Feasibility of hyperthermia as a purging modality in autologous bone marrow transplantation
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CHAPTER FIVE

Studies on the Hyperthermic Sensitivity of the Murine Hematopoietic Stem Cell Compartment. II. Heat Effect on Donor Stem Cells With Long-Term Repopulating Ability

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ABSTRACT

Variations in hyperthermic sensitivity among different hematopoietic progenitor and stem cell populations of the bone marrow have been previously described for clonogenic subsets responsible for short-term hematopoiesis. However, less is known of the heat sensitivity of more primitive stem cells capable of long-term repopulation in irradiated recipients. In the present study, control and heat-treated (60 minutes 43°C) donor bone marrow cells from congenic B6-Gpi-1α mice were transplanted at different cell doses (10^4, 10^5, 10^6 and 10^7 nucleated cells) in pre-irradiated (6 Gy) B6-Gpi-1β mice. The development and levels of donor marrow engraftment was determined from blood Gpi phenotyping and the bone marrow dose required for equivalent long-term engraftment at 20 weeks provided an estimate of the surviving fraction corresponding to primitive stem cells of long-term repopulating ability (LTRA). Comparison with previous bone marrow cell survival values demonstrates that LTRA cells are less sensitive to hyperthermic treatment than other hematopoietic subsets confirming a relationship between the heat sensitivity and the hierarchical structure of the hematopoietic stem cell compartment.
INTRODUCTION

In the ex vivo treatment of hematopoietic malignancies, an effective purging protocol requires not only the elimination of leukemic cells from the bone marrow transplant but also the survival of a sufficient number of normal hematopoietic progenitors and stem cells. This is particularly important for the reconstitution of the hematopoietic system in recipients aggressively pretreated with chemotherapy and/or radiotherapy. Existing data on the differential heat sensitivity of normal and malignant hematopoietic cells has encouraged attempts to apply hyperthermia as a potential purging agent in the removal of leukemic cells from the donor bone marrow inoculum prior to autologous transplantation (Symonds et al., 1981; Robins et al., 1983; Flentje et al., 1984; Da et al., 1989; Murphy and Richman, 1989; Gidali et al., 1990; Iwasawa et al., 1991; Moriyama et al., 1992). Of the studies that have focused on the normal hematopoietic stem cell compartment, most concern the hyperthermic effect on the committed progenitor pool (Elkon et al., 1981; Van Zant et al., 1983; Wang et al., 1985; Baeza et al., 1989; O’Hara et al., 1990). It is now widely appreciated that the short-term hematopoietic recovery after bone marrow transplantation is dependent on the number of progenitors (CFU-S, CFU-GM, BFU-E and CFU-E) in the transplant while the more primitive stem cells with extensive proliferative potential are responsible for the long-term reconstitution of the hematopoietic system (Ploemacher and Brons, 1989; Jones et al., 1990). Thus, in order to investigate the possible use of hyperthermia as a purging agent in the treatment of leukemia, it is important to characterize the hyperthermic sensitivity of all the subsets within the hematopoietic stem cell compartment. Studies on human bone marrow are limited by the fact that no functional assays are yet available for the primitive subsets responsible for maintained hematopoietic engraftment. Bone marrow chimera models in mice, however, offer us the possibility to study effects on these primitive subsets of the hematopoietic stem cell compartment on the basis of long-term competition between congenically marked marrow cells (Harrison et al., 1988; Down et al., 1991).

In previous studies from our laboratory the hyperthermic characteristics of the clonogenic subsets of the murine hematopoietic stem cell compartment were reported (Wierenga and Konings, 1990, 1991, 1993). It was demonstrated that a relationship exists between susceptibility towards hyperthermic cell killing and the position of cells within the hematopoietic hierarchy. Here progression of early clonogenic stem cells with marrow repopulating ability (MRA) towards the differentiated progenitors was accompanied by an increase in hyperthermic sensitivity. In the present study, the hyperthermic sensitivity of cells with long-term repopulating ability (LTRA) in vivo was investigated in a congenic murine bone marrow transplant model. Based on our
previous studies (Wierenga and Konings, 1990, 1991, 1993), 60 minutes at 43°C was chosen as an appropriate time-temperature treatment that shows a wide variation in surviving fractions ranging from 30% for MRA down to 2% for CFU-E. The aim of this study was to see whether the relationship between thermal sensitivity and stem cell hierarchy could be extended to include data on primitive hematopoietic stem cells.

**MATERIALS AND METHODS**

*Mice.* Throughout this study female C57Bl/6-Gpi-1/Gpi-1 (B6-Gpi-1) mice were used as recipients and female congenic C57Bl/6-Gpi-1a/Gpi-1a (B6-Gpi-1a) mice as donors. The recipient mice were bred under SPF conditions in the Central Animal Unit of the Faculty of Medicine, University Groningen. The congenic mouse strain was originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and then bred in the animal facility of the Department of Radiobiology. All mice were maintained free of pathogenic viruses (Sendai, MHV, PVM, GD, VII, REO III, EMC, LMC, MVM, K and Mycoplasma pulmonis) in clean conventional rooms in our own animal facility. The mice were fed *ad libitum* with food pellets and acidified water (pH = 2.8).

*Hyperthermic treatment of bone marrow cells.* A suspension of bone marrow cells was harvested from the femora and tibiae of B6-Gpi-1a donor mice by grinding the cleaned bones in a mortar using alpha-medium (GIBCO Europe, Paisley, Scotland) buffered with 20 mM morpholinopropane sulphonic acid (Janssen Chimica, Beerse, Belgium) and in the presence of 5% fetal calf serum (GIBCO Europe, Paisley, Scotland). The cell suspension was filtered and counted on a Coulter Counter Model ZF (Coulter Electronics, Hialeah, FL, USA). Bone marrow cells were placed in tightly capped tubes (Greiner Labortechnik, Nurtingen, Germany) at a concentration of 2-3x10⁶/ml. The tubes were immersed in a precision waterbath (Julabo Labortechnik, Seelbach, Germany) maintained at the required temperature within 0.1°C. The tubes were gently shaken during the incubation. The hyperthermic treatment was interrupted by placing the cell suspension on ice. Control and heat-treated cells were diluted with alpha-medium plus 5% fetal calf serum to the appropriate concentration and injected intravenously in pre-irradiated mice at the different cell doses for the chimerism assay (see below).

*Chimerism assay.* The donor engraftment assay was performed according to the technique of van Os *et al.* (1992). Recipient mice were irradiated at a dose of 6 Gy with a ⁶⁰Co-source (Siemens Gammatron 3 unit) at a dose rate of 45 cGy/min. This radiation dose ensures long-term survival of all recipient mice regardless of the amount of transplanted viable donor marrow cells. Control and heat-treated cell
susensions were injected via the orbital vein under halothane anesthesia within 3-6 hours after irradiation into different bone marrow dose groups consisting of 5 mice. At the indicated time intervals after transplantation, blood was collected by bleeding 25 µl from the orbital vein and the percentage of donor Gpi-1<sup>+</sup> erythrocytes was determined as previously described (Wierenga and Konings, 1993).

**Statistical analysis.** Dose-response relationships for donor bone marrow cell dose and the levels of donor erythroid chimerism were fitted by least squares linear regression analysis following logit transformation of percent (P) engraftment (logit P = ln (P/100-P) and logarithmic transformation of bone marrow cell input. In addition, the 95% confidence limits were estimated. The comparison of the two regression lines given by the control and heat-treated group was performed using the parallel line assay (Woodward, 1980).

**RESULTS**

The development of erythroid donor engraftment up to 20 weeks after transplantation is shown in Figure 5.1 for each bone marrow dose and treatment group. Control bone marrow provided moderate levels of early engraftment during the

![Figure 5.1. Development of erythroid engraftment of control and heat-treated (HT) bone marrow cells transplanted at different cell doses. Data points are for 10 mice pooled from two separate experiments. Error bars represent ±1 SEM.](image-url)
first two months. Sustained chimerism was achieved at doses of $10^6$ and $10^7$ nucleated cells while at the lower cell doses of $10^4$ and $10^5$ the donor component subsided during the later post-transplant period. Heat-treated bone marrow exhibited less engraftment at all cell doses and at all time points after transplantation with no evidence for an early wave of engraftment. Hence the difference between control and heat-treated groups appeared more marked for short-term than long-term engraftment.

An estimate of the cell surviving fraction remaining after a hyperthermic treatment can be obtained from dose-response curves with respect to control and heat-treated bone marrow dose. Such an estimation is important for comparison with the clonogenic subsets of the hematopoietic stem cell compartment. In Figure 5.2, these logit-fitted dose-response curves are displayed at the early and late times of 8 and 20 weeks post-transplant. During the period of early donor chimerism at 8 weeks post-transplant the slope as well as the position of the regression lines from control and heat-treated dose-response curves differs appreciably and therefore complicates any calculation of the surviving fraction of the cells responsible for the short-term engraftment. At 20 weeks post-transplant the dose-response curve for the hyperthermic treatment was closer to control and shared a similar slope. Thus, the difference in bone marrow dose that separate these curves appears independent of

**Figure 5.2.** Logit regression analysis on bone marrow cell dose-response curves for engraftment 8 weeks and 20 weeks after transplantation of normal and hyperthermic treated bone marrow cells. C= control bone marrow, HT= heat-treated bone marrow. 95% confidence limits are presented at the level of 30% donor engraftment. Data points are for 15 mice pooled from three separate experiments. Error bars represent ±1 SEM.
the measured engraftment levels. At a given iso-effect level, the number of surviving LTRA cells present in the bone marrow graft should theoretically be the same. The cell dose ratio then measures the surviving fraction of the LTRA remaining after the hyperthermic treatment. The surviving fraction was calculated to be 34.3% (with 95% confidence limits of 32.1% and 36.7%) after a hyperthermic treatment of 60 minutes at 43°C.

**DISCUSSION**

Concepts revolving around the hierarchical organization of functionally distinct hematopoietic cell populations, whether they be for those remaining in the host after intensive conditioning therapy or for those present in the donor bone marrow inoculum after ex-vivo manipulations, are now becoming central to the role that bone marrow transplantation plays in the treatment of various genetic and malignant diseases. The timing and magnitude of blood chimerism using the Gpi-1 congenic marker to distinguish donor from host cells in mice have recently been compared to the frequency of hematopoietic subpopulations as defined using both in vitro and in vivo clonogenic assays (Down and Ploemacher, 1993). These studies have demonstrated that while progenitor and CFU-S-like subsets govern the early period of donor engraftment, a separate primitive pre-CFU-S stem cell population dictates the long-term fate of the marrow graft. In the present study, hyperthermic treatment of donor bone marrow prior to transplant had the most profound effect on short-term engraftment potential. As stated before, the calculation of an unique value of the surviving fraction of the cells responsible for the short-term engraftment is complicated because of the different slope of the two dose-response curves (see Figure 5.2a). At engraftment levels ranging from 50% to 10%, the calculated values vary between 8% and 1%, respectively. These surviving fractions, however, are consistent with the highest thermal sensitivity previously found for hematopoietic progenitor cell populations (Wierenga and Konings, 1993).

More valuable information can be obtained from the lasting supply of donor blood cells originating from pluripotent stem cells of high self-renewal. Although in this study chimerism is determined in erythrocytes, there is a good correlation between erythroid chimerism and long-term engraftment in other tissues such as bone marrow, spleen and thymus (Harrison et al., 1988; Van Os et al., 1992; Down and Ploemacher, 1993; Van Os et al., 1993). It appears that bone marrow dose-response relationships are best resolved at times that are late enough for clearance of mature host cells and emergence of circulating blood cells that arise from steady-state competition between donor and host LTRA stem cells. Displacement of the bone marrow cell dose-response
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dimensions: 595.0x842.0

curve reflects the amount of hyperthermic cell killing and the calculated surviving fraction of 34% allows comparisons with other treatments and other endpoints. In our previous study, the relationship between the heat sensitivity and the hierarchical structure of the clonogenic subsets within the hematopoietic stem cell compartment was demonstrated (Wierenga and Konings, 1993). Cell survival data for each subset and for the same heat treatment (60 minutes at 43°C) are summarized in Table 5.1 in an ascendency of primitiveness. A relationship with proliferative activity as measured using hydroxyurea cell kill is also shown. The 34% survival obtained in our present study is indeed similar to that previously reported for MRA, the primitive subset that appears closely related to the LTRA cells. The higher thermal sensitivity of the committed hematopoietic descendants follows an order of increased cell cycling. MRA was found to be very resistant to hydroxyurea-killing (Wierenga and Konings, 1993) and hence the slow or resting cycling state of the primitive stem cell population as demonstrated by others (Hodgson et al., 1982; Ross et al., 1982; Harrison and Lerner, 1991) may underlie the principle reason for their resistance to hyperthermia.

Table 5.1. Relationships between heat sensitivity and proliferative activity of the different subsets in the hematopoietic cell hierarchy.

<table>
<thead>
<tr>
<th>Subset</th>
<th>% survival</th>
<th>% cells in S-phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTRA</td>
<td>34 ± 2</td>
<td>n.d.</td>
</tr>
<tr>
<td>MRA</td>
<td>29 ± 7</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>CFU-S-12</td>
<td>15 ± 3</td>
<td>11.0 ± 3.7</td>
</tr>
<tr>
<td>CFU-S-8</td>
<td>3.5 ± 1.5</td>
<td>29.3 ± 4.5</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>3.7 ± 0.4</td>
<td>28.5 ± 0.7</td>
</tr>
<tr>
<td>BFU-E</td>
<td>2.2 ± 0.3</td>
<td>35.0 ± 4.2</td>
</tr>
<tr>
<td>CFU-E</td>
<td>1.8 ± 0.7</td>
<td>54.3 ± 9.9</td>
</tr>
</tbody>
</table>

* = subsets are placed in order of hierarchy
** = % survival after 60 minutes at 43°C are given ± 1 SEM
*** = data from hydroxyures-kill experiments (Wierenga and Konings, 1993)
n.d. = not determined in this study

It can be concluded that the LTRA is, together with the MRA, the most heat resistant subset of the hematopoietic stem cell compartment. Differential heat effects on donor stem cell subsets capable of short- or long-term engraftment in irradiated recipients confirm the relationship between the hyperthermic sensitivity and the hierarchical structure within the hematopoietic stem cell compartment based on differences in proliferative activity. Although this conclusion may seem encouraging with respect to the long-term engraftment potential of hyperthermic purged marrow transplant, we have to bear in mind that another important requirement is its provision for short-term hematopoiesis arising from proliferating progenitor cell populations. The
high heat sensitivity of these cell types could therefore extenuate the acute period of pancytopenia and become the limiting factor in attempts to safely eradicate malignant cells, if present in the transplanted marrow inoculum. Problems related to depletion of critical proliferating hematopoietic cells may similarly arise following transplant of bone marrow grafts purged with certain cytostatic drugs (Emminger et al., 1989; Rowley et al., 1989, 1991). Experiments are now in progress in our laboratory to explore new ex-vivo purging modalities that encompass strategies aimed at moderating the cycling activity of normal hematopoietic progenitors and conferring the type of heat resistance shared by quiescent primitive stem cell subsets.