Application of Hyperthermia to the Treatment of Human Acute Leukemia: Purging Human Leukemic Progenitor Cells by Heat

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The application of hyperthermia to the treatment of neoplastic disease has focused on solid tumors. Since the hyperthermic sensitivity of human acute leukemia cells is not known, we have studied the in vitro response of human leukemic progenitor cells (L-CFU) to hyperthermia using a quantitative assay system for L-CFU. Human L-CFU were found to be more sensitive than committed normal myeloid progenitor cells to hyperthermic killing (41 to 42 °C). In addition, in the five acute myelogenous leukemic patients studied, it was shown that their leukemic progenitor cells—all types were studied according to the French-American-British diagnosis—were unable to form colonies when exposed to a temperature of 42 °C for 60 minutes, whereas the residual normal clones suppressed by the leukemic cell population were found to recover and to form more colonies in vitro as compared with untreated leukemic marrows. This strongly suggests that in vitro hyperthermia may selectively purge residual leukemic cells, especially L-CFU in stored remission bone marrow before autologous bone marrow transplantation.

Materials and Methods

Leukemic cells. Human leukemia cells (57% to 95%) for L-CFU assay were obtained from five patients with AML prior to chemotherapy (Table 1). Normal marrow cells used as controls were obtained from two normal volunteers and from three patients with acute leukemia in complete remission. The marrow cells were aspirated from the sternum or posterior iliac crests using conventional techniques and diluted immediately in McCoy's 5A medium (GIBCO, Grand Island, NY) containing heparin (10 U/mL). The buffy coat was obtained by centrifugation for five minutes at 200 g and washed twice with McCoy's 5A medium. In one patient studied, peripheral blood was obtained by venipuncture into heparinized syringes. Mononuclear cells were then separated by layering the blood over Lymphoprep (Nyegaard, Oslo) and centrifuging for 25 minutes at 400 g. Cells in the interface band were harvested and washed twice with McCoy's 5A medium. For heating, the cells obtained were adjusted to 2 × 10^6/mL.

Heating. Cells suspended in 1 mL of McCoy's 5A medium in Falcon 3033 culture tubes (Falcon Labware, Oxnard, Calif) were immersed in a constant water bath (±0.1 °C) and exposed to temperatures of 41 and 42 °C for 15 to 60 minutes. Control cells were kept at room temperature for the same time period. After hyperthermic treatment, the cell suspension was immediately cooled in water (4 °C) and prepared for L-CFU and CFU-c assay.

Stem cell assays. Basically, our PHA assay for L-CFU consists of two phases: an initial liquid phase of 15 to 24 hours at 37 °C and a semisolid phase for seven days of incubation. Briefly, in the liquid phase, 0.1 mL (for one plate) of McCoy's 5A medium containing 2 × 10^6 marrow or peripheral mononuclear cells was cultured without any serum in a Falcon 3033 culture tube to which 0.05 mL PHA-LCM was added. After preincubation, the cells were not washed, but diluted with 0.9 mL of 0.3% agar medium (enriched McCoy's 5A medium + 15% fetal calf serum) and pipetted carefully to create a single-cell suspension. After being resuspended in an agar medium, the cells were plated into 35-mm Falcon Petri dishes and cultured in a solid phase without any other stimulators.

The CFU-c assay was performed according to the method of Burgess et al using human placenta-conditioned medium (HPCM) as a source of colony-stimulating factor (CSF) to compare to the thermal sensitivity of L-CFU. All cultures were done in duplicate or triplicate.

After seven to ten days of incubation in a 5% CO₂ gas-controlled humidified incubator at 37 °C, colonies containing 40 or more cells were counted with an inverted microscope. For determining stem cell survival, the ratio of the colony count in each heated sample to the mean control colony count was calculated.

Results

A comparison between the thermal sensitivity of human L-CFU and CFU-c is illustrated in Fig. 1. The survival of leukemic progenitor cells in AML (patient T.O.) decreased exponentially with continuous heating at temperatures of 41 and 42 °C. At both temperatures the survival curves for L-CFU had an initial shoulder region. At 42 °C the survival of CFU-c also decreased exponentially, but was not affected at the lower temperature of 41 °C. The terminal slopes of the curves clearly demonstrated greater hyperthermic sensitivity of L-CFU when compared to CFU-c at both temperatures studied. The slope of the exponential portion of the curves for L-CFU increased over ten times with a 1 °C rise in tempera-
ture, whereas that for CFU-c increased only about 1.8 times.

FAB diagnosis,10 the clinical outline of five patients with AML, and the thermal sensitivity of L-CFU in each patient at 42 °C are summarized in Table 1. All leukemic progenitor cells (L-CFU) studied, even those producing any type of leukemic cells according to FAB diagnosis, were found to be very sensitive to heat at 42 °C. In addition, L-CFU obtained from four out of five patients were completely killed by heat for 60 minutes at 42 °C and therefore unable to form colonies in vitro, whereas 54% of normal CFU-c survived for the same time period.

To see whether hyperthermic treatment could save normal clone but selectively eradicate leukemic cell populations in leukemic marrows containing normal clone, the possibility that residual normal clones suppressed by the leukemic environment could be detected after purging the leukemic cell population by hyperthermia was examined using a standard agar method.9 Table 2 is based on the data of an experiment in which four leukemic marrows containing 57.5% to 98.8% blasts were cultured for a CFU-c assay using HPCM as a source of CSF before and after heating for 60 minutes at 42 °C, and three out of these four leukemic marrows were also investigated by an L-CFU assay. The four untreated leukemic marrows obtained from three patients with AML and one with acute lymphocytic leukemia (ALL) grew none or a few CFU-c-derived colonies in an agar medium. After hyperthermic treatment, on the other hand, the L-CFU population disappeared (in three out of four patients, see Table 1), but the residual normal CFU-c in all of the four leukemic marrows studied remained active and appeared to form more colonies in vitro.

**DISCUSSION**

Human acute leukemia is considered to be a clonal hemopathy originating in pluripotential stem cells, and leukemic cells are produced by L-CFU, which are able to self-renew and proliferate in vitro.11,12 Therefore, it is necessary to eradicate the L-CFU population in the bone marrow of patients for a successful treatment of acute leukemia. In humans, attempts have been made to purge malignant cells from marrow using 4HC, ASTA-Z-7557, and monoclonal antibodies.13-15 Experience is still too limited to evaluate the success of these agents.

In this study, using a quantitative assay system for L-CFU, we clearly demonstrated that the survival of human L-CFU decreases exponentially with continuous heating at temperatures of 41 and 42 °C and that L-CFU in AML are more sensitive than CFU-c to hyperthermic killing. In addition, the majority of the L-CFU obtained from the marrow or peripheral mononuclear cells of five patients with AML appeared to be unable to form colonies and were eradicated by heat at 42 °C for 60 minutes, whereas over half of the normal CFU-c remained active and survived for the same time period.

### Table 1. Clinical Data and L-CFU Survival of Five Patients With AML by Heat at 42 °C

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age and Sex</th>
<th>FAB Diagnosis</th>
<th>Hb (g/dL)</th>
<th>Platelets (× 10^11/L)</th>
<th>WBC (μL^3)</th>
<th>Blasts in Marrow (%)</th>
<th>Survival of L-CFU at 42 °C (0 min, 30 min, 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T.O.</td>
<td>51M</td>
<td>M1</td>
<td>8.1</td>
<td>4.1</td>
<td>1760</td>
<td>80.2</td>
<td>182 ± 18 31 ± 4.2 0.3 ± 0.5</td>
</tr>
<tr>
<td>Y.A.</td>
<td>34F</td>
<td>M2</td>
<td>11.2</td>
<td>5.9</td>
<td>2400</td>
<td>57.5</td>
<td>46 ± 8 4 ± 0.5 0</td>
</tr>
<tr>
<td>T.K.</td>
<td>65M</td>
<td>M2</td>
<td>5.2</td>
<td>2.4</td>
<td>11000</td>
<td>95.0</td>
<td>202 ± 14 28 ± 5.0 0</td>
</tr>
<tr>
<td>S.I.</td>
<td>56F</td>
<td>M4</td>
<td>8.2</td>
<td>0.8</td>
<td>23600</td>
<td>86.2</td>
<td>62 ± 8t 4 ± 0.9t 0t</td>
</tr>
<tr>
<td>K.K.</td>
<td>38M</td>
<td>M5</td>
<td>6.3</td>
<td>3.6</td>
<td>34800</td>
<td>88.6</td>
<td>314 ± 23 18 ± 2.2 0</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>116 ± 27 108 ± 15 63 ± 14</td>
</tr>
</tbody>
</table>

Abbreviation: FAB, French-American-British.

*Mean ± SD colonies/2 × 10^6 bone marrow cells.

†Assayed for L-CFU of peripheral mononuclear cells (blasts, 75%).

**Table 2. Survival and Recovery of Residual Normal Clones (CFU-c) in Four Leukemic Marrows Before and After Heating for 60 Minutes at 42 °C**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age and Sex</th>
<th>FAB Diagnosis</th>
<th>No. of CFU-c-Derived Colonies *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y.A.</td>
<td>34F</td>
<td>M2</td>
<td>2.5 ± 0.7 48.0 ± 4.5</td>
</tr>
<tr>
<td>T.K.</td>
<td>65M</td>
<td>M2</td>
<td>1.0 ± 0 2.0 ± 1.0</td>
</tr>
<tr>
<td>K.K.</td>
<td>38M</td>
<td>M5</td>
<td>0 3.0 ± 0</td>
</tr>
<tr>
<td>H.M.</td>
<td>32F</td>
<td>L2</td>
<td>0 3.6 ± 0.6</td>
</tr>
</tbody>
</table>

*Mean ± SD/2 × 10^6 bone marrow cells.

†See Table 1.

‡Patient with ALL (blasts in bone marrow, 98.8%).

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![Survival curves for human L-CFU and CFU-c heated for varying lengths of time at different temperatures](image-url)
In general, leukemic marrows in humans contain normal clones, but these normal clones are usually suppressed by a leukemic cell population, showing almost no growth of CFU-c-derived colonies in vitro. However, after heating leukemic marrow cells in vitro, residual normal clones (CFU-c) appeared and formed more colonies than untreated marrow cells, indicating the possibility of a division delay of CFU-s, which mostly remain in the G_0 phase. This finding seems to be important for keeping the CFU-s population, which is essential to hematopoietic regeneration after bone marrow transplantation, from being killed while purging residual leukemic cells in stored bone marrow by heat.

From these and our previous results, we emphasize that the in vitro treatment of stored remission bone marrow with hyperthermia may selectively eradicate the L-CFU population before autologous bone marrow transplantation. Thus, in vitro hyperthermia may be a useful tool in clinical bone marrow transplantation for the treatment of human acute leukemia as an alternative to monoclonal antibody treatment for purging leukemic cells in vitro.

REFERENCES

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