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 v 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 granulocytepoiesis. © 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 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 al. 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. 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, 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. 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, 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. 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 medium5,10 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 mL of NCTC 109 supplemented with 10% (vol/vol) fetal calf serum10 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.11 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 antiamouse Thy-1.1 MoAb 1A14, an IgG2a, and H12C12, a murine monoclonal IgM against the mouse Thy-1.2 antigen.11 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 µg/mL) and p67.5 ascites (1:10 dilution). Control cells were incubated with: (1) H12C12 (25 µg/mL) and 1A14 ascites (1:10); (2) H12C12 (25 µg/mL) and p67.5 (1:10); or (3) 12.8 (25 µg/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 antiserum (µ-chain specific; Tago Inc, Burlingame, CA) and a 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (µ-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 then analyzed and sorted using either a FACScan or FACS II (Becton Dickinson) and collected in tissue culture medium with 10% fetal calf serum (FCS). Quantitation of CD34+ cells into CD33+ and CD34− 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 reported5,10 and colonies counted on days 7 and 21. Adherent-cell-depleted BMMCs were cultured at between 5 x 105 and 2 x 106 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, Amgen, Inc, Thousand Oaks, CA) or by phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM).9 Sorted CD34+ cells were plated at between 1 and 4 x 104 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 patients 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/105 marrow cells) did not significantly differ from normal subjects (107 ± 25 colonies/105 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
different from those for their “neutropenic” marrows
and were therefore pooled (5 patients, 7 marrows). Interest-
ingly, 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 prep-
arations 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+ cell populations 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,
N < .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 in-
creased 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 some-
how 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 accompa-
nied 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 abnormal-
ality persisted in the CD34+ cell populations. At the 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 dif-
gerent 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 hematopo-
ietic 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 granu-
lopoietic 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 transduc-
tion system that drives granulocytopoiesis.

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