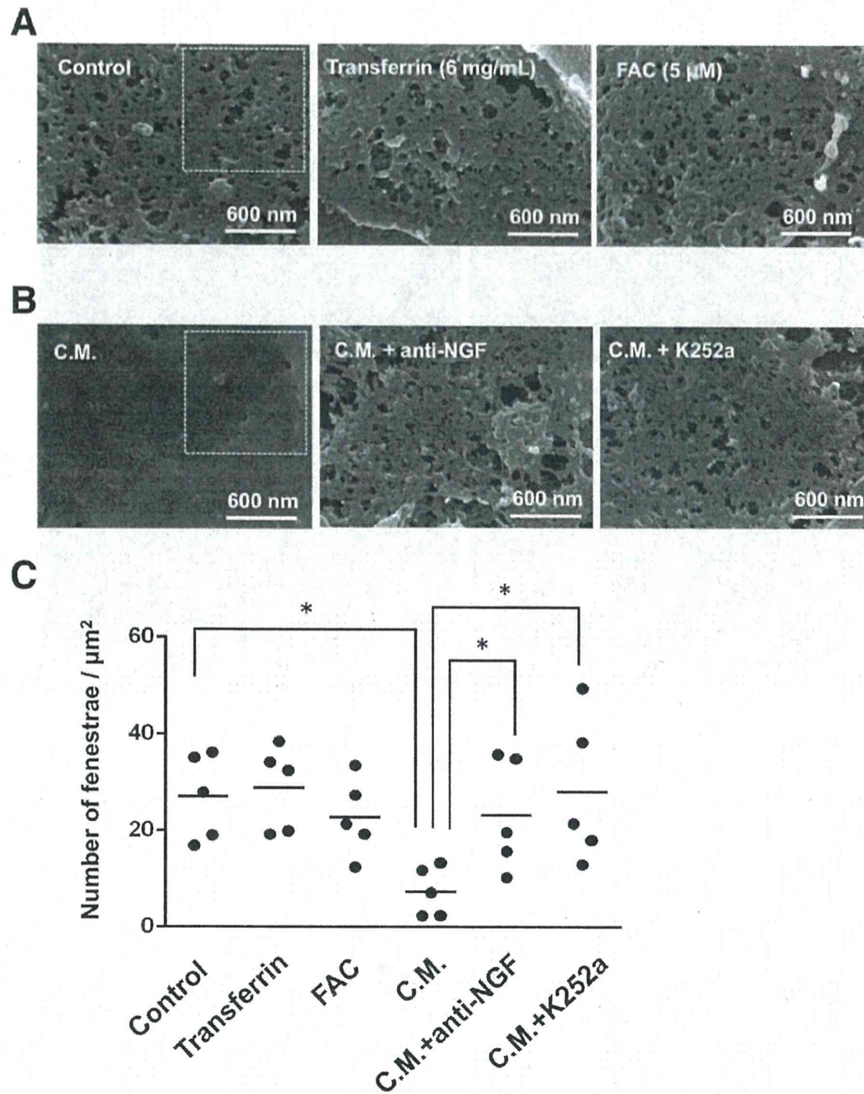

Fig. 7. SEM in isolated LSECs. (A) LSEC fenestration was unaffected after treatment with fresh iron-overloaded medium (holo-transferrin 6 mg/ml and 5 μM FAC). The white dotted line in the control image represents a 1 μm^2 area. (B) LSEC fenestration was reduced after culture in supernatant iron overload medium (holo-transferrin 6 mg/ml) obtained from pre-culture with mouse primary hepatocytes. Treatment with an anti-NGF neutralizing antibody or TrkA inhibitor, K252a, reversed this effect (SEM x50,000 magnification). The white dotted line in the control image represents a 1 μm^2 area. (C) Graph showing the number of fenestrae per μm^2. The pictures shown for each treatment group represent one picture selected from a total of five representative images. C.M.: conditioned medium from iron-overloaded primary hepatocytes.

the major site of NGF expression under hepatic stress conditions [27]. Several growth factors regulate liver regeneration after exposure to hepatotoxins; however, the expression of two such important growth factors, HGF and VEGF, in the iron-loaded liver showed no significant change in expression, compared to that of NGF. This indicates that NGF is one of the growth factors that is secreted early in the development of liver iron loading. Furthermore, the high-affinity NGF receptor, TrkA, was found to be expressed in LSECs of both control and iron-overloaded mice. While different cellular populations including hepatocytes, biliary epithelial cells, Kupffer cells, and stellate cells make up the intact liver, this finding suggests that LSECs are the main target for NGF and suggests a possible paracrine mode of action for NGF in the liver. Thus, our study demonstrates the localization of TrkA in LSECs of control and iron-overloaded mice *in vivo*.

Most importantly, endothelial cell defenestration was observed in both the severe as well as slight iron overload models, a clear indication that defenestration occurs early in the development of iron overload. One critical step to understanding the potential relevance of this observation was determining the factor responsible for this occurrence. As iron in excess represents a potential hepatotoxin capable of influencing endothelial cell function and defenestration, the possibility that iron itself may have induced the defenestration was considered. We also considered the fact that the deleterious effects of oxidative damage due to reactive oxygen species could also, at least in part, have been responsible for the defenestration of LSECs [23]. Surprisingly, however, we found that iron itself did not directly affect LSEC defenestration. To further investigate the relevance of this finding, we also considered NGF as the factor responsible because it was highly expressed, and we found that when mouse primary endothelial cells were cultured with mouse recombinant NGF, defenestration was increased as compared to the controls. To further confirm this finding, we observed that subsequent incubation with an anti-NGF neutralizing antibody or the TrkA inhibitor (K252a) reversed this defenestration effect. Taken together, these data provide clear evidence that under conditions of iron overload, NGF is expressed and released from hepatocytes, which then induces a defenestration response in LSECs via TrkA signaling. The data also demonstrate that the expression of NGF and subsequent defenestration occur early in the development of iron overload, which is possibly aimed at reducing

the exposure of cells in the space of Disse to the accumulating iron, similar to the response of endothelial cells to other agents [14–22]. This phenomenon may therefore contribute to the defensive machinery employed by the liver to counter iron accumulation during periods of overload and may represent an early part of the sequence of events that precede eventual liver disease. It is not yet clear how iron induces NGF expression in hepatocytes, but epigenetic regulation may be responsible. A recent study implicated epigenesis in the control of NGF during alcohol withdrawal [34], whereas another reported frequent hypermethylation of six genes (RASSF1A, cyclinD2, $p16^{INK4a}$, GSTπ1, SOCS-1, and APC) in patients with hereditary hemochromatosis, with an elevated risk of developing HCC [35]. Taken together, the epigenetic regulation of NGF by iron is likely to occur, although further studies are needed to clarify this issue.

In this report, we have provided evidence indicating that NGF mediates the regulation of LSEC fenestration during the development of iron overload, a phenomenon that may contribute to the defense of the liver to protect against iron excess, even in the early stages of the development of iron overload. This newly demonstrated link between iron and NGF on endothelial cell defenestration may contribute to further broaden our scope of understanding regarding the likely role of NGF in the interplay between iron loading and endothelial cell function. Future studies will be required to further elucidate the mechanism by which iron increases the expression of NGF in iron overload.

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INTERNAL MEDICINE

□ CASE REPORT □

Effective Control of Relapsing Disseminated Intravascular Coagulation in a Patient with Decompensated Liver Cirrhosis by Recombinant Soluble Thrombomodulin

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Abstract

A 70-year-old Japanese man was hospitalized for expanding purpura and chronic disseminated intravascular coagulation (DIC) caused by decompensated liver cirrhosis. As there are no effective treatments for chronic DIC caused by liver cirrhosis, we decided to administer recombinant human soluble thrombomodulin (rhsTM) after he provided informed consent. The DIC was rapidly improved; however, the purpura and coagulopathy recurred after two months, and repeated rhsTM treatments were required. The rhsTM treatment sufficiently controlled the coagulopathy for two years, without any complications, including bleeding. This is the first report demonstrating that rhsTM can be administered safely and repeatedly to a patient with decompensated liver cirrhosis, and that it appears to be associated with a favorable outcome.

Key words: recombinant human soluble thrombomodulin, chronic disseminated intravascular coagulation, liver cirrhosis

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Introduction

Disseminated intravascular coagulation (DIC) is a life-threatening disease that can cause organ failure or bleeding. It is caused by various underlying conditions, such as infection, malignant tumors or leukemia, and treating these diseases is considered to be the cornerstone of DIC treatment (1). In contrast, chronic DIC is often associated with an aortic aneurysm or malignant tumors (2). Moreover, chronic DIC is a late-stage complication of decompensated cirrhosis; however, no effective methods of controlling or curing this type of chronic DIC have been reported, because liver dysfunction strongly affects the coagulation state of these patients.

Recombinant human soluble thrombomodulin (rhsTM) is a promising product, that can significantly improve DIC and alleviate bleeding symptoms, as compared with generic heparin therapy (3, 4). Recent reports demonstrated that rhsTM is effective for treating the DIC caused by infections, malignant tumors and leukemia; however, treatment of chronic DIC caused by decompensated liver cirrhosis using rhsTM has not been reported. We herein present the case of a patient with chronic DIC caused by decompensated liver cirrhosis who was successfully treated with rhsTM.

Case Report

A 70-year-old Japanese man with liver cirrhosis due to hepatitis C was hospitalized with a tendency for bleeding. Interferon treatment had not been previously administered because of thrombocytopenia (approximately 65×10^9/L) caused by the liver cirrhosis. Since 2007, the patient's hepatic edema had been treated with spironolactone. In 2008, gastroscopy revealed mild esophageal varices. Subsequently, in February 2009, expanding purpura appeared with no apparent causes, such as infection, trauma or skin infarction, and the patient was referred to our hospital. A physical ex-

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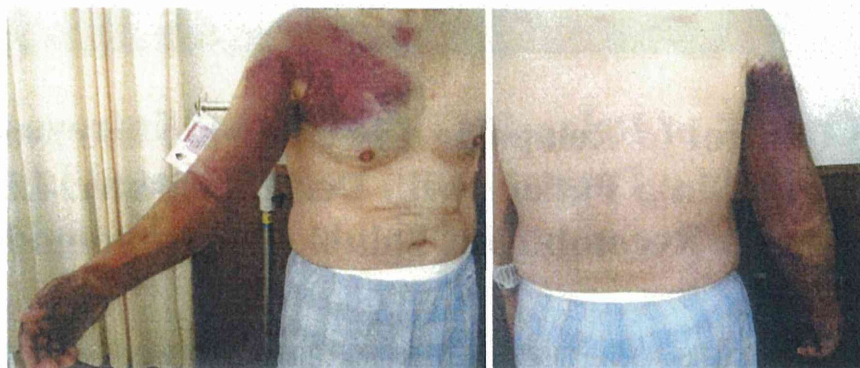

Figure 1. **Images of the patient during the first admission. The purpura was present on the entire right arm and, the arm showed pitting edema.**

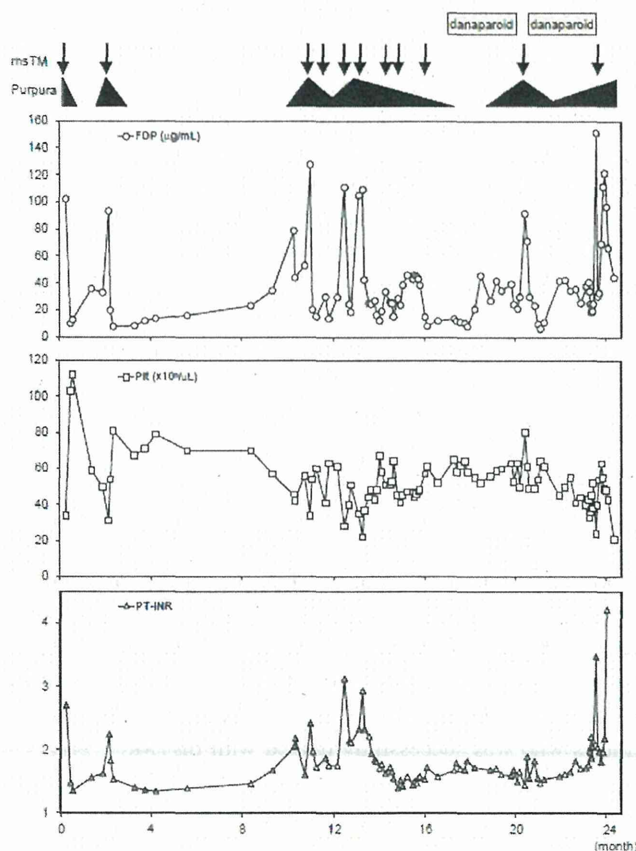

Figure 2. **The entire two year clinical course of rhsTM treatment. The purpura improved after rhsTM treatment; however, it gradually relapsed with a corresponding increase of the FDP levels and PT-INR and decrease of the Plt. The FDP levels, Plt and PT-INR rapidly improved with the administration of each of the 11 courses of rhsTM. The administration of danaparoid was performed when the purpura or other bleeding symptoms were less severe. FDP: fibrin degradation products, rhsTM: recombinant human soluble thrombomodulin, Plt: platelet count, PT-INR: prothrombin time-international normalized ratio**

amination revealed that the purpura appeared on his entire right arm and some portions of both legs (Fig. 1). The laboratory investigation revealed a markedly decreased platelet count (34×10^9/L) and increased prothrombin time-international normalized ratio (PT-INR; 2.7) and levels of fibrin degradation products (FDP; 102.2 μg/mL) (Table). The diagnosis of DIC was made according to the criteria established by the Japanese Ministry of Health and Welfare (5), and the patient was hospitalized for treatment.

Because the patient had widespread and expanding purpura, anticoagulation therapies, including heparin or similar agents which could worsen his bleeding tendency, were not used. Considering its low potential to cause bleeding (4), we decided to administer rhsTM at a dose of 25,600 U/day (approximately 380 U/kg/day) for seven days. Within a week, the patient's platelet count had recovered to 112×10^9/L, his PT-INR to 1.35 and the FDP levels to 12.6 μg/mL (Fig. 2), thus indicating that this treatment modality was highly effective for his DIC. The purpura had gradually improved and exhibited near-complete resolution within two weeks, and the patient could be discharged. However, five weeks after discharge, another area of purpura appeared on his left lateral chest, and computed tomography (CT) revealed a hematoma within the muscle of the same side (Fig. 3). Moreover, his platelet count had decreased to 31×10^9/L, whereas the PT-INR and FDP levels had increased to 2.24 and 93.3 μg/mL, respectively. Because these findings indicated a recurrence of DIC, he was administered rhsTM treatment again; continuing this treatment for a week significantly improved the hematologic abnormalities (Fig. 2) and the hematoma on the left lateral chest (Fig. 3).

There was no evidence of any of the major causes of DIC, including infection, malignancy and trauma. Laboratory data revealed no deficiency of antithrombin III (AT III) or protein C activity, and the serum anticardiolipin antibody level was 3.0 IU/mL (normal 0.0-9.9 IU/mL) (Table). In addition, the CT image did not show any portal thrombosis or aortic aneurysms. Consequently, liver cirrhosis was diagnosed to be the cause of the chronic DIC.

Six months after the second discharge from the hospital, the purpura and coagulopathy gradually developed again, necessitating another round of treatment for the DIC. The patient was hospitalized and treated with rhsTM for one

Table. Laboratory Data of First Admission

WBC	4190	/μL	T.P.	6.7	g/dL	HBs-Ag	(−)	
RBC	3.18	$\times 10^6$/μL	Alb	3.1	g/dL	HCV-Ab	(+)	
Hb	10.4	g/dL	T.Bil	2.4	mg/dL			
Ht	31.4	%	D.Bil	1.0	mg/dL	AFP	9	ng/mL
PLT	34	$\times 10^9$/L	AST	69	IU/L	PIVKA-II	39	mAU/mL
			ALT	37	IU/L			
PT%	35	%	ALP	309	IU/L	aCL	3.0	IU/mL
PT-INR	2.7		LDH	397	IU/L	β_2GPI	<1.3	IU/mL
APTT	46.8	sec	γGTP	13	IU/L			
Fibrinogen	22	mg/dL	ChE	84	IU/L			
FDP	102.2	μg/mL	BUN	18	mg/dL			
AT-III	53	%	Cr	0.63	mg/dL			
PCA	26	%	Na	142	mEq/L			
TAT	50.1	ng/mL	K	3.7	mEq/L			
PIC	6.1	μg/mL	Cl	105	mEq/L			

RBC, red blood cell count; Ht, hematocrit; PLT, platelet count; PT%, prothrombin time; PT-INR, prothrombin time-international normalized ratio; APTT, activated partial thromboplastin time; FDP, fibrin degradation products; AT-III, anti thrombin III activity; PCA, protein C activity; TAT, thrombin antithrombin complex; PIC, plasmin α2-plasmin inhibitor complex; T.P., total protein; Alb, Albumin; T.Bil, total bilirubin; D.Bil, direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactose dehydrogenase; γGTP, γ-glutamyl transpeptidase; ChE, cholinesterase; BUN, blood urea nitrogen; Cr, creatinine; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus antibody; AFP, α fetoprotein; PIVKA-II, protein induced by Vitamin K absence or antagonists-II; aCL, anticardiolipin antibody; β_2GPI, anti-β_2 glycoprotein I

week. Following a brief remission of a couple of weeks, the patient experienced another purpura relapse along with a decrease in the platelet count (45×10^9/L) and an increase in the PT-INR and FDP levels (2.05 and 78.8 μg/mL, respectively), which necessitated treatment for the DIC. We attempted outpatient maintenance therapy for coagulopathy using danaparoid (1,250 U/day), but this resulted in the worsening of the hematological parameters for four months. Because of the gradual expansion of purpuric lesions, discontinuation of danaparoid and the administration of rhsTM were necessary.

The patient eventually died of hepatic failure. However, for two years from the onset of his first bleeding episode, he received 11 courses of rhsTM, which were highly effective and did not his worsen bleeding tendency.

Discussion

We herein described the treatment of a patient with liver cirrhosis who had recurrent DIC using rhsTM. To the best of our knowledge, this is the first case in which rhsTM was repeatedly administered, and which resulted in remarkable improvement of the chronic DIC caused by severe liver cirrhosis.

Coagulopathy frequently occurs in patients with chronic liver disease, particularly in those with end-stage liver cirrhosis; this can be attributed to the fragile nature of the coagulation system, which results from the reduction of procoagulants and anticoagulants (6). As the Child-Pugh score of patients with cirrhosis becomes greater, the levels of anticoagulants such as protein C decrease, and those of procoagulants, such as factor VIII, increase (7, 8). However, recent studies have revealed that increased thrombin generation contributes to the coagulopathy in patients with chronic liver disease (7, 9). Thrombomodulin is a protein that activates protein C, and the activated protein C inhibits the activated forms of factor VIII, and this consequently inhibits the thrombin formation (10). Patients with liver cirrhosis usually demonstrate prolonged PT-INR and decreased AT III. The anticoagulant effect of rhsTM is not influenced by the plasma level of AT III (11). This patient showed a prolonged PT-INR and decreased AT III (approximately 50% of normal), but rhsTM was effective without requiring the administration of fresh-frozen plasma and AT III concentrate. The plasma level of TM is sometimes increased due to damage to the sinusoidal endothelial cells in patients with liver cirrhosis. However, this increased plasma level of TM has low activity. The activity of TM on endothelial cells is decreased due to dysfunction of endothelial cells in patients with liver cirrhosis. Therefore, it is reasonable to administer rhsTM, which has the same activity as native TM, to patients with liver cirrhosis. Because the plasma activity of AT III is usually decreased in patients with liver cirrhosis and liver failure, treatment with anticoagulant agents such as heparin, whose effect is dependent on AT III, may cause a further decrease in the plasma activity of AT III and worsen the DIC (12). Therefore, anticoagulant agents such as rhsTM and recombinant tissue factor pathway inhibitor (TFPI), whose effects are independent of the activity of AT III, should be used to treat patients with DIC caused by liver cirrhosis and failure. Of note, only minimal expressions of TM and TFPI are observed in hepatic sinusoidal endothelial cells compared with those in endothelial cells of other

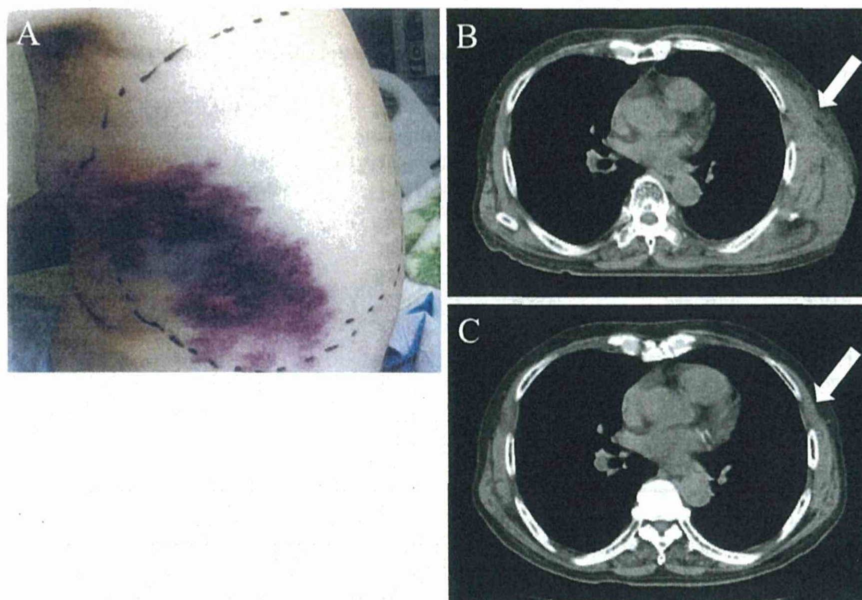

Figure 3. **Images obtained from the physical examination and the CT images obtained during the second admission. The purpura appeared on the left lateral chest (A). A CT image of the left lateral chest showed hematoma within the muscle layer (arrow) (B). A CT image taken eight months later showed an improvement of the hematoma (C) (arrow).**

organs (13). The expression levels of TM and TFPI increase due to the capillarization in the hepatic sinusoid in the cirrhotic liver. However, these changes in expression in the cirrhotic liver are smaller than those that occur in other organ (14). In addition, rhsTM has anti-inflammatory activity via the activation of protein C, which inhibits high mobility group box 1 (15) and lipopolysaccharide [LPS (16)]. The plasma level of LPS has been reported to increase in patients with liver cirrhosis (17). Therefore, it is reasonable to use rhsTM to treat DIC caused by liver cirrhosis. The Child-Pugh score of our patient before the appearance of the initial bleeding symptoms was grade B; however, after the purpura relapse, it became grade C because of the worsening of his bilirubin or albumin levels due to the deterioration of his hepatic functional reserve. However, repeated treatments with rhsTM were effective, and antibodies against rhsTM were not detected, indicating that, regardless of the Child-Pugh score, rhsTM can be an effective and safe treatment for DIC in patients with liver cirrhosis. The anticoagulant effect of rhsTM is influenced by the plasma level of protein C (being especially effective when the level is >10% of the normal level) (11). The plasma level of protein C in this patient decreased to 19-32% before the initiation of the administration of rhsTM, and the administration of rhsTM improved the DIC without the need for additional administration of fresh-frozen plasma. In patients with severe renal failure or hemodialysis due to renal excretion of rhsTM, it is advisable to decrease the dose administered by one-third. In this case, a full dose of rhsTM was regularly administered, because there was no evidence of renal dysfunction due to hepatorenal syndrome or multiple organ failure due to DIC before or during the administration of rhsTM.

In a phase III clinical trial, no pharmacokinetic difference in rhsTM was observed in patients with hepatic dysfunction (4). However, one should be careful while administering rhsTM to these patients, because their general condition could easily worsen. We administered 11 courses of rhsTM (85 days) to our patient under sufficient informed consent, and no side effects such as worsening of the bleeding, hematuria or proteinuria were observed.

Taken together, our case findings suggest that rhsTM can be repeatedly and safely administered to patients with liver cirrhosis. Repeated administration of rhsTM did not exacerbate the bleeding symptoms, nor did it negatively affect the renal functions or cause other organ dysfunctions. These may be considered as positive prognostic factors for a patient. Therefore, it is especially promising that during the treatment of DIC using rhsTM, we could control the bleeding symptoms in a patient with severely reduced hepatic reserve for two years without any apparent side effects.

Our case suggests that rhsTM treatment can sufficiently control the chronic DIC caused by coagulopathy in patients with end-stage liver cirrhosis, and that it may also be able to serve as a bridge to subsequent liver transplantation. However, further studies in a large number of patients with cirrhosis are required.

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

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ORIGINAL ARTICLE

Hepcidin production in response to iron is controlled by monocyte-derived humoral factors

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Abstract Hepcidin, which is mainly produced by the liver, is the key regulator in iron homeostasis. Hepcidin expression is up-regulated by iron loading in vivo, but the mechanism underlying this process is not completely understood. In the present study, we investigated the mechanism, following the hypothesis that hepcidin production in response to iron loading is regulated by extra-hepatic iron sensors. We measured serum hepcidin concentrations and iron indices in Wistar rats treated with saccharated ferric oxide (SFO). Human hepatoma-derived HepG2 cells were stimulated using SFO-administered rat sera, and co-cultured with rat spleen cells, human monocyte-derived THP-1 cells, or human monocytes with diferric transferrin (holo-Tf), and hepcidin concentrations in the conditioned media were measured. SFO elevated rat serum hepcidin concentrations. SFO-treated rat sera increased hepcidin production from HepG2 cells, and this induction correlated with serum hepcidin levels, but not with iron indices. Holo-Tf up-regulated hepcidin concentrations in media from HepG2 cells co-cultured with rat spleen cells, THP-1 cells, or human monocytes with or without cell-to-cell contacts, while holo-Tf did not up-regulate hepcidin from HepG2 cells alone. Our results suggest the existence of humoral factors capable of inducing hepcidin production that are secreted by extra-hepatic cells, such as reticuloendothelial monocytes, in response to iron.

Keywords Hepcidin · Iron · Monocyte · Extra-hepatic iron sensor

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Introduction

Iron is an essential metal for hemoglobin synthesis, many oxidation–reduction reactions, cellular proliferation, and more, while excess iron accumulation may cause organ dysfunction through the production of reactive oxygen species (ROS) and redox reactions. Iron is strictly conserved by recovering and recycling about 20 mg/day of iron from hemoglobin of senescent red blood cells. Dietary iron is absorbed predominantly in the duodenum to replace small daily losses of about 1–2 mg/day. The absorption of iron is tightly regulated by several factors including hepcidin [1]. Hepcidin is a 25 amino acid peptide hormone mainly produced by the liver, and it is thought to be the key regulator in iron homeostasis. Hepcidin is produced as precursor protein which undergoes proteolytic processing resulting in the active 25 amino acid protein [2–4].

Hepcidin regulates intestinal iron absorption and iron release from reticuloendothelial cells by causing the internalization and degradation of the cellular iron exporter ferroportin [5]. Hepcidin is involved in various disorders, such as the anemia of chronic disease (ACD), in which inflammatory cytokines such as interleukin (IL)-6 and IL-1β up-regulate hepcidin expression and thus cause iron-deficiency anemia [6, 7].

The regulatory mechanism of hepcidin production is complicated and still under investigation. Hepcidin transcription is regulated by stimuli such as inflammation, erythropoietic activity, and iron loading. Inflammation has a potent effect on iron homeostasis, reducing intestinal iron absorption, sequestering iron in macrophages, and thereby decreasing serum iron levels. The stimulatory effect of IL-6 on hepcidin is transcriptional and depends on a signal transducer and activator of transcription (STAT) 3 interactions with a STAT3-binding element in the hepcidin promoter [8, 9]. The erythroid regulator pathway also has a strong effect on hepcidin expression [10]. Several groups reported that the administration of erythropoietin (EPO) decreased urinary hepcidin or circulating hepcidin levels in healthy volunteers, patients with chronic kidney diseases (CKD), and patients on hemodialysis (HD) [11–13]. There is evidence in a mouse study that bone marrow cells are involved in suppression of hepcidin after EPO treatment [14–16], but the molecular events are not yet clear. Patients with β-thalassemia have ineffective erythropoiesis, and this is involved with iron overload resulting from increased gastrointestinal iron absorption due to low hepcidin levels. The molecule responsible for hepcidin down-regulation in β-thalassemia was identified as growth differentiation factor (GDF)-15, a transforming growth factor (TGF)-β super family, but GDF-15 is not responsible for physiologic hepcidin regulation [11, 12, 17].

Hepcidin production is regulated by iron levels in the body. The mechanism of this regulation has been very difficult to determine and is under investigation currently. In humans, oral administration of iron increases urinary hepcidin excretion [18], and in a mouse model hepcidin-1 mRNA expression was induced by iron loading [19]. Although hepcidin induction after in vivo iron loading has been observed, inconsistent findings have been reported in experiments in vitro. Addition of diferric transferrin (holo-Tf) did not up-regulate hepcidin production or hepcidin mRNA expression in hepatoma-derived cell lines or primary hepatocytes [18, 20]. Limitations of these experiments weaken their significance. For example, transcriptional experiments would not be expected to cause active hepcidin production because of the complicated processing pathway. The mechanisms by which hepatocytes sense iron and control hepcidin expression are not completely understood, although a recent report suggests that epithelial cells of the small intestine may be one of the iron sensors [21]. Bone morphogenetic protein (BMP) 6, the endogenous regulator of hepcidin expression [22, 23], has been reported to be expressed by small intestinal cells in response to iron loading and induce hepcidin production in the liver, but other groups insisted that iron overload induces BMP6 expression in the liver but not in the duodenum [24]. It seems likely that small intestinal enterocytes sense iron and regulate hepatic hepcidin production because the small intestine is the only organ which absorbs iron, but this hypothesis is controversial. Another possibility is that extra-hepatic cells that store iron, such as reticuloendothelial cells, sense body iron status and regulate hepatic hepcidin production.

We, therefore, hypothesized that sensors to detect body iron status are located in extra-hepatic sites and these sensors mediate hepatic hepcidin production to maintain iron homeostasis.

To test the hypothesis, we developed a quantitative method for measuring levels of hepcidin concentrations in rat serum and culture media of human cell lines by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS). These methods were used to investigate the mechanism of hepcidin production in response to iron in iron-loaded rats and cultured human hepatoma-derived cells and monocyte-derived cells in vitro.

Materials and methods

Hepcidin standards

Human hepcidin-25 was obtained from the Peptide Institute, Inc. (Osaka, Japan). Rat hepcidin and [$^{13}C_{18}$, $^{15}N_3$]-human hepcidin were synthesized at the Peptide Institute, Inc.

Chemicals and antibodies

Holo-Tf was purchased from R&D Systems (Minneapolis, MN, USA). Human IL-6 was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Japan Bioserum Co., Ltd. (Hiroshima, Japan). Minimum essential medium eagle (E-MEM), L-glutamine, sodium bicarbonate, and albumin solution from bovine serum (BSA) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Penicillin streptomycin solution, sodium pyruvate, and non-essential amino acids (NEAA) were provided by Life Technologies Corporation (Carlsbad, CA, USA). Saccharated ferric oxide (SFO) was from Nichi-Iko Pharmaceutical Co., Ltd. (Toyama, Japan). Otsuka glucose injection 10 % was from

Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Tocilizumab, humanized anti-human IL-6 receptor antibody [25], was produced by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade.

Animals

Nine-week-old male Wistar rats were purchased from Japan SLC., Inc. (Shizuoka, Japan). All animals were allowed to acclimatize and recover from shipping-related stress for 1 week prior to the study. Rats were fed rodent chow and water ad libitum. All studies were approved by our Institutional Animal Care and Use Committee and conducted according to guidelines for the care and use of laboratory animals from Chugai Pharmaceutical Co., Ltd.

Animal treatment

SFO was diluted in Otsuka glucose injection 10 % used as a vehicle in appropriate concentrations. Two or 5 mg/kg of SFO or equal volumes of vehicle was administered intravenously into rats which were sacrificed 1, 3, 6, 12, 18, 24 and 30 h after SFO injection. Three rats from each group were used.

Specimen collection

Rats were anesthetized with isoflurane and blood was collected into evacuated blood-collecting tubes (TERUMO Corporation, Tokyo, Japan), and serum was isolated according to the manufacturer's instructions. Spleen cells were isolated from Wistar rats.

Measurement of iron indices

Serum iron was measured using TBA-120FR biochemistry automatic analyzer (Toshiba Medical Systems, Tochigi, Japan). Non-transferrin bound iron (NTBI) was determined by metal-free high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA) [26].

Cell cultures

Human hepatocellular carcinoma cell line, HepG2 and human monocytic cell line, THP-1 were obtained from American Type Culture Collection (Manassas, VA, USA). Human peripheral blood monocytes were purchased from Biopredic international (Rennes, France).

HepG2 cells were cultured at 37 °C in a humidified incubator with 5 % CO_2 in E-MEM with 10 % (v/v) FBS supplemented with 0.1 mM NEAA, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. HepG2 cells were seeded at a density of 1.0×10^6 cells/well in 6-well plates (Corning Incorporated, Corning, NY, USA), and the cells were stimulated with 2.4 mg/mL holo-Tf, 20 ng/mL IL-6, or 10 % (v/v) rat serum in 2 mL of growth medium for 48 h. After incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed.

Each treatment was performed in triplicate.

Cell co-culture system

All cells were cultured at 37 °C in a humidified incubator with 5 % CO_2 in E-MEM with 2 % (v/v) FBS and 1 % (v/v) BSA supplemented with 0.1 mM NEAA, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. HepG2 cells were seeded at a density of 1.0×10^6 cells/well in 6-well plates, and the medium in each well was replaced by 2 mL of growth medium containing 1.0×10^6 of normal rat spleen cells, THP-1 cells or human peripheral blood monocytes. To investigate the effect of IL-6 signaling on hepcidin production, cells were incubated with 100 μg/mL tocilizumab. After incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed.

To inhibit cell–cell contact in cell co-culture system, we used cell culture inserts (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). HepG2 cells were seeded at a density of 1.0×10^6 cells/well in 6-well plates, and the medium in each well was replaced by 2 mL of growth medium. HepG2 cells were overlaid by cell culture inserts with additional 1 mL growth medium containing THP-1 cells or not. After incubation, total 3 mL cultured media were collected and human hepcidin concentrations in the media were analyzed.

Each treatment was performed in triplicate.

Sensitive LC/ESI–MS/MS analysis of human and rat hepcidin

In this study, concentration of human hepcidin-25 was specifically determined as previously reported, and that of rat hepcidin with the following modification [27]. Human hepcidin was used as an internal standard to measure rat hepcidin. LC/ESI–MS/MS was performed using an AB SCIEX Triple Quad™ 5500 System (AB SCIEX, Foster City, CA, USA) equipped with prominence $UFLC_{XR}$ systems (Shimadzu corporation, Kyoto, Japan). Analytical chromatography of human and rat hepcidin was performed on a PLRP-S (5 μm, 300 Å, 150 mm × 2.1 mm i.d.; Polymer Laboratories Ltd., Shropshire, UK). Instrument control and data processing were run by Analyst™ software version 1.5.1 (AB SCIEX). Selected reaction monitoring (SRM)