CONCISE REPORT

Effects of Recombinant Erythropoietin on the Concentration and Cycling Status of Human Marrow Hematopoietic Progenitor Cells In Vivo

By Emmanuel N. Dessypris, Stanley E. Graber, Sanford B. Krantz, and William J. Stone

The concentration of human marrow progenitors CFU-E, BFU-E, CFU-GM, and CFU-Mk and the percentage of these progenitor cells in DNA synthesis were studied in nine patients with transfusion-dependent anemia of end-stage renal failure before and 2 weeks after treatment with human recombinant erythropoietin (Epo) at a dose of 150 to 300 U/kg intravenously three times per week. The concentration of CFU-E in the posttreatment marrow increased by a mean of 4.15-fold, BFU-E by 3.37-fold, CFU-GM by 1.86-fold, and CFU-Mk by 1.96-fold as compared with their respective concentrations in the pretreatment marrows. This increase in the concentrations of marrow progenitors was accompanied by almost a doubling of the percentage of these cells in DNA synthesis as assessed by the "H-thymidine suicide technique. These observations demonstrate that at the progenitor cell level the human marrow responds to therapeutic doses of Epo as an organ rather than by a selective expansion of the erythroid cell line.

DURING the last few decades the role of erythropoietin (Epo) as the major humoral regulator of red cell mass has been well established. The recent cloning of the human Epo gene and its expression in CHO cells have resulted in the production of quantities of recombinant human Epo (rHuEpo) that allowed its use for therapeutic purposes. Administration of rHuEpo to patients with end-stage renal failure has proven to be highly effective in correcting the anemia of advanced renal disease.

The purpose of this study was to investigate the change that may occur in the concentration and cycling status of bone marrow hematopoietic progenitors in patients with transfusion-dependent anemia of chronic renal failure who have been maintained by chronic hemodialysis and treated with rHuEpo. A significant increase in the concentration of erythroid, myeloid, and megakaryocytic progenitors was detected in the marrow of these patients 2 weeks after the initiation of treatment with rHuEpo. Moreover, this increase in concentration was accompanied by a significant elevation in the percentage of these progenitor cells in DNA synthesis as assessed by "H-thymidine suicide. These observations suggest that at the progenitor cell level the bone marrow responds to therapeutic doses of rHuEpo as an organ rather than by a selective and isolated expansion of the erythroid cell line.

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METHODS

Patients. Nine patients undergoing chronic hemodialysis with transfusion-dependent anemia of chronic renal failure were included in this study. These patients received rHuEpo (AmGen Corp, Thousand Oaks, CA) at a dose of 150 to 300 U/kg intravenously three times weekly. Bone marrow progenitor cell assays were performed before and 2 weeks after the initiation of treatment. During this period patients continued to receive hemodialysis and all medications that they had been taking before treatment with rHuEpo. Normal adult volunteers served as controls during the period of this study. Normal volunteers had two marrow aspirations for progenitor cell assays within a period of 2 to 6 weeks apart. These studies were approved by the Vanderbilt University Committee for the Protection of Human Subjects.

Progenitor cell assays. After informed consent was obtained, 6 ml of bone marrow was aspirated from the posterior iliac crest and collected in Iscove's medium containing 5 U/mL of heparin. Light-density cells were separated by centrifugation over Ficoll-Hypaque (density, 1.077), and they were further depleted of T lymphocytes and adherent cells by sheep erythrocyte rosetting and double one-hour adherence to plastic. All progenitor cell assays were performed by the plasma clot method. Sera, plasmas, conditioned media, and Epo were stored before the initiation of the study in quantities sufficient to complete all assays. CFU-E and BFU-E were plated at concentrations of 2 x 10^6 cells/ml in medium containing 30% heat-inactivated human AB serum and 1 or 3 U/ml of rHuEpo, respectively. In another set of cultures, 1% (vol/vol) of serum-free medium conditioned by T lymphocytes stimulated with phytohemagglutinin (PHA-LCM) was also added. CFU-GM were assayed in medium containing 20% heat-inactivated human AB serum, 10% PHA-LCM, and 2 x 10^6 cells/ml. CFU-Mk were plated at a concentration of 2 x 10^7/ml in medium containing 30% human citrated AB plasma and 5% PHA-LCM. Enumeration of CFU-E and BFU-E was performed on days 7 and 15, respectively, after staining the clots with benzidine-hematoxylin. Scoring of CFU-GM colonies was performed on day 15 after staining the clots for myeloperoxidase. Collections of 50 or more cells containing granulocytes, macrophages, eosinophils, or any combination of them were scored as single colonies. CFU-Mk were enumerated on day 15 after staining the clots by an indirect immunoperoxidase method using the monoclonal antibody Tab.3

Determination of the percentage of progenitor cells in DNA synthesis was performed by the titrated thymidine suicide method as previously described.6

All cultures were performed in four to six replicates, and the results are expressed as means ± SEM. Statistical analysis was performed by the Wilcoxon signed rank test for pairs.
normal volunteers showed no statistically significant changes in the numbers of progenitor cell–derived colonies between the first and the second assay (P = .75). The percentages of progenitor cells in DNA synthesis in the marrow obtained before and after treatment as assessed by the 3H-thymidine suicide method are presented in Table 2. Significant increases in the percentage of progenitor cells killed by 3H-thymidine suicide were noted in all classes of hematopoietic progenitors. In contrast, the mean percentage of progenitor cells in DNA-synthesis in the group of seven normal volunteers did not change significantly between the first and the second marrow aspirates (P = .81).

DISCUSSION

The increase in the concentration of CFU-E in the marrow of patients treated with rHuEpo for 2 weeks is reminiscent of previous investigations performed in animals with acute anemia characterized by high Epo levels. In the current study not only did the concentration of CFU-E increase significantly, but the percentage of these mature erythroid progenitors killed by 3H-thymidine almost doubled in the

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### Table 1. Changes in the Concentration of Hematopoietic Progenitors in the Bone Marrow of Patients Treated With rHuEpo

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CFU-E</th>
<th>BFU-E*</th>
<th>BFU-E†</th>
<th>CFU-GM</th>
<th>CFU-Mk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>1</td>
<td>178 ± 10</td>
<td>1,052 ± 11</td>
<td>20 ± 2</td>
<td>139 ± 19</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>162 ± 13</td>
<td>933 ± 45</td>
<td>24 ± 3</td>
<td>81 ± 4</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>3</td>
<td>399 ± 38</td>
<td>1,903 ± 82</td>
<td>89 ± 7</td>
<td>319 ± 15</td>
<td>186 ± 11</td>
</tr>
<tr>
<td>4</td>
<td>705 ± 52</td>
<td>1,620 ± 40</td>
<td>57 ± 4</td>
<td>115 ± 14</td>
<td>83 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>475 ± 2</td>
<td>1,723 ± 111</td>
<td>99 ± 6</td>
<td>190 ± 10</td>
<td>202 ± 10</td>
</tr>
<tr>
<td>6</td>
<td>713 ± 42</td>
<td>1,958 ± 126</td>
<td>64 ± 5</td>
<td>280 ± 19</td>
<td>136 ± 12</td>
</tr>
<tr>
<td>7</td>
<td>158 ± 12</td>
<td>993 ± 31</td>
<td>59 ± 6</td>
<td>292 ± 16</td>
<td>178 ± 9</td>
</tr>
<tr>
<td>8</td>
<td>887 ± 13</td>
<td>1,944 ± 45</td>
<td>121 ± 11</td>
<td>186 ± 10</td>
<td>236 ± 10</td>
</tr>
<tr>
<td>9</td>
<td>286 ± 17</td>
<td>1,442 ± 35</td>
<td>59 ± 5</td>
<td>105 ± 12</td>
<td>268 ± 14</td>
</tr>
</tbody>
</table>

Mean ± SEM 440 ± 91 1,506 ± 140 66 ± 11 190 ± 29 169 ± 26 331 ± 30

P values† .003 .003 .007 .003 <.003

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### Table 2. Percentage of Marrow Hematopoietic Progenitors in DNA Synthesis Before and After Treatment With rHuEpo

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CFU-E</th>
<th>BFU-E*</th>
<th>BFU-E†</th>
<th>CFU-GM</th>
<th>CFU-Mk</th>
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<tr>
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<td>Pre</td>
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<td>69</td>
<td>21</td>
<td>54</td>
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<td>9</td>
<td>22</td>
<td>56</td>
<td>32</td>
<td>51</td>
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</tr>
</tbody>
</table>

Mean ± SEM 31 ± 3 69 ± 4 18 ± 3 52 ± 4 9 ± 2 38 ± 4 27 ± 1 51 ± 2 15 ± 3 41 ± 4

P values† .018 .0018 .043 .027 <.018

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All numbers represent means ± SEM from four to six replicates and express numbers of progenitor cell–derived colonies per 10⁶ bone marrow cells, except for CFU-Mk, which express colonies per 2 × 10⁶ cells.

*BFU-E grown in the presence of Epo.
†BFU-E grown in the presence of Epo and PHA-LCM.
‡P values by the Wilcoxon test.
posttreatment marrows. It should be noted that the percentage of these cells in DNA synthesis in the pretreatment marrow was found to be much lower than what has been previously reported for normal marrow CFU-E, a finding similar to a preliminary report from other investigators. It is possible that the very low levels of Epo present in the plasma of patients with end-stage renal disease are reflected in the marrow CFU-E compartment by a decline in the percentage of cells in DNA synthesis that can be restored to normal by the administration of rHuEpo. Since the CFU-E are probably the most Epo-sensitive cells having Epo receptors, the observed changes could be attributed to a direct effect of rHuEpo on these cells. The increase in the concentration and the percentage of BFU-E killed by $^{3}$H-thymidine is not surprising since at least a proportion of marrow BFU-E seem to be Epo dependent and pure Epo has been shown to induce DNA synthesis by human marrow BFU-E in vitro. An increase in the percentage of BFU-E killed by $^{3}$H-thymidine suicide has also been detected in a preliminary report by other investigators. The finding of increased BFU-E concentrations in the posttreatment marrows cannot be compared with studies that showed a lack of change in the BFU-E pools in experimental animals rendered acutely anemic or injected with Epo since both the doses of Epo and the duration of exposure of marrow cells to Epo are widely different between the present study in humans and previous studies in animals. 

The changes observed in the concentration and the percentage of megakaryocytic progenitors in DNA synthesis along with a mild increase in the peripheral blood platelet count may be related to the various effects of rHuEpo on human and murine megakaryocytopoiesis in vitro and in vivo. 

The increase in the concentration and cycling status of CFU-GM was a totally unexpected finding, the significance of which is poorly understood at the present time. The fact that similar changes were observed in all erythroid and nonerythroid progenitors suggests that induction of erythroid differentiation by the administered rHuEpo may lead to activation of stem cells, which in a stochastic manner results in an increase of all committed progenitor cells in the marrow or alternatively to an indirect stimulation of nonerythroid progenitors through the liberation of growth factors by erythroid or nonerythroid cells. A similar observation has been made with another differentiation factor, G-CSF, on murine bone marrow and splenic hematopoiesis in vivo. The administration of G-CSF led to an increase not only of granulopoiesis but of erythropoiesis and megakaryocytopoiesis as well as in the spleens of the treated animals, and this was accompanied also by a significant elevation of the number of CFU-S. The precise mechanism through which a specific differentiation-promoting factor might lead to the activation of pluripotent stem cells in vivo and whether, in the case of Epo, this directly involves the Epo-responsive cells or other nonerythroid cells remain to be determined. Nevertheless, the results of these studies demonstrate that at the progenitor cell level the bone marrow responds to Epo as an organ rather than by an isolated and selective expansion of the erythroid cell line.

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Effects of recombinant erythropoietin on the concentration and cycling status of human marrow hematopoietic progenitor cells in vivo

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