction

Myelodysplastic syndromes (MDS) are characterized as clonal disorders of immature hematopoietic cells, and they exhibit various degrees of refractory cytopenia and dysplasia. Clinically, they are usually classified into two categories, i.e., higher-risk and lower-risk groups, according to the international prognostic scoring system (IPSS) or revised IPSS.

In higher-risk MDS, most patients have increasing numbers of blasts or unfavorable chromosomal abnormalities, and they are exposed to high risks of leukemic evolution. Therefore, it has been recommended that patients with higher-risk MDS be treated by anticancer drugs, such as azacitidine; and if possible, early hematopoietic stem cell transplantation should be conducted.

On the other hand, the pathological condition of lower-risk MDS is mostly characterized by inefficient hematopoiesis with refractory cytopenia. Therefore, treatments for patients with lower-risk MDS mainly aim at improving hematopoiesis. For this purpose, patients are treated by various cytokines, immunosuppressive agents, lenalidomide, anabolic steroids and azacitidine. Among the available cytokines, granulocyte-colony stimulating factor (G-CSF) and erythropoiesis-stimulating agents (ESAs) are commonly used, but ESAs have not been approved for MDS in Japan.

A clinical trial of darbepoetin α, a synthetic erythropoietin, for lower-risk MDS is currently underway in Japan, and its approval is expected in the near future. It has been reported that lower serum erythropoietin (EPO) levels, i.e., below 200 or 500 IU/L, are one of the favorable predictive factors for the efficacy of ESAs [1–7], but very little data are available about the distribution of serum EPO levels in Japanese MDS patients [8, 9]. Therefore, we examined serum EPO in Japanese MDS patients, to estimate the proportion of patients to

whom ESAs may be beneficial. In this research, we included patients without anemia to investigate how EPO levels change according to the decrease in hemoglobin (Hb) levels, and we also investigated EPO levels in higher-risk MDS patients, to check if risk categorization relates to EPO levels.

**Table 1** Patient characteristics

| | | |
|---|---|---|
| Males/females | | 26/17 |
| Age, median (range) | | 66 (38–81) |
| IPSS | Low | 7 |
| | Intermediate-1 | 23 |
| | Intermediate-2 | 6 |
| | High | 7 |
| FAB classification | RA | 26 |
| | RARS | 2 |
| | RAEB | 13 |
| | RAEB-t | 2 |
| WHO classification | RCUD | 11 |
| | RARS | 0 |
| | RCMD | 12 |
| | RAEB-1 | 5 |
| | RAEB-2 | 8 |
| | MDS-U | 4 |
| | del (5q) | 1 |
| | AML | 2 |
| Renal function (eGFR) (mL/min) | eGFR $\geq$ 90 | 10 |
| | 90 > eGFR $\geq$ 60 | 20 |
| | 60 > eGFR $\geq$ 30 | 13 |
| Number of transfusion-dependent patients | | 22 |
| Hemoglobin, median (range) (g/dL) | | 7.7 (4.4–13.6) |
| Hematocrit, median (range) (%) | | 23 (12.8–40.5) |
| WBC, median (range) (/μL) | | 2600 (1000–9300) |
| Platelet count, median (range) ($\times 10^4$/μL) | | 10.1 (0.7–101.3) |
| Reticulocyte count, median (range) (/μL) | | 38375 (2160–94000) |
| Bone marrow erythroblasts, median (range) (%) | | 27 (1.4–84.6) |

Transfusion dependency was defined as requirement of at least two JPN units of red blood cells transfused per month. One JPN unit of red blood cells is derived from 200 mL of blood

*IPSS* international prognostic scoring system, *eGFR* estimated glomerular filtration rate

## Patients and methods

From June 2011 to August 2014, patients older than 20 years with MDS in all risk categories were enrolled. In this study, patients diagnosed with MDS by FAB classification were eligible. As EPO levels are influenced by renal dysfunction, patients with estimated glomerular filtration rate (eGFR) less than 30 mL/min were excluded from the study. After obtaining written informed consent, serum EPO levels were examined by radioimmunoassay or chemiluminescent enzyme immunoassay (SRL Inc., Tokyo). The study was approved by the ethical committee of Jichi Medical University.

Correlations between EPO and other hematopoietic parameters were analyzed by Spearman's rank correlation method, and the difference of serum EPO and hemoglobin levels between transfusion-dependent and -independent groups was analyzed by Welch's *t* test.

## Results

Forty-three patients (26 males and 17 females, median age 66 years) were analyzed in the study (Table 1). According to the IPSS classification, 30 patients were categorized as lower-risk (low and intermediate—1) and 13 patients were higher-risk (high and intermediate—2); all of the higher-risk patients had excess blasts (>5 % in the bone marrow). The median level of Hb for all patients was 7.7 g/dL (range 4.4–13.6). Twenty-two patients were transfusion dependent (TD); they required at least two JPN units of packed red blood cells per month. All patients with Hb less than 7.0 g/dL were TD, 14 in total; and the other 8 TD patients had Hb levels less than 8.5 g/dL. Renal function was estimated by eGFR. Among the 43 patients, 10 had normal renal function (eGFR $\geq$ 90), 20 had mild renal dysfunction

**Table 2** Correlation between Serum EPO levels and hematopoietic parameters in all and lower-risk patients

| Hematopoietic parameters | All MDS | | | Lower-risk MDS | | | Higher-risk MDS | | |
|---|---|---|---|---|---|---|---|---|---|
| | *n* | Correlation coefficient | *p* value | *n* | Correlation coefficient | *p* value | *n* | Correlation coefficient | *p* value |
| Hemoglobin (Hb) | 43 | −0.69 | $9.0 \times 10^{-6}$ | 30 | −0.87 | $3.0 \times 10^{-6}$ | 13 | −0.15 | 0.59 |
| Hematocrit (Ht) | 43 | −0.70 | $5.2 \times 10^{-6}$ | 30 | −0.89 | $1.6 \times 10^{-6}$ | 13 | −0.22 | 0.43 |
| Reticulocytes | 38 | −0.36 | 0.029 | 25 | −0.53 | 0.010 | 13 | −0.14 | 0.61 |
| WBC | 43 | −0.38 | 0.014 | 30 | −0.45 | 0.015 | 13 | −0.37 | 0.20 |
| Platelets | 43 | −0.13 | 0.40 | 30 | −0.28 | 0.13 | 13 | 0.28 | 0.34 |
| BM erythroblasts | 42 | 0.07 | 0.64 | 29 | 0.22 | 0.25 | 13 | −0.35 | 0.23 |

*BM* bone marrow

(90 > eGFR ≥ 60), and 13 had moderate renal dysfunction (60 > eGFR ≥ 30). No patients were given ESAs.

The median level of serum EPO was 254 IU/L (range 16.4–23000).Age and sex had no significant impact on EPO levels (data not shown). Among the hematopoietic parameters analyzed, i.e., Hb, hematocrit (Ht), reticulocytes, WBC, platelets and erythroblasts in the bone marrow, serum EPO was strongly correlated with Hb and Ht;

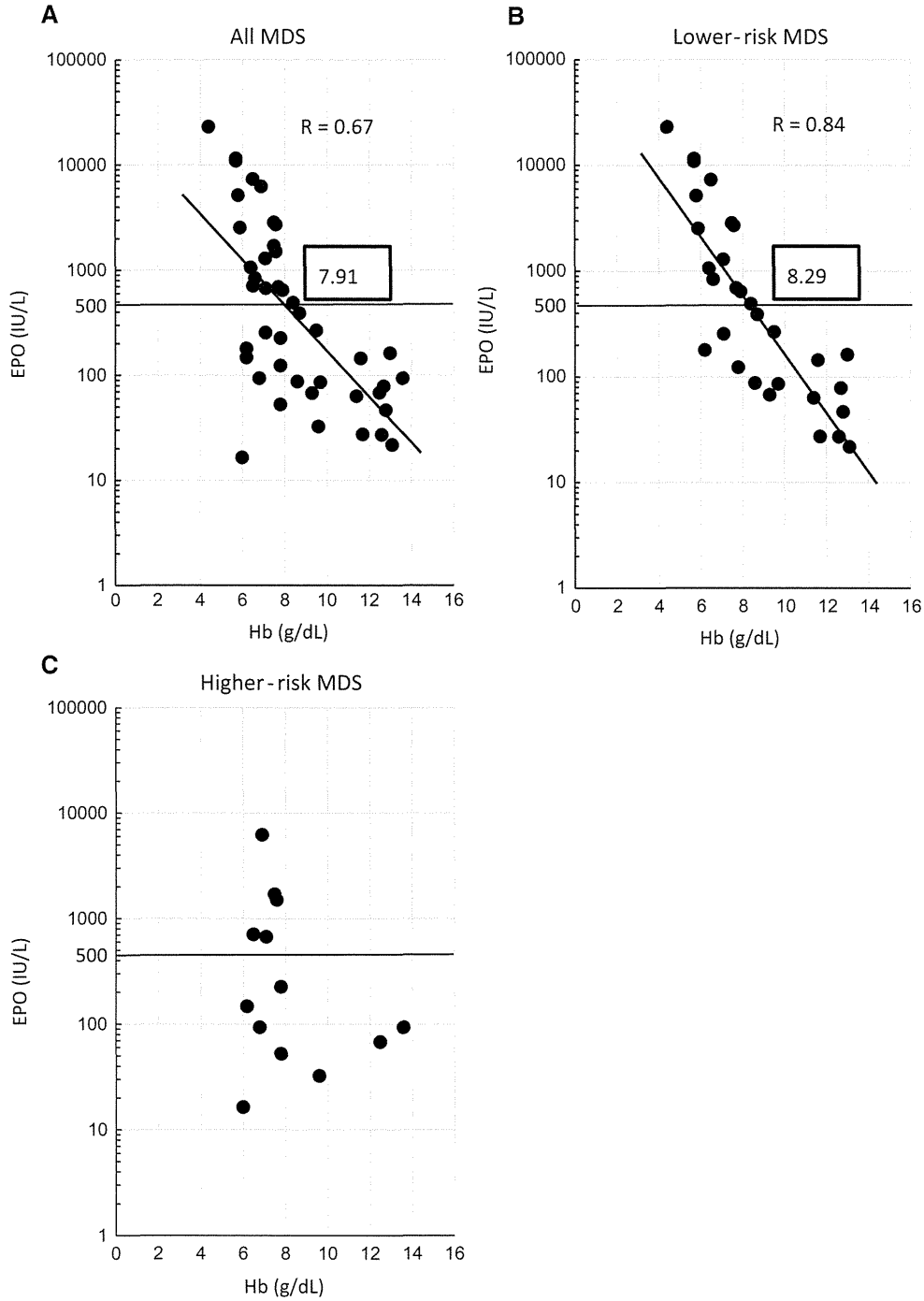


**Fig. 1** Relationship between serum erythropoietin and hemoglobin levels. Serum EPO levels were highly correlated with hemoglobin levels in all patients (**a**) and lower-risk patients (**b**), but not in higher-risk patients (**c**). Logarithmic values of serum EPO showed linear inverse correlation with Hb, and the EPO value of 500 IU/L corresponds to 7.91 g/dL and 8.29 g/dL of Hb in all (**a**) and lower-risk (**b**) patients, respectively. The corresponding values of Hb are shown in *central rectangles*. *EPO* erythropoietin, *Hb* hemoglobin, *R* correlation coefficient

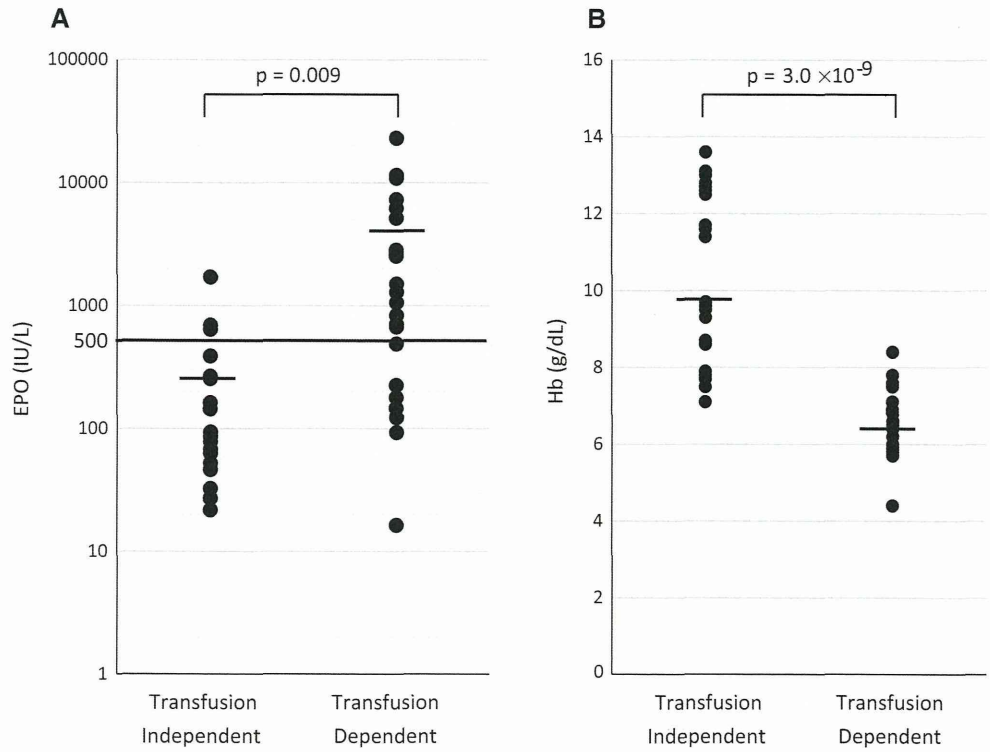


**Fig. 2** Serum erythropoietin and hemoglobin levels in transfusion-dependent and independent patients. Serum EPO levels were significantly lower in transfusion-independent patients than in transfusion-dependent patients (**a**). In contrast, Hb levels were significantly higher in transfusion-independent patients (**b**).*Short horizontal bars* indicate mean values of EPO or Hb in each group. *EPO* erythropoietin, *Hb* hemoglobin

correlation coefficients were $-0.69$ ($p = 9.0 \times 10^{-6}$) and $-0.70$ ($p = 5.2 \times 10^{-6}$), respectively (Table 2). Serum EPO levels were also correlated with reticulocytes and WBC, but the correlations were weak.

Logarithmic values of serum EPO in all patients were inversely correlated to Hb and Ht, and single regression analyses demonstrated very strong linear correlation; the coefficients were 0.67 ($p = 7.9 \times 10^{-7}$) for Hb (Fig. 1a) and 0.69 ($p = 2.8 \times 10^{-7}$) for Ht. The analyses showed that serum EPO is estimated by the following functions: "log EPO $= -0.22 \times$ Hb $+ 4.44$ ($p = 7.9 \times 10^{-7}$)" and "log EPO $= -0.077 \times$ Ht $+ 4.49$ ($p = 2.8 \times 10^{-7}$)".

Interestingly, significant correlations between EPO and Hb or Ht were not observed in higher-risk patients ($p = 0.59$ and $p = 0.43$, respectively) (Table 2; Fig. 1c), and the correlations in lower-risk patients were stronger than those in all MDS patients (Table 2; Fig. 1b). As a result, in lower-risk patients, serum EPO was estimated by "log EPO $= -0.28 \times$ Hb $+ 5.02$ ($p = 8.5 \times 10^{-9}$)" and "log EPO $= -0.096 \times$ Ht $+ 5.09$" ($p = 8.7 \times 10^{-10}$)".

According to these formulas, the serum EPO value of 500 IU/L corresponds to 7.91 g/dL of Hb and 23.3 % of Ht in all MDS patients, and 8.29 g/dL of Hb and 24.9 % of Ht in lower-risk patients.

Serum EPO had a strong correlation with transfusion dependency. Its levels in TD patients were significantly higher than those in transfusion-independent (TID) patients ($p = 0.009$) (Fig. 2a), and a significantly larger proportion of patients showed serum EPO levels higher than 500 IU/L in TD patients (68 % of TD vs. 14 % of TID patients, $p = 0.0004$). Hb levels were significantly lower in TD patients than in TID patients ($p = 3.0 \times 10^{-9}$) (Fig. 2b).

Most of the patients in the lower-risk group were with RCUD or RCMD in the WHO classification, and there was no significant difference in the Hb and serum EPO levels between RCUD and RCMD patients ($p = 0.39$ for Hb and $p = 0.42$ for EPO).

## Discussion

Lower-risk MDS is characterized by inefficient hematopoiesis, and most treatments aim at ameliorating cytopenias. ESAs are used to improve anemia, and many studies have demonstrated their effectiveness, sometimes combined with G-CSF [1–7, 10–12]. According to these studies, major predictive factors for response are lower serum EPO levels and minimal requirement of red blood cell transfusion; consequently, serum EPO levels less than 500 IU/L have been widely accepted as a standard for high probability of response [13].

In this study, we evaluated serum EPO levels in MDS patients in all risk categories, and we found a very strong linear correlation between logarithmic values of EPO and Hb or Ht. Since Hb and Ht are mutually dependent, we conclude that Hb is the most reliable factor for the prediction of serum EPO levels. Our results are generally similar

to those presented in previous reports [8, 9], and our data validate these reports. Unexpectedly, a significant correlation between Hb or Ht with serum EPO was not observed in the higher-risk patients. Although currently we have no explanation for this phenomenon, it may simply be a statistical aberration due to the small sample size of the higher-risk population. A study with a larger number of patients would elucidate this issue.

Serum EPO also correlated well with transfusion dependency. EPO levels in the TD patients were significantly higher than those in TID patients, and a larger proportion of the former had serum EPO levels greater than 500 IU/L. This correlation can be explained by the fact that Hb levels in the TD patients were significantly lower than those in TID patients. WHO subtypes did not have significant impact on serum EPO levels in lower-risk MDS.

Regression analyses showed strong inverse linear correlation between log EPO and Hb. According to the regression formulas, the serum EPO value 500 IU/L corresponds to 8.29 g/dL of Hb in lower-risk patients. As serum EPO level below 500 IU/L is a major predictive factor for response, this result indicates that patients with Hb levels of 8.0 g/dL or more, who are still transfusion independent, are good candidates for ESA treatment. Because minimal requirement of transfusion is another predictive factor for response [3, 5, 6, 11], these TID patients can expect the highest probability of response by ESA therapy. Some reports have described prolonged overall survival [2, 6, 11], improvement of quality of life [3], and extended time to transfusion requirements [7] in patients who were treated by or responded to ESAs (with G-CSF in some studies). Although it must be validated whether these favorable outcomes are true in the Japanese population, ESA therapy might be a good treatment option for the lower-risk Japanese patients with mild or moderate anemia, especially those who are transfusion-independent.

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# Leukemia Cells Directly Phagocytose Blood Cells in AML-Associated Hemophagocytic Lymphohistiocytosis: A Case Report and Review of the Literature

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Hemophagocytic lymphohistiocytosis (HLH), also called hemophagocytic syndrome (HS), is characterized by phagocyte activation caused by uncontrolled hyperinflammation due to immune disturbance. The malignancy-associated type (malignancy-associated HS, MAHS) is the most common type of reactive HLH in adults. Intravascular B cell lymphoma and T cell/natural killer cell lymphoma are the major background diseases associated with MAHS [1, 2]. In patients with MAHS, both proliferation and activation of normal phagocytes in the monocyte-macrophage lineage are generally found. Consequently, activated phagocytes phagocytose blood cells, resulting in the development of cytopenia. MAHS is also found in patients with other hematopoietic tumors including acute myelogenous leukemia (AML), but less frequently than lymphoma-associated HLH. In some patients with AML-associated HLH, leukemia cells instead of activated phagocytes directly phagocytose blood cells, thereby playing an essential role in phagocytosis. Interestingly, most of those patients have specific chromosomal abnormalities involving 8p11 and 16p13 (table 1). Here, we present a case of HLH associated with AML (M5b) with no chromosomal abnormality, in which leukemia cells primarily phagocytosed blood cells.

A 64-year-old man was admitted to our hospital with a 3-day history of high fever and oral bleeding. Hematological examination revealed pancytopenia: his white blood cell count was $0.7 \times 10^9$/l with 39% monocytoid blasts, hemoglobin 9.6 g/dl and a platelet count of $21 \times 10^9$/l. Bone marrow aspiration disclosed hypocellular bone marrow with 77.2% monocytoid blasts (fig. 1a). The blasts were positive for CD13, CD33, CD56, CD15, CD36, CD34 and CD14, but negative for myeloperoxidase on flow cytometric analysis. Cytochemical and immunohistochemical studies revealed that the blasts were positive for KP1/CD68 (fig. 1b), CD31 and α-naphthyl butyrate esterase and negative for peroxidase and CD34. Chromosomal analysis showed no abnormal karyotype. The level of serum lysozyme was high (108.9 μg/ml). Based on these results, we diagnosed this case as AML (M5b) with no chromosomal abnormality. The patient showed high levels of serum ferritin (77,505.8 ng/ml), lactate dehydrogenase (9,008 U/l), soluble IL-2 receptor (4,040 U/ml) and triglycerides (261 mg/dl). Coagulation examination revealed a reduced fibrinogen level (74 mg/ml) along with elevated levels of D-dimer (538.4 μg/ml) and FDP (1,176.0 μg/ml). In addition, computed tomography showed splenomegaly, ascites and cervical lymphadenopathy. Taken

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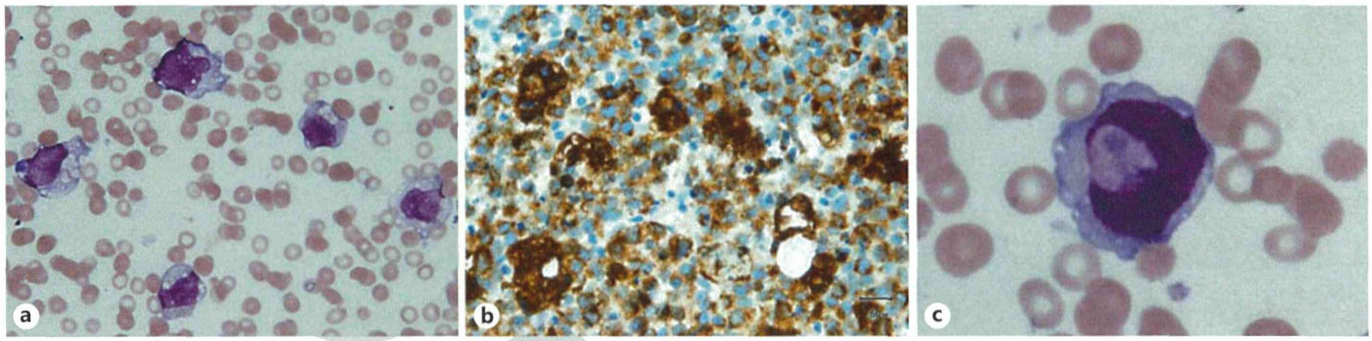


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**Fig. 1.** Bone marrow aspiration revealed phagocytosis by leukemia cells with monocyte-macrophage features. **a** The bone marrow shows hypocellularity with monocytoid blasts. Wright-Giemsa. ×600. **b** Immunohistochemical studies on bone marrow aspiration clots show positive results for KP-1. ×600. **c** A monocytoid blast, which directly phagocytosed blood cells. Wright-Giemsa. ×600.

**Table 1.** Chromosomal abnormalities in AML-associated HLH with direct hemophagocytosis of leukemia cells

| Karyotype | FAB diagnosis (number of cases) | Reference number |
|---|---|---|
| t(16;21)(p11;q22) | M1 (12), M2/M1 (2), M1/M7 (1), M4 (1), M5a (1), M5b (2) | 3, 5 |
| t(8;16)(p11;p13) | M0 (1), M4 (11), M4/M5 (2), M5a (3), M5b (5), M5 (3), Histiocytosis (1) | 6, 7 |
| t(8;19)(p11;q13.2) | M4 (1), M5a (1) | 8, 9 |
| inv(8)(p11;q13) | M4 (2), M5a (3) | 10 |
| t(8;18;16)(p11;q21;p13) | M5b | 11 |
| t(6;8)(q27;p11) | M5b | 9 |
| t(8;22)(p11;q13) | M5 | 12 |
| t(3;8;17)(q27;p11;q12) | M5 | 13 |
| t(10;17)(p15;q22) | M7 | 14 |
| t(1;22)(p13;q13) | M7 | 15 |
| del(20)(q11) | M0 | 16 |
| normal | Histiocytic leukemia (M5c?) | 17 |
| normal | M5b | our case |

FAB = French-American-British classification.

together with these results, a diagnosis of HLH was made according to the 2004 diagnostic guidelines for HLH [2]. Importantly, in addition to a few mature histiocytes, 27.6% of the monocytoid blasts directly phagocytosed blood cells in bone marrow (fig. 1c).

Treatment with etoposide (100 mg/body weight) was started for HLH. The fever disappeared the next day and the serum ferritin level declined gradually to 2,619.6 ng/ml on day 8. Subsequently, the patient underwent induction chemotherapy consisting of cytarabine and daunorubicin, and he achieved complete remission. At that time, the serum ferritin level remained high (1,104.3 ng/ml), but the HLH-related clinical symptoms and other laboratory abnormalities completely disappeared. Four further courses of chemotherapy were given as postremission therapy, and there has been no recurrence of AML or HLH since.

AML-associated HLH is rare. In fact, Ishii et al. [1] reported that in only 9 of 132 patients was the MAHS associated with AML. In our case, there was no other possible cause for secondary HLH, e.g. an infectious or autoimmune disease or a drug allergy. Furthermore, the patient had no malignant diseases except for AML. We therefore concluded that the patient had AML-associated HLH.

It has been reported that, in some patients with AML-associated HLH, leukemia cells directly phagocytose blood cells in the bone marrow. Interestingly, most patients in this group have specific chromosomal abnormalities involving 8p11 and 16p13 (table 1), though it has still to be clarified how such abnormalities are involved in the phagocytic activation of leukemia cells. Since many of these patients have acute myelomonocytic leukemia (M4) or acute monocytic leukemia (M5), it is possible that genetic alterations due to chromosomal abnormalities increase the phagocytic ability of monocytic leukemia cells. In fact, t(8;16)(p11;p13) and inv(8)(p11;q13) result in the formation of MOZ-containing fusion genes, and it is therefore possible that MOZ is involved in such genetic alterations. Our patient, however, showed no abnormal chromosomes. This is a very rare case. Indeed, only 1 patient with a normal karyotype was previously reported (table 1). It is noteworthy that the leukemia cells in our patient also showed monocyte-macrophage features; it is likely that these cells primarily had a strong phagocytic

ability independent of the chromosomal abnormalities. Jekarl et al. [3] showed that the expression level of CD56 is associated with the percentage of hemophagocytic leukemia cells in AML patients with t(16;21)(p11;q22). Since the leukemia cells in our case were positive for CD56, the involvement of CD56 expression in the ability to phagocytose is of great interest.

Jordan et al. [2] reported that patients with MAHS should be initially treated with immunochemotherapy for suppressing hyperinflammation and subsequently undergo therapy specific for a background malignant disease. In addition, it has been shown that Epstein-Barr virus-HLH patients receiving etoposide within 4 weeks after the diagnosis of HLH have a good prognosis [4]. In our case, the initial administration of etoposide resulted in an improvement of HLH-related clinical symptoms, and the patient was treated successfully with subsequent chemotherapy for AML.

We have described a case of AML-associated HLH with a normal karyotype, in which leukemia cells phagocytosed blood cells. Although AML-associated HLH is rare, it is potentially fatal. It is therefore important to elucidate the pathophysiology of this disease.


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