Recombinant Human Granulocyte Colony-Stimulating Factor Increases Circulating Burst Forming Unit-Erythron and Red Blood Cell Production in Patients With Severe Human Immunodeficiency Virus Infection

By Steven A. Miles, Ronald T. Mitsuyasu, Kyoung Lee, Jaime Moreno, Kirby Alton, Joan C. Egie, Lawrence Souza, and John A. Glaspy

Erythropoietin (EPO) is a major regulatory factor controlling red blood cell (RBC) production in humans. Although other humoral factors can alter the proliferation of committed early erythroid progenitors in vitro, no factor other than EPO has been clearly shown to induce proliferation of these cells in vivo. In a clinical trial of recombinant granulocyte colony-stimulating factor (G-CSF) and recombinant EPO in patients with advanced human immunodeficiency virus (HIV) infection, we noted reticulocytosis and increases in hemoglobin when G-CSF was administered before the administration of EPO. Subsequent studies demonstrated a significant increase in circulating burst forming unit-erythron (BFU-E) during daily recombinant G-CSF therapy. This increase was both time- and dose-dependent. The magnitude of increase in BFU-E correlated with the magnitude of increase in neutrophils and was associated with a mean increase in reticulocytes of 32.363/µL and a significant increase in mean hemoglobin of 1.04 ± 0.34 g/dL over an 18-day interval. There was a significant increase in iron binding capacity and decreases in iron saturation and ferritin levels. In patients who were not recently transfused, there was an associated fall in endogenous erythropoietin levels. The increase in RBC production was most marked in patients who were severely anemic, transfusion-dependent, and who had elevated pretreatment EPO levels. There was no correlation between the increase in BFU-E and endogenous EPO levels or the time since last dose of zidovudine. The addition of recombinant EPO therapy three times weekly to patients did not result in further significant increases in BFU-E but did significantly increase hemoglobin. Our data suggest that recombinant G-CSF may be one of the hematopoietic factors that influences production of BFU-E and RBCs in humans.

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REGULATION OF hematopoiesis in humans involves the interaction of several interdependent humoral factors. The production of red blood cells (RBCs) requires an orderly proliferation and differentiation of pluriptotent stem cells into committed erythroid progenitors. In a process of progressive lineage restriction and loss of self-renewal capacity, these committed progenitors proliferate and differentiate into mature RBCs. The hormone erythropoietin (EPO) is the primary factor that regulates this process in vivo.

Previous in vitro studies have identified the major target of action of EPO activity as the late RBC precursor, the colony-forming unit erythron (CFU-E). Under the influence of EPO, the CFU-E proliferates and differentiates into mature RBCs. Studies of recombinant EPO therapy in patients with chronic renal failure demonstrate that treatment with recombinant EPO is associated with increases in circulating CFU-Es and, to a lesser degree, earlier RBC precursors, including the burst forming unit erythron (BFU-E). EPO therapy also increased circulating myeloid precursors, including colony-forming unit-granulocyte monocyte (CFU-GM) and colony-forming unit-granulocyte megakaryocyte (CFU-GEMM). However, since EPO therapy is not associated with increases in white blood or platelet counts and in vitro studies fail to detect an effect of EPO on proliferation of highly enriched adult BFU-E in the absence of interleukin-3 (IL-3) or granulocyte monocyte colony stimulating factor (GM-CSF), the physiologic significance of these observations is unclear. It is possible that the increases in BFU-E, CFU-GM, and CFU-GEMM are a result of a nonspecific feedback stimulation of hematopoiesis or a local crowding effect in the bone marrow rather than a specific effect of EPO on committed hematopoietic progenitor cells. The failure to detect a direct effect of EPO on the earliest RBC precursors makes it doubtful that EPO is the sole factor regulating the earliest stages of erythropoiesis.

The role of cytokines other than EPO in regulating the early stages of erythropoiesis is poorly understood. Other factors, including IL-3, G-CSF, and GM-CSF, have been shown to increase the proliferation of early erythroid progenitors in vitro. One of these factors, G-CSF, has effects on early stem cells and may be involved in the production of multiple cell types. Studies using serum-free conditions demonstrate that G-CSF acts synergistically with IL-6 to decrease the cycling time of the hematopoietic stem cell between Go and GI, facilitating entry of larger numbers of cells into the pool of proliferating hematopoietic progenitors. Thus, it is possible that G-CSF might increase production of early RBC precursors. In mice, the administration of human G-CSF results in an eight- to 15-fold increase in splenic BFU-E and a fivefold increase in splenic pluripotent stem cells. However, the increase in splenic BFU-E was associated with a decrease in marrow BFU-E resulting in no overall increase in erythropoiesis. In humans, one study has reported on the effects of G-CSF on BFU-E formation.

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Similar to the mouse studies, this study reported increases in circulating BFU-E in two patients but there were corresponding decreases in bone marrow BFU-E. There was no increase in reticulocytes or hemoglobin, suggesting that the detection of increased numbers of BFU-E in the circulation might be due to premature release of erythroid precursors and not due to an increase in RBC production.

In a clinical trial of recombinant human G-CSF and EPO in patients with advanced human immunodeficiency virus (HIV) infection, which was designed to study the impact of these two hormones on the anemia and leukopenia of acquired immunodeficiency syndrome (AIDS) and zidovudine therapy, we noted reticulocytosis and increased hemoglobin levels when patients were administered G-CSF alone. To study the mechanism of reticulocytosis induced by G-CSF, we measured peripheral blood BFU-E before and during daily subcutaneous administration of recombinant G-CSF.

**MATERIALS AND METHODS**

**Patient population.** Twenty-two patients with severe HIV infection, leukopenia, and anemia were enrolled in a clinical study of two recombinant human hematopoietins, recombinant human G-CSF (AmGen, Thousand Oaks, CA) and recombinant human EPO (AmGen). There were no competing trials and all eligible patients were enrolled. Patients were required to discontinue all bone marrow suppressive drugs, including zidovudine and cotrimoxazole, for more than 4 weeks before study entry and to have a neutrophil count of <2,500/μL, hemoglobin of <11.5 g/dL, and platelet count of >50,000/μL after this washout period. Patients self-administered recombinant human G-CSF at 3 μg/kg by daily subcutaneous injection. This was continued until an absolute neutrophil count of >6,000/μL was achieved and maintained for 2 weeks. Patients whose neutrophil counts did not increase to >6,000/μL had their G-CSF dose escalated to a maximum of 6 μg/kg/d. At the completion of this phase, patients were administered recombinant human EPO starting at 150 IU/kg three times a week by subcutaneous injection. This was continued with increases of the daily dose by 50 to 100 IU/kg every other week until the patient’s hemoglobin level increased by at least 1.5 g/dL over the baseline value. Successful groups of eight patients were then placed on zidovudine at 1,000, 1,500, or 2,000 mg/d.

BFU-Es were measured for the first four patients at the completion of the recombinant human G-CSF phase. Subsequent patients had baseline, post G-CSF, and post G-CSF/EPO determinations of peripheral blood BFU-E performed when possible.

Because of the significant elevations of white blood cells seen with modest doses of recombinant G-CSF, the protocol was later amended to allow decreases of the G-CSF dose to maintain absolute neutrophil counts between 1,500 and 5,500/μL. Thus, patients received less recombinant G-CSF than the first group of patients studied.

**BFU-E assay.** Assays for BFU-E were performed in a standard protocol using normal human bone marrow as the control. Heparinized blood was diluted with an equal volume of Hank’s Balanced Salt Solution (GIBCO, Grand Island, NY) layered over Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at 400 g for 30 minutes at room temperature. Light density cells (specific gravity <1.077) were collected and washed twice in Iscove’s Modified Dulbecco’s Medium (IMDM) (GIBCO). Cells were resuspended in IMDM with 10% fetal calf serum (FCS) (GIBCO) at a concentration of 1 x 10^7/mL. Cells (1 x 10^7 cells/mL) were cultured in IMDM supplemented with 5 x 10^{-5} mol/L 2-mercaptoethanol (2ME) (Sigma, St Louis, MO), 0.9% methylcellulose (Dow Chemical Co), 30% FCS, and either 1 and 4 IU of human recombinant EPO (AmGen). Zidovudine (AZT) was added to the mixture and resulted in final concentrations of 0, 0.01 μmol/L, 0.1 μmol/L, and 1.0 μmol/L. Control cultures were simultaneously initiated using normal donor’s bone marrow light density cells. Erythroid burst colonies were scored after 14 days of culture in a humidified atmosphere containing 5% CO2. In our assays, peripheral blood from normals generally yielded approximately 75% of the bone marrow level of BFU-E.

**Statistical methods.** Data was entered into Dbase IV (Ashton-Tate, Carson, CA) files on an IBM PC (Armonk, NY). These files were checked by three individuals with the original data to ensure accuracy. The database was analyzed using PC-SAS version 6.02 statistical computing program (SAS Institute, Raleigh, NC). The two-sided sign rank test was used for analysis of pre- and posttherapy continuous variables, and the Spearman correlation coefficient was used for correlation analysis of continuous variables.

**RESULTS**

Data on the first 13 evaluable patients are summarized in Table 1. Two patients were removed from study, one because of death (patient no. 2), and one because of noncompliance (patient no. 3). These patients did not complete the combined treatment phase and are not evaluable for the effects of both hormones. Four patients were transfused within 4 weeks of starting G-CSF therapy. Because transfusions artificially alter endogenous EPO, reticulocyte, and hemoglobin levels making interpretation of the effects of G-CSF on these parameters difficult, we chose to use the nadir hemoglobin value after initiation of G-CSF and before EPO therapy as the baseline value. For all patients, the post G-CSF hemoglobin values were used for comparison. This technique correctly demonstrates the effect of G-CSF on the hemoglobin values.

There was a significant correlation between the measured BFU-E in the peripheral blood of patients treated with G-CSF using normal human bone marrow as a control and the dose of G-CSF at the time of measurement (r = .65, P = .02, see Fig 1). There was no correlation with the time since last dose of zidovudine or other hematopoietic suppressive agents, baseline hemoglobin, reticulocyte count, or endogenous EPO level.

In patients with both baseline and post G-CSF BFU-E determinations, there was a statistically significant increase in circulating BFU-E during G-CSF therapy (median increase, 34.5% of bone marrow normals, range -29% to 144%, n = 8, P = .039, sign rank test) (Fig 2). This increase correlated directly with both the dose of G-CSF at the time of second measurement (r = .75, P = .03) and the cumulative dose of G-CSF (r = .69) (Fig 3), and correlated inversely with the baseline BFU-E level (r = -.76, P = .028). Those patients who received the highest doses of G-CSF and/or had the lowest levels of circulating BFU-E at baseline had the largest increases with therapy.

For the entire cohort, the increase in circulating BFU-E with G-CSF was associated with a trend toward increased reticulocytes (mean increase ± SEM, 32,363 ± 17,186/μL, P = .08) and significant increases in hemoglobin levels.
G-CSF increases BFU-E in HIV infection

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Table 1. Summary of Laboratory Studies

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G-CSF doses are in micrograms per kilogram. The times since discontinuation of AZT are in days. BFU-E values are percent of a normal bone marrow control. The value in parenthesis are the mean value of the patient over the mean value of the control. The remainder of the values are expressed as percent microliter except as noted. As noted in the text, the nadir hemoglobin value after G-CSF therapy is used as the baseline for the four patients who received transfusions. The EPO levels were determined by radioimmunoassay (Smith Kline Laboratories, Van Nuys, CA) and confirmed in batch with an EPO enzyme linked immunosorbent assay. (AmGen Inc).

*Patient was transfusion-dependent before study but did not receive blood within 1 week.
†Patient was transfused within 1 week of study.

The biggest increases in hemoglobin were generally seen in the patients administered the highest cumulative G-CSF dose and the magnitude of increase in hemoglobin correlated with the magnitude of increase in absolute neutrophils (Table 1, r = .64, P = .026). As expected, the magnitude of increase in reticulocytes and hemoglobin correlated with the magnitude of decline in endogenous EPO levels (r = -.55, P = .052 and r = .71, P = .007, respectively). However, there was no correlation between increases in hemoglobin levels or reticulocytes and baseline endogenous EPO levels, suggesting that a factor other than EPO may have been responsible for the increase in RBC production. As further evidence of an increase in RBC production and, as a result, an increase in iron utilization, there was a significant increase in iron binding capacity with G-CSF therapy (mean increase 177 ± 47.7, sign rank P < .001). Because there was no significant change in iron levels (P = .29) there was a corresponding decrease in iron saturation and ferritin levels, although these were not statistically significant. Recombinant G-CSF therapy was associated with an increase in erythropoiesis in the four patients who were transfusion-dependent and had received transfusions within 4 weeks of the start of G-CSF therapy. All four of these patients became transfusion independent with G-CSF treatment.

Subsequent treatment with EPO resulted in a further increase in BFU-E formation (median 27, range 50% to 125%, P = .438), but this was not significant and may have been the result of continued G-CSF therapy (Fig 2). As expected, the addition of EPO to G-CSF therapy resulted in
significant increases in hemoglobin (1.97 g/dL ± 1.78, P = .007) and reticulocytes (126,836 ± 143,512, P = .008).

**DISCUSSION**

Regulation of hematopoiesis involves a complex, incompletely understood network of interdependent humoral growth and inhibitory factors.17-19 EPO is the major hormone that regulates erythropoiesis in humans. Treatment with EPO results in correction of anemia in patients with renal insufficiency and AIDS.5,6,20 Patients with normal or relatively low endogenous levels of EPO appear to be the most responsive to this agent.20 Because the major site of action of EPO is on the late stage committed erythroid progenitor cell (CFU-E), additional, as yet unidentified factors are likely to be involved in the recruitment of pluripotent stem cells into erythropoiesis.

In our study, treatment of HIV-infected patients with recombinant G-CSF resulted in a rapid, statistically significant increase in circulating BFU-E. Unlike other settings where BFU-E have been reported to be increased,8,16 the stimulation by G-CSF resulted in the full range of expected associated physiologic responses. We observed significant increases in hemoglobin levels and iron binding capacity with corresponding, but not significant, decreases in iron and ferritin levels. As a result of the increase in hemoglobin levels, the endogenous EPO levels decreased in those patients in whom the levels were not altered by prior transfusions.

These data demonstrate that G-CSF may directly or indirectly stimulate the production of circulating BFU-E and RBCs in humans. While circulating BFU-E were stimulated by G-CSF well above normal circulating levels in several patients, corresponding increases in reticulocytes were not uniformly observed. This suggests that, in the absence of sufficient endogenous EPO, these RBC progenitors may not proliferate and differentiate into mature RBCs. Thus, there may be a coordination of activity between G-CSF and EPO in erythropoiesis.

The mechanism by which G-CSF may increase circulating BFU-E formation is not known. In vitro studies suggest that both G-CSF and IL-6 may facilitate the entry of pluripotent CD34+ stem cells into active differentiation.13,14 These hematopoietins appear to decrease the Go resting time and may facilitate entry into the G1 phase. Once in the G1 phase, the stem cells can be stimulated by other hematopoietic factors including IL-6, IL-3, and perhaps IL-4 and IL-2 to differentiate into a variety of committed progenitor cells.21,22 The increases in bone marrow cellularity, bone marrow megakaryocytes, and circulating lymphocytes in our study suggest that
the increase in BFU-E observed was just one part of the overall stimulation of hematopoiesis that occurred with G-CSF.24

Alternative hypotheses for the increases in erythropoiesis that were seen while patients were on G-CSF include a clearing of subclinical infections by G-CSF with a resultant increase in iron mobilization for subsequent erythropoiesis, or premature release of RBC precursors into the circulation. The former hypothesis is unlikely as these patients had no evidence of infections before therapy despite extensive evaluations, including bone marrow sampling, and the rate of acquisition of new infections while on G-CSF was very high. Overall, 10 of 13 patients developed serious infections while on G-CSF. However, it is possible that G-CSF caused premature release of RBC precursors. In fact, previous groups have demonstrated that increases in circulating BFU-E in animals and patients treated with G-CSF15,16 were associated with corresponding decreases in bone marrow BFU-E. As a result, the increases in circulating BFU-E were not associated with an overall increase in erythropoiesis. Our data is only partially in agreement with these in vitro and in vivo observations. In contrast to the previous reports, we noted increases in reticulocytes and significant increases in hemoglobin levels. These changes were accompanied by changes in EPO levels and other indirect measures of iron utilization. Thus, our data do not support the hypothesis of premature release of RBC precursors into the circulation. Instead, our data suggest that G-CSF may have a physiologic role in erythropoiesis.

Moreover, of the myeloid factors studied in vivo to date, this effect appears to be unique to G-CSF. Previous studies of other myeloid factors, including GM-CSF and IL-3, have demonstrated an increase in BFU-E formation in vitro, but this has not been reported in human clinical trials. Our previous trial of GM-CSF in patients with AIDS did not demonstrate an increase in RBC production.25 A trial of recombinant GM-CSF in patients with myelodysplasia suggested a decrease in transfusion requirements in some patients during therapy, but this has not been verified in other trials in similar patients.26,27 In a trial of GM-CSF in aplastic anemia patients at our institution, no consistent increase in reticulocyte counts or circulating BFU-E were observed.28 In contrast, studies of recombinant G-CSF in aplastic anemia patients have shown an increase in reticulocytosis and hemoglobin in a minority of patients.29 In trials of G-CSF in patients with congenital neutropenia or myelodysplasia, some patients have had significant increases in their reticulocyte count and hemoglobin levels.30,31

In conclusion, our data support earlier reports of additional biologic activities both in vitro and in vivo for recombinant human G-CSF. We have demonstrated that G-CSF increases circulating BFU-E and hemoglobin levels in patients with advanced HIV infection. Additional clinical trials with G-CSF in which cytotoxic chemotherapy is not used are necessary to validate our observation that G-CSF may be involved in the regulation of erythropoiesis in humans. G-CSF has previously been shown to increase circulating hematopoietic progenitors, increase circulating neutrophils, and increase neutrophil number and function in humans.14,32 Studies in patients with Kostmann's syndrome, hairy cell leukemia, cyclic neutropenia, and chemotherapy-related neutropenia have been reported.33,34 We suggest that G-CSF may also have a role in other clinical situations such as aplastic anemia and other chronic anemia states where BFU-E formation is decreased. Clinical trials in these settings are warranted.

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Recombinant human granulocyte colony-stimulating factor increases circulating burst forming unit-erythron and red blood cell production in patients with severe human immunodeficiency virus infection

SA Miles, RT Mitsuyasu, K Lee, J Moreno, K Alton, JC Egrie, L Souza and JA Glaspy