In Vivo Erythropoietin Requirements of Regenerating Erythroid Progenitors (BFU-e, CFU-e) in Bone Marrow of Mice

By Kodethloor B. Udupa and Kurt R. Reissmann

Erythroid progenitors (B-8, B-4, CFU-e) in the femoral marrow of polycythemic mice were measured by in vitro culture assays after a single administration of BCNU or Myleran. BCNU reduced pluripotent stem cells to 40% and erythroid progenitors to less than 5% of normal. B-8, the earliest erythroid progenitors, regenerated without erythropoietin (Epo) completely within 5 days. At 14 days after BCNU, intermediate progenitors (B-4) attained 60% of their normal numbers and CFU-e attained approximately 30%. Daily injections of Epo promptly restored normal B-4 numbers and near-normal CFU-e numbers in BCNU-treated mice. After Myleran, CFU-e remained below 2% of normal for 14 days, and no regeneration of the B-8 occurred with or without daily Epo injections. The findings suggest that regeneration of B-8 was dependent on cell inflow from the pluripotent stem cell compartment but was independent of the presence of Epo. Intermediate progenitors (B-4) required Epo and the presence of B-8 for complete and permanent regeneration. CFU-e were the most Epo-dependent of the three progenitors. B-4, recruited by Epo, required after their formation a second exposure to the hormone in order to progress into the CFU-e stage.

IN VITRO CULTURE TECHNIQUES have provided direct evidence of morphologically unidentifiable erythroid progenitors whose existence had been postulated earlier on theoretical grounds and on the basis of indirect evidence. This multistage age-structured compartment is interposed between pluripotent stem cells (CFU-s) and proerythroblasts. Its function lies in the multiplication of a relative small number of committed erythroid (stem) cells. This adaptable multiplication maintains at all rates of erythropoiesis a sufficiently large number of precursors (ERC) for transformation into proerythroblasts. Three murine erythroid progenitors can be quantitated by in vitro culture assays. They represent sequential maturation stages and comprise, in order of immaturity, day-8 BFU-e (referred to as B-8), which form large erythroid colonies after 8 days of culture, day-4 BFU-e (B-4), an intermediate stage that forms clusters of small erythroid colonies after 3–4 days of culture, and CFU-e, a late progenitor stage including most likely the immediate precursors of proerythroblasts.

In vitro, the formation of colonies of erythroblasts derived from BFU-e requires the presence of a relatively large concentration of erythropoietin (Epo), whereas the growth of CFU-e requires only one-tenth of that Epo concentration. The present study is concerned with the in vivo Epo requirements of the three erythroid
Epo regulation of BFU-e SNF CFU-e

progenitors and seeks information on the following questions: (1) Is Epo required for the restoration of normal population size of the three progenitors after severe reduction of their numbers by cytotoxic drugs? (2) Is such restoration dependent on cell inflow from the pluripotent stem cell compartment, or can the populations of the erythroid progenitors be restored for any length of time by their self-replication? (3) Is repeated exposure to Epo required for an in vivo progression of BFU-e into CFU-e, or is one-time contact of early BFU-e with Epo sufficient to activate the maturation process?

By in vitro assays, measurements were obtained on the numbers of the three progenitor types present in the femoral marrow of mice whose endogenous Epo was suppressed by posthypoxic polycythemia. Each mouse received a single administration of either 1,3-bis(2-chlorethyl)-1-nitrosourea (BCNU) or Myleran, which reduced the erythroid progenitors to less than 5% of normal. BCNU reduced the pluripotent stem cells (CFU-s) to about 40% of normal, whereas Myleran induced long periods of near-absence of CFU-s. A comparison of erythroid progenitor restorations in these two models provided information on the role of cell inflow from the CFU-s compartment in this process. Effects of Epo on progenitor formation and progression were assessed by injecting the polycythemic mice with Epo at various times after administration of the cytotoxic drug.

MATERIALS AND METHODS

Animals and Injections

All experiments were carried out on female CF1 mice (25 ± 3 g) made polycythemic by 3 wk of exposure (16 hr/day) at an atmospheric pressure of 370–340 mm Hg. On the third day after return to a normal environment, each mouse received either BCNU (35 mg/kg) i.p. or Myleran (44 mg/kg) by stomach tube. The Myleran was dissolved in acetone (11 mg/0.5 ml) and then diluted with corn oil. Only mice whose hematocrits had remained above 60% during the 7 days following drug administration were included in the results. In the longer experiments the mice received intravenous injections of 0.4 ml of washed red cells (buffy coat removed) on day 6, and their hematocrits remained above 60% through day 14. Human urinary Epo (pool 13, 57 U/mg, kindly supplied through the Erythropoietin Committee, National Heart, Lung and Blood Institute) was injected subcutaneously in doses as indicated.

Assays for CFU-e, BFU-e, CFU-c, and CFU-s

Mice were killed at daily intervals after BCNU or Myleran administration. The femur was removed, and its marrow was flushed out with Eagle’s MEM (GIBCO). Marrow particles were drawn back and forth through a 19- and 21-gauge needle. An aliquot of each suspension was used for cell counting in a Coulter counter and other aliquots for the progenitor assays. CFU-e were measured by a modification10 of the 2-day plasma-clot method of McLeod et al.1 The medium contained 10⁻¹⁰ M thioglycerol and 0.3 U of human Epo per milliliter. BFU-e were cultured in methyl cellulose according to the method of Iscove and Sieber.1 The medium contained 2 U of step-three sheep Epo (Connaught Laboratories, Willowdale, Ont.) and was plated in 0.1-ml aliquots in the wells of flat-bottomed Microtiter plates (8 wells per sample). By use of criteria formulated by Gregory,7 the erythroid colonies were scored in situ by two observers under an inverted microscope on day 4 for B-4 and on day 8 for B-8. CFU-c were assayed according to the method of Bradley and Metcalf as described earlier.11 CFU-s were assayed by the exocolonization method.12 The femoral marrows from 3 mice were pooled, and suitable aliquots of each sample were injected into groups of 10 irradiated mice (850 rads, linear accelerator). Their spleen colonies were scored on day 9.

RESULTS

Is Epo Required for Regeneration of BFU-e and CFU-e?

Figure 1 shows the percentile decrease in hematopoietic progenitor cells resulting from a single injection of BCNU in posthypoxic polycythemic mice. At 48 hr after
BCNU the CFU-s were reduced to 40% of normal, and they showed progressive regeneration thereafter. The granulocytic progenitors (CFU-c) were 25% of normal at 48 hr after BCNU, and they returned to normal values on day 5. The lower panel shows the effects of BCNU on the three erythroid progenitor stages and their regeneration in the absence of Epo. All three stages were reduced to less than 5% of normal on day 2 after BCNU, but their subsequent regeneration revealed marked differences among the three progenitors. The early precursors B-8 returned to normal values on day 5 after BCNU administration. The intermediate erythroid progenitors B-4 regenerated to only 40% of normal on day 5, and after a dip on day 7 (p < 0.02), they reached 60% of normal 14 days after BCNU administration. The late progenitors (CFU-e) showed little regeneration in the absence of Epo.

Table 1. Regeneration of Erythroid Progenitors in Femoral Marrow of Polycythemic Mice With or Without Daily Injections of 1 U of Epo After Administration of BCNU on Day 0; Means and SEM of 6 Mice in Each Group

<table>
<thead>
<tr>
<th>Days After BCNU</th>
<th>Day-8 BFU-e/Femur</th>
<th>Day-4 BFU-e/Femur</th>
<th>CFU-e/Femur ( \times 10^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal*</td>
<td>2563 ± 207</td>
<td>11,854 ± 1286</td>
<td>169 ± 12</td>
</tr>
<tr>
<td>Polycythemic+</td>
<td>3031 ± 580</td>
<td>9,188 ± 1200</td>
<td>45 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Epo</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>112 ± 54</td>
<td>16 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>132 ± 36</td>
<td>168 ± 61</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>362 ± 94</td>
<td>497 ± 78</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1166 ± 147</td>
<td>1469 ± 393</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2421 ± 234</td>
<td>4079 ± 933</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2426 ± 372</td>
<td>4625 ± 456</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2090 ± 190</td>
<td>2875 ± 808</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2130 ± 275</td>
<td>2063 ± 406</td>
</tr>
</tbody>
</table>

*Means and SEM in 10 nonpolycythemic and in 10 posthypoxic (day 6) polycythemic mice without BCNU.
†Significantly (p < 0.01) different from group without Epo.
Table 2. Regeneration of CFU-s and of Erythroid Progenitors in Femoral Marrow of Polycythemic Mice With or Without Daily Injections of 5 U Epo After Administration of Myleran on Day 0; means and SEM of 6 Mice in Each Group

<table>
<thead>
<tr>
<th>Days After</th>
<th>Epo</th>
<th>O</th>
<th>Epo</th>
<th>O</th>
<th>Epo</th>
<th>O</th>
<th>Epo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myleran</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38 ± 21</td>
<td>70 ± 51</td>
<td>106 ± 19</td>
<td>37 ± 23</td>
<td>575 ± 221</td>
<td>0.6 ± 0.1</td>
<td>16 ± 31</td>
</tr>
<tr>
<td>3</td>
<td>9 ± 4</td>
<td>33 ± 29</td>
<td>47 ± 30</td>
<td>38 ± 11</td>
<td>1208 ± 2131</td>
<td>1.0 ± 0.4</td>
<td>31 ± 13</td>
</tr>
<tr>
<td>4</td>
<td>21 ± 6</td>
<td>44 ± 27</td>
<td>67 ± 21</td>
<td>25 ± 14</td>
<td>1369 ± 3044</td>
<td>0.8 ± 0.4</td>
<td>51 ± 94</td>
</tr>
<tr>
<td>5</td>
<td>35 ± 29</td>
<td>79 ± 19</td>
<td>81 ± 37</td>
<td>2463 ± 2014</td>
<td>4.0 ± 2.0</td>
<td>68 ± 12</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>58 ± 7</td>
<td>16 ± 6</td>
<td>143 ± 42</td>
<td>156 ± 94</td>
<td>3969 ± 6401</td>
<td>1.7 ± 1.5</td>
<td>127 ± 34</td>
</tr>
<tr>
<td>7</td>
<td>71 ± 36</td>
<td>62 ± 10</td>
<td>179 ± 11</td>
<td>2230 ± 1944</td>
<td>4.2 ± 1.6</td>
<td>160 ± 12</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>56 ± 10</td>
<td>32 ± 18</td>
<td>104 ± 82</td>
<td>188 ± 97</td>
<td>1193 ± 1514</td>
<td>9.2 ± 3.4</td>
<td>131 ± 36</td>
</tr>
<tr>
<td>10</td>
<td>108 ± 11</td>
<td>10 ± 6</td>
<td>94 ± 74</td>
<td>63 ± 36</td>
<td>1688 ± 3114</td>
<td>5.7 ± 3.4</td>
<td>116 ± 12</td>
</tr>
<tr>
<td>12</td>
<td>161 ± 16</td>
<td>16 ± 5</td>
<td>91 ± 36</td>
<td>31 ± 14</td>
<td>406 ± 170</td>
<td>5.5 ± 2.1</td>
<td>39 ± 51</td>
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<tr>
<td>14</td>
<td>169 ± 12</td>
<td>47 ± 16</td>
<td>219 ± 91</td>
<td>250 ± 114</td>
<td>813 ± 238</td>
<td>10 ± 6.5</td>
<td>58 ± 16</td>
</tr>
</tbody>
</table>

*Means and SEM in 10 nonpolycythemic and in 10 posthypoxic (day 6) polycythemic mice without Myleran.
†Pooled marrow of 3 mice.
‡Significantly (p < 0.01) different from group without Epo.

Daily injections of Epo accelerated the recovery of B-8 on day 2 and 3 after BCNU, but they had no significant effect on the number of B-8 on subsequent days (Table 1). In contrast, the numbers of B-4 and CFU-e were significantly greater in the Epo-injected BCNU mice, and normal B-4 numbers were attained on day 5.

**BFU-e and CFU-e Regeneration During Near-Absence of CFU-s**

A single oral administration of Myleran (44 mg/kg) reduced the femoral CFU-s to about 1% of their normal number. There was no significant difference in CFU-s between the Epo-injected and noninjected groups, and only values in the former are presented in Table 2. The B-8 were suppressed 24 hr after the drug to approximately the same level (2% of normal) as those found in the BCNU-injected mice. In sharp contrast to the latter, no regeneration of B-8 was seen in the Myleran-treated mice either with or without daily injections of Epo. Without Epo injections, the B-4 and CFU-e also remained at levels of a few percent of normal throughout the observation period of 14 days. Daily injections of Epo resulted in wavelike increases in B-4 and CFU-e, illustrated in Fig. 2. In these mice the B-4 reached 40% of normal on day 6 after Myleran, and the CFU-e rose to 88% of normal on day 7. The increases were transient, and the numbers of both progenitor types decreased rapidly from day 8 to 14 after Myleran.

**Effect of Epo on Progression of BFU-e into CFU-e**

As seen in the third column of Table 3, a single Epo injection into polycythemic mice without prior cytotoxic drug administration resulted within 24 hr in an increase in CFU-e from $43 \times 10^3$ to $164 \times 10^3$ per femoral marrow. A single injection of 4 U of Epo given 24 hr after BCNU raised femoral marrow B-4 from 112 to 7048 within 48 hr, but this very large increase in B-4 was not accompanied or followed by a proportional rise in the number of CFU-e. In order to test whether or not an additional exposure to Epo would produce a greater recruitment of CFU-e, a second injection of Epo was given on day 3 after BCNU. It increased the
Fig. 2. Transient regeneration of B-4 and CFU-e in near-absence of CFU-s in B-8 in polycythemic mice receiving daily injections of 5 U of Epo after Myleran administration on day 0. Means and SEM of 6 mice in each group.

CFU-e by 500% within 24 hr, and the time course of the increase was similar to that seen after Epo injection in polycythemic mice without BCNU.

DISCUSSION

Accepting nearly complete suppression of Epo formation in the polycythemic mouse, our findings in the BCNU-treated mice clearly show that restoration of early erythroid progenitors (B-8) does not require the presence of Epo. Our results are in agreement with those of Greenberg et al., who observed a similar time course in B-8 regeneration in polycythemic mice after marrow suppression by repeated injections of ara-C. In our experiments the numbers of femoral B-8 rose in the absence of Epo from 56 ± 42 on day 1 to a normal range of 242 ± 234 on day 5 after BCNU. The results represent strong evidence that Epo is not required in the

Table 3. Recruitment of CFU-E by One or by Two Epo Injections in the Femoral Marrow of Polycythemic Mice After BCNU Administration on Day 0. Third Column Shows Effect of Epo on CFU-E in Polycythemic Mice Without BCNU. Means and SEM of 6 Mice in Each Group

<table>
<thead>
<tr>
<th>Day</th>
<th>BCNU on Day 0</th>
<th>No BCNU</th>
<th>BCNU on Day 0</th>
<th>No BCNU</th>
<th>BCNU on Day 0</th>
<th>No BCNU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 U of Epo on Day 1</td>
<td></td>
<td>4 U of Epo on Day 1</td>
<td></td>
<td>1 U of Epo on Day 1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>112 ± 54</td>
<td>43 ± 5</td>
<td>16 ± 3</td>
<td>164 ± 5</td>
<td>102 ± 7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1894 ± 381</td>
<td>56 ± 8</td>
<td>28 ± 2</td>
<td></td>
<td>36 ± 4</td>
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<tr>
<td>3</td>
<td>7048 ± 501</td>
<td></td>
<td>26 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4406 ± 914</td>
<td>127 ± 12</td>
<td>25 ± 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4140 ± 463</td>
<td>98 ± 9</td>
<td>24 ± 5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>6</td>
<td>3583 ± 611</td>
<td>34 ± 6</td>
<td>17 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CFU-e per femoral marrow × 10⁻³.
formation of B-8. The same conclusion has been suggested by several investigators on the basis of small increases rather than decreases in the numbers of B-8 in mice made polycythemic by hypertransfusion. No B-8 regeneration took place in the Myleran-treated mice. Their pluripotent stem cells (CFU-s) remained at levels below 3%, in contrast to the level of above 40% found in the BCNU-treated mice. This difference points toward the essential role of cell inflow from the CFU-s compartment in the restoration of erythroid progenitors. One could argue that the Myleran damaged B-8 proliferation for prolonged periods, as well as that of CFU-s, and that the lack of B-8 regeneration was thus caused by prevention of their self-replication rather than by lack of cell inflow from the CFU-s compartment. This explanation is not supported by the fact that B-8 that were present in Myleran-treated mice formed in vitro erythroid colonies of normal size and within the usual culture period. The combined results of the BCNU and Myleran experiments are thus consistent with the concept that cell inflow from CFU-s into the committed erythroid (stem) cell compartment is essential for restoration of a normal B-8 population and that this process does not require the presence of Epo.

Although Epo was not required for complete restoration of a normal B-8 population, it nevertheless induced, on days 2 and 3 after BCNU, significant increases in B-8 over those in controls without Epo injections (Table 1). This stimulatory effect was no longer discernible after restoration of a normal B-8 population. The mechanism underlying this different response remains to be clarified.

The intermediate progenitors B-4 regenerated in the BCNU-treated mice in the absence of Epo to only 40%–60% of normal in spite of the presence of normal numbers of B-8. Daily injections of 1 U of Epo promptly restored normal numbers. A very different pattern of regeneration was seen in the Myleran-injected mice. Without Epo injections no significant regeneration of B-4 took place in these animals, most likely as a result of the very low precursor population of B-8, which remained at levels below 2% of normal. However, daily injections of 5 U of Epo induced a wave of increases in B-4 and in CFU-e. These findings confirm and extend earlier work from our laboratory showing that daily Epo injections in Myleran-treated mice induced a wave of erythropoiesis as measured by incorporation or reticulocyte counts. In view of the near absence of CFU-s in these mice, it was suggested at that time that the Epo injections stimulated the proliferation of morphologically unrecognizable erythroid progenitors. The present results strongly suggest that Epo stimulated the proliferation of B-4 and that an increase in B-4 thus initiated the wave of erythropoiesis noted in the earlier experiments. These findings and the large and rapid increases in B-4 in the Epo-injected BCNU-treated mice indicate the B-4 and possibly their precursors, i.e., intermediate between B-8 and B-4, as the primary sites of the proliferation-stimulation effect of Epo.

The CFU-e were found to be the most Epo-dependent of the three erythroid progenitors. Regeneration of the B-4 population on day 10 to 40% of normal in the polycythemic BCNU-treated mice without Epo injections was accompanied by increases in CFU-e to only 15% of their normal number. Polycythemia alone suppressed the number of CFU-e to about 25% of normal without a reduction in the number of B-4 or B-8 (Table 1). It remains undecided whether this residual CFU-e formation in polycythemic mice represents a basal rate that is independent of Epo.
or whether it is sustained by undetected low Epo levels in these mice. Injection of 1 U of Epo into polycythemic mice raised their suppressed numbers of CFU-e to normal levels within 24 hr (Table 3), and the effect can be attributed to recruitment of precursors into the CFU-e stage. In BCNU-treated polycythemic mice, injection of Epo on day 1 resulted in large increases in B-4 but not in CFU-e (Table 3). At the time of this injection the number of B-4, and presumably that of any intermediates between B-4 and CFU-e, was about 1% of normal, and the failure to induce CFU-e is very likely the result of a near absence of their precursors. However, aside from this lack of direct recruitment, no significant CFU-e increase developed after the increase in B-4, indicating that these Epo-induced B-4 did not progress into the CFU-e stage without further exposure to Epo. A second small dose of Epo injected 48 hr after the first promptly recruited large numbers of CFU-e. The plasma half-life of Epo is rather short, and there is evidence that Epo molecules become attached to Epo membrane receptors of the target cells. The need for a second exposure to Epo in order to effect the transformation of B-4 into CFU-e suggests that the Epo molecules from the first injection are rendered ineffective on the newly formed B-4 or that the latter develop new receptors that require activation by a second exposure to the hormone.

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