RAPID COMMUNICATION

Long-Term Bone Marrow Culture in Persons With Fanconi Anemia and Bone Marrow Failure

By Anna Butturini and Robert Peter Gale

Fanconi anemia is an autosomal recessive disease characterized by a high risk of developing bone marrow (BM) failure and acute myelogenous leukemia. We studied growth of hematopoietic progenitor cells in long-term BM culture (LTBMC) in 8 persons with Fanconi anemia and BM failure. Although LTBMC were initiated with very few BM cells, an adherent layer formed in cultures from 7 persons. In these cultures, the number of nonadherent cells increased for 10 to 15 days. Cell growth continued until cultures were terminated at day 35 to 40. During the first 2 weeks of culture, most nonadherent cells were differentiated myeloid cells. By days 35 to 40, the adherent layer contained cells able to initiate secondary LTBMCs. These data indicate that hematopoietic precursors cells able to proliferate and differentiate in vitro are present in the BM of persons with Fanconi anemia and BM failure. They suggest that mechanisms other than absent precursor cells are responsible for BM failure in Fanconi anemia.

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MATERIALS AND METHODS

Heparinized BM (0.5 to 4 mL) was obtained on one or more occasions from 8 persons with Fanconi anemia confirmed by diepoxylbutane (DEB) testing and BM failure. Informed consent, approved by the institutional review board, was obtained from all subjects. Median age at study was 12 years (range, 0.5 to 32 years). Median duration of BM failure before study was 57 months (range, 1 to 158 months). Prior treatment of BM failure included androgens and transfusions. Cytogenetic studies were successful in 6 persons: 4 had two BM cells with the same abnormality (clonal cytogenetic abnormalities) on at least one occasion of these had increased proportion of BM undifferentiated blasts at least in one study (Table 1).

Histocompatible BM samples were obtained from donors and BJMC samples were obtained from healthy donors and self-donor by blood transfusion. Buffy coat cells were centrifuged on ficoll-hypaque density gradients and subjected to nylon-wool columns to remove T lymphocytes and monocytes. They were then cultured in semi-solid medium containing 12.5% fetal calf serum (FCS), 12.5% horse serum, 1% glutamine, 1% sodium pyruvate, 1% antibiotics, 1% nonessential amino acids, 1% vitamins, 1% sodium bicarbonate, and 10 mol/L hydrocortisone (LTBMC medium). In some experiments, stem cell factor (SCF), 10 ng/mL, interleukin-1 (IL-1; 50 ng/mL), and IL-3 (50 ng/mL) were added to LTBMC medium alone or in combination (SCF, IL-1, and IL-3 were gifts of Dr. M.A.S. Moore). Cultures were incubated at 37°C in 5% O2, 5% CO2, and 90% nitrogen. After 4 to 7 days, nonadherent cells were centrifuged on a ficoll-hypaque gradient to remove red blood cells. Beginning on day 10, one-half of the supernatant was removed every 5 days and replaced by fresh LTBMC medium. The nonadherent cells were counted and assessed for morphology, cytogenetics, and/or colony-forming unit-granulocyte-macrophage (CFU-GM). After 35 to 40 days, adherent layers were harvested by trypsinization and then incubated for 24 hours on a plastic surface. Nonadherent cells were tested for their capability to initiate secondary LTBMC and for CFU-GM.

From the Department of Pediatrics, University of Parma, Parma, Italy; and the Department of Internal Medicine, UCLA School of Medicine, Los Angeles, CA.

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Address reprint requests to Anna Butturini, MD, Department of Pediatrics, University of Parma, via Gramsci 14, 43100 Parma, Italy.

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Cells capable of initiating secondary LTBMCs were assayed by culturing on irradiated (20 Gy) ST stromal cell line (gift of Dr C. Gasparetto) in a 50-ml flask. Cultures were inspected visually for formation of “cobblestone” areas by inverted microscope, for production of nonadherent cells, and for CFU-GM in the adherent layer at day 35.

CFU-GM were performed by plating 10^5 cells in 1 ml of Iscove’s medium with 0.36% agar, 20% FCS, 1% glutamine, 10^{-4} mol/L non-essential amino acids and 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). In some experiments, LTBMC medium was substituted with or without SCF (10 ng/ml), IL-1 (50 ng/ml), IL-3 (50 ng/ml), 10^{-4} mol/L hydrocortisone, or 10^{-3} mol/L testosterone (gift of Dr K. Sundaram, Rockefeller University, New York, NY). After 7 days, colonies (>40 cells) and clusters (>5 to 40 cells) were enumerated using an inverted microscope.

Experiments were performed under laminar flow hoods at 5% O_2. Cultures were performed in duplicate (LTBMC) or triplicate (CFU-GM) and incubated at 37°C in 5% O_2 and 5% CO_2. Results are expressed as mean ± 2 standard deviations. Results were compared by t-test.

RESULTS

BM samples contained 0.4 to 7 × 10^6 cells/ml (median, 1.6 × 10^6 cells/ml). Fifty percent to 70% of these cells were erythroid precursors, 5% to 10% were monocyte-macrophages, 1% to 15% were myeloblasts or promyelocytes, 0% to 25% were myelocytes or granulocytes, and 5% to 40% were lymphocytes. In 2 persons, 7% to 20% of cells were undifferentiated blasts.

LTBMCs were initiated with 0.2 to 5 × 10^6 cells/flask (median, 0.9 × 10^6 cells/flask). Adherent layers with “cobblestone” areas formed in cultures from 7 persons by day 15.

The number of nonadherent cells increased in all the cultures with an adherent layer by day 10 (Fig 1). Between days 5 and 15, nonadherent cells were predominantly monocyte-macrophages, granulocytes, erythroid blasts, and myelocytes (Table 2). In 8 experiments from 6 persons, production of nonadherent cells continued until cultures were terminated at day 35 to 40. In 2 experiments, cultures were continued until they stopped growing at day 62 and 74, respectively. From day 20, nonadherent cells were mostly monocyte-macrophages and small round cells with scarce cytoplasm and dense chromatin.

In experiments in which the adherent layer was harvested on day 35 to 40, adherent cells were 4.5 ± 3.1 × 10^7/flask (mean ± 2 SD). These cells formed “cobblestone” areas when cultured on irradiated ST cells and produced nonadherent cells of myeloid morphology in 5 of 7 experiments.

CFU-GM colonies and clusters in uncultured BM were 5 ± 28/10^5 cells. LTBMCs were initiated with 12 ± 52 CFU-GM per flask. CFU-GM significantly increased in the non-adherent cell compartment after 10 to 15 days of cultures (903 ± 1,200/flask; P = .04), declining thereafter. At day 35 to 40, the adherent layer contained 204 ± 328 CFU-GM/flask. In adherent layers obtained at day 35 of secondary LTBMC, CFU-GM were 89 ± 68/flask. Size and morphology of CFU-GM were abnormal; most were clusters and small, irregularly shaped colonies. Changes in medium and addition of horse serum, growth factors, androgens, or hydrocortisone did not affect CFU-GM growth.

In experiments from 4 persons, addition of growth factors to primary LTBMCs did not affect formation of adherent layer, production of nonadherent cells or CFU-GM, culture survival, or presence of cells able to initiate secondary LTBMCs at day 35.

DISCUSSION

We used LTBMCs to study hematopoietic cells in persons with Fanconi anemia and BM failure. Because BM samples contained few cells, cultures were initiated with numbers of cells far below (1% to 10%) those commonly used. Nevertheless, apparently normal adherent layers formed in cultures from 7 of 8 persons and large numbers of differentiated myeloid cells were generated within 1 to 2 weeks. By day 35, the adherent layer of most cultures contained cells able to initiate secondary LTBMCs. However, growth of CFU-GM was impaired.

These data suggest that early hematopoietic precursors are present in the BM of persons with Fanconi anemia and BM failure. These cells are able to proliferate and differentiate in LTBMC but not in vivo and do not form colonies.
in the CFU-GM assay. The reason for these disparities is unknown. It is possible that in vitro conditions correct abnormalities present in vivo. For example, the culture medium may supply nutrients or growth factors absent in vivo. However, this does not explain the disparity between results of LTBMC and the CFU-GM assay, even when they were performed using the same culture medium. Also, addition of testosterone, hydrocortisone, or growth factors did not correct the deficit of CFU-GM; similar data are reported for BFU-E and CFU-GEMM (Alter et al4 and B. Rotoli and R. Notaro, personal communication).

Another possible explanation of the disparity between in vivo and in vitro hematopoiesis is the absence of toxins that operate in vivo but not in vitro. For example, some data suggest that Fanconi anemia cells are especially sensitive to oxygen-mediated damage. In this study, we avoided exposing cells to oxygen concentrations greater than 5%. However, a low O\textsubscript{2} concentration is present in the BM in vivo. Also, LTBMC and CFU-GM were performed under similar conditions.

An alternative explanation is that in vivo hematopoiesis is suppressed by mechanisms not operating in LTBMC.

### Table 2. Stroma and Nonadherent Cell Production in LTBMC

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Cells/Flask* Day 0 (\times 10^6)</th>
<th>Cells/Flask* (\pm 2) SD</th>
<th>G</th>
<th>M</th>
<th>Day</th>
<th>Culture Length (d)</th>
<th>LTBM C IC Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>147.4 ± 66</td>
<td>5%</td>
<td>30%</td>
<td>15</td>
<td>40</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>1.4</td>
<td>12.7 ± 2.8</td>
<td>15%</td>
<td>53%</td>
<td>10</td>
<td>20</td>
<td>NT</td>
</tr>
<tr>
<td>3a</td>
<td>0.6</td>
<td>6.3 ± 0.8</td>
<td>15%</td>
<td>53%</td>
<td>10</td>
<td>35</td>
<td>Yes</td>
</tr>
<tr>
<td>3b</td>
<td>0.2</td>
<td>6.6 ± 1.2</td>
<td>15%</td>
<td>53%</td>
<td>10</td>
<td>40</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>13.2 ± 2</td>
<td>48%</td>
<td>15%</td>
<td>5</td>
<td>35</td>
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</tr>
<tr>
<td>5a</td>
<td>5</td>
<td>73.2 ± 36</td>
<td>75%</td>
<td>6%</td>
<td>5</td>
<td>40</td>
<td>Yes</td>
</tr>
<tr>
<td>5b</td>
<td>0.8</td>
<td>12.7 ± 2.8</td>
<td>45%</td>
<td>12%</td>
<td>10</td>
<td>40</td>
<td>No</td>
</tr>
<tr>
<td>6a</td>
<td>1.2</td>
<td>62.5 ± 24</td>
<td>18%</td>
<td>40%</td>
<td>10</td>
<td>35</td>
<td>Yes</td>
</tr>
<tr>
<td>6b</td>
<td>0.8</td>
<td>12.7 ± 2.8</td>
<td>45%</td>
<td>12%</td>
<td>10</td>
<td>62t</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>4.7 ± 1.2</td>
<td>10%</td>
<td>60%</td>
<td>10</td>
<td>62t</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>0.9</td>
<td>7.4 ± 1.8</td>
<td>28%</td>
<td>15%</td>
<td>10</td>
<td>35</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Abbreviations: G, granulocytes; M, monocytes; NT, not tested.

* Mean ± 2 SD of results of replicate cultures.

† Cultures continued until less than 5 \(\times 10^6\) nonadherent cells/flask in at least one culture replicate.

Fig 1. Production of nonadherent cells in LTBMCs in which an adherent layer formed. (---) Mean values of replicate in individual experiments; (- - -) mean of all experiments. Bars represent ±2 standard deviations. Arrows indicate splitting of nonadherent cells.
This is compatible with the hypothesis that BM failure in Fanconi anemia is a phase of leukemia transformation. Considerable data suggest that leukemia is a multistep process and early phases of transformation are characterized by the expansion of a "preleukemia" clone that may retain the ability to differentiate. In Fanconi anemia, this "preleukemia" clone (and possibly normal hematopoiesis) may be inhibited by specific or nonspecific mechanisms. This hypothesis is supported by clinical observations. For example, in a substantial proportion of persons with Fanconi anemia, the same cytogenetically abnormal clone was detected during BM failure and after developing leukemia. Also, in some persons with Fanconi anemia and BM failure, development of clonal cytogenetic abnormalities was associated with transient hematopoietic recovery. Some of these persons eventually developed leukemia.

Several of our observations support the notion that in Fanconi anemia, BM failure is related to leukemia transformation. For example, 2 of 6 persons with BM failure and less than 5% myeloblasts had clonal cytogenetic abnormalities similar to those reported in MDS and AML. The disparity between capability to differentiate in LTBMC and results of CFUGM may also mirror the disparity between ability to differentiate in vivo and impaired growth in clonogenic assays typical of persons with MDS. Mechanisms that might suppress hematopoiesis in persons with Fanconi anemia and BM failure are unknown. Experimental data suggest that several phases of leukemia transformation including clonal expansion, inhibition of normal hematopoiesis, and possibly progression from "preleukemia" to leukemia, are regulated by complex mechanisms involving stromal, immune, and hematopoietic cells. In preliminary experiments, we found that autologous blood mononuclear cells inhibit the growth of nonadherent cells in LTBMC (data not shown). Further studies of early hematopoietic precursors in Fanconi anemia are needed. These studies may explain early phases of leukemia transformation and cellular and humoral mechanisms regulating normal and leukemia hematopoiesis.

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A Butturini and RP Gale

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