Diamond-Blackfan Anemia: Heterogenous Response of Hematopoietic Progenitor Cells In Vitro to the Protein Product of the Steel Locus

By Nancy F. Olivieri, Tom Grunberger, Yaacov Ben-David, Judy Ng, Douglas E. Williams, Stewart Lyman, Dirk M. Anderson, Arthur A. Axelrad, Paulo Correa, Alan Bernstein, and Melvin H. Freedman

Diamond-Blackfan anemia is a congenital disorder of erythropoiesis in humans, characterized by a macrocytic anemia often associated with physical anomalies. Mutations at either the W or Steel loci in the mouse also lead to a severe macrocytic anemia, as well as other developmental abnormalities. The W locus encodes the proto-oncogene c-kit, a member of the receptor tyrosine kinase family, while the Steel locus encodes a potent hematopoietic growth factor that is the ligand for c-kit. Growth of clonogenic marrow erythroid progenitor cells in vitro in the presence of the recombinant hematopoietic growth factors interleukin-3 (IL-3) and Steel was used to characterize this disease at the cellular level. Three patterns of in vitro marrow response to both recombinant IL-3 or Steel were observed among 10 Diamond-Blackfan patients: those that responded quantitatively and qualitatively almost as well as cells from normal marrow, those that responded at an intermediate level, and those that did not respond at all. These results provide evidence for cellular heterogeneity underlying the pathogenesis of this disorder and therefore raise the possibility that there may be more than one underlying molecular basis for the disease. No gross abnormalities in the structure of either the c-kit or Steel loci were observed in these patients. The normal response in culture of the progenitor cells from at least some patients to Steel with or without IL-3 raises the possibility of using this novel growth factor as a therapeutic agent in Diamond-Blackfan anemia.

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CONSTITUTIONAL PURE red blood cell (RBC) aplasia, or Diamond-Blackfan anemia, is a severe congenital abnormality of erythropoiesis characterized by a macrocytic anemia developing early in childhood. The bone marrows of affected individuals have markedly reduced or absent RBC precursors, with normal marrow cellularity and preservation of other hematopoietic cell lineages. Although most cases are sporadic, there is evidence for both autosomal dominant and recessive inheritance. Associated anomalies, one or more of which has been described in 70% of reported cases of Diamond-Blackfan anemia, include abnormalities of the skeletal system, jaw and mouth, eyes, kidneys, and heart; dwarfism, cartilage-hair hypoplasia, and hypogonadism have also been reported. The molecular basis for this syndrome has not been determined, nor is it clear, given the heterogeneity in associated abnormalities, whether Diamond-Blackfan anemia is a homogeneous disorder resulting from mutations in a single gene, or several distinct diseases that result from mutations in different genes.

In the mouse, mutations at either the W or Steel (S1) loci also lead to a severe macrocytic anemia that can be inherited in either a dominant (eg, the W/+ allele) or recessive (eg, W/) manner. In addition, these mice have associated abnormalities, including defects in mast cells, coat color, and germ cells. Previous marrow transplantation and embryonic chimera experiments have shown that W mutants have a cell-autonomous, intrinsic defect in the stem cells that gives rise to erythrocytes, melanocytes, and germ cells, whereas S1 mice have a defect in the microenvironment in which these cells develop and function. Recently, molecular analyses of these loci have established that W encodes the c-kit growth factor receptor,6, 10 while S1 encodes the ligand that binds to the Kit receptor,11-16 resulting in the activation of the intrinsic tyrosine kinase activity of c-kit. As expected from the anemic phenotype of mice with mutations at the S1 locus, the protein product of the S1 gene, alternatively called mast cell growth factor, stem cell factor, or the Kit ligand, and referred to in this report as Steel, has profound biologic effects on hematopoietic progenitor cells both in vitro and in the intact animal.11-16

Furthermore, while mast cells or marrow cells from normal or mutant S1 mice proliferate in response to Steel factor in vitro, cells from mice with severe mutations at the W/c-kit locus do not proliferate in response to Steel.11-16 Given the overlap in phenotypes of W and S1 mutant mice with that of individuals with Diamond-Blackfan anemia, we sought to determine whether this disease might arise as the result of mutation in some step in the c-kit signal transduction pathway by investigating the ability of hematopoietic progenitor cells from affected patients to proliferate in response to Steel factor in culture. We show that patients with this disease can be grouped into three categories on the basis of the ability of their marrow cells to respond to Steel in cultures. This heterogeneity in response to Steel may serve as a useful parameter to characterize and categorize Diamond-Blackfan anemia. In addition, these data suggest that this new hematopoietic growth factor either alone, or in concert with other factors, may be a powerful therapeutic agent for this disease.
MATERIALS AND METHODS

Hematopoietic colony assays. Patients with Diamond-Blackfan anemia were from the Transfusion Clinic, Hospital for Sick Children, Toronto, Canada. Marrow samples were drawn in preservative-free heparin, performed with slight modifications of the method described by Isacoe et al. Briefly, neutrophils and RBCs were removed from heparinized (10 U/mL) bone marrow by centrifugation (800g at 4°C 10 minutes) over Percoll (density: 1.077 g/mL). After Percoll separation, mononuclear bone marrow cells (at 1 x 10^6 cells/mL) were suspended in α medium plus 30% fetal calf serum, 1% bovine serum albumin (BSA), and methylcellulose at a final concentration of 0.84% in 35-mm Petri dishes. Partially purified (25- to 50-fold) human erythropoietin (EPO; Terry Fox Laboratories, Vancouver, Canada) was added at a final concentration of 2 U/mL. Where indicated, recombinant interleukin-3 (IL-3), kindly provided by S. Clark (Genetics Institute, Cambridge, MA) was added. The Steel factor was expressed in yeast and purified as described. The cultures were incubated at 37°C with 5% CO2 and high humidity. Colonies were counted on day 7 (CFU-E) and 14 (BFU-E) using an inverted microscope. A BFU-E colony was defined as a cluster of eight or more hemoglobinized erythroid cells. Colonies that were scored as BFU-E were confirmed as BFU-E by growth in the presence of purified human EPO. Normal marrow cells showed no increase in the number of BFU-E compared to cultures with EPO alone assayed on day 7, and a 41% increase in the number of BFU-E assayed on day 14 (Table 2). These observations are in agreement with previous results on the growth of normal marrow progenitor cells in the presence of these growth factors.

Results

In vitro response of normal and Diamond-Blackfan anemia marrow cells to Steel. To assess the ability of hematopoietic cells to respond to Steel, we obtained marrow samples from 10 steroid-unresponsive, transfusion-dependent patients with Diamond-Blackfan anemia (Table 1). Also, control bone marrow was aspirated from one healthy adult volunteer. The ability of these cells to respond to either Steel alone, or in combination with the recombinant hematopoietic growth factor IL-3, was determined by performing clonogenic assays (CFU-E and BFU-E assays) in semisolid methylcellulose medium in vitro. Cell cultures of bone marrow from a normal adult volunteer gave rise to mean CFU-E and BFU-E colony numbers (±SD) of 135 ± 1 and 123 ± 13 per 10^5 cells plated, respectively (Table 2) in the presence of purified human EPO. Normal marrow cells grown in both EPO and recombinant IL-3 (10 U/mL) showed no increase in the number of CFU-E compared to cultures with EPO alone assayed on day 7, and a 41% increase in the number of BFU-E assayed on day 14 (Table 2).

When normal marrow cells were plated in increasing concentrations of recombinant soluble Steel (ranging from 12.5 to 100 ng/mL), there was an increase in the number of BFU-E, with no dose response observed (Table 2). Growth in the presence of both Steel and IL-3 resulted in a large increase in the number of BFU-E observed on day 14 (Table 2). More striking than the increase in numbers of BFU-E (and mixed-lineage colonies) was the very dramatic increase in the size and degree of hemoglobinization of the colonies. Very large, macroscopically visible pure and mixed lineage colonies containing between 10^3 and 10^4 cells of erythroid, granulocytic, macrophage, and undifferentiated morphology were also observed in response to growth in Steel plus IL-3 plus EPO (Fig 1).

We next examined the response of marrow progenitor cells from patients with Diamond-Blackfan anemia to the recombinant Steel protein. As described previously, the number of erythroid progenitor cells from patients with Diamond-Blackfan anemia was, in general, markedly reduced when compared with normal marrow (Table 2). Patients D.B., C.B., R.T., and J.R. had low, but detectable, numbers of CFU-E and BFU-E when cultured in the presence of EPO. The colony numbers in these patients increased markedly when IL-3 was added to the culture media (Table 1). Patients K.N. and De.B. had lower numbers of colony-forming cells when assayed in the absence or presence of IL-3, while patients B.S., D.C., N.M., and D.Ba. had very low numbers of progenitors in the absence of IL-3 and no increase when IL-3 was added (Table 2).

These same marrow samples were simultaneously plated in the presence of recombinant Steel protein, either with EPO alone, or in combination with IL-3. Table 2 shows that three discrete patterns of response were observed. The same four patients that responded well to IL-3 also showed a marked increase in the number of BFU-E when grown in Steel, two patients had a more moderate response, with only a slight elevation of their BFU-E numbers, and the remaining four patients that failed to respond to IL-3 also had no response to Steel.

Despite these quantitative differences between normal and Diamond-Blackfan marrow, the addition of Steel resulted in the same dramatic increase in colony size and hemoglobinization that was observed for normal marrow.

### Table 1: Clinical Characteristics of Patients With Diamond-Blackfan Anemia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Associated Abnormalities</th>
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<tbody>
<tr>
<td>D.B.</td>
<td>17.5</td>
<td>F</td>
<td>Duplicated terminal phalanx of thumb, growth and pubertal delay, duplicated ureter</td>
</tr>
<tr>
<td>C.B.</td>
<td>10.0</td>
<td>F</td>
<td>Microcephaly, bifid uvula, webbed neck, triphalangeal thumbs, congenital hip dislocation, underrotated kidney, growth retardation</td>
</tr>
<tr>
<td>R.T.</td>
<td>9.0</td>
<td>M</td>
<td>Triphalangeal thumb and upper limb bony abnormalities</td>
</tr>
<tr>
<td>J.R.</td>
<td>15.0</td>
<td>F</td>
<td>Abnormality, abnormal dermatoglyphics, duplicated terminal phalanx of thumb, growth retardation</td>
</tr>
<tr>
<td>K.N.</td>
<td>13.0</td>
<td>M</td>
<td>Growth retardation</td>
</tr>
<tr>
<td>De.B.</td>
<td>19.5</td>
<td>F</td>
<td>Hypoplastic teeth and nails, congenital glaucoma, growth retardation, developmental delay</td>
</tr>
<tr>
<td>B.S.</td>
<td>16.0</td>
<td>M</td>
<td>None</td>
</tr>
<tr>
<td>D.C.</td>
<td>16.5</td>
<td>M</td>
<td>Duplicated ureter</td>
</tr>
<tr>
<td>N.M.</td>
<td>12.5</td>
<td>F</td>
<td>None</td>
</tr>
<tr>
<td>D.Ba.</td>
<td>3.0</td>
<td>F</td>
<td>None</td>
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Table 2. Response of Progenitor Cells (BFU-E) From Patients With Diamond-Blackfan Anemia to Steel Factor

<table>
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</thead>
<tbody>
<tr>
<td>Control (EPO 2 U/mL)</td>
<td>123 ± 13</td>
<td>13 ± 2</td>
<td>29 ± 2</td>
<td>32 ± 1</td>
<td>37 ± 2</td>
<td>29 ± 2</td>
<td>9 ± 2</td>
<td>3 ± 2</td>
<td>5 ± 2</td>
<td>0 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>IL-3 (40 U/mL)</td>
<td>165 ± 9</td>
<td>62 ± 2</td>
<td>80 ± 4</td>
<td>54 ± 2</td>
<td>86 ± 16</td>
<td>22 ± 1</td>
<td>20 ± 1</td>
<td>1 ± 1</td>
<td>6 ± 3</td>
<td>2 ± 1</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Steel (12.5 ng/mL)</td>
<td>194 ± 40</td>
<td>43 ± 0</td>
<td>50 ± 3</td>
<td>73 ± 2</td>
<td>102 ± 10</td>
<td>36 ± 4</td>
<td>24 ± 2</td>
<td>5 ± 3</td>
<td>5 ± 1</td>
<td>0 ± 1</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>Steel (50 ng/mL)</td>
<td>168 ± 28</td>
<td>80 ± 2</td>
<td>86 ± 2</td>
<td>71 ± 9</td>
<td>117 ± 17</td>
<td>41 ± 2</td>
<td>42 ± 4</td>
<td>7 ± 1</td>
<td>7 ± 0</td>
<td>3 ± 1</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>Steel (100 ng/mL)</td>
<td>182 ± 35</td>
<td>69 ± 9</td>
<td>91 ± 9</td>
<td>71 ± 2</td>
<td>93 ± 2</td>
<td>44 ± 1</td>
<td>46 ± 4</td>
<td>8 ± 1</td>
<td>9 ± 2</td>
<td>3 ± 0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>IL-3: Steel (20 U/mL: 50 ng/mL)</td>
<td>233 ± 22</td>
<td>121 ± 4</td>
<td>142 ± 4</td>
<td>96 ± 4</td>
<td>148 ± 9</td>
<td>53 ± 9</td>
<td>71 ± 8</td>
<td>12 ± 4</td>
<td>12 ± 2</td>
<td>4 ± 0</td>
<td>6 ± 1</td>
</tr>
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Bone marrow cells from a healthy volunteer and 10 patients with Diamond-Blackfan anemia were grown in semisolid culture media containing fetal calf serum and the growth factors indicated. At day 14, the numbers of BFU-E were determined.

(Fig 1). In addition, the addition of Steel promoted the growth of multilineage colonies from cells derived from patients with Diamond-Blackfan anemia.

Response to Steel in serum-free medium. The experiments presented in Table 2 were performed in the presence of fetal calf serum which might contain growth factors that interact synergistically with Steel or IL-3 to produce the results shown in Table 1. Therefore, we repeated these clonogenic assays in an improved serum-free medium, as described in Materials and Methods. As shown in Table 3, bone marrow cells from patient R.T. who responded to Steel in medium containing serum (Table 2) also showed an increase in the numbers of erythroid colonies under serum-free conditions.

Southern blot analysis of the c-kit and Sl genes in Diamond-Blackfan patients. It is interesting to speculate whether any of the patients examined in this study have anemia as the result of mutation in either the human W or Sl loci. For example, those patients who responded normally in culture to Steel might have a mutation in the Sl locus itself. To determine whether any of the individuals in our study had gross rearrangements in either the Sl or c-kit gene, Southern blot analysis was performed on DNA isolated from Epstein-Barr virus (EBV)-immortalized lymphoblastoid cells derived from seven patients with Diamond-Blackfan anemia. As shown in Fig 2, no alterations in either gene was detected at the level of resolution of genomic Southern blot analysis.

Fig 1. Erythroid colony growth from patient D.B. (see Table 1). The colony on the left was grown in medium containing EPO while the colony on the right was grown in EPO + IL-3 (20 U/mL) + Steel (50 ng/mL). Original magnification × 10.
Table 3. Growth of Erythroid Progenitor Cells From a Patient With Diamond-Blackfan Anemia Under Serum-Free Conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Normal</th>
<th>Patient R.T.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d7</td>
<td>d14</td>
</tr>
<tr>
<td>Epo (2 U/mL)</td>
<td>46</td>
<td>14</td>
</tr>
<tr>
<td>Epo + Steel factor (50 ng/mL)</td>
<td>450</td>
<td>125</td>
</tr>
</tbody>
</table>

Bone marrow cells from a healthy volunteer and a child with Diamond-Blackfan anemia were grown as described. The numbers of CFU-E and BFU-E were determined on day 7 and 14, respectively.

DISCUSSION

The results presented here suggest that Diamond-Blackfan patients can be divided into three discrete groups: those that respond well to the addition of Steel in culture, those that respond only moderately well, and those that do not appear to respond at all. Of the 10 patients that we examined in this series of experiments, six fell into the first and second categories, while the remaining four fell into the third category. These differences in colony number in response to Steel cannot be accounted for by the age of the patient at the time of study, nor can we identify any other variable that might explain these observations. Thus, the differences in erythroid progenitor proliferation in bone marrow cultures may reflect a true heterogeneity in the pathogenesis of Diamond-Blackfan anemia. This conclusion extends earlier observations showing heterogeneity in the response of marrow cells from these patients to IL-3.

Strikingly, there was an excellent correlation between those patients that responded to IL-3 and those that responded to Steel in culture. In contrast, murine W mutants fail to respond normally to Steel, but retain their ability to respond to IL-3. Thus, one interpretation of our results is that the primary defect in these patients leads to a partial or complete absence of progenitor cells, and hence an inability to respond in culture to any hematopoietic growth factor. This primary defect could be in the c-kit signaling pathway itself or, in a second, unrelated, pathway that also controls the proliferation and differentiation of erythroid progenitor cells.

Because the SI defect in the mouse leads to a defect in the environment in which hematopoietic stem cells function, their anemia cannot be cured by bone marrow transplantation. There have been a number of cases in which patients with Diamond-Blackfan anemia have been successfully cured by bone marrow transplantation, suggesting that, in these patients, the defect is not in the hematopoietic microenvironment and hence does not appear to be in the human SI locus itself. It clearly would be of interest to determine whether there is any relationship between the ability to respond to Steel in culture and the success of bone marrow engraftment after transplantation.

Although the cellular characterization of marrow progenitor cells described in this report does not provide direct information concerning the molecular basis of the defect in Diamond-Blackfan disease, two important conclusions can be drawn from these experiments. First, Diamond-Blackfan anemia is a heterogeneous disorder at the hematopoietic progenitor cell level. This heterogeneity may indicate that this disorder is not a single entity, but rather can arise as the result of mutation in one of several genes. Second, a significant proportion of patients with this disease responded almost normally to Steel factor in culture. The administration of recombinant Steel factor to either mice

![Fig 2. Southern blot analysis of the c-kit and SI genes in Diamond-Blackfan patients. High molecular weight DNA (10 µg) was isolated from EBV-immortalized lymphoblastoid cell lines from a normal individual and patients with Diamond-Blackfan anemia. The DNA was digested with the restriction enzymes BamHI (A) or HindIII (B), fractionated by electrophoresis in an 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized with 32P-random primed human c-kit and SI probes. Both 3-kb c-kit cDNA (A) and 0.9-kb SI (B) cDNA used for hybridization contained the entire coding regions for c-kit and SI transcripts, respectively. Lanes: 1, D.B.; 2, D.E.; 3, R.T.; 4, J.R.; 5, C.B.; 6, D.C.; 7, N.M.; 8, normal.](image)
or nonhuman primates leads to an elevation in the number of hematopoietic cells of both the myeloid and lymphoid lineages. In addition, recombinant soluble Steel protein can at least partially correct the macrocytic anemia in mutant Sl mice. The in vivo biologic effects of this new hematopoietic growth factor, together with the characterization of Diamond-Blackfan patients by the in vitro colony assays described here, may provide a means for identifying those individuals who might be suitable candidates for therapy with recombinant Steel.

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REFERENCES

Diamond-Blackfan anemia: heterogeneous response of hematopoietic progenitor cells in vitro to the protein product of the steel locus

NF Olivieri, T Grunberger, Y Ben-David, J Ng, DE Williams, S Lyman, DM Anderson, AA Axelrad, P Correa and A Bernstein