The murine growth differentiation factor 15 is not essential for systemic iron homeostasis in phlebotomized mice

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ABSTRACT

In conditions of increased erythropoiesis, expression of hepcidin, the master regulator of systemic iron homeostasis, is decreased to allow for the release of iron into the blood stream from duodenal enterocytes and macrophages. It has been suggested that hepcidin suppression is controlled by growth differentiation factor 15 (GDF15), a member of the transforming growth factor-β superfamily of cytokines that is secreted from developing erythroblasts. In this study, we analyzed iron-related parameters in mice deficient for GDF15 under steady-state conditions and in response to increased erythropoietic activity induced by blood loss. We demonstrate that GDF15 suppresses the hepatic mRNA expression of some BMP/TGFβ target genes but not of hepcidin, and show that GDF15 is not required to balance iron homeostasis in response to blood loss.

Introduction

Most iron available in the mammalian system is required for the synthesis of red blood cells. As a consequence, erythropoietic iron consumption dramatically increases following blood loss. Erythropoietic, hypoxic and/or iron-related signals repress the synthesis of hepcidin, a hepatic peptide hormone that orchestrates the release of iron into the blood stream from duodenal enterocytes, hepatocytes and macrophages.[1] Hepcidin binds to the iron exporter ferroportin, which is expressed in these cell types, to trigger its internalization and degradation.[2,3] While the molecular mechanisms involved in hepcidin suppression in response to increased erythropoietic activity remain unclear, it is believed that they may include soluble proteins secreted from developing erythroblasts in the bone marrow. Growth differentiation factor 15 (GDF15, also named MIC1, PDF or PLAB), a member of the transforming growth factor-β superfamily of cytokines, was suggested to be such a protein. It is produced in erythroid precursor cells, and high concentrations of GDF15 down-regulate hepcidin mRNA expression in primary human hepatocytes.[4] Furthermore, GDF15 concentrations are strongly increased in disorders hallmarked by increased ineffective erythropoiesis, such as β-thalassemia, congenital dyserythropoietic anemias or, to a lesser extent, pyruvate kinase deficiency.[4-7]

GDF15 is expressed in several organs, including liver, lung, kidney, and exocrine glands.[8,9] In mice, GDF15 acts as a cardioprotective factor released by the myocardium, and as a neurotrophic factor for midbrain dopaminergic neurons.[10-12] Mice treated with systemic GDF15 and transgenic mice overexpressing GDF15 show hypophagia and reduced body weight.[13] Conversely, homozygous GDF15 null mice exhibit increased body weight, combined with a progressive postnatal loss of motoneurons.[14] To date, no studies on the role of GDF15 in maintaining iron homeostasis have been reported in mice.

Design and Methods

Mice

The generation of mice lacking the complete coding sequence of GDF15 has been described previously.[14] All mice analyzed were 8-12 weeks old, male and maintained on a C57BL/6N genetic background. Age-/sex-matched wild-type mice were used as controls throughout the study. Mice were euthanized by CO_2 inhalation. Heparinized blood was collected by cardiac puncture. Hematologic parameters were measured by the Central Laboratory services of University Hospital, Heidelberg, Germany. For the study of β-thalassemic animals, 5-month old *Th3/+* and wild-type mice were maintained on a C57BL/6 genetic background. Spleen and bone marrow RNAs from wild-type and *Th3/+* age- and sex- (female) matched animals were compared. Four animals per group were used. All animal experiments were approved and conducted in compliance with the guidelines of the European Molecular Biology Laboratory and Weill Cornell Medical College Institutional Animal Care and Use Committee.

Phlebotomies

On two consecutive days, 0.4 mL of blood were withdrawn via retroorbital puncture using a heparinized capillary tube. Animals were

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euthanized 24 h after the last phlebotomy for blood and tissue collection. Blood was collected in heparin tubes and analyzed for hematologic parameters and iron levels. Tissue samples were snap-frozen and used for measuring tissue iron content and RNA extraction.

Iron measurements

Plasma iron concentrations and non-heme iron levels in liver and spleen were measured as described previously.[15,16] Results are reported as micrograms of iron per gram of dry tissue.

Quantitative real-time PCR

Tissue total RNA was prepared by phenol-chloroform extraction (TRIzol®, Invitrogen) and relative mRNA levels of iron-related genes were analyzed by quantitative real-time polymerase chain reaction (PCR) in 20 μL reaction volumes using SYBR Green I dye on an ABI Prism 7500 (Applied Biosystems). Primers were designed to specifically amplify the murine Hamp, Gdf15, Twsg1, activin, Col4a1, Gadd45b, Id1, Smad6, Smad7, Skil, Tgfb1, Gapdh and Actb genes. Primer sequences are shown in *Online Supplementary Table S1*. Abundance of each specific mRNA was calculated relative to the expression of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and β-actin (Actb), assuming exact doubling of amplified DNA in each PCR cycle.

RNA samples extracted from the spleen and bone marrow of wild-type and *Th3/+* mice were prepared by phenol-chloroform extraction (TRIzol®, Invitrogen). They were retrotranscribed using SuperScript II First Strand Kit (Invitrogen). cDNAs were analyzed by Q-PCR with primers specific for Gdf15 (*Online Supplementary Table S1*). Q-PCR reactions were performed by using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), with TaqMan (TaqMan PCR 2X Master Mix; Applied Biosystems) and SYBR Green (iTaq SYBR Green Supermix; Bio-Rad Laboratories) chemistry.

Statistical analysis

Data are presented as mean±SD or as median (interquartile range) as appropriate. N represents the number of animals used for each experiment. Statistical significance of hematologic parameters was determined by non-parametric two-tailed Mann-Whitney test. The Relative Expression Software Tool (REST, *http://rest.gene-quantification.info*)[17] was used to analyze the quantitative PCR data. The mathematical model is based on the mean crossing point (Cp) deviation between sample and control groups of target genes, normalized by the mean Cp deviation of the reference genes Gapdh and Actb. For the analysis of Gdf15 quantitative PCR data in thalassemic animals, an unpaired two-tailed Student's t-test was performed using Microsoft Excel, Mac 2011 software. $P<0.05$ was considered statistically significant.

Results and Discussion

In this study, we investigated whether ablation of GDF15 in the mouse affects the control of iron homeostasis under steady state conditions or as a consequence of blood loss. A total of 16 wild-type (WT) and 16 Gdf15 knockout (KO) mice were analyzed. To increase erythropoietic activity, half of the mice of each genotype were subjected to phlebotomies via retroorbital puncture. An average of 0.4 mL of blood was removed on two consecutive days and mice were sacrificed 24 h after the last phlebotomy.

Consistent with previous reports,[14] untreated Gdf15 homozygous null mice showed no obvious abnormalities other than a significant increase in body weight compared to wild-type mice (wild-type 26.3±2.1 g, n=16, *vs.* knockout 28.5±2.6 g, n=16; 8.2% increase, $P=0.012$;). There was no difference in hematologic parameters, plasma iron concentrations, or liver or spleen non-heme iron content between the two genotypes (Table 1). As expected, two consecutive rounds of bleeding induced severe iron-deficiency anemia both in wild-type and GDF15-deficient mice, with strongly decreased hemoglobin concentration and erythrocyte counts (Table 1). Despite a major increase in erythropoiesis, as indicated by increased reticulocyte numbers in phlebotomized mice, no significant differences were detected between the hematologic parameters of wild-type and Gdf15 knockout mice (Table 1). Taken together, these results suggest that GDF15 is not required to mediate the response to phlebotomy-induced anemia.

To satisfy the increased iron demand for erythropoiesis following blood loss, iron is mobilized from the plasma as well as from iron storage tissues such as the spleen and the liver. The two consecutive phlebotomies depleted plasma iron levels in wild-type and Gdf15$^{-/-}$ mice, while the non-heme iron content of liver and spleen showed a trend toward being reduced or remaining unchanged in both genotypes (Table 1). This suggests that under these experimental conditions the iron content of the plasma (and possibly its replenishment from the diet) is sufficient to satisfy erythropoietic demands.

High serum GDF15 levels have been reported in patients suffering from β-thalassemia and congenital dyserythropoietic anemias type I and II,[4,6,7] and to a lesser extent, in patients with pyruvate kinase deficiency.[5] In all cases, increased GDF15 levels were associated with inappropriately low hepcidin concentrations, given the severe hepatic iron overload that hallmarks these disorders.[4-7] In WT mice, acute blood loss significantly increased Gdf15 mRNA expression in the bone marrow (3.4-fold; $P=0.027$; Figure 1), and effectively reduced hepatic hepcidin mRNA

Table 1. Hematologic and iron parameters in control and phlebotomized wild-type and Gdf15$^{-/-}$ mice. Data are shown as mean ± standard deviation. Statistical significance was assessed between phlebotomized (phleb) and control (ctrl) mice by Mann Whitney test: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

	WT (ctrl) n = 7	Gdf15-/- (ctrl) n = 8	WT (phleb) n = 8	Gdf15-/- (phleb) n = 8
Hemoglobin (g/dL)	11.2±1.7	11.9±1.7	5.7±1.2***	6.6±2.1***
Erythrocytes (10^{12}/L)	7.5±1.2	7.9±1.3	3.9±0.9***	4.4±1.5**
Hematocrit (%)	36.0±6.0	39.0±6.0	18.5±4.9***	21.6±6.6***
MCV (fL)	48.0±1.6	49.0±1.0	47.8±1.6	49.1±2.2
MCH (pg)	15.1±0.4	15.1±0.6	15.0±0.5	15.0±0.9
MCHC (g/dL)	31.3±1.5	30.9±1.1	31.0±1.7	30.9±2.2
RDW (%)	12.2±0.3	12.5±0.3	14.8±1.7**	15.2±2.1**
Reticulocytes (‰)	8.9±4.7	12.9±4.4	67.0±36.3***	68.0±33.4**
Hemoglobin in reticulocytes (pg)	14.9±0.4	15.6±0.8	17.2±0.8***	17.8±1.2**
Leukocytes (/nL)	140±155	72±117	83±140	92±179
Thrombocytes (/nL)	596±211	650±197	678±216	761±144
Plasma iron (μg/dL)	313±111	373±197	218±38*	238±43
Liver iron (μg/g)	192±52	171±58	139±31	176±49
Spleen iron (μg/g)	2793±560	3165±606	2274±416	2624±427

MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean corpuscolar hemoglobin concentration; RDW: red blood cell distribution width.

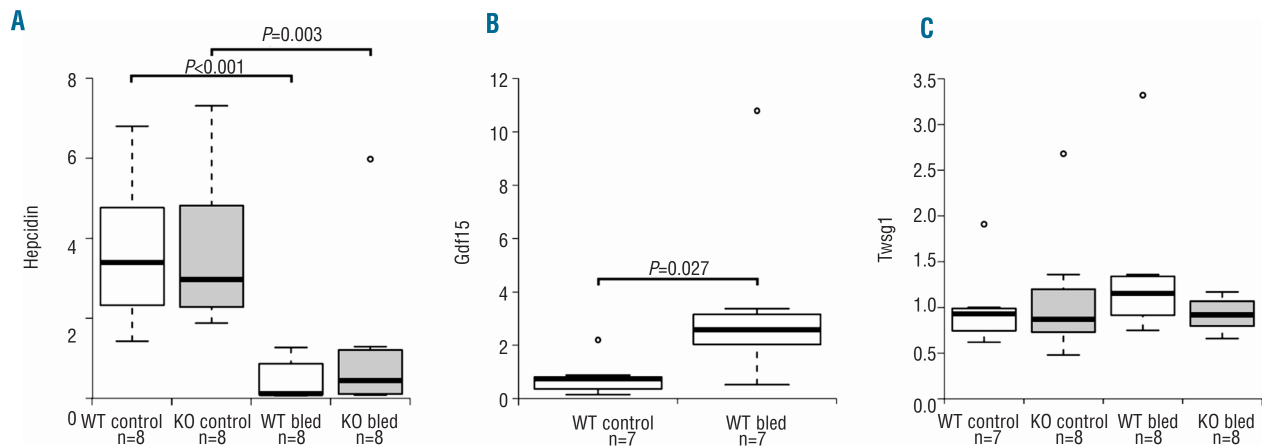

Figure 1. Hepcidin, Gdf15 and Twsg1 mRNA expression in control and phlebotomized wild-type and Gdf15$^{-/-}$ mice. (A) Hepatic hepcidin. (B) Bone marrow Gdf15. (C) Bone marrow Twsg1. Relative mRNA expression of each gene *versus* Actb is shown as median ± interquartile range. Statistical significance *versus* corresponding control group was calculated with REST, using Gapdh and Actb as reference genes. Circles (○) mark the outlying values, as determined by 1.5 times the interquartile range distance from the upper and lower quartile. n: number of animals analyzed in each group.

levels (Figure 1). Surprisingly, hepatic hepcidin mRNA expression was not altered in Gdf15$^{-/-}$ mice under steady-state conditions, and was reduced to equal levels upon phlebotomy in Gdf15$^{-/-}$ and wild-type mice (Figure 1). This contrasts with the mRNA expression of some TGFβ/Bmp/Smad-target genes (activin, Col4a1 and Smad6), which showed increased mRNA levels in Gdf15$^{-/-}$ mice, while others (gadd45b, Id1, Smad7, SnoN) did not (*Online Supplementary Figure S1*). Interestingly, the mRNA expression of the inhibitory Smad6, a well known regulator of the BMP/SMAD pathway,[18,19] was increased in wild-type mice upon phlebotomy, suggesting that it might play a role in hepcidin suppression (*Online Supplementary Figure S1*). The increase in Smad6 mRNA levels in untreated Gdf15$^{-/-}$ mice may indicate a compensatory role for Smad6 in GDF15 deficiency. Smad6 mRNA levels are not further increased in phlebotomized Gdf15$^{-/-}$ mice, which may be attributed to a saturation of the signaling pathways that activate Smad6 mRNA expression. By contrast, the mRNA levels of Smad7, a recently identified suppressor of hepcidin expression,[20] were reduced upon phlebotomy in wild-type mice, suggesting that it does not contribute to the reduction of hepcidin levels in response to acute anemia.

In addition to GDF15, the twisted gastrulation protein (TWSG1) was proposed to mediate suppression of hepcidin transcription in response to increased erythropoietic activity.[21,22] TWSG1 is produced during early erythroblast maturation, and suppresses hepcidin mRNA expression in cultured hepatocytes by interfering with the BMP/HJV pathway. Mice suffering from thalassemia show increased levels of TWSG1, arguing in favor of a function as an erythropoietic signal also in mice.[22] Interestingly, our analysis of wild-type and GDF15 knockout mice revealed no alterations of Twsg1 mRNA levels in the murine bone marrow following phlebotomies, suggesting that this factor is not involved in the downregulation of hepcidin as a result of acute blood loss (Figure 1).

Taken together, our data do not support an essential role for GDF15 in regulating iron homeostasis under steady-state conditions or in response to blood loss in the mouse. It remains possible that GDF15 may exert effects on iron homeostasis under conditions of increased erythropoiesis, as would be observed in response to long-term phlebotomies, or in congenital disorders such as thalassemia, sickle cell disease, or congenital dyserythropoietic anemias.[4,6,7,23] It is, however, interesting that, in a mouse model for β-thalassemia (*th3*/$^+$) hallmarked by low hemoglobin levels, reticulocytosis, increased production of immature erythroid cells and elevated tissue iron levels due to low hepcidin expression,[24-26] GDF15 mRNA expression is unaltered in the bone marrow (0.013±0.018 in wild-type *vs.* 0.030±0.002 in *th3*/$^+$ mice, P=0.33) and even decreased in the spleen (0.036±0.013 in wild-type *vs.* 0.015±0.006 in *th3*/$^+$ mice, P=0.03). These data suggest that GDF15 is unlikely to contribute to hepcidin suppression in *th3*/$^+$ mice. Thus, crosses between Gdf15$^{-/-}$ and *th3*/$^+$ mice to assess whether murine GDF15 recapitulates the functions of its human homolog in thalassemia are not likely to be useful. Rather, the analysis of transgenic mice with constitutive GDF15 or TWSG1 overexpression may be interesting to assess their long-term roles in iron metabolism.

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