Reduction of Tumor Burden in a Murine Osteosarcoma following Hyperthermia Combined with Cyclophosphamide

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ABSTRACT

A radiation- and chemotherapy-resistant murine osteosarcoma was used to investigate the effect of local hyperthermia (42.5 ± 0.1°, 30 min) alone and in combination with cyclophosphamide. The cytotoxicity of cyclophosphamide on murine osteosarcoma was established previously in our laboratory. Local hyperthermia (42.5 ± 0.1°, 30 min) had little or no effect on the 16-day-old (206 × 10^6 osteosarcoma tumor cells/mouse) tumor as shown by the changes in the tumor cell marker, alkaline phosphatase. A 2.5 ± 3.5% reduction in the number of tumor cells was seen. Large tumors treated at 21 days postimplantation (357 × 10^6 tumor cells) showed a reduction of 24 ± 14%. The effect of combination treatment with cyclophosphamide and hyperthermia produced greater reduction in the numbers of tumor cells than did either treatment used alone.

INTRODUCTION

The majority of patients with osteogenic sarcoma will eventually have systemic involvement, primarily due to lung metastases. Thus, the optimum treatment for this cancer will probably involve both local and systemic therapies. Current evidence does not conclusively support the usefulness of adjuvant chemotherapy following amputation (17). Furthermore, loss of a limb poses major physical and emotional challenges for the predominantly young patients affected with this disease. Thus, improvements are needed in both the local and systemic management of osteosarcoma.

We present studies on a murine osteosarcoma which, in many respects, resembles its human counterpart (6). We have examined a combination of systemic chemotherapy with an investigational local therapy, hyperthermia, in an attempt to improve local and systemic tumor control.

MATERIALS AND METHODS

Mice. Six-week-old female C3H/HeN mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. The mice were maintained on standard laboratory rodent pellets and water ad libitum.

Tumor. The osteosarcoma arose spontaneously in the tail vertebra of a C3H/HeN mouse in the laboratory of Dunn and Andervont (3) at the National Cancer Institute. The tumor was maintained by serial passages in C3H/HeN mice every 2 to 3 weeks. Single-cell suspension of the tumor was prepared according to the procedure described previously (5). One-tenth ml containing 1 × 10^6 viable osteosarcoma cells was injected into the femoral muscle area.

Measurement of Tumor Marker and Number of Tumor Cells.

The tumor synthesizes and releases a nonspecific enzyme marker, AP. We related the total number of viable tumor cells recovered from solid osteosarcoma to total AP levels in the plasma of individual mice (9). A plot of the lognormal AP versus number of tumor cells per mouse for animals with tumors of different sizes gave a straight line which was described by the equation

\[ C = -483 + 124 \ln AP \]

where \( C \) is the number of tumor cells multiplied by \( 10^6 \), AP is the total AP given at \( A_{90} \), and -483 (intercept) and 124 (slope of the line) are experimentally derived constants (9). This equation is useful to plot the lognormal AP of 3.91; below this value, the experimental equation would give negative numbers of tumor cells. All animals were monitored individually, and the number of tumor cells was calculated for each animal during the course of tumor growth and treatment.

Treatment. Mice were grouped into early (16-day-old) and late (21-day-old) tumor. This was an arbitrary division to evaluate the effectiveness of treatment on differently sized tumors. There is probably a greater number of hypoxic cells in the later group which had 2 times more tumor cells than did the early group. The mice were anesthetized with pentobarbital, and the tumor limb was gently wrapped with heavy gauge aluminum foil to keep the limb extended. It should be noted that pentobarbital can modify the thermal response of tumors. Under Nembutal anesthesia, the heating of the tumor was more uniform, and the temperatures were much closer to the water bath temperatures (19). Mice were placed on a rack, and only the tumor-bearing limb was immersed into a 42.5 ± 0.1° water bath. The body of the mouse was placed on a slanted Plexiglas so that it remained out of water. The tumor limb was immersed in the water bath maintained at 42.5 ± 0.1° for 30 min. The core temperature was measured in mice with large tumors in a separate experiment. A type IT-21 thermocouple microprobe was passed completely through the tumor, exiting into the water bath. The probe was then slowly pulled through the tumor while continuous measurements were made. Core temperature may vary as much as 0.5°, even though the water bath accuracy is ± 0.1°. In many cases, the temperature was entirely homogeneous.

Cyclophosphamide (200 mg/kg) was given i.p. one time on Day 16 or 21 post-tumor implantation, 2 hr prior to hyperthermia. Hyperthermia was given 3 times on Days 16, 18, and 20 for early tumor and on Days 21, 23, and 25 for late tumor. The mice were bled every 4 to 6 days, and the plasma was assayed for the changes in the AP values. Because cyclophosphamide requires time for conversion to the active form, cyclophosphamide was given 2 hr prior to heating to give a chance for the drug to react before hyperthermia was instituted.

RESULTS

Evidence for Sensitivity of Late Murine Osteosarcoma to Hyperthermia. Early (Day 16) and late (Day 21) osteosarcoma-bearing mice were treated for 30 min at 42.5 ± 0.1° every other day, 3 times. The average number of tumor cells at the time...
Table 1
Summary data of average mouse weight, tumor cell number, tumor weight, and percentage of body weight of tumor (tumor burden)

<table>
<thead>
<tr>
<th>Age of tumor</th>
<th>Treatment</th>
<th>Av. no. of tumor cells/ mouse x 10^6</th>
<th>Av. mouse wt (g)</th>
<th>Av. tumor wt (g)/ mouse</th>
<th>Av. % of body wt of tumor at time of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 16</td>
<td>Hyperthermia</td>
<td>5</td>
<td>32.1 ± 0.9</td>
<td>206 ± 49</td>
<td>0.824</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>5</td>
<td>30.0 ± 1.2</td>
<td>229 ± 32</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide + hyperthermia</td>
<td>6</td>
<td>31.0 ± 1.0</td>
<td>169 ± 25</td>
<td>0.676</td>
</tr>
<tr>
<td>Day 21</td>
<td>Hyperthermia</td>
<td>5</td>
<td>30.5 ± 1.8</td>
<td>357 ± 41</td>
<td>1.428</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>5</td>
<td>29.0 ± 2.3</td>
<td>389 ± 18</td>
<td>1.556</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide + hyperthermia</td>
<td>5</td>
<td>30.1 ± 0.7</td>
<td>357 ± 41</td>
<td>1.428</td>
</tr>
</tbody>
</table>

* One g tumor is equivalent to 2.5 x 10^6 viable tumor cells as determined experimentally.
* Hyperthermia treatment is given at 42 ± 0.1° for 30 min every other day for 3 times.
* Average ± S.D.
* Cyclophosphamide was given at 200 mg/kg.

Treatment was initiated ranged from 169 ± 25 to 229 ± 32 x 10^6 and 357 ± 41 to 389 ± 18 x 10^6/mouse, for 16- and 21-day groups, respectively (Table 1). The mice bearing 16-day-old tumors had a body burden of 2.18 to 3.05% of body weight. The late-tumor group had a body burden of 4.74 to 5.36% of body weight. When the early tumor group was treated with hyperthermia, only 2 mice had a slight decrease in AP values (Chart 1). All mice with late tumors showed some decrease in AP value (Chart 2). The decline in the AP values of the group with 21-day-old tumor was greater than in the group with 16-day-old tumor. However, the effect was transient and lasted roughly for the length of treatment only.

Sensitivity of Osteosarcoma to Cyclophosphamide. Our earlier results and the results reported here indicate that the murine osteosarcoma showed moderate sensitivity to high-dose cyclophosphamide (200 mg/kg). As determined by the changes in AP values, the therapeutic effect of the drug lasted for 12 days in the early group and 15 days for the late group. One hundred % of the osteosarcoma-bearing mice responded to the treatment. In mice whose body burden of tumor was 3.05% of body weight (early group), the percentage of reduction in tumor cell number was 62.0 ± 16.1. In mice whose body burden of tumor was 5.36% (late tumor), a 51.0 ± 17.1 reduction in tumor cell number was observed (Table 2). No long-term remission or cures were obtained by this treatment.

Effect of Cyclophosphamide and Hyperthermia Combination. Early (16-day-old) and late (21-day-old) osteosarcoma tumor-bearing mice were given cyclophosphamide (200 mg/kg) 2 hr prior to hyperthermia. Hyperthermia was given 3 times on alternate days. The mice were monitored for changes in plasma AP every 4 to 6 days (Charts 3 and 4). At the time treatment was initiated, the late (Day 21) group had 2.17 times more tumor than did the early group (Table 1). The combination treatment produced 95 and 99% reduction of tumor cell number in the early and late-tumor groups, respectively. If the reduction of tumor obtained with hyperthermia and cyclophosphamide used singly is compared with the combination treatment, the results show the use of combined therapy had a better than additive effect on the early and late tumor (Table 2). The percentage of body weight of tumor was drastically reduced by the combined treatment. For example, the percentage of reduction in number of tumor cells in the 21-day-old tumor after heat or drug is 24.0 or 51%, respectively (Table 2). This means a surviving fraction of tumor cells after drug or heat would be 0.76 (1.0 minus 0.24) or 0.49 (1.0 minus 0.51). If the drug plus heat produces only additive effect, surviving fraction should be 0.76 x 0.49 = 0.37. However, we observed a surviving fraction of 0.01 (1.0 minus 0.99), which is obviously greater than an additive effect. Based on the above observation, we explored the possibility...
perthermia on osteosarcoma. Mice were treated with cyclophosphamide (200 mg/kg) on Day 16, followed by hyperthermia on Days 16, 18, and 20 (arrows). Each line represents individual perthermia. Cyclophosphamide was given at 200 mg/kg on Day 21, followed by hyperthermia on Days 21, 23, and 25 (arrows). Each line represents individual perthermia.

Cyclophosphamide was administered and every 2 days thereafter at 42.5 ± 0.1° for 1 hr for a total of 3 times. Because of the stress on the animals from the 1 hr of heating, hyperthermia was applied after resting the animals for 2 days between treatment rather than every other day as in the previous experiment. Several of the mice died during the course of the treatment from the effects of drug (cyclophosphamide, pentobarbital) toxicity and combined effects of hyperthermia and stress. These were not included in the data. Hyperthermia alone caused <50% reduction in body burden of tumor (data not shown), and cyclophosphamide alone produced approximately 50% reduction in body burden. The individual responses are shown in Table 3. Of the 6 mice that survived the combination treatment, >99% reduction of body weight of tumor was achieved in 5 animals, and 2 mice were cured of disease.

**DISCUSSION**

The murine osteosarcoma is treatment resistant in that treatment of large tumors (~21-day-old) with cyclophosphamide (200 mg/kg) every 12 days (optimal schedule) does not bring about cure, even when initial kill of tumor cells and transient regression can be demonstrated. The tumor recurs in the animals, leading to death. We have used this treatment-resistant murine osteosarcoma to investigate the interaction between hyperthermia and chemotherapy. Hyperthermia is an experimental form of tumor therapy which is now being investigated in a variety of human cancers, including osteosarcomas (16). Hyperthermia by itself can produce partial and temporary responses in as many as half of adequately treated masses (15). However, durable complete remissions are rare. For this reason, much current effort is directed towards combination therapy, including heat. A rationale for this approach is that hyperthermia may kill the acidic, poorly proliferating tumor core, while radiation or chemotherapy controls the well-vascularized, rapidly proliferating tumor rim (21).

Heat has been combined with a variety of systemic drugs in an attempt to improve tumor control (7). Prominent among such drugs are alkylating agents. These agents have radiomimetic properties and might be expected to show some synergism with hyperthermia similar to that seen with ionizing radiation and heat. Mustard-type alkylators studied include melphalan (12), thiotapec (11), and cyclophosphamide (10). All have shown some favorable interaction with heat. Other alkylator-like drugs which produce enhanced cell kill with hyperthermia include 1,3-bis(2-chloroethyl)-1-nitrosourea (14), 1,3-(2-chloroethyl)-3-cyclohexyl-1-nitro-

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**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of reduction in no. of tumor cells</th>
<th>% of body wt of tumor after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperthermia, x 3 treatments</td>
<td>2.5 ± 3.5</td>
<td>2.50</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>62 ± 16</td>
<td>1.16</td>
</tr>
<tr>
<td>Cyclophosphamide + hyperthermia, × 3 treatments</td>
<td>95 ± 3</td>
<td>0.0011</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperthermia, × 3 treatments</td>
<td>24 ± 14</td>
<td>3.56</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>51 ± 17</td>
<td>2.6</td>
</tr>
<tr>
<td>Cyclophosphamide + hyperthermia, × 3 treatments</td>
<td>99 ± 1</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

* Percentage of reduction in number of tumor cells was obtained from calculating the number of tumor cells at maximum and the number remaining following treatment using the equation, C = -483 + 124 In AP.

**Discussion**

The murine osteosarcoma is treatment resistant in that treatment of large tumors (~21-day-old) with cyclophosphamide (200 mg/kg) every 12 days (optimal schedule) does not bring about cure, even when initial kill of tumor cells and transient regression can be demonstrated. The tumor recurs in the animals, leading to death. We have used this treatment-resistant murine osteosarcoma to investigate the interaction between hyperthermia and chemotherapy. Hyperthermia is an experimental form of tumor therapy which is now being investigated in a variety of human cancers, including osteosarcomas (16). Hyperthermia by itself can produce partial and temporary responses in as many as half of adequately treated masses (15). However, durable complete remissions are rare. For this reason, much current effort is directed towards combination therapy, including heat. A rationale for this approach is that hyperthermia may kill the acidic, poorly proliferating tumor core, while radiation or chemotherapy controls the well-vascularized, rapidly proliferating tumor rim (21).

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**Table 3**

<table>
<thead>
<tr>
<th>Animal</th>
<th>% of body wt of tumor at beginning</th>
<th>% of body wt of tumor after treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.1</td>
<td>54.3</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>0.03b</td>
</tr>
<tr>
<td>7</td>
<td>8.1</td>
<td>0.04</td>
</tr>
<tr>
<td>11</td>
<td>4.1</td>
<td>0.04</td>
</tr>
<tr>
<td>13</td>
<td>5.0</td>
<td>0.04b</td>
</tr>
<tr>
<td>14</td>
<td>3.5</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* This was determined after treatment was completed and at the time when the number of tumor cells calculated from AP values had reached a minimum in the animal.

**Figure 3**

Effect of addition of cyclophosphamide chemotherapy along with hyperthermia on osteosarcoma. Mice were implanted with tumor into the leg and were treated with cyclophosphamide (200 mg/kg) at a time when the body weight of tumor ranged from 3.5 to 8.1%. Animals were given hyperthermia on the day cyclophosphamide was administered and every 2 days thereafter at 42.5 ± 0.1° for 1 hr for a total of 3 times. Because of the stress on the animals from the 1 hr of heating, hyperthermia was applied after resting the animals for 2 days between treatment rather than every other day as in the previous experiment. Several of the mice died during the course of the treatment from the effects of drug (cyclophosphamide, pentobarbital) toxicity and combined effects of hyperthermia and stress. These were not included in the data. Hyperthermia alone caused <50% reduction in body burden of tumor (data not shown), and cyclophosphamide alone produced approximately 50% reduction in body burden. The individual responses are shown in Table 3. Of the 6 mice that survived the combination treatment, >99% reduction of body weight of tumor was achieved in 5 animals, and 2 mice were cured of disease.
combination therapy, we are encouraged because both local and systemic tumor control was seen in some animals. This treatment combination may, in fact, be superior to a chemotherapy-surgery combination. In a study of similar-sized tumors, we found no cures following a single treatment with cyclophosphamide and subsequent amputation (8). Our current results raise the possibility that local hyperthermia and chemotherapy may diminish the extent of surgery needed for control of human osteosarcomas and lead to some limb salvage.

Our finding of enhanced thermal effects against larger tumor is consistent with previous reports (23). Acidotic cells are more sensitive to heat than those at normal pH (4). As tumors grow, they experience progressive impairment of vascular supply and hypoxia, which may lead to a decrease in extracellular pH (22). This phenomenon may account for the enhanced thermal sensitivity of large tumors. Our data are in agreement with the detailed studies of Magin and Johnson (13). Their results point out that the slower-growing tumors were most responsive to the heat treatment and that the faster-growing tumors with larger growth fractions of cells are least responsive.

Cyclophosphamide has been combined with whole-body hyperthermia in several tumor systems. While tumor cell kill was enhanced, marrow toxicity was also increased, leading to no real therapeutic gain (10). Cyclophosphamide metabolism was also impaired, with an increase in free cyclophosphamide and a decrease in urinary alkylating activity being seen at temperatures above 40.5°C (2). Similar perturbation of cyclophosphamide metabolism has been seen following whole-body hyperthermia of humans (18). We feel that these drawbacks may not be applicable to the combination of cyclophosphamide and local or regional hyperthermia. While mice receiving combined therapy did show exaggerated mortality, we feel this is due primarily to the stresses of general anesthesia for treatment and skin breakdown leading to infection. Such toxicity is unlikely to be as important in human treatment for the following reasons. Supportive care (fluids, antibiotics, pressors, etc.) would be available if similar events happened in human treatment, but no such care was provided for mice. Furthermore, the combination of regional heating with melphalan, another mustard-type alkylator, has been shown to be an effective and reasonably safe local therapy for limb cancers (20). We believe that our data support the development of combined therapies using cyclophosphamide and local hyperthermia for the treatment of bulky cancers, including osteosarcoma.

REFERENCES


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