RAPID COMMUNICATION

Abnormal Responsiveness of Granulocyte-Committed Progenitor Cells in Cyclic Neutropenia

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The mechanism(s) driving cyclic hematopoiesis in human cyclic neutropenia remains unknown. Clinical trials suggest that an abnormal responsiveness of bone marrow progenitor cells to hematopoietic growth factors might cause oscillatory blood counts. Studies were performed to determine whether an abnormal responsiveness to multiple growth factors exists in this disorder and whether the defect could be shown in highly enriched populations of marrow progenitor cells. Bone marrow mononuclear cells from patients with congenital cyclic neutropenia required higher concentrations of added granulocyte-colony-stimulating factor (G-CSF) to achieve half-maximal colony growth than cells from normal subjects (478 ± 90 pmol/L vs 53 ± 12 pmol/L, P < .01). Patients also differed in requirement for granulocyte-macrophage-CSF (P < .05), but not for interleukin-3 (P > .30). CD34+ bone marrow cells from three patients also showed this difference in G-CSF responsiveness (P < .05). These data suggest that the defect in congenital cyclic hematopoiesis lies in growth factor receptor binding or the postreceptor signal transduction system that drives granulocytopoiesis. © 1992 by The American Society of Hematology.

HUMAN CYCLIC neutropenia (cyclic hematopoiesis) is a rare disorder in which regular 21 day oscillations of blood cell counts occur.1-3 The periodic severe neutropenia is associated with fever, malaise, aphthous stomatitis, pharyngitis, cervical lymphadenopathy, and, occasionally, very severe infections. The cycling of blood cells is attributable to oscillation in leukocyte, erythrocyte, and platelet production, but the basic mechanism(s) driving these oscillations remains unknown.

As one possible mechanism for hematopoietic cycling, Wright et al4 suggested that there was an abnormal responsiveness of marrow precursors to hematopoietic growth factor. They found diminished colony formation (colony-forming unit granulocyte-macrophage [CFU-GM]) in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of cryopreserved marrow cell populations obtained from four patients with congenital cyclic neutropenia.4 No assessment of responsiveness to other growth factors was made and the potential contribution of accessory cell response alterations in the marrow cell populations was not determined. Subsequently, Wright et al have reported that recombinant human GM-CSF (rhGM-CSF) treatment in two of these patients was relatively ineffective in increasing blood neutrophil counts,5 suggesting that a simple lack of this growth factor is not a sufficient explanation for the disease.

We have examined the therapeutic response of patients with cyclic neutropenia to granulocyte-CSF (G-CSF) and the response of dogs with a very similar disease to several recombinant growth factors. In grey collie dogs, neither interleukin-3 (IL-3) nor GM-CSF altered the cyclic fluctuations of the blood counts, whereas high doses of G-CSF obliterated all of the cyclic fluctuations.6,7 The degree of neutrophilia induced for a given dose was substantially less in the grey collies compared with normal dogs. When the dose of G-CSF was reduced to levels that drive marked neutrophilia in normal dogs, however, the oscillatory behavior persisted, although the degree of neutrophilia was reduced. Thus, pharmacologic but not physiologic doses of recombinant G-CSF can eliminate the cyclical oscillations in dogs with this disorder.

Further, we observed that rh G-CSF ameliorated cyclic neutropenia clinically, but failed to eliminate the cyclic fluctuations of cell counts in five patients with the congenital disease,5 suggesting that the underlying cause of their disorder had not been eliminated. In aggregate, these data suggest that some component of decreased responsiveness to hematopoietic growth factors is a pathophysiologically important step in congenital cyclic hematopoiesis.

The present studies were undertaken to specify the abnormal responsiveness of cells from patients with cyclic neutropenia by examining responsiveness to G-CSF and IL-3 as well as GM-CSF, and to determine whether this abnormality is still evident in populations of cells enriched for committed progenitor cells.

MATERIALS AND METHODS

Human subjects. Blood sampling and bone marrow (BM) aspirations were performed with informed consent under protocols approved by the University of Washington’s Human Subjects Review Committee. Five patients with congenital and one with acquired cyclic neutropenia were studied before initiation of rhG-CSF treatment, and are the same patients reported in our clinical report.8 Five normal volunteers were studied for comparison. Heparinized marrow cell samples were harvested during neutropenic periods in all patients and during neutrophilia in two.
patients and processed fresh without freezing; all normal subjects had normal blood counts at the time of sampling.

Cell preparation. Heparinized marrow cell suspensions were diluted 1:1 with NCTC 109 tissue culture medium and the marrow cells dispersed by repipetting. The light-density cell fraction was separated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation as previously described to obtain BM mononuclear cells (BMMCs). Adherent cells were removed by two 1-hour incubations of the cell suspensions in plastic flasks (T-75; Falcon) containing 2 \times 10^5 cells in 20 mL of NCTC 109 supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO2. These adherent cell-depleted, light-density marrow cell suspensions were used for colony assays as described below.

Staining and cell sorting. Cells were stained using indirect immunofluorescent antibody staining techniques and separated by fluorescence-activated cell sorter (FACS) as previously described. Antibody 12.8, a murine IgM monoclonal antibody (MoAb) that identifies CD34, and antibody p67.5, a murine IgG2a MoAb that identifies the CD33 antigen, were used for positive sorting. As isotype controls for staining, we used the antimouse Thy-1.1 MoAb 1A14, an IgG2a, and H12C12, a murine monoclonal IgM against the mouse Thy-1.2 antigen. All staining was performed with cells suspended in sterile phosphate-buffered saline (PBS) supplemented with 2% human AB serum. For two-color staining, BMMCs were incubated with both antibody 12.8 (25 \mu g/mL) and p67.5 (1:10 dilution). Control cells were incubated with: (1) H12C12 (25 \mu g/mL) and 1A14 ascites (1:10); (2) H12C12 (25 \mu g/mL) and p67.5 (1:10); or (3) 12.8 (25 \mu g/mL) and 1A14 (1:10). Control and experimental cells were incubated with the primary antibodies for 30 minutes at 4°C, washed twice, then incubated with a 1:80 dilution of biotin-conjugated goat antimouse IgM antisera (Tago Inc, Burlingame, CA) and a 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (γ-chain specific; Southern Biotechnology Associates, Inc, Birmingham, AL) for 30 minutes at 4°C, and then washed twice. Finally, cells were incubated with a 1:20 dilution of phycoerythrin-conjugated avidin (Becton Dickinson Co, Oxnard, CA) for 30 minutes at 4°C and washed once. Cells were analyzed and sorted using either a FACS-440 or FACS-II (Becton Dickinson) and collected in tissue culture medium with 10% fetal calf serum (FCS). Quantitation of CD34+ cells into CD33+ and CD33- cell populations was performed; sorting was performed using only CD34 positivity for cell culture studies.

Colony assays. In vitro colony formation from granulocytic committed progenitor cells was performed in a serum-containing system as previously reported and colonies counted on days 7 and 21. Adherent-cell-depleted BMMCs were cultured at between 5 \times 10^4 and 2 \times 10^5 cells/mL in 0.3% agar containing 20% FCS and various concentrations of individual growth factors. Maximal control growth was stimulated by a mixture of the three recombinant hematopoietic growth factors IL-3 (40 pmol/L), GM-CSF (50 pmol/L), and G-CSF (282 pmol/L) (kindly provided by Dr. L. Souza, Angen, Inc, Thousand Oaks, CA) or by phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM). Sorted CD34+ cells were plated at between 1 and 4 \times 10^5 cells/mL with otherwise identical culture conditions. Dose-response curves were constructed by representing each individual marrow's growth as the proportion of maximal growth for that particular growth factor and averaging across patients. In addition, for each individual patient the curve for each growth factor was graphed and the growth factor concentration at which 50% of maximal growth occurred (ED50) estimated graphically. The ED50s for normal and cyclic neutropenic subjects were then tested for statistically significant differences by Wilcoxon rank sum tests.

RESULTS

BMMC colony formation. The total granulocytic colony formation of patient BMMCs (110 ± 24 [±1 SEM] colonies/10^5 marrow cells) was not significantly different from normal subjects (107 ± 25 colonies/10^5 marrow cells) when stimulated by maximal concentrations of recombinant hematopoietic growth factors in combination (IL-3 plus GM-CSF plus G-CSF) and counted at day 21. The response to added concentrations of individual growth factors in this serum-containing system showed significant differences between patients with congenital cyclic neutropenia and normal volunteers (Fig 1A through C). For colony formation in response to G-CSF, patients required significantly higher concentrations of added factor to produce colonies.
than normals. When individual dose-response curves were plotted to generate an ED50 and group means for ED50s were calculated, patients had higher ED50s for G-CSF (478 ± 90 pmol/L v 53 ± 12 pmol/L, P < .01) and GM-CSF (114 ± 32 pmol/L v 48 ± 7 pmol/L, P < .05), whereas the ED50s for IL-3 were not different (36 ± 6 pmol/L v 35 ± 9 pmol/L, P > .30). Of note, the ED50s for “neutrophilic” marrow samples in two patients appeared no different from those for their “neutropenic” marrows and were therefore pooled (5 patients, 7 marrows). Interestingly, the one patient with acquired cyclic neutropenia showed dose-response curves to G-CSF, GM-CSF, and IL-3 that were indistinguishable from the normals (data not shown).

Enriched progenitor cell colony formation. BMMC preparations were subjected to sorting by FACS. The cell population of interest was gated by a combination of forward scatter and right angle light scatter and then sorted by fluorescence-staining intensity into CD34+ and CD34− subpopulations. The CD34+ cells were enumerated for CD33 positivity, and no significant change in proportions of doubly labeled (CD34+, CD33−) cells of CD34+ cells was evident.

Colony formation by CD34+ cells was evaluated in response to G-CSF at days 7 and 21 in culture in three patients and three normal volunteers. For day 7 colonies there was a fivefold increase in ED50 for patients compared with normals (1,507 ± 807 pmol/L v 340 ± 163 pmol/L, N = 3), whereas for day 21 colonies there was a sevenfold increase in ED50 (333 ± 82 pmol/L v 44 ± 4 pmol/L, P < .05, N = 3) (Fig 1D). Control cultures of CD34− cells grew negligible numbers of granulocytic colonies.

DISCUSSION

The data presented here show that maximal granulocytic colony formation in BM cell populations from patients with congenital cyclic neutropenia requires abnormally increased concentrations in vitro of G-CSF and GM-CSF, but not IL-3. This implies that these patients’ marrow cells either require increased growth factor stimulation or somehow block growth factor action on the progenitor cells within the marrow. This study attempted to localize the defect to the progenitor cell itself by examining highly enriched populations of progenitor cells and showing that the same abnormality was demonstrable in CD34+ cells.

These data extend the report by Wright et al4 by showing that the abnormal responsiveness to GM-CSF is accompanied by an apparently larger abnormality in response to G-CSF. Furthermore, they show that the defect is probably expressed in the progenitor cell itself because the abnormality persisted in the CD34+ cell populations. At least, this latter finding implies that morphologically identifiable neutrophilic precursors (eg, promyelocytes, myelocytes, metamyelocytes, and bands) are not the cause of the abnormal responsiveness of unseparated marrow cells (eg, by excessive binding of G-CSF), because these cells are not seen in the CD34+ cell populations. Whether a small population of CD34+ marrow “accessory” cells contributes to the abnormal response remains unknown.

Because we found abnormal responsiveness to two different growth factors, we must consider different possible explanations. It is probable that the in vitro culture system requires multiple different growth factors acting in concert for optimal colony formation and that serum in the system provides a limiting quantity of each factor.10,12 In that case, a defect in responsiveness to one factor for which a second factor was required, would result in apparent abnormalities in response to each. Hence, the finding of two abnormalities could be an in vitro artifact of a single abnormality in factor responsiveness. If both abnormalities are due to an in vitro requirement for two factors, these data might be important pieces of evidence for the hierarchical action of hematopoietic growth factors.

Alternatively, the existence of abnormal response to two factors could mean that the defect in cyclic neutropenia lies in a common stimulatory pathway shared by these granulopoietic factors. In this case, elucidation of the specific defect in cyclic neutropenia could demonstrate critical features of as yet unidentified mechanisms underlying homeostasis in hematopoiesis. In either event, these data suggest that the abnormality causing cyclic hematopoiesis in patients with congenital cyclic neutropenia lies in growth factor receptor binding or the postreceptor signal transduction system that drives granulopoiesis.

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