Original Paper

Associations among Darbepoetin- α , CD34⁺ Cells and Cardiovascular Disease Events in Patients on Hemodialysis

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Key Words

Erythropoiesis-stimulating agent • Cardiovascular disease • Hematopoietic stem cells

Abstract

Background and Objectives: Erythropoiesis-stimulating agents (ESAs) might moderate circulating CD34-positive hematopoietic stem (CD34⁺) cells. We assessed associations between ESA therapy and CD34⁺ cells and their impact on cardiovascular disease (CVD) events in patients on prevalent hemodialysis (HD). **Design, Setting, Participants and Measurements:** We analyzed 95 patients on prevalent HD who received the ESAs epoetin- β (n = 22), darbepoetin- α (n = 60), or neither (control; no ESA, n = 13). Baseline values for CD34⁺ cells, high-sensitivity C-reactive protein, interleukin-6, vascular endothelial growth factor, inter-cellular adhesion molecule-1, and carotid intima-media thickness were determined. The numbers of CD34⁺/erythropoietin receptor (EPOR)⁺ cells were determined in 35 and 8 patients in the darbepoetin- α and control groups, respectively. CD34⁺ cells were counted after 6 and 12 months of darbepoetin- α treatment (n = 35). All patients were followed up for a mean of 28 months. **Results:** Hemoglobin levels were lower, carotid intima-media thickness was more pronounced, and the ESA dose was higher in patients with a low, than with a high, CD34⁺ cell count. The ratio of CD34⁺/EPOR⁺ to CD34⁺ cells positively correlated with the darbepoetin- α dose. A low, but not a high, dose of darbepoetin- α for 6 and 12 months was associated with more CD34⁺ cells. Although high-dose darbepoetin- α therapy was an independent predictor of composite CVD events, this association disappeared when adjusted for the CD34⁺ cell count with other confounders. **Conclusions:** High-dose ESA therapy is associated with a low CD34⁺ cell count and comprises a risk factor for CVD events in patients on prevalent HD.

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Introduction

Accumulating evidence indicates a high prevalence of cardiovascular disease (CVD) among patients with chronic kidney disease (CKD), especially those under dialysis, and this remains the most common cause of premature death in such patients [1]. Several factors, such as oxidative stress, chronic inflammation as well as CKD mineral-bone disorder, influence the high prevalence of CVD [2–5].

In this setting, endothelial dysfunction that causes accelerated atherosclerosis is a hallmark of patients on hemodialysis (HD) [6, 7]. A peripheral blood mononuclear cell population, namely CD34-positive hematopoietic stem (CD34⁺) cells, can differentiate into endothelial progenitor cells, become incorporated into ischemic tissue during angiogenesis and lead to endothelial regeneration, and thus become an important contributor to vascular homeostasis [8, 9]. The production of CD34⁺ cells tends to decrease in patients on HD [10, 11] and decreased CD34⁺ cell trafficking in the peripheral blood is a risk for CVD and increased CVD-related mortality [12].

Erythropoiesis-stimulating agents (ESAs) have revolutionized the management of the anemia associated with CKD. However, ESAs stimulate the production of not only red blood cells (RBCs), but also CD34⁺ cells. The ESAs epoetin and darbepoetin- α increase the number and improve the function of CD34⁺ cells regardless of dialysis [13, 14]. Thus, ESAs might protect against damage to the vascular endothelium.

On the other hand, a large prospective study has confirmed that high-dose ESA therapy confers an increased risk of CVD events and mortality in patients under HD or with end-stage renal disease (ESRD) who respond poorly to these drugs [15–17]. These findings suggest that the benefits of ESAs are diminished in patients who require high-dose ESA therapy. Therefore, the present study investigated associations among ESA therapy, CD34⁺ cells, atherosclerosis, and CVD events in patients on prevalent HD.

Materials and Methods

Patients

This study enrolled 95 outpatients who had been undergoing HD at two clinics for ≥ 6 months and who were ≥ 20 years old. Patients who did not provide blood samples, or whose anticipated life expectancy was < 6 months, or who presented with clinical signs of overt infection, acute vasculitis, or liver disease at the time of recruitment were excluded from the study. Written informed consent was obtained from all enrolled patients and the ethics committee of our institute approved the study protocol.

All patients were similarly managed in terms of treatment protocols for HD including prescribed dialysis dose, medical treatment, and type of medication [18]. Basically, all recruited patients underwent three 3- to 4-hour routine HD sessions each week using a conventional bicarbonate or acetate-free dialysate and standard high-flux cellulose triacetate, polysulfone, or other dialysis membranes. Patients were cautioned to maintain a daily protein intake of > 1.0 – 1.2 g/kg body weight. Dialysis dosage was calculated from second-generation Kt/V, which was estimated using the Daugirdas formula.

Study Design

This prospective cohort study included a cross-sectional analysis, which assessed associations among CD34⁺ cells, biomarkers associated with CD34⁺ cells and patient characteristics, and between ESA therapy and CD34⁺ cells ($n = 95$; control group, $n = 13$; epoetin- β

group, $n = 22$; darbepoetin- α group, $n = 60$), and the impact of CD34⁺ cells on atherosclerosis estimated as carotid intima-media thickness (CIMT; $n = 95$).

The association between the ESA, darbepoetin- α , and CD34⁺/erythropoietin receptor (EPOR)⁺ cells was assessed in 43 patients who were randomly selected from the control ($n = 8$) and darbepoetin- α ($n = 35$) groups, and the effect of the darbepoetin- α dose on CD34⁺ cells was prospectively assessed at baseline and after 6 and 12 months of therapy in these 35 patients. Anemia was managed according to the guidelines of the Japanese society for dialysis therapy [18]. During the 1-year follow-up period, darbepoetin- α dose as well as hemoglobin, ferritin, and transferrin saturation (TSAT) was measured at baseline, 3, 6, 9, and 12 months to distinguish high and low responders to darbepoetin- α therapy. Patients were placed in the high-dose group if $\geq 0.65 \mu\text{g/kg/week}$ of darbepoetin- α was required to maintain target hemoglobin levels at >3 of the 5 time points.

After baseline measurement, patients in the control and darbepoetin- α groups were followed up for 36 months to estimate associations among darbepoetin- α dose, the CD34⁺ cell count, and composite CVD events ($n = 73$).

The following clinicopathological factors were recorded at baseline: cause of ESRD, presence of diabetes mellitus (DM), and history of CVD and smoking. A history of CVD was defined based on medical history, clinical symptoms, or findings of cerebrovascular (stroke) and/or peripheral vascular disease.

Routine biochemical parameters, CD34⁺ and CD34⁺/EPOR⁺ cells, and high-sensitivity C-reactive protein (hs-CRP) were measured in non-fasting venous blood samples collected immediately before a HD session and 3 days after the most recent session. Serum was separated by centrifugation and stored at -80°C . Baseline levels of interleukin (IL)-6, inter-cellular adhesion molecule (ICAM)-1, and vascular endothelial growth factor (VEGF) were also measured.

Survival analysis included elapsed time to CVD-related events and mortality (mean follow-up period: 28 months). A major CVD-related death was defined as a non-fatal myocardial infarction (MI), non-fatal stroke, or death from CVD causes. Elapsed time to each composite CVD event was determined in the analysis of events, and composite CVD events comprised non-fatal CVD death, fatal MI, angina pectoris (AP), fatal cerebral infarction, or arterial or peripheral artery disease. Fatal and non-fatal CVD events were defined by findings of electrocardiography and cardiac angiography for MI or AP, brain CT or MRI for cerebral infarction, and enhanced CT scanning or arterial or peripheral angiography for arterial or peripheral artery disease.

Assessment of Nutritional Status

Nutritional status was evaluated using the subjective global assessment (SGA) and the geriatric nutritional risk index [19]. Fully trained physicians assessed SGA according to K/DOQI recommendations; 4 items (weight loss during the preceding 6 months, anorexia, subcutaneous fat, and muscle mass) were scored on a 7-point Likert scale [20]. Actual body mass index was calculated as $\text{weight (kg)}/\text{height (m)}^2$. Normalized protein catabolic rate was also calculated.

Carotid Ultrasound

Transverse and longitudinal views of the walls of the left and right carotid arteries of 94 patients were obtained at baseline using a B-mode ultrasound scanner equipped with a 14-MHz linear probe. Values for CIMT in both arteries are expressed as mean CIMT of both sides [21]. The inter-observer technical error of measurement was $<5.5\%$ [21].

Fig. 1. Quantitation of CD34⁺/EPOR⁺ cells by flow cytometry. Initial gate R1 was set on a FSC versus SSC dot plot to contain lymphomononuclear cell population (a). Events in gate R1 area displayed in CD34⁺ versus SSC dot plot (b) and CD34⁺ cells were adjusted from a SSC versus FSC plot to determine true CD34⁺ cell counts (c). CD34⁺/EPOR⁺ cells were then identified in the upper right quadrant of the dot plot (control (d); CD34⁺/EPOR⁺ cells (arrow; e)).

CD34⁺ and CD34⁺/EPOR⁺ Cell Counting

Circulating CD34⁺ cells were counted as described [22]. Briefly, 50 μ l of blood samples was collected before a HD session into BD Trucount tubes and incubated with antibodies against FITC-labeled CD45 and PE-labeled CD34, as well as BD ViaProbe™ (7-AAD; BD Biosciences, San Diego, Calif., USA). The RBCs were then lysed and the total number of circulating CD34⁺ cells was measured using a FACSCalibur flow cytometer.

The total number of circulating CD34⁺/EPOR⁺ cells was measured by FACS analysis. Briefly, heparinized peripheral blood (2 ml) was obtained before a HD session and incubated with antibodies against FITC-labeled EPOR (R&D Systems Inc., Minneapolis, Minn., USA) and PerCP-labeled CD34 (BD Biosciences) for 40 min at 4°C, and then RBCs were lysed. Respective FITC- or PerCP-conjugated isotype control antibodies served as a control. An initial gate R1 was set on a forward light scatter (FSC) versus side light scatter (SSC) dot plot to contain the lymphomononuclear cell population (fig. 1a). The events in gate R1 were displayed in a CD34 versus SSC dot plot (fig. 1b) and the CD34⁺ cell count was adjusted from a SSC versus FSC plot to determine true CD34⁺ cells (fig. 1c). The CD34⁺/EPOR⁺ cells were then counted by flow cytometry using the FACSCalibur (fig. 1e).

All samples were analyzed within 2 h of sample preparation. The cumulative intra-assay coefficient of variation (CV) in these analyses was <7.0%.

Fig. 2. CD34⁺ cell count according to control (n = 13), epoetin- β (n = 22), and darbepoetin- α (n = 60) groups.

Biochemical Methods

Creatinine, hs-CRP urea, and albumin (Bromocresol Purple) were measured by routine immunonephelometry. Levels of IL-6 were measured using an ELISA (QuantiGlo Human IL-6 ELISA Kit; R&D Systems Inc.); the sensitivity was 0.16 pg/ml, with an intra-assay CV <4.8% and an inter-assay CV <7.8%. Serum ICAM-1 levels were measured using an ELISA kit (Human Soluble ICAM-1 (CD54); Thermo Scientific, Rockford, Ill., USA); the sensitivity was 0.3 ng/ml, with an intra-assay CV <10.0% and an inter-assay CV <10.0%. Serum VEGF levels were measured by ELISA (Quantikine Human VEGF; R&D Systems Inc.); the sensitivity was 9.0 pg/ml, with an intra-assay CV <6.7% and an inter-assay CV <8.8%.

Statistical Analyses

Data are presented as means $\pm$ standard deviation or median (range) unless otherwise indicated and values of $p < 0.05$ were considered to indicate statistical significance. Normally distributed variables were compared between two groups using Student's *t* test, and non-normally distributed variables were analyzed using the Wilcoxon rank-sum test. Nominal variables were compared between two groups using Fisher's exact test, and more than two groups were compared using the χ^2 test. Correlations were calculated using Pearson's correlation test (r^2) for parametric data and paired samples were compared using the Wilcoxon signed-rank test. Survival was assessed by the Kaplan-Meier method. Independent predictors of composite CVD events were determined using the Cox proportional hazards model, and interaction between two independent variables and composite CVD events was estimated. Data were analyzed using JMP version 9.0.2 (SAS Institute, Cary, N.C., USA) and STATA 12.0 (Stata Corp, Lakeway, Tex., USA).

Results

Of the 95 enrolled patients, 37 had DM and 32 had a history of one or more CVDs including MI or clinical signs of ischemic heart disease such as AP (n = 11), peripheral artery disease (n = 9), or a history of stroke or cerebral bleeding (n = 12), valvular disease (n = 7), and aortic aneurysm (n = 1). Causes of ESRD were chronic glomerulonephritis (n = 18, 19%), diabetic nephropathy (n = 38, 41%), nephrosclerosis (n = 14, 15%), other diseases (n = 5, 5%), and unknown (n = 19, 20%). None of the patients had type 1 DM. No patients were newly diagnosed with DM during follow-up examination. Table 1 shows the characteristics of the patients according to ESA therapy. The CD34⁺ cell count was decreased in the darbepoetin- α group compared to the control group but did not differ between the epoetin- β and darbepoetin- α groups (fig. 2).

Table 1. Patient characteristics according to ESA treatment

	rHuEPO (n = 22)	Darbepoetin- α (n = 60)	Control (n = 13)	p value
HuEPO dose, U/week	4,500 (3,000–7,500)	–	–	
Darbepoetin- α dose, μ g/week	–	30 (10–60)	–	
Age, years	60 $\pm$ 14	69 $\pm$ 11	56 $\pm$ 17	0.001
Male gender, %	81	50	93	0.005
Body mass index	21.3 $\pm$ 3.5	21.0 $\pm$ 3.6	23.0 $\pm$ 4.0	0.09
DM, %	22	48	23	0.06
CVD, %	14	45	14	0.003
Protein-energy wasting, %	22	31	7	0.12
Geriatric nutritional risk index	95.0 $\pm$ 7.3	93.6 $\pm$ 8.3	100.3 $\pm$ 9.9	0.05
Smoking, %	23	14	50	0.02
Normalized PCR, g/kg/day	0.99 $\pm$ 0.22	0.88 $\pm$ 0.15	0.95 $\pm$ 0.19	0.04
HD duration, months	47 (10–378)	55 (6–285)	69 (13–285)	0.36
Kt/V	1.33 $\pm$ 0.19	1.29 $\pm$ 0.27	1.25 $\pm$ 0.16	0.92
Use of ACE-I or ARB, %	26	38	17	0.31
Use of statin, %	10	18	17	0.59
White blood cell count, / μ l	6,154 $\pm$ 1,534	5,618 $\pm$ 1,788	6,335 $\pm$ 1,801	0.19
Hemoglobin, g/dl	10.2 $\pm$ 0.8	10.1 $\pm$ 0.9	10.6 $\pm$ 0.6	0.09
Albumin, g/dl	3.7 $\pm$ 0.4	3.6 $\pm$ 0.3	3.8 $\pm$ 0.3	0.18
Creatinine, mg/dl	12.2 $\pm$ 2.3	11.2 $\pm$ 2.5	14.4 $\pm$ 3.8	0.01
Ferritin, ng/ml	23.6 (5.0–118.0)	24.7 (5.9–260.4)	17.1 (11.6–57.5)	0.45
TSAT, %	20.5 $\pm$ 7.7	19.7 $\pm$ 8.3	19.4 $\pm$ 8.4	0.82
hs-CRP, mg/dl	0.009 (0.018–1.86)	0.072 (0.008–4.76)	0.082 (0.018–2.33)	0.84
IL-6, pg/ml	2.6 (1.3–10.9)	3.1 (1.2–55.6)	2.1 (1.3–14.7)	0.007
VEGF, ng/ml	0.44 (0.14–1.39)	0.39 (0.05–1.27)	0.23 (0.07–0.56)	0.33
ICAM-1, ng/ml	219.0 (135.5–369.6)	209.6 (128.1–438.3)	204.9 (155.7–316.3)	0.75
CIMT, mm	0.86 $\pm$ 0.20	1.01 $\pm$ 0.19	0.94 $\pm$ 0.23	0.04
Composite CVD events, %	14	38	7	0.01

Values are median (range) or mean $\pm$ SD. rHuEPO = Recombinant human erythropoietin; PCR = protein catabolic rate; TSAT = serum Fe/total iron-binding capacity.

Association between CD34⁺ Cells and Various Factors

The CD34⁺ cell count positively correlated with hemoglobin (fig. 3) and was inversely associated with weekly epoetin- β dose (units/week) (Pearson r = -0.45 , p = 0.03, n = 22, fig. 4a), weekly darbepoetin- α dose (μ g/week) (Pearson r = -0.51 , p < 0.0001, n = 60, fig. 4b), and darbepoetin- α dose including the control (Pearson r = -0.46 , p = 0.0002, n = 73, fig. 4c). Hemoglobin levels tended to inversely correlate with weekly doses of epoetin- β (units/week) (Pearson r = -0.37 , p = 0.08, n = 22), darbepoetin- α (μ g/week) (Pearson r = -0.29 , p = 0.03, n = 60), and darbepoetin- α (μ g/week) with the control group representing 0 μ g/week of darbepoetin- α (Pearson r = -0.32 , p = 0.006, n = 73).

Associations between the CD34⁺ cell count and related factors were estimated in the control and darbepoetin- α groups (n = 73, table 2). The CD34⁺ cell count correlated with age, SGA status, geriatric nutritional risk index, hemoglobin, and albumin as well as with the darbepoetin- α dose. Multivariate models independently associated the darbepoetin- α dose as well as a tendency for ICAM-1 to be inversely associated with the CD34⁺ cell count (table 2).

The log CD34⁺ cell count and darbepoetin- α dose (μ g/kg/week) were inversely (Pearson r = -0.23 , p = 0.03) and positively (Pearson r = 0.25, p = 0.04) associated with CIMT, respectively.

Table 2. Factors contributing to CD34⁺ cell counts in controls and patients administered darbepoetin- α

	Crude		Multivariate model 1 $r^2 = 0.44$		Multivariate model 2 $r^2 = 0.43$	
	β , SE	p value	β , SE	p value	β , SE	p value
Intercept	–	–	–2.98, 1.44	0.04	–3.06, 1.42	0.03
Age (10-year increase)	–0.12, 0.03	0.001	–0.07, 0.04	0.06	–0.06, 0.04	0.09
Gender (men vs. women)	–0.05, 0.08	0.55	0.09, 0.07	0.18	0.11, 0.06	0.10
DM (yes vs. no)	–0.005, 0.08	0.95	–	–	–	–
CVD (yes vs. no)	–0.11, 0.08	0.17	–	–	–	–
Smoking (yes vs. no)	0.17, 0.09	0.08	–	–	–	–
Protein-energy wasting (yes vs. no) ^a	–0.17, 0.08	0.05	–	–	–	–
Geriatric nutritional risk index	0.03, 0.008	0.001	0.02, 0.008	0.02	0.02, 0.009	0.07
HD duration, months	0.0008, 0.0008	0.31	–	–	–	–
Kt/V	0.11, 0.28	0.69	–	–	–	–
Normalized PCR, g/kg/day	1.13, 0.45	0.02	0.67, 0.39	0.09	–	–
Darbepoetin- α dose, μ g/week	–0.02, 0.004	<0.0001	–0.02, 0.005	0.001	–0.02, 0.004	0.0003
Hemoglobin, g/dl	0.30, 0.08	0.0008	0.08, 0.09	0.37	0.02, 0.04	0.09
Albumin, g/dl	0.75, 0.26	0.005	–	–	0.34, 0.28	0.23
log hs-CRP	–0.02, 0.05	0.71	–	–	–	–
log IL-6	–0.17, 0.10	0.10	–	–	–	–
log VEGF	0.16, 0.09	0.08	–	–	–	–
log ICAM-1	–0.66, 0.32	0.05	–	–	–	–

Crude and multivariate analyses included log CD34⁺ cells/ μ l as a dependent factor. ^a Protein-energy wasting defined as subjective global assessment >1. PCR = Protein catabolic rate.

Association between ESA Therapy and CD34⁺/EPOR⁺ Cells

The numbers of CD34⁺/EPOR⁺ cells tended to correlate with the darbepoetin- α dose (μ g/kg/week), but the values did not reach statistical significance (Pearson $r = 0.26$, $p = 0.10$). However, the ratio of CD34⁺/EPOR⁺ to CD34⁺ cells positively correlated with darbepoetin- α dose (fig. 5a, b) and log IL-6 (Pearson $r = 0.36$, $p = 0.03$).

Association of ESA Therapy with Change in CD34⁺ Cells

We assessed the influence of longitudinal ESA therapy on CD34⁺ cells in patients who were selected for the ESA and CD34⁺/EPOR⁺ study ($n = 35$). A blood sample was not collected from 1 patient at 12 months because a CVD event developed. The CD34⁺ cell count was significantly increased at 6 and 12 months compared with baseline in patients with a good ESA response in whom a low dose of darbepoetin- α ($<0.65 \mu$ g/kg/week) was required to maintain target hemoglobin levels at >3 of 5 time points in the first year compared with a high dose (fig. 6a). During the 1-year follow-up period, changes in hemoglobin, ferritin, and TSAT did not significantly differ between the high- and low-dose darbepoetin- α groups (fig. 6).

ESA Therapy, CD34⁺ Cells and Composite CVD Events

Composite CVD events developed in 22 (30%) patients during the follow-up period. Patients were assigned to control, epoetin- β , and high- or low-dose darbepoetin- α groups as described for the study of changes in CD34⁺ cells. Kaplan-Meier analysis indicated that high-dose darbepoetin- α was significantly associated with increased composite CVD events (fig. 7).

Fig. 3. Correlation between hemoglobin and CD34⁺ cells (n = 95).

Fig. 4. Correlation between ESAs and CD34⁺ cells.
a Epoetin- β (n = 22). **b** Darbepoetin- α (n = 60).
c Darbepoetin- α and control (n = 73).

Table 3 shows the characteristics of the patients who received high and low doses of darbepoetin- α . A high dose was associated with an increased number of composite CVD events. Crude and adjusted Cox hazard ratios for composite CVD events were significantly elevated in patients on high-dose darbepoetin- α , even after adjustment for age, gender, and DM status (table 4). However, the statistical significance of the high-dose darbepoetin- α therapy relative to the composite endpoint disappeared when adjusted for CVD history or the CD34⁺ cell count (table 4). Interaction between the CD34⁺ cell count and high-dose darbepoetin- α therapy was significantly associated with composite CVD events (table 5).

Fig. 5. Association between ESA therapy and ratio of CD34⁺/EPOR⁺ to CD34⁺ cells. Darbepoetin- α dose positively correlated with ratio of CD34⁺/EPOR⁺ to CD34⁺ cells.

Fig. 6. Association between darbepoetin- α dose and changes in CD34⁺ cells (a), hemoglobin levels (b), TSAT (c), and ferritin levels (d). Significantly increased CD34⁺ cell counts in good responders to ESA in whom a low dose of darbepoetin- α (<0.65 μ g/kg/week) was required to maintain target hemoglobin levels at >3 of 5 time points comprising baseline, 3, 6, 9, and 12 months during the first year of follow-up compared to a high dose ($\geq$ 0.65 μ g/kg/week; solid and dashed lines, respectively) (a).

Fig. 7. Kaplan-Meier curves of lapsed time to composite CVD events according to controls (black dashed line) and groups given epoetin- β (gray dashed line), low (gray line) ($<0.65 \mu\text{g/kg/week}$) and high (black line) darbepoetin- α doses ($\geq 0.65 \mu\text{g/kg/week}$).

Table 3. Patient characteristics according to darbepoetin- α dose^a

	High-dose darbepoetin- α (n = 32)	Low-dose darbepoetin- α (n = 28)	p value
Age, years	70 $\pm$ 10	68 $\pm$ 12	0.63
Male gender, %	52	48	0.79
Body mass index	20.6 $\pm$ 3.5	21.3 $\pm$ 3.7	0.33
DM, %	52	45	0.61
CVD, %	55	38	0.19
Protein-energy wasting, %	40	22	0.13
Geriatric nutritional risk index	92.8 $\pm$ 7.3	94.3 $\pm$ 9.0	0.47
Smoking, %	15	13	0.83
Normalized PCR, g/kg/day	0.88 $\pm$ 0.15	0.88 $\pm$ 0.16	0.73
HD duration, months	65 (6–255)	40 (6–285)	0.86
Kt/V	1.23 $\pm$ 0.16	1.35 $\pm$ 0.34	0.37
Use of ACE-I or ARB, %	42	32	0.43
Use of statin, %	12	23	0.27
White blood cell count, / μl	5,401 $\pm$ 1,641	5,487 $\pm$ 1,947	0.74
Hemoglobin, g/dl	9.9 $\pm$ 0.9	10.1 $\pm$ 0.6	0.22
Albumin, g/dl	3.6 $\pm$ 0.3	3.6 $\pm$ 0.3	0.96
Creatinine, mg/dl	11.5 $\pm$ 2.2	10.8 $\pm$ 2.5	0.23
Ferritin, ng/ml	23.6 (8.4–144.1)	24.7 (5.9–260.4)	0.98
TSAT, %	18.2 $\pm$ 6.6	21.0 $\pm$ 9.5	0.22
hs-CRP, mg/dl	0.06 (0.01–3.89)	0.09 (0.008–4.76)	0.70
IL-6, pg/ml	4.2 (1.5–14.2)	2.6 (1.2–55.6)	0.05
VEGF, ng/ml	0.30 (0.05–1.06)	0.42 (0.07–1.27)	0.23
ICAM-1, ng/ml	223.7 (128.1–438.3)	199.3 (142.2–276.6)	0.07
CIMT, mm	1.06 $\pm$ 0.20	0.96 $\pm$ 0.19	0.04
CD34 ⁺ cell count, / μl	0.60 $\pm$ 0.41	0.93 $\pm$ 0.55	0.007

Values are median (range) or mean $\pm$ SD. PCR = Protein catabolic rate; TSAT = serum Fe/total iron-binding capacity. ^a Patients were divided by darbepoetin- α dose of $0.65 \mu\text{g/kg/week}$.

Table 4. Hazard ratios of darbepoetin- α dose to composite CVD events

	Crude	Model 1	Model 2	Model 3	Model 4	Model 5
High darbepoetin- α dose	10.3 (2.1–186.7)	6.7 (1.0–125.5)	5.2 (0.93–97.0)	6.3 (1.1–120.6)	6.0 (1.1–111.4)	4.0 (0.6–79.9)
Low darbepoetin- α dose	4.1 (0.8–75.9)	2.9 (0.5–55.5)	2.8 (0.5–54.0)	2.1 (0.3–41.6)	2.7 (0.5–51.2)	2.1 (0.3–41.6)
Non-ESA therapy	Ref	Ref	Ref	Ref	Ref	Ref
CVD (yes vs. no)	4.4 (2.0–10.4)	–	2.4 (0.93–7.3)	–	–	–
Mean CIMT, mm	24.5 (3.8–158.0)	–	–	2.3 (0.24–19.0)	–	–
Hemoglobin, g/dl	0.6 (0.4–0.9)	–	–	–	0.8 (0.5–1.3)	–
log CD34 ⁺ cell count, / μ l	0.3 (0.1–0.6)	–	–	–	–	0.6 (0.2–1.3)

Values are hazard ratios (95% CI). Crude model included control, low (<0.65 μ g/kg/week) and high (≥ 0.65 μ g/kg/week) darbepoetin- α dose groups as independent variables. Model 1 included variables in crude model, age (years), gender (men vs. women), and DM (yes vs. no) as independent variables. Models 2, 3, 4, and 5 included variables in model 1 and CVD history, mean CIMT (mm), hemoglobin and log CD34⁺ cell count, respectively, as independent variables.

Table 5. Impact of interaction between CD34⁺ cell count and high-dose darbepoetin- α therapy on composite CVD events

	Crude		Model 1	
	Coefficient, SE	p value	Coefficient, SE	p value
Composite CVD events	89.3, 187.1	0.03	121.7, 219.4	0.04

Model 1 included age (years), gender (male vs. female) and DM (yes vs. no) as independent variables.

Discussion

The present study showed that high-dose ESA therapy could be a risk factor for CVD events among patients on prevalent HD. Although ESA therapy might have increased the number of CD34⁺ cells, a high dose was associated with a low CD34⁺ cell count.

While the CD34⁺ cell count did not differ between the epoetin- β and darbepoetin- α groups (fig. 2), the ESA dose was inversely associated with the CD34⁺ cell count in patients on prevalent HD (table 2, fig. 4) and the number of CD34⁺ cells was less increased in patients on a high, than on a low, ESA dose (fig. 6a). Bahlmann et al. [13, 14] reported that ESAs stimulate CD34⁺ cell production together with functional improvement. On the other hand, Kohagura et al. [23] demonstrated that a high dose of epoetin is associated with a lower CD34⁺ cell count in patients on prevalent HD. Overall, both epoetin- β and darbepoetin- α improved the number and function of CD34⁺ cells in patients on prevalent HD. However, the benefit of ESAs on CD34⁺ cells might be decreased in patients requiring a high ESA dose to maintain hemoglobin levels.

On the other hand, the association between ESA dose and EPOR expression in CD34⁺ cells considerably differed; the ratio of CD34⁺/EPOR⁺ to CD34⁺ cells positively correlated with darbepoetin- α dose (fig. 5). The reason why the numbers of CD34⁺ and CD34⁺/EPOR⁺ cells were decreased in patients and increased, respectively, in patients receiving high-dose ESA therapy remains uncertain. The detection of functional EPOR in humans remains controversial due to the specificity of the antibodies used to detect them [24]. However, ESA therapy might be associated with the upregulation of proinflammatory cytokines in patients with CKD [25], and levels of proinflammatory cytokines such as TNF- α and IL-6 are quite frequently increased in ESA hyporesponsiveness in terms of RBC production [26, 27]. Thus,

we speculate that factors associated with inflammation, e.g. proinflammatory cytokine, might also influence the ESA-EPOR pathway that leads to the proliferation and differentiation of CD34⁺ cells, because the IL-6 level correlated with the ratio of CD34⁺/EPOR⁺ to CD34⁺ cells.

The present study confirmed that high-dose ESA can predict an increase in CVD events (fig. 7, table 4). However, the predictive ability of high-dose ESA for CVD events disappeared when adjusted for the CD34⁺ cell count as well as CVD history in multivariate models (table 4). The significant effects of interaction between the CD34⁺ cell count and high-dose darbepoetin- α on composite CVD events (table 5) indicated that the predictive value of ESA therapy for CVD events depends on CD34⁺ cell count. Moreover, the ESA dose positively correlated with CIMT and the CD34⁺ cell count was lower in patients on high- than on low-dose ESA (fig. 6). A recent study has shown that treatment with 60 μ g/injection of darbepoetin- α , which is a higher dose than in the present study, improves flow-mediated dilation and increases peripheral blood EPCs in patients with coronary artery disease [28]. Thus, high-dose darbepoetin- α might benefit EPC production in good responders. On the other hand, the advantage of high-dose ESA might be blunted or absent in uremic patients as shown herein and according to Kohagura et al. [23]. Studies of uremic patients have shown that ESAs increase the number and improve the function of CD34⁺ cells [13, 14] and the doses of ESA in these studies were normal or not particularly high. Moreover, high-dose ESA therapy did not provide a significant non-hematopoietic effect, such as a protective function or a change in histology, among patients with transplanted kidneys or acute kidney damage [29–31]. Alternatively, patients that are hyporesponsive to ESA often require a high dose of ESA, and the mortality rates in such patients are quite high [15–17]. Decreased CD34⁺ cells in the peripheral blood is a risk for CVD and increased CVD-related mortality [12]. The overall disadvantage of ESA in patients with a poor response to ESA, or requiring a high dose of ESA, might be that a reduced effect on CD34⁺ cells is associated with pronounced CIMT and an increase in the number of CVD events.

However, the results of our study must be considered with the following caveats. Firstly, the patient cohort was relatively small. Secondly, the association between darbepoetin- α and CD34⁺ cells was estimated as changes in CD34⁺ cells measured at three time points in the selected population. Thirdly, differences in backgrounds among the control and high- or low-dose darbepoetin- α groups might influence survival and composite CVD events, although confounders were adjusted in our models. Finally, we did not assess changes in the CD34⁺ cell function in patients receiving high or low doses of ESA. Nevertheless, the impact of ESA dose on CD34⁺ cells among patients on prevalent HD should be clarified in a large prospective cohort study.

Conclusion

High-dose ESA therapy is apparently associated with CD34⁺ cell counts and is a risk factor for CVD events in patients on prevalent HD, especially in those with low CD34⁺ cell counts. High doses of ESA in this setting might influence the ability of ESA to reduce the numbers of CD34⁺ cells.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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