Alteration of mRNA expression of molecules related to iron metabolism in adenine-induced renal failure rats: a possible mechanism of iron deficiency in chronic kidney disease patients on treatment

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Abstract

Background. Recombinant human erythropoietin (rHuEpo) is a definitive treatment for anaemia in chronic kidney disease (CKD). During long-term rHuEpo treatment most patients develop and show persistent iron deficiency in spite of oral iron supplementation. Abnormalities of iron absorption and transport in the duodenum may contribute to this deficiency.

Methods. To investigate changes in iron absorption and transport in CKD and iron deficiency against the background of rHuEpo treatment, we used severely anaemic rats with adenine-induced renal failure (adenine rats) and sham-treated control rats given only the vehicle. After 4 weeks on adenine or the vehicle, the rats were divided into four groups according to whether or not they received rHuEpo for the next 4 weeks: rHuEpo(-)-adenine, rHuEpo(-)-control, rHuEpo(+)-adenine and rHuEpo(+)-control. We evaluated the effects of rHuEpo treatment on iron balance, duodenal mRNA expression of molecules related to iron absorption and transport and hepatic mRNA expression of hepcidin.

Results. Treatment with rHuEpo improved anaemia and induced iron deficiency only in the adenine rats, in whom the expression of mRNAs for ferroportin 1 and hephaestin 1 increased and for divalent metal transporter 1 (DMT1) was unchanged. In contrast, control rats treated with rHuEpo showed no changes. Hepcidin mRNA expression was greater in adenine rats than in control rats.

Conclusions. In the adenine rats, rHuEpo treatment improved renal anaemia and induced persistent iron deficiency. An alteration of mRNA expression of molecules related to iron metabolism in renal insufficiency may be one of the reasons for this iron deficiency.

Keywords: adenine; anaemia; chronic kidney disease; erythropoietin; iron

Introduction

A common complication of chronic kidney disease (CKD) is anaemia, a result primarily of erythropoietin deficiency, and often a contributor to the poor functional status and quality of life of patients with renal insufficiency. Erythropoietin is a 34-kDa glycoprotein that is indispensable for the proliferation, differentiation and survival of erythroid precursor cells [1]. Since the cloning of human erythropoietin gene [2] and the commercial release of recombinant human erythropoietin (rHuEpo), rHuEpo has been a definitive therapy for anaemia in CKD [3,4]. This therapy results in clinical and symptomatic improvements in patients with renal anaemia and improves their quality of life. However, most patients on long-term rHuEpo treatment develop iron deficiency. Interestingly, rHuEpo-treated patients may continue to have iron deficiency even when iron is supplemented orally. Patients with CKD may have an abnormality of iron absorption and transport, which may contribute to iron deficiency when they are treated with rHuEpo.

In mammals, the excretory mechanism for iron is not regulated and body iron levels appear to be regulated primarily at the level of absorption [5,6]. The absorption of ingested iron occurs primarily in the duodenum [7,8]. Whether ingested in food or as nutritional supplement, dietary iron is transported through the apical membrane of enterocytes by the divalent metal transporter 1 (DMT1) [9,10]. It is then exported through the basolateral membrane of enterocytes by ferroportin 1 (FRN1) [11,12]. In addition, hephaestin is also considered a key component of the transport of iron from duodenal enterocytes into circulation [13]. Moreover, identified recently is hepcidin, a key regulator of iron metabolism produced in the liver [14]. However,
in patients with CKD the behaviour of hepcidin and other molecules related to iron absorption and transport remains unexplored.

To investigate these effects and the responses of these components, an animal model that represents renal anaemia as seen in patients with CKD is necessary. Therefore, we focused on rats with adenine-induced renal failure [15]. These rats are considered to be a model of the rapidly progressive type of chronic renal failure, and they exhibit severe anaemia when treated with adenine about 4 weeks [16].

In this study, we (i) examined the progression of adenine-induced renal failure in rats and the development of severe anaemia in them, (ii) used rHuEpo to treat severe anaemia associated with adenine-induced renal failure, (iii) assessed the effects of this treatment and (iv) evaluated the expression of mRNAs for molecules related to iron absorption and transport in the duodenum and of hepcidin mRNA in the liver.

**Subjects and methods**

**Animals and experimental design**

Eight-week-old Wistar male rats (Charles River Japan Inc., Yokohama, Japan) had equal amounts of standard chow. The control group (n = 11) was given a commercial powdered diet (CE-2) containing 1.0% calcium, 1.0% phosphorus, 0.035% iron and 0.0008% copper (Nihon Clea Inc., Tokyo, Japan) and the rats in which renal failure was to be induced (termed ‘adenine rats’; n = 26) were given CE-2 with the addition of 0.75% adenine (Sigma, St Louis, MO, USA) in addition to 1.0% calcium, 1.0% phosphorus, 0.035% iron and 0.0008% copper. After 4 weeks on one or the other of these two diets, the rats were randomized into four groups that continued to receive the above-described CE-2-based adenine-enriched or normal diets together with intraperitoneal injections of either rHuEpo, 50 IU/kg, three times weekly for 4 weeks [rHuEpo(+)-adenine-induced renal failure rats and rHuEpo(+)-control rats] or vehicle [phosphate buffered saline containing 0.01% Tween 20 (pH 7.4)] alone [rHuEpo(−)-adenine-induced renal failure rats and rHuEpo(−)-control rats]. The rHuEpo and vehicle treatments were given for 4 weeks. rHuEpo was purchased from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). Body weight was checked twice weekly throughout the study.

**Blood and biochemical parameters**

At intervals of 2 weeks during the 8-week study, 700 μl blood samples were obtained from the jugular veins of rats under diethyl ether anaesthesia. At the time the animals were killed under diethyl ether anaesthesia, blood samples were taken for biochemical analysis by puncturing the abdominal aorta. Haematocrit and haemoglobin concentrations and red blood cell counts were determined with an F-820 electronic cell counter (Sysmex, Hyogo, Japan). Serum urea nitrogen and creatinine were measured with an autoanlyser (Hitachi 7170; Hitachi Co. Ltd, Tokyo, Japan). Serum interleukin-6 (IL-6) was quantified using enzyme-linked immunosorbent assay kits (Biosource International, Camarillo, CA, USA).

**Determination of iron status**

Serum iron was measured using a Hitachi 7170 autoanalyser (Hitachi Co. Ltd), and serum ferritin and transferrin with enzyme-linked immunosorbent assay kits (Panapharm Laboratories Co. Ltd, Kumamoto, Japan).

**Duodenal and liver tissue sample preparation**

Eight weeks after being started on the adenine diet, rats were killed by inhalation of diethyl ether. The duodenum and liver were quickly removed from the animals, washed in sterile saline solution and immediately frozen in liquid nitrogen for analysing gene expression. All procedures were approved by the Institutional Animal Care and Use Committee and were performed according to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources.

**Duodenal and liver tissue RNA extraction and quantitative real-time PCR analysis**

We evaluated the following: (i) duodenal mRNA expression of the genes for DMT1 [both iron-responsive element (IRE)-containing splice variant of DMT1 (IRE-DMT1) and the non-iron-responsive element-containing splice variant of DMT1 (non-IRE-DMT1)] and of the genes for FRN1 and hephaestin, which are molecules related to duodenal iron absorption and transport and (ii) liver mRNA expression of the genes for hepcidin, which is a key regulator of iron metabolism. Total RNA was extracted from the duodenum or the liver using RNaseasy (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The RNA was dissolved in RNase-free water and stored at −80°C until it was used. Total RNA was reverse-transcribed to evaluate gene expression levels. Equal amounts of total RNA from each sample were converted to cDNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s manual. Real-time quantification of the target genes was performed using a LightCycler® system (Roche Diagnostics, Basel, Switzerland). Reaction volume was 20 μL. Forty cycles of PCR amplification were run at 95°C for 10 s for denaturation, 62°C for 10 s for annealing and 72°C for 7 s for extension. The following respective sense and antisense primers were used: 5’-AAGTCTCTGCTGAGCGAAAGAT-3’ and 5’-TGTCCTAAAATGCCAGTCTG-3’ for IRE-DMT1; 5’-TCTACCTCTGAAACCCGTTG-3’ and 5’-CGTAGCTTTACCCGAATCC-3’ for non-IRE-DMT1; 5’-ACTGGCTACTAGAAAT-3’ and 5’-CTGCTCTCTGTAATTAC-3’ for FRN1; 5’-AAGAGAGCTGTACAACATG-3’ and 5’-CATATCATCCCCTTGCGATATTC-3’ for hephaestin; and 5’-GGACACACACGACGACGACT-3’ and 5’-ATGACACAGAGCCACAGG-3’ for hepcidin. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (housekeeping genes) were LightCycler primer probe sets purchased from Roche Diagnostics. The relative differences in gene expression between groups were expressed using cycle time values. For each individual reaction, the values for the genes of interest were normalized with those of GAPDH and...
Fig. 1. Red blood cell analysis. Rats with adenine-induced renal failure showed anaemia associated with chronic renal failure and a significantly lower red blood cell count (A), haemoglobin (B) and haematocrit (C) than the control rats. rHuEpo treatment completely restored anaemia associated with renal insufficiency in the adenine rats. Data are mean ± SD. Open circles: control rats without rHuEpo treatment (n = 6); closed circles: control rats with rHuEpo treatment (n = 5); open squares: adenine rats without rHuEpo treatment (n = 13); closed squares: adenine rats with rHuEpo treatment (n = 13). *P < 0.01 versus controls.

β-actin for each individual reaction. Each amplification was repeated three times for each different reverse transcription reactions, and the resultant were averaged. Then, the relative differences between control rats and other groups were calculated and expressed as relative increases, setting the value for control rats to 1.00.

Statistical analysis
Results were expressed as mean ± SD. SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, and data were examined by one-way ANOVA followed by Tukey's HSD test. P < 0.05 was considered statistically significant.

Results
Body weight and renal function
Body weight increased steadily in the control groups over the 8-week experimental period. In contrast, the rats with adenine-induced renal failure showed little gain in weight 8 weeks after the start of the study. At the end of the experiment, the average body weight was 441.9 ± 18.3 g in the rHuEpo(−) control rats, 453.4 ± 20.2 g in rHuEpo(+) controls, 408.7 ± 30.0 g in the rHuEpo(−)-adenine-induced renal failure rats and 416.2 ± 25.9 g in the rHuEpo(+) adenine-induced renal failure rats. The average value of serum urea nitrogen concentration was 25.0 ± 2.1 mg/dl in rHuEpo(−) control rats, 24.9 ± 2.7 mg/dl in rHuEpo(+) controls, 94.8 ± 17.2 mg/dl in the rHuEpo(−)-adenine-induced renal failure rats and 89.6 ± 27.6 mg/dl in the rHuEpo(+) adenine-induced renal failure rats. These results are similar to those of a previous report [15]. On the other hand, rHuEpo did not affect renal function in either the control or the adenine-fed group throughout the experimental period. Serum IL-6 levels of all groups were below the detectable range throughout the study.

Red blood cell analysis
The adenine rats showed anaemia associated with chronic renal failure. They had a significantly lower haematocrits, haemoglobin levels and red blood cell counts than the controls (Figure 1). Treatment with rHuEpo improved anaemia associated with renal insufficiency in the adenine rats. In addition, rHuEpo treatment induced increases in haematocrit, haemoglobin and red blood cell count in the control rats (Figure 1).
Iron status

At the end of the study, serum iron levels in the rHuEpo-treated adenine rats were significantly lower than in the other three groups (Figure 2A). Serum ferritin levels in the rats treated with rHuEpo were significantly higher than in the untreated rats (Figure 2B). There were no significant differences in serum transferrin levels between the four groups throughout the rHuEpo treatment period (Figure 2C).

DMT1, FRN1 and hephaestin mRNA expression in the duodenum and hepcidin mRNA expression in the liver

Figure 3 shows gene expressions as determined by quantitative real-time PCR after comparison with the expression of GAPDH as an internal control. Gene expression normalized to the value of β-actin manifested the same tendencies (data not shown). We measured expression of mRNAs for DMT1 (both IRE-DMT1 and non-IRE-DMT1), FRN1 and hephaestin, which are molecules related to iron absorption and transport in the duodenum. The mRNA expression levels of IRE-DMT1 and non-IRE-DMT1, which transport dietary iron through the apical membrane of enterocytes, were not different between the four groups (Figure 3A, B). On the other hand, the mRNA expression of FRN1, which exports iron through the basolateral membrane of enterocytes, was significantly higher in the rats treated with adenine and rHuEpo than in the other three groups (Figure 3C). In addition, quantitative analysis of mRNA for hephaestin, which is also recognized as a key component of iron transport from duodenal enterocytes into the circulation, showed the same tendency (Figure 3D). The hepatic mRNA expression of hepcidin, which is a key regulator of iron metabolism, was significantly higher in the adenine rats than in controls. Moreover, treatment with rHuEpo induced an increase in hepcidin mRNA expression in both control and adenine rats (Figure 3E).

Discussion

In this study, we demonstrated that rats with adenine-induced renal failure exhibit severe anaemia associated with renal failure and that treatment with rHuEpo improves severe anaemia. Our results suggest that rats with adenine-induced renal failure are an ideal animal model for the anaemia associated with renal insufficiency that is seen in humans with chronic renal failure.

CKD patients receiving rHuEpo often develop iron deficiency. In the present study, rHuEpo given to the adenine rats improved anaemia and this was accompanied by decreased serum iron levels. In contrast, the control rats treated with rHuEpo had increase in haematocrit, haemoglobin and red blood cell count with no decrease in serum iron. Because there was no iron deficiency in the adenine rats not receiving rHuEpo, the iron deficiency in renal failure seems to be caused by the treatment with rHuEpo. On the other hand, serum ferritin levels were higher in the rHuEpo-treated group than in the group not treated with rHuEpo. Ferritin is an iron storage compound with a large capacity to store iron, and serum ferritin levels correlate directly with the body’s iron stores. In the present study, data on serum ferritin levels seem to contradict the data on serum iron levels. However, some investigators have reported that, in patients with renal anaemia, the most reliable indicator of the availability of iron to the bone marrow is the saturation level of transferrin [17,18]. Functional iron deficiency may have developed in the subjects of the present study even though serum ferritin concentrations were normal or high. Perhaps serum ferritin levels were not a reliable marker of iron status in the present study—although serum levels of IL-6, which is a marker of inflammation, were below the detectable range. Taken together, our results indicate that the iron deficiency associated with rHuEpo treatment occurred only in rats with adenine-induced renal failure.
This suggests that there may be an abnormality of iron metabolism in renal failure and that this abnormality may contribute to iron deficiency in patients treated with rHuEpo.

Hepcidin, a small peptide synthesized in the liver, has recently been identified as a key regulator of iron metabolism [14]. In our present study, adenine-induced renal failure increased the expression of hepcidin mRNA in the liver. In addition, rHuEpo treatment additively increased the mRNA expression of hepcidin. Although our subjects did not have iron overload and the serum levels of IL-6, a marker of inflammation, were below the detection range, hepcidin mRNA expression and serum ferritin levels were increased in the rHuEpo-treated control and adenine rats. The increase noted in the present study may have occurred if treatment with rHuEpo induced excessive haematopoiesis, which leads to excessive destruction of red blood cells. On the other hand, however, despite the absence of iron overload, the increased expression of hepcidin mRNA in the rats with adenine-induced renal failure may have been involved in the anaemia associated with renal failure.

In mammals, body iron appears to be regulated primarily at the level of absorption [5,6]. Moreover, nutritional iron is absorbed mainly in the duodenum [7,8]. DMT1, FRN1 and hephaestin are molecules related to this process. In our present study, rHuEpo treatment did not change the
expression of these molecules in control rats although the mRNA expression of hepcidin was increased in the rHuEpo-treated control rats. On the other hand, although mRNA expression of DMT1 did not differ significantly between any of the study groups, the expression of mRNAs for FRN1 and hephaestin, which are key participants in iron transport from duodenal enterocytes into circulation, was greater in the group of rats treated with both rHuEpo and adenine than in the other groups. In our study, although hepcidin expression increased in the rHuEpo(+)adenine-treated rats, FRN1 mRNA levels also increased. Canonne-Hergaux have reported that increased FRN1 was seen when mice were fed a low iron diet and that FRN1 is predominantly regulated by systemic signals in response to iron-restricted erythropoiesis [19]. In addition, Chen reported that copper deficiency in vivo restricted erythropoiesis [19]. In addition, Chen reported inantly regulated by systemic signals in response to iron-mice were fed a low iron diet and that FRN1 is predom-

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