Evidence for the Presence of CFU-E With Increased In Vitro Sensitivity to Erythropoietin in Sickle Cell Anemia

By R. Pennathur-Das, E. Alpen, E. Vichinsky, J. Garcia, and B. Lubin

To investigate the cellular events that accompany erythroid hyperplasia, we studied several effects of erythropoietin (Epo) on marrow CFU-E in sickle cell anemia (SCA). We measured CFU-E number, CFU-E growth as a function both of Epo exposure time and of Epo concentration, and suppression of Epo-induced CFU-E formation by anti-Epo antiserum. With 0.5 U Epo/ml, the number of CFU-E was elevated in SCA (1,087 ± 520) compared to normal (430 ± 130). CFU-E were formed even when Epo was immediately neutralized by a 1/150 dilution of anti-Epo. After 40 hr of Epo exposure, only 2% of total CFU-E were expressed in normal marrow, whereas 12%-40% of CFU-E were expressed in SCA. Inhibition of CFU-E growth required at least 1/50 dilution of anti-Epo in SCA and a 1/300 dilution in normal marrow. In contrast to normal, a small number (5%-20%) of CFU-E were expressed in the absence of added Epo in SCA, and this pool required a 1/150 dilution of anti-Epo for inhibition. The Epo dose–response curve in SCA revealed a peak in colony formation around 0.1 U Epo/ml and 0.5 U Epo/ml, whereas only one peak at 0.5 U Epo/ml was seen in normals. These data strongly suggest that, in response to the demands of chronic erythroid hyperplasia in SCA, a pool of CFU-E is present characterized by increased in vitro sensitivity to Epo.

SICKLE CELL ANEMIA (SCA) is a hereditary disease in which hemolytic anemia, bone marrow erythroid hyperplasia, and elevated levels of serum erythropoietin (Epo) are observed. Studies on in vitro erythroid hyperplasia and elevated levels of serum Epo concentration were observed. In order to investigate this erythroid colony formation in SCA have shown increased numbers of both CFU-E and BFU-E when compared to normal. In SCA, the number and size, CFU-E growth as a function of Epo exposure time, suppression of Epo-induced CFU-E formation by anti-Epo, and Epo dose–response of CFU-E. The results of these studies suggest increased in vitro sensitivity of CFU-E to Epo in SCA.

MATERIALS AND METHODS

Human bone marrow aspirates were obtained, after informed consent, from four patients with SCA and from 7 control subjects. The serum Epo concentration was measured by radioimmunoassay, as described by Garcia et al. The method used [125I]-labeled pure human Epo and anti-Epo antiserum prepared from rabbits immunized against human Epo. The assay is sensitive to an absolute amount of Epo equivalent to 0.4 mU.

The anti-Epo antiserum was prepared by immunizing rabbits with low specific activity human urinary Epo prepared in this laboratory. A volume of 0.1 ml was placed in a multiwell tissue culture dish with bovine citrated plasma and allowed to clot. For timed Epo exposure studies, the Epo present in culture was neutralized by addition of 0.15 ml of partially purified anti-Epo serum (1/150 dilution) to the plasma clots at specified times.

For studies on the suppression of Epo-induced CFU-E formation as a function of anti-Epo dilution, we initiated plasma clots as described above and overlaid them with 0.15 ml of various dilutions of anti-Epo at 0 time.

All cells were allowed to grow in culture for 7 days at 5% CO₂ and 37°C, after which the clots were fixed in situ with 5% glutaraldehyde for 6 min and then stained with benzidine. The numbers and size distribution of colonies were then determined by counting benzidine-positive colonies containing either >8 cells, >16 cells, or >32 cells.

RESULTS

The hemoglobin concentration, reticulocyte count, serum Epo concentration, and in vitro response to Epo in SCA are summarized in Table 1. All four patients were anemic (mean Hb 8.3 g/dl) and had high reticulocyte counts (mean 17.5%), which is consistent with erythroid hyperplasia. The serum Epo level was elevated in all patients, with a mean of 137 mU/ml.
compared to a normal level of 18 mU/ml. The number of CFU-E obtained with 0.5 U/ml Epo was elevated in all patients (mean 1,087 ± 520) compared to normal (mean 430 ± 130). CFU-E in SCA consisted mostly of 8–16 cells (73%), whereas such small colonies represented only 45.5% of the total colonies in normal marrow CFU-E.

The results of timed exposure studies on four patients are shown in Fig. 1, where both ≥8 cell (Fig. 1A) and ≥16 cell (Fig. 1B) colonies are represented as a function of Epo exposure time. From 5% to 25% of the CFU-E were expressed in SCA, even when Epo was immediately neutralized (Fig. 1A); this type of response was never observed in normal marrow. In SCA, 12%–40% CFU-E were expressed after only 40 hr of Epo exposure, whereas only 0%–4% CFU-E were expressed after 40 hr of Epo exposure in normal. About 8%–20% of large colonies were expressed when Epo was present for 70 hr in SCA, compared to 0%–1% observed in normal (Fig. 1B).

We conducted anti-Epo titration experiments in order to see if we could entirely suppress CFU-E formation with higher concentrations of anti-Epo. As shown in Fig. 2, a 1/300 dilution of anti-Epo completely suppressed normal CFU-E expression, whereas a much higher concentration, i.e., at least 1/50 dilution of anti-Epo, was required for complete suppression of CFU-E in SCA.

The Epo dose–response curves obtained with normal and SCA marrow CFU-E are shown in Fig. 3. CFU-E formation in SCA was unique, in that 5%–25% of the maximum response was obtained in the absence of added Epo; a shoulder in CFU-E response was noted around 0.05–0.1 U/ml Epo and another peak, similar to that in normals, around 0.5 U/ml Epo. In 1 of the 4 patients so studied, CFU-E expressed in the absence of added Epo, could be completely suppressed by anti-Epo (1/150 dilution), demonstrating their Epo requirement.

DISCUSSION

We measured CFU-E formation in SCA, a disease characterized by marrow erythroid hyperplasia, in order to investigate potential alterations in the in vitro response of erythroid-committed stem cells to Epo as a consequence of chronic in vivo hyperproliferation. At a fixed Epo concentration, CFU-E growth was greater in SCA than in normal marrow. This finding is similar to that reported by Lutton et al. and is consistent with an
increased number of erythroid progenitor cells and/or increased sensitivity of CFU-E to Epo. The predominance of small colonies in SCA contrasted sharply to colony size in normals, which suggests a pool of mature CFU-E in SCA. The relationship between colony size and maturity in CFU-E is much like that reported for BFU-E.7,11

Our results demonstrate heterogeneity in the CFU-E pool in SCA that is characterized by a subset of CFU-E with increased in vitro sensitivity to Epo. We can support this conclusion by a number of observations. First, considerable numbers of CFU-E are formed when the Epo is neutralized by anti-Epo shortly after initiation of culture. Second, very high anti-Epo concentrations are necessary to cause complete suppression of Epo-induced CFU-E formation. Third, a small percent of CFU-E, which can be completely suppressed by anti-Epo, is formed in the absence of added Epo. Such endogenous colonies were never observed in normal marrow. Finally, Epo dose-response curves demonstrate considerable colony formation at low Epo concentrations. These observations are all distinct from the CFU-E response in normal marrow. Taken together, these results very strongly suggest the presence of a "highly sensitive" CFU-E pool in SCA.

The increased in vitro Epo sensitivity of the CFU-E pool in SCA may arise from conditioning of the cells by high in vivo Epo concentration, from increased number of Epo receptors, and/or from increased binding affinity of Epo to these receptors. Although we cannot distinguish between these alternatives from our experiments, it is likely that the high in vitro sensitivity of CFU-E to Epo in SCA arises from prior in vivo conditioning of the CFU-E. In polycythemias (PV), where such highly sensitive CFU-E have been reported,12,15 the situation is likely to be very different. Because the in vivo Epo concentration in PV is low, the high in vitro sensitivity most likely reflects an increased number of EPO receptors or an increased affinity of these receptors to Epo. Although in vitro erythroid colony formation in the absence of Epo has been previously demonstrated in other disease states, such as iron deficiency,16 megaloblastic anemia,16 and SCA,1 increased sensitivity of CFU-E to Epo has only been previously demonstrated in PV.12,13

The amount of anti-Epo needed to completely suppress CFU-E formation in the presence of 0.5 U/ml Epo is at least 6 times greater in SCA (1/50) than that required for normals (1/300). We have demonstrated that the anti-Epo is specific and reversible at concentrations required for suppression of CFU-E in SCA. The requirement of high anti-Epo concentrations suggests that the CFU-E in SCA may already have Epo bound on their receptors, perhaps due to high in vivo Epo concentrations. The cell-bound Epo may have its anti-Epo binding locus hidden, such that it is inaccessible for anti-Epo. The excessive anti-Epo then serves to displace Epo from the erythroid-committed cell and to shift the equilibrium toward free Epo by binding to the free Epo. Alternatively, cell-bound Epo may have a weaker binding affinity to anti-Epo than free Epo.

Erythroid progenitor cells with increased sensitivity to Epo have been demonstrated in several animal models.17-22 Using timed Epo exposure studies, similar to those employed in this investigation, Kennedy and Alpen have demonstrated an Epo-sensitive CFU-E pool in murine marrow.18 Monnet et al. have identified erythroid clusters that have increased sensitivity to Epo in mice.19,20 Roodman et al. have observed results similar to those of Kennedy et al. in the ovine system.21 Finally, Macklis et al. have observed a highly Epo-sensitive CFU-E pool in simian marrow.22 Although we were unable to identify such an Epo-sensitive pool of CFU-E in normal human marrow, this pool was consistently identified in SCA. Whether this is a common occurrence in all states of erythroid hyperplasia needs to be tested.

ACKNOWLEDGMENT

We thank Marion Douglass for typing this manuscript and Mary Pirruccello for editorial review. We acknowledge the help of Tamlyn Yee and Charlotte Smith in counting the colonies.

REFERENCES

Evidence for the presence of CFU-E with increased in vitro sensitivity to erythropoietin in sickle cell anemia

R Pennathur-Das, E Alpen, E Vichinsky, J Garcia and B Lubin