CONCISE REPORT

Effects of Recombinant GM-CSF on the Blast Cells of Acute Myeloblastic Leukemia

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The effects of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) were compared to those of media conditioned by the continuous bladder carcinoma line, HTB9 (HTB9-CM), using three criteria. First, both GM-CSF and HTB9-CM stimulated blast colony formation in methylcellulose cultures, patient-to-patient variations were seen in the dose-response curves, and GM-CSF was effective, but less so than HTB9-CM. Second, GM-CSF also enhanced growth of blast progenitors in suspension culture, indicating its capacity to support self-renewal. GM-CSF was as effective as HTB9-CM in the production of adherent cells during the growth of blast cells in suspension, a finding that is interpreted to mean that GM-CSF also supports postdeterministic events in blast differentiation. Finally, colonies growing in the presence of GM-CSF were not phenotypically different than those stimulated by HTB9-CM.

Source of Growth Factors

Conditioned medium from HTB9 and recombinant GM-CSF. HTB9-CM was harvested on day 7, 2 to 3 days after the cells became confluent. The HTB9-CM was sterilized by filtration (Millipore, 22 μm) and stored at 4°C. One batch, selected for its potency as a stimulator of colony formation by AML blasts, was used without purification as a standard in all experiments. Recombinant GM-CSF was obtained from the Genetics Institute, Boston, as unpurified supernatant from GM-CSF-transfected CHO cells. This material contains 1% bovine serum and ~10^9 U of GM-CSF.

Cell cultures. Recombinant GM-CSF was compared with HTB9-CM in cultures of blast cells from patients with AML and marrow cells from normal donors. The effect of growth factors on blast colony formation was tested by plating cells in 96-microwell plates (Linbro, Flow Lab, McLean, Va) as described previously, in α-MEM supplemented by 10% FCS and 0.8% methylcellulose. Each well contained from 10^2 to 10^4 cells in 100 μL of medium; these cell concentrations were selected to yield a minimum number of colonies in the absence of added factor. The putative stimulators to be tested were added to the wells in dilutions ranging from 10^{-1} to 10^{-7}. The cultures were incubated for 5 to 7 days in a moist atmosphere containing 3% CO_2, and colonies were enumerated using an inverted microscope.

Blast cells were cultured in suspension in 35-mm Petri dishes (Lux) in α-MEM and 20% FCS as described previously. Cells from patient 1 can be maintained for long periods in suspension; their exponential growth for 224 days has been described and still continues; growth remains dependent on the addition of HTB9-CM to the cultures. Growth factors were added at the same range of dilution given above. After 7 days, the cells were harvested and plated in methylcellulose with an optimal concentration of HTB9-CM.

MATERIALS AND METHODS

Cells

With the patients' informed consent, cells were obtained from the peripheral blood of three patients with AML. The sample from patient 1 (French-American-British classification, FAB 4) was obtained at relapse following treatment with high-dose cytosine arabinoside. Patient 2 (FAB 1) was seen at first occurrence and was treated with a combination of Adriamycin, cytosine arabinoside, and thioguanine. Patient 3 (FAB 4) was in relapse following treatment with high-dose cytosine arabinoside.

The suspensions were depleted of T cells by sheep erythrocyte rosetting as described previously. Normal bone marrow cells were obtained from marrow transplant donors; these populations were depleted of adherent cells by overnight incubation in Iscove's medium (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS, GIBCO). The human bladder carcinoma cell line (HTB9) was obtained from Dr Robert Phillips of this institute; the cells are maintained in α-medium (α-MEM) (GIBCO) supplemented with 4% FCS.

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CM (10%). The recovery of clonogenic cells was obtained by multiplying the plating efficiency in methylcellulose by the number of cells harvested from the suspension.

The assay for adherent cells was a minor modification of that described by Langley and colleagues in which blast cells were cultured in suspension in Lux tissue culture microwells for 7 days. The nonadherent cells were then removed, and the residual adherent cells were counted using an inverted microscope. Based on cell sources, the kinetics of development of the adherence property, and the cell phenotypes, we concluded that the adherent cells belonged to the blast population.

Normal nonadherent bone marrow cells were plated at a concentration of \(5 \times 10^4\) in 35-mm Petri dishes in Iscove's medium supplemented with 10% FCS, deionized bovine serum albumin (BSA) (Hoechst, Calbiochem-Behring, La Jolla, Calif, electrophoretically pure, 10 mg/mL), iron-saturated human transferrin (Hoechst, 360 mg/mL), \(\alpha\)-thioglycerol (10\(^{-4}\)mol/L), purified homogeneous erythropoietin (obtained from the Terry Fox Laboratory, University of British Columbia, 1 U/mL), and methylcellulose.

As in the previous assays, various concentrations of growth factors were added; colonies were assessed both for number and morphology with an inverted microscope after 7 and 14 days of incubation. The concentration required for half-maximal stimulation of granulocyte and macrophage colonies was defined as 1 U of activity. The batch of recombinant GM-CSF used for the present study contained 1.2 \(\times 10^3\) U/mL. At plateau concentration, it stimulated the same number of granulocyte and macrophage colonies as HTB9-CM. In the presence of erythropoietin, it also supported the growth of multipotential and large erythroid colonies.

**Monoclonal antibodies.** Phenotypes of blast cells were determined before and after culture, using indirect immunofluorescence, as described previously. The antibodies used were My-1, My-7, and My-9 that recognize granulopoiesis-associated antigens, and MO1 that binds markers of both granulopoietic cells and macrophages.

**RESULTS**

**Titrations**

Titration of GM-CSF on blast colony formation in methylcellulose, growth of clonogenic blast progenitors in suspension, and the generation of adherent blast cells are shown in Fig 1. Panel A depicts the increase in blast colonies observed with increasing concentration of GM-CSF. Data from three patients are shown. For patient 1, the cells were obtained after frozen cells had been maintained in suspension culture for 3 weeks. Cells from the other patients were tested when obtained or from cryopreserved samples. Patient-to-patient variation is evident. However, in each instance, the dose–response curve extended over several tenfold dilution steps. The maximum values for colony formation obtained with a standard HTB9-CM preparation are given in the legend to Fig 1. In no instance was this value obtained with GM-CSF, although no obvious differences in colony size were noted. Indeed, for patient 1, only 19% of the colonies stimulated by HTB9-CM were seen with a concentration of GM-CSF much greater than that required for maximum stimulation of granulopoietic colonies (8,000 U/mL).

Panel B shows the effects of increasing GM-CSF concentration on recovery of clonogenic blasts after 7 days in suspension culture. Again, a dose-related increase was seen, although the form of the dose–response curve was different for the two samples depicted in panel B of Fig 1. Cells from patient 1 were tested after almost 1 year in continuous suspension culture; their response to recombinant GM-CSF was similar to that seen with HTB9-CM (see legend to Fig 1). For patient 2, GM-CSF was less effective than HTB9-CM.

The generation of adherent blast cells in suspension cultures is shown in panel C as a function of GM-CSF concentration. The cells were from patient 1, tested after many months in suspension culture. Again a dose response was obtained, reaching almost the same value as that seen with HTB9-CM, even though the cells were those that responded poorly to GM-CSF in the assays based on proliferation (patient 1). The same increase in adherent cells in the presence of GM-CSF was seen with samples 2 and 3 (data not shown).

**Phenotypes**

Using monoclonal antibodies directed against granulopoietic and monocytic differentiation antigens, the pheno-
types of blast cells were determined before plating and from blast colonies. Results of an experiment representative of four experiments are given in Table 1. In agreement with past findings, myelopoietic markers increased during colony formation. Evidence of granulopoietic maturation was not seen. No difference was observed between colonies grown in the presence of GM-CSF as compared with those grown in the presence of HTB9-CM.

DISCUSSION

The major finding of this article is that recombinant GM-CSF is able to stimulate blast colony formation in methylcellulose cultures and the growth of blast progenitors in suspension. The latter assay is considered to reflect the renewal potential of blast stem cells; furthermore, colonies grown in methylcellulose cultures in the presence of recombinant GM-CSF were replated successfully (data not shown), confirming the ability of GM-CSF to support self-renewal. Finally, the capacity of GM-CSF to increase the number of adherent cells is evidence for its support for differentiation-like events, since adherent blast cells usually show increased expression markers often associated with macrophages as well as those characteristic of the blast population.

Recently, we reported that supernatants from cultures of the continuous cell line HTB9 (HTB9-CM) were effective stimulators of blast colony formation. On the basis of partial separation of Sephadex G100, we suggested that the active growth factor for blast colony formation (leukemic blast growth factor or LBGF) might not be identical to GM-CSF. The present study does not contradict that suggestion, since GM-CSF was usually a much less powerful stimulator than HTB9-CM. Rather, the data is now consistent with the possibility that colony formation or growth of blast progenitors may require more than one factor, working singly or in combination.

Our data confirm the findings of others that GM-CSF will stimulate multilineage colonies as well as colonies containing only granulocytes and macrophages, a finding that prevents the use of GM-CSF response for lineage definition. In the context of this report, the finding that GM-CSF is active on AML blast progenitors is not evidence that these cells belong exclusively to a lineage related to granulopoietic or macrophage differentiation. In the experiment reported here, it is unlikely that adherent cells not from the leukemic clone were detected, since the blasts had been maintained for 1 year with repeated subcultures.

Finally, our results may have practical significance. GM-CSF has been considered as a putative therapeutic agent. Indeed, preclinical trials have been reported. The ability of GM-CSF to stimulate the growth of blast progenitors suggests that it may be contraindicated in the treatment of any condition related to AML or capable of progression to that disease.

REFERENCES


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