In Vitro Purging of Human Rhabdomyosarcoma Cells Using 4-Hydroperoxycyclophosphamide

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ABSTRACT

The outcome of patients with advanced stage rhabdomyosarcoma is extremely poor, with a disease-free survival of <20% at 3 years. Autologous bone marrow transplantation for patients with Clinical Group IV rhabdomyosarcoma may be an effective therapy. The bone marrow involvement diagnosed by light microscopy is 29% for patients with advanced disease. The present study was performed to test the ability of 4-hydroperoxycyclophosphamide (4-HC) to eliminate clonogenic rhabdomyosarcoma cells. Four different human rhabdomyosarcoma cell lines were treated in vitro with 4-HC at a concentration of 100 ng/ml. Limiting dilution analysis was performed to detect surviving clonogenic tumor cells. Treatment with 4-HC resulted in 1.7-5.7 log of elimination of tumor cells. Survival analysis of patients with localized disease, the survival rate for patients with advanced disease has been poor with the conventional multidisciplinary approach (17 to 23% survival rate at 3 years for Clinical Group IV patients) (2). In addition, if relapse occurs in any Clinical Group of patients with rhabdomyosarcoma, the long term disease-free survival, with conventional chemotherapy, is extremely poor (5.5%) (3). BMT is a therapeutic option for patients with various malignancies, who are either refractory to the “first line” therapy or in whom relapse occurs. Unfortunately, only 20-25% of children who might otherwise benefit from a BMT are eligible, since the majority of patients do not have an appropriate donor. ABMT avoids the problem of donor availability. The principles which apply to the utilization of ABMT with purging in leukemia can be applied to the treatment of pediatric solid tumors (4-6). Several studies have documented improved disease-free survival with ABMT in patients with advanced stage neuroblastoma, as compared with conventional therapy (7, 8). Also, there are reports of ABMT in pediatric patients with advanced rhabdomyosarcoma (9-11).

INTRODUCTION

Rhabdomyosarcoma is the most common soft tissue sarcoma in childhood and represents 4 to 8% of all malignant diseases under 15 years of age (1). Although there has been improvement in the treatment of rhabdomyosarcoma, especially in patients with localized disease, the survival rate for patients with advanced disease has been poor with the conventional multidisciplinary approach (17 to 23% survival rate at 3 years for Clinical Group IV patients) (2). In addition, if relapse occurs in any Clinical Group of patients with rhabdomyosarcoma, the long term disease-free survival, with conventional chemotherapy, is extremely poor (5.5%) (3). BMT is a therapeutic option for patients with various malignancies, who are either refractory to the “first line” therapy or in whom relapse occurs. Unfortunately, only 20-25% of children who might otherwise benefit from a BMT are eligible, since the majority of patients do not have an appropriate donor. ABMT avoids the problem of donor availability. The principles which apply to the utilization of ABMT with purging in leukemia can be applied to the treatment of pediatric solid tumors (4-6). Several studies have documented improved disease-free survival with ABMT in patients with advanced stage neuroblastoma, as compared with conventional therapy (7, 8). Also, there are reports of ABMT in pediatric patients with advanced rhabdomyosarcoma (9-11).

One of the potential problems associated with ABMT is the reinfusion of viable tumor cells with the bone marrow. Bone marrow involvement, diagnosed by light microscopy, has been reported as 29% in patients with advanced rhabdomyosarcoma (12). Therefore, in order to minimize the risk of tumor recurrence from ABMT, it is necessary to have an effective means of selective removal of tumor cells from the marrow before cryopreservation.

4-HC, a congener of cyclophosphamide, is one of the most commonly used drugs for pharmacological purging in ABMT (13-15). The evidence from animal models showed that 4-HC can purge marrow of clonogenic leukemia cells while sparing the pluripotential stem cells (16). Furthermore, 4-HC-treated human marrow cell suspensions are able to reconstitute the hematopoietic system after ablative therapy, despite a marked reduction in detectable progenitor cells (17, 18). The efficacy of cyclophosphamide as part of the combination chemotherapy regimens used in the treatment of rhabdomyosarcoma has been well established. Hence, it is logical to expect 4-HC to be an effective agent in purging rhabdomyosarcoma cells from the bone marrow. The present study was designed to test the ability of 4-HC to eliminate clonogenic rhabdomyosarcoma cells, initially on human rhabdomyosarcoma cell lines and then from a mixture of rhabdomyosarcoma and normal HBMMC. For this purpose, an in vitro clonogenic assay that could measure elimination of 6 log of malignant cells was used.

MATERIALS AND METHODS

Human Rhabdomyosarcoma Cell Lines. A204, RD, CB-NJR, and SMS-CTR were studied. A204 and RD were purchased from American Type Culture Collection, whereas CB-NJR and SMS-CTR were kindly supplied by Dr. Reynolds, Childrens Hospital of Los Angeles. Histologically, A204 and CB-NJR are of alveolar subtype of rhabdomyosarcoma, and RD and SMS-CTR are embryonal subtype (19, 20). A204, RD, and CB-NJR/SMS-CTR cells were maintained in McCoy 5A, Dulbecco’s modified Eagle’s high glucose, and RPMI 1640 media, respectively, with the addition of 10% FCS, 1% penicillin-streptomycin (10,000 units), 1% l-glutamine, an 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and were routinely grown at 37°C in a humidified incubator with 5% CO2. The cells were all adherent and were maintained in exponential growth. The viability of all cell lines was 90%, as judged by trypsin blue dye exclusion.

Preparation of Bone Marrow Suspensions. Bone marrow specimens were aspirated into heparinized syringes from the posterior iliac crests of healthy volunteers, after obtaining their informed consent. The mononuclear marrow cell fraction was prepared by centrifugation on Ficoll-Paque/Percoll gradients.

Purging Technique. 4-HC was kindly supplied by Dr. O. Michael Colvin from the Johns Hopkins Oncology Center (Baltimore, MD). It was dissolved in calcium- and magnesium-free Dulbecco’s phosphate-buffered saline, pH 7.4, to a concentration of 1 mg/ml, immediately before use. Tumor cells or mixtures of normal marrow and tumor cells were incubated with four different concentrations of 4-HC (12.5, 25, 50, and 100 ng/ml), at a cell concentration of 2 x 107/ml in medium, at 37°C for 30 min and were cooled rapidly to 4°C for 10 min. The cells were then washed twice in medium. Normal marrow cells were mixed with tumor cells in a ratio of 19:1. The hematocrit of the mixture

Received 8/22/89; revised 11/6/89; accepted 11/14/89.

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1 Supported in part by a grant from the Children’s Hospital of Los Angeles, Me Allister Research Fellowship.

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3 The abbreviations used are: BMT, bone marrow transplantation; 4-HC, 4-hydroperoxycyclophosphamide; ABMT, autologous bone marrow transplantation; HBMMC, normal human bone marrow mononuclear cells; CFU-GM, colony-forming unit-granulocyte-macrophage; BFU-E, burst-forming unit-erythroid; CFU-GEMM, colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte; FCS, fetal calf serum; ANLL, acute nonlymphoblastic leukemia; BMM, bone marrow metastases; CR, complete response; IR, Intergroup Rhabdomyosarcoma Study.
was kept constant throughout these experiments and it was <1%.

Clonogenic Assay and Data Analysis. After treatment, rhabdomyosarcoma cells were assayed for clonogenic growth by the limiting dilution technique. Following the 4-HC treatment, a series of dilutions of the control and test cell suspensions were prepared in medium supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-glutamine, and 1% 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid buffer solution. The initial undiluted tube contained 10⁴ to 10⁷ tumor cells/ml. In all these experiments, a dilution factor of 5 was used. Then from each dilution, 100 µl were plated in 96-well, flat bottomed tissue culture plates. Six wells were plated for each dilution, and usually eight dilutions were made. One hundred µl of medium was then added to each well. No feeder cells were used. After 14 days of culture at 37°C in a humidified atmosphere of 5% CO₂, the wells were examined for clonogenic growth, using an inverted microscope. A group of 40 or more cells was scored as a "colony." Each well was scored as positive or negative for the presence of colonies. The frequency of wells with growth was used to estimate the most probable number of remaining clonogenic cells in the original suspension, by a modification of the Spearman-Karber method (21, 22). The extent of clonogenic rhabdomyosarcoma cell elimination was expressed as log kill = log (Ocontrol/θₐ₀), where θ is the most probable number of clonogenic units as estimated by the modified Spearman-Karber method.

Normal Progenitor Cell Toxicity Assay. CFU-GM, BFU-E, and CFU-GEMM from normal human marrow were assayed as described by Fauser and Messner (23). Each 35-mm Petri dish contained 10⁴ nucleated marrow cells and 1 ml of Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) supplemented with 30% FCS, 0.9% methyleneblue, 10⁻⁴ mol/liter 2-mercaptoethanol, 10% bovine serum albumin, 5% phytohemagglutinin-stimulated leukocyte-conditioned medium, 1% L-glutamine, 1% penicillin-streptomycin, 10⁻⁴ mol/liter hydrocortisone, 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (SCHERING), and 1.3 units/ml erythropoietin (AMGEN, Thousand Oaks, CA), added on day 4. Cultures were incubated for 14 days at 37°C in a humidified atmosphere of 5% CO₂ in air. Colonies were counted in situ with an inverted microscope on days 14 and 30 and scored positive if they contained >40 cells.

RESULTS

All four human rhabdomyosarcoma cell lines were sensitive to 4-HC. The dose-response curve for each cell line was established by incubating tumor cells with 12.5, 25, 50, and 100 µg/ml 4-HC. Fig. 1 shows the ability of 4-HC to kill clonogenic rhabdomyosarcoma cells in a dose-dependent manner. A204 was the most responsive cell line, which was followed by RD, SMS-CTR, and CB-NJR in order of sensitivity. A204 and CB-NJR, respectively, were of alveolar histology. Table 1 demonstrates the range of log cell kill for each cell line with 100 µg/ml 4-HC. The mean cell kill for these four cell lines was 3.5 log, ranging from 1.7 to 5.7. Cell lines with alveolar histology, A204 and CB-NJR, showed 4.8 and 2.3 log of cell kill, whereas cells with embryonal histology, SMS-CTR and RD, demonstrated similar responses with 3.6 and 3.5 log of cell kill, respectively. There were no changes in the tumor cell count immediately following treatment, indicating no lysis of tumor cells (data not shown).

In a different set of experiments, normal HBMMC were mixed with rhabdomyosarcoma cells to see if normal HBMMC would interfere with the effect of 100 µg/ml 4-HC on clonogenic tumor cells. Under the culture conditions described above, HBMMC did not alter the effect of 4-HC on rhabdomyosarcoma cells, and exactly the same log cell kill was obtained, as shown in Table 2. Tumor colonies were easily discriminated from normal bone marrow progenitor colonies.

Then, we observed the effect of 100 ng/ml 4-HC on the growth of normal immature committed progenitor cells. Table 3 shows the effect of 100 ng/ml 4-HC on normal progenitor cells, as assayed in continuous marrow culture. There were a few detectable CFU-GM, CFU-GEMM, or BFU-E colonies on day 14 following 4-HC treatment. However, CFU-GM, CFU-GEMM, and BFU-E recoveries on day 30 were 15.2, 13.5, and 19.8% of control, respectively. This concentration of 4-HC permits the growth of immature committed progenitors, as determined by the 30-day colony growth.

DISCUSSION

Patients with advanced stage rhabdomyosarcoma continue to do poorly despite the use of intensive multiagent chemotherapy as part of the multidisciplinary approach (24). Ruymann et al. reported BMM at diagnosis in 30 of 103 (29%) children and

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Table 1 In vitro clonogenic rhabdomyosarcoma cell kill with 100 µg/ml 4-HC

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clonogenic cell kill (log)</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>A204</td>
<td>4.8</td>
</tr>
<tr>
<td>CB-NJR</td>
<td>2.3</td>
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<tr>
<td>SMS-CTR</td>
<td>3.6</td>
</tr>
<tr>
<td>RD</td>
<td>3.5</td>
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<tr>
<td>Overall</td>
<td>3.5</td>
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Table 2 In vitro clonogenic rhabdomyosarcoma cell kill with 100 µg/ml 4-HC using a mixture of human bone marrow mononuclear cells and 5% tumor cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Clonogenic cell kill (log)</th>
</tr>
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<tbody>
<tr>
<td>A204</td>
<td>4.2</td>
</tr>
<tr>
<td>CB-NJR</td>
<td>3.3</td>
</tr>
<tr>
<td>SMS-CTR</td>
<td>3.0</td>
</tr>
<tr>
<td>RD</td>
<td>3.0</td>
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Table 3 Recovery of normal progenitor cells after treatment with 4-HC

<table>
<thead>
<tr>
<th>Concentration of 4-HC (µg/ml)</th>
<th>Day of assessment</th>
<th>Recovery (% of control)</th>
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<tbody>
<tr>
<td>100</td>
<td>14</td>
<td>0.7</td>
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<tr>
<td></td>
<td>30</td>
<td>15.2</td>
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<tr>
<td></td>
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<td>13.5</td>
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<tr>
<td></td>
<td>30</td>
<td>19.8</td>
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</table>
adolescents with rhabdomyosarcoma entered on IRS-I with Clinical Group IV disease (12). However, the incidence of BMM may be even higher if aggressive sampling and more sensitive diagnostic techniques like immunohistochemistry are used, as seen with neuroblastoma (25, 26). The overall CR rate for these patients was 60% using vincristine, actinomycin-D, and cyclophosphamide or vincristine, actinomycin-D, and cyclophosphamide plus Adriamycin. Relapse after achieving CR in patients with BMM at diagnosis occurred in 16 of 18 (89%) and was significantly higher than the 17 of 29 (59%) observed in Clinical Group IV patients without BMM (P < 0.035).

There is limited experience with the use of ABMT in pediatric patients with advanced rhabdomyosarcoma. Ekert et al. (9) reported their experience with ABMT in eight children with Group III and IV rhabdomyosarcoma without bone marrow involvement. Only one remained in CR; seven of eight relapsed. Hartman et al. (10) reported a 14-year-old patient with Group III rhabdomyosarcoma who died from toxicity 1.5 month after the BMT, while he was free of tumor. Miser et al. (11) treated 68 newly diagnosed, high risk, sarcoma patients, 18 of whom had rhabdomyosarcoma, in their first CR with an intensive consolidation regimen followed by ABMT (11). Thirty-five patients had metastases at diagnosis, of which 15 had disease involving the bone marrow. The overall actuarial survival and disease-free survival at 24 months were approximately 60 and 40%, respectively. They concluded that one of the most important negative prognostic factors for outcome was metastatic disease at diagnosis. However, in these studies, no attempt was made to cleanse the marrow of tumor cells at the time of harvesting.

The role of in vitro purging of potentially contaminating tumor cells in the use of ABMT is still a controversial area. When there is tumor recurrence following ABMT, it is difficult to determine if it occurs because of persistence of tumor cells in the patient or in the reinfused marrow. Based on results of syngeneic and allogeneic BMT for hematological malignancies, many patients relapse because of the inability of the conditioning regimen to ablate tumor within the patient (27). The second observation is that infusion of untreated autologous remission marrow does not inevitably lead to relapse (28). However, several observations argue that tumor cells given with an ABMT can be tumorigenic. First, animal studies have clearly shown a therapeutic benefit to purging marrow (16). Second, neuroblastoma patients receiving nonpurged marrow have shown relapse patterns suggestive of tumor embolization from the marrow infusion, such as diffuse malignant pulmonary infiltration (29, 30). Third, continuous tumor cell lines have been initiated from apparently uncontaminated marrows in patients with Burkitt’s lymphoma and breast and lung cancer (31–34). Fourth, one recent study identified a group of ANLL patients with insufficient purging, according to the percentage of CFU-GM survival as a measure of 4-HC cytotoxicity (35). Twenty-three ANLL patients with a CFU-GM content after 4-HC purging of >1% of the pretreatment value had an actuarial disease-free survival of 12%, compared to 36% for 22 patients with a ≤1% CFU-GM content after purging (P < 0.006). The only way to resolve this issue and demonstrate the efficacy of purging is to do randomized trials. The main obstacle in this area is the small number of patients, especially with pediatric solid tumors. Since the minimal number of malignant cells that will give rise to a tumor after i.v. infusion is unknown and since this number may vary with different malignancies and for different patients with the same malignancy, it is prudent to attempt to remove all detectable tumor cells provided that there is no significant morbidity or mortality associated with purging.

Several centers in North America and Europe have reported encouraging clinical results in ANLL with the use of cyclophosphamide derivatives to purge marrow of residual leukemic cells. These drugs are not selective for malignancy and eliminate large fractions of normal stem cells. In a phase I trial, a concentration of 100 μg/ml 4-HC was determined to be the maximum that would allow consistent hematological reconstitution (17). Recent studies have shown that 4-HC- or mafosfamide-treated human marrow suspensions are able to reconstitute the hematopoietic system, within an acceptable period, following ablative therapy, despite a marked reduction in detectable progenitor cells (17, 35–37). Our normal progenitor cell toxicity assay results are compatible with the observations made in these clinical studies.

In this study, we have shown that a concentration of 100 μg/ml 4-HC is capable of eliminating 1.7–5.7 log of human rhabdomyosarcoma cells from the human bone marrow mononuclear cells. It is also encouraging to note that 4-HC is effective in both alveolar and embryonal human rhabdomyosarcoma cell lines. The nondiscriminatory effect of 4-HC on histological subgroups is important because, of the children and adolescents presenting with rhabdomyosarcoma with BMM in IRS-I, half had alveolar histology although only 19% of the total patients in the same study had alveolar rhabdomyosarcoma (12). Hence, our results suggest that in vitro treatment with 4-HC could be an effective and safe method of eliminating clonogenic rhabdomyosarcoma cells from the human bone marrow and support its use in ABMT for advanced stage rhabdomyosarcoma patients.

ACKNOWLEDGMENTS

We are grateful to Anne I. Goldman for statistical assistance and to Dr. Jorge A. Ortega for helpful comments.

REFERENCES

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