Free Radical Research, 2002 Vol. 36 (11), pp. 1155–1161

Reduced Serum Hydroxyl Radical Scavenging Activity in Erythropoietin Therapy Resistant Renal Anemia

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Accepted by Professor N. Taniguchi

(Received 4 June 2002; In revised form 17 June 2002)

Relation between anemia resistant to recombinant human erythropoietin (rHuEPO) therapy and the oxidative stress in hemodialysis (HD) patients was investigated. Stable HD patients who had consistent hemoglobin concentrations on a constant dose of rHuEPO were studied. Patients were excluded if there were factors that might affect hemopoiesis or administration of antioxidant supplements. Patients were classified into three groups: High (9000 U/week), Low (1500–4500 U/week) and No rHuEPO group. Thiobarbituric acid reactive substances (TBARS) of sera and erythrocyte were examined. Serum superoxide and hydroxyl radical scavenging activities were measured using electron spin resonance.

TBARS in the erythrocyte was higher in High rHuEPO group compared with No rHuEPO group, though the serum TBARS were similar. A diminution of serum hydroxyl radical scavenging activity was observed in High rHuEPO group. Hydroxyl radical signal intensity showed a strong correlation with the serum ferritin in High rHuEPO group, although ferritin concentrations were not different among the 3 groups. Superoxide scavenging activity showed no differences. These results indicate that increased lipid peroxidation in erythrocyte, raised by decreased serum hydroxyl radical scavenging activity, is one cause of rHuEPO resistant anemia. Serum ferritin may be involved in this hydroxyl radical production.

Keywords: Hemodialysis; Lipid peroxidation; Hydroxyl radical; Erythropoietin; Renal anemia

INTRODUCTION

Since the advent of human recombinant erythropoietin (rHuEPO) therapy, the management of renal anemia has greatly improved.[1,2] However, there are large individual differences in the doses of rHuEPO given, even to patients on the same hemodialysis (HD) regimen, and some patients show a strong resistance to rHuEPO treatment. Long-standing anemia in HD patients is associated with an increased risk of cardiovascular complications as well as reducing their quality of life.[3] Many factors including chronic inflammation, iron deficiency, hyperparathyroidism, vitamin deficiency, aluminum toxicity and usage of particular medication such as angiotensin converting enzyme inhibitors, are known to be causes of this rHuEPO resistance.[4] However, in some patients no satisfactory explanation can be found.

HD patients are exposed to high oxidative stress.[5–8] This high oxidative status deeply concerned to cardiovascular complications in HD patients,[7] and a control of oxidative stress now becomes an important strategy to improve their mortality rate.[8] In consideration of anemia, the oxidative denaturation of hemoglobin or the modification of erythrocyte membrane promotes erythrophagocytosis.[9] Therefore, oxidation of erythrocyte membrane lipid bilayer may lead to anemia.

In this study, we propose that the increased oxidative stress is a cause of rHuEPO resistant renal anemia, i.e. patients who require high dose rHuEPO to keep a target hemoglobin concentration show increased erythrocyte lipid peroxidation. To investigate this hypothesis, we have examined serum and

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/1071576021000016418

erythrocyte membrane lipid peroxidative products in hemoglobin and rHuEPO dose matched HD patients, and analyzed the relationship between rHuEPO requirement dose and serum and erythrocyte oxidative stress. Since many factors affect hemoglobin concentration, we selected stable HD patients who showed constant hemoglobin concentration with constant dose rHuEPO. As markers of lipid peroxidation, we have measured thiobarbituric acid reactive substances (TBARS) as a lipid peroxidative product. In addition, we tried to identify the reactive oxygen species (ROS), which increases lipid peroxidation. For this investigation, we measured the $^{\bullet}OH$ and superoxide $(O_2^{\bullet -})$ scavenging activities of sera from these patients using electron spin resonance (ESR), which was a direct way to measure a scavenging activity to specific ROS.

PATIENTS AND METHODS

Subjects

To close up the participation of oxidative stress among many factors to affect to rHuEPO resistant anemia, patients who satisfied the following conditions were subjected to this study. We selected stable HD patients who showed stable hemoglobin concentration and were treated with constant dose of rHuEPO (0–9000 units/week) for more than 3 months. The hemoglobin levels of patients were varied from 8.5 to 13.0 g/dl, however, the changes of hemoglobin concentrations were limited within ±12% in this 3 months. The patients were treated with different doses of rHuEPO, yet this dose was constant and no changes of rHuEPO dose were made in the 3 months. All of these patients had been on HD for more than 1 year, were in a stable condition, and had no history of inflammation or hemorrhagic disorders in this 3 months period. The causes of end stage renal failure of these patients were chronic glomerulonephritis, polycystic kidney, nephrosclerosis and diabetic nephropathy. Patients who suffered from chronic inflammation such as lupus erythematosus were excluded. Oral or venous iron, or antioxidative drugs such as Vitamin E or eicosapentaenoic acid, were not administrated to the patients. All patients were dialyzed with polysulfone membranes. Total 29 patients of about 400 patients from 3 different dialysis centers satisfied these conditions and subjected to the study. Informed consent was obtained from all patients subjected to this study.

Classification

Patients were classified into 3 groups according to their weekly rHuEPO dose. Nine patients with weekly dose of 9000 units rHuEPO were grouped as High rHuEPO group. Ten patients with weekly dose of 1500–4500 units rHuEPO were grouped as Low rHuEPO group. Ten patients received no rHuEPO treatment classified as No rHuEPO group.

Regents

The spin trapping reagent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Labotec Company (Tokyo, Japan), and xanthine oxidase (from cow milk) from Boehringer-Mannheim Company (Indianapolis, IN). Methanol, n-butanol and pyridine were purchased from Wako Chemical (Tokyo, Japan). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and were of analytical grade.

Sampling

Samples were collected, before HD sessions, into heparinized syringes and separated into serum and erythrocytes by centrifugation. Butylated hydroxytoluen (BHT) of 0.5 μg/ml was added to serum samples. Erythrocyte samples were washed three times with normal saline with BHT and kept at −80°C. All samples were analyzed within 2 weeks.

TBARS Measurement

Serum and erythrocyte TBARS were measured by the method previously described[10] with minor modifications. For erythrocytes, after hemolysis with ice-cold distilled water, samples were mixed with 9 times of sample weight of 1.15% potassium chloride solution and homogenized on ice. Both serum and erythrocyte samples were added with BHT to avoid autooxidation when exposed to high temperature.

ESR Measurement of Hydroxyl Radical Scavenging Activity

$^{\bullet}OH$ scavenging activity was measured by the described method[11] using JEOL TE-25X ESR equipment (JEOL, Tokyo, Japan). DMPO was used for a spin trapping reagent in both $^{\bullet}OH$ and $O_2^{\bullet -}$ scavenging study. ESR measurements were made with the following conditions: magnetic field: 335.6 ± 5 mT, power: 4 mW, modulation frequency: 100 kHz, modulation amplitude: 0.1 mT, time constant: 0.03 sec, gain: × 250 and the sweep time: 1 min. A measured amount of 20μl of serum sample with 50 μl of 50 mM phosphate buffer pH 7.4, 50 μl of 0.1 mM diethylene triaminepenta acetic acid (DETAPAC), 20 μl of 50 μM $FeSO_4$ (dissolved in distilled water) and 20 μl of 0.1 M DMPO were mixed. To start $^{\bullet}OH$ production, 40 μl of H_2O_2 was added and the mixture was put into a quartz flat cell (130 μl of capacity). ESR spectrum of DMPO–OH was recorded

exactly 45 sec after mixing. The $^{\bullet}OH$ relative intensities (OHRI) were determined by the relative height of the first peak of 4-line DMPO–OH signal to the peak of MnO internal standard signal.

ESR Measurement of Superoxide Anion Scavenging Activity

Serum $O_2^{\bullet -}$ scavenging activity was determined by the method previously described.[11] ESR condition was same as $^{\bullet}OH$ experiments except modulation amplitude, 0.079 mT. $O_2^{\bullet -}$ was generated using xanthine oxidase (XOD)-hypoxanthine (HOX) system. A measured amount of 50 μl of serum sample with 50 μl of 2 mM hypoxanthine, 35 μl of 0.1 mM DETAPAC, 35 μl of 9.2 M DMPO was mixed with 50 μl of 0.4 U/ml XOD. The mixture was put into a quartz flat cell and ESR spectrum of DMPO–OOH was recorded exactly 30 sec after mixing. Serum $O_2^{\bullet -}$ scavenging activity was converted into superoxide dismutase (SOD) equivalent activity.[11] SOD dissolved in 100 mM phosphate buffer pH 7.4 for 0–50 U/ml as the final concentration was used for the standard.

Statistical Analysis

Statistical analysis was performed using Statview 5.1 computer software (Abacus Concepts Incorporation, USA). Data are presented as the mean ± SD and analyzed by Student's *t*-test. In correlation studies, Pearson's correlation coefficient was calculated.

RESULTS

Clinical Data of the Patients

Clinical data of the patients are shown in Table I. No significant differences in age, hemoglobin concentration, hematocrit value, total protein and albumin concentration were observed among the 3 groups. The duration of HD was significantly longer in No rHuEPO group patients. Serum total cholesterol, triglyceride and low density lipoprotein levels of the 3 patients groups also showed no significant differences. Serum ferritin level in No rHuEPO group was 52.8 ± 23.8 ng/ml (mean ± SEM) and was not significantly different with that of Low rHuEPO group 62.8 ± 50.2 ng/ml and High rHuEPO group 46.2 ± 19.1 ng/ml. Serum iron concentrations also showed no differences. Despite the differences of administered rHuEPO doses, serum erythropoietin concentrations were not different among the 3 groups.

Serum and Erythrocyte Lipid Peroxide

Serum concentrations of lipid peroxide are shown in Fig. 1a. TBARS of sera was 6.4 ± 1.6 nmol/ml in No rHuEPO group, 5.6 ± 1.5 nmol/ml in Low rHuEPO group and 5.4 ± 1.1 nmol/ml in High rHuEPO group. This serum lipid peroxidative product showed no significant differences among the 3 groups and no correlation with the age or the duration of HD in this study. There were also no significant differences of TBARS level classified by etiology of end stage renal failure (data not shown). In erythrocyte membrane, TBARS of High rHuEPO group was 142.0 ± 15.3 nmol/gRBC and significantly higher than that of No rHuEPO group, 117.2 ± 16.4 nmol/gRBC ($p < 0.05$, Fig. 1b). Low rHuEPO group showed slightly increased membrane TBARS (124.8 ± 26.1 nmol/gRBC) compared with No rHuEPO group, however, this was not significant. TBARS of erythrocytes showed no correlation with hemoglobin concentration (Fig. 1c).

Hydroxyl Radical Scavenging Activity

$^{\bullet}OH$ produced by the Fenton reaction system gave a typical 4-line DMPO–OH ESR signal which *g* value

TABLE I Clinical parameters of the patients

Group	No	Low	High
Dose of rHuEPO (units/week)	0	1500–4500	9000
N	10	10	9
Age (yrs)	59.5 ± 11.9	65.9 ± 10.5	64.3 ± 7.1
Male/Female	7/3	8/2	8/1
HD Duration (yrs)	10.2 ± 5.8*	2.9 ± 1.5	3.5 ± 2.1
Hb (mg/dl)	10.7 ± 1.6	11.3 ± 1.3	11.4 ± 1.4
Ht (%)	33.3 ± 5.6	32.8 ± 4.0	33.1 ± 4.1
Total protein (g/dl)	6.5 ± 0.5	6.5 ± 0.3	6.8 ± 0.4
Albumin (g/dl)	3.9 ± 0.3	4.0 ± 0.2	4.0 ± 0.2
Total cholesterol (mg/dl)	148.1 ± 24.4	150.9 ± 28.7	138.6 ± 33.6
Triglyceride (mg/dl)	87.8 ± 42.5	102.9 ± 34.0	89.0 ± 40.9
LDL (mg/dl)	70.3 ± 23.2	86.4 ± 24.0	77.1 ± 21.8
Fe (mg/dl)	52.8 ± 23.8	62.8 ± 50.2	46.2 ± 19.1
Ferritin (ng/ml)	70.7 ± 40.3	107.7 ± 66.9	58.1 ± 33.9
Erythropoietin (mU/ml)	18.0 ± 8.2	10.6 ± 3.8	13.7 ± 3.1

All laboratory data were obtained at the start of hemodialysis sessions. *$p < 0.05$ versus No rHuEPO groups.

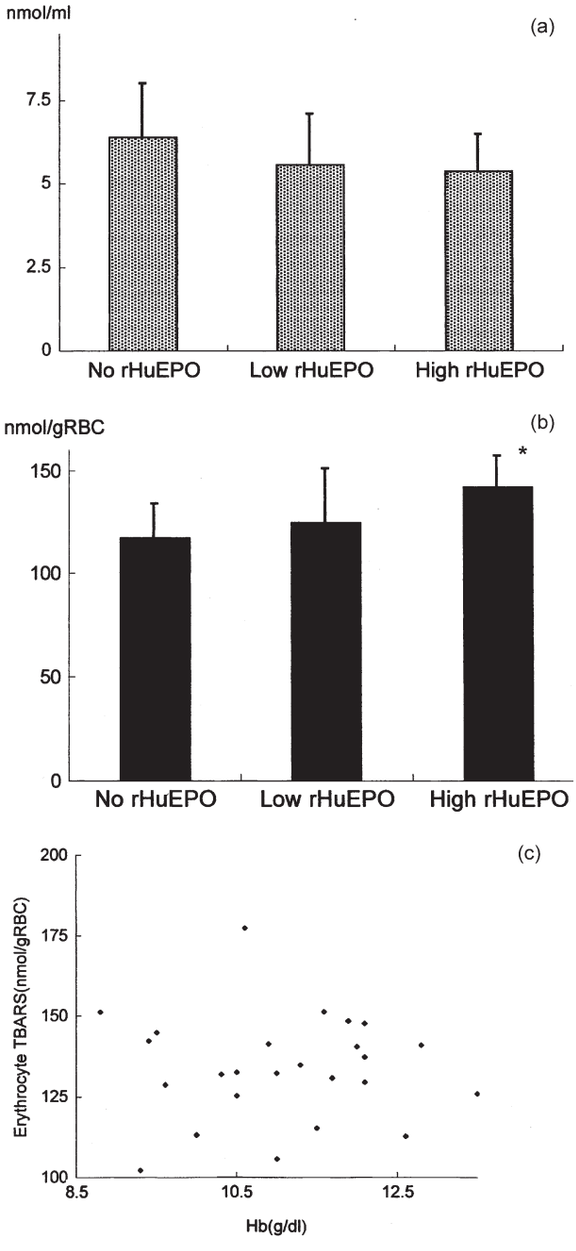

FIGURE 1 TBARS concentration in sera (a) and erythrocyte membrane (b), and correlation between hemoglobin and erythrocyte membrane TBARS (c). All samples were obtained at the start of each HD session. Each value is expressed as mean ± SEM. The difference of erythrocyte membrane TBARS between No rHuEPO and High rHuEPO groups is statistically significant ($p < 0.05$, expressed as * in (b)). No statistical correlation was observed between erythrocyte membrane TBARS concentration and hemoglobin level in (c).

was 2.006 and hyperfine coupling constant (hfc) were $aN\alpha = aH\beta = 1.49\,mT$. This signal was reduced by addition of serum sample. OHRI from sera of the 3 patient groups are shown in Fig. 2. Sera from No rHuEPO group patients mostly depressed OHRI to 88.9 ± 1.8% of serum free blank, which meant the highest $^{\bullet}OH$ scavenging activity.

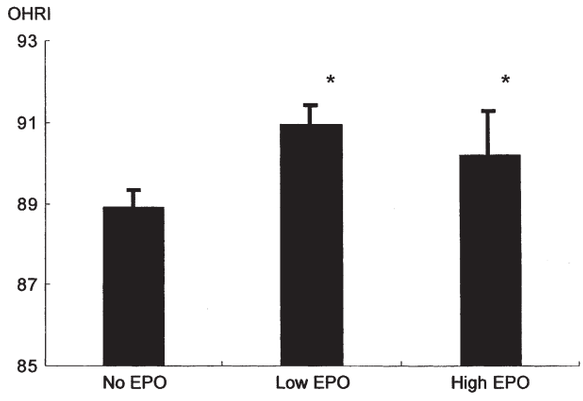

FIGURE 2 Serum hydroxyl radical scavenging activity. The values indicate OH signal intensities (OHRI). OH was generated by Fenton reaction and trapped with DMPO in 10% serum. Note that the lowest OHRI in No rHuEPO group indicates the highest OH scavenging activity. Each value is the mean ± SEM. The difference between No rHuEPO and High rHuEPO groups is statistically significant ($p < 0.05$, expressed as *).

In contrast, OHRI were 91 ± 1.4% in Low rHuEPO group and 90.4 ± 0.8% in High rHuEPO group, respectively, and both showed significant increase compared with No rHuEPO group ($p < 0.05$). OHRI showed a strong positive correlation with serum ferritin level in High rHuEPO group ($r = 0.767$, $p = 0.01$), but not in Low and No rHuEPO group (Fig. 3A–C). Serum iron level showed a similar correlation as ferritin though it was not significant (data not shown). Serum ferritin or iron level showed no significant difference among the 3 groups.

Superoxide Radical Scavenging Activity

Addition of DMPO to XOD-HOX generated $O_2^{\bullet -}$ to produce an ESR signal which g value was 2.006 and hfc were $aN\alpha = 1.39\,mT$, $aH\beta = 1.158\,mT$ and $aH\gamma = 0.121\,mT$, determined as DMPO–OOH. Sera from the patients reduced this signal intensity. $O_2^{\bullet -}$ scavenging activity expressed as SOD equivalent were shown in Fig. 4. Sera from No rHuEPO group patients had 4.85 ± 0.45 U/ml of SOD equivalent activity and showed no difference with that of Low rHuEPO (5.6 ± 1.1 U/ml) or High rHuEPO group (4.7 ± 0.4 U/ml). Contrast to $^{\bullet}OH$, $O_2^{\bullet -}$ scavenging activity had no relations with serum ferritin or iron (Fig. 5A–C).

DISCUSSION

In this study, we have proposed that lipid peroxidation of erythrocyte membrane induced by free radical contributes to rHuEPO resistant renal anemia. Our finding of significantly greater concentrations of TBARS in the erythrocyte membrane of the High

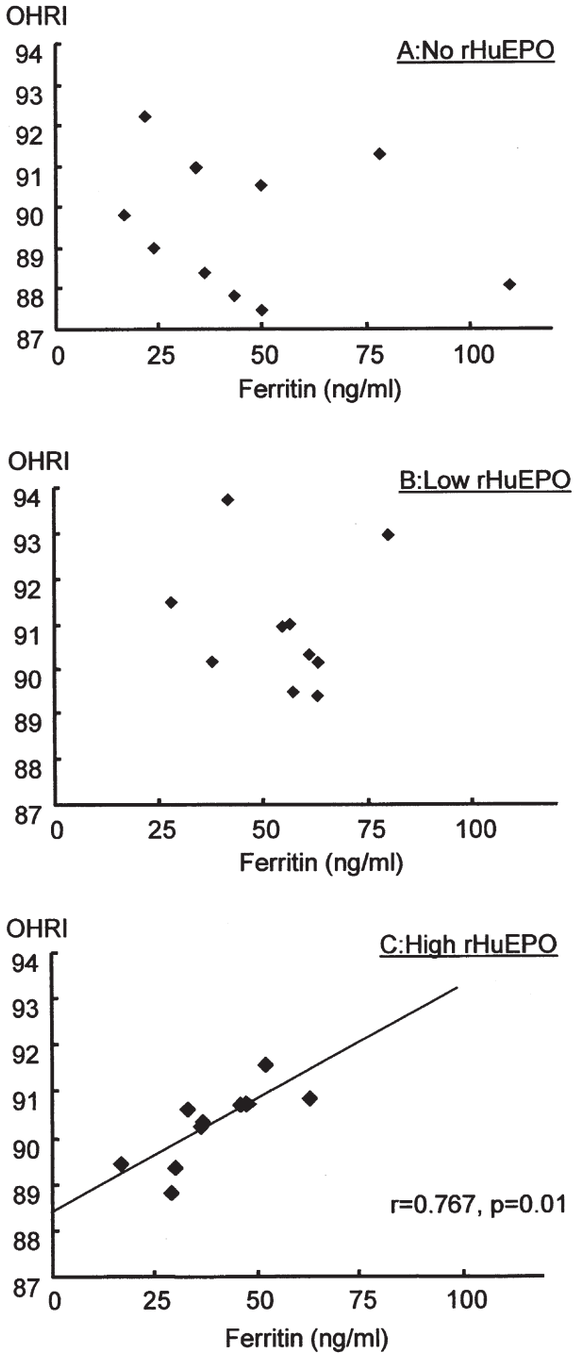

FIGURE 3 Correlation between serum ferritin level and hydroxyl radical signal intensity in No rHuEPO group (A), Low rHuEPO group (B) and High rHuEPO group (C). All samples were obtained at the start of each HD session. Significant correlation with serum ferritin level was found only in High rHuEPO group ($r = 0.767$, $p < 0.01$).

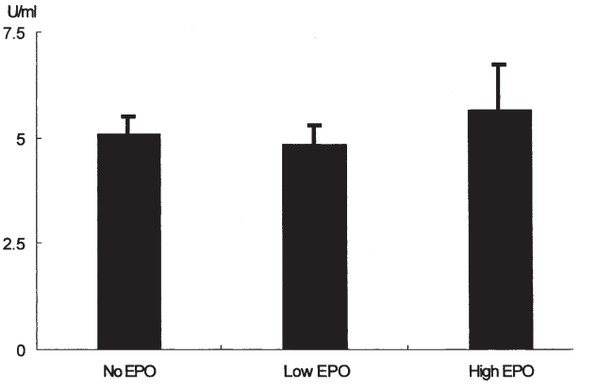

FIGURE 4 Serum superoxide anion scavenging activity. $O_2^{\bullet -}$ was generated by xanthine oxidase-hypoxanthine system and trapped with DMPO. The activity was expressed as SOD equivalent values (U/ml, see details in "Method" section). Each value is the mean ± SEM. No statistical differences were observed among the 3 groups.

rHuEPO group compared with No rHuEPO group supports this hypothesis. Many factors which cause rHuEPO resistant renal anemia are already known, and several reports already detected the relation between renal anemia and oxidative stress.[12,13] Sommerburg *et al.* suggested that renal anemia itself might cause radical generation, and the increase in the number of red blood cells might decrease oxidative stress.[12] Gallucci *et al.* reported an increase of erythrocyte membrane TBARS and an inverse correlation between hemoglobin concentration and erythrocyte TBARS.[13] These studies are done with a wide range of hemoglobin concentration or erythrocyte counts, and their rHuEPO dose was not kept stable to keep same hemoglobin concentration. Our study was performed during a stable period while patients had a constant range of hemoglobin concentration, not with hemoglobin increasing (improving anemia) period by rHuEPO, because we tried to extract the effect of oxidative stress on renal anemia among many factors to affect hemoglobin concentration. This strict condition turned out to the relatively small number of patients in every classified group, however, in consequence allowed us to draw out lipid peroxidative effect on erythrocyte membrane more specifically.

Because of well-developed antioxidant mechanism, lipid peroxides in healthy subjects are usually low. In contrast, uremic patients have higher levels of lipid peroxides than healthy subjects do.[6] In our current study, although serum TBARS of the HD patients showed higher level than that of healthy subjects, there were no differences in the 3 groups according to rHuEPO dose. On the other hand, in High rHuEPO group, i.e. most rHuEPO resistant group, increased lipid peroxide was found in erythrocyte membranes. These results indicate that increase of lipid peroxidation in erythrocyte membrane but not in sera, is an essential cause of oxidative stress related rHuEPO resistant anemia.

In the next step, using ESR technique, we investigated ROS that might cause this membrane lipid peroxidation. $^{\bullet}OH$ is known as an initiator of lipid peroxidation. Lipid peroxidation requires both

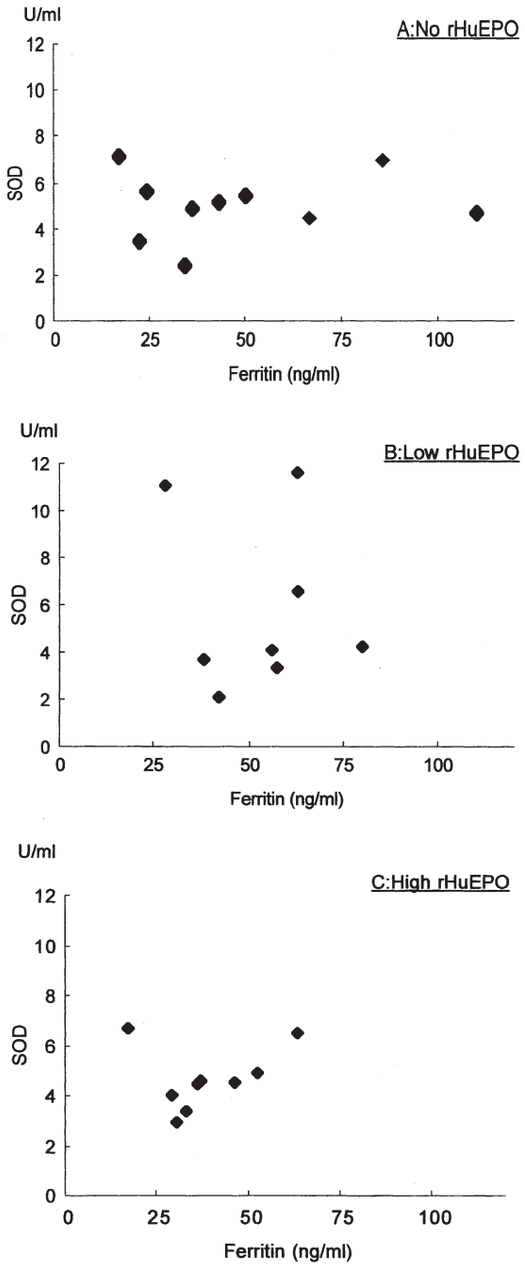

FIGURE 5 Correlation between serum ferritin level and superoxide anion scavenging activity in No rHuEPO group (A), Low rHuEPO group (B) and High rHuEPO group (C). The activity was expressed as SOD equivalent values. Contrast to OH, no statistically significant correlations were observed in the 3 groups.

ferrous and ferric iron, probably as a dioxygen-iron complex. $O_2^{\bullet -}$ and H_2O_2 are converted to $^{\bullet}OH$ via the Fenton reaction in which chelated iron is used as a catalyst.[14] In the presence of iron, $O_2^{\bullet -}$ reduces ferric iron to ferrous iron, and hydrogen peroxide reacts with this ferrous iron and produces $^{\bullet}OH$. Our results show a diminution of $^{\bullet}OH$ scavenging activity in the High rHuEPO group (but not $O_2^{\bullet -}$ scavenging activity), which suggests that the $^{\bullet}OH$ is the major factor causing increased erythrocyte membrane lipid peroxidation.

By a process to search for the cause of the diminution of $^{\bullet}OH$ scavenging activity, we found that serum ferritin level showed significant correlation with OHRI only in the High rHuEPO group. Ferritin can be a source of mobilisable iron to increase $^{\bullet}OH$ production within erythrocyte membrane during oxidative stress.[15] Our measurement of OHRI is based on the production of $^{\bullet}OH$ via the Fenton reaction. Therefore, our results raise a possibility that patients with rHuEPO resistant anemia, have reduced antioxidant activity against $^{\bullet}OH$ formed by an iron-catalyzed reaction whose source of iron in ferritin, but this activity is preserved in No rHuEPO group. Hydroxyl radical scavenging activity of Low rHuEPO group was reduced, however, there were no relations between ferritin and OHRI in this group. These results may also suggest that $^{\bullet}OH$ producing mechanism linking ferritin is crucial for this oxidative stress related anemia. However, the relation between serum ferritin and "membrane iron compartment" is not clear yet and further investigations are required. In contrast, scavenging activity against $O_2^{\bullet -}$ was not different among the 3 groups and retained in High rHuEPO group. These findings suggest (1) the abnormality of catalyst(s) of $^{\bullet}OH$ producing reaction, but not increase of $O_2^{\bullet -}$, or (2) $^{\bullet}OH$ production via other reactions such as peroxynitrite formation, leads to augmentation of $^{\bullet}OH$ production.

Since the National Kidney Foundation—Dialysis Outcomes Quality Initiative (NKF-DOQI) guidelines have been published,[1] iron repletion by parenteral or oral route is widely recommended.[16] This therapy has major benefits in both patients' care and medical economy,[16] however, in theory intravenous iron injection may increase oxidative stress via the Fenton reaction.[17,18] Our results suggest a potential risk for overproduction of hydroxyl radical after administration of iron in the patients who require high dose rHuEPO to keep a certain hemoglobin level. Thus, administration of iron to the patients who require high dose rHuEPO, or rHuEPO resistant anemia, should be done under constant observation for general complications, or accompanied with proper antioxidant therapies.

Acknowledgements

We thank Professor Guy H. Neild, University College London, and Dr Toshiki Masumizu, JEOL, for their assistance. This study was supported by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture, Japan and Japan Society for the promotion of Science, University of Tsukuba Research Project and the Renal Anemia Foundation, Japan.

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