MEMBRANES REPLACE IRRADIATED BLAST CELLS AS GROWTH REQUIREMENT FOR LEUKEMIC BLAST PROGENITORS IN SUSPENSION CULTURE

By NOBUO NARA AND E. A. McCULLOCH

From the Ontario Cancer Institute, Toronto, Ontario M4X 1K9, Canada

Recently (1), we described a suspension culture procedure that permitted the exponential growth of the progenitors of the blast population in acute myeloblastic leukemia (AML). Changes in the number of progenitor cells in such cultures was assessed using a colony assay for clonogenic cells (2). Increases in blast progenitors were seen regularly, and in some instances, the cells could be recultured, with the maintenance of exponential growth, for periods of weeks.

In addition to standard growth medium (fetal calf serum [FCS] and α minimum essential medium [αMEM]), there were two requirements for growth. These were, first, the presence of one or more growth factors present in media conditioned either by leukocytes stimulated by phytohemagglutinin (PHA-LCM) (3), or by bladder carcinoma cells from the continuous line HTB9 (HTB9-CM) (4). Second, the cell density of the cultures had to be maintained at 10⁶ cells/ml; the latter requirement could be met using irradiated cells added to smaller numbers of intact blasts.

In this paper, we describe experiments designed to characterize the feeding role of blast cells. We found that the capacity of blasts to support the growth of small numbers of progenitors was distributed heterogeneously among the population. We also found that cell membranes from autologous or homologous AML blasts could replace intact cells in supporting the growth of blast progenitors in suspension culture. We suggest that these findings may have an important bearing on the regulation of blast cell proliferation.

Materials and Methods

Cell Sources. Blasts were obtained with informed consent from the peripheral blood of five patients with AML, one with acute lymphoblastic leukemia (ALL), and one with chronic lymphocytic leukemia (CLL). The diagnosis was based on cell morphology using a previously-defined classification system (5); normal marrow cells were obtained from healthy marrow transplantation donors.

Mononuclear cell fractions were prepared as described previously by separation through Ficoll-hypaque (density = 1.077 g/cm³; Winthrop Laboratories, Aurora, Canada). The

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Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CLL, chronic lymphocytic leukemia; EGF, epidermal growth factor; FCS, fetal calf serum; LCM, leukocyte-conditioned medium; α-MEM, α-minimum essential medium; PBS, phosphate-buffered saline; PHA, phytohemagglutinin.

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suspension was then depleted of T lymphocytes using a second Ficoll-Hypaque separation after erythrocyte rosette formation. The T cell–depleted cells were either cultured immediately or preserved by freezing in liquid nitrogen in 10% dimethylsulfoxide and 50% fetal calf serum.

**Blast Colony Assay.** The capacity of blast progenitors to form colonies in culture was assessed as reported previously (1, 6), except that media conditioned by cells of the continuous bladder carcinoma line HTB9 (American Type Culture Collection, Rockville, MD) were used rather than PHA-LCM (4). Blast cell suspensions, at a concentration of 4 × 10⁵ cells/ml in 1 ml of α-MEM, 20% FCS, 10% HTB9-CM, and 0.8% methylcellulose were cultured in 35-mm Lux culture dishes (Miles Laboratories, Inc., Naperville, IL). After 7 d incubation at 37°C in a humidified atmosphere of 5% CO₂ in air, colonies of >20 cells were counted.

**Liquid Suspension Culture.** Blast progenitors were cultured in suspension as described previously (1). T cell–depleted mononuclear cells from AML patients, at a concentration of 10⁶ cells/ml were suspended in 3 ml of α-MEM supplemented with 20% FCS and 10% HTB9-CM. The cells were harvested and counted at day 7. The blast plating efficiency was determined using the blast colony assay. The total yield of blast progenitors per dish was calculated from plating efficiency and cell recovery.

**Separation of Cells by Velocity Sedimentation.** Blast cells were separated by velocity sedimentation as previously described (7). Cells suspended in phosphate buffered saline (PBS) supplemented with 0.2% bovine serum albumin (BSA) were added to a shallow buffered gradient consisting of 1–2% BSA in PBS. After 4 h sedimentation at 4°C, the cone volume was discarded, and 15.5-ml fractions were collected. Each fraction was concentrated to 1 ml by centrifugation, and the cells were counted.

**Cell Membrane Preparation.** Cells were fractionated by a modification of the method of Bell et al. (8). Cells were washed in PBS and incubated in lysis buffer (10 mM KCl, 1.5 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 10 mM Tris-HCl, pH 7.4) at 4°C for 30 min. Meanwhile, cells were mixed at 10-min intervals. Cells were ruptured in a Ten Broeck homogenizer (Kontes Co., Vineland, NJ). Complete rupture was confirmed using phase-contrast microscopy. The homogenate was centrifuged at 400 g for 10 min. The pellet obtained consisted of nuclei. The supernatant was centrifuged again at 35,000 g for 40 min. This pellet contained membranes and microsomes (referred to hereafter as the membrane fraction), and the supernatant was considered as fluid cytoplasm. The three fractions were resuspended quantitatively in α-MEM, so that the number of cell equivalents per milliliter was known.

**Statistics.** Group data were compared by Student’s t test. Linear regression was determined by the method of least squares. Data are presented as the mean ± SE.

**Results**

Under optimal conditions, there is a linear relation between the number of intact blast cells added to suspension cultures and the recovery of clonogenic cells 7 d later. This relationship is shown in Fig. 1; where cell density was maintained by adding irradiated cells and growth factors, supplied either as HTB9-CM or PHA-LCM, clonogenic cell recovery increased linearly with the number of intact cells in the cultures. HTB9-CM was, however, more effective than PHA-LCM. In contrast, removing either HTB9-CM or irradiated cells from the cultures greatly reduced the yield of clonogenic progenitors; intact cells by themselves, without either feeders or a source of growth factors, were ineffective in generating new clonogenic cells. It is evident from the figure that both a high cell concentration and the presence of a source of growth factors were required; adding increased numbers of irradiated cells did not substitute for growth factors, nor was HTB9-CM sufficient by itself when irradiated cells were omitted from the cultures. Thus, the data depicted in Fig. 1 lead to three conclusions: (a)
HTB9-CM can replace PHA-LCM as a source of growth factors in suspension culture system; (b) both growth factors and a high cell density are required for optimal growth, and (c) the linear relationship between input blast cell number and clonogenic cell recovery allows the method to be used in quantitative experiments.

**Media Conditioned by Blast Cells.** Previously (1), we observed that the addition of methylcellulose to suspension cultures of blast cells reduced the yield of new clonogenic progenitors, even when cell density was maintained. This observation was interpreted to indicate that cell contact was required. Alternatively, blast cells might secrete or shed growth factors that could act synergistically with those present in HTB9-CM. For the populations used in this study, we tested for this possibility by using supernatants from 7-d blast cultures in the clonogenic assay. Results of a typical experiment are given in Fig. 2. It is evident that HTB9-CM was a very effective growth factor for blast colonies. The addition of media conditioned by blast cells also increased colony formation; however, the cultures used to produce conditioned media contained HTB9-CM. Accordingly, as a control, HTB9-CM was incubated for 7 d in the absence of cells. It may be seen from the figure that this material was as effective as blast cell-conditioned medium in stimulating colony formation. It seems unlikely, therefore, that blast
cell-derived growth factors play an important role in the generation of new blast progenitors in suspensions. Rather, the data support the view that cell contact is necessary.

Separation of Blast Cells by Sedimentation Velocity. In the next series of experiments, we asked whether all blasts were equally effective in providing support for growth. Blast populations were separated by sedimentation velocity. Fractions were assayed for their content of blast cells and their plating efficiency in the blast assay. Other aliquots of each fraction were adjusted to a concentration of $5 \times 10^5$ cells/ml, irradiated, and added to suspension cultures containing $5 \times 10^5$ autologous intact blast cells. After 7 d, these cultures were harvested, washed, and the blast plating efficiency was determined. The data from one of two similar experiments are presented in Fig. 3. Fig. 3a shows the distribution of nucleated cells and clonogenic cells; these results are similar to those obtained in previous experiments. Fig. 3b shows the distributions obtained when cells from the fractions were tested for their capacity to support the generation of new clonogenic cells. The recoveries of blast progenitors when intact cells only were cultured and when $5 \times 10^5$ unseparated cells were included are shown in the figure as dotted lines. It is evident that the fractions differed widely in their capacity to stimulate blast progenitor growth. However, maximum stimulation was seen in regions of the gradient that contained the largest numbers of nucleated cells. Thus, in addition to showing heterogeneity, the separation experiments provided evidence that the majority of blast cells were active in providing support for growth.

Blast Cell Fractionation. If the role of blast cells in supporting growth depends
FIGURE 3. Fractionation of blasts by velocity sedimentation. Blasts were separated, and the numbers of nucleated cells (○) and clonogenic cells (●) per fraction were counted (a). 5 × 10⁵ cells/ml from each fraction were irradiated and added to a suspension culture of 5 × 10⁵ intact cells/ml. The recovery of clonogenic cells is shown in b. The control consisted of 5 × 10⁵ intact cells cultured with 5 × 10⁵ irradiated unfractionated cells. Values for cultures above the upper dotted line indicating this control were enriched for feeding function. The second control is the recovery of clonogenic cells in the absence of added irradiated cells (lower dotted line).

upon cell contact, this contact might be a function of cell membranes. To test this hypothesis, cells from two patients were fractionated as described in Materials and Methods. Then, the three fractions, consisting respectively of membranes, nuclei, and cytoplasm, were tested for their capacity to promote growth of 1.5 × 10⁶ intact cells in suspension culture. The fractions were added to the cultures in amounts equivalent to 1.5 × 10⁶ cells; that number of irradiated, nondisrupted cells served as a positive control. The data are given in Table I. It is evident that the membrane fraction was almost equivalent to intact cells in capacity to stimulate the production of clonogenic progenitors. In contrast, fractions containing principally nuclei and cytoplasm were almost inactive.

Any further study of the membrane effect would require a quantitative assay. Accordingly, we added varying concentrations of membrane fractions to a small number of target cells (3 × 10⁵ cells/dish) to observe the effects of membrane specifically, HTB9-CM was not added. The data from a typical experiment are shown in Fig. 4. It is evident that the yield of clonogenic progenitors increased linearly with the addition of membrane fraction until a plateau was reached at a level of membrane equivalent to 6 × 10⁶ cells/dish.

Specificity. The availability of a quantitative assay for membrane function
Table 1

Comparison of Supportive Activity of Irradiated Cells and Cellular Components on Blasts' Growth

| Addition to intact blasts in suspension | Recovery of clonogenic cells/dish |
|----------------------------------------|---------------------------------|
|                                        | Patient 1                        | Patient 2                        |
| Irradiated cells                       | 13,926 ± 1,907                  | 114,405 ± 12,499                 |
| Membranes                              | 13,058 ± 503                    | 112,092 ± 9,759                  |
| Nuclei                                 | 6,509 ± 1,118                   | 84,669 ± 5,635                   |
| Cytoplasm                              | 5,029 ± 666                     | 91,776 ± 7,296                   |
| None                                   | 4,233 ± 758                     | 75,240 ± 3,510                   |

1.5 × 10^6 intact blasts were cultured in suspension. 1.5 × 10^6 irradiated blasts or cell components derived from the same number of blast were added, and the recovery of clonogenic cells was determined.

Figure 4. Titration of cell membranes for their capacity to promote the growth of blast progenitors. Varying concentrations of membrane fractions were added to a small number (3 × 10^5 cells/dish) of autologous blasts in suspension culture without other growth promoters. The recovery of clonogenic cells was assessed. The yield of clonogenic progenitors increased linearly with the addition of membrane fraction until a plateau was reached at a membrane equivalent to 6 × 10^6 cells/dish. The recovery of clonogenic cells was 696 ± 169, 2,978 ± 346, or 8,877 ± 970 when 2.7 × 10^5 irradiated cells, HTB9-CM, or both irradiated cells and HTB9-CM were added to the culture, respectively.

made it feasible to examine the issue of specificity, since homologous cells under test did not enter the calculation of the yield of clonogenic cells. Fig. 5 contains the results of two experiments. In each, different patients provided target cells. It is evident that those of patient A had a higher basal blast progenitor plating efficiency than those of patient B. In both instances, membrane preparations from autologous blasts were effective stimulators, in a dose-related fashion. Further, homologous blast membrane preparations were also active. In the experiment shown at the top, the homologous membrane preparation was less effective stimulator than the autologous preparation; however, it is evident that the target cells, rather than the membrane preparations, are the major determinants of the extent of response. Thus, cells of patient A cultured with a membrane
FIGURE 5. Data from two experiments designed to test the specificity of the stimulation of blast progenitor growth by membrane preparations. Patient-to-patient variation is apparent by comparing top to bottom. In both, autologous and homologous AML blasts were effective stimulators. In contrast, controls, obtained from cells of normal marrow, ALL blasts, or CLL lymphocytes, were inactive. The effect of membrane preparations from cells of Patient C on autologous targets is shown in Fig. 4, where the response is similar to that seen for homologous targets shown here. Top: autologous cells (patient A) (○); homologous cells (patient B) (□); normal marrow cells (▲); ALL blast cells (△). Bottom: autologous cells (patient B) (○); homologous cells (patient C) (□); CLL leukemic cells (□).

preparation from patient B (top) yielded many more blast progenitors than patient B targets stimulated by an autologous membrane preparation (bottom).

In both experiments, control membrane preparations, derived either from normal marrow, ALL blasts, or CLL lymphocytes, were inactive. Thus, the data provide preliminary evidence that the stimulation of blast progenitor growth in suspension cultures by membrane preparations may have AML specificity.

Discussion

In patients with AML, blast cells grow without apparent restraint; their numbers can only be controlled by cytotoxic chemotherapy. In contrast, in culture, the proliferation of AML blasts depends on a number of factors. These include the usual nutrients supplied by fetal calf serum and α-MEM. Of greater interest, diffusible growth factors are needed both for colony formation in viscid cultures and blast progenitor self-renewal in suspension. For exponential blast cell expansion in suspension cultures, there is an additional requirement for cell-cell contact.

In this paper, we have provided evidence that growth promotion by cell contact may be a function of one or more components of cell membranes or
microsomes. The cell fractionation experiments showed that suspensions containing membranes were much more effective in promoting blast cell growth than fractions enriched for nuclei or fluid cytoplasm. The observations that the membrane-associated cellular function was distributed heterogeneously among blast cells, and the preliminary finding of AML blast specificity combine to make it unlikely that blast membranes were acting passively to protect cells. Rather, the data encourage us to suggest the presence of a defined membrane structure, perhaps with the characteristics of a receptor.

The relationship between blast cell membrane-associated structures and growth factors from media conditioned by HTB9 cells or normal mononuclear cells is unclear. These experiments indicate that neither source of growth stimulation can substitute for the other (see Fig. 1). In normal hemopoiesis, where molecular clones have been obtained for growth factors, these have been found to be unrelated products of single genes (9–11). It is also known that membrane-bound receptors may be encoded by oncogenes, providing a possible link between the malignant behaviour of AML blasts and membrane-associated structures promoting growth. Further, structural relationships have been found between the cell receptor for low density lipoprotein and a precursor molecule for epidermal growth factor (EGF); these data may mean that an EGF precursor molecule may be a membrane component (12). This is, of course, reminiscent of lymphopoiesis, where immunoglobulin molecules serve both as receptors on B cells and secreted functional products.

Resolution of these uncertainties will require analysis of membrane-associated and cell-secreted growth factors at both the amino acid and nucleotide level. Fortunately, the methods for such studies are now available.

In conclusion, AML blasts provide a readily available source of clonal (13) human leukemic cells that may have many of the regulatory features characteristic of the malignant phenotype. AML blast cells have the advantage over cell lines that findings obtained with them may be correlated with the clinical course of patients (14). It is even possible that regulatory mechanisms, discovered using the methods of cellular and molecular biology, may prove accessible targets for novel therapies.

Summary

The blast cells of acute myeloblastic leukemia (AML) may be considered as a renewal population, maintained by blast stem cells capable of both self-renewal and the generation of progeny with reduced or absent proliferative potential. Blast progenitor renewal is manifested in suspension culture by an exponential increase in clonogenic cells. This growth requires that two conditions be met: first, the cultures must contain growth factors in media conditioned either by phytohemagglutinin (PHA)-stimulated mononuclear leukocytes (PHA-LCM), or by cells of the continuous bladder carcinoma line HTB9 (HTB9-CM). Second, the cell density must be maintained at $10^6$ blasts/ml; this may be achieved by adding irradiated cells to smaller numbers of intact blasts. We are concerned with the mechanism of the feeding function. We present evidence that (a) cell-cell contact is required. (b) Blasts are heterogeneous in respect to their capacity to support growth. (c) Fractions containing membranes from blast cells will
substitute for intact cells in promoting the generation of new blast progenitors in culture. (d) This membrane function may be specific for AML blasts, since membranes from blasts of lymphoblastic leukemia or normal marrow cells were inactive.

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