CHAPTER EIGHT

Enhanced Selectivity of Hyperthermic Purging of Human Progenitor Cells Using Goralatide, an Inhibitor of Cell Cycle Progression

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ABSTRACT

Recurrence of leukemia is a major problem in autologous bone marrow transplantation settings. One potential means of reducing this risk is to purge the autologous transplant in vitro by hyperthermia. We have demonstrated that after a hyperthermic treatment of 120 minutes at 43°C, the leukemic progenitor cells (CFU-AML) are decreased by 5-log but the normal hematopoietic committed progenitor cells (CFU-GM, BFU-E and CFU-E) are reduced by only 1-log. Moreover, the hyperthermic sensitivity coincides with the stem cell hierarchy, i.e. CFU-GM are less heat sensitive than BFU-E, while CFU-E are the most sensitive. The impact of pretreatment with the tetrapeptide AcSDKP (Goralatide) on the proliferative activity and heat sensitivity of the normal and leukemic progenitor cells was determined. An incubation of 21 hours at 37°C with $10^{-9}$ M Goralatide reduces the number of normal hematopoietic progenitor cells in S-phase and concomitantly decreases their hyperthermic sensitivity. This effect implies that the proliferative activity is the major determinant for the detected differences in hyperthermic sensitivity of the subsets in the normal hematopoietic stem cell compartment. In contrast, the cell cycle progression of leukemic progenitor cells is not affected and hence these cells are not protected from a hyperthermia-induced cell kill after a preincubation with Goralatide. Thus, the treatment with Goralatide increases the therapeutic window of hyperthermia and increases the potential of this physical purging protocol.
INTRODUCTION

An increasing number of patients with hematological malignancies who enter a complete remission are being treated with autologous stem cell transplantation. However, many of these patients will relapse (Zittoun et al., 1995; Imrie et al., 1996). Recurrence might occur from residual cells in the patient, from contaminating leukemic cells in the transplant, or from both. Although the beneficial effect of purging is controversial, recent evidence for the contribution of the stem cell product to relapse (Gribben et al., 1991a,b; Rill et al., 1992; Brenner et al., 1993) strengthens the use of purging modalities in clinical settings.

Hyperthermia can be considered as a potent in vitro purging agent as demonstrated in murine and human normal bone marrow and leukemic cell suspensions (Higuchi et al., 1991; Herrmann et al., 1992; Wierenga and Konings, 1996; Wierenga et al., submitted). In the murine system, it was shown that the differences in heat sensitivity of the normal subsets in the hematopoietic stem cell compartment are related to the differences in proliferative activity (Baeza et al., 1987; Wierenga and Konings, 1990, 1993; Wierenga et al., 1995). The primitive hematopoietic stem cell with long-term repopulating ability, which has a quiescent cell cycle status in normal bone marrow, is less sensitive to hyperthermic treatments than the active proliferating committed progenitor subsets. In addition, it could be shown that an increase in the proliferative activity of a subset, increases the hyperthermic cell killing effect on that particular subset (Baeza et al., 1987; Wierenga and Konings, 1990).

Hematopoietic cell proliferation is modulated by a series of positive and negative regulators (Moore, 1991). The tetrapeptide AcSDKP (Goralatide) belongs to the family of negative regulators (Lenfant et al., 1989). This peptide inhibits entry into the S-phase of the cell cycle (Monpezat and Frindel, 1989; Guigon et al., 1990; Cashman et al., 1994). Such proliferative arrest might decrease the hyperthermic sensitivity of human hematopoietic cells, an effect already demonstrated in murine bone marrow (Wierenga and Konings, 1996; Wierenga et al., submitted). By contrast, leukemic cells have abnormal cell cycle regulation and may therefore be less sensitive to inhibition by a negative regulator such as Goralatide. We therefore determined the effect of proliferative activity on the heat sensitivity of the committed granulocyte-macrophage progenitors (CFU-GM) and the late and early erythroid progenitor cells (BFU-E and CFU-E) in human bone marrow. We also compared the effect of Goralatide on the cell cycle activity of normal and malignant hematopoietic progenitors and ascertained its effect on the hyperthermic sensitivity of these cells.
Our results show that the differences in hyperthermic sensitivity of the human normal progenitor cells are related to their proliferative activity, and that the differences in heat sensitivity between normal and leukemic progenitor cells confirm the feasibility of hyperthermia as purging modality. Moreover, because of a differential effect on cell cycle progression Goralatide has a protective effect on the hyperthermic sensitivity of the normal hematopoietic subsets but does not protect leukemic progenitor cells. This enhanced selectivity allows an increase in the therapeutic window of hyperthermic purging protocols for leukemia.

**MATERIALS AND METHODS**

*Hematopoietic cells.* Normal bone marrow cells were collected from patients in complete remission and from BMT donors. Informed consent was obtained for the use of cells for research purposes. Mononuclear cells were separated on Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden). The cells were resuspended in RPMI-1640 medium (GIBCO BRL, Grand Island, NY) by dispersion through a 21-gauge needle. Nucleated cells were counted in a hemacytometer.

*AML cells.* Bone marrow cells from five patients with AML classified according to the FAB Committee criteria were obtained from the St. Jude Children’s Research Hospital bone marrow cell bank. Four patients were classified as M2 and one as M4. Cells were thawed rapidly at 37°C, washed twice with RPMI-1640 medium and counted. Viability was always >95%.

*Goralatide treatment.* Goralatide (kindly provided by IPSEN-BIOTECH, Paris, France) was dissolved in RPMI-1640 medium as a stock solution of 10^{-7} M. Normal bone marrow cells or AML cells were plated out at a concentration of 2x10^6/ml in culture flasks (Falcon, Becton- Dickinson, Lincoln Park, NJ, USA) and incubated for 21 hours ± 10^{-9} M Goralatide in RPMI-1640 medium supplemented with 10% fetal calf serum. Captopril (1 μM) was added to inhibit the degradation of Goralatide by serum during the incubation. After the incubation the cells were washed twice and immediately heat treated.

*Proliferative activity.* The proliferative activity of the leukemic cells and hematopoietic progenitor cells was determined by the hydroxyurea-kill assay (Wierenga and Konings, 1993). In brief, hydroxyurea (200 μg/ml) was added to aliquots of cell suspensions ± Goralatide after zero and 20 hours, respectively, and incubated for another 60 minutes at 37°C. The cells were then washed and plated out for the colony-forming assays.
Effect of Heat and Goralatide on human normal and leukemic progenitors

Hyperthermic treatments. The cell suspensions were placed in culture tubes at a concentration of 2-3x10⁶/ml in RPMI-1640 medium with or without 10% fetal calf serum. The hyperthermic treatments were performed at 43±0.1°C up to 120 minutes and interrupted by chilling. Cells were diluted to the appropriate concentration and immediately used for the colony-forming assays.

Colony-forming assays. Normal bone marrow and AML cells were plated out in duplicate in methylcellulose medium (StemCell Technologies Inc., Vancouver, B.C.) at concentrations varying from 5x10⁴ to 5x10⁶ nucleated cells/ml. In this culture system colony growth is stimulated by the addition of agar leukocyte conditioned medium and erythropoietin. Cultures were grown in 35-mm polystyrene culture dishes (Falcon, Becton-Dickinson, Lincoln Park, NJ, USA) at 37°C in a 5% CO₂ humidified atmosphere. In normal bone marrow, the colonies (>20 cells) containing hemoglobinized cells were scored as CFU-E at day 8 of culture. After 14 days of culture, colonies (>50 cells) containing hemoglobinized cells were scored as BFU-E and those without any sign of hemoglobin as CFU-GM. In leukemic bone marrow, the blast colonies (>20 cells) were scored after 14 days.

RESULTS

Hyperthermic sensitivity of normal and AML progenitors. Figure 8.1 shows the effect of hyperthermic treatment at 43°C on normal hematopoietic and leukemic progenitor cells.

Figure 8.1. Survival curves of normal and leukemic progenitors at 43°C.
Data (mean ± SEM) from at least three separate experiments. If not present, error bars are smaller than the symbols. See Materials and Methods for details.

CFU-GM are less sensitive to heat than the erythroid progenitors BFU-E and CFU-E. The heat-induced cell kill of the leukemic progenitor CFU-AML is about 4-log after 90 minutes at 43°C while normal subsets are reduced by less than half. With the clonogenic assay the detection
limit is a 4-log reduction in cell viability. By extrapolation, however, an increase to 5-log leukemic cell depletion after 120 minutes at 43°C is feasible, at which point the normal subsets are killed by approximately 1-log. To investigate a possible protective effect of serum, the heat treatment was also performed without serum. As can be seen in Figure 8.2, a moderate protection of the normal subsets is observed in the presence of serum. However, the heat-induced cell killing of leukemic progenitor cells is not reduced.

*Figures 8.2. Effect of serum on the hyperthermic sensitivity of normal and leukemic progenitors.* Cells were treated at 43°C in the absence (dashed curves) or presence (solid curves) of 10% fetal bovine serum. *N* = 3. Bars = SEM.
Effect of Heat and Goralatide on human normal and leukemic progenitors

Effect of Goralatide on the proliferative activity of normal and AML progenitors. Figure 8.3 shows the effect of Goralatide on the proliferative activity of the normal and leukemic progenitor cells. In the controls, the erythroid progenitor cells are actively proliferating since respectively 38% and 30% of the CFU-E and BFU-E, are in S-phase. CFU-GM showed the lowest proliferative activity, with only 10% in S-phase. A 21-hour incubation with $10^{-9}$ M Goralatide reduced the proliferative activity of all three progenitor subsets, so that < 7% were in S-phase. In contrast to the CFU-GM, the leukemic CFU-AML shows an extremely high proliferative activity (>60% S-phase cells) but appears to be insensitive to Goralatide. Table 8.1 shows the effect of the incubation ± Goralatide on the total number of progenitor cells. In normal bone marrow almost all nucleated cells are recovered. Although Goralatide reduces the proliferative activity of normal progenitors, it has no effect on viability except the CFU-E. This subset decreases to ± 50% of the initial number. The number of nucleated cells and progenitors in leukemic bone marrow are slightly decreased after the incubation period.

Table 8.1. Recovery of the number of nucleated cells and progenitor subsets in normal and AML bone marrow after an incubation ± Goralatide

<table>
<thead>
<tr>
<th></th>
<th>Nucl. cells</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-E</th>
<th>CFU-AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal b.m.</td>
<td>97±9</td>
<td>89±6</td>
<td>107±14</td>
<td>105±4</td>
<td>104±11</td>
</tr>
<tr>
<td>AML b.m.</td>
<td>79±14</td>
<td>79±6</td>
<td>79±14</td>
<td>79±6</td>
<td></td>
</tr>
</tbody>
</table>

Bone marrow cells were incubated for 21 hours at 37°C ± $10^{-9}$ M Goralatide.
- = without Goralatide,
+ = with Goralatide.
Data are expressed as % (± SEM) of the initial number of nucleated cells or progenitor subsets.

Figure 8.3. Effect of Goralatide on the proliferative activity of normal and leukemic progenitors. Cells were incubated for 21 hours at 37°C in the
absence (dashed bars) or presence (black bars) of $10^{-9}$ M Goralatide. $N = 3$. See Materials and Methods for details.

Impact of Goralatide on the heat sensitivity of normal and AML progenitors. The contrasting effect of Goralatide on the proliferative activity of normal versus malignant progenitor cells has a major impact on the effects of hyperthermia. Figure 8.4 shows the overall effect of the Goralatide pretreatment on the heat sensitivity of the normal and leukemic progenitors. A marked protective effect was seen for all normal subsets due to the decrease in proliferative activity. For the leukemic progenitor cells, the hyperthermic sensitivity remains unchanged after a Goralatide pretreatment. Hence, Goralatide effectively increases the therapeutic index of hyperthermic purging.

Figure 8.4. Effect of Goralatide on the hyperthermic sensitivity of normal and leukemic progenitors. Cells were incubated for 21 hours at $37^\circ$C in the absence (dashed curves) or presence (solid curves) of $10^{-9}$ M Goralatide. A) normal hematopoietic subsets. B) leukemic progenitor cells. $N = 3$. Bars = SEM.

DISCUSSION
Recent evidence for the contribution of the transplant to relapse after autologous stem cell transplantation (Gribben et al., 1991a,b; Rill et al., 1992; Brenner et al., 1993) has intensified studies aimed at improving purging techniques. Of the physical techniques used to eliminate residual leukemic cells from a transplant, hyperthermia has shown promising clinical results (Higuchi et al., 1991; Herrmann et al., 1992). In this article we describe a means by which the safety and efficacy of such purging may be greatly increased by taking advantage of the differing biological response of normal and malignant progenitor cells to Goralatide, a tetrapeptide that modulates the cell cycle. In most studies, the effect of purging on the normal progenitor cells is compared with the effects on its leukemic counterpart CFU-AML. In the clinical studies on the feasibility of hyperthermia as purging modality, remission bone marrow was treated at 42°C (Higuchi et al., 1991; Herrmann et al., 1992). The treated patients engrafted promptly. We would predict a similar response for patients receiving marrow treated as described here. Although no clinical trials have been designed to document the minimal number of progenitor cells needed for short-term engraftment, evidence exists that the CFU-GM content can be decreased to 1-5% of the initial value without slowing hematological recovery (Douay et al., 1986; Rowley et al., 1987). Our data show that a hyperthermic treatment of 120' at 43°C results in 1-log decrease in CFU-GM survival (Figure 8.1). Moreover, based on our murine data (Wierenga and Konings, 1993; Wierenga et al., 1995) and those presented in this study, we can conclude that the primitive hematopoietic stem cell will be less likely to be in cell cycle and therefore will be less sensitive to heat than the CFU-GM.

An efficient purging therapy must not only spare sufficient normal hematopoietic cells to allow engraftment, it must also be highly toxic to leukemic cells. Our data already show a 4-log reduction in a tumor burden after 90 minutes at 43°C. In combination with the reduction of normal progenitor cells by less than half, this indicates the feasibility of hyperthermia as purging modality. Our data on the heat sensitivity of the normal human hematopoietic progenitors are consistent with those reported by others (Da et al., 1989; Murphy and Richman, 1989; Moriyama et al., 1990). Moriyama et al. (1990) showed that in human bone marrow the multi lineage progenitor cell is less heat-sensitive than the lineage restricted progenitor cells. However, in that study no attempts were made to correlate the heat sensitivity with the proliferative activity of the subsets. It should be noted that these experiments were performed in the presence of 10% fetal bovine serum. It was reported that serum has a protective effect on the heat-induced cell kill of normal and leukemic cells (Moriyama et al., 1990). However, in the current study no protective effect of serum on the heat-induced cell kill of the leukemic progenitor could be demonstrated (Figure 8.2). In the study of Moriyama et al. (1990) a hyperthermic temperature of 42°C was used. It is
well known that continuous exposure of cells to a relative low hyperthermic temperature (at or below 42.5°C) results in the development of thermotolerance (Urano, 1986). The data of Moriyama et al. (1990) indeed suggest the occurrence of thermotolerance in K-562 cells after one hour at 42°C in the presence of serum. Usually, thermotolerance is observed after a few hours of heat treatment (Urano, 1986). Serum might, however, improve the environmental conditions, thereby enhancing the kinetics of thermotolerance development. At 43°C, chronic thermotolerance induction will be decreased which might explain the absence of an effect of serum on the leukemic progenitor in our study. Nevertheless, a protection of serum on the heat sensitivity of normal progenitor subsets could be demonstrated but this protective effect does not show thermotolerance kinetics (Figure 8.2). Because of the enhanced survival of the normal subsets, we have used serum during the hyperthermic treatment.

Although these results offer encouragement for the use of hyperthermia, it would be highly desirable to obtain a further decrease in the hyperthermic sensitivity of the normal progenitor cells which in turn would allow an increase in the hyperthermic dose and augment the elimination of leukemic cells. We have now shown that in humans as in mice (Baeza et al., 1987; Wierenga and Konings, 1990, 1993, 1996) the hyperthermic sensitivity of normal subsets is related to their cycling status. For leukemic subsets a comparable relationship might exist (Wierenga et al., submitted). Decreasing the proliferative activity is a logical approach to reduce the heat-induced killing of progenitor cells, but will only result in benefit if the anti-leukemic effect is maintained. In other words, the proliferative activity and consequent hyperthermic sensitivity of the leukemic progenitor subset should not be affected by the efforts to reduce the hyperthermic sensitivity of the normal hematopoietic subsets. Goralatide has proven to be a selective protector of normal murine progenitor subsets (Guigon et al., 1990; Cashman et al. 1994; Wierenga and Konings, 1996; Wierenga et al., submitted). In this study we could confirm the selective inhibitory effect of Goralatide on human progenitor cells (Figure 8.3). The number of S-phase cells in all three normal subset compartments decreased to below 7% after a 21-hour incubation with 10^9 M Goralatide at 37°C. Concomitantly the hyperthermic sensitivity of all subsets decreased to comparable levels (Figure 8.4). This finding is further evidence that the differences in heat sensitivity of the hematopoietic subsets is determined by the differences in their proliferative activity. Based on these findings and on our murine data (Wierenga et al., submitted), we assume that the survival data of the real stem cell responsible for long-term repopulation, approximately fits these curves. Furthermore, the appearance of a shoulder region in the survival curves after a pretreatment with Goralatide is seen. This phenomenon was also observed for the
Effect of Heat and Goralatide on human normal and leukemic progenitors

quiescent subsets in the murine stem cell compartment (Wierenga and Konings, 1993). The interpretation of the shoulder region might be that in resting hematopoietic subsets more damage can be accumulated before lethality becomes manifest. The reason the heat sensitivity of leukemic progenitor cells is not decreased by Goralatide appears to be due to the insensitivity of these cells to the cell cycle inhibitory action of Goralatide, since the number of S-phase cells of the leukemic cells before and after the Goralatide treatment remains >60% (Figure 8.3). Hence, the overall effect of the Goralatide pretreatment is a reduction in the heat-induced cell kill of normal progenitors. Since no effect on the leukemic progenitor is detected, an increase in the therapeutic window is obtained (Figure 8.4).

The mechanism of inhibition by Goralatide is as yet unknown. It might act through the inhibition of cyclin D$_1$ synthesis as is described for TGF-β1 modulated inhibition of cell cycle progression (Ko et al., 1995). These D-type cyclins are essential for the cell cycle transition from G$_i$ to S-phase. In malignant cells these D-type cyclins may be abundantly present and an inhibition of cyclin D$_1$ synthesis might not affect the cell cycle status of leukemic cells. By contrast, in normal progenitor subsets this inhibition leads to cyclin D$_1$ levels below the threshold value needed for cell cycle progression.

Taken together, we can conclude that the differences in heat sensitivity between the normal progenitor and its leukemic counterpart makes hyperthermia an effective purging modality. The hyperthermic sensitivity of the progenitor subsets in human bone marrow is related to the proliferative activity. The negative regulator of hematopoiesis Goralatide decreases the proliferative activity of the progenitor subsets and hence their hyperthermic sensitivity, but has no activity on leukemic progenitor cells. As a consequence, the therapeutic window of this purging modality can be increased by pretreatment with Goralatide.