Three-Lineage Hemopoietic Precursor Cells and Effectiveness of Recombinant Human Erythropoietin in Patients with Myelodysplastic Syndromes

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FUKUSHIMA, Y., KANNO, Y. and MIURA, A.B. Three-Lineage Hemopoietic Precursor Cells and Effectiveness of Recombinant Human Erythropoietin in Patients with Myelodysplastic Syndromes. Tohoku J. Exp. Med., 1992, 166 (3), 375-385 — Four-stem-cell assays, which evaluate megakaryocytic (CFU-Meg), immature and mature erythropoietic (BFU-E, CFU-E), and granulocyte-macrophage (CFU-GM) colony formation, were performed in nine patients with myelodysplastic syndromes (MDS). The CFU-Meg, BFU-E, and CFU-E colony growths were disturbed more often than the CFU-GM colony formation. A CFU-E increase was not recognized in most MDS patients, but a dose-dependent increase of bone marrow CFU-Es in response to erythropoietin (EPO) was recognized only in two refractory anemia (RA) patients whose CFU-Es were more than one tenth of normal controls. One patient with RA and the other with chronic myelomonocytic leukemia (CMML), both of whose bone marrow CFU-Es did not increase at the higher dose of EPO in vitro, were treated with recombinant human EPO (rHuEPO), resulting in no effects. The responsiveness of patients with MDS to various recombinant hemopoietic factors might be predicted by both the residual degree of bone marrow hematopoietic precursor cells and the response of stem cells to the higher doses of each hemopoietic factor. —— myelodysplastic syndromes; CFU-Meg; BFU-E; CFU-GM; recombinant human erythropoietin

The myelodysplastic syndromes (MDS) represent a preleukemic state where a clonal abnormality of the hematopoietic stem cell is characterized by an ineffective hemopoiesis. Although many studies on the in vitro precursor cells of patients with MDS have been done, three-lineage stem cell studies which include megakaryocytic colony growth in MDS, have been few. Therefore, we have studied CFU-Meg (megakaryocyte colony forming unit), BFU-E (burst forming unit, erythroid), CFU-E (colony forming unit, erythroid), CFU-GM (granulocyte-macrophage colony forming unit) colony growth, and have compared the number of CFU-Meg colony growths with those of BFU-E, CFU-E, and CFU-GM colony formation. Recombinant human erythropoietin (rHuEPO) was administered to
our two patients, and we examined the relationship between the effectiveness of rHuEPO and erythroid colony growth.

**MATERIALS AND METHODS**

**Subjects**

Nine patients with MDS, as defined in the FAB classification (Bennett et al. 1982), were studied (Table 1). None of the patients had been exposed to any prior cytotoxic treatment or irradiation except for case 8, and there were no inhibitory of any other predisposing toxic agents. Case 8 had been treated with aclacinomycin and VP-16 two months before this study. The 9 patients consisted of four with refractory anemia (RA), one with refractory anemia with excess blasts (RAEB), one with chronic myelomonocytic leukemia (CMML), and three with RAEB in transformation (RAEB-T). Normal bone marrow cells were obtained from healthy control volunteers.

**Colony-forming assays**

Bone marrow mononuclear cells (BMMNC) were separated by Ficoll-Conray density gradient centrifugation at 400 g for 30 min, washed three times and resuspended in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY, USA). MNC were further separated into non-adherent and adherent cells after incubation in a 60 x 15 mm plastic tissue culture dish (Becton Dickinson Labware, Lincoln Park, NJ, USA) at 37°C for 60 min in an atmosphere of 5% CO₂. Bone marrow non-adherent mononuclear cells (BMNAMNC) were collected by gently swirling the dishes and carefully aspirating the floating cells. All cultures for CFU-Meg, BFU-E, CFU-E, and CFU-GM were performed using a methylcellulose system, and the results represent the mean of duplicate cultures.

For CFU-Meg assay, phytohemagglutinin (PHA-P, Difco, Detroit, MI, USA) stimulated leukocyte-conditioned medium (PHA-LCM) and normal human plasma were used as the

<table>
<thead>
<tr>
<th>Case (years)</th>
<th>Sex</th>
<th>FAB</th>
<th>Hb (g/100 ml)</th>
<th>Granulocyte (/μl)</th>
<th>Plt (×10⁴/μl)</th>
<th>Karyotype</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Y.I.</td>
<td>M</td>
<td>RA</td>
<td>7.5</td>
<td>2035</td>
<td>1.3</td>
<td>46XY</td>
<td>Primobolan + STH*</td>
</tr>
<tr>
<td>2. A.S.</td>
<td>F</td>
<td>RA</td>
<td>11.4</td>
<td>1400</td>
<td>10.0</td>
<td>ND⁹</td>
<td>(—)</td>
</tr>
<tr>
<td>3. A.S.</td>
<td>F</td>
<td>RA</td>
<td>6.0</td>
<td>1178</td>
<td>2.0</td>
<td>ND</td>
<td>STH</td>
</tr>
<tr>
<td>4. H.H.</td>
<td>F</td>
<td>RA</td>
<td>7.4</td>
<td>1015</td>
<td>0.8</td>
<td>46XX</td>
<td>Anadrol + V.D₃</td>
</tr>
<tr>
<td>5. S.I.</td>
<td>M</td>
<td>RAEB</td>
<td>8.8</td>
<td>1353</td>
<td>5.3</td>
<td>46XY</td>
<td>(—)</td>
</tr>
<tr>
<td>6. K.G.</td>
<td>M</td>
<td>CMML</td>
<td>6.0</td>
<td>1232</td>
<td>4.6</td>
<td>ND</td>
<td>(—)</td>
</tr>
<tr>
<td>7. S.Y.</td>
<td>F</td>
<td>RAEB-T</td>
<td>7.9</td>
<td>810</td>
<td>13.9</td>
<td>46XX</td>
<td>(—)</td>
</tr>
<tr>
<td>8. K.C.</td>
<td>M</td>
<td>RAEB-T</td>
<td>6.4</td>
<td>240</td>
<td>1.0</td>
<td>46XY, 20q-(10/20)</td>
<td></td>
</tr>
</tbody>
</table>

| 9. C.Y.     | F   | RAEB-T | 7.4           | 5220              | 6.3          | 40XX, 1p−, 3p−, 19p+, +mar | (—) |

*STH, steroid hormone; ACR, aclacinomycin. ⁹ND, not done.
source of megakaryocytic colony-stimulating activity (Meg-CSA). $2 \times 10^5$ MNC were suspended in 1 ml of a solution consisting of 0.96% methylcellulose (Shin-etsu Chemical Co., Tokyo), 30% normal human plasma, $5 \times 10^{-5}$ M 2-mercaptoethanol (Sigma Chemical company, St. Louis, MO, USA), and IMDM in a 35 x 10 mm plastic tissue culture dish (Lux, Nunc Inc, Naperville, IL, USA) with 5% PHA-LCM. After incubation in 5% CO$_2$, 95% humidified air at 37°C for 14 days, cell clusters consisting of at least 3 cells as a colony from CFU-Meg were counted under an inverted microscope. The cells in the colony were frequently checked by positive immunofluorescence labelling with anti-human GPIIb/IIIa mouse monoclonal antibody (Clone P2, Cosmo BIO Co., Ltd., Tokyo).

For most of the experiments, erythroid colonies were assayed by culturing non-adherent marrow cells at a concentration of $10^5$/ml in IMDM, made semisolid with 0.96% methylcellulose, and supplemented with the following: 30% fetal calf serum (FCS, Hyclone, Logan, UT, USA), 1% deionized bovine serum albumin (Armour Pharmaceutical Company, Kankakee, IL, USA), $10^{-4}$M 2-mercaptoethanol, penicillin-streptomycin, and 2 U/ml rHuEPO (Chugai Pharmaceutical Co., Ltd., Tokyo). One ml volumes were cultured in 35 mm plastic tissue culture dishes (Lux, Miles Laboratories, Naperville, IL, USA) at 37°C in 5% CO$_2$ in air. Erythroid colonies (derived from CFU-E) and bursts (from BFU-E) were counted with an inverted microscope 7 and 14 days in culture, respectively. Aggregates of 8 or more hemoglobinized cells were scored as CFU-E-derived colonies. BFU-E colonies were identified as having at least 100 hemoglobinized cells that were aggregates of two or more hemoglobinized subcolonies, and BFU-E clusters were defined as being aggregates of under 100 hemoglobinized cells.

Granulocyte-macrophage colony-forming cells (CFU-GM) were assayed by culturing non-adherent marrow cells at a concentration of $10^5$/ml in IMDM, made semisolid with 0.96% methylcellulose, and supplemented with 20% FCS, penicillin-streptomycin, and 10% colony-stimulating factor (CSF). CSFs used were CSF-"Chugai" (crude CSF, Chugai Pharmaceuticals, Tokyo), recombinant human granulocyte-CSF (rhG-CSF, Kirin-Amgen Inc., Thousand Oaks, CA, USA), and recombinant human granulocyte-macrophage-CSF (rhGM-CSF, Behringwerke AG, Marburg, Germany). CFU-GM colonies were identified by inverted microscopy as aggregates of 50 or more cells, and CFU-GM clusters were defined as aggregates having less than 50 cells.

The administration of rHuEPO

rHuEPO (Chugai Pharmaceutical Co., Ltd., Tokyo) was administered to two patients (case 1 and 6) after informed consent. The dose of rHuEPO was 12,000 U x 3/week for 8 weeks in case 1, and 6,000 U x 3/week for 4 weeks in case 6.

Plasma EPO levels

Plasma EPO levels were measured by radioimmunoassay (RIA).

Data analysis

The Student's $t$-test and the F test, for the effect of repeated measures, were used to determine $p$ values for statistical significance. All results are given as means $\pm$ S.E.

Results

The CFU-Meg colony formation was both higher in one patient with RAEB-T and lower in one patient with CMML when compared to normal controls, but was absent in the other seven (Table 2). Case 7 had normal platelet counts but even higher CFU-Meg numbers, while case 2 had an almost normal platelet count but CFU-Meg was absent. A positive correlation between CFU-Meg numbers and the
platelet count does not seem to exist. Under various concentrations of PHA-LCM (2.5, 5.0, 10%), CFU-Meg colony growths reached a peak when PHA-LCM was 5% (Fig. 1). CFU-Meg colony growths in four patients with RA and one patient with RAEB were not found in any of the concentrations of PHA-LCM. A patient with CMML had dose-dependent CFU-Meg growths in the range studied. The highest number of CFU-Meg growths were found in a patient with RAEB-T (case 7). This patient’s bone marrow cell numbers were so low that CFU-Meg colony formation added various concentrations of PHA-LCM could not be examined.

BFU-E colony growth was normal in one RAEB-T patient, and reduced in 4 RA patients and one RAEB patient. It was absent in one CMML patient, and in two RAEB-T patients (Table 2). The number of BFU-E cluster formations was almost the same or higher than that of the BFU-E colony growth of each patient except case 7. The number of BFU-E cluster growths in case 7 was nearly half that of BFU-E colony growths. The response of BFU-E to various doses of rHuEPO (2, 5, 20 U/ml) was variable in normal controls, and the mean BFU-E numbers were 80 ± 6 at 2 U/ml, 91 ± 6 at 5 U/ml, and 85 ± 9 at 20 U/ml of rHuEPO (Fig. 2). Regarding the mean values, a plateau in the increase of BFU-E colonies was observed at 5 U/ml of rHuEPO concentration. Changes in the number of BFU-E colonies in relation to increasing doses of rHuEPO were not detected in any MDS patients except case 7. The increase of BFU-E growths in case 7 was observed at 20 U/ml of rHuEPO concentration.

CFU-E colony growth was normal in one patient with RAEB-T and reduced

<table>
<thead>
<tr>
<th>Case</th>
<th>CFU-Meg</th>
<th>BFU-E</th>
<th>CFU-E</th>
<th>CFU-GM</th>
<th>EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>21</td>
<td>2730</td>
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<td>2</td>
<td>28</td>
<td>67</td>
<td>380</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>3</td>
<td>67</td>
<td>ND</td>
<td>1560</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
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<td>28</td>
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<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>23</td>
<td>147</td>
</tr>
<tr>
<td>Normal</td>
<td>16 ± 2</td>
<td>73 ± 9</td>
<td>322 ± 35</td>
<td>113 ± 11</td>
<td></td>
</tr>
<tr>
<td>± s.e.</td>
<td>(n = 4)</td>
<td>(n = 18)</td>
<td>(n = 17)</td>
<td>(n = 18)</td>
<td></td>
</tr>
</tbody>
</table>

The numerals show the number of CFU-Meg colony growths per 2 × 10^6 MNC at the concentration of 5% PHA-LCM, the number of erythropoietic (BFU-E, CFU-E) colony growths per 10^6 NAMNC at 2 U/ml of EPO concentration, and the number of CFU-GM colony growths at 100 U/ml of CSF “Chugai”. The normal range of plasma EPO levels is 8 to 30 U/ml. ND = not done.

**Table 2. Three-lineage precursor cells in bone marrow and plasma EPO titers**
Precursor Cells of MDS Patients

Fig. 1. Dose-response curves between CFU-Meg numbers and PHA-LCM concentrations. Except for one patient with RAEB-T, and another with CMML, a response of CFU-Meg to the concentration of PHA-LCM was not found among most MDS patients.

Fig. 2. Dose-response curves between BFU-E numbers and EPO concentrations. The increase of BFU-E colony growth was not detected in any patient except one with RAEB-T. A and B in the figure show the patients treated with high-dose rHuEPO.
in the other 8 patients; Cases 1, 6, 8, and 9 scarcely had any CFU-E colony growth (Table 2). The mean CFU-E numbers for the normal controls were 259 ± 25 at 2 U/ml, 267 ± 27 at 5 U/ml, and 225 ± 34 at 20 U/ml of EPO concentrations (Fig. 3). Dose-response increases between CFU-E colony numbers and EPO concentrations were not observed. CFU-E colony numbers of the patient with RA (case 1) were 8 at 2 U/ml, 10 at 5 U/ml, and 13 at 20 U/ml, but the increase of CFU-E colony growth was only slight. CFU-E colony growth from two patients (cases 2 and 3) with RA increased as EPO concentrations increased. The number of CFU-E colony growth in a patient with CMML (case 6) were 1 at 2 U/ml, 2 at 5 U/ml, and 2 at 20 U/ml concentrations of EPO. Cases 1 and 6 were treated with rHuEPO, but their hematocrit levels did not increase; neither did the frequency of transfusion decrease. The normal colony growth pattern of CFU-E was found in a patient with RAEB-T (case 7).

CFU-GM colony growth, added with 100 U/ml CSF- "Chugai", was normal in cases 2, 5, and 7, and reduced in cases 1, 4, and 9. It was absent in case 8, and increased in case 6 (Table 2).

CFU-GM colony formation in normal controls (n = 7) was 106 ± 13 at 10 ng/ml of G-CSF, and was 88 ± 8 at 100 ng/ml of G-CSF. The colony growth was likely to decrease at higher concentrations (Fig. 4). The four patients with RA and one patient with RAEB did not show significant increases of CFU-GM colony growth at each higher concentration. The patient with CMML shows the promi-
Precursor Cells of MDS Patients

Fig. 4. Dose-response curves between CFU-GM numbers and G-CSF concentrations. One patient with CMML and another with RAEB-T showed significant increases of colony growth in inverse relation to the response of normal controls.

Fig. 5. Dose-response curves between CFU-GM numbers and GM-CSF concentrations. The colony increase pattern added with GM-CSF in MDS was nearly the same as that added with G-CSF.
nent increase at 20 ng/ml of G-CSF. In RAEB-T, two patients (cases 8, 9) showed no increase in colony growth at each higher concentration, while one patient (case 7) showed a significant increase at 100 ng/ml of G-CSF.

CFU-GM colony formations added with GM-CSF in normal controls (n=10) were 118±16 at the concentrations of 10 ng/ml of GM-CSF, and 127±21 at 100 ng/ml of GM-CSF. The colony growth pattern was likely to increase slightly at higher concentrations (Fig. 5). The colony increment pattern added with GM-CSF in MDS was nearly the same as that added with G-CSF. In one of the patients with RAEB-T (case 7), the response of CFU-GM growth was slightly less than that added with G-CSF. The number of CFU-GM cluster formations that were added with G-CSF or GM-CSF varied greatly compared to the BFU-E cluster formations, and was approximately one-tenth to over one hundredth that of CFU-GM colony growth. When the colony formations were fewer, the cluster formations were likely to be greater.

The percentage of each colony growth in the MDS patients compared with normal controls was 27.8±25.5% of CFU-Meg, 9.0±7.5% of BFU-E, 11.1±5.8% of CFU-E, and 98.0±51.3% of CFU-GM (Table 2). The degree of precursor cell deficit, from larger to smaller, is as follows: BFU-E, CFU-E, CFU-Meg, CFU-GM.

Plasma EPO levels were very high in some patients (median 970 mU/ml: range, 40 to 6,960 mU/ml, Table 2). Two parameters between the hemoglobin concentration and plasma EPO levels did not attain a statistical inverse relationship (r = −0.405, p > 0.10). A wide range of EPO levels among patients with similar hemoglobin levels was found.

**DISCUSSION**

Although MDS are a heterogenous group of disorders, the pattern of in vitro growth in cultures of CFU-Meg, BFU-E, and CFU-E was very similar. Only CFU-GM colony growth in CMML was different from that of other MDS.

We demonstrated that the CFU-Meg, BFU-E, and CFU-E colony growths were less than the CFU-GM colony formation in MDS. These results seem to be appropriate, since the degree of thrombocytopenia and anemia is greater than that of leukopenia. Juvonen et al. (1989) demonstrated that the CFU-Meg colony formation reduced more often than the CFU-GM colony formation and less frequently than the growth of erythroid progenitors. Geissler et al. (1989) also reported that defects in CFU-Meg colony formation seem to be more comparable to alterations of BFU-E derived colonies than to CFU-GM. In the former report, the number of CFU-Meg colony growths of normal controls were relatively high (10-300/2×10⁵), and the highest limit of CFU-Meg colony growth was nearly three times greater than the highest limit of BFU-E colony formation. The range of CFU-Meg colony growths in our normal controls was relatively low, since we used normal human plasma. As expected, the CFU-Meg colony numbers were higher in aplastic anemia plasma, when compared with those of normal controls as the
Precursor Cells of MDS Patients

source of the megakaryocyte colony stimulating factor (Meg-CSF) (Kimura et al. 1984; Juvonen et al. 1989). We were therefore not able to clarify which colony growth reduced more often — the CFU-Meg or erythroid (BFU-E, CFU-E) colony formation. The number of CFU-Meg colony formations were not dose-dependent, since PHA-LCM contained not only Meg-CSF but also a Meg-CSF inhibitor (Kimura et al. 1984). It is also reported that the defect of megakaryocytic cell lineage in patients with MDS is due to, not only an intrinsic megakaryocytic progenitor cell defect, but also a deficiency in the megakaryocytic colony stimulating activity (Geissler et al. 1989).

Nowadays, clinical trials using recombinant products containing hemopoietic factors such as G-CSF, GM-CSF, EPO, and interleukin-3 (IL-3) have been done in MDS. Generally speaking, the responsiveness to G-CSF and GM-CSF was good (Vadhan-Raj et al. 1987; Antin et al. 1988; Ganser et al. 1989; Kobayashi et al. 1989; Negrin et al. 1989, 1990; Thompson et al. 1989), but to EPO was not good (Bessho et al. 1990; Stebler et al. 1990; Bowen et al. 1991). We tried rHuEPO treatment on one patient with RA, and on another patient with CMML. Both had few CFU-E & BFU-E colonies, resulting in no effects. Bessho et al. (1990) reported that a high-dose of rHuEPO was effective in 3 out of 8 patients with MDS. Two of the three demonstrated a significant Hb increase, and had 88 and 157 bone marrow CFU-Es, respectively. Although there were no effective cases in which CFU-E colony growths were not detected, there were some non-responders in which CFU-E colony growths were detected (Bessho et al. 1990). Bowen et al. (1991) reported that rHuEPO was effective in two out of 10 patients. The peripheral blood BFU-E of the two patients was 20 and 12/ml blood, respectively (normal controls: 58–632/ml blood). Therefore, the effectiveness of rHuEPO may be due to the number of residual CFU-E or BFU-E, although there are some exceptions. We found responses of CFU-Es to higher concentrations of EPO in two patients with RA (cases 2, 3) in vitro. The CFU-E numbers of those patients were 28 and 67 at 2 U/ml of EPO concentration. Therefore, CFU-E colony growths greater than at least 1/10 that of normal controls may be necessary in order for MDS patients to respond to a large dose of rHuEPO.

In addition, it is reported that the response of CFU-E to EPO was relatively good in RA and sideroblastic anemia (SA) between 1 to 500 U/ml of EPO concentrations in vitro (Aoki et al. 1990). However, the precursor cells of our two patients treated with rHuEPO did not increase between 2–20 U/ml of EPO concentrations in vitro. Therefore, the effectiveness of recombinant hemopoietic factors might be due to, not only the residual degrees of precursor cells, but also the responses of committed stem cells to each hemopoietic factor.

Having few CFU-Es does not appear to be an indication for high-dose rHuEPO therapy. Although erythropoiesis is rarely improved by the use of recombinant human IL-3 (rhIL-3) alone (Ganser et al. 1990), combinations of rhIL-3 and rHuEPO may be good therapy for severe anemic patients who have
few erythroid colonies, since IL-3 promotes the development of multipotential hematopoietic stem cells.

Plasma EPO levels in patients with MDS were noticeably high, and the variability of EPO levels for each MDS patient has been demonstrated (Jacobs et al. 1989; Vadhan-Raj et al. 1990; Bowen et al. 1991). The EPO levels of our patients were consistent with these observations. Bowen et al. (1991) reported that serum concentrations of two patients who responded to rHuEPO treatment were relatively low. The plasma EPO titers of our two rHuEPO-treated patients were very high. Aside from BFU-E, CFU-E colony growth in bone marrow cells, circulating EPO levels may also be worth consideration in estimating the effectiveness of rHuEPO administration.

In conclusion, the CFU-Meg, BFU-E, and CFU-E colony growths were less than the CFU-GM colony formation in MDS. The residual degrees of precursor cells and the responses of committed stem cells to each hemopoietic factor in patients with MDS may be helpful in predicting the effectiveness of recombinant hemopoietic factors.

References


