Growth Factors Influence the Sensitivity of Leukemic Stem Cells to Cytosine Arabinoside in Culture

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We have proposed that the blasts in acute myeloblastic leukemia (AML) are renewal populations maintained by a small subpopulation of stem cells. The balance between self-renewal and differentiation in blast stem cells may be an important attribute contributing to treatment outcome. Cytosine arabinoside (ara-C) is included in most chemotherapeutic regimens for the treatment of AML. When ara-C survival curves are constructed, the drug appears to be more toxic when an assay is used that detects principally self-renewing divisions, compared with a procedure that depends on terminal divisions. AML blasts usually respond in culture to myelopoietic growth factors; their response often includes a change in self-renewal, differentiation, or both. These features of the model for AML blasts led to the prediction that growth factors would alter ara-C survival curves in a way that depended on the effects of the culture conditions on self-renewal and differentiation. Four AML blast populations were chosen to test this prediction on the basis of our ability to manipulate them by adding or withholding one or more growth factors. Highly significant changes were seen in the ara-C survival curves, depending on the growth factors present in the cultures as was predicted by the observed effects of the factors on renewal and differentiation.

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CANCERS MAY BE CONSIDERED as clonal populations consisting of stem cells, terminally dividing transitional cells, and their final descendants, proliferatively inert end cells. Clonal survival depends on the capacity of a minority population of stem cells to undergo self-renewal. Most of the cells in tumors may be nonrenewing or inert; as such they may be considered analogous to the differentiated cells of normal renewal tissues even if they do not achieve morphological maturity. It is important, therefore, to distinguish between stem cell proliferative cycles leading to self-renewal and others initiating terminal divisions, since stem cells, and particularly their capacity for self-renewal, are the appropriate targets for curative chemotherapy.

The blast cells of acute myeloblastic leukemia (AML) provide a model system that displays the stem cell functions of self-renewal and differentiation, the latter followed by terminal divisions. AML blast cells can be grown in tissue culture. Patterns of growth reflect a balance between renewal and determination. The former leads to an increase in clonogenic cells; the rate of this increase is reduced by determination, which removes cells from the renewing population.

Two culture methods are available; with them an operational distinction can be made between self-renewing and terminal divisions; when used together the two methods permit an estimation of the balance between renewal and determination. In the first assay, blast cells form colonies in methylcellulose, the result expressed as a plating efficiency (PEmc); PEmc reflects principally terminal divisions. In contrast, in the second method, a suspension assay, changes are measured in numbers of clonogenic cells; an increase in this population (clonogenic cell recovery) and the plating efficiency after suspension culture (PEs) are measures of self-renewal. Some terminal divisions can be detected in the suspension assay by observing the generation of adherent cells. These have many morphological and immunophenotypic characteristics of macrophage/monocyte differentiation and have not been seen to divide. Growth of blasts in either culture technique usually is influenced by the myeloipoietic growth factors GM-CSF, G-CSF, and IL-3. Moreover, the balance between stem cell renewal and differentiation can be influenced by such glycopeptide hormones.

The sensitivity of blast cells to chemotherapeutic drugs varies greatly from patient to patient. The differences cannot be explained by changes in cell cycle parameters, since blast stem cells are uniformly in an active proliferative state and since the cells are tested when in exponential growth. Certain chemotherapeutic drugs appear to be more lethal for either self-renewing or differentiating divisions as compared with cell-cycle events common to both. Cytosine arabinoside (ara-C) for example, may have selective toxicity for self renewal while 5-azacytidine (5-aza) appears to have the opposite effect. The contrast can be seen in the survival curves for these drugs measured in suspension or using the clonogenic assay. For ara-C the D10 value (the drug concentration required to reduce survival to 10% of control) is smaller in suspension (D10s) than in the clonogenic assay in methylcellulose (D10mc); in contrast, the D10s is greater than the D10mc for 5-aza. Further, sensitivities to ara-C10 or 5-aza12 measured in suspension, but not in methylcellulose, are attributes associated with outcome following chemotherapy.

In this article we report experiments designed to test the hypothesis that the balance between self-renewal ("birth") and determination ("death") in blast populations affects the sensitivity of such cells to ara-C; specifically we ask whether the ara-C survival curves measured in suspension can be changed by manipulating the culture conditions to favor either self-renewal or determination.
MATERIALS AND METHODS

Cells. Two blast populations were selected because they could be maintained in culture and because of their reproducible responses to growth factors. OCI/AML1 was considered a cell strain, since after many weekly passages growth slowed and the population had to be recovered from cryopreserved stock; two such populations were used, one after culture for 8 months and the other after 4 weeks in culture following thawing. Medium conditioned by the continuous bladder carcinoma cell line 5637 (5637-CM), known to contain at least GM-CSF, G-CSF, interleukin-1 (IL-1), and IL-6, was required to maintain growth of OCI/AML1; further, exposure of OCI/AML1 to recombinant GM-CSF in suspension results in the production of adherent cells incapable of further division.13 Subsequently, OCI/AML1 has been established as a line, but retains its characteristic response to growth factors. OCI/AML2, has been maintained in suspension culture for 10 months originally in the presence of 5637-CM and recently in the absence of added growth factors. The cells now grow in the absence of added factor; thus OCI/AML2 is considered to be an established line whose growth pattern changes on exposure to growth factors (see text below).

Blast cells were obtained from two AML patients. Patient A was a 61-year-old woman, with a diagnosis of AML, FAB M4. The peripheral leukocyte count was 29.4 x 10⁶/L, consisting of 69% blast cells. Patient B was a 35-year-old woman, with a diagnosis of AML, FAB M1 and a peripheral leukocyte count of 60.7 x 10⁶/L with 98% blasts. Blast cells were isolated and partially purified to remove T cells as described previously14 and cryopreserved immediately. After thawing the cells were maintained in suspension culture for ten days in fetal calf serum (FCS) with αMEM (growth medium) and 10% 5637-CM. During this period growth was exponential as assessed by total nucleated cell counts and clonogenic cell assays (see below).

Culture Methods. Both a clonogenic assay and a suspension culture system were used. Blast colony formation was assessed as previously described.15 Cells from suspension culture were washed with αMEM and plated in 6 mm Linbro/Titertek microtitration multi-well plates (Flow Laboratories, McLean, VA) at a concentration of 10⁶ cells/well (OCI/AML1) or 5 x 10⁵ cells/well (OCI/AML2) in 0.1 mL of 10% (OCI/AML1) or 20% FCS and 10% 5637-CM (OCI/AML1) or without added growth factor (OCI/AML2) and 0.8% methylcellulose. After five to seven days of incubation, PE was determined by counting colonies consisting of more than 20 cells using an inverted microscope. For the suspension assay, the blast cells were cultured in either 24 mm Linbro tissue culture multi-well plates (OCI/AML1 and OCI/AML2) or 2 cm² Linbro tissue culture multi-well plates (blasts from patients A and B) (Flow Laboratories) at a concentration of 10⁶ (OCI/AML1) or 3 x 10⁵ (OCI/AML2) cells/mL in 2 mL (for 24 mm plates) or 1 mL (2 cm² plates) of growth medium and 5637-CM, rGM-CSF, rG-CSF, and rG-CSF or ara-C as required by the experimental design. Nonadherent cells were harvested after seven days, counted, washed with αMEM, and tested for their ability to form blast colonies with 5637-CM (PE). The yield of clonogenic cells per milliliter of

Fig 1. Simple negative exponential survival curves16 for recovery of clonogenic cells from suspension cultures of OCI/AML1 (A) and OCI/AML2 (B) exposed to increasing concentrations of ara-C under different growth conditions. For OCI/AML1 (A), survival curves obtained in the presence of 5637-CM are shown as open symbols and with GM-CSF as closed symbols; additional experimental points obtained with GM-CSF or GM-CSF plus G-CSF are shown as closed and open squares, respectively. For OCI/AML2 (B), open symbols represent curves obtained in cultures without added growth factors and closed symbols for curves obtained in the presence of 5637-CM. In each instance, replicate experiments are shown as triangles or circles. For each panel, two superimposed star diagrams are included; scales for each axis are shown in the figure: for panel A, the up axis is PE (colonies/10⁴ cells), the down axis is clonogenic cell recovery (×10⁴/mL), the right horizontal axis is adherent cell number (×10⁴/culture), the left horizontal axis is PE (colonies/10⁴ cells). For panel B, the up axis is nonadherent cell number (×10⁴/mL) after suspension culture, the down axis is clonogenic cell recovery (×10⁴/mL), the right horizontal axis is PE (colonies/3 x 10⁵ cells) and left horizontal axis is PE (colonies/3 × 10⁷ cells). As noted in the text, the axes in both panels have been chosen so that a movement to the left and down indicates a shift towards birth probabilities and a movement to the right and up a shift towards death probabilities.
Table 1. Clonogenic Cell Recovery of Blasts Exposed to ara-C and Either G-CSF or GM plus G-CSF

<table>
<thead>
<tr>
<th>ara-C (μmol/L)</th>
<th>GM-CSF</th>
<th>G-CSF + GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4,949 ± 957</td>
<td>20,900 ± 3,795</td>
</tr>
<tr>
<td>1</td>
<td>2,918 ± 385</td>
<td>3,573 ± 1,179</td>
</tr>
<tr>
<td>2*</td>
<td>919 ± 400</td>
<td>330 ± 110</td>
</tr>
<tr>
<td>4</td>
<td>186 ± 122</td>
<td>0</td>
</tr>
</tbody>
</table>

Response to growth factors. The effects of growth factors on AML blasts can be inferred from the multiple parameters obtained from cultures in methylcellulose or in suspension. These measurements may conveniently be integrated and described using star diagrams. The diagrams consist of axes, radiating from a central point. Each axis is scaled for one of the growth parameters that characterize blast populations, such as PE, PE, clonogenic cell recovery, or the number of adherent and nonadherent cells after suspension. Values are then plotted on each axis at the appropriate distance from the central point; the plotted values are joined to form a star diagram, a multi-sided figure, where the number of sides is determined by the number of parameters depicted. By assigning appropriate orientations to the axes scaled for properties reflecting renewal or differentiation, the figures become quantitative reflections of the balance between the two, as this balance is determined for several parameters measured in cell culture. Stars can be prepared for a variety of growth conditions and then compared by superimposition or other means. Examples of star diagrams are shown in Fig 1, comparing (A): the growth of OCI/AML1 in 5637-CM or GM-CSF and (B): OCI/AML2 growing with or without added factor 5637-CM. Since adherent cells were found in suspension cultures of OCI/AML1 but not in cultures of OCI/AML2, different axes were chosen to construct stars for these populations. However, in each instance the axes were chosen so that a star predominantly to the left and down is an indication that "birth" is favored, while a star to the right and up signals increased "death" probability (see Fig 1 legend for details). The star diagrams were found to be reproducible when the two samples of OCI/AML1 were compared.

Survival curves. Ara-C survival curves were obtained by the method of Nara et al, based on a linear relationship between number of blast cells cultured in suspension and recovery of clonogenic blast cells after seven days; linearity is maintained in the presence of ara-C and a negative exponential relationship is found between ara-C dose and clonogenic cell recovery.

Aliquots of blast cell suspensions, containing the same number of clonogenic cells, were cultured in suspension without drug or in the presence of increasing concentrations of ara-C. After seven days the cultures were harvested, nonadherent cells were counted, washed with MEM and the plating efficiency measured in methylcellulose in the presence of 5637-CM. Clonogenic cell recoveries were then calculated as the product of nucleated cells recovered multiplied by the plating efficiency. Clonogenic cell recoveries obtained with varying drug concentrations are presented as a percentage of clonogenic cells in cultures without drug. Drug sensitivities can then be expressed for each curve as the D10, or dose required to reduce survival to 10% of control, and compared directly.

OCI/AML1 and OCI/AML2. Table 1 contains star diagrams depicting the responses of OCI/AML1 (A) and OCI/AML2 (B) to different culture conditions and ara-C survival curves for the blasts exposed to drug under those conditions.

For OCI/AML1, 5637-CM favored self-renewal, as evident from a star showing high values for the birth-related parameters PE and clonogenic cell recovery, although PE was also stimulated. In contrast, GM-CSF favored death probabilities; the star shows the generation of adherent cells and decreases in the birth-related functions. The survival curves show that OCI/AML1 blasts were more sensitive to ara-C in 5637-CM than in GM-CSF (P < .001) as anticipated by the balance between birth and death depicted in the star diagrams. On the basis of earlier studies with OCI/AML1 we expected that the effects of 5637-CM on growth, and hence on ara-C survival, were the consequence of the synergistic activities of GM-CSF and G-CSF both present in the conditioned medium. Accordingly the survival curves were repeated using a different OCI/AML1 strain obtained 4 weeks after recovery from cryopreservation and tested in suspension either with GM-CSF alone or in combination with G-CSF at a dilution of 10^-2. The data are shown in Table 1. The calculated survival curve obtained with GM-CSF was not different from that obtained earlier, using OCI/AML1 cells maintained in culture for 8 months (P = .29). The survival curve obtained with GM-CSF and G-CSF was not different from that obtained with an earlier sample of OCI/AML1 and tested in 5637-CM (P = .75). For comparison, the points obtained for these experiments are combined in Fig 1 with the previous data and are shown as squares.
star moved toward parameters indicating death; that is, increased values of PE_{MC} and large numbers of nonadherent cells with low PE. Again the survival curves are consistent with the star diagrams. OCI/AML2 are more sensitive to ara-C in suspension cultures without added factors than in suspension cultures in the presence of 5637-CM (P = .001). Thus, changes were seen in ara-C survival curves consistent with the growth parameters shown in the star diagrams regardless of the great difference in the sensitivities of OCI/AML1 and OCI/AML2 to ara-C when each population of cells was tested in the presence of 5637-CM (note different horizontal scales for Fig 1A and B).

**Studies with fresh blast cells from patients A and B.** The data obtained for blasts from Patient A are shown in Fig 2. The cells were examined under four growth conditions, no added factor, rG-CSF, rG-CSF plus rGM-CSF, and 5637-CM. Adherent cells were not seen under any of the culture conditions. Accordingly values obtained in each were plotted as star diagrams, consisting of nucleated cell number plotted on the up vertical axis, PE_{MC} on the right horizontal axis, clonogenic cell recovery on the down vertical axis and PE on the left horizontal axis, the same arrangement used for OCI/AML2 (Fig 1).

Ara-C survival curves were obtained under each of the conditions; the ara-C D10 values for each condition are shown in Fig 2. The relevant summaries for these survival curves are the control value, the D10 values, and the confidence intervals for the D10 values. The control values for clonogenic cell recovery for no added factor, G-CSF, GM-CSF plus G-CSF, and 5673-CM were 3.9 × 10^4, 3.4 × 10^4, 1.7 × 10^4, and 4.0 × 10^4, respectively. The ara-C DI0 values and their confidence intervals are shown in Fig 2, where their relationship to the balance between birth and death can be assessed from inspection of the star diagrams. It is evident that the shape of the stars, for no added factor, G-CSF and 5637-CM were very similar; the stars, therefore may be interpreted to show very similar balances between self-renewal and determination, although the area of the figures varied, indicating quantitatively different responses. The D10 values for these three conditions were not different (P = .14). In contrast, the star diagram for G-CSF plus GM-CSF is larger than the others, perhaps indicating an additive or synergistic effect of the two factors together; this star diagram also has a different shape from those shown above it. It appears to be shifted in the direction of the death probability. As predicted, the D10 value was significantly larger (P = .0001).

The results of applying the same experimental design to patient B are shown in Fig 3, using the same format as for Fig 2. In this instance, the control values for clonogenic cell recovery for no added factor, G-CSF, G-CSF plus GM-CSF, and 5637-CM were 1.03 × 10^3, 7.8 × 10^3, 8.9 × 10^3, and 7.6 × 10^3, respectively. The star diagrams for no added

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**Fig 2.** Data for patient A. Star diagrams with the same axes as in Fig 1B (for description, see text). The diagrams for no added factor, G-CSF, and 5637-CM are superimposed at the top of the figure; each is associated with an ara-C D10 value; these values are not significantly different (P = .14). The star diagram for G-CSF plus GM-CSF is at the bottom of the figure. Its associated ara-C D10 value is significantly larger than that for the other three conditions (P < .001).

**Fig 3.** Data for patient B. Star diagrams with the same axes as in Figs 1 and 2 (for description see text). At the top of the figure the diagrams for no added factor and 5637-CM are superimposed, and associated with ara-C D10 values that are not significantly different (P = .33). At the bottom, the diagrams for G-CSF and GM-CSF are superimposed; D10 values for these two conditions differ (P < .013) and both are different from those for 5637-CM or no added factor (P = .007, P < .001, respectively).
factor and 5637-CM are similar in shape, although the response to 5637-CM is evident. The D10 values were also similar under these two conditions ($P = .93$). The star diagrams for G-CSF and G-CSF plus GM-CSF were different in shape from each other and from those of the other two growth conditions. For both G-CSF and G-CSF plus GM-CSF the star diagrams showed increased death probabilities associated with larger D10 values. The ara-C D10 value for G-CSF was significantly larger than those for no added factor and 5637-CM ($P = .007$), and so was the D10 value for G-CSF plus GM-CSF ($P < .001$). In addition, the balance between birth and death was different for cells grown in G-CSF compared with G-CSF plus GM-CSF. The D10 values were also different; that is the D10 value for GM-CSF plus G-CSF was larger than that for G-CSF ($P = .013$).

The statistical comparisons of the D10 values shown in Figs 3 and 4 are given in Table 2.

Adriamycin survival curves. The interpretation of the survival curves in Fig 1 and the D10 values in Fig 2 depend on other data indicating that ara-C is more toxic for renewal than terminal divisions. Figure 4 shows Adriamycin survival curves for the second OCI/AML1 strain tested in GM-CSF alone or in GM-CSF and G-CSF. It is apparent that Adriamycin, a drug that is equally toxic to blasts in suspension (renewing) or in methylcellulose (terminal divisions), gives very similar survival curves under the two growth conditions. It follows that the effects of growth conditions on drug dose response curves are drug-specific; differences were seen for ara-C, a drug with some specificity for self-renewal, but not with Adriamycin, a drug for which no such specificity for self renewal has been demonstrated.

**DISCUSSION**

The experiments reported in this article were designed to test and amplify a model of the blast population in AML.
The model has two components: first, that the blast population is a cellular hierarchy, maintained by blast stem cells with self-renewal capacity; second, that ara-C is more toxic for renewing stem cells than for their descendants undergoing terminal divisions. The first part of the model, the lineage concept of blast cells, is based largely on cell culture studies; a minority of blasts are capable of colony-formation in viscous media and self-renewal during colony-formation has been shown by replating experiments. Recently, we have provided evidence that an operational quantitative distinction can be made between renewing and terminal divisions; the former are the basis for increases in clonogenic cells in suspension culture; the latter dominate quantitative distinction can be made between renewing and terminal divisions. The second component of the model, increased ara-C sensitivity of renewing as compared with terminal dividing blast cells, is based on measuring dose-response curves in the two assays. For both, the cells were exposed to the drug continuously over seven days. This prolonged exposure led to dose-response curves that were simple negative exponentials in both assays in contrast with the sharp fall followed by a plateau that is found when proliferating cells are exposed briefly to DNA-specific agents such as tritiated thymidine. The negative exponentials could be characterized by their slopes or equivalently by their D10 values. Great patient-to-patient variation was found in D10 values regardless of whether the suspension or methylcellulose assay was used. However, regularly, the blast cells were found to be more sensitive to ara-C when measured in suspension rather than using the clonogenic methylcellulose assay. This result is consistent with the assumption that the ara-C dose survival curves measured in suspension reflect, at least in major part, the sensitivity of self-renewing divisions to the drug.

The studies reported here became feasible when it was demonstrated that recombinant growth factors could affect the balance between self-renewal and terminal divisions in AML blast cells. Then, either growth factors or conditioned media containing such factors could be used to perturb the growth characteristics of blast populations; the effects of such perturbation on ara-C sensitivity in culture could be measured. The prediction from the model was that any conditions that favored self-renewal would lead to increased ara-C sensitivity while, in contrast, a change in the direction of terminal divisions would decrease ara-C sensitivity. The major finding of the report is that this prediction was correct in that the expected associations were found between measures of growth parameters and ara-C sensitivity for the four blast populations studied and under the specific conditions used. Any conclusion based on association must be guarded. It is possible that the changes in D10 observed under different growth conditions might be the consequence of biological effects not detected by the culture conditions used. For the various parameters measured, however, we did not observe any association other than that described above.

Some details of the experimental results deserve emphasis. First, experiments were done on cells that had been maintained for months in suspension culture and on populations freshly obtained from AML patients. Regardless, the D10 value could be altered by changing the growth conditions and in a direction predicted by measurements of the balance between self-renewal and differentiation depicted in the star diagrams. Second, D10 values could be manipulated by changing growth culture conditions regardless of the overall ara-C sensitivity. These are seen by comparing the ara-C sensitivities of OCI/AML1 and OCI/AML2. The D10 values for the blasts from the two patients were also different.

As discussed above, the experimental design depended on continuous exposure of blasts to ara-C in order to detect drug sensitivity rather than cell-cycle specificity. The patient-to-patient variation observed with this technique is evidence that the object is achieved, a view supported further by the observation of an association between ara-C sensitivity in suspension and response to remission-induction therapy. We took great care to test populations under conditions of exponential growth. However, it might still be argued that the D10 changes observed under different growth conditions were secondary to changes in the percentage of cells in cycle. Direct studies of blast cells using the tritiated thymidine "suicide" technique showed that all populations studied contained a high and uniform proportion of cells in the S-phase of the cycle. Nevertheless, if variations were occurring in response to growth conditions, one would expect that these might be related to the extent of the response, and that the ara-C sensitivity would be directly associated with increased growth as suggested recently by Lista et al. Such a relation was not obvious in our data. For example, in the cells from patient A the conditions that gave the greatest response were associated with the highest ara-C D10 value.

While we consider that the data presented in the report support the proposed model, more studies are needed; specifically other drugs should be tested for specificity similar to that of ara-C and then for interactions with growth conditions; other methods should be used to change the balance between renewal and differentiation, and then see if these perturbations were also associated with changes in drug sensitivity.

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