CONCISE REPORT

Cell-Cycle Manipulation of Human Leukemic Progenitor Cells With Humoral Adjustment In Vitro

By Chan H. Park

The cell-cycle change of human leukemic colony-forming cells was studied using a new agar culture method featuring daily feeding of new culture medium with or without leukocyte conditioned medium (LCM). Leukemic cells could be kept out of cycle by withholding LCM from daily feeding and put back into cycle by adding LCM to the daily feeding.

RESULTS

No colonies grew in those cultures that never received LCM, despite daily feeding and long-term maintenance of the cultures. However, a substantial number of colonies could easily be grown if LCM was added 1–2 wk later (Fig. 1). In fact, normal-size colonies were occasionally observed even when the delay in LCM addition was as long as 3 wk. The cells composing these colonies with delayed addition of LCM were characteristic of leukemic cells on the basis of morphological and cytochemical criteria (Fig. 2), which was substantiated by chromosomal study. Multiple observations of cultures through the culture period are shown in Fig. 3. Provided cultures were fed daily, single cells persisted without LCM addition (B1 of Fig. 3) and, upon addition of LCM, these cells were capable of proliferating to form colonies (BII and BIII ) similar to the control cultures in which LCM was added from the day of plating (A1 and AII ). The delay in the addition of LCM did not decrease the proliferative potential of individual colony-forming cells as shown by the sizes of colonies, BIII compared to AIII of Fig. 3. This observation indicated that leukemic cells capable of colony formation remained alive without LCM, but in a resting state, and started to proliferate upon exposure to LCM. Direct evidence for the fact that leukemic cells were alive but indeed were in a resting state was obtained by exposing cultures to hydroxyurea between days 2 and 4 before adding LCM on day 5 of culture. In 2 cases studied (cases 3 and 4), 39% and 4.5% of colony-forming cells, respectively, survived this hydroxyurea exposure, while only

MATERIALS AND METHODS

Bone marrow aspirates for this study were obtained from five patients with acute nonlymphocytic leukemia at the time of initial diagnosis and prior to treatment. Informed consent was obtained from all patients in accordance with guidelines established by the University of Kansas Human Subject Committee.

The culture system, described in detail elsewhere, consisted of two 0.3% agar layers of 1 ml each in a 35-mm plastic Petri dish. Both agar layers were suspended in alpha medium containing 15% fetal calf serum, with leukemic bone marrow cells added in the upper layer. These cultures were fed daily from the top with 0.5 ml of new alpha medium containing fetal calf serum. This feeding medium also contained 15% LCM, except when its addition to the culture was to be delayed. LCM was prepared by incubation of normal human peripheral leukocytes with phytohemagglutinin as described. Old medium to be removed drained out of holes at the bottom of culture dish. Colonies of 50 cells or more were counted after 14–21 days of culture with LCM at 37°C with 7% CO2 in air.

A sharpened Pasteur pipette was used to pick up colonies for morphological and cytochemical characterization of cells as described. Colonies grown in this culture system with no delay in LCM addition were shown to be leukemic in origin, characterized by chromosomal, morphological, and cytochemical studies.

For the estimation of the colony-forming cells in resting state, hydroxyurea was used to kill cycling cells. Hydroxyurea was added to culture at 4 x 104 M with feeding on day 2 of culture, and 48 hr later on day 4, this was washed out by immersing the whole culture system in a beaker containing 100 ml of medium without hydroxyurea for 24 hr. Cultures were then taken out of the beakers on day 5, excess medium removed, and the daily feeding resumed. Control cultures were set up concurrently and treated in identical fashion except that hydroxyurea was not used. The proportion of colonies surviving this hydroxyurea exposure was considered to represent the proportion of colony-forming cells in resting state.

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Culture time before LCM addition (days)

Fig. 1. Survival of leukemic colony-forming cells with delayed addition of leukocyte conditioned medium (LCM) to culture. Five patients were studied: case 1 with acute myelomonocytic leukemia; cases 2, 4, and 5 with acute myelocytic leukemia; and case 3 with erythroleukemia. For each of these patients a group of dishes were plated with bone marrow cells but without LCM in identical fashion. These dishes were randomly divided into 2–5 subgroups. One subgroup received LCM from the day of plating with daily feeding (control subgroup). Other subgroups also received daily feeding but LCM was deleted from the feeding medium for the first 3, 4, 7, 10, or 14 days of culture. Colonies were counted after cultures were exposed to LCM for 14–21 days. There was no change in colony number during this interval. Each datum point represents the mean of 4 dishes. All the data were normalized to unity for the control subgroups. At least 2 separate experiments were done for 2 datum points on case 3 and 3 datum points on case 4, and these data were averaged. Six experiments were done for day 7 datum point on case 3, ranging 9.3% and 79% with mean of 45%, SD of 29% and SE of 12%. Because of this variation within a single case, a significance of difference between cases cannot be determined. However, it was apparent that a substantial proportion of leukemic colony-forming cells survived at least for a week without LCM in all cases. Raw data (number of colonies/number of cells plated), for the control subgroups were 305/5 \times 10^4, 148/10^4, 828/8 \times 10^4, 149/3 \times 10^4, and 61/4 \times 10^4 for cases 1–5, respectively.

0.5% and 0% of colonies, respectively, survived the same exposure in cultures performed in an identical fashion except that LCM was added from the day of plating. It should be noted, however, that these survival fractions may not be an accurate estimate for the resting cell proportions at the time of drug exposure, because there may have been residual drugs remaining after the wash that may have been inhibitory to colony formation later. However, for each of these 2 cases, the experiment was controlled concurrently for the groups with delayed and immediate exposure to LCM. Therefore, the relative meaning of these survival fractions, a substantial survival for the former and a negligible survival for the latter, should be valid, indicating higher proportion of cells in resting state for the former than the latter.

DISCUSSION

There are a number of implications from this study. First, human leukemic cells can stay viably dormant for a significant period of time. In this culture system, leukemic cells were seen to survive dormant up to 3 wk, but this limitation might merely reflect an unfavorable in vitro environment. The cell that can survive in vitro for 3 wk may survive much longer in more favorable in vivo conditions. This then may explain some of the patients with leukemia in whom recurrence ensues at long intervals after complete remission. Also, because this study was done with cells taken directly from patients as opposed to cell lines in long-term cultures, it therefore is likely to have greater clinical relevance. This is supported by the fact that in vitro chemotherapy sensitivity assessment using this culture system correlates very well with the clinical response to chemotherapy. The more important implication of this study is that the cell-cycle states of human leukemic cells can be manipulated by the adjustments of humoral factor(s). This finding may then be exploited in the treatment of leukemia; as long as leukemic cells are kept out of cell-cycle, there will

Fig. 2. Myeloperoxidase stain of a leukemic colony cultured for 3 wk with no leukocyte conditioned medium added for the first 1 wk of culture (case 3). The top panel shows a whole colony (\times 40), and the bottom panel a magnified view of a portion of the colony (\times 1000). There are cells that are strongly (A) or intermediately (B) positive, and cells that are negative (C) for the enzyme. This pattern of variation in the enzyme activity has been shown to be characteristic of leukemic colonies as substantiated by chromosome study.\footnote{1}
Fig. 3. Appearance of cells from a leukemic patient (case 3) with or without delay in the addition of leukocyte conditioned medium (LCM). Two dishes of culture, $2 \times 10^4$ bone marrow cells per dish, were plated without LCM in identical fashion. One dish, control culture, received LCM from the day of plating with daily feeding (A). The other also received daily feeding, but LCM was deleted from the feeding medium for the initial 7 days of culture (B). The photomicrographs were taken at 1 wk ($A_1$ and $B_1; \times 50$), 2 wk ($A_2$ and $B_2; \times 200$), and 4 wk ($A_4$ and $B_4; \times 100$) after plating. Only single cells are seen in $B_1$, clusters of 20–40 cells are seen in $A_1$ and $B_1$, and colonies of 200–500 cells are seen in $A_1$,$ A_2$, and $B_4$.

be no progression of the disease, and the induction of cells to cycling state can make them more susceptible to cycle-dependent cytotoxic treatments. The nature of the humoral factor(s) present in the LCM that are necessary for leukemic cell growth is not known, although this factor appears similar to the colony-stimulating factor (CSF) for normal myeloid colonies, commonly called CFU-C.⁴⁵ The role of CSF for CFU-C is generally regarded as a specific growth stimulator,⁶ but there is no definite evidence to exclude the alternative possibility that CSF is merely a factor that allows CFU-C to retain viability; and the proliferation of CFU-C is then an expression of an intrinsic property of these cells. In this leukemic system, the humoral factor was shown to be a growth factor and leukemic cells survived without its addition. Finally, this culture system, with or without delayed addition of LCM, provides a matched pair of cycling and resting states within an in vitro model of a human malignant cell system that can be useful in various
studies, including the test of cycle dependency of new chemotherapeutic agents.

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REFERENCES


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