Temperature-dependent Inhibition of Murine Granulocyte-Monocyte Precursors

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ABSTRACT

The response of nucleated bone marrow cells from C3H mice to hyperthermic temperatures of 41.5 to 49.5° for a fixed heating period of 30 min has been determined. The threshold temperatures for cell lysis, loss of trypan blue exclusion, and histological evidence of cell injury were > 49.5°, 45.5° and 43.5°, respectively. Growth of mature granulocyte-monocytes from precursors was evaluated in Millipore diffusion chamber culture. There was a biphasic decrease in granulocyte-monocyte growth after exposure to temperatures of 41.5 to 45.5°. The ratio of granulocytes to monocytes in proliferated cultures was not altered after hyperthermia. Pluripotential and committed granuloid stem cells were assayed by the spleen colony technique. These also showed a biphasic decrease with increase in temperature, with committed stem cells having a greater thermal sensitivity at lower temperatures.

INTRODUCTION

In the past decade, there has been considerable interest in the use of hyperthermia to treat cancer. Elevated temperatures of 41 to 46° have been amply demonstrated to inhibit proliferation of malignant cells (8-10, 14). However, there has been conflicting evidence on the relative intrinsic thermosensitivity of malignant and normal tissues (2, 5, 6). Since there is a great variation in this sensitivity between different tissues (3, 11), the therapeutic ratio (i.e., relative sensitivity between normal tissues and malignant tumors) will depend on the specific tissues being tested.

Hyperthermic injury has been determined in a relatively limited range of normal tissues (3), and there is little information on the sensitivity of normal hematopoiesis. We report studies on the thermal sensitivity of murine hematopoietic bone marrow. Specifically, the heat sensitivity of nucleated bone marrow cells to cell lysis, trypan blue dye exclusion, and changes on light microscopy has been determined as well as the thermal inhibition of granulocyte-monocyte proliferation in Millipore diffusion chambers. In order to approximate conditions pertaining to the clinical application of hyperthermia, a standard heating period of 30 min has been used.

MATERIALS AND METHODS

Heating

The bone marrow suspension was heated in a constant-temperature water bath. The cell concentration during heating ranged between 6 x 10^3 and 1 x 10^4 in different experiments, and the cells were kept at relatively constant pH. There was no color change of the pH indicator (phenol red) in the medium during heating, and direct measurement showed a pH range of 7.0 to 7.6.

Temperature measurements in the bath were made with a mercury thermometer (National Bureau of Standards standardization), and the temperature of the bath was maintained to ±0.2°. Preliminary measurements showed that the temperature of the cell suspension was ±0.2° of the temperature of the bath in 3 to 4 min.

Isolation of Nucleated Hematopoietic Precursors

Bone marrow cell suspensions were obtained by flushing the marrow from the tibiae and femurs of 3 donor C3H mice (Flow Laboratories, Inc., Dublin, Va.). The medium was Eagle’s minimal essential medium with 15% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). After flushing, the cell suspension was agitated and flushed 3 times with a Pasteur pipet to break up cell clumps. It was allowed to stand for 5 min to sediment any tissue fragments, and the supernatant was used to prepare the final cell suspension.

Cell Lysis and Trypan Blue Exclusion Experiments

Preliminary experiments to determine nucleated bone marrow cell viability after hyperthermic exposure (41.5–49.5°) were performed as follows. Total nucleated cell counts of the final suspension were performed in duplicate with a hemocytometer. The cells were then exposed to 30 (or 60-min temperatures of 41.5–49.5°, while control cells were kept at 25° for the same time period. After hyperthermia, a total cell count was performed. Slides for differential counts on samples before and after heating were prepared with a cytocentrifuge and stained with Wright’s stain.

Trypan blue exclusion was performed by exposing the final cell suspension to 0.05% trypan blue for 2 min after hyperthermic exposure of 30 min at 41.5–49.5°. One hundred to 300 cells were scored, and the proportion of cells stained with trypan blue was determined.

Granulocyte-Monocyte Precursor Proliferation

Control Experiments. The proliferation of granulocyte-monocyte precursors in diffusion chambers was assessed by a modification of the method of Benestad and Reikvam (1). The diffusion chambers were made up to 2 Millipore filters (13-mm diameter; 0.22-µm pore size) cemented onto a plastic ring. Nucleated bone marrow cells (150,000) were introduced into the chamber and sealed with a nylon plug. The chambers were implanted into the peritoneal cavity of outbred ICR mice (Flow Laboratories, Inc.,). Two chambers were implanted per mouse. The recipient mice were pretreated with 500 rads whole-body radiation.
irradiation 24 hr prior to implantation of the chambers. The chambers were removed at intervals up to 7 days after implantation, and the cells were harvested by the method of Benestad and Reikvam (1) using a 0.05% pronase-5% Ficoll solution. The total nucleated cell counts in the chamber were obtained, and slides for differential counts were prepared. Occasional chambers with evidence of leakage or contamination were discarded. Six to 10 chambers were removed per day, and the mean ± S.E. of cell counts were calculated. Incubation of chambers filled only with culture medium demonstrated no ingress of cells from the peritoneal cavity into the chamber.

Hyperthermia Experiments. After isolation of nucleated hematopoietic cells, the cell suspension was divided into 2 parts (control and hyperthermia). The cells for hyperthermia were exposed to 30-min hyperthermia at 41.5 and 45.5°. Control cells were maintained at 25°. At the completion of each treatment, the cell suspension was counted, and 1.5 × 10^5 nucleated cells in 100 μl of suspension were inserted into diffusion chambers and incubated as described above. Six to 8 chambers were removed at Days 1, 2, 6, and 7, and the mean ± S.E. of cell counts were determined.

In order to assess cellular proliferation in the chamber, a proliferative index ΔH was defined such that ΔH = ΔP/ΔC, where ΔP was the ratio of the mean cell count on Day 7 to the mean cell count on Day 1 in heated samples; ΔC was a similar ratio for control chambers. Two to 4 experiments were performed at each temperature, and ΔH for each experiment was calculated.

Differential cell counts were obtained for each sample by pooling 6 to 8 chambers for each group on each day. One hundred to 300 cells were scored for the differential counts.

Stem Cell Assays. The thermal sensitivities of pluripotential and committed myeloid stem cells in the final cell suspension were done as follows. (a) Pluripotential stem cells (CFU-S) were measured by the spleen colony technique of Till and McCulloch (15). Recipient mice were pretreated with 700 rads whole-body irradiation 6 hr prior to injection of the cell suspension. Hyperthermia was administered in vitro, as in the diffusion chamber experiments; and 8 × 10^5 nucleated cells were injected into 8 to 10 recipient mice in each of 3 experiments. (b) Committed myeloid stem cells (CFU-DG) were measured in plasma clot diffusion chamber culture (13). The 1.5 × 10^5 nucleated cells were inserted into the chambers. Colonies comprised at least 20 myeloid precursors and were scored on Day 3. Recipient mice were preirradiated with 500 rads whole-body treatment. Duplicate hyperthermia experiments were performed at each temperature with 8 plasma clots per experiment.

RESULTS

There was no increase in nucleated bone marrow cell lysis after hyperthermia of 41.5–49.5° for a 30-min heating period. After 60 min of heat, a progressive increase in the proportion of cells undergoing lysis began at 47.5° (Chart 1). After a 60-min heating period, 8% of cells were lysed at temperatures of 47.5°, and 40% were lysed at 49.5°. At 45.5° for 30 min, a significant number of cells developed cell membrane changes, demonstrated by an increase in the proportion of cells staining with trypan blue dye, compared to cells at 25° (Chart 1). Eleven % of nucleated cells stained with trypan blue at 45.5°, and 20% stained at 47.5°.

Light microscopy of cytocentrifuged cell samples immediately after heating showed histological evidence of cellular injury starting at 43.5° for 30 min. There was clumping of the nuclear chromatin which was particularly evident in segmented granulocytes where segmented nuclei coalesced. The nuclear to cytoplasmic ratio appeared to decrease. These changes are illustrated in Fig. 1.

Granulocyte-monocyte precursor proliferation in diffusion
chamber cultures from control experiments is shown in Chart 2. There was an initial decrease in total nucleated cell number on Day 1, followed by an increase of 3.5 to 4.8 times the initial cell number by Day 7. The cells on Day 7 mainly comprised mature granulocytes and monocyte-histiocytes.

After the cells were exposed to temperatures of 41.5–45.5°C for 30 min, there was a decrease in cellular proliferation at Day 7 compared to controls (Chart 3). Because of some variation in proliferation in control experiments, representative heating experiments where control proliferation was similar have been shown in Chart 3. The decrease in cell number at Day 1 was similar for temperatures of 41.5–44.5°C. There was a greater reduction in cell number at this time after 45.5°C.

In Chart 4, the proliferative index (ΔH) has been plotted against heating temperature. There was a biphasic decrease in the relative proliferative index (ΔH) with increasing temperature. For each 1°C rise in temperature, the fractional reduction in ΔH was 0.3 and 0.8 above and below 43.5°C, respectively.

The differential cell counts at Day 7 demonstrated no change in the ratio of mature granulocytes to monocyte-histiocytes after heating (Table 1). The percentages of these cells in the hyperthermia experiments are shown in Table 1.

In Chart 5, the survival of CFU-S and CFU-DG was plotted against heating temperature for 30 min. There was a biphasic reduction of both stem cell elements with a more rapid decrease in the surviving fraction at temperatures above 42.5°C. At temperatures above 42.5°C with each 1°C rise in temperature, CFU-S and CFU-DG decreased by 0.85 and 0.75, respectively.

- **DISCUSSION**

Few previous reports have documented the thermal sensitivity of hematopoietic precursors. Klein et al. (7) showed no inhibition of erythroid colony-forming units and burst-forming units after exposure to 41.5°C for 60 min. Tribukait et al. (16) reported heating time-dependent inhibition of the murine pluripotential stem cell CFU-S after heating to temperatures between 37 and 43°C.

In the present study, we have demonstrated the temperature-dependent (for a fixed heating period of 30 min) inhibition of hematopoietic bone marrow. A significant number of nucleated cells develop membrane lesions after heating to 45.5°C. At this
The rate of cell death is greater for CFU-DG than CFU-S at lower temperatures.

The progeny of hyperthermic diffusion chamber cultures have a normal microscopic appearance, and the ratio of mature granulocytes to monocyte-histiocytes is similar to that of control cultures. These observations suggest that the precursors differentiate normally after hyperthermia. However, more precise evaluation of mature cell function will be required to determine whether differentiation is proceeding normally.

The present study does provide evidence that hyperthermia causes changes in both the histological appearance and precursor proliferation of hematopoietic bone marrow cells at relatively low levels of thermal energy input, and care should be exercised when large volumes of bone marrow are included in the therapy of malignant tumors.

REFERENCES

Fig. 1. Photomicrographs of murine bone marrow subjected to hyperthermia of 41.5° for 30 min (A), 43.5° for 30 min (B), 49.5° for 30 min (C), and unheated bone marrow (D).
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