The Effect of Recombinant Human Erythropoietin on Circulating Hematopoietic Progenitor Cells in Anemic Premature Infants

Bernhard Meister,[a] Heiner Maurer,[a] Burkard Simma,[a] Hannelore Kern,[a] Hanno Ulmer,[b] Anton Hittmair,[c] Franz-Martin Fink[a]

[a]Department of Pediatrics, [b]Department of Biostatistics and [c]Department of Pathology, University of Innsbruck, Innsbruck, Austria

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Abstract

In vitro and animal studies suggest that high concentrations of recombinant human erythropoietin (rHuEPO) might divert multipotent progenitors into erythroid maturation at the expense of granulocyte production. We determined whether changes of number and lineage commitment of peripheral blood progenitor cells occur in premature infants during therapy with rHuEPO. Thirty preterm infants were randomly assigned either to receive 300 IU of eopoetin alpha s.c. per kilogram body weight three times a week for four weeks or to a control group. At study entry and after two weeks of treatment the numbers of circulating BFU-E, granulocyte-macrophage colony-forming units (CFU-GM) and granulocyte-erythrocyte-macrophage-megakaryocyte CFU (CFU-GEMM) were analyzed by semisolid culture technique, $CD34^+$ cells and early myeloid $CD34^+CD45RA^-$ progenitors by flow cytometry. As compared with the control group, rHuEPO treatment did not exert any significant modulatory effect on numbers of CFU-GM, nor was there a significant change in numbers of BFU-E, CFU-GEMM, total-CFU, percentage of $CD34^+$ or $CD34^+CD45RA^-$ cells. Mean neutrophil count was not significantly reduced at any period during the study. Compared with the control group, the infants receiving rHuEPO had higher hematocrit values ($p = 0.003$) and absolute reticulocyte counts ($p < 0.001$). The median cumulative volume of blood transfused per kilogram per day was 0.86 ml (first quartile 0.5 ml; third quartile 1.1 ml) in the control group and 0 ml (first quartile 0 ml; third quartile 0.47 ml) in the rHuEPO group ($p = 0.038$). We conclude using a relatively high dose of rHuEPO in premature infants, no significant in vivo effect on circulating peripheral blood progenitor or neutrophil count could be detected. ***Stem Cells*** *1997;15:359-363*

Introduction

During recent years several studies have been performed to evaluate the efficacy and possible toxicity of recombinant human erythropoietin (rHuEPO) in anemic premature infants. During anemia of prematurity, circulating progenitor cells committed to erythroid differentiation are present [1]. In vitro studies have indicated that rHuEPO stimulates circulating and marrow erythroid progenitors from preterm infants [1, 2]. In vitro studies [3, 4] as well as one animal experiment [5], however, suggest that high concentrations of rHuEPO might divert multipotent progenitors into erythroid maturation at the expense of granulocyte production. Neutropenia, presumably due to stem-cell competition [6], has been observed in small numbers of infants given EPO in uncontrolled trials [7-10]. Controlled randomized clinical studies, however, reported no reduction in absolute neutrophil counts [11-13]. To achieve sufficient erythropoietic response the dose of rHuEPO administered to premature infants has been increased during the past five years. Thus, from the above-mentioned animal and in vitro data, one would assume an increased risk of neutropenia after administration of higher doses of rHuEPO.

In vivo effects of rHuEPO on circulating hemopoietic progenitor cells during therapy with rHuEPO were reported for adults [14-17], but no data are yet available on the effects in premature infants. We hypothesized that circulating

Correspondence: Dr. Bernhard Meister, Department of Pediatrics, Innsbruck University Hospital, Anichstr. 35, A-6020 Innsbruck, Austria. *Accepted for publication May 27, 1997.*

hematopoietic progenitors might reflect changes of lineage commitment. We therefore analyzed whether the application of a relatively high dose of rHuEPO (900 IU per kilogram body weight administered s.c. per week) could influence the formation of granulocyte-macrophage colonies (CFU-GM), early $CD34^+CD45RA^-$ myeloid progenitors [18] and the number of granulocytes. The response to therapy was evaluated by the volume of blood transfused, reticulocyte count, hematocrit, hemoglobin and erythrocyte values.

METHODS

Study Population

A randomized trial was conducted at Innsbruck University Hospital, Innsbruck, Austria, with the approval of the local ethics committee and with the informed consent of the infants' parents. Criteria for enrollment and withdrawal were adopted from the study published by *Maier et al.* [11] with the exception that infants with ventilation or continuous positive airway pressure with a fraction of inspired oxygen above 0.40 after day 6 were not excluded from the study. Thirty preterm infants, at an age of five to ten days, were randomly assigned to an rHuEPO-treated group or to a control group using a computerized random numbers generator. Those in the control group did not receive the drug. The study was terminated after four weeks. One patient (control group) was withdrawn from the study because of development of intraventricular hemorrhage grade IV on study day 6. Medical records were coded without knowledge of laboratory results.

Transfusions

Guidelines for transfusions were derived from the largest multicenter trial [11]. Infants more than two weeks old who have been breathing spontaneously and whose fraction of inspired oxygen was less than 0.4 were given transfusions if they had signs of anemia and their hematocrit fell below 11 g per deciliter (6.8 mmol per liter); if they had no signs of anemia, the corresponding cutoff values were 27 percent and 9 g per deciliter (5.6 mmol per liter).

Hematologic Measurements

Leukocyte, erythrocyte and platelet counts were determined with automated blood cell counters. The neutrophil count was calculated from the number of leukocytes and the percentage of neutrophils in a blood smear. The reticulocyte count was corrected to a hematocrit of 45 percent by multiplying the count by the actual hematocrit and dividing by 45 [11].

Erythropoietin Administration

Infants assigned to the rHuEPO group received 300 IU of eopoetin alpha per kilogram body weight s.c. three times a week for four weeks. The rHuEPO (2,000 U/ml) was supplied by JANSSEN-CILAG Pharmaceuticals (Vienna, Austria). Oral iron administration was started with a dose of 6 mg/kg/day and increased after two weeks to 8 mg/kg/day. Control group patients received iron alone. For ethical reasons an s.c. placebo treatment was not conducted.

Media and Reagents

Nycoprep (specific gravity 1.077 g/cm^3) was obtained from Nycomed (Oslo, Norway); powdered Iscove's modified Dulbecco's medium, antibiotic antimycotic solution (100×) and L-glutamine solution (100×) were from GIBCO (Paisley, Scotland, UK). Fetal bovine serum albumin was supplied by Sebak (Aidenbach, Germany), and bovine serum albumin by Sigma (St. Louis, MO). rHuEPO was from Cilag (Vienna, Austria) and methylcellulose stock solution from Terry Fox Laboratories (Vancouver, BC, Canada). Cells were cultured in 35-mm polystyrene dishes (Costar; Cambridge, MA). Recombinant human interleukin 3 and rHuGM-CSF were purchased from Genzyme (Boston, MA). Fluorescein isothiocyanate-labeled CD34 monoclonal antibody was from Becton-Dickinson (Sunnyvale, CA; clone 8, G12) and CD45-RA phycoerythrin-labeled (clone 2H4) antibody from Coulter (Krefeld, Germany).

Clonogenic Assay

The methylcellulose-based semisolid culture medium was prepared as described elsewhere [19]. The final concentrations were: 30% (vol/vol) fetal bovine serum, 1% (wt/vol) bovine serum albumin and 0.9% (wt/vol) methylcellulose. One milliliter of culture medium contained 2.5 U of rHuEPO, 100 U of rHuGM-CSF, 10 U of recombinant human interleukin 3. One milliliter of soft gel was used per 35-mm dish, and each test was performed in duplicate. Depending on the flow cytometric CD34 analysis performed before plating, the number of unsorted mononuclear cells plated per milliliter of culture medium ranged between 1×10^3 and 1×10^5. Three different cell concentrations were plated for each mononuclear cell sample. The cultures were incubated at 37°C in a humidified atmosphere and in the presence of 5% CO_2 and 3% O_2 in N_2. The proportions of CFU-GM, CFU-mix, and BFU-E (>50 cells per colony) were evaluated on day 14. Day 14 of the study was chosen to investigate the effects of rHuEPO since in the large European multicenter trial the stimulation of reticulocyte counts was best in premature infants two weeks after onset of treatment [11]. Furthermore, in adult patients mobilization of peripheral blood progenitors was observed after the first week of onset of rHuEPO treatment [15-17]. Throughout the study a total of 3 ml blood per infant was removed for clonogenic assays and flow cytometric analysis.

Table 1. Characteristics and laboratory data of patients at study entry

Group	rHuEPO	Control
Number of patients	15	15
Number of females	8	7
	mean ± SE	
Birth weight (grams)	1,100 ± 63	1,078 ± 63
Gestational age (weeks)	29.7 ± 0.4	28.8 ± 0.5
Age at entry (days)	6.6 ± 0.5	7.5 ± 0.4
Reticulocytes (%)	3.1 ± 0.7	2.2 ± 0.3
Hematocrit (I/l)	0.46 ± 0.05	0.46 ± 0.07
Hemoglobin (g/l)	157 ± 4	151 ± 6
Red cells ($\times 10^{12}$ cells/l)	4.4 ± 0.1	4.3 ± 0.2
White cells ($\times 10^{9}$ cells/l)	18.4 ± 3.0	18.9 ± 3.6
Neutrophils ($\times 10^{9}$ cells/l)	8.7 ± 2.0	10.1 ± 2.2
Lymphocytes ($\times 10^{9}$ cells/l)	5.3 ± 0.7	4.2 ± 0.6
Monocytes ($\times 10^{9}$ cells/l)	2.7 ± .04	2.8 ± 0.6

Flow Cytometry

Flow cytometric analysis was performed on a FACScan flow cytometer, calibrated with CaliBRITE Beads and AutoCOMP software. Forward light scattering, orthogonal light scattering and fluorescence signals were acquired and stored in listmode data files. Data were analyzed using the PAINT-A-Gate software.

Statistical Analysis

Analysis was performed with the SPSS Statistical System. Peripheral blood progenitor data had a Gaussian distribution. Significance of differences between means was estimated with the Student's *t* test. The Mann-Whitney U test was used when distributional assumptions of parametric statistics were not met, particularly for data on numbers of transfusions per infant, cumulative volume of blood transfused and cumulative blood loss. Chi^2 testing was used to compare discontinuous variables. Repeated-measures analysis of variance was used to compare weekly observations of hematologic indices between the treatment groups. Similar to a pilot study and previous studies of rHuEPO effects on peripheral blood progenitor cells in adults [14-17], we used a fixed sample size of 30 patients. All *p* values are two-tailed. A *p* value below 0.05 was considered statistically significant.

Results

Treatment and control groups were comparable at study entry. There were no significant differences with respect to the features shown in Table 1, numbers of baseline peripheral blood progenitor cells, $CD34^+$ cells or early $CD34^+CD45RA^-$ myeloid progenitors. As compared with the control group, rHuEPO treatment did not exert any significant modulatory effect on numbers of peripheral blood CFU-GM ($p = 0.11$), nor was there a significant change in numbers of BFU-E ($p = 0.35$), CFU-GEMM ($p = 0.45$) or total CFU ($p = 0.62$) (Fig. 1). Furthermore, there was no significant change in percentage of $CD34^+$ cells ($p = 0.80$) and $CD34^+CD45RA^-$ ($p = 0.76$) between the two groups (Fig. 2).

At no time during the study was the mean neutrophil count significantly reduced in patients treated with rHuEPO ($p = 0.46$) (Fig. 3). Mononuclear cells did not significantly differ between rHuEPO-treated infants and control infants (Table 2).

Median cumulative blood loss due to diagnostic routine and study tests was 0.34 ml kg/day in the rHuEPO group (first quartile 0.31 ml; third quartile 0.43 ml) and 0.44 ml (0.34 ml and 0.58 ml, respectively) in the control group (not significant). Compared with the control group, the infants receiving rHuEPO had higher absolute reticulocyte counts ($p < 0.001$), hematocrit ($p = 0.003$),

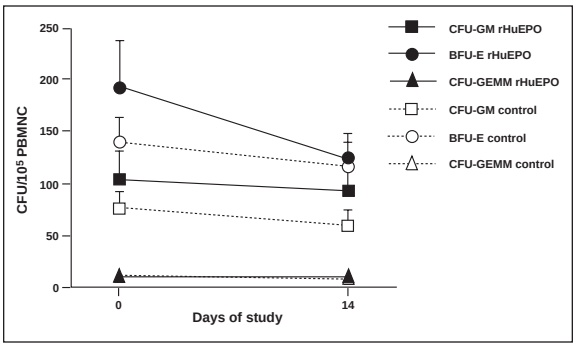

Figure 1. Peripheral blood hematopoietic progenitor cell growth per 10^5 peripheral blood mononuclear cells in premature infants treated with 300 IU/kg rHuEPO and control infants. *The values represent mean ± SE.*

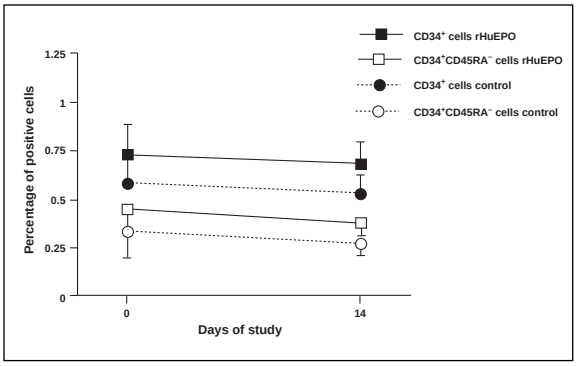

Figure 2. Peripheral blood CD34⁺ and early myeloid CD34⁺RA⁻ cells in premature infants treated with 300 IU/kg rHuEPO and control infants. *The values represent mean ± SE.*

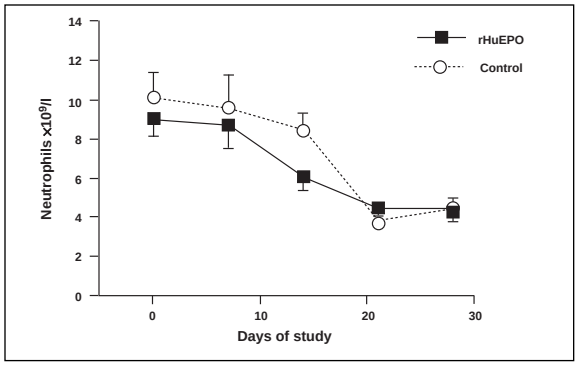

Figure 3. Course of absolute neutrophil counts in premature infants treated with 300 IU/kg rHuEPO and control infants. *The values represent mean ± SE.*

Table 2. Mean (±SE) values for total white cell numbers and absolute neutrophil, lymphocyte, monocyte counts at the end of study weeks 1, 2, 3 and 4 in preterm infants receiving recombinant human erythropoietin (rHuEPO) and in control patients

	Week 1	Week 2	Week 3	Week 4
White cells ($\times 10^9$ cells /l)				
rHuEPO	22.5 ± 2.9	16.5 ± 1.4	15.3 ± 1.6	13.9 ± 1.4
Control	20.3 ± 2.1	16.8 ± 1.3	11.9 ± 1.1	12.3 ± 1.4
Lymphocytes ($\times 10^9$ cells/l)				
rHuEPO	8.4 ± 1.4	6.8 ± 0.8	7.0 ± 1.0	7.1 ± 0.9
Control	5.6 ± 0.7	5.1 ± 0.5	4.9 ± 0.6	5.1 ± 0.8
Monocytes ($\times 10^9$ cells/l)				
rHuEPO	2.6 ± 0.7	1.6 ± 0.2	2.0 ± 0.4	1.8 ± 0.3
Control	2.3 ± 0.3	2.1 ± 0.3	1.6 ± 0.3	1.6 ± 0.2

Values represent mean ± SE.

erythrocyte ($p = 0.008$) and hemoglobin ($p = 0.004$) values. The median cumulative volume of blood transfused per kilogram per day was 0.86 ml (first quartile 0.5 ml; third quartile 1.1 ml) in the control group and 0 ml (first quartile 0 ml; third quartile 0.47 ml) in the rHuEPO group ($p = 0.038$).

Discussion

Treatment of premature infants with 900 IU rHuEPO per kilogram body weight s.c. per week did not result in reduced formation of peripheral blood CFU-GM, early myeloid CD34⁺CD45RA⁻ progenitor cells [18] or absolute neutrophil counts. Thus, there is no evidence of rHuEPO-mediated diversion of multipotential hemopoietic stem cells into committed erythroid precursors at the expense of myeloid precursors in premature infants at this dose level. *Mizuno et al.* [13] similarly reported in a retrospective analysis that the difference in neutrophil counts between an rHuEPO-treated group and a control group was not significant. The weekly administered dose in his study, however, was less than one-fourth the dose administered in our study.

In vivo effects of rHuEPO on circulating human hemopoietic progenitor cells have been reported for adults with anemia of chronic renal failure treated with far lower dosages (40 and 120 U/kg/week) [14, 15]. *Ganser et al.* [14] and *Geissler et al.* [15] both reported an increase of BFU-E and CFU-GEMM after onset of treatment. Whereas *Geissler et al.* also observed an increase of CFU-GM incidence within one week of supplementation therapy, *Ganser et al.* reported the incidence of CFU-GM to not be significantly altered. In adult lymphoma patients 300 IU/kg rHuEPO s.c. thrice weekly similarly induced a fivefold colony-forming cell increase over baseline and a 4.6-fold increase in CD34⁺ cells [16]. In contrast to these studies in adults, we observed no significant increase of peripheral blood BFU-E, CFU-GM or CFU-GEMM after onset of treatment in premature neonates. Furthermore, the percentage of CD34⁺ cells, increasingly used to quantify

the hematopoietic precursor population [20], did not increase after onset of treatment.

In vitro and animal investigations of rHuEPO on CFU-GM formation and neutrophil generation in progenitor cell cultures have been controversial. *Christensen et al.* [3] reported that in vitro high concentrations (4 U/ml) of rHuEPO reduced the formation of CFU-GM colonies and diminished the number of granulocytes. Fetal progenitors were more sensitive to these effects of EPO than were adult progenitors. *Mizuno et al.* [13] and *Ward et al.* [21], however, reported that even administration of rHuEPO doses up to 10 U/ml did not exert any significant modulatory effects on the number of CFU-GM in vitro. Administration of high doses of recombinant EPO to newborn rats resulted in diminished neutrophils (liver, spleen), CFU-GM and CFU-GM titrated thymidine suicide rates, whereas EPO injections in two-week-old rats did not result in reduced neutrophil production [22].

Conclusion

Erythropoietic response to therapy in the present study was significant, comparable with that observed in the largest European multicenter trial [11]. Regarding the safety aspects of rHuEPO, high dose administration of 900 IU/kg does not reduce absolute neutrophil counts in preterm infants and has no significant in vivo effects on number or lineage commitment of peripheral progenitor cells.

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