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 hematopoietic 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.

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.

Moreover, synergism occurs when GM-CSF and G-CSF are used together. In some instances, the effect of the combination of factors is the equivalent of HTB9-CM alone. Sensitivity to each factor, singly or in combination, however, varied from patient to patient.

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) and the combination was equivalent to HTB9-CM alone. Sensitivity to each factor, singly or in combination, however, varied from patient to patient.

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overnight incubation. The cultures consisted of 2 x 10^6 cells/well clonogenic cells in each dish that was recovered in suspension earlier. This procedure provided a measurement of the number of cloned cDNAs in COS cells and testing the supernatants using of factor needed to produce half-maximum colony formation in CSF-transfected Chinese hamster ovary (CHO) cells. This material and growth medium with or without recombinant growth factors or consisted of high-dose cytosine arabinoside for induction and mainte-

-200 colonies 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 transfectected COS cells, the activity of recombinant growth factors on colony numbers is illustrated in Fig 3. Purified recombinant 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^-3. Colony formation with these dilutions of G-CSF alone were 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/10^4 cells and 31 ± 4 colonies/10^4 cells, respectively. In combination, the highest tested concentrations of GM-CSF and G-CSF resulted in 92 ± 12 colonies/10^4 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

Table 1. Clinical Characteristics of the Patient Population

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>FAB Classification</th>
<th>Age</th>
<th>Blood (%)</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>1</td>
<td>M4</td>
<td>67</td>
<td>90</td>
<td>Failure</td>
</tr>
<tr>
<td>2</td>
<td>M4</td>
<td>40</td>
<td>60</td>
<td>Remission</td>
</tr>
<tr>
<td>3</td>
<td>M4</td>
<td>56</td>
<td>57</td>
<td>Second remission</td>
</tr>
<tr>
<td>3b</td>
<td>M4</td>
<td>56</td>
<td>53</td>
<td>Third remission</td>
</tr>
<tr>
<td>4</td>
<td>M4</td>
<td>70</td>
<td>72</td>
<td>Partial remission</td>
</tr>
<tr>
<td>5</td>
<td>M4</td>
<td>76</td>
<td>79</td>
<td>Remission</td>
</tr>
<tr>
<td>6</td>
<td>M4</td>
<td>17</td>
<td>80</td>
<td>Remission</td>
</tr>
<tr>
<td>7</td>
<td>M4</td>
<td>66</td>
<td>63</td>
<td>Failure</td>
</tr>
<tr>
<td>8</td>
<td>M4</td>
<td>65</td>
<td>78</td>
<td>Remission</td>
</tr>
</tbody>
</table>

Blood was obtained at initial visit except for patient 3. Treatment consisted of high-dose cytosine arabinoside for induction and maintenance. 

Activity of recombinant growth factors in methylcellulose. Because the source of G-CSF was an unpurified supernatant from transfectected COS cells, the activity of recombinant growth factors on colony numbers is illustrated in Fig 3. Purified recombinant 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^-3. Colony formation with these dilutions of G-CSF alone were 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/10^4 cells and 31 ± 4 colonies/10^4 cells, respectively. In combination, the highest tested concentrations of GM-CSF and G-CSF resulted in 92 ± 12 colonies/10^4 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...
cells or after propagation of the cells in suspension. In each instance, the growth factor used in the early part of the experiment (colony formation only or suspension culture followed by colony formation) was included in the medium for the PE2 measurement. The data are shown in Table 4. Neither recombinant growth factor was as effective as HTB9-CM; but, as in other experiments, synergism occurred when both recombinants were included in the cultures. Indeed, the PE2 values were greater than those that occurred with HTB9-CM alone.

Morphology. Cells were examined by light microscopy under oil immersion after staining with Wright's stain. The predominant appearance was that of blast cells. For populations recovered from methylcellulose, numerous vacuoles were observed, presumably the result of ingestion of methylcellulose. From cells that had been exposed to G-CSF,
responses to these two growth factors show the patient-
to-patient variation that has occurred when other biological
parameters of AML blasts were measured.15 The signifi-
cance of this variability with respect to the biology of the
tumor cells or clinical response is unknown.

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.14 The blast
population in AML is considered to be maintained indepen-
dently 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 differentiation 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-
mation 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 3. Maximum Effect of Recombinant Growth Factors on
Blast Progenitors in Suspension Cultures

<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 3</td>
<td>8.1</td>
<td>14.8</td>
<td>312</td>
</tr>
<tr>
<td>3b†</td>
<td>18.1</td>
<td>22.9</td>
<td>142.9</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2.2</td>
<td>58.7</td>
</tr>
<tr>
<td>Class 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 7</td>
<td>70</td>
<td>72</td>
<td>90</td>
</tr>
<tr>
<td>Class 4</td>
<td>Factor independent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 8</td>
<td></td>
<td></td>
<td></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.

occasional forms had band-shaped nuclei; in these cells,
characteristic granules were not observed.

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 are classified FAB M4, patient-to-patient varia-
tion 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 synergis-
tic and together did not approach the stimulation observed
with HTB9-CM (class 2). Furthermore, in cultures with
G-CSF, colonies 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-
mation in these populations of leukemic cells; any differen-
tiation occurred only during postdeterministic divisions.

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

<table>
<thead>
<tr>
<th>Cells</th>
<th>Growth Conditions</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GM-CSF</td>
</tr>
<tr>
<td>From suspension</td>
<td></td>
</tr>
<tr>
<td>Patient 3b</td>
<td>19 ± 3</td>
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<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>336 ± 38</td>
</tr>
<tr>
<td>From methylcellulose</td>
<td></td>
</tr>
<tr>
<td>Patient 5</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>3b</td>
<td>2 ± 0.5</td>
</tr>
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</table>
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REFERENCES


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