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

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1997

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER TWO

Effect of a Hyperthermic Treatment on the Pluripotent Hematopoietic Stem Cell in Normal and Anemic Mice

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From: Int. J. Hyperthermia (1990) 6:793-800
ABSTRACT

Up to now, the hyperthermic sensitivity of pluripotent hematopoietic stem cells is unknown, and the few existing data from reports in the literature are conflicting. There are two main drawbacks in the set-up of those studies: 1) only CFU-S-day 8 results were presented, whereas it is questionable if this assay gives a true reflection of the pluripotent stem cell, and 2) no attention has been paid to heat effects on the seeding efficiency, i.e. the amount of stem cells which will lodge in the spleen. The present study focused on the procedural differences and compared the results of a hyperthermic treatment (60 minutes at 42°C) on the stem cells, assayed with the CFU-S-day 8 and the CFU-S-day 12 method, using the following three stem cell suspensions, all differing in their proliferative activity: bone marrow from normal mice and bone marrow and spleen cells from anemic mice. Furthermore, we investigated the seeding efficiency before and after the heat treatment. Resting stem cells, assayed with the CFU-S-day 12 method, turned out to be resistant to hyperthermia as compared with the active cycling stem cells, while with the CFU-S-day 8 assay the stem cells showed the same thermosensitivity in the two bone marrow suspensions. The active cycling stem cells do not significantly differ in thermosensitivity, in CFU-S-day 8 and day 12 assays, although there is a difference between bone marrow and spleen. Hyperthermia appears to influence the seeding efficiency for spleen CFU-S; an increase of 1.73 was observed. The difference in heat sensitivity between the resting and the active cycling stem cells, assayed with both in vivo methods, however, cannot be explained by a change in the seeding efficiency only. Comparing the amount of cycling cells in the three stem cell suspensions and their thermosensitivity leads to the conclusion that the differences in heat sensitivity might be fully explained by the cycling status of the stem cell.
INTRODUCTION

Blood cell formation depends on the proliferation and differentiation of hematopoietic pluripotent stem cells. Because of the dependence of all the different hematopoietic cell lineages on this stem cell the hyperthermic effect on the pluripotent hematopoietic stem cell is of great interest. There have been some studies on the effect of heat on the hematopoietic stem cell compartment. The main purpose of those studies was to investigate the possibility of purging the bone marrow from leukemic cells with the aid of hyperthermia in order to make the marrow feasible for autologous transplantation (Symonds et al., 1981; Robins et al., 1983; Flentje et al., 1984; Moriyama et al., 1986; Okamoto et al., 1988; Robins et al., 1988;). Furthermore this type of studies is important because the hematopoietic stem cell compartment may be the limiting normal tissue during cancer treatment with combined modalities including hyperthermia. Up to now, the results of the different heat treatments are not unanimous in elucidating the thermosensitivity of the resting and active cycling hematopoietic stem cells.

Pluripotent hematopoietic stem cells are in general assayed by the CFU-S (Colony Forming Unit in the Spleen) method (Till and McCulloch, 1961). To translate the CFU-S values into pluripotent hematopoietic stem cell numbers the fraction of stem cells lodging in the spleen must be known: the seeding efficiency expressed as the \( f \)-factor. One has to be aware of a possible change of the seeding efficiency due to the hyperthermic treatment, and as a consequence of its implications on the outcome of the experiments. In the hyperthermic studies on bone marrow cells performed up to now, little or no attention has been paid to the seeding efficiency.

Furthermore, in all reports published, the effect of heat was studied with the CFU-S-day 8 (CFU-S-8) assay. It is questionable however, if the colony appearing at day 8 after transplantation is a real reflection of the pluripotent hematopoietic stem cell (Magli et al., 1982; Harris et al., 1984; Priestly and Wolf, 1985). Instead of the CFU-S-8 assay, which probably detects committed stem cells, it is claimed in those studies that the CFU-S-day 12 (CFU-S-12) assay is a better method to measure the pluripotent hematopoietic stem cell.

The aim of the present study was to address these issues in the light of clarifying the effect of heat on resting and active cycling pluripotent hematopoietic stem cells. We determined the seeding efficiency before and after the heat treatment and investigated possible differences when using the CFU-S-8 or CFU-S-12 assay.
MATERIALS AND METHODS

Mice. Male and female C57Bl mice (9-12 wk) were used throughout the study and were obtained from the Central Animal Facility of the Medical Faculty, State University Groningen. The mice were bred under SPF conditions and kept in clean conventional rooms during the experiments.

Cells. Normal bone marrow cells were isolated by flushing the two femora of untreated mice with alpha-medium (GIBCO Laboratories, Grand Islands, NY, USA) buffered with 20 mM morpholinopropane sulfonic acid (Janssen Chimica, Beerse, Belgium). The medium was supplemented with 10% fetal calf serum (Hy Clone, Logan, UT, USA).

Bone marrow cells from anemic mice were isolated as described for the normal untreated mice. The mice were made anemic by bleeding them on three consecutive days by orbital puncture (0.5 ml per day). Four days after starting the bleeding procedure, cells were harvested.

The isolation of spleen cells was the same as previously described (Nijhof et al., 1982). Briefly, mice were treated with thiamphenicol for 5 days via a subcutaneously implanted dialysis bag. The mice were bled on 2 consecutive days as described above. Three days after removal of the drug the spleen was squeezed through a fine mesh metal screen into 3 ml of alpha-medium. As a result of the thiamphenicol treatment, hematopoiesis is highly stimulated in the spleens of the mice.

All three cell suspensions were dispersed through a 21 Gauge needle. Cell counts were performed on a Coulter Counter - Model ZF (Coulter Electronics Inc., Hialeah, FL, USA).

Heat Treatment. The isolated cells were heated at a cell concentration of 2-3 x 10^7/ml in the same medium as listed to prepare the cell suspensions. The hyperthermic treatment was performed in a precision water bath, maintained at the desired temperature within 0.1 °C. After the heat treatment, cells were put on ice and diluted to the appropriate cell concentration for the CFU-S assay.

Spleen Colony Assay. To determine the amount of pluripotent hematopoietic stem cells the spleen colony assay was used (Till and McCulloch, 1961). The recipient mice were irradiated with a dose of 9 Gy by an X-ray machine (Philips MG 300, 200 kV, 15 mA, 0.5 mm Al, 0.5 mm Cu, 0.5 Gy.min^-1). For each series of experiments the following procedure was used. Control and heated cell suspensions were intravenously (orbital) injected in a group of 20 mice, 24 hours after irradiation. After eight days, 10 of the mice were killed by cervical dislocation and the spleens were fixed in
Bouin's solution. The other 10 mice were sacrificed after 12 days and their spleens were treated as the day 8 group. The number of macroscopic colonies on the surface of each spleen was counted. The endogenous spleen colonies turned out to be zero as a result of the relatively high X-ray dose used.

**Seeding Efficiency Assay.** The seeding efficiency (f-factor) was measured with the double-transplant technique (Siminovitch, 1963). All recipients received a 9 Gy X-ray dose. As for the spleen colony assay, it was decided to irradiate the recipients 24 hours before the transplantation. This procedure was chosen, because of the fact that the f-factor should be determined from the number of CFU-S recoverable from fully shrunken spleens (Lord and Hendry, 1973). After an irradiation dose of >8.5 Gy the shrinkage of the spleen is completed within 24 hours (Testa et al., 1972; Monette and DeMello, 1979). The first transplant consisted of 500-1000 times the number of cells injected into a parallel series of mice, according to the method mentioned in the previous section. The amount of CFU-S, which will lodge in the spleen of the recipient, reaches a plateau 2-3 hours after injection and then remain constant afterwards (Testa et al., 1972).

![Schematic representation of the Seeding Efficiency Assay.](image)

So, after a time interval of 2-3 hours the spleen was removed and dispersed to form a single cell suspension. The second transplant consisted of a known number of this spleen cell suspension (between 5-10% of the total spleen mass per mouse) into a group of irradiated recipients in order to determine the CFU-S content. It should be noted that in the double transplant technique it is assumed that the f-factor in both transplants is the same (Hendry, 1971). Figure 2.1 shows the method for the determination of the seeding factor.
RESULTS

It was decided to give hyperthermic treatments at 42°C for the sake of comparison, because this temperature was used in earlier reports revealing thermosensitivity differences between resting and regenerating bone marrow cells (Flentje et al., 1984; Baeza et al., 1987). Since all the studies reported up to now used the CFU-S-8 values to determine the heat sensitivity of the hematopoietic stem cell, we also employed this assay and compared the results with those obtained from the CFU-S-12 experiments. Figure 2.2 shows the results of the hyperthermic treatment on stem cells when the CFU-S-8 and CFU-S-12 are compared. In normal bone marrow we found a surviving fraction of 49 ± 4% for the CFU-S-8, comparable to the bone marrow from anemic mice showing a survival of 52 ± 6%. The surviving fraction of the spleen cells from anemic mice turned out to be 24 ± 1%. The incubation of cells for 60 minutes at 37°C has no effect on cell survival (data not shown). As can be seen, the heat effect on the CFU-S-12 is not significantly different from the CFU-S-8 as far as the anaemic bone marrow and spleen cells are concerned: 62 ± 1% and 28 ± 3%, respectively. For normal bone marrow, however, there is a significant difference detectable between the two ways of assaying heat sensitivity, i.e. 98 ± 4% versus 49 ± 4% when CFU-S-12 and CFU-S-8 is compared.

Figure 2.2. The effect of a hyperthermic treatment (60 minutes at 42°C) on CFU-S-8 and CFU-S-12. Hyperthermia was performed as described in Materials and Methods (n=3). The results are related to the surviving fraction of the untreated cells (=100%). Bars=SD. Statistical analysis: student t-test, p<0.05.

A change in seeding efficiency (f-factor) of the transplanted CFU-S, due to the heat treatment, would lead to a misinterpretation of the obtained data. To examine this change, if any, we determined the f-factor before and after the heat treatment. As can be seen in Figure 2.3, there is no significant change in f-factor detectable in normal and anemic bone marrow CFU-S, whereas the spleen CFU-S demonstrated an increase of 1.73 compared with the untreated spleen CFU-S. These data appear to be
the same for the CFU-S-8 and CFU-S-12 assays. The main reason for determining the seeding efficiency is the need to estimate the amount of pluripotent hematopoietic stem cells in the used cell suspensions. Adjusting the CFU-S-12 values in Figure 2.2 for the $f$-factor results in the following surviving fraction of the pluripotent hematopoietic stem cell in normal bone marrow, anemic bone marrow and anemic spleen: 100±13%, 59±8% and 15±3%, respectively. It is clear from these data that besides a

![Change in f-factor (untreated=1.0)](image)

**Figure 2.3.** Change in seeding efficiency after a hyperthermic treatment (60 minutes at 42°C). The change in seeding efficiency is expressed as a factor, related to the seeding efficiency of the untreated cells. Bars= SD. Statistical analysis: see Figure 2.2.

difference in heat sensitivity between pluripotent hematopoietic stem cells under normal and anemic condition, there is also a significant difference in thermal effect in the two anemic situations. This difference might be caused by a difference of proliferative activity of the cells in suspension.

In a previous study we determined the percentage of CFU-S present in the S-phase in different cell suspensions using a ‘thymidine-suicide’ technique (Nijhof and Wierenga, 1985). In normal bone marrow only 8 ± 0.1% of the CFU-S is killed by the $^3$H-thymidine incorporation. This value increases to 34 ± 8% when the mice are made anaemic. In the ex-TAP spleens the thymidine kill even reaches up to 65 ± 4%. Figure 2.4 shows the relation between thymidine kill and the surviving fraction after the heat treatment. It is obvious that there is a strong correlation ($r=0.9995$) between the decrease in survival and the increase in percentage S-phase cells in the stem cell suspensions.
DISCUSSION

Whereas S-phase cells are more radioresistant than cells in the other phases of the cell cycle, the S-phase and the M-phase appear to be the most heat-sensitive stages of the cell cycle (Dewey et al., 1977; Hahn, 1982). If the cycle state is a factor in explaining the difference in thermosensitivity one would expect differences between resting and active cycling hematopoietic stem cells. In reports in which the hematopoietic stem cell compartment was used as a model for studying normal tissue damage in hyperthermia, results are conflicting. It was observed that active cycling hematopoietic stem cells in the bone marrow are more sensitive to a heat treatment at 42°C than the resting stem cell (Flentje et al., 1984) while this difference was not observed by others (Baeza et al., 1987). At 43°C no difference in heat sensitivity was found between proliferating and resting bone marrow stem cells (Wang et al., 1985). In contrast to the situation in bone marrow, Baeza et al. (1987) did find a difference between active cycling and resting stem cells in the spleen.

There is substantial evidence at the moment that, instead of the CFU-S-8 cells, CFU-S-12 cells are derived from the pluripotent stem cell, at least in normal bone

\[ \text{Surviving fraction (\%)} \]

\[ \text{Thymidine-kill (\%)} \]

Figure 2.4. The relationship between heat sensitivity (surviving fraction) and proliferative state (thymidine kill) of the pluripotent hematopoietic stem cells from bone marrow of normal mice (□), bone marrow from anaemic mice (■) and from spleens of anaemic mice (●).
marrow. Magli et al. (1982) observed that 7-9 days after the transplantation recognizable cells of one hematopoietic lineage were present in the colonies, whereas at day 14 most of the spleen colonies contained cells of more than one hematopoietic cell line. Harris et al. (1984) reported antigenic differences between CFU-S-8 and CFU-S-day 14 cells. Priestly and Wolf (1985) used a surgical technique which enabled them to follow the colony development of the CFU-S from the moment of transplantation until day 14. They found that the CFU-S-12 is a late-appearing colony, not predominantly present at day 8. Data from Molineux et al. (1986) show no persistence of colonies from day 7-9 to day 12-14. In their paper it is also shown that the CFU-S-12 colonies are novel colonies appearing between day 8 and 11 after injection. All these studies suggest that only when colonies are scored between days 11-14, the CFU-S technique provides a measure of the pluripotent hematopoietic stem cell and spleen colonies between day 7-9 are assumed to be representative of committed stem cells. Our results (Figure 2.2) indicate a difference in heat sensitivity between CFU-S-8 and CFU-S-12 in normal bone marrow. This can be explained by the fact that in normal bone marrow the CFU-S-8 is already active proliferating (Shibagaki et al., 1986), in contrast to the CFU-S-12. In the anemic bone marrow we did not find a significant difference in heat sensitivity between CFU-S-day 8 and CFU-S-12. Because of the hematopoietic pressure in the anemic situation, the resting CFU-S-12 will, like the CFU-S-8, become active proliferating and as a consequence more heat-sensitive.

If it is true, as stated by Magli et al. (1982) and Harris et al. (1984), that in normal bone marrow the CFU-S-8 assay appears to detect the BFU-E or CFU-GM, it is interesting to compare our results with those of others, performed on the committed hematopoietic stem cell compartment (Bromer et al., 1982; Elkon et al., 1981; Mivechi and Li, 1986; Van Zant et al., 1983)). In those studies the surviving fraction of the committed stem cells decreases to values comparable to our CFU-S-8 results. In a recent paper by Goris et al. (1989) it is shown that in normal bone marrow the committed stem cells are active proliferating, which is in agreement with the detected increase in heat sensitivity of the CFU-S-8 in normal bone marrow. So, our in vivo results correlates very well with results from the regular in vitro culture assay for committed stem cells.

Up to now little or no attention has been paid to the seeding efficiency of the injected cells. Because the heat treatment may alter the plasma membrane (Konings, 1988) this might influence the lodging ability of the cells in the spleen. This possibility was only investigated for leukemic cells (Flentje et al., 1984) and appeared not to change during hyperthermic treatment. We too found no significant alterations in the seeding efficiency of bone marrow stem cells. The spleen CFU-S, however, behaved
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quite differently and showed an increase in the \( f \)-factor. At the moment the divergent behavior of the spleen CFU-S is unclear. Knowledge of the \( f \)-factor before and after the heat treatment is essential to translate the CFU-S-12 numbers into pluripotent hematopoietic stem cell numbers. The aggregated results lead to the following conclusions: using CFU-S-12 data there is a difference in heat sensitivity between resting and cycling pluripotent hematopoietic stem cells, and there is a strong correlation between the proliferating status and thermal sensitivity.

Several mechanisms by which hyperthermia might cause cell death are proposed (Konings, 1987). The proliferative activity as well as the organ source are believed to be important factors in the heat sensitivity of the cells (Baeza et al., 1987). Our results imply, that the difference in heat sensitivity between hematopoietic stem cells mainly depends on the amount of proliferative stem cells. Because of the low incidence of the stem cells in the bone marrow and spleen, the accessory cells may also play an important role (Symonds et al., 1981). To elucidate a possible role of these accessory cells, as well as the origin of the organ source, we are performing hyperthermic experiments on purified pluripotent and progenitor hematopoietic stem cells at the moment.