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Significance of Content of the Reticulocyte Hemoglobin in the Management of Renal Anemia

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Abstract
Optimal iron level in hemodialysis patients remains a subject of debate. The reticulocyte hemoglobin content (CHr) is believed to reflect the concentration of iron required in the most recent hematopoiesis in the bone marrow. CHr is not influenced by any factors that measure direct hemoglobin (Hb) of reticulocytes and is considered a reliable indicator. The supply of iron for Hb synthesis is regulated by hepcidin-25 (Hep-25). However, CHr and Hep-25 measurements are not covered by the Japanese medical insurance system. Serum ferritin (s-ft) and transferrin saturation (TSAT) are used mainly as indicators of internal iron status. Therefore, we examined the relationships among CHr, s-ft, TSAT, and Hep-25 to determine the optimal iron level. This report presents the clinical significance of CHr, the potential use of CHr for the diagnosis of iron deficiency, and tests for optimal iron level using CHr as performed in our hospital.

Clinical Significance of Reticulocyte Hemoglobin Content

Of the total 3–5 g of iron in the body, 70% is present in the reticuloendothelial system. Iron is used for hemoglobin (Hb) synthesis in the bone marrow at an hourly rate of 0.8–1.0 mg. Because 3–4 mg of iron circulates in the blood, the recycling of iron represents a primary source. Therefore, maintenance of Hb synthesis requires an efficient use of iron to avoid iron deficiency or iron overload.

The hematopoiesis of red blood cells (RBCs) starts with erythropoietin acting on erythroblast precursor cells to aid their differentiation into proerythroblasts. Proerythroblasts finally differentiate into erythroblasts after 4–5 divisions. After 3–5 days of differentiation, erythroblasts undergo enucleation through chromatin condensation and become reticulocytes. Hb synthesis starts at the proerythroblast stage and increasingly accelerates with differentiation into basophile erythroblasts, polychromatophilic erythroblasts, and orthochromatic erythroblasts while Hb is being accumulated. After their production in the bone marrow, the reticulocytes are released 2–3 days later into the peripheral blood. Their

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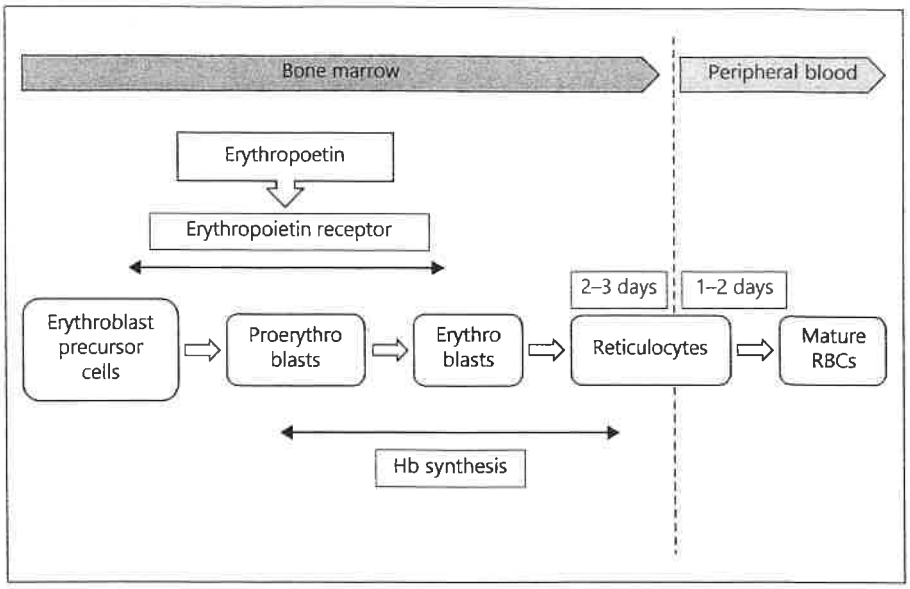

Fig. 1. The hematopoiesis of RBCs. Hb synthesis starts at the proerythroblast stage and increasingly accelerates in erythroblasts. The reticulocytes are released 2–3 days later from the bone marrow into the peripheral blood and differentiate in mature RBCs 1–2 days later. RBCs, red blood cells; Hb, hemoglobin.

reticular components are removed at each of their passage in the spleen in a cycle of 1–2 days to produce mature RBCs (Fig. 1). Because reticulocytes remain in the peripheral blood only for a short period, reticulocyte hemoglobin content (CHr) is believed to accurately reflect the concentration of iron required in the most recent hematopoiesis.

To date, optimal iron level in hemodialysis (HD) patients has been examined with the aim of raising Hb levels and reducing erythropoiesis-stimulating agents (ESAs) [1]. The survival time of mature RBCs is 120 days in healthy people. Although decreased RBC survival has been reported in HD patients, it is difficult to assess hematopoietic efficacy based on Hb in mature RBCs [2]. Therefore, the optimal iron level in hematopoiesis is examined more accurately using CHr as an index rather than using Hb in mature RBCs.

CHr Measurement

CHr is measured by the flow cytometry technique using a laser beam. First, a surfactant is applied to spheroidize and immobilize RBCs without changing their volume. Next, the RNA in the reticulocyte is stained in blue using Oxazin 750. Subsequently, mature RBCs and reticulocytes are distinguished based on the concentration of RNA calculated using absorbance values, and their volumes and Hb levels are calculated using their scattering light measurements. Therefore, as a reticulocyte index, a mean corpuscular volume of reticulocyte and a corpuscular Hb concentration mean of reticulocyte can be directly measured. CHr is a mean value of reticulocyte Hb level, which is calculated based on the cell volume and Hb level of each reticulocyte. These measurements are highly stable, with 20-time repeated measurements resulting in a coefficient of variation of 0.8–1.6% [3], with stable CHr measurements also observed in HD patients [4]. When samples were preserved for a longer time, CHr presented the highest stability: while mean corpuscular volume of reticulocyte and concentration mean of reticulocyte remained stable only for 8 h at room temperature and for 24 h of refrigeration, the results for CHr did not vary after 24 h at room temperature and 72 h of refrigeration [5].

Previous Examinations of CHr in HD Patients

Based on its characteristics to reflect the concentration of iron required in hematopoiesis in real time, CHr has been considered useful for diagnosing absolute, functional iron deficiency. In particular, HD patients suffer from dialysis-related blood loss and use ESA under chronic inflammatory conditions; therefore, they are more likely to present absolute, functional iron deficiency and their diagnosis of iron deficiency is considered crucial. Maximizing the effectiveness of high-cost ESAs being used by HD patients by avoiding iron deficiencies will also help to reduce medical expenses.

Currently, the optimum cutoff values for CHr of iron deficiency in HD patients are determined and examined using a technique wherein the "iron deficiency criteria" are

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established via a ROC curve, which is used to determine both sensitivity and specificity. For the iron deficiency criteria, a few methods have been used, including one in which intravenous chalybeate is administered to observe increased responses in CHr, Hb, and Hct [6–10] and another in which serum ferritin (s-ft) <100 ng/mL and transferrin saturation (TSAT) <20% are used as the criteria [11]. In the intravenous chalybeate administration method, CHr significantly rises at 48 h after the administration and peaks at 96 h in the iron-deficient group, whereas little variability is observed in the iron-sufficient group [9]. These data show that CHr responds to the chalybeate administration in the iron-deficient condition but not once the concentration of iron reaches a certain level. In the present study, wherein we used ADVIA 120 for measurement, the cutoff value was 29–32.4 pg. A sensitivity of 43–100% and a specificity of 80–93.2% have been reported in the literature. These results showed that despite a high specificity, the sensitivity varied in various reports, suggesting that further examination is required. Nevertheless, many of these results were superior to the sensitivity and specificity compared to the s-ft and TSAT tested at the same time, indicating the usefulness of CHr [7, 8, 10, 11].

Examination of the Optimal Iron Level using CHr in HD Patients

CHr has previously been shown to be useful in diagnosing iron deficiency. However, because CHr measurements are not covered by the Japanese medical insurance system, they are not widely used. In this study, we examined the correlation of CHr with s-ft and TSAT, which are routinely used as iron parameters, to express the optimal iron content for hematopoiesis in HD patients using s-ft and TSAT.

Previous reports have shown that CHr is positively correlated with s-ft and TSAT [9, 12], but in iron-sufficient conditions, CHr has been shown to remain unchanged even with chalybeate administration. Therefore, we propose that CHr is positively correlated with s-ft and TSAT up to certain s-ft and TSAT values but is not affected beyond these values. Our aim was to identify the optimum cutoff values for which the relationship of CHr with s-ft and TSAT changes. In addition, we investigated the relationship between CHr and hepcidin-25 (Hep-25) measured at the same time. Iron absorption from the intestinal tract and the iron supply from cells to the blood are regulated by Hep-25 [13]. Because iron in the blood is used for hematopoiesis and is depleted within 4–5 h unless supplied, iron metabolism by Hep-25 is likely regulated during hematopoiesis.

This is a retrospective observational study using the data of 181 HD outpatients receiving recombinant human erythropoietin. All patients provided informed consent authorizing data sampling and analysis at the initiation of dialysis therapy. We measured CHr using ADVIA 120 (Bayer Diagnosis, Tarrytown, NY, USA) and Hep-25 using LC–MS/MS.

To determine optimal cutoff values, correlation coefficients were analyzed using the change sensitivity analysis method. As a result, the optimal cutoff value for s-ft was 50 ng/mL (≤50 ng/mL, $r = 0.47$ vs. >50 ng/mL, $r = 0.22$; F value = 17.64) and that for TSAT was 24% (≤24%, $r = 0.58$ vs. >24%, $r = 0.08$, F value = 34.45). Using these optimum cutoff values, we classified the patients by iron levels to additionally examine the relationship between the number of reticulocytes and CHr. As a result, the CHr in the s-ft >50 ng/mL and TSAT >24% group did not decrease even if the number of reticulocytes increased. Therefore, under the condition of s-ft >50 ng/mL and TSAT >24%, the concentration of iron required in hematopoiesis is likely sufficient even if the number of reticulocytes has increased. In addition, we found that in the relationship between CHr and Hep-25, there were two cutoff values, with optimal Hep-25 cutoff values of 20 ng/mL (≤20 ng/mL, $r = 0.52$ vs. >20 ng/mL, $r = -0.01$; F value = 21.12) and 70 ng/mL (≤70 ng/mL, $r = 0.36$ vs. >70 ng/mL, $r = -0.45$; F value = 24.52). Hep-25 is induced by iron and inflammatory signals [14], but in the patient groups examined in this study, CRP levels were low (median: 0.06 [interquartile range 0.03–0.27] mg/dL), indicating that inflammation was well controlled. Therefore, we believe the Hep-25 level depends on the concentration of iron in many patients. These results suggest that the concentration of iron equivalent to 20 ng/mL of Hep-25 is insufficient for hematopoiesis and that the concentration of iron equivalent to 21–70 mg/mL of Hep-25 is sufficient, and the concentration of iron equivalent to >70 mg/mL develops negative iron metabolism.

Hep-25 and s-ft showed a strong, significant correlation ($r = 0.731$, $R^2 = 0.535$, $p < 0.001$). The s-ft values equivalent to 20 and 70 ng/mL of Hep-25, which were predicted based on an approximate straight line, were 59.7 (95% CI 48.5–70.9) and 155.1 (95% CI 142.6–167.5), respectively.

Conclusions

Currently, iron level in HD patients is managed using s-ft, which is an index of stored iron, and TSAT, which is an index of available iron, in accordance with

relevant guidelines. However, both indices are affected by factors such as inflammatory status, infectious diseases, liver diseases, malignant tumors, and nutritional status. Furthermore, TSAT is subject to a diurnal variation. Therefore, their reliability as indices for iron is debatable [15, 16]. Only CHr directly reflects the concentration of iron required in hematopoiesis. In HD patients, many factors influence iron-related data, such as chronic inflammatory conditions, undernutrition, frequent chalybeate administration, and ESA use. However, CHr levels are almost equal to those of healthy people in the iron-sufficient state [12]; CHr represents a stable marker for the concentration of iron required in hematopoiesis.

The results of the present study suggest that in patients receiving recombinant human erythropoietin, the concentration of iron of Hep-25 >70 ng/mL is the lower limit of iron sufficiency and that negative iron metabolism occurs at this level in hematopoiesis. Because it that been shown that the long-acting ESA uses a greater concentration of iron during hematopoiesis and it suppresses Hep-25 [17, 18], further studies are warranted.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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