Kit Ligand Improves In Vitro Erythropoiesis in Myelodysplastic Syndrome

By Bianca Backx, Lianne Broeders, and Bob Löwenberg

Erythropoiesis in response to erythropoietin (Epo) in myelodysplastic syndrome (MDS) in vitro and in vivo is severely impaired. We investigated the stimulative effect of c-kit ligand (KL) on the erythroid colony-forming abilities of bone marrow cells from 17 patients with MDS. The effects of normal donor-derived marrow were examined in comparison. Suppression of erythroid colony formation in MDS in response to Epo could not be restored by the addition of interleukin-3 (IL-3) to culture. In cultures dishes supplemented with KL, erythroid colony formation was dramatically enhanced, regarding both colony number and size. Colony-forming abilities by MDS progenitors were improved following costimulation with KL, particularly in refractory anemia (RA) and refractory anemia with ring sideroblasts (RARS); however, little enhancement was apparent following KL stimulation of marrow from patients with refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t), and chronic myelomonocytic leukemia (CMML). These results suggest that KL responsiveness of patients with low-risk MDS may still be intact, and that with progression to high-risk MDS, erythroid progenitors lose proliferative reactivity to both KL and Epo stimulation. KL may have a therapeutic role in restoring erythropoiesis in a subset of patients with MDS.

THE MYELODYSPLASTIC syndromes (MDS) constitute a heterogeneous group of clonal disorders of hematopoietic pluripotent stem cells characterized by ineffective hematopoiesis resulting in anemia, neutropenia, and/or thrombocytopenia.1,4 The in vitro erythroid and myeloid colony formation is reduced and the addition of high doses of growth factors can augment colony formation only in infrequent cases of MDS.5-7 Clinical trials of MDS patients have shown that the response to treatment with recombinant erythropoietin (Epo) is generally poor.8-12 Furthermore, Epo levels in the serum of MDS patients are normal or increased,13 indicating that ineffective erythropoiesis is not the result of a lack of Epo production. Recently a new growth factor, designated c-kit ligand (KL), mast cell growth factor (MGF), or stem cell factor (SCF),14,15 has been isolated and cloned. KL alone has little colony-stimulating activity, but it considerably enhances the in vitro colony formation induced by Epo, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), or interleukin-3 (IL-3).16-18 We report our evaluation of the effects of KL on the in vitro colony-forming ability of marrow cells from 17 patients with different cytology types of MDS.

MATERIALS AND METHODS

Patients and Preparation of Cell Suspension

Bone marrow cells were obtained from 17 patients with MDS. The patients were classified according to French-American-British (FAB) criteria19 as refractory anemia (RA; n = 5), refractory anemia with ring sideroblasts (RARS; n = 2), refractory anemia with excess of blasts (RAEB; n = 4), refractory anemia with excess of blasts in transformation (RAEB-t; n = 3), and chronic myelomonocytic leukemia (CMML; n = 3). Clinical characteristics are summarized in Table 1. For comparison, marrow was obtained from hematologically normal adults following informed consent. The marrow cells were collected in Hanks’ balanced salt solution (HBSS) supplemented with heparin.

Purification of progenitor cells. Mononuclear cells were collected after Ficoll Isopaque density centrifugation as previously described.20 Cells were either used fresh or after cryopreservation21 and then enriched by a two-step cell purification procedure as described previously.22 In brief, cells were depleted of accessory cells by complement mediated cytolysis using monoclonal antibodies (MoAbs) against the antigens CD3 (T3, IgG1, final dilution 1:10), CD14 (B44,1, IgM, monocytes, final dilution 1:1,000),23 and CD15 (B4.3, IgM, granulocytic cells, final dilution 1:500).24 For the second purification step, cells were labeled with anti-CD34 MoAb (B13C5, IgG1, Sera-Lab, Crawley Down, UK)25 and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1 (GAM-FITC, Nordic, Tilburg, The Netherlands), and sorted using a fluorescence-activated cell sorter (FACS) as described.22

Recombinant human cytokines. Recombinant human interleukin-3 (IL-3; Gist Brocades, Delft, The Netherlands) was used at the optimum concentration of 40 ng/mL. Recombinant human KL (MZF, Immunex, Seattle, WA) was used at concentrations of 10 to 100 ng/mL. Recombinant human Epo (Dr S. Clark, Genetics Institute, Cambridge, MA) was used at optimal concentration of 1 U/mL. The optimum stimulatory concentrations were determined separately for each growth factor batch (data not shown).

Colony Assay

CFU-GEMM assay. Cells after cytolysis with CD3, CD14, and CD15 (1 × 10^6 cells/mL; cases 1-4, 7), or CD34-enriched cells (0.5 to 1 × 10^6 cells/mL; cases 5, 6, 8-17), were cultured in duplicate for 15 days in the presence of growth factors (as indicated) in a humidified atmosphere of 5% CO2 in air at 37°C and erythroid colony numbers were scored as described.20

Determination of colony size. To determine the mean number of erythroid cells per burst, bursts were individually removed by Pasteur pipette from culture dishes. Colonies were collected in phosphate-buffered saline. For each culture condition, three pools of five randomly picked bursts were counted. Results are expressed as the mean of the total number of pooled colony cells per experiment divided by the number of bursts.

Cytogenetic analysis. Marrow cells were cultured in the presence of Epo plus IL-3 or Epo plus IL-3 and KL. After 7 days in culture, the metaphases were harvested according to standard techniques on glass slides.26 The spread metaphases were stained with 10 mg Atebrin (G.T. Gurr) in 100 mL ethanol.

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Table 1. Characteristics of Patients With MDS

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Karyotype</th>
<th>FAB Subtype</th>
</tr>
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<tr>
<td>1</td>
<td>75/F</td>
<td></td>
<td>46,XX</td>
<td>RA</td>
</tr>
<tr>
<td>2</td>
<td>72/F</td>
<td></td>
<td>46,XX</td>
<td>RA</td>
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<tr>
<td>3</td>
<td>37/M</td>
<td></td>
<td>46,XY</td>
<td>RA</td>
</tr>
<tr>
<td>4</td>
<td>18/F</td>
<td></td>
<td>46,XX</td>
<td>RA</td>
</tr>
<tr>
<td>5</td>
<td>73/M</td>
<td></td>
<td>46,XY(63%); 46,XY,18q+(37%)</td>
<td>RA</td>
</tr>
<tr>
<td>6</td>
<td>36/M</td>
<td></td>
<td>46,XY(29%); 43,XY,mar1q−, −7,17p+, −18; −21(71%)</td>
<td>RARS</td>
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<tr>
<td>7</td>
<td>38/M</td>
<td></td>
<td>46,XY</td>
<td>RA</td>
</tr>
<tr>
<td>8</td>
<td>33/F</td>
<td></td>
<td>46,XX,t(3;12),del(7)(100%)</td>
<td>RAEB</td>
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<tr>
<td>9</td>
<td>63/M</td>
<td></td>
<td>46,XY</td>
<td>RAEB</td>
</tr>
<tr>
<td>10</td>
<td>67/F</td>
<td></td>
<td>46,XX(6%); 47,XX, +8(91%); 48,XX, +8; +21(3%)</td>
<td>RAEB</td>
</tr>
<tr>
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<td>46,XY</td>
<td>RAEB</td>
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<tr>
<td>12</td>
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<td>46,XY(3%); 44-48,XY, −4; −16, −18(50%); 46-49,XY, −4; −16, −18, 5q−, 7q−, 13q−(47%)</td>
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<td>46,XY(9%); 46,XY,9q−(91%)</td>
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<tr>
<td>17</td>
<td>74/F</td>
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RESULTS

Effect of KL on Erythroid Colony Formation

KL (10 ng/mL) was rarely capable of inducing BFU-E formation in MDS. Following stimulation with KL (with no Epo), scarce erythroid colonies (<3 burst-forming units-erythroid [BFU-E]/10⁴ cells) were formed in only three of 17 cases. KL alone induced BFU-E (31 ± 16 BFU-E/10⁴ cells; n = 3) from normal marrow. Stimulation with Epo alone gave rise to small numbers of erythroid colonies in MDS (Fig 1). Addition of KL to Epo cultures often considerably enhanced the appearance of erythroid colony numbers in MDS (Fig 1). In normal marrow, KL similarly enhanced Epo-induced erythroid colony formation (Fig 2). Besides the effect on number of erythroid colonies, KL also favorably influenced the size of erythroid colonies both in MDS and normal marrow (Figs 3 through 5). In the presence of KL and Epo, the mean erythroid colony size increased more than 10-fold as compared with erythroid

Fig 1. Effect of KL on erythroid colony formation in MDS. (A) KL (10 ng/mL) enhances the erythroid colony formation in Epo− (1 U/mL) or Epo + IL-3− (40 ng/mL) induced cultures from RA and RARS marrow. (B) Minimal or no enhancement of erythroid colony growth by KL is observed in marrow from patients with RAEB, RAEB-t, and CMML. Data are expressed as number of erythroid colonies per 10⁴ enriched marrow cells. Open bars, Epo; hatched bars, Epo plus KL; dotted bars, Epo plus IL-3; black bars, Epo plus IL-3 plus KL.

Fig 2. Effect of KL on erythroid colony formation in normal marrow. Results of three normal marrows are shown. For explanation, see Fig 1. Open bars, Epo; hatched bars, Epo plus KL; dotted bars, Epo plus IL-3; black bars, Epo plus IL-3 plus KL.

Fig 3. Effect of KL on size of erythroid colonies in MDS. Erythroid colony size was determined in cultures with Epo (1 U/mL), Epo plus KL (10 ng/mL), Epo plus IL-3 (40 ng/mL), or Epo plus IL-3 plus KL from three cases of MDS (see Materials and Methods). On the vertical axes, the average cell number of a pool of five collected colonies is plotted. For each of three cases of MDS, three such estimates were obtained. Horizontal line represents the mean of three estimates of three patients. △, Case 1; ○, case 2; □, case 3.
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Fig 4. Effect of KL on size of erythroid colonies in normal marrow. Marrow cultures from one normal donor. For explanation, see Fig 3.

Fig 5. Erythroid colonies derived from a patient with MDS. Marrow cells (case 2) were stimulated with (A) Epo or (B) Epo plus KL. (Original magnification x 100.)

colony size induced by Epo alone, and more than threefold as compared with size of erythroid colonies induced by Epo plus IL-3. In certain cases of MDS, numbers and size of erythroid colonies following stimulation with the combination of KL and Epo were elevated toward normal. The positive effect of KL was particularly apparent in patients with RA and RARS (low-risk MDS). The addition of IL-3 to cultures supplemented with KL plus Epo did not further augment the efficiency of erythroid colony growth (number or size). In contrast, in RAEB, RAEB-t, and CMML forms of MDS, the addition of KL to Epo-induced or Epo + IL-3–induced cultures promoted erythroid colony formation minimally. When 10-fold greater concentrations of KL (100 ng/mL) were added, identical results were obtained (data not shown). In patient 5, showing an 18q+ abnormality in 37% of metaphases (Table 1), the majority of erythroid colony formations with and without IL-3 were absolutely KL-dependent. Cells were incubated with KL plus Epo and IL-3 and after 7 days of culture cytogenetic analysis was performed. Of nine cells examined, five showed an 18q+ abnormality and four a normal karyotype. Among 11 cells in Epo + IL-3–induced cultures, seven 18q+ cells and four normal cells were identified. These results are consistent with the stimulation of erythroid colonies from the MDS clone.

DISCUSSION

The abilities of in vitro erythroid colony formation by marrow of patients with MDS are severely suppressed.5–7 The in vivo findings would suggest that inappropriate erythropoiesis in these individuals can not be explained by an Epo deficiency, since Epo levels are usually normal or even elevated.13,27 Furthermore, anemia of the majority of patients with MDS fails to improve following treatment with Epo.8,9 Our results demonstrate that KL in combination with Epo, significantly promotes the in vitro erythroid colony formation. This effect, an increase in both number and size of colonies, is most likely the consequence of direct stimulation of marrow progenitor cells by KL, since the marrow cells were depleted of accessory cells.20 The effect of KL depends on the cytological type of MDS. Improvement of erythroid growth in vitro by KL is especially seen in patients with RA and RARS, and minimal or no responses of erythroid growth in culture to KL are apparent in RAEB, RAEB-t, and CMML cases. The notable increase of BFU-E numbers in the marrow of RA patients, stimulated with KL and Epo, may result in BFU-E numbers comparable to normal marrow values (cases 1 and 2). The low number of BFU-E in RA and RARS is probably not due to a diminished population of erythroid progenitor cells, but to a qualitative inability of these cells to properly respond to stimulation by Epo.28 Thus, in RA and RARS, impaired in vitro erythroid colony formation can to a large extent be overcome by costimulation with KL (in combination with Epo). Since the doses of KL used were comparable to concentrations resulting in optimal stimulation of normal marrow, we assume that KL-receptor function in these MDS cases is intact. These in vitro data suggest that KL would be a good candidate for use in clinical trials to...
restore defective erythropoiesis in MDS in vivo. The variability of the stimulatory effect of KL among MDS cases potentially provides a means for identifying individuals who would most likely respond to treatment with KL. In high-risk MDS (eg, RAEB, RAEB-t, CMML), erythroid progenitor cells that would normally respond to stimulation by KL (in combination with Epo) can no longer be demonstrated. Impaired in vitro erythropoiesis in these cases of MDS may thus be caused by progressive loss of responsiveness to KL. As a result, MDS progenitor populations that would respond to Epo, as well as those inducible by KL, are quantitatively depressed in high-risk MDS. Whether these progenitors are unable to react properly to KL due to an abnormal functioning KL-receptor or an intrinsic cell defect more downstream to the receptor is presently unclear. Of note is the striking similarity in the response of MDS patients and patients with Diamond-Blackfan anemia (DBA) to KL. In DBA, a selective deficiency of red blood cell precursors exists, resulting in defective erythropoiesis. Analogous to MDS, erythroid progenitors in DBA fail to respond to growth factors like Epo and IL-3, but the addition of KL permits a dramatic in vitro increase in both size and number of erythroid colonies. These results stress the central role of KL in erythropoiesis.

We conclude that in RA and RARS, the population of Epo-responsive target cells in the marrow is severely reduced, but KL-responsive precursors are maintained at normal or near-normal levels. On the other hand, in high-risk MDS types (RAEB, RAEB-t, CMML), both Epo-responsive and KL-responsive subsets among the erythroid progenitor cell compartment are greatly reduced. These findings suggest that evolution of MDS and impaired hematopoiesis are associated with progressive loss of Epo and KL responsiveness, probably due to intrinsic inabilities of the cells to respond.

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REFERENCES


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