Serum hepcidin level and erythropoietic activity after hematopoietic stem cell transplantation

Junya Kanda,[1,3] Chisaki Mizumoto,[1] Hiroshi Kawabata,[1] Hideyuki Tsuchida,[2] Naohisa Tomosugi,[2] Keitaro Matsuo,[3,4] and Takashi Uchiyama[1]

[1]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto; [2]Proteomics Research Unit, Division of Advanced Medicine, Medical Research Institute, Kanazawa Medical University, Ishikawa; [3]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, and [4]Department of Epidemiology, Nagoya University Graduate School of Medicine, Nagoya, Japan

ABSTRACT

The relationship between serum hepcidin, a key regulator of body iron homeostasis, and erythropoiesis was investigated before and after stem cell transplantation in 31 patients with hematopoietic malignancies. Serum hepcidin-25 was monitored using a liquid chromatography-tandem mass spectrometry-based assay system. Other iron- and erythropoiesis-related parameters and known hepcidin regulators, such as interleukin-6 and growth differentiation factor-15, were also monitored. The serum hepcidin level peaked one week after stem cell transplantation, followed by a gradual decrease with a parallel change in interleukin-6 and a reciprocal change in reticulocyte count. Multivariate regression analysis demonstrated that the serum hepcidin level at four weeks after stem cell transplantation showed significant inverse correlations with erythropoietic activity markers, such as the soluble transferrin receptor, but not with growth differentiation factor-15. These results indicate the existence of an unknown functional erythropoiesis-associated circulating factor, other than growth differentiation factor-15, that negatively regulates hepcidin production in stem cell transplantation settings.

Key words: hepcidin, iron metabolism, stem cell transplantation, erythropoiesis.

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Introduction

Hepcidin, first identified in human urine as a small bactericidal peptide,[1,2] is now considered to be a central molecule regulating iron metabolism. Hepcidin decreases iron absorption in the intestine and blocks iron release from its stores by down-regulating the expression of the iron exporter ferroportin.[3,4] The hepatic expression of hepcidin can be up-regulated by at least 2 signals - iron loading[5,6] and inflammatory stimuli, including interleukin-6 (IL-6),[6,7] IL-1[7] and lipopolysaccharide (LPS).[8] The former signal is related to the machinery involved in maintaining body iron homeostasis, and the latter is regarded as one of the etiological mechanisms underlying the development of anemia in inflammatory diseases. In addition to these signals, the existence of erythropoiesis-associated regulatory factors of hepcidin has been hypothesized.[9-12] Consistent with this hypothesis, hepcidin expression increased when hematopoiesis in mice was blocked by irradiation or chemotherapeutic agents. However, this increase was not suppressed by erythropoietin administration or anemia caused by phenylhydrazine or phlebotomy, indicating that neither erythropoietin nor anemia *per se* was the hepcidin regulator.[13,14] Recently, growth differentiation factor-15 (GDF-15), a member of the transforming growth factor-β superfamily, was proposed to be one such factor in β-thalassemia patients.[15] However, to date, most information regarding such putative hepcidin regulators have been derived from animal experiments or *in vitro* studies with patient sera, and the association between erythropoiesis and hepcidin production has not been well documented in various clinical settings.

Hematopoietic stem cell transplantation (SCT) is a potentially curative intervention for malignant and intractable non-malignant hematologic diseases. Prior to SCT, hematopoiesis in the recipient should be eliminated by conditioning treatments, including high-dose chemotherapy and total body irradiation. After SCT, hematopoiesis is restored by donor stem

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Correspondence: Hiroshi Kawabata, Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan. E-mail: hkawabat@kuhp.kyoto-u.ac.jp

cells. Drastic changes in haematopoiesis and iron metabolism occur during and after SCT.[16] Since erythropoiesis is completely suppressed by conditioning treatments, SCT is an ideal model for investigating the relationship between iron homeostasis and erythropoiesis in clinical settings. Here, we monitored the pre- and post-SCT serum hepcidin levels, as well as factors possibly affecting hepcidin expression, and explored factors significantly associated with the serum hepcidin level in SCT settings.

Design and Methods

The study group comprised 31 consecutive adult patients with hematologic malignancies undergoing autologous or allogeneic SCT at Kyoto University Hospital from July 2006 to 2007. At approximately 8 am, their serum samples were obtained and stored in tubes at –80°C until analysis. Serum samples were also collected from 17 healthy control volunteers, who did not present anemia or C-reactive protein (CRP) elevation (median age, 31, range 27–44; male/female, 16/1; median serum ferritin: 84.4, range 14.5–279 ng/mL), at approximately 9 am. This study was approved by the ethics committee of Kyoto University Graduate School and the Faculty of Medicine. All patients provided their written informed consent. Serum levels of iron, total iron-binding capacity, ferritin, IL-6, CRP, erythropoietin, GDF-15 and hepcidin-25 (the major form of active hepcidin peptide) were monitored weekly, beginning from one week before SCT or conditioning to four weeks after SCT. The serum soluble transferrin receptor (sTfR) was analyzed four weeks after SCT. Serum levels of IL-6, erythropoietin, GDF-15 and sTfR were assayed using enzyme-linked immunosorbent assay kits (IL-6, GDF-15 and sTfR: Bender MedSystems, Vienna, Austria; erythropoietin: Roche, Mannheim, Germany) according to the manufacturers' protocols. Serum hepcidin-25 was quantified using a liquid chromatography-tandem mass spectrometry-based assay system following the method described by Murao *et al.*[17] Other serum parameters were measured using standard laboratory techniques. A non-parametric test was used to compare data between the 2 groups. The correlation between hepcidin and variables of interest was tested by Spearman's correlation coefficient. We performed uni- and multivariate linear regression analyses to clarify the factors associated with hepcidin production, using the bootstrap method with resampling performed 1,000 times. The bootstrap method is a general approach to statistical inferences and falls within a broader class of resampling methods.[18] Here, we estimated the measure of association with the resampled data repeatedly drawn from the original data with replacement. p values less than 0.05 were considered significant. All statistical analyses were performed using Stata software version10 (Stata Corp., College Station, Texas, USA).

Results and Discussion

Patient diagnoses revealed 13 acute myeloid leukemia, 3 myelodysplastic syndrome (MDS), 2 acute lymphoblastic leukemia, 8 non-Hodgkin's lymphoma, 1 Hodgkin's lymphoma, 1 multiple myeloma and 3 adult T-cell leukemia cases. The median age of these patients was 51 years (range, 23–63 years). Total body irradiation ranging from 4 to 12 Gy was used in 21 cases in combination with chemotherapeutic agents as conditioning regimens. Five patients received autologous SCT. Among the 26 allogeneic SCTs performed, the stem cell sources used were cord blood (8 cases) and bone marrow (18 cases). The pre-SCT disease status for these included 16 complete remissions, 11 partial remissions, 1 non-remission and 3 untreated MDS. At four weeks after SCT, complete remission was obtained in all patients except 2 who died.

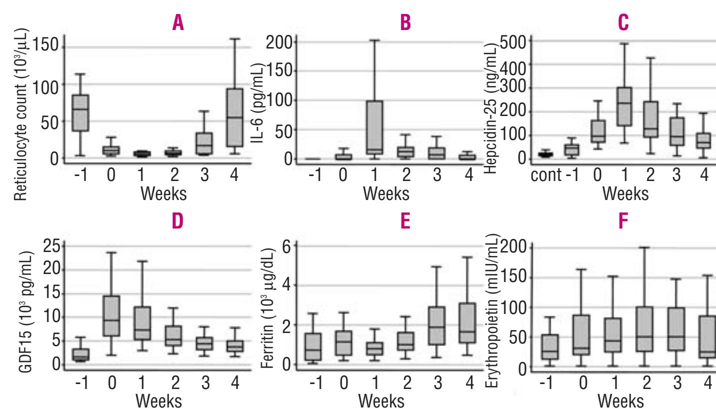

Figure 1. Sequential changes in each parameter. Sequential changes in reticulocyte count, serum levels of IL-6, hepcidin-25, GDF-15, ferritin and erythropoietin are shown in a box plot. Each box extends from approximately the first to third quartiles. The median values are shown as bars in the middle of the boxes and the ends of the whiskers indicate the upper and lower adjacent values.

Outliers are not shown in the figures. We found 4 patients to be outliers for serum hepcidin levels at one week before stem cell transplantation (SCT) (144, 371, 219.5, and 301 ng/mL). All these patients were diagnosed with acute myelocytic leukemia, and the disease was not in a state of complete remission (CR) at the time of SCT. Due to the presence of disease, the reticulocyte count was extremely low in 3 cases (< 10,000/μL). High IL-6 levels (15.842 pg/mL) were detected in 1 case, even at one week before SCT. We found 2 patients to be outliers for hepcidin levels (700 and 590 ng/mL) at two weeks after SCT; both patients developed severe infection and died four weeks after SCT. The IL-6 levels at two weeks after SCT were found to be extremely high in both these patients (1984 and 754 pg/mL). IL-6: interleukin-6; GDF-15: growth differentiation factor-15. *Weeks* indicates the span of weeks after SCT. *Cont* indicates control experiments with healthy volunteers.

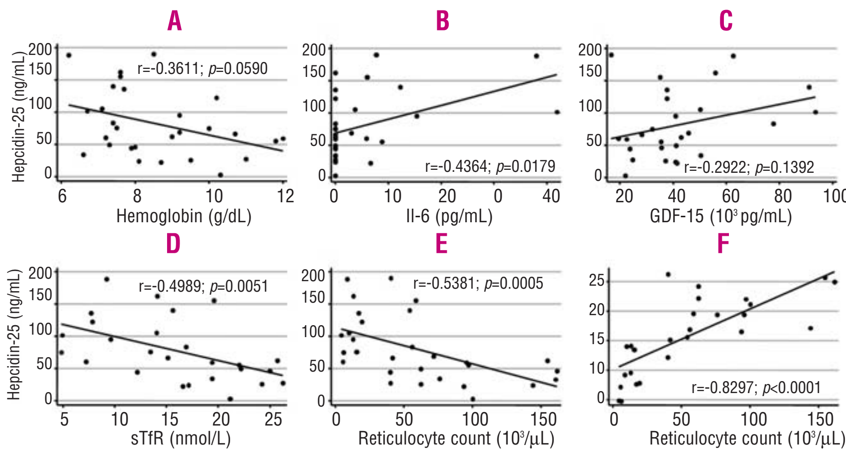

Figure 2. Correlations between the serum hepcidin level and factors potentially associated with hepcidin production. Panels A–E show correlations between the serum hepcidin level and hemoglobin (A), serum levels of IL-6 (B), GDF-15 (C), sTfR (D) and the reticulocyte count (E), and F shows correlation between sTfR and reticulocyte count at four weeks after SCT. The analysis was performed for 29 cases that could be evaluated. IL-6: interleukin-6; GDF-15: growth differentiation factor 15; sTfR: soluble transferrin receptor.

The changes in the parameters involved in iron metabolism and erythropoiesis during SCT are shown in Figure 1. The reticulocyte count decreased rapidly after the conditioning treatment and began increasing three or four weeks after SCT (Figure 1A). The serum IL-6 level peaked one week after SCT (median, 15.56 pg/mL; normal range, <4 pg/mL) in almost all cases (Figure 1B). The serum hepcidin-25 level at one week before SCT was higher (median, 42.8 ng/mL) than that in the control sera of healthy volunteers (median, 19.05 ng/mL) (p=0.011); it further increased after the day of SCT (Figure 1C). The mean hepcidin level peaked one week after SCT (median, 232.5 ng/mL), followed by a gradual decrease until four weeks after SCT. This pattern was consistent regardless of the conditioning regimens (myeloablative or reduced intensity) or stem cell sources (autologous or allogeneic) (*data not shown*). The GDF-15 level peaked on the day of SCT (median, 9337.7 pg/mL) and gradually decreased thereafter (Figure 1D). Transferrin saturation increased up to nearly 100% on the day of SCT and gradually decreased thereafter (*data not shown*). The serum ferritin level at one week before SCT was high in almost all cases (median, 726.3 μg/dL; normal range, <150 μg/dL) and tended to increase after transplantation (Figure 1E). In a majority of the cases, the serum erythropoietin level was elevated one, two and three weeks after SCT (two weeks after SCT; median, 49.2 mIU/mL; normal range, 8–36 mIU/mL) (Figure 1F).

The serum hepcidin peak at one week after SCT can partially be explained by the elevation in IL-6 levels caused by conditioning treatments and/or infections. At four weeks after SCT, when the IL-6 levels normalized in most cases, some cases continued to show relatively high serum hepcidin levels. Therefore, we analyzed the factors associated with the elevation in serum hepcidin levels at four weeks after SCT. As shown in Figure 2, by univariate analyses, we identified inverse correlations between the hepcidin level and both sTfR (r =–0.4989, p=0.0051) and reticulocyte count (r=–0.5381, p=0.0005), and a positive correlation between the hepcidin and IL-6 levels (r = 0.4364, p=0.0179). In contrast, no significant correlation was observed between the hepcidin level and either of the following parameters: hemoglobin (r=–0.3611, p=0.0590), serum levels of GDF-15 (r=0.2922, p=0.1392), erythropoietin (r=0.2511, p= 0.1888), ferritin (r=0.0654, p=0.7562) or transferrin saturation (r=0.0038, p=0.9854) (Figure 2 and *data not shown*).

Pairwise correlation tests for all pairs revealed a strong correlation between the reticulocyte count and sTfR as expected (Figure 2F; r = 0.8297, p<0.0001) since both reflect erythropoietic activity. We included sTfR as a representative indicator of erythroid recovery in uni- and multivariate regression analyses together with other factors reported to be associated with the hepcidin level. As shown in Table 1, among the factors analyzed, sTfR and hemoglobin were significantly associated with the hepcidin level in the univariate analysis. Multivariate analysis revealed that only sTfR was independently associated with the hepcidin level (p=0.019). When we used the reticulocyte count instead of sTfR in multivariate analysis, only the reticulocyte count was observed to be associated with the hepcidin level (p=0.017).

Table 1. Uni- and multivariate linear regression analyses for factors independently associated with the hepcidin level.

Independent variables	Univariate Coefficient	p	Multivariate Coefficient	p
sTfR (nmol/L)	-3.733	0.001	-5.531	0.019
Ferritin (μg/dL)	0.006	0.356	0.011	0.305
Hemoglobin (g/dL)	-12.172	0.023	-13.777	0.335
IL-6 (pg/mL)	2.163	0.083	0.121	0.955
GDF-15 (10³ pg/mL)	0.008	0.127	0.005	0.661
Erythropoietin (mIU/mL)	0.045	0.622	-0.142	0.493
Transferrin saturation (%)	-0.216	0.681	-1.055	0.165

The linear regression analyses were made using the bootstrap method with resampling performed 1,000 times. The value of adjusted R2 in multivariate analysis was 0.5936. Adjusted R2 indicates the proportion of the variance of dependent variable (hepcidin levels) explained by explanatory variables included in the model. Maximum of R2 is 1 and 0.5936 here indicates fairly good fitting of the models. The analysis was performed for the 29 cases that could be evaluated. sTfR: soluble transferrin receptor; IL-6: interleukin-6; GDF-15: growth differentiation factor-15.

In this study, we demonstrated that the serum hepcidin-25 level of our SCT patients was high before the conditioning treatments and peaked one week after SCT followed by a gradual decrease; this pattern was strikingly reciprocal to that of the reticulocyte count, an indicator of erythropoiesis. In addition, among the factors reported to be associated with the hepcidin production, only the serum sTfR level and reticulocyte count, indicators of the erythropoietic activity, were strongly associated with the serum hepcidin level at four weeks after SCT. To our knowledge, this is the first report assessing the association between serum hepcidin levels and various parameters, including those of erythropoiesis and iron homeostasis, in clinical settings of SCT.

Among the known signals that stimulate hepcidin synthesis, the contribution of the iron signal to the elevated hepcidin levels was probably minimal in our SCT cases since the serum hepcidin level did not significantly correlate with either the serum ferritin level or transferrin saturation. The inflammatory IL-6 pathway has also been regarded as an important pathway for the induction of hepcidin synthesis,[6,7] leading to microcytic anemia with low serum iron levels observed in chronic inflammation. The association between the hepcidin level in urine or serum and IL-6 level was observed in healthy individuals after LPS injection[8] and patients with acute inflammation.[19] A concurrent increase in hepcidin and IL-6 levels at one week after SCT in our cases suggested that IL-6 contributed to the high hepcidin level in SCT to some extent. Recently, several investigators have indicated the existence of an erythropoiesis-associated humoral factor that negatively regulates hepcidin synthesis, mainly based on animal experiments and *in vitro* experiments using sera of β-thalassemia patients.[9-14] These patients usually show severe microcytic anemia, increased iron stores and ineffective erythropoiesis with relatively low hepcidin production, and a clear inverse correlation was observed between the urinary hepcidin level and levels of both erythropoietin and sTfR.[20] Kemna *et al*. demonstrated that patient sera containing high sTfR levels could suppress hepcidin mRNA expression in human hepatoma cells.[21] In our study, sTfR showed a significant inverse correlation with the serum hepcidin level at four weeks after SCT, in accordance with their observations. However, Flanagan *et al*. showed that hepcidin mRNA expression was not downregulated by sTfR1 overexpression in mice,[22] indicating that sTfR *per se* is not a hepcidin regulator but an indicator of erythropoietic activity.

More recently, Tanno *et al*. have proposed that GDF-15 is one of the erythropoietic regulators in β-thalassemia patients.[15] GDF-15 concentrations in their sera were abnormally high (10,000–100,000 pg/mL), and addition of such high concentrations of recombinant GDF-15 into human primary hepatocytes or hepatoma cell cultures suppressed hepcidin mRNA expression. In our study, no significant correlation was observed between the serum levels of hepcidin and GDF-15 probably because the levels of GDF-15 were considerably lower than those observed in β-thalassemia patients.

In the clinical management of SCT patients, the assessment of body iron status is important because a growing body of evidence suggests that iron overload has a strong negative impact on clinical outcomes.[23,24] Dynamic changes in the hepcidin level could be a key to understanding the mechanism of iron homeostasis in SCT settings. Among the factors potentially associated with hepcidin regulation, we showed that erythropoietic activity represented by the reticulocyte count and sTfR exerted the strongest inverse correlation with the serum hepcidin-25 level in SCT, indicating the existence of an erythropoiesis-associated circulating hepcidin regulator, other than GDF-15, in SCT settings.

Since the sample size used in our study was considerably small, we focused on the relationship between the serum level of hepcidin and erythropoietic recovery, and we performed statistical analyses only at four weeks after SCT, instead of a time series analysis. Studies involving a considerably larger sample size and a time series analysis might provide additional information on the serum levels of hepcidin following SCT. Furthermore, future studies should evaluate the clinical relevance of hepcidin monitoring during SCT.

Authorship and Disclosures

JK and HK designed and performed the experiments, analyzed the data and prepared the manuscript. CM and HT performed the experiments. NT designed and performed the experiments. JK and KM performed the statistical analysis. HK and TU supervised the overall study and edited all drafts of the manuscript.

NT declares that he is the President of Medical Care Proteomics Biotechnology Co. Ltd. (Ishikawa-ken, Japan), a start-up company, the stock of which is not publicly traded. The other authors declare that they have no conflicts of interest relevant to this paper.

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