Synergism Between Recombinant Growth Factors, GM-CSF and G-CSF, Acting on the Blast Cells of Acute Myeloblastic Leukemia

By Colm Kelleher, Jun Miyauchi, Gordon Wong, Steven Clark, Mark D. Minden, and E.A. McCulloch

The genes for the hemopoietic growth factors, GM colony-stimulating factor (CSF) and G-CSF have been cloned, and recombinant material is available for both. We tested these recombinant factors for their effects on the blast cells of acute myeloblastic leukemia (AML). Culture methods are available that support both colony formation by AML blasts and the growth of blast stem cells in suspension. Recombinant GM-CSF is active in both culture systems, although to a varying degree. We found that recombinant G-CSF was also effective; however, the two recombinant factors showed striking synergism for the stimulation of blast growth of cells from five of eight AML patients. In these cases, the combination was equivalent to the stimulating activity of supernatants from the continuous cell line 5637.

The blast cells of acute myeloblastic leukemia (AML) proliferate in suspension cultures or form colonies in cultures made viscid with methylcellulose if a suitable source of growth factor(s) is included in the medium. This requirement has often been met using crude supernatants, either from phytohemagglutinin-stimulated leukocytes (PHA-LCM) or a high factor-producing subclone of the continuous bladder carcinoma cell line 5637 (hereafter called HTB9-CM, a name used in the publication describing the clone and its active supernatants). Recently, we and others showed that recombinant GM-CSF, first described as a requirement for granulocyte/macrophage colony formation in culture, is an active growth factor for blast cells. The recombinant material was not as effective as HTB9-CM in promoting colony formation, however.

G-CSF is a growth factor described originally as specific for granulopoiesis in culture. Recently, molecular clones coding for this growth factor have been isolated; their protein products proved to be effective stimulators not only of granulocyte progenitors, but also of other early hemopoietic cells. Recombinant G-CSF induced morphological differentiation in the murine continuous cell line WEHI13 and is reported to have a similar although less marked effect on freshly obtained human AML blast cells.

We report the effects of recombinant G-CSF on AML blasts using clonogenic and suspension culture assays. We find that G-CSF is an effective stimulator of blast growth. This conditioned medium (HTB9-CM) is considered the standard for blast growth. Blasts from one of the patients grew without added factor. In another instance, recombinant GM-CSF alone was almost as effective as HTB9-CM. In the third case, both recombinant factors were active, but synergism was not observed and their combined effect was not equivalent to that of HTB9-CM. Both GM-CSF and G-CSF were active on normal bone marrow granulopoietic progenitors, but synergism was not observed. We conclude that the marked heterogeneity observed when AML blasts are examined by other criteria is also observed when their response to growth factors is evaluated.

MATERIALS AND METHODS

Cells. Normal marrow was obtained with informed consent from transplant donors. Peripheral blood was obtained with informed consent from eight patients with AML undergoing treatment at the Princess Margaret Hospital. The diagnosis was made on clinical and morphological grounds, using published criteria (French-American-British classification, FAB). The clinical data. Blasts were separated using the two-cycle Ficoll density-gradient separation procedure described earlier. The cells were either placed in culture at once or preserved by freezing at -70°C in 50% fetal calf serum (FCS) and 10% dimethylsulphoxide (DMSO). Thawed cells were cultured in suspension containing 10% HTB9-CM for at least 3 days prior to plating in methylcellulose. Some experiments used cells that had been maintained continuously in suspension culture from 6 to 12 months (patients 5 and 8).

Cell culture methods. The clonogenic assay was done as described previously using HTB9-CM or recombinant growth factors. Blast populations were cultured in microwells (Linbro/Tittertek, Flow Laboratories, McLean, VA) in a medium consisting of α-minimum essential medium (MEM), 10% heat-inactivated FCS (growth medium) 0.8% methylcellulose and 10% HTB9-CM, or recombinant factors at various concentrations. The cultures were incubated for 7 days in a humidified atmosphere of 5% CO2 in air at 37°C, after which time colonies containing >20 cells were counted with an inverted microscope. The secondary plating efficiency (PE2) was measured using the pooled colony technique described previously. The cells were harvested from primary colonies, pooled, washed, and replated in microwells at a cell density of 2 × 10^4/well with media of the same composition used for the primary cultures. Colonies with >20 cells were counted after 7 days of incubation. PE2 was expressed as colonies per 10^4 cells plated. Blast cells were cultured in suspension in 1.7-cm tissue-culture wells (Linbro multiwell plates, Flow Laboratories). As in the clonogenic assay, HTB9-CM (10%) or various concentrations of recombinant growth factors were used as a source of stimulation. The cells were suspended in growth medium at a concentration of 10^6/mL, with growth factor added. After 7 days, the cells were harvested and counted. The cells recovered from the suspensions were washed and replated in methylcellulose in microwells for blast colony formation as described.
overnight incubation. The cultures consisted of 2 x 10^6 cells/well culture. The calculation consisted of multiplying the number of cells earlier. This procedure provided a measurement of the number of clonogenic cells in each dish that was recovered in suspension culture. The calculation consisted of multiplying the number of cells recovered in each dish by the plating efficiency in methylcellulose. Normal marrow was cultured after removing adherent cells by overnight incubation. The cultures consisted of 2 x 10^6 cells/well and growth medium with or without recombinant growth factors or cytosine arabinoside for induction and maintenance.

Recombinant growth factors. Purified recombinant GM-CSF was produced at the Genetics Institute (GI), Boston, from GM-CSF–transfected Chinese hamster ovary (CHO) cells. This material has a potency of 1,000 U/μg, where a unit is defined as the amount of factor needed to produce half-maximum colony formation in methylcellulose. Recombinant G-CSF was also made at GI. The published sequence of G-CSF was used to prepare oligonucleotides. A mammalian expression cDNA library was prepared from RNA from TPA30-1, a SV40-transformed human trophoblast cell line, and was screened using these oligonucleotides as probes. Positive colonies were tested for biological activity by expressing the cloned cDNAs in COS cells and testing the supernatants using chronic myelogenous leukemia (CML) cells sensitive to growth factors (G. Wong and S. Clark, unpublished observations). The potency of these preparations was ~200 U/μL. In our experiments, supernatants were used from GM-CSF–transfected COS cells; controls using supernatants from mock-transfected COS cells did not stimulate colony formation in excess of background. A result typical of these controls is shown in Fig 1.

Morphology. Cells were obtained either from colonies in methylcellulose or from suspension cultures, placed on coverslips by cytospin, and stained with Wright’s stain. The cells were examined by light microscopy using oil immersion.

RESULTS

Activity of recombinant growth factors in methylcellulose. Because the source of G-CSF was an unpurified supernatant from transfectcd COS cells, this test was performed on responsive blasts (patient 5) at a number of dilutions. The titration curve is shown in Fig 1 and compared with a supernatant from mock-transfected COS cells. Maximum activity occurred at a dilution of 10^-3, and the control COS supernatant was inactive.

In the next series of experiments, GM-CSF and G-CSF alone were equivalent to HTB-9, however. When the two recombinant factors were used together, synergism with respect to colony size and numbers was observed in only 3 of eight patients. The augmentation of colony size is shown in Fig 2, a photomicrograph comparing cultures with G-CSF alone (panel B), GM-CSF and G-CSF in combination (panel D), GM-CSF alone (panel A), and HTB-9 (panel C). The two recombinant factors, combined in the same concentrations that gave maximum stimulation for each alone, supported the growth of colonies equivalent in size to those seen in cultures with HTB9-CM. As a further control, a supernatant from mock-transfected COS cells (dilution, 10^-2) was mixed with 2,000 pmol of GM-CSF and tested for its effect on colony formation in methylcellulose. No stimulation over that obtained with GM-CSF alone occurred, indicating that COS cells alone do not secrete an activity capable of a synergistic effect with GM-CSF.

The synergistic effect of the two recombinant growth factors on colony numbers is illustrated in Fig 3. Purified GM-CSF, ranging in concentration from 0.2 to 2,000 pmol/L, was tested for its effect on colony formation with and without the addition of G-CSF at a dilution of 10^-2 or 10^-3. Colony formation with these dilutions of G-CSF alone are given at the left (Fig 3). Colony formation with 10%, 20%, and 30% HTB9-CM are shown at right. The synergism between the two recombinant factors is evident. Maximum stimulation by GM-CSF, or G-CSF alone, gave 11 ± 3 colonies/10^6 cells and 31 ± 4 colonies/10^6 cells, respectively. In combination, the highest tested concentrations of GM-CSF and G-CSF resulted in 92 ± 12 colonies/10^6 cells.

In addition to the synergistic effect, three other patterns of responsiveness to recombinant growth factors were observed. In one case, blast cells were independent of added stimulator whether in the form of HTB-9 CM or either of the recombinant factors. In a second case, nearly maximum colony
formation occurred with GM-CSF alone. The addition of G-CSF was additive, resulting in the same degree of stimulation as was obtained with HTB9-CM. In one other case, neither GM-CSF nor G-CSF was a very potent stimulator of growth in methylcellulose. The combination of the two factors was additive and not synergistic. These results are summarized in Table 2; colony formation with the various recombinant factors is shown normalized against growth with the effective stimulator HTB9-CM.

Two similar experiments were done with normal bone marrow cells, previously depleted of adherent cells. These were exposed to GM-CSF and G-CSF alone or in combination. In 7 days, when used alone, G-CSF stimulated more colony formation than GM-CSF (Fig 4). After 14 days, the number of colonies in culture with GM-CSF was approximately equal to that with G-CSF (data not shown). There was no evidence of synergism at either time in methylcellulose.

**Activity of growth factors in suspension culture.** Parallel experiments were done using the suspension culture method. Figure 5 shows a titration of GM-CSF, with or without the addition of G-CSF. The data are presented in the same form as the titration in methylcellulose shown in Fig 3 and include a comparison with HTB9-CM. Although both GM-CSF or G-CSF alone had small effects on the increase of clonogenic cells in suspension, a combination of the two was highly effective, yielding a maximum recovery exceeding that obtained with HTB9-CM.

Patient-to-patient variation observed with the suspension assay was similar to that which occurred with the methylcellulose assay. The data are presented in Table 3. Unfortunately, cells were not available from patient 6, the only representative of class 2. For the other classes, the growth responses were similar to those seen in methylcellulose.

**Secondary plating efficiencies.** Cells from blast colonies were replated in methylcellulose to measure PE2. The colonies were grown either directly from fresh or cryopreserved

---

**Figure 2.** A photomicrograph of blast colonies formed with different growth factors. (A) G-CSF alone at a maximum stimulatory concentration of 2 nmol/L. (B) G-CSF alone at maximum stimulatory concentration of 200 U/ml. (C) 10% HTB-9 CM. (D) A mixture of GM-CSF and G-CSF, both at maximum stimulatory concentrations.

---

**Figure 3.** Center panel: titration of blast cells with recombinant GM-CSF alone or with two different concentrations of G-CSF, with colony formation as an endpoint. Left panel: values for G-CSF alone, with dilution of COS supernatants shown between error bars. Right panel: colony formation stimulated by HTB9-CM alone. SD is shown.
Table 2. Maximum Stimulation of Colony Formation by Recombinant Growth Factors

<table>
<thead>
<tr>
<th>Class and Patient No.</th>
<th>GM-CSF*</th>
<th>G-CSF*</th>
<th>GM-CSF + G-CSF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>25.6</td>
<td>62.2</td>
<td>121</td>
</tr>
<tr>
<td>2</td>
<td>24.3</td>
<td>59.5</td>
<td>167.5</td>
</tr>
<tr>
<td>3</td>
<td>14.7</td>
<td>42.9</td>
<td>128</td>
</tr>
<tr>
<td>4</td>
<td>16.5</td>
<td>22.5</td>
<td>87.9</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>35.7</td>
<td>80.3</td>
</tr>
<tr>
<td>Class 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 6</td>
<td>12.5</td>
<td>13.9</td>
<td>32.3</td>
</tr>
<tr>
<td>Class 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 7</td>
<td>82.3</td>
<td>30</td>
<td>105</td>
</tr>
<tr>
<td>Class 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 8</td>
<td>Factor independent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values were normalized to HTB9-CM by the formula: n = no. of colonies with growth factor/no. of colonies with HTB9-CM x 100.

Fig 4. Center panel: titration of normal bone marrow cells with recombinant GM-CSF alone or with 10^-3 dilution of G-CSF with colony formation as an endpoint. Left panel: values for G-CSF alone with dilution of COS supernatants shown between error bars. Right panel: colony formation stimulated by HTB9-CM alone. SD is shown.

Fig 5. Effect of recombinant GM-CSF and G-CSF on growth of clonogenic cells in suspension. Left panel: values for G-CSF alone, with dilution of COS cells in suspension. Supernatants shown between error bars. Right panel: colony formation stimulated by HTB9-CM alone. SD is shown.
thereafter when maintained in suspension (class 4). Thus, almost equivalent to that which occurred with HTB9-CM second patient, GM-CSF alone was sufficient to give growth with G-CSF alone. G-CSF, colonies were usually less compact than when with HTB9-CM (class 2). Furthermore, in cultures with tic and together did not approach the stimulation observed G-CSF stimulated growth, but the effects were not synergis-
tically on AML blast cells whether measured by the blast colony assay or in suspension; furthermore, similar effects occur on the PE2, which, like the suspension system, reflects blast stem cell renewal. Even though all but one of the patients were classified FAB M4, patient-to-patient variation is evident. For most patients (class 1), a combination of GM-CSF and G-CSF is as effective a stimulator as media conditioned by the continuous bladder cell line, 5637. Blasts from three patients responded to the growth factors in conditions by blast stem cells; these may either undergo self-renewal or pass through an event similar to differentiation, followed by a few terminal maturation divisions. These maturation steps may be considered to be part of differentia-
tion. GM-CSF has been suggested to promote blast cell growth whereas G-CSF is a more potent differentiating agent. Indeed, in our experiments, some segmented forms were observed in cultures that had been exposed to G-CSF but not to GM-CSF. Care must be used, however, in assigning significance to such observations. An increase in the probability of determination as opposed to self-renewal may be a more important aspect of the differentiation process; it is certainly more relevant clinically since an increase in determination events would decrease the chance of survival of malignant clones. In our experiments, both GM-CSF and G-CSF were equally efficient in stimulating increases of blast clonogenic cells in suspension; recombinant GM-CSF was an effective stimulator of PE2 only in patient 7; neither recombinant factor was highly effective in this assay applied to other patients, although in combination they were usually more effective than HTB9-CM alone. Both growth in suspension and PE2 may be considered to reflect blast stem cell renewal; the data might be interpreted, therefore, as indicating that neither of the two recombinant factors affected the balance between self-renewal and deter-
mination in these populations of leukemic cells; any differen-
tiation occurred only during postdeterministic divisions.

The data may be considered in light of a model for the organization of the cellular populations in AML clones and the possible clinical significance of the model. The blast population in AML is considered to be maintained independ-
ently by blast stem cells; these may either undergo self-renewal or pass through an event similar to determination, followed by a few terminal maturation divisions. These maturation steps may be considered to be part of differentia-
tion. GM-CSF has been suggested to promote blast cell growth whereas G-CSF is a more potent differentiating agent. Indeed, in our experiments, some segmented forms were observed in cultures that had been exposed to G-CSF but not to GM-CSF. Care must be used, however, in assigning significance to such observations. An increase in the probability of determination as opposed to self-renewal may be a more important aspect of the differentiation process; it is certainly more relevant clinically since an increase in determination events would decrease the chance of survival of malignant clones. In our experiments, both GM-CSF and G-CSF were equally efficient in stimulating increases of blast clonogenic cells in suspension; recombinant GM-CSF was an effective stimulator of PE2 only in patient 7; neither recombinant factor was highly effective in this assay applied to other patients, although in combination they were usually more effective than HTB9-CM alone. Both growth in suspension and PE2 may be considered to reflect blast stem cell renewal; the data might be interpreted, therefore, as indicating that neither of the two recombinant factors affected the balance between self-renewal and deter-
mination in these populations of leukemic cells; any differen-
tiation occurred only during postdeterministic divisions.

The synergism between GM-CSF and G-CSF observed in most AML blast populations was not observed in the two normal bone marrow specimens tested under conditions that would support granulocyte/macrophage colony-formation (Fig 4). For this limited sample population, it would not be appropriate to consider the data evidence for a leukemia-
associated growth-factor response. Moreover, the cells of origins of such colonies (CFU-C) may not be comparable to blast stem cells. Resolution of these issues will require measurements on defined cell populations together with determination of receptor densities for both factors. Such studies are now in progress.

<table>
<thead>
<tr>
<th>Class and Patient No.</th>
<th>GM-CSF (%)</th>
<th>G-CSF (%)</th>
<th>GM-CSF + G-CSF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1 Patient 3</td>
<td>8.1</td>
<td>14.8</td>
<td>312</td>
</tr>
<tr>
<td>Class 4 Patient 8</td>
<td>Factor independent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class 3 Patient 7</td>
<td>70</td>
<td>72</td>
<td>90</td>
</tr>
</tbody>
</table>

*HTB9-CM is 100%; values were normalized to HTB9-CM as in Table 1.
†A second sample from patient 3, taken 2 months after the first, after a brief remission.

**Table 3. Maximum Effect of Recombinant Growth Factors on Blast Progenitors in Suspension Cultures**

**Table 4. Maximum Effects of Recombinant Growth Factors on Secondary Plating Efficiencies**

**Discussion**

We showed that recombinant GM-CSF and G-CSF act synergistically on AML blast cells whether measured by the blast colony assay or in suspension; furthermore, similar effects occur on the PE2, which, like the suspension system, reflects blast stem cell renewal. Even though all but one of the patients were classified FAB M4, patient-to-patient variation is evident. For most patients (class 1), a combination of GM-CSF and G-CSF is as effective a stimulator as media conditioned by the continuous bladder cell line, 5637. Blasts from three patients responded to the growth factors in different manners. In one instance, both GM-CSF and G-CSF stimulated growth, but the effects were not synergistic and together did not approach the stimulation observed with HTB9-CM (class 2). Furthermore, in cultures with G-CSF, colonies were usually less compact than when HTB9-CM was used; indeed, even with HTB9-CM, colonies usually disperse after 7 days, and this tendency is marked with G-CSF alone.

This result suggests that yet other growth factors responsible for blast colony formation exist in HTB9-CM. In a second patient, GM-CSF alone was sufficient to give growth almost equivalent to that which occurred with HTB9-CM (class 3). Finally, the blasts from one patient were growth factor independent at the time of the initial study and thereafter when maintained in suspension (class 4). Thus, responses to these two growth factors show the patient-to-patient variation that has occurred when other biological parameters of AML blasts were measured. The significance of this variability with respect to the biology of the tumor cells or clinical response is unknown.

<table>
<thead>
<tr>
<th>Growth Conditions</th>
<th>PE2 (Colonies/10^4 Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>G-CSF</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>From suspension</td>
<td></td>
</tr>
<tr>
<td>Patient 3b</td>
<td>19±3</td>
</tr>
<tr>
<td>7</td>
<td>336±38</td>
</tr>
<tr>
<td>From methylcellulose Patient 5</td>
<td>2±0.5</td>
</tr>
<tr>
<td>3b</td>
<td>2±0.5</td>
</tr>
</tbody>
</table>
REFERENCES


C Kelleher, J Miyauchi, G Wong, S Clark, MD Minden and EA McCulloch