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Research Paper

Pharmacodynamics of Recombinant Human Erythropoietin in Murine Bone Marrow

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Purpose. Originally approved for three times/week dosing, recombinant human erythropoietin (rhEPO) is now often used at weekly intervals. We have studied rhEPO in mice to better understand why the extended dosing interval retains efficacy.
Methods. C57Bl/6 mice received a single sc. dose of rhEPO (3,000 IU/kg). Bone marrow and blood were collected at 8 h and 1, 2, 5 and 7 days. Staining for TER-119 and CD71, pulse labeling with bromodeoxyuridine, annexin-V binding and vital staining with 7-aminoactinomycin D were used cell cycle and apoptosis in erythroblasts by four color flow cytometry.
Results. A wave of proliferation and/or maturation progressed through all erythroblasts, resulting in the emigration of immature reticulocytes into the periphery. An increase in the fraction of erythroblasts in S and G2M was found, but suppression of apoptosis was not.
Conclusions. Most of the effects of rhEPO occurred 48 h after dosing, when the concentration of rhEPO was less than 1% of Cmax, suggesting that the processes set in motion by rhEPO can continue after rhEPO concentrations fall. Our observation of apoptosis in erythroblasts even when rhEPO concentrations were high suggests that regulatory mechanisms which down-regulate erythropoiesis are also engaged.

KEY WORDS: cytometry; erythropoiesis; erythropoietin; hematology; pharmacodynamics.

INTRODUCTION

Erythropoiesis is the process of forming red blood cells (RBC) from multipotent stem cells in which erythropoietin (EPO) plays a central role (1). EPO is a 34 kDa glycoprotein whose amino acid sequence is highly conserved (~80% identical in humans and mice) (2). EPO acts by binding and activating EPO receptors (EPO-R) on the surface of populations of erythroid precursor cells. Like that of EPO, the amino acid sequence of EPO-R is also highly conserved (3). Thus, it is not surprising that recombinant human EPO (rhEPO) has been shown to be pharmacologically active in most species. Activation of EPO-R results in intracellular signaling which, in turn, stimulates these cell populations to expand, survive and ultimately mature into red blood cells.

Erythropoiesis can be thought of in two parts, early and late. In the early stages, hematopoietic stem cells give rise to burst forming cells-erythroid (BFU-e), colony forming units-erythroid (CFU-e), and finally the phenotypically identifiable late stage erythroid precursors: pro-erythroblasts (ProEB); basophilic erythroblasts (BasoEB); polychromic erythroblasts (PolyEB) and orthochromic erythroblasts (OrthoEB). At the earliest stages, colony stimulating factor (CSF) plays a major role in controlling cell growth. Subsequently, EPO is believed to play a major role in determining the fate of CFU-e, ProEB and BasoEB, increasing the number of erythroid precursors but without altering the length of the cell cycle or number of mitotic divisions involved in the differentiation process (4, 5). In the later stages of erythropoiesis, BasoEB mature into PolyEB which, in turn, mature into the non-dividing OrthoEB, reticulocytes and finally mature RBC. The role, if any, of EPO in determining the fate of the PolyEB and OrthoEB is less well understood.

Erythropoiesis can be recapitulated *in vitro* and studies of BFU-e and CFU-e in semi-solid cell cultures have added immeasurably to our understanding of this process. *In vivo*, however, bone marrow stromal cells and macrophages play an important role in creating microenvironments for stem cells and erythroblastic islands, respectively (6). Depending on the preparation and culture techniques, the function of these cells and their cytokines may be lost in *in vitro* systems. This was demonstrated recently by Chang *et al.* (7) who showed that rather than the expected inhibition of apoptosis, three doses of recombinant murine EPO resulted in an increase in apoptosis in erythroblasts from both normal and mice made anemic by infection with Plasmodium chabaudi.

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Recently, a monoclonal antibody to TER-119 (which binds a glycophorin A associated protein) that can discriminate erythroid precursors from other bone marrow cells in mice has been described (8). Moreover, as described by Chang *et al.* and Socolovsky *et al.* (7, 9), when coupled with expression of transferrin receptor (CD71), expression of TER-119 can be used to enumerate the various late stage erythroid precursors. In this study, we have coupled staining for CD71/TER-119 with staining for bromodeoxyuridine and 7-aminoactinomycin-D (7AAD), and annexin-V binding and 7AAD uptake to study cell cycle and apoptosis in these cells by four color flow cytometry. We have found that treatment of normal mice with a single pharmacologic dose EPO caused a transient increase in the fraction of Poly/OrthoEB and BasoEB and an increase in the fraction of these cells in S and G2M phase. We also found a decrease in the fraction of Poly/OrthoEB, consistent with a pro-differentiation effect. And, rather than the expected net inhibition, we found a transient increase of apoptosis in late-stage erythroid precursors.

MATERIALS AND METHODS

Reagents

rhEPO (epoetin-α) was obtained from Ortho Biologics (Raritan, NJ). Doses are expressed as IU/kg (The activity of rhEPO was ≈120 IU/μg.). The following monoclonal antibodies (mAbs) against mouse cell surface markers were purchased from BD-Pharmingen (BD Biosciences, San Jose, CA): PE-conjugated anti-TER-119, biotinylated anti-CD71 (clone C2), APC-conjugated streptavidin; FITC conjugated anti-BrdU, FITC conjugated annexin-V, and the viability/DNA probe 7-amino-actinomycin D (7AAD). Appropriately labeled isotype-matched IgG controls were also used (BD Biosciences, San Jose, CA).

Study Designs

Mice. Eight to 10 week old female C57Bl/6 mice were obtained from Charles River Laboratories (Kingston, NY). Mice were acclimated for at least 2 weeks and group housed with a 12 h light/dark cycle. Food and water were provided *ad libitum*. All mice were maintained in the pathogen-free animal facility at Centocor, Radnor PA. The Centocor Animal Care and Use Committee approved all procedures.

Pharmacokinetics. The subcutaneous route of administration was used in these studies. In humans, this route has been shown to more effective in stimulating erythropoiesis than the intravenous route (10). The pharmacokinetics of rhEPO in mice have been described previously (11–14). Following a subcutaneous dose, a Tmax of ≈2 h, a $t_{1/2}$ of ≈3 h, a mean residence time of ≈4 h and a systemic bioavailability was of ≈70% was reported. To study the pharmacokinetics of rhEPO under our experimental conditions, mice received a single subcutaneous dose of rhEPO at a dose of 30,000 IU/kg (300 μg/kg). This dose was chosen to ensure that the plasma concentration would be above the lower limit of quantitation for at least 24 h. Blood samples were collected from mice (three/time point) at 2, 4, 6, 8, 16, 24 and 48 h after dosing. These data were used to calculate standard pharmacokinetics parameters using non-compartmental analysis (WinNonlin version 5.1, Pharsight Corporation, Mountain View, CA).

Dose Range. To determine an appropriate dose for subsequent pharmacokinetic and pharmacodynamic studies, groups of mice (four/group) received as single subcutaneous dose of rhEPO (30, 100, 300, 1,000, 3,000 or 10,000 IU/kg) or phosphate buffered saline and femoral bone marrow was collected 48 h after dosing and analyzed by flow cytometry to enumerate erythroid precursors. This time point was chosen to give sufficient time for clearance of rhEPO from a subcutaneous injection and to allow time for cell cycling in the marrow. Based on the results of the dose range study (see "Results") a dose of 3,000 IU/kg was chosen for the pharmacodynamic study.

Pharmacodynamics. Groups of mice (eight/group) received a single subcutaneous injection of 3,000 U/kg rhEPO or phosphate buffered saline 8, 24, or 48 h or 5 or 7 days prior to sacrifice. All dosing and collection of bone marrow samples was at approximately 8 A.M. Mice for the 8 h time point had been acclimated to an adjusted light/dark cycle to eliminate possible circadian effects. To study the cell cycle, mice were exposed to bromodeoxyuridine (BrdU) for the last 8 or 24 h prior to collection of bone marrow. Mice received a single intraperitoneal injection of BrdU (0.6 mg in 0.2 mL) and were offered BrdU in drinking water (1 mg/mL) from time of BrdU injection until time of bone marrow collection.

Analysis of Reticulocytes. Blood was collected from mice anesthetized with a CO_2 mixture via open chest cardiac puncture into commercially prepared EDTA coated microtubes. Blood analyses were performed on whole blood using the Advia 120 blood analyzer (Bayer Diagnostics, Tarrytown, NY). Total reticulocytes and high RNA (premature), mid RNA (immature) and low RNA (mature) reticulocytes (15) were enumerated according to manufacturer's directions. Data are expressed as absolute reticulocytes count or as % red blood cells.

Staining for Flow Cytometry

Discrimination of Erythroid Precursors. Femoral bone marrow was obtained by flushing both femurs and dispersed mechanically. Mature erythrocytes and reticulocytes were removed by hypotonic lysis. To label erythroid precursors, cell suspensions were stained for CD71 and TER-119 (7, 9). Erythroid precursors were classified according to the nomenclature of Metcalf and Moore (16) and enumerated as follows: cells that were CD71hi/TER-119lo were counted as ProEB; cells that were CD71hi/TER-119hi counted as BasoEB and cells that were CD71mid-lo/TER119hi counted as Poly/OrthoEB.

Cell Cycle. To discriminate S and G2M phase cells, bone marrow suspensions were simultaneously stained for CD71, TER-119, BrdU and 7AAD (17) using a kit obtained from BD Biosystems (San Jose, CA) according to the manufacturer's instructions. Cells that were polyploid and stained strongly for BrdU were counted as S Phase and cells that were polyploid and stained weakly for BrdU were counted as G2M.

Apoptosis and Cell Death. Apoptosis and cell death were studied by simultaneously staining unfixed cells for

CD71, TER-119, binding of annexin-V and uptake of 7AAD using a kit obtained from BD Biosystems (San Jose, CA) according to the manufacturer's instructions. Cells that were annexin-V+ and 7AAD lo-mid were counted as apoptotic and cells that were 7AAD bright were counted as dead (18). We have previously validated this assay for the study of apoptosis in EPO dependent cells *in vitro* and in murine bone marrow (19).

Flow Cytometry

Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 15 mW air-cooled 488 nm argon ion laser for excitation of FITC, PE, and 7AAD and a 635 nm diode laser for excitation of APC. Electronic compensation was used among the fluorescence channels to reduce residual spectral overlap. Photomultiplier tube voltage and spectral compensation were established using cells single-stained with FITC alone, PE alone, 7AAD alone or with streptavidin APC alone. FITC, PE, and APC fluorescence were acquired on 4-decade log scales. 7AAD fluorescence for analysis of ploidy was acquired on a linear scale and for analysis of apoptosis/cell death on a log scale. Analysis of the multivariate data was performed using CellQuest Pro™ software (BDIS, BD Biosciences, San Jose, CA). Data for at least 10,000 cells/sample were collected. To discriminate lineage and functional phenotypes, a nested analysis strategy was used. First, debris and aggregates were excluded using low angle and orthogonal light scatter. Next, gates that delimited the functional phenotypes: S and G2M phase; apoptotic and dead cells, were drawn for the entire bone marrow. Then, these gates were combined with the lineage phenotypes using the Boolean "and" operator to enumerate the populations of interest.

To establish a normal range for erythroid cells in the bone marrow (see Table 2), data for 32 control animals were evaluated. Data for each cell type are expressed as % total nucleated cells in the bone marrow or as % total erythroid (TER-119+) cells. Data for cell cycle and apoptosis are expressed as % of each cell type.

Data from the dose range and pharmacodynamic studies for cell type are expressed as % total bone marrow. Because there was considerable day-to-day variation in the control values for S Phase, apoptotic and dead cells, values for these parameters for individual rhEPO treated mice were normalized to the mean control value for each day and the data expressed as % control for that day.

Statistical Analysis and Data Presentation

Because the data for a number of the parameters were non-normally distributed or showed unequal variance, all data are shown as median ± interquartile range. Statistical differences were determined using Mann–Whitney *U*-tests or analysis of variance (ANOVA) based on ranks with Dunn's correction for multiple comparisons. P values less than 0.05 were accepted as significant. For graphical presentation, data were fitted to a fourth order polynomial equation. This equation facilitates visualizing trends in the data visually, but is not intended to model the data.

Table 1. Pharmacokinetics Parameters for rhEPO Following a Single Subcutaneous Dose in Mice

Parameter	(Mean ± SD)
Cmax (μg/mL)	1.7±0.5
Tmax (h)	6.0±2.0
AUCt (μg •h/mL)	17.9±3.9
AUC (μg •h/mL)	20.6±4.9
$t_{1/2}$ (h)	6.9±2.0
Vz/F (mL/kg)	147.4±40.0
CL/F (mL/h/kg)	15.2±4.1

RESULTS

Pharmacokinetics of rhEPO in Mice

rhEPO was detectable in mouse plasma at 2, 4, 6, 8, 16 and 24 h after dosing. Values for 48 h were below the lower limit of quantitation. These data were used to calculate standard pharmacokinetics parameters using non-compartmental analysis (Table 1). Individual and mean (± SD) serum concentrations-time profiles are shown in Fig. 1. rhEPO was rapidly absorbed into the systemic circulation with a Tmax of 6 hrs and was rapidly eliminated, with a terminal $t_{1/2}$ of approximately 6 h. At 24 h, less than 10% of the initial plasma concentration remained.

Normal Range for Erythroid Precursors

Representative contour plots of the flow cytometric methods used to discriminate Pro-, Baso- and Poly/Ortho-EB is shown in Fig. 2a, to discriminate S and G2M phase cells is shown in Fig. 2b and to discriminate apoptotic and dead cells is shown in Fig. 2c. The composite results for the 32 control mice are summarized in Table 2. Erythroid precursors accounted for approximately 18% of the bone marrow and the proportion of bone marrow erythroid precursors increased as the cells matured from ProEB to Poly/OrthoEB. The values for the individual cell types are consistent with those reported previously for bone marrow smears from

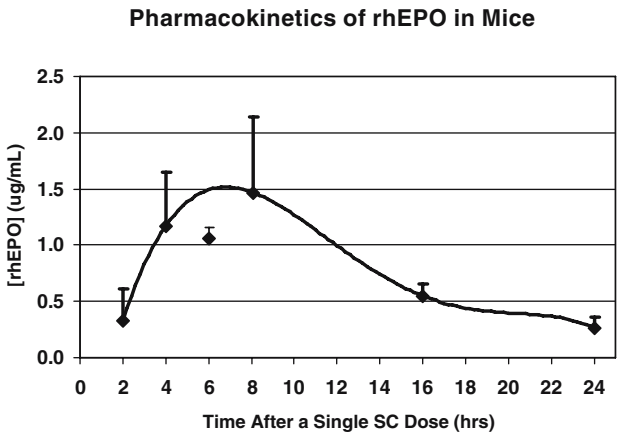

Fig. 1. Pharmacokinetics of rhEPO in mice. Mice received a single subcutaneous dose of rhEPO of 0.3 μg/kg and serum samples were collected by open chest cardiac puncture at 2, 4, 6, 8, 16 and 24 h after dosing. Data are shown as mean ± standard deviation, three mice per time point.

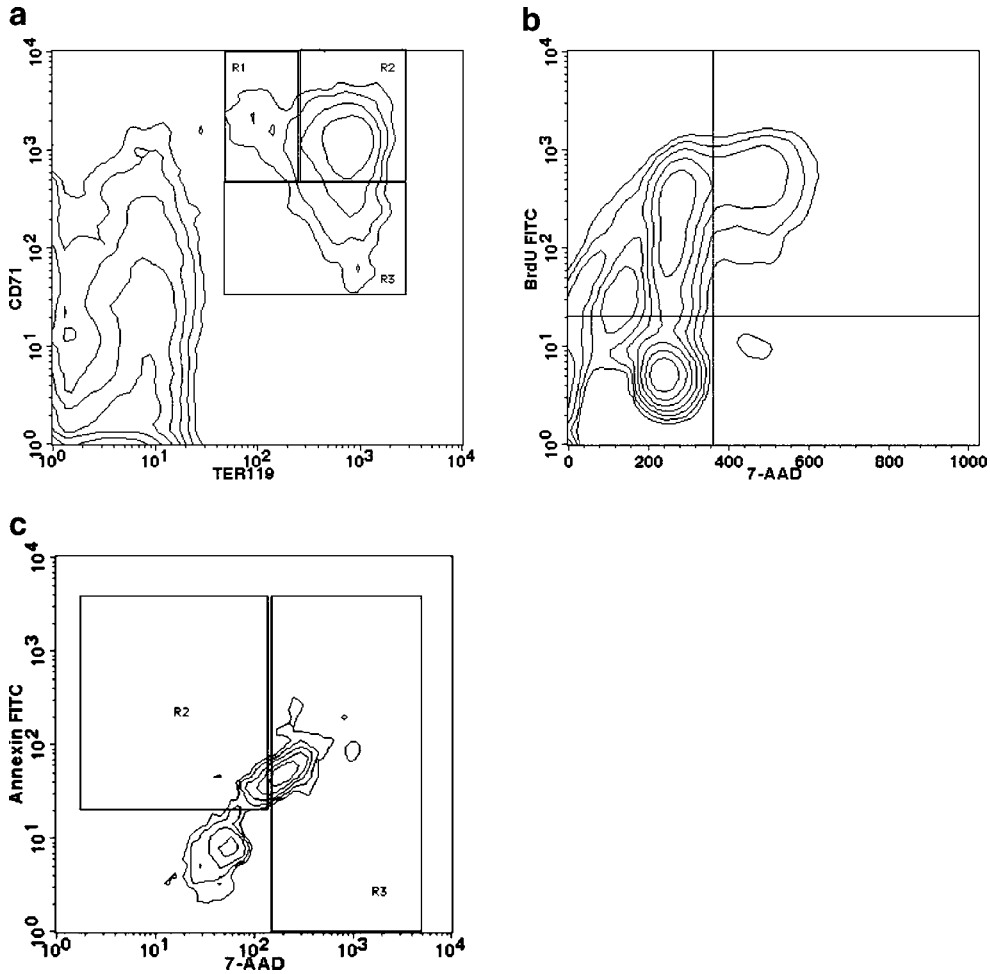

Fig. 2. Analysis strategy for flow cytometry. **a** A contour plot showing CD71 vs. TER-119 and the analytical strategy for enumeration and study of proerythroblasts (R1), basophilic erythroblasts (R2), poly-orthochromic erythroblasts (R3). **b** A contour plot showing the analytical strategy for study of cell cycle. Upper right quadrant S Phase, lower right quadrant G2M Phase. **c** A contour plot showing the analytical strategy for study of live, apoptotic cells (R2) and dead cells (R3).

Table 2. Characterization of Erythroid Precursors Control Murine Bone Marrow

Cell Type	Parameter	% (Median)	(Interquartile Range)
Pro Erythroblasts	% Bone Marrow	3	1–5
	% Erythroid	17	8–25
	% S Phase	23	14–30
	% G2M Phase	17	6–27
	% Annexin V+	14	7–21
	% 7AAD+	10	6–28
Basophilic Erythroblasts	% Bone Marrow	7	4–9
	% Erythroid	36	22–45
	% S Phase	54	41–62
	% G2M Phase	25	11–42
	% Annexin V+	4	3–7
	% 7AAD+	21	13–28
Poly/Orthochromic Erythroblasts	% Bone Marrow	5	3–10
	% Erythroid	28	18–51
	% S Phase	3	1–10
	% G2M Phase	2	0.4–6
	% Annexin V+	5	1–11
	% 7AAD+	11	8–19

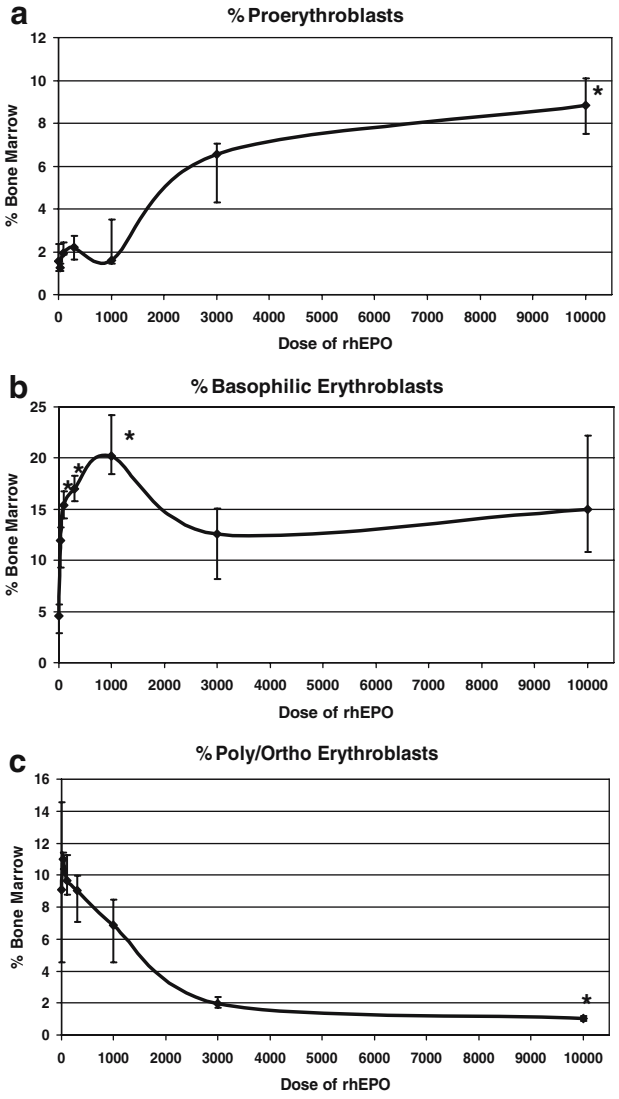

Fig. 3. Results of the dose range experiment. **a** Shows the number of proerythroblasts as % total cells in the bone marrow as a function of dose of rhEPO. **b**, **c** Show the data for basophilic erythroblasts and poly/orthochromic erythroblasts. A dose-dependent expansion of the proerythroblasts, a bimodal effect on basophilic erythroblasts and a dose-dependent decrease in poly/orthochromic erythroblasts are evident.

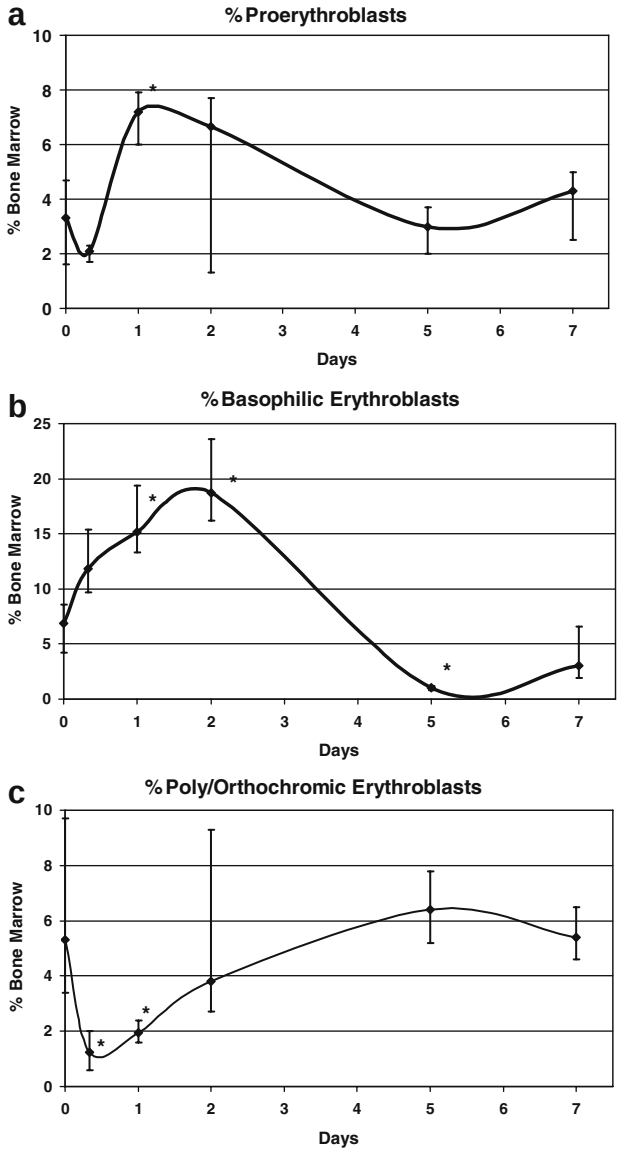

Fig. 4. Pharmacodynamic effects of rhEPO in bone marrow. This figure shows the median ± interquartile range for erythroid precursors (as % bone marrow) as a function of time. **a** Proerythroblasts. **b** Basophilic erythroblasts. **c** Polychromic and orthochromic erythroblasts. Expansion of the erythroid precursors that moves as a wave through the bone marrow is evident.

normal mice (20, 21). An appreciable fraction of Pro- and Baso-EB were in S and G2M phase while only a small fraction of Poly/OrthoEB were in S or G2M. Also as reported previously (7), an appreciable fraction of all stages of erythroblasts were apoptotic or dead. The method used to define dead cells, staining with 7-AAD, cannot discriminate between cells that died as a result of preparation of bone marrow cell suspensions. Thus, an unknown fraction of the dead cells may have been the result of experimental handling.

Dose Range

The composite results for the dose-dependent effects of rhEPO on Pro- Baso- and Poly/OrthoEB 48 hrs after dosing are shown in Fig. 3a–c. rhEPO caused a dose-dependent increase in the % of the bone marrow occupied by ProEB, had a bimodal effect on BasoEB and a dose-dependent decrease in Poly/OrthoEB. Because there was little difference in the response to 3,000 or 10,000 IU/kg, 3,000 IU/kg was chosen for the pharmacodynamic experiments.

Pharmacodynamics

Enumeration of Late Erythroid Precursors and Reticulocytes

Time course data for ProEB, BasoEB and Poly/OrthoEB expressed as % bone marrow are shown in Fig. 4a–c. rhEPO caused a statistically significant (approximately twofold) increase in the ProEB that peaked by 24 h then returned to the control range by day 5. There was appreciable increase in

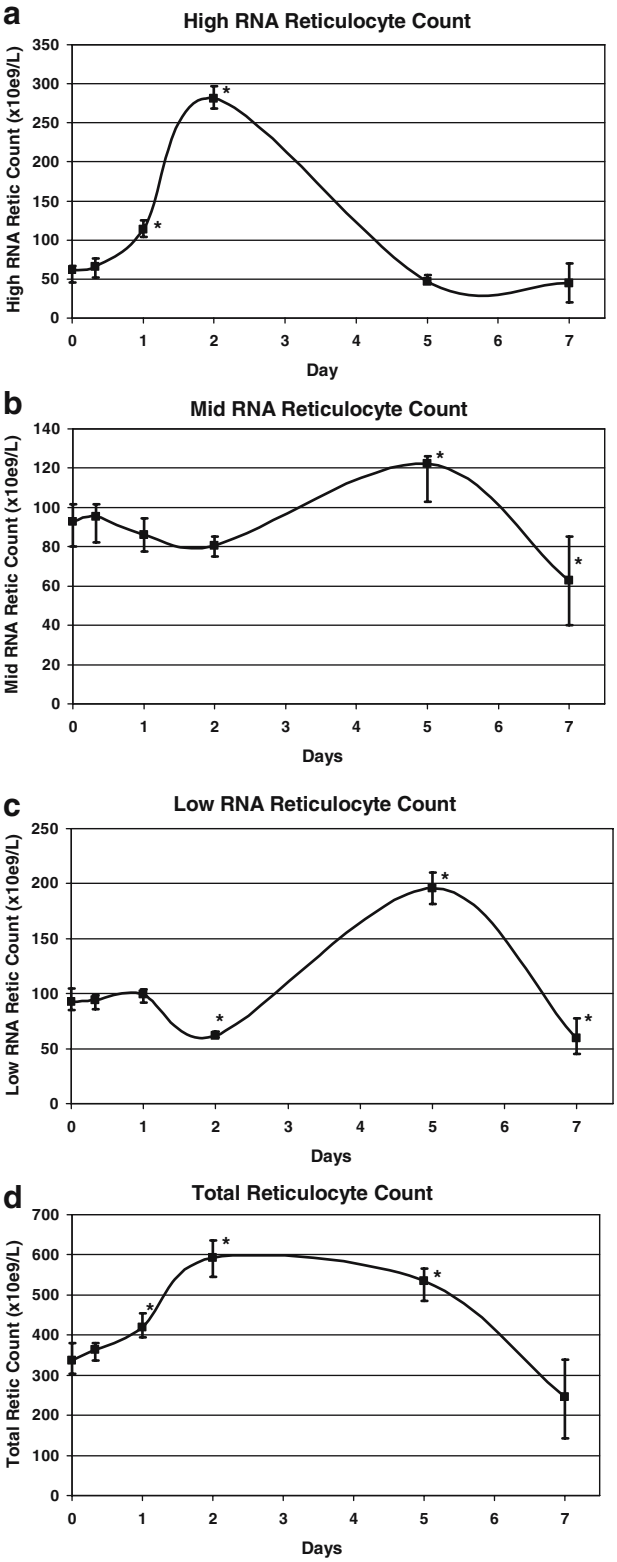

Fig. 5. Pharmacodynamic effects of rhEPO on reticulocytes in peripheral blood. This figure shows the median ± interquartile range for reticulocytes (as absolute number) as a function of time. **a** High-RNA (premature) reticulocytes. **b** Mid-RNA (immature) reticulocytes. **c** Low-RNA (mature) reticulocytes. **d** Total reticulocytes. Expansion of the reticulocytes that moves as a wave is evident.

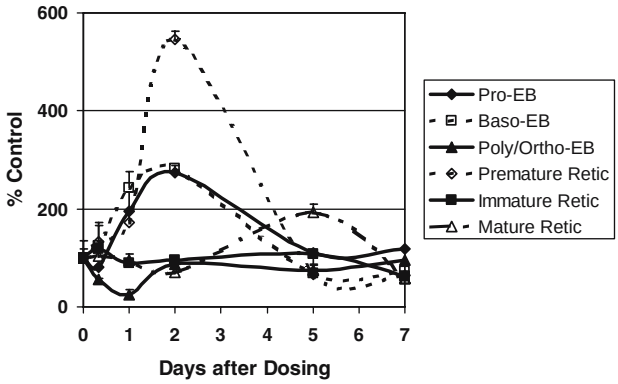

Fig. 6. Composite data for enumeration of erythroid cells in bone marrow and peripheral blood. This figure shows the median ± interquartile range for all erythroid cells normalized to % control to allow simultaneous plotting as a function of time. The sequential waves of expansion and contraction of the various populations is evident.

BasoEB by 8 h that was statistically significant at 24 h, peaked by 48 h (an approximately fourfold increase) and subsequently fell below the control range by day 5 (an approximately 50% decrease) before rebounding to the control range by day 7. Coincident with the increases in ProEB and BasoEB, there was a statistically significant decrease in Poly/OrthoEB that reached a nadir by 8 h (approximately 80% decrease), remained below the control range at 24 h and then returned to the control range by 48 h.

The results for the reticulocyte subsets and total reticulocytes are shown in Fig. 5a–d. Coincident with the decrease in Poly/OrthoEB in bone marrow, there was an increase in premature reticulocytes in the periphery by 24 h that peaked by 48 h (approximately fivefold) before returning to the control range by day 5. The increase in premature reticulocytes was followed in time by an increase in immature reticulocytes that peaked by day 5 (approximately 1.3 fold) and returned to the control range by day 7. Mature reticulocytes initially decreased between 24 and 48 h (approximately 40%) then increased (approximately twofold) before again falling below the control range (approximately 40%) by day 7. Total reticulocytes were increased by 24 h, peaked by 48 h (approximately twofold), were still elevated on day 5 and returned to the control range by day 7.

To better allow appreciation of the temporal relationships among the changes in the various cell types, data expressed as % control for all six cell types studied are shown in Fig. 6. The initial decrease in ProEB may reflect conversion from ProEB to BasoEB faster than the proliferation of ProEB and/or production of ProEB from erythroid progenitors which is responsible for the following increase in ProEB. The early increase observed in BasoEB probably resulted from this initial decrease in ProEB and was followed by a subsequent increase by continuous conversion to BasoEB from increased ProEB. Similarly, the conversion of Poly/OrthoEB to reticulocyte appeared to occur first before an increase in production from their predecessor cell, which was clearly seen in the rapid decrease in Poly/OrthoEB. The sequential expansion (or contraction) of each stage of erythopoiesis suggests that the effects of rhEPO move as a wave through the erythroid populations.

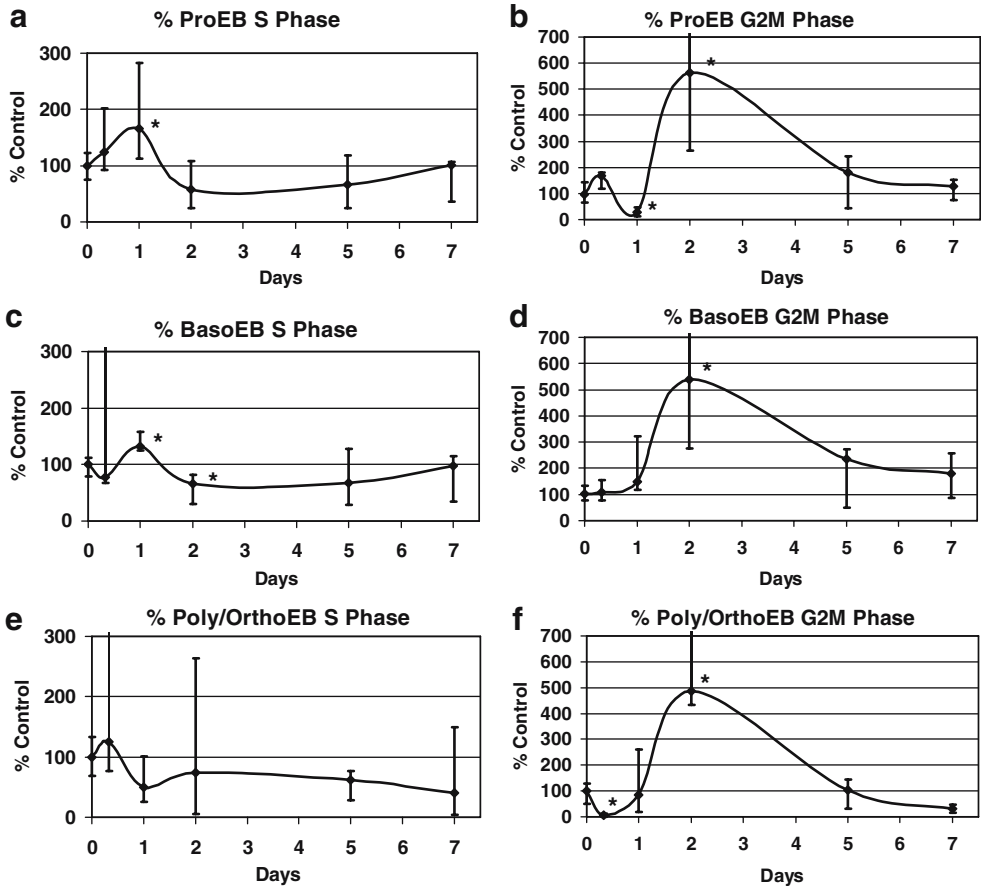

Fig. 7. Flow cytometric analysis of cell cycle in erythroid precursors in murine bone marrow. This figure shows the median ± interquartile range for S Phase and G2M Phase cells (as % cell type) as a function of time. **a**, **b** % proerythroblasts in S Phase and G2M. **c**, **d** % basophilic erythroblasts in S Phase and G2M. **e**, **f** % polychromic and orthochromic erythroblasts in S Phase and G2M. Sequential waves of expansion of the erythroid precursors in S Phase and G2M are evident.

Cell Cycle

Flow cytometric data for S Phase and G2M Phase the erythroid populations over time are summarized in Fig. 7a–f. A single dose of rhEPO caused a statistically significant increase in the fraction of Pro- and BasoEB in S Phase on day 1 and a statistically significant decrease in the fraction of BasoEB in S Phase on day 2. In contrast, there was no statistically significant effect on the fraction of Poly/OrthoEB in S Phase. There were statistically significant decreases in the fraction of ProEB and Poly/OrthoEB in G2M on day 1 and statistically significant increases in the fraction of all three stages in G2M on day 2.

Apoptosis and Cell Death

Flow cytometric data for annexin V and 7AAD for the erythroid populations over time are summarized in Fig. 8a–f. There was a statistically significant increase in apoptosis in ProEB and a trend toward an increase in apoptosis in BasoEB and on day 1. Similarly, the fraction of Poly/OrthoEB that were apoptotic was significantly increased on day 5. The effect of rhEPO on the fraction of dead cells followed a similar pattern. rhEPO caused a transient increase in the fraction of all three stages that were dead that peaked on day 1.

DISCUSSION

In this study, a four color flow cytometer and a flow based hematology analyzer were used to study the pharmacodynamic effects of a single dose subcutaneous dose of 3,000 U/kg of rhEPO on erythropoiesis in the bone marrow of normal mice. This study is the first to systematically and simultaneously evaluate the effects of a single dose of rhEPO on cell number, cell cycle and apoptosis and cell death in all morphologically defined erythroid precursors in the bone marrow as well as the stages of reticulocyte maturation in peripheral blood in intact mice. Although the effects of rhEPO have been studied in the past, prior work on EPO has by and large, studied the effects of multiple doses (22), used partially purified, natural EPO (22, 23) or have approached the issue in a piece meal, rather than a simultaneous analysis of cell cycle and apoptosis

In our study, we have found that in steady state murine bone marrow a sizable fraction of the Pro- and BasoEB fractions, are in S Phase. We have also found that considerable fractions of these cells as well as Poly/OrthoEB are either apoptotic (as measured by annexin-V binding) or dead (as measured by permeability to 7AAD). The results of this study have also shown that in normal mice, a single pharmacologic dose of rhEPO mediates time-dependent effects on cell number, cell cycle, apoptosis and maturation,

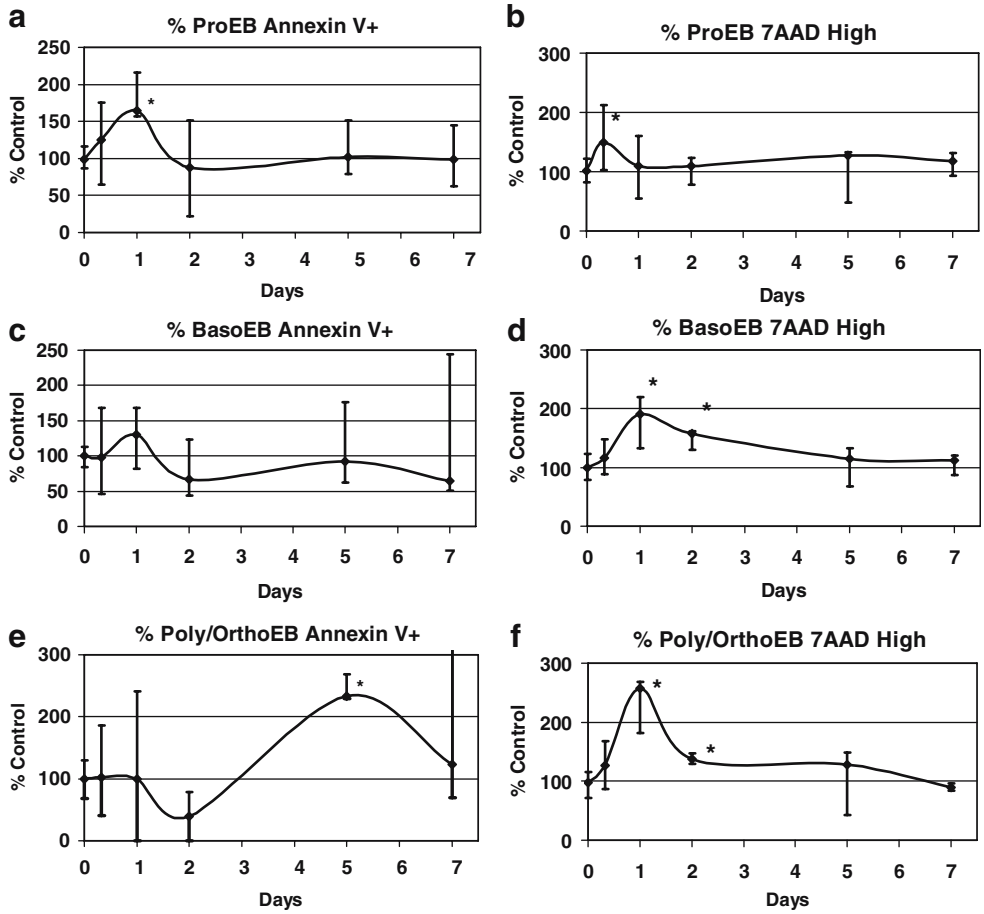

Fig. 8. Flow cytometric analysis of apoptosis and cell death in erythroid precursors in murine bone marrow. This figure shows the median ± interquartile range for apoptotic and dead cells (as % cell type) as a function of time. **a**, **b** % apoptotic and % dead proerythroblasts. **c**, **d** % apoptotic and % dead basophilic erythroblasts. **e**, **f** % apoptotic and % dead polychromic and orthochromic erythroblasts. Waves of expansion of the % apoptotic and % dead erythroid precursors are evident.

and that these effects move as a wave through the late-stage erythroid precursors and reticulocytes (Fig. 6).

In this study, a transient increase in ProEB and BasoEB that peaked 24 and 48 h after dosing, respectively was observed. Similar results were reported previously in mice made polycythemic (to suppress endogenous EPO and erythropoiesis) that received up to 4.5 cobalt units of sheep plasma EPO (23) and in normal mice that received 5 U of human urinary EPO every 12 h for 3–5 days (22). We also observed a transient decrease in Poly/OrthoEB followed by a step-wise increase in premature, immature and mature reticulocytes. Premature reticulocytes were significantly increased 24 h after injection of rhEPO and peaked at 48 h while immature and mature reticulocytes peaked 5 days after dosing with rhEPO. Similar results have been reported previously after a single intravenous injection of 50 U rhEPO or multiple injections of 5 U human urinary EPO in normal mice (21, 24) and in polycythemic mice receiving a single dose of 4.5 cobalt units of sheep EPO (23). Because it is generally accepted that it requires several days for a ProEB to mature into a reticulocyte (23, 24) this observation suggests that pharmacologic doses of EPO may influence maturation of the lattermost stages of erythroid precursors and release of reticulocytes. Although this has been suggested previously (25, 26), this observation is in conflict with the generally accepted conclusion that EPO does not act past the BasoEB stage of erythropoiesis. As shown by Broudy *et al.* (27), the number of EPO receptors on a cell surface peaks at about 1,000 receptors per cell at the CFU-e/ProEB stages and progressively declines as cells mature. However, although Broudy *et al.* were unable to detect EPO-R on reticulocytes, Lawrence *et al.* (28) have shown that EPO decreases Ca^{2+}-ATPase in rabbit reticulocyte membranes, suggesting that these cells may express active EPO-R, albeit in very low numbers.

In our study of the cell cycle, trends for an early increase and then a decrease in the fraction of erythroblasts in S Phase were observed. These trends moved as a wave through the Pro- and BasoEB and a statistically significant increase in the fraction of all three stages of erythroblasts in G2M, that peaked 48 h after dosing, was also observed. EPO is believed to be a growth factor for erythroid precursors, mediating proliferation these cells (29), but without altering the length of the cell cycle (22) or number of mitotic divisions involved in the differentiation process [reviewed in (4)]. As reported

previously, EPO *in vitro* (30) stimulates proliferation of Pro- and Baso-EB and EPO has also been shown to increase DNA synthesis concomitant to the increase in erythropoiesis in the spleen of polycythemic mice (31). Mirroring the effects of rhEPO on S phase, was an increase in the fraction of Pro- and BasoEB in G2M. These results are consistent with those of Fibach and Rachmilewitz (32) who studied the effects of EPO on cell cycle and differentiation of erythroblasts derived from human peripheral blood in a two-stage culture system. They found that when cultured with physiologic concentrations of EPO (50 mU/mL) erythroblasts differentiated to the orthochromic stage and arrested in G1. In contrast, when cultured with supraphysiologic concentrations (2 U/mL) erythroid cells arrested in G2.

One of the more surprising results of our study was the observation of a wave of increase in the fractions of erythroid precursors that were in apoptotic or dead. At the later time points studied (> 48 h) when rhEPO blood concentrations were low (>7 half-lives), our observations are consistent with the model proposed by Koury and Bondurant (33) that predicts that erythroid precursor populations that have expanded when EPO concentrations are high will involute by apoptosis when EPO concentrations fall. As recently reviewed by Testa (34), it is well established that depriving erythroid precursors of EPO results in induction of apoptosis and that replenishment of EPO can rescue cells starved for EPO from apoptosis. However, in our study, even when blood concentrations of rhEPO were at their highest (8 h after dosing), we found no evidence of a suppression of apoptosis or cell death. Thus, our results suggest that it may be inaccurate to categorize EPO as a simple inhibitor of apoptosis in erythropoiesis. Rather, the fate of any given erythroid precursor is likely the net result of an interplay among numerous factors: the density of EPO-R on the cells, and the concentration of EPO (35), feed-back inhibition of early precursors by more mature erythroid cells (5, 36–38) as well as cytokines (39, 40), transcription factors (41) and expression of pro- and anti-apoptotic factors by erythroid precursors, e.g., TNF-α, TRAIL, Fas, Fas ligand and Bcl-x (37, 42–44).

In conclusion, we have used four-color flow cytometric analysis to simultaneously study the effects of a single, clinically relevant, pharmacologic dose of rhEPO on cell numbers, cell-cycle and apoptosis in normal murine bone marrow. We have observed that a wave of proliferation and/or maturation progresses through all morphologically definable erythroid precursors resulting in the emigration of immature reticulocytes from the bone marrow into the periphery. With the exception of the decrease in the fraction of Poly/OrthoEB observed at 8 h, the maximal effects seen in our study occurred when blood concentrations of rhEPO were less than 1% of their maximal value, suggesting that the processes set in motion by pharmacologic doses of EPO can continue after EPO concentrations fall. Somewhat surprisingly, we failed to observe the suppression of apoptosis observed previously *in vitro*. As Epo is well known to be able to suppress apoptosis, these data suggest that concomitant with the stimulatory effects of EPO on proliferation and maturation, regulatory mechanisms are also engaged which down regulate erythropoiesis. This finding supports a role for feed-back inhibition in the control of erythropoiesis in the marrow stimulated by pharmacologic doses of rhEPO.

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