ABSTRACT

The response of murine granulocyte-macrophage progenitor cells to hyperthermia was examined using normal and regenerating marrow. Hyperthermic exposure was given in vitro at 41–44 °C for periods of up to 60 min, and the results were compared between the 2 groups. Although almost no difference in percentage of survival was observed between them at 41 °C, murine granulocyte-macrophage progenitor cells of regenerating marrow showed markedly increased thermal sensitivity at and above 42 °C in comparison with that of normal marrow. Hydroxyurea suicide experiments revealed that the proportion of cells in S phase increased in the same order of CFU-S, CFU-C, BFU-E, and CFU-E. This implies a close relationship between stem cell differentiation pathway. On the other hand, it was also shown by hydroxyurea suicide experiments that the proportion of cells in S phase increased in the same order of CFU-S, CFU-C, BFU-E, and CFU-E. This implies a close relationship between the thermal sensitivity and the cell cycle characteristics of hematopoietic stem cells. The present study extends our investigation to regenerating marrow, in which stem cells in S phase are known to increase greatly.

INTRODUCTION

In recent years, there has been growing interest in the application of hyperthermia for the treatment of advanced cancers (13, 17, 18). Since the hyperthermic effect is not selective for malignant cells, due attention must be paid to the possible heat damage of normal tissues, especially those undergoing rapid division including the bone marrow. We have previously reported the effect of hyperthermia on normal hemopoiesis using stem cell assay techniques (12). In mice, pluripotent stem cells (CFU-S) were most heat resistant, and myeloid committed progenitors (CFU-C) were more heat resistant than were early erythroid progenitors (BFU-E). Late erythroid progenitors (CFU-E) were the least heat resistant. These results suggest increased thermal sensitivity along the stem cell differentiation pathway. On the other hand, it was also shown by hydroxyurea suicide experiments that the proportion of cells in S phase increased in the same order of CFU-S, CFU-C, BFU-E, and CFU-E. This implies a close relationship between the thermal sensitivity and the cell cycle characteristics of hematopoietic stem cells. The present study extends our investigation to regenerating marrow, in which stem cells in S phase are known to increase greatly.

MATERIALS AND METHODS

Mice. Female JCL/ICR mice (8 to 12 weeks old) were used throughout these experiments.

Preparation of Cell Suspensions from Normal Marrow. Bone marrow cells from mice were flushed from femurs or tibiae with a 25-gauge needle into α-medium (Flow Laboratories, McLean, VA). The cells were then dispersed by repeated pipettings until single-cell suspensions were obtained. The cell concentrations were adjusted to 1 x 10⁶ cells/ml.

Mice with Regenerating Marrow. Nucleated marrow cells (10⁶) were injected i.v. into syngeneic mice that had received 800 rads of irradiation from a 137Co source immediately prior to marrow transfusion. The femoral and tibial marrow cells from these mice were harvested 5 or 6 days later.

Preparation of Cell Suspensions from Regenerating Marrow. The lower limbs (femurs and tibiae) of the mice with regenerating marrow were extirpated and cut into pieces with a nail clipper in α-medium, and the marrow cells were washed out. The marrow cell suspensions were filtered through a stainless steel screen (100 mesh) and then dispersed by repeated pipettings until single-cell suspensions were obtained. The cell concentrations were adjusted to 1 x 10⁶ cells/ml.

Heating. Sealed glass tubes containing 1 ml of cell suspensions were exposed to heat in a circulating water bath (Model BT-46; Yamato Scientific Co., Tokyo, Japan), for 0, 5, 15, 30, and 60 min at 41–44 °C at 1 °C increments [temperature variation, ±0.1 °C (SD)]. Before heating, they were kept at room temperature (25°C); after immersion in the water bath, the temperature of the cell suspensions rose to the preset temperature ±0.1 °C in 90 s with a half-time of 15 s for the transition. Since freshly prepared medium was used in most experiments, the pH monitored in representative tubes was fairly constant (about 7.2) and showed little change during hyperthermia (ΔpH < 0.2). As soon as incubation was terminated, each glass tube was transferred to ice water. Stem cell assay was performed at the same time for all 5 specimens.

CFU-C Assay. CFU-C were assayed in methylcellulose by a modification of the technique first described by Iscove et al. (8). Marrow cells (10⁵) suspended in 1 ml of mixture containing α-medium, 0.8% methylcellulose, 20% fetal calf serum, and 5% peritoneal conditioned medium were plated in 35- x 10-mm Lux plastic dishes (Lab-Tek Division, Miles Laboratories, Naperville, IL). Peritoneal conditioned medium was prepared as a potent source of colony-stimulating activity from the 3-day culture supernatant of peritoneum obtained from mice 6 h after i.v. injection of 5 μg of lipopolysaccharide (Escherichia coli 026:B6; Difco Laboratories, Detroit, MI). The cultures were done in duplicate and incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in air. After 7 days, colonies containing more than 50 cells were counted under an inverted microscope.

BFU-E Assay. BFU-E were assayed in methylcellulose according to the method of Iscove et al. (9) with some modifications. Marrow cells (10⁵) suspended in 1 ml of mixture containing α-medium, 0.8% methylcellulose, 30% fetal calf serum, 1% deionized bovine serum albumin (Fraction V; Armour Subdivision, Nakarai Chemicals, Kyoto, Japan), 1.0 unit of the Step III preparation of erythropoietin per ml (Connaught Laboratories, Willowdale, Ontario, Canada), and α-thioglycerol at a final concentration of 1 x 10⁻⁴ M were plated in 35- x 10-mm plastic dishes. The cultures were done in duplicate and incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in air. After 7 days, erythroid bursts were counted using an inverted microscope.

CFU-E Assay. CFU-E were assayed in a plasma clot system according to the method of Stephenson et al. (22) with slight modifications. Cells (10⁵) were suspended in 1 ml of mixture containing α-medium, 20% fetal calf serum, 2% bovine embryo extract (Flow Laboratories, Stanmore,

1 Supported in part by Grant-in-Aid for Scientific Research No. 57480407 from the Ministry of Education, Japan.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CFU-S, spleen colony-forming units; CFU-C, in vitro colony-forming units; BFU-E, erythroid burst-forming units; CFU-E, erythroid colony-forming units.
Australia), 1% bovine serum albumin, erythropoietin (1.0 unit/ml), thrombin (1.0 unit/ml; Mochida Pharmaceutical Co., Tokyo, Japan), α-thioglycerol at a final concentration of 1 × 10⁻⁴ M, and 10% citrated bovine plasma. Following repeated pipettings, 0.4 ml of cell suspensions was plated in 35- x 10-mm plastic dishes. After clotting, 0.6 ml of α-medium was added around the plasma clot. The cultures were done in duplicate and incubated at 37 °C in a fully humidified atmosphere containing 5% CO₂ in air. After 2 days, plasma clots were fixed with glutaraldehyde and stained with benzidine. Cell aggregates containing 8 or more benzidine-positive cells were scored for colonies.

Hydroxyurea Suicide Experiment (14, 21). An equal volume of marrow cell suspensions was placed into 2 test tubes. Hydroxyurea (Sigma Chemical Co., St. Louis, MO) was added to one tube at a final concentration of 1 × 10⁻³ M, 0.9% NaCl solution was added to the control tube, and both tubes were incubated for 1 h at 37°C. After 3 washes with α-medium, stem cell assay was performed. The results were expressed as the percentage of the control values.

Statistical Analysis. The statistical significance of the difference of the thermal sensitivity between CFU-C of normal and regenerating marrow was determined by Student’s t test.

RESULTS

Proliferative State of CFU-C of Regenerating Marrow. CFU-C began to proliferate at Day 2 and reached a plateau at Day 9 (Chart 1). We used the Day 5 or Day 6 marrow cells for experiments because they were at an exponential phase of maximal CFU-C growth with a doubling time of about 20 h.

Chart 2 shows the results of hydroxyurea suicide experiments. The percentage of survival of CFU-C was 82 ± 8% in normal marrow and 34 ± 4% in regenerating marrow. Since hydroxyurea is considered to eliminate selectively the cells in S phase, the proportion of CFU-C in this phase was calculated to be 18 ± 8% in normal marrow and 66 ± 4% in regenerating marrow.

Effect of Hyperthermia on CFU-C of Normal and Regenerating Marrow. Chart 3 shows the results of hyperthermia on CFU-C of normal and regenerating marrow. At 41 °C, almost no difference of percentage of survival was observed between the 2 groups; however, at and above 42 °C, a significant difference (P < 0.05 to < 0.001) was seen (Table 1).

Effect of Hyperthermia and Hydroxyurea Treatment on CFU-C of Regenerating Marrow. In one experiment, regenerating marrow cells were treated with hydroxyurea and then exposed to hyperthermia at 42 °C for 60 min. As shown in Chart

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Table 1

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>5 min</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>42</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>43</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>44</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS, not significant.

4, the percentage of survival of CFU-C of regenerating marrow was 69 ± 10% in the hydroxyurea-treated group, whereas it was only 21 ± 9% in the non-hydroxyurea-treated group.

In another experiment, hyperthermic exposure was given to the regenerating marrow cells at 42 °C for 60 min, and these marrow cells were then treated with hydroxyurea. As shown in Chart 5, the percentage of survival of CFU-C of regenerating marrow was 97 ± 5% in the heated group, whereas it was only 34 ± 4% in the nonheated group.

Comparison of the Percentage of Survival among 4 Types of Stem Cells. Chart 6 shows the percentage of survival of 4 types of stem cells after hyperthermia at 41 and 42 °C. At 41 °C, no significant difference of the percentage of survival of CFU-C was observed between normal and regenerating marrow. However, at 42 °C, the percentage of survival of CFU-C of regenerating marrow decreased to the level of BFU-E of normal marrow, and further decreases were seen at 43 and 44 °C (Chart 7).

DISCUSSION

Although there have been many reports on the effect of hyperthermia on malignant cells (1, 6, 15, 20), a few papers to date have studied the heat-induced damage of normal hemo-

4. NS, not significant.

poiesis. Tribukait et al. (23) first reported the influence of hyperthermia on murine and human hemopoietic stem cells. Elkon et al. (4) showed temperature-dependent inhibition of murine stem cells. Zant et al. (25) found that murine erythroid progenitors are much more heat sensitive than are those of granulocyte-macrophage or megakaryocyte lineages. We have reported the in vitro effect of hyperthermia on normal hemopoietic stem cells (12). The thermal sensitivity of stem cells increased as the proportion of cells in S phase became greater (Table 2). Since S-phase cells are known to be the most sensitive to heat of all cell populations.
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Charts 7. Comparison of the percentage of survival among 4 types of stem cells after hyperthermia at 43 °C (A) and 44 °C (B). Each point represents the mean of 4 or more separate assays; bars, SD.

Table 2

Results of hydroxyurea suicide experiments for murine hemopoietic stem cells

<table>
<thead>
<tr>
<th></th>
<th>% of survival</th>
<th>% in S phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-S</td>
<td>83.0 ± 5.9°</td>
<td>7.0 ± 5.9</td>
</tr>
<tr>
<td>CFU-C</td>
<td>82.4 ± 7.7</td>
<td>17.8 ± 7.7</td>
</tr>
<tr>
<td>BFU-E</td>
<td>70.0 ± 11.7</td>
<td>30.0 ± 11.7</td>
</tr>
<tr>
<td>CFU-E</td>
<td>33.1 ± 6.1</td>
<td>66.9 ± 6.1</td>
</tr>
</tbody>
</table>

° Mean ± SD of 4 separate assays.
°° P < 0.05.
°°° P < 0.01.

(1, 2), this heat sensitization might be explained by cell cycle effects. In the present study, this relationship was examined by comparing regenerating marrow with normal marrow. The thermal sensitivity and the proportion of cells in S phase were greatly increased in CFU-C of regenerating marrow (Charts 2 and 3). These results suggested a direct cause-and-effect relationship between them.

Shortly before submitting this manuscript, Flentje et al. (5) reported experiments and results similar to ours, showing the increased thermal sensitivity of CFU-S in regenerating marrow. They suggested the importance of S-phase cells; however, the possibility that regenerating marrow cells might be more sensitive to heat irrespective of the position in cell cycle was not ruled out. To test this, we attempted to eliminate the cells in S phase by hydroxyurea treatment. Our results showed that the percentage of survival of CFU-C after hyperthermia (42 °C, 60 min) was much higher in hydroxyurea-treated regenerating marrow than in nontreated regenerating marrow (Chart 4). Therefore, it would appear that heat sensitization occurs because of cell cycle effects, when they apparently mean the relative proportion of cells in S phase.

Since about 30% of the cells in non-S phase were also damaged after 60 min heat exposure at 42 °C (Chart 4), the hyperthermic effect was not necessarily selective for the cells in S phase. In order to obtain a more direct evidence that the cells in S phase are more heat sensitive than are those in non-S phase, we exposed the regenerating marrow to heat at 42 °C for 60 min to remove the heat-sensitive cells (mostly in S phase) and then treated the remaining cells (mostly in non-S phase) with hydroxyurea. We found that 97 ± 5% of CFU-C survived after hyperthermic treatment of regenerating marrow which had been preexposed to heat (Chart 5). These results indicated that the cells in S phase are more heat sensitive than are those in non-S phase. The possibility that prior heat may be inhibiting DNA synthesis, thus preventing cell killing by hydroxyurea, may be excluded as follows. Prior heat (42 °C, 60 min) reduces the percentage of survival of CFU-C to 21 ± 9% (a) (Chart 4). On the other hand, 34 ± 4% (b) of CFU-C are in non-S phase (Chart 2), and 69 ± 10% (c) of these cells survive after hyperthermia (42 °C, 60 min) (Chart 4). The overall CFU-C in non-S phase surviving after prior heat are calculated by b x c, i.e., 24 ± 6% (d). Since these 2 data (a and d) are almost identical, it may be that CFU-C surviving after prior heat are mostly in non-S phase.

Although our results showed that the thermal sensitivity was directly related to the cell cycle, it is possible that factors other than the cell cycle are involved. Since our previous data (12) suggested the probable participation of differentiation in the thermal sensitivity of stem cells, we further examined these points. The proportion of cells in S phase was 65 ± 4% in CFU-C of regenerating marrow (Chart 2) and 67 ± 6% in CFU-E of normal marrow (Table 2). However, the percentage of survival of CFU-C of regenerating marrow showed temperature-dependent changes from the level of CFU-C (at 41 °C) to the level of CFU-E (at 44 °C) of normal marrow (Charts 6 and 7). Therefore, the thermal sensitivity is determined not by the cell cycle alone but also by the degree of commitment in terms of stem cell differentiation.

Many reports have been published about the kinetics of cell kill by hyperthermia (1, 2, 7, 16, 24), using tumor cell lines such as Chinese hamster ovary (24), L1210 (2), and HeLa cells (16). In these experiments, the selective removal of the mitotic cells from an asynchronous population clearly showed that the cells in S phase were more sensitive to hyperthermia. Our results further indicated that this relationship could be applied not only for malignant cells but also for normal hemopoietic stem cells. However, the majority of experiments have revealed that nonproliferating tumor cells with decreased proportions of cells in S phase may be more sensitive to heat than are those in active growth with increased proportions of cells in S phase. Hahn (7) found that HA-1 cells in plateau phase were much more sensitive to heat than were those in exponential growth phase. Bhuyan et al. (2) also showed, using L1210 cells in culture and L1210 ascites, that exposure to hyperthermia at 43°C for 60 min was more lethal to stationary-phase cells than to exponentially growing cells. These results cannot be explained on the basis of distribution of cells in different phases of the cell cycle. On the other hand, Power and Harris (19) and Douple (3) reported that hyperthermia killed exponentially growing Chinese hamster V79 cells more efficiently than it killed plateau-phase cells. Kase and Hahn (11) found the highest sensitivity in the exponential fraction of their transformed human fibroblast line. Our experiments showed increased sensitivity of CFU-C in exponential growth (regenerating marrow) than in stationary phase (normal marrow), and the identical result was also reported by Flentje et al. (5). The discrepancies concerning the heat sensitivity of cells at the different growth phases reported by others (2, 7) and our results remain unsettled. Although many factors may affect the heat...
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sensitivity of these partially synchronized populations, such as cell types used (20), environmental factors (especially nutrients) (7), and the methods used for achieving synchrony, further works are awaited to define the heat sensitivity of normal and neoplastic mammalian cells.

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Cell Cycle-dependent Heat Sensitization of Murine Granulocyte-Macrophage Progenitor Cells in Regenerating Marrow

Eiji Kobayashi, Morihisa Yamagishi, Takayuki Kamamoto, et al.


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