Survival of Highly Proliferative Colony Forming Cells after Treatment of Bone Marrow Cells with 4-Hydroperoxycyclophosphamide

Norio Komatsu,1 Toshio Suda, Junko Suda, and Yasusada Miura

Division of Hematology, Department of Medicine, Jichi Medical School [N. K., T. S., Y. M.] and The Second Department of Pediatrics, Dokkyo University School of Medicine [T. S.]

ABSTRACT

We investigated the in vitro effects of 4-hydroperoxycyclophosphamide (4-HC) on human hemopoietic stem cells. Marrow cells were exposed to 4-HC and then assayed for mixed (CFU-GEMM), erythroid (BFU-E), megakaryocyte (CFU-M), and granulocyte-macrophage (CFU-GM) colony forming cells. We found that highly proliferative colony forming cells, especially CFU-GEMM and BFU-E, were relatively spared by 4-HC treatment. One third of the surviving progenitors formed large colonies, some of which contained more than 50,000 cells. By sequential examination of the formation of these large colonies, we found immature colonies consisting of blasts at the early stage of culture. The morphology of these "blast cell colonies" in situ was arbitrarily classified into four types. Among them were the blast cell colonies consisting of the individual cells that were dispersed and had a few granules within the cytoplasm (type A); these cells finally formed very large colonies on day 22 of culture. Approximately 70% of the single cells derived from type A blast cell colonies produced secondary colonies consisting of erythroblasts, macrophages, eosinophils, and/or basophils. These results show that the blast cells in type A colonies have a highly proliferative capacity. The availability of a highly enriched population of primitive hemopoietic progenitors will provide us with a unique opportunity to study the interactions between a single stem cell and purified hemopoietic factors.

INTRODUCTION

Hemopoietic blast cell colonies with high replating efficiency provide a unique opportunity to study the interaction between single stem cells and purified hemopoietic factors or defined microenvironments. Pretreatment of mice by injection of high dose 5-FU2 enriches marrow and spleen cells for blast cell colony-forming units (1, 2). Several laboratories have attempted to enrich human hemopoietic progenitors. Some used single or combinations of monoclonal antibodies (3-6). Nakahata and Ogawa obtained human blast cell colonies in cultures of umbilical cord blood cells (7). By the combination of drug treatment in vitro and blast cell colony assay, we attempted to obtain the colonies which would allow consistent isolation of colony-forming cells.

4-HC, a derivative of cyclophosphamide, exhibits in vitro chemical and biological properties similar to those of microsomal activated cyclophosphamide (8, 9). It was reported that 4-HC can selectively kill murine leukemic cells but does not impair the repopulating ability of pluripotent stem cells (10, 11). Based on this observation, 4-HC has been applied to autologous bone marrow transplantation for patients with leukemia (12-14) and lymphoma (15), because autologous bone marrow harvested in remission has a high risk of being contaminated with microscopically undetectable malignant cells. Recent clinical studies showed that satisfactory hematological recovery can be obtained by 4-HC-treated bone marrow cells, despite elimination of detectable hemopoietic progenitors (12-14). It was demonstrated that murine day 12 CFU-S, which has been proposed to represent pluripotent stem cells, was relatively spared compared with day 7 CFU-S and committed hemopoietic progenitors (16). In contrast, in humans, it was recently reported that the progenitor cells most sensitive to 4-HC were CFU-GEMM, which were thought to be more immature than BFU-E and CFU-GM (17, 18).

To estimate the number of mixed colonies more accurately, we observed the colony formation for a longer period and identified further by lifting colonies, preparing as cytopsin and staining. By this method, we studied the effect of 4-HC on human pluripotent progenitors. During the daily observation of the dishes, we obtained colonies consisting mostly of immature blast cells surviving 4-HC treatment. We assayed proliferation capacity of these blast cells by using a replating experiment and a culture of single progenitors derived from blast cell colonies.

MATERIALS AND METHODS

Bone Marrow Cells. Bone marrow cells were obtained by aspiration from the sternum of healthy volunteers who gave informed written consent. Preservative free heparin was used as an anticoagulant. Mononuclear cells separated by centrifugation of Ficoll-Metrizoate (Lymphoprep; Nyegaard, Oslo) were washed three times in IMDM (GIBCO Laboratory, Grand Island, NY) and resuspended in IMDM containing 20% autologous or AB blood type plasma.

Drug Treatment. Bone marrow mononuclear cells were resuspended at 1 x 10^7/ml in IMDM containing 20% plasma and incubated with 4-HC at various concentrations. 4-HC was generously provided by Shionogi and Company, Ltd. (Osaka, Japan). Incubation was performed for 30 min in a 37°C waterbath with frequent agitation and then washed three times with IMDM. Since 4-HC is unstable in solution, we prepared the 4-HC solution just before each experiment.

 Colony Assays. The colony assay was carried out according to a modification of the method of Fauser and Messner (19). Briefly, the cells were suspended in medium containing 0.9% methylcellulose (Dow Chemical Co., Midland, MI), 30% autologous or AB blood type platelet poor plasma, 5% PHA-LCM prepared as previously described (20), 5 x 10^{-3} M 2-mercaptoethanol (Eastman Organic Chemical, Rochester, NY), 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), and 1 U/ml human urinary Ep (Toyobo, Osaka, Japan). Kimura et al. and the authors of this study confirmed that the human platelet-poor plasma supports megakaryocytic colony formation better than serum (20, 21). PHA-LCM was prepared by the method described by Kimura et al. (20). Briefly, peripheral blood mononuclear cells from normal individuals were incubated at a concentration of 10^6/ml in IMDM, 5% plasma, and 0.5% PHA (Difco Laboratories Inc., Detroit, MI). After 6 days, the supernatant was filtered through a 0.45-μm filter and frozen. One-mll aliquots of culture medium containing 5 x 10^3 or 1 x 10^4 control cells and 2 x 10^3 4-HC-treated cells were cultured in 35-mm nonmottle culture dishes (Falcon, Oxnard, CA) at 37°C in a 5% CO_2 humidified atmosphere. Incubated plates were cultured for 22 days and individual colonies were identified further by lifting them with a 3-μl Eppendorf pipet, preparing as cytopsin and staining with May-Grunwald-Giemsa. The size of small colonies (<500 cells) was estimated in situ, and that of large colonies, by use of a counting chamber.

Received 12/15/86; revised 5/1/87; accepted 8/26/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1To whom requests for reprints should be addressed, at Division of Hematology, Department of Medicine, Jichi Medical School [N. K., T. S., Y. M.]

2The abbreviations used are: 5-FU, 5-fluorouracil; 4-HC, 4-hydroperoxy cyclophosphamide; IMDM, Iscove's modified Dulbecco's medium; PHA-LCM, phytohemagglutinin-stimulated leukocyte conditioned medium; Ep, erythropoietin.
Sequential Examination of Maturation of Blast Cell Colonies. Cell culture dishes containing $2 \times 10^5$ 4-HC-treated bone marrow cells were examined daily under an inverted microscope. When small colonies consisting usually of 50 or more cells were identified, the location of the colony and the number of cells in situ were recorded. Subsequently, proliferation and expression of differentiation of the developing colonies were recorded daily. When colonies appeared mature, individual colonies were lifted and stained with May-Grunwald-Giemsa.

Replating Experiments. On days 7 to 9 of incubation, blast cell colonies were individually lifted from the methycellulose medium and suspended in 0.1 ml IMDM. After gentle pipeting, the samples were individually added to 0.9 ml methycellulose culture medium containing 5% PHA-LCM and 1 U Ep in the culture dishes. The preparation was again thoroughly mixed by gentle pipeting. Cultures of the replated cells were incubated at 37°C and the colonies scored in the same manner as in primary culture.

Culture of Single Progenitors. On day 7 of incubation, blast cell colonies consisting of 20–100 cells were identified, and lifted from the methycellulose medium using a 3-µl Eppendorf pipet under direct microscopic observation, and individually suspended in 0.1 ml of IMDM. Each sample was then added to 0.9 ml of methycellulose medium in a second 35-mm culture dish, and the dish was agitated gently to disperse the cells. Transfer of a single cell to a third 35-mm culture dish containing 1 ml of methycellulose medium was accomplished by using a fine Pasteur pipet (with a diameter of approximately 30 µm) attached to a micromanipulator (Narishige Scientific Instrument Lab., Tokyo) under direct microscopic observation. Approximately one-third of the cells from a single blast cell colony were identified and transferred to the third dish. After confirmation of the presence of a single cell in each culture dish, incubation was carried out at 37°C in 5% CO₂/95% air. On days 6 through 15 of incubation, when the secondary colonies appeared to have matured, individual colonies were lifted from the methycellulose medium and stained with May-Grunwald-Giemsa for differential counting.

RESULTS

In Vitro Effect of 4-HC on the Survival of Colony Forming Cells. To determine the appropriate time for identifying human multilineage colonies, we first observed the growth of colonies formed by untreated human bone marrow cells. The colonies in situ were observed on 14, 18, and 22 days under an inverted microscope and each colony was lifted from plates on day 22 of culture, suspended in IMDM containing 20% fetal calf serum prepared as cytopsins, and stained with May-Grunwald-Giemsa to verify the cell composition of individual colonies. We noticed that some of the day 14 erythroid bursts, which consisted of both hemoglobinized and nonhemoglobinized cells, looked like "mixed colonies." Therefore, we decided to observe them for a longer period and identify further by picking up colonies and preparing as cytopsins and staining. Moreover, to avoid the overlap of the colonies, we plated the appropriate number of cells to form less than 20 to 40 colonies.

To study the drug toxicity of 4-HC to hemopoietic progenitors, colony assay was performed following the treatment of bone marrow cells with 4-HC at various concentrations of 30, 60, 120, and 240 µg/ml. As shown in Fig. 1, colony-forming cells were completely eliminated at the concentration of 120 and 240 µg/ml. At the concentration of 60 µg/ml, approximately 4.5% of colony forming cells survived. This concentration of 4-HC has been reported to kill most of the leukemic cells, still preserving the ability of successful recovery of the normal hemopoiesis after bone marrow transplantation (22).

So we decided to use the 60 µg/ml dose to analyze the nature of surviving progenitors by 4-HC treatment. Table 1 shows the effect of 4-HC on the survival of each colony forming cell. We observed the tendency that CFU-GM were more sensitive to 4-HC treatment than CFU-GEMM or BFU-E. We compared the size of colonies derived from 4-HC-treated marrow cells with normal control. The size of colonies obtained from 4-HC-treated marrow cells were apparently larger than those from nontreated marrow cells. Of a total of 121 colonies from 4-HC-treated cells, 19 colonies contained more than 50,000 cells, while only nine colonies out of a total of 179 colonies from nontreated cells consisted of 50,000 cells. There was a significant difference between them (Table 2). These results indicate that highly proliferative progenitors are relatively resistant to 4-HC.

Table 3 shows the differential count of large GEMM colonies (>50,000 cells) derived from 4-HC-treated bone marrow cells. However, in this experiment, megakaryocytic colonies were not formed since a lower number of untreated bone marrow cells (2.0-3.5 x 10⁶ cells) were plated. In our culture system, 24 ± 16 megakaryocytic colonies were usually formed by 1 x 10⁵ untreated bone marrow cells. However, in this experiment, megakaryocytic colonies were not formed since a lower number of untreated bone marrow cells (2.0-3.5 x 10⁶ cells) were plated.

![Fig. 1. Toxicity of 4-HC on colony forming cells. Marrow cells at a concentration of 1 x 10⁵/ml were incubated with 30, 60, 120, and 240 µg/ml, washed, and assayed for colony forming cells. Values, percentage of control (0 µg/ml). We observed colonies "in situ" after days 18 through 22 of incubation.](image-url)
Table 2 Comparison of cell numbers in colonies formed by 4-HC-treated and nontreated bone marrow cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total colonies</th>
<th>Colonies containing more than 50,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HC Treatment</td>
<td>121</td>
<td>19*</td>
</tr>
<tr>
<td>Normal control</td>
<td>179</td>
<td>9*</td>
</tr>
</tbody>
</table>

*Results show the number of colonies per 4 x 10^6 plated cells from two dishes.

Table 3 Differential count of very large GEMM colonies derived from 4-HC treated and untreated bone marrow cells in Table 2

<table>
<thead>
<tr>
<th>Colony number</th>
<th>Cell count</th>
<th>Colony type</th>
<th>n</th>
<th>m</th>
<th>e</th>
<th>b</th>
<th>E</th>
<th>M</th>
<th>Blast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88,000</td>
<td>EM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>98.5</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>120,000</td>
<td>neE</td>
<td>2.0</td>
<td>1.0</td>
<td>0</td>
<td>97.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>92,000</td>
<td>neE</td>
<td>4.0</td>
<td>18.0</td>
<td>1.5</td>
<td>74.0</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>80,000</td>
<td>neE</td>
<td>4.5</td>
<td>7.5</td>
<td>1.0</td>
<td>87.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>122,000</td>
<td>neE</td>
<td>4.5</td>
<td>3.0</td>
<td>0</td>
<td>92.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>85,000</td>
<td>eE</td>
<td>0</td>
<td>0</td>
<td>17.0</td>
<td>0</td>
<td>83.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>98,000</td>
<td>neE</td>
<td>3.0</td>
<td>1.5</td>
<td>0</td>
<td>95.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>143,000</td>
<td>neE</td>
<td>16.0</td>
<td>11.0</td>
<td>0</td>
<td>73.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>73,000</td>
<td>nneEbi</td>
<td>3.5</td>
<td>2.5</td>
<td>4.0</td>
<td>87.5</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>56,000</td>
<td>EM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>99.5</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>119,000</td>
<td>eE</td>
<td>0</td>
<td>0</td>
<td>7.0</td>
<td>0</td>
<td>93.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>81,000</td>
<td>ebE</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
<td>2.0</td>
<td>97.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The abbreviations are based on the recommendation at a workshop at a University of California at Los Angeles Symposium: n, neutrophil; m, macrophage; e, eosinophil; b, basophil; E, erythrocyte; and M, megakaryocyte.

Though their major component was erythroid cells.

Sequential Examination of Maturation of Blast Cell Colonies.

As shown in Table 2, large-sized colonies were 16% among a total of colonies formed after treatment with 4-HC. To identify the blast cell colonies which develop large colonies, we carried out the sequential observation of the colony formation. Bone marrow cells treated with 4-HC in vitro were cultured at a concentration of 2 x 10^5 cells per ml in the presence of Ep and PHA-LCM. Subsequently, the dishes were examined daily under an inverted microscope for assessment of colony growth and maturation. Blast cell colonies were classified into four types: type A, individual cells dispersed with few granules within cytoplasm; type B, individual cells aggregated compactly; type C, individual cells dispersed with a translucent cytoplasm; and type D, others. A type A blast cell colony is shown in Fig. 2A. A blast cell in type A had a few granules in the basophilic cytoplasm and several "folds" in the nucleus (Fig. 2). Fig. 3 shows the cell counts and morphology of each type of blast cell. From 48 type A colonies, 14 GEMM colonies and 32 erythroid bursts were formed. In contrast to this finding, from 20 type B colonies small erythroid bursts (N = 14) and GM colonies (N = 5) were formed. From type C colonies, mainly GM were formed (Fig. 3). Table 4 summarizes the colony size data. The formation of large colonies occurred primarily with type A blast cell colonies (Table 4); especially very large colonies consisting of more than 50,000 cells were obtained only from type A colonies. Fig. 2C shows a large colony derived from a blast cell colony that is shown in Fig. 2A. This indicates that type A blast cell colonies contained cells with higher proliferative capacity than those in other types.

Since the type A blast cell colonies were dispersed, it was possible to estimate cell numbers during the early phase of colony growth. A graphic analysis of a total of eight large colonies (>50,000 cells) is presented in Fig. 4. Colony formation began at various times (from day 7 through 16) after incubation.

Secondary Replating Capabilities of the Blast Cell Colony.

From the above observations, it was speculated that blast cells in type A colonies contained primitive hemopoietic progenitors. To investigate whether cells obtained from type A blast cell colonies have a proliferative capacity, replating experiments were performed. Sixty-five out of 66 blast cell colonies produced secondary colonies, indicating that most colonies contained hemopoietic progenitor cells. Total replating efficiency (number of secondary colonies per number of cells in a primary blast colony) ranged from 0 to 100%; the mean ± SD was 58 ± 29%. In other words, more than half of the cells in the primary colonies yielded the secondary colonies. We observed various sizes of secondary colonies. Some colonies contained less than 20 cells, and others contained more than 5000 cells. The majority of secondary colonies contained a single cell lineage,
mainly consisting of erythroblasts. Small numbers of neutrophil, macrophage, basophil, eosinophil, and megakaryocyte colonies were also seen which have been confirmed by cytospin preparation. Larger colonies usually consisted of macrophages, neutrophils, eosinophils, and/or basophils, whereas smaller ones of erythroblasts or megakaryocytes. A few secondary colonies contained mixed colonies consisting of eosinophil-basophils or neutrophil-macrophages.

Culture of Single Progenitors. Eighty-three single cells derived from the blast cell colony were individually transferred to 35-mm culture dishes using a micromanipulator. Sixty cells (or 72%) produced secondary colonies, of which 55 colonies consisted mainly of erythroblasts. Small numbers of neutrophils, eosinophils, and megakaryocytes were also seen which have been confirmed by cytospin preparation. Larger colonies usually consisted of macrophages, neutrophils, eosinophils, and/or basophils, whereas smaller ones of erythroblasts or megakaryocytes. A few secondary colonies contained mixed colonies consisting of eosinophil-basophils or neutrophil-macrophages.

DISCUSSION

We studied the in vitro effects of 4-HC on human hemopoietic progenitors. Some investigators reported that CFU-GEMM is more sensitive to 4-HC than BFU-E and CFU-GM (17, 18). This finding is not in accord with the hierarchy of hemopoietic differentiation from pluripotent stem cells to monopotent progenitors. To clarify this point, we improved the culture technique for CFU-GEMM by prolongation of the culture time to 22 days for colony identification. Moreover, we found that colony types determined by in situ observation under an inverted microscope were unreliable (data not shown). Pharr et al. have already reported that in situ identification of murine mixed colonies is not dependable (23). Therefore, all the colonies in cultures were individually picked up for analysis by May-Grunwald-Giemsa staining when the colonies appeared to be mature. Our results clearly demonstrated that CFU-GEMM and BFU-E were relatively spared by 60 μg/ml 4-HC compared with CFU-GM. This finding is supported by successful bone marrow reconstitution in recipients of 4-HC-treated grafts in which almost all CFU-GM were undetectable (12-14).

We found a relative sparing of progenitors having highly proliferative capacity. Bradley et al. described that primitive macrophage progenitor cells with high proliferative potentials survive 5-fluorouracil treatment in mice (1). Subsequently, Suda et al. reported that only the primitive population of stem cells survive after such manipulation (2). Beyer et al. found that colonies grown from marrow obtained following chemotherapy were frequently macroscopic and were composed of thousands of cells (24). Our observations are in agreement with their findings.

We could identify human blast cell colonies having a high proliferative potency by the time course observation of colony formation by 4-HC-treated bone marrow cells. Serial observations of the development of blast cell colonies in the course of cultures of 4-HC-treated human marrow cells showed that the highly proliferative progenitors had variable lag times before the cells commence to cell division. These data confirmed the findings observed in the murine blast cell colony formation from 5-FU-treated spleen cells (2), although 4-HC is not an S-phase-specific cytotoxic drug as is 5-FU.

The blast cell colony consists of individual cells that are dispersed and have a few granules within the cytoplasm when observed in situ under an inverted microscopy. The morphology in situ of the blast cell is similar to that obtained by panning
with anti-My-10 monoclonal antibody reported by Leary et al. (3). The total replating efficiency of the blast cell colony was 58 ± 29%. The majority of the secondary colonies were composed of erythroblasts but macrophage, neutrophil, eosinophil, basophil, and megakaryocyte colonies were also observed. In addition, about 70% of single cells formed secondary colonies consisting of erythroblasts, macrophages, eosinophils, and/or basophils. These findings confirmed that the blast cell has a highly proliferative capacity and at least, in part, the ability to differentiate to multilineage pathways. Blast cell colonies obtained from 5-FU-treated mice can differentiate to multilineage pathways. Compared with this, 4-HC-treated bone marrow cells showed the predominant erythroid nature of the mixed colonies. The majority of secondary colonies contained the erythroid single lineage. Further exploration to obtain the blast cell colonies which contained more multilineage progenitors should be continued. Human blast cell colonies obtained after such a simple manipulation promise to be important for studies of the mechanisms of proliferation and differentiation of hemopoietic progenitors.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Cancer Research and Scientific Research from the Ministry of Education, Science and Culture, Japan. We wish to thank Professor K. Tsujibayashi for his valuable suggestions on statistical analysis.

REFERENCES

15. Gulati, S., Gandola, L., Vega, R., Yopp, J., Chang, T. T., Ibrahim, S., Siena, S., Castro-Malaspina, H., Colvin, M., and Clarkson, B. Chemopurification...


Survival of Highly Proliferative Colony Forming Cells after Treatment of Bone Marrow Cells with 4-Hydroperoxycyclophosphamide

Norio Komatsu, Toshio Suda, Junko Suda, et al.


Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/47/23/6371

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.