RAPID COMMUNICATION

Selective Hypersensitivity to Granulocyte-Macrophage Colony-Stimulating Factor by Juvenile Chronic Myeloid Leukemia Hematopoietic Progenitors

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Juvenile chronic myelogenous leukemia (JCML) is a good model for the study of myeloproliferation because JCML hematopoietic progenitor cells grow in vitro at very low cell densities without the addition of exogenous stimulus. Previous studies have demonstrated that this proliferation is dependent on granulocyte-macrophage colony-stimulating factor (GM-CSF), and that removal of monocytes from the cell population before culture eliminates this "spontaneous" myeloproliferation, suggesting a paracrine role of monocyte stimulation. However, subsequent studies have shown that increased GM-CSF production from the JCML monocytes is not a consistent finding and therefore not a plausible sole mechanism. In examining hematopoietic growth factor dose-response curves, both JCML GM and erythroid nonadherent progenitor cell populations displayed a marked and selective hypersensitivity to GM-CSF. Responses to interleukin-3 and G-CSF were identical to control dose-response curves. This is the first demonstration of a myeloid leukemia in which hypersensitivity to a specific growth factor appears to be involved in the pathogenesis of the disease.

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JUVENILE CHRONIC myelogenous leukemia (JCML) is a myeloproliferative disorder that primarily affects patients less than 4 years of age. It is clearly a different disease from and is distinguished from adult-type CML by absence of the Philadelphia chromosome, elevated fetal hemoglobin, thrombocytopenia, prominent monocytosis, and usually a more moderate leukocytosis, and a normal karyotype. JCML patients rarely undergo transformation to a blast crisis, and death is usually a result of infection or organ failure or obstruction due to infiltration by monocytes and macrophages. In vitro, JCML peripheral blood granulocyte-macrophage progenitors (CFU-GM) demonstrate the unique ability to proliferate "spontaneously" at very low cell densities in the absence of exogenous stimulus. Peripheral blood mononuclear cells from normal donors and patients with other myeloproliferative disorders virtually never show "spontaneous" proliferation, whereas bone marrow mononuclear cells can show small amounts of "spontaneous" CFU-GM formation when cultured at higher cell densities. The "spontaneous" proliferation in JCML can be abolished by prior depletion of adherent cells (monocytes) from the cultures. We have demonstrated previously that the "spontaneous" proliferation in vitro is regulated primarily by granulocyte-macrophage colony-stimulating factor (GM-CSF), because only specific antibodies to GM-CSF, but not antibodies to other cytokines, were able consistently to inhibit CFU-GM proliferation. Because monocytes are necessary for the myeloproliferation, at least in vitro, a likely mechanism could be an increased production of GM-CSF by the JCML monocytes. However, we recently have reported that increased GM-CSF production by JCML cells is not a consistent finding. Another plausible explanation could be JCML progenitor cell hypersensitivity to low concentrations of GM-CSF and/or other hematopoietic growth regulatory molecules.

MATERIALS AND METHODS

Sample acquisition and preparation. With parental consent and the approval of the respective Institutional Review Boards, peripheral blood samples from children with either JCML or adult-type CML were collected into preservative-free heparin (100 U/mL) and shipped at ambient temperature overnight to the University of Alabama at Birmingham. Normal control bone marrow samples were obtained by bone marrow aspirate from the posterior iliac spine from normal, paid, consenting adults, as approved by the University of Alabama at Birmingham Institutional Review Board. Mononuclear cells (MNC) were obtained by Ficoll-Hypaque separation of peripheral blood or bone marrow. Adherent cells were depleted by incubating MNC in the presence of human AB serum in tissue culture dishes overnight, followed by a second 90-minute adherence depletion. T lymphocytes were removed by incubation of MNC with anti-Leu-1 and anti-Leu-5b antibodies (Becton-Dickinson, Mountain View, CA), followed by panning in sheep antinmouse IgG-coated tissue culture dishes.

CFU-GM assays. CFU-GM assays were established as described previously. Briefly, we used 0.3% agar in McCoy's 5A medium supplemented with nutrients, 15% fetal bovine serum, and added 2 to 5 x 10^5 cells per 1 mL culture. Recombinant human G-CSF (R & D, Minneapolis, MN), GM-CSF (Amgen Biologicals, Thousand Oaks, CA), or interleukin-3 (IL-3) (Amgen Biologicals) was added at the time the cultures were initiated. Cultures were performed in triplicate, and colonies of ≥ 40 cells were scored after 14 days of incubation. Recombinant human erythropoietin (Amgen Biologicals) was included at 1U/mL. Cultures were performed in triplicate with 2 to 5 x 10^5 cells per 0.3 mL of plasma clot culture. BFU-E-derived colonies containing ≥ 64 benzidine-positive cells and/or > 2 subcolonies were scored after 12 to 14 days of incubation.

Erythroid burst-forming unit (BFU-E) assays. BFU-E were assayed by establishing plasma clot cultures as described previously. Recombinant human erythropoietin (Amgen Biologicals) was included at 1U/mL. Cultures were performed in triplicate with 2 to 5 x 10^5 cells per 0.3 mL of plasma clot culture. BFU-E-derived colonies containing ≥ 64 benzidine-positive cells and/or > 2 subcolonies were scored after 12 to 14 days of incubation.

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Statistical analysis. The Student's t-test was used to determine the significance of differences among groups of unpaired samples.

RESULTS

We examined in vitro proliferation of JCML CFU-GM and BFU-E in the presence of escalating doses of GM-CSF, IL-3, or G-CSF in separate culture systems. The progenitor cells were obtained from adherent cell-depleted peripheral blood and bone marrow mononuclear cells from JCML patients. Control cultures used adherent cell-depleted peripheral blood mononuclear cells from children with adult-type CML (Philadelphia chromosome positive; Ph+), as well as adherent and T-lymphocyte cell-depleted mononuclear cells from normal adult bone marrow donors. Peripheral blood mononuclear cells from normal donors cannot be used as control cells because they do not show consistent CFU-GM proliferation, even with maximal hematopoietic growth factor (HGF) stimulation (P.D.E. and K.S.Z., unpublished observations). As seen in Fig 1A, JCML hematopoietic progenitor cells showed no spontaneous proliferation when adherent cells were depleted and in the absence of exogenous growth factor. Because there was very minimal colony growth at 0.1 U GM-CSF/mL, the cultures could not have contained more than 0.1 U of endogenously produced GM-CSF. However, in the presence of escalating doses of exogenous CM-CSF, the JCML progenitor cells demonstrate a marked hypersensitivity to GM-CSF as compared with the two control populations. With as little as 1 U GM-CSF/mL of agar culture, JCML CFU-GM showed 44% of maximal growth (±10 SEM) as compared with 4% (±4) of maximal growth for normal donors and 2% (±4) for children with adult-type CML ($P = 3.8 \times 10^{-4}$). This hypersensitivity was observed throughout the dose-response curve, as illustrated in Fig 1A. On the other hand, as seen in Figs 1B and 1C, response of JCML progenitors to IL-3 and G-CSF was similar to the two control populations. It is critically important to note that all eight JCML patients tested demonstrated a very similar level of hypersensitivity to GM-CSF, despite varying levels of GM-CSF production from their monocytes, ranging from 80 to greater than 1,000 pg/mL (normal value, 0 to 200 pg/mL).12 Of further importance, four JCML patients had peripheral blood and bone marrow progenitors cultured and showed identical dose responses of progenitors from both sources. In addition to these JCML patients, samples were also available from four other JCML patients in which we obtained only bone marrow from one patient and only peripheral blood from three patients.

We also examined BFU-E responsiveness to GM-CSF and IL-3, in the presence of erythropoietin (EPO). Adherent cell-depleted peripheral blood JCML progenitor cells

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig 1. Hematopoietic growth factor dose-response curves for CFU-GM. The data are expressed as percent of maximal numbers of CFU-GM. This more accurately reflects changes in sensitivity and does not bias the results as compared with graphing actual counts because most JCML samples had considerably higher total numbers of CFU-GM than the normals, although there was considerable patient-to-patient variability. CFU-GM responsiveness to (A) GM-CSF, (B) IL-3, and (C) G-CSF. Mean actual maximal CFU-GM counts per 10⁵ cells were (A) JCML = 166, normal = 77, ACML = 84, (B) JCML = 171, normal = 60, ACML = 65; and (C) JCML = 135, normal = 57, ACML = 21.
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from three patients were compared with three adherent and T-lymphocyte cell-depleted peripheral blood normal donor samples as well as three similarly treated normal marrow samples. Again, no difference was observed in the dose response between peripheral blood versus bone marrow samples. In numerous previous studies in our laboratory there is no colony growth difference between adherent cell-depleted and adherent plus T-cell–depleted mononuclear cells. As seen in Fig 2, JCML BFU-E displayed 50% (±13) of maximal growth at 1 U GM-CSF/mL plasma clot culture as compared with 13% (±8) of maximal growth from normal populations (P = .001). JCML BFU-E responsiveness to IL-3 was similar to controls. In our erythroid culture studies we also examined three JCML peripheral blood samples without added EPO (data not shown) and saw no EPO-independent colony growth.

DISCUSSION

We have demonstrated a marked leftward shift of the dose-response curve, representing an approximate 10-fold, selective hypersensitivity of JCML hematopoietic progenitors, both CFU-GM and BFU-E, to GM-CSF. Because of a strikingly similar degree of hypersensitivity, both from peripheral blood and bone marrow JCML samples, and in JCML patients with widely varying levels of GM-CSF production, we believe this to be a real phenomenon that is independent of such potential culture artifacts as derivation of target cells from different sources or the presence or absence of T lymphocytes.

Hematopoietic growth factor mRNA expression or actual factor production in an autocrine manner in acute myeloid leukemia (AML) cells has been demonstrated previously for IL-1, IL-6, GM-CSF, G-CSF, M-CSF, and tumor necrosis factor-α (TNFα). However, apart from a preliminary report before the advent of recombinant growth factor production, we believe this to be a real phenomenon that is independent of such potential culture artifacts as derivation of target cells from different sources or the presence or absence of T lymphocytes.

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BFU-E also show hypersensitivity to GM-CSF, the final control on production of mature erythroid cells is EPO, regardless of the number of BFU-E that may be present. We found no EPO-independent growth by JCML erythroid progenitors (data not shown), in agreement with previous reports that demonstrated normal, EPO-dependent erythropoiesis in JCML patients. Therefore, while primitive erythroid progenitors may be dysregulated, the final steps of erythroid differentiation show normal EPO regulation, which may explain why JCML patients do not have erythroid hyptisensitivity to growth factors? responsiveness of myeloid leukemic cells to CSFs previously for IL-1, IL-6, GM-CSF, G-CSF, M-CSF, and tumor necrosis factor-α (TNFα). However, apart from a preliminary report before the advent of recombinant growth factor production, we believe this to be a real phenomenon that is independent of such potential culture artifacts as derivation of target cells from different sources or the presence or absence of T lymphocytes.

The hypothesis of the mechanism of JCML being based on a pluripotent stem cell disorder was first advanced by Altman et al in 1974 and later by others. This hypothesis was based on indirect evidence of involvement of more than one cell lineage based on the following: (1) "spontaneous" myeloproliferation, (2) dyserythropoiesis with some reversion to the fetal type, and (3) qualitative abnormalities of platelets and lymphocytes, as well as hypergammaglobulinemia. Our data provide the first direct evidence that the mechanism in JCML is a disorder involving at least the
myeloid and erythroid lineages, and therefore supports and confirms the original hypothesis that JCM involves a pluripotent stem cell disorder, as is the case in adult type (Ph\(^+\)) CML, although the pathogenesis of the two diseases is clearly different.

Defining the mechanisms of the hypersensitivity may greatly advance the understanding of normal and abnormal hematopoiesis. However, judging from the numerous previous reports of autocrine production of various HGFs, the AMLs are likely to involve a more complex situation. Likewise, we cannot rule out a secondary contribution of increased GM-CSF production by the JCM monocytes in some cases. A previous report suggested that IL-1 may induce CSF production in JCML monocytes. Nevertheless, we have examined GM-CSF levels in the monocyte conditioned medium from 15 JCML patients and have found increased levels in only seven of them. Additionally, four of these seven patients with increased GM-CSF production were also analyzed in the current studies, and all four demonstrated similar GM-CSF hypersensitivity to the four patients whose monocytes produced normal levels of GM-CSF. Further, the clinical histories of the two groups show no obvious differences.

The exact mechanism of the selective GM-CSF hypersensitivity may occur at a receptor, intracytoplasmic, or nuclear signalling level. Receptor studies in the JCM progenitor cells have been hampered by the inability to identify and isolate sufficient numbers of hematopoietic progenitors, which demonstrate the GM-CSF hypersensitivity, from the relatively small amount of blood or bone marrow that can be obtained from these very small children. Further, there usually is no chromosomal marker in JCM and the hematopoietic cells morphologically appear normal, which makes it difficult to distinguish malignant from residual normal progenitors. Recently, however, there have been several studies investigating GM-CSF receptors. There are at least two classes of GM-CSF receptor. There I receptors are found on neutrophils and other more mature cells. These receptors are downregulated by GM-CSF, and show no competitive binding with IL-3. Class II receptors are found on myeloblasts and other more immature cells, as well as monocytes. They show a slightly higher affinity, are not down-regulated by GM-CSF, but do show cross-competition of binding by IL-3 and GM-CSF. Monocytes and other cells and cell lines may express both classes of receptors. There may exist several other forms of GM-CSF receptor on hematopoietic and other cells with varying affinities, and the interaction of GM-CSF and IL-3 with receptors is clearly not yet well understood. Despite these shortcomings, a low-affinity human GM-CSF receptor has been cloned and more recently has been shown to have the ability to deliver a proliferative signal to hematopoietic cells. This signalling mechanism appears not to be species-specific, although GM-CSF binding to the receptor is species-specific. With the isolation and characterization of this receptor, studies examining qualitative or quantitative GM-CSF receptor changes in JCM hematopoietic progenitor cells may now be more readily accomplished.

In summary, JCM is a pediatric malignancy in which the abnormal myeloproliferation appears to be due nearly exclusively to dysregulation of GM-CSF signalling. The mechanism does not appear to be excessive production of GM-CSF, but rather is a selective hypersensitivity of the hematopoietic progenitor cells to GM-CSF. The cells are not autocrine stimulated but instead are dependent on low basal levels of GM-CSF. This hypersensitivity is likely to be the unifying mechanism that explains previous reports by us and others suggesting possible paracrine stimulatory roles for GM-CSF or inductive effects by IL-1 or TNF in the JCM myeloproliferation. This is the first demonstration of a myeloid leukemia in which hypersensitivity to a specific growth factor appears to play a major role in the pathogenesis of the disease.

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