ABSTRACT

To study the effects of total-body hyperthermia (TBH) on metastases from malignant tumors, Lewis lung carcinoma (LLC)-bearing C57BL/6 mice and mouse ascites hepatoma 134-bearing C3H/He mice were used. After treatment, the incidence of lung metastasis was analyzed in LLC-inoculated mice, and the presence or absence of metastasis in affiliated lymph nodes was determined in mouse ascites hepatoma-134-inoculated mice. A significant inhibition in primary tumor growth in LLC- and mouse ascites hepatoma-134-bearing mice treated with 42 °C TBH was noted. The incidence of lung metastasis was increased from the control level of 1.6 ± 0.63 (SD) to 2.4 ± 0.98 in the 42 °C TBH (P < 0.01) groups but not in the 40 °C TBH group. Metastasis to affiliated lymph nodes was similar for the controls and the 40 °C and 42 °C TBH groups. The increase in lung metastasis in LLC-treated mice subjected to 42 °C TBH could be prevented by the combined use of anticancer drugs such as cis-diamminedichloroplatinum(II) (1.0, 3.0 mg/kg) or mitomycin C (0.3, 1.0 mg/kg). Furthermore, the combined use of 42 °C TBH and anticancer drugs showed the inhibition of primary tumor growth to a greater degree than did 42 °C TBH alone or anticancer drugs alone. Since 42 °C TBH may induce tumor metastasis, especially hematogenous metastasis, it seems advisable to use anticancer drugs in combination with clinical thermal applications.

INTRODUCTION

Due to a better understanding of the antitumor action of heat (3, 9, 11, 12, 15) and progress in the development of clinically applicable heating methods, hyperthermia has become a promising modality in cancer therapy (1, 4, 8, 16). However, the question of whether hyperthermia enhances tumor metastasis remains unanswered (2, 17-21, 23). Our study was designed to clarify the effects of hyperthermia, especially TBH, on tumor metastasis.

MATERIALS AND METHODS

Animals and Tumors. Male C57BL/6 and C3H/He mice, purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), were used at 9 weeks of age. Groups of 5 mice were kept in different cages and provided a standard laboratory diet and tap water ad libitum. To produce the inocula, one group of mice (C57BL/6) was transplanted i.m. with LLC, and the other group (C3H/He) received an i.p. transplant of MH-134. Because LLC usually metastasizes only to the lungs, the LLC recipients were used as the hematogenous metastasis model; their lymph nodes were examined to also study the presence of metastases in the lymph nodes. Because MH-134 has a high rate of lymph node metastasis, the MH-134 recipients were used as the lymphogenous metastasis model; their lungs and livers were examined to also study the presence of hematogenous metastases in these organs.

To prepare the LLC inoculum, the tumor was removed sterilely, cut up with scissors, and washed twice in phosphate buffer solution. Tumor sections were passed through stainless steel mesh (80 μm) and stained with 0.5% trypan blue, and the number of viable cells was determined. To obtain the MH-134 inocula, ascites was withdrawn and stained with 0.5% trypan blue, and viable cells were counted. LLC cells (1 x 10⁶) and MH-134 cells (1 x 10⁶) were injected into the right hind leg muscle of C57BL/6 and C3H/He mice, respectively. For both inocula, the rate of tumor implantation was 100%.

Hyperthermic Treatment Apparatus and Temperature Measurement. Chart 1 illustrates the apparatus used for TBH induction. Before the water bath was filled and during TBH induction, the rectal, intratumor, and lung temperatures of LLC-bearing mice were recorded. A Nihon Koden Model MGA-III electric thermistor (type 219; Shibaura Electronics, Tokyo, Japan) was used. The tip of the thermistor was placed 2 cm into the anus to monitor the rectal temperature, 3 mm into the tumor to obtain the intratumor temperature, and 5 mm past the thoracic wall to measure the lung temperature. Within ± 0.3 (SD) min from the start of TBH induction, these 3 temperatures were equilibrated with the water temperature.

Thermal Treatment. On the basis of preliminary experiments, we determined the water temperatures to be 40 °C and 42 °C. The mice were exposed to TBH 5 days after tumor inoculation. LLC-bearing mice were treated with one 30-min heating session (40 °C or 42 °C) at 5 days after tumor inoculation; in the 42 °C TBH group, immersion was repeated at 10 days postinoculation. After their rectal temperature reached the desired level, it was maintained at that level for 30 min; the degree of temperature variation was ±0.1 °C. The electric thermistor was used to monitor temperature.

Drug Treatment. Tumor growth and the development of lung metastases examined in LLC-bearing mice exposed to 42 °C TBH treatments alone or concomitantly treated with cis-DDP (0.3, 1.0, or 3.0 mg/kg) or with MMC (0.1, 0.3, or 1.0 mg/kg). Five days after tumor inoculation, 42 °C TBH was induced within 15 min after the i.p. administration of the drug.

Inhibition of Tumor Growth. To examine the effect of each treatment on tumor growth, the tumor volume was measured at predetermined times, using a slide caliper. The tumor volume was calculated by the formula

\[ V = a \times b \times c \times \pi/6 \text{ (ml)} \]

where \( V \) is tumor volume, \( a \) is the longest line of the tumor, \( b \) is the axis transecting \( a \) at right angles, and \( c \) is the axis of a plane vertical to another plane including \( a \) and \( b \).

Lung Metastasis. LLC-bearing C57BL/6 mice were killed 17 days after tumor inoculation; MH-134-bearing C3H/He mice were killed at 21 days. Their lungs were stained according to the method of Wexler (22) by injecting 2 ml India ink through the trachea; they were washed with
water and allowed to stand for 24 h at room temperature in Fekete's solution. The number of lung metastases was examined under a dissecting microscope (10-fold magnification). The grade of metastasis was recorded according to the number of metastatic foci observed; Grade 0, no macroscopic metastases; Grade 1, 1 to 9 colonies; Grade 2, 10 to 29 colonies; Grade 3, 30 to 69 colonies; Grade 4, more than 70 colonies; Grade 5, total replacement of the lung with tumors.

**Lymph Node Metastasis.** LLC-bearing C57BL/6 mice were killed 17 days after tumor inoculation; MH-134-bearing C3H/He mice were killed at 21 days. Their bilateral inguinal, axillary, and lumbar lymph nodes were resected and studied histologically to obtain evidence of metastasis to these lymph nodes.

**Statistical Analysis.** All experimental results were analyzed by Student's t test and subjected to analysis of variance (2-layout method).

**RESULTS**

**Inhibition of Primary Tumor Growth in Heat-treated Mice**

Chart 2A shows the changes of primary LLC tumor volume over time. At 17 days after tumor inoculation, there was no difference between control mice and those exposed to one 40 °C TBH treatment. However, we noted a significant delay in tumor growth in mice treated with one or two 42 °C TBH sessions (P < 0.05). Two 42 °C TBH treatments were somewhat more effective than one 42 °C TBH treatment in inhibiting tumor growth, although the difference was not statistically significant.

In MH-134-inoculated mice, tumor volume on Day 21 was significantly inhibited in the 42 °C TBH group (P < 0.001) but not in the 40 °C TBH group (Chart 2B). Thermal treatment was not curative in any of the experimental groups.

**Thermal Effects on Metastasis**

**Lung Metastasis.** LLC-bearing mice were killed at 17 days after tumor inoculation, and MH-134-bearing mice were killed at 21 days. As shown in Table 1, in LLC-bearing mice, lung metastasis was neither potentiated nor inhibited by 40 °C TBH treatment. However, in mice treated once or twice with 42 °C TBH, we noted a significant (P < 0.01) increase in the average grade of lung metastasis. Two 42 °C TBH sessions tended to increase the grade of lung metastasis more than did a single session; however, the difference was not statistically significant. In MH-134-bearing mice, we observed no macroscopic evidence of lung metastasis in either the control or the TBH groups.

**Lymph Node Metastasis.** LLC-bearing mice were killed on
Day 17, and MH-134-bearing mice were killed on Day 21. The incidence of lymph node metastasis in 40 °C and 42 °C TBH-treated MH-134-bearing mice did not differ statistically from the control (Table 2). None of the LLC-bearing mice manifested lymph node metastasis.

Effects of Combined Thermal (42 °C) and Chemotherapeutic Treatments

Because 42 °C TBH facilitated the spread of LLC metastases into the lungs (Table 1), we examined the effects of combined chemotherapy (Table 3).

cis-DDP (0.3 mg/kg) and MMC (0.1 mg/kg) failed to inhibit the heat-induced metastatic spread. However, at 1.0- and 3.0-mg/kg doses of cis-DDP and at 0.3- and 1.0-mg/kg doses of MMC, combined chemotherapy inhibited the development of lung metastases. Most effective was the concomitant administration of cis-DDP (3.0 mg/kg); 4 of 19 (21%) mice were free of lung metastases. Most effective was the concomitant administration of cis-DDP (3.0 mg/kg); 4 of 19 (21%) mice were free of lung metastases. When MMC was the combination anticancer drug used, the 1.0-mg/kg dose level was most effective.

The antitumor effects of different treatment modalities on primary tumor growth are shown in Chart 3. At 0.3 mg/kg, cis-DDP showed no antitumor effect with or without combined TBH at 42 °C. However, at the higher doses (1.0 and 3.0 mg/kg) of this drug, significant tumor growth inhibition was noted in mice also subjected to TBH (P < 0.001). Similarly, in combination with TBH, the higher dose (1.0 mg/kg) of MMC inhibited tumor growth significantly (P < 0.001).

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inguinal Right</th>
<th>Inguinal Left</th>
<th>Axillary Right</th>
<th>Axillary Left</th>
<th>Lumbar Right</th>
<th>Lumbar Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (12)^a</td>
<td>67</td>
<td>17</td>
<td>50</td>
<td>17</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>40 °C (11)</td>
<td>45</td>
<td>0</td>
<td>82</td>
<td>0</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>42 °C (15)</td>
<td>60</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>93</td>
<td>0</td>
</tr>
</tbody>
</table>

a Positive metastatic rate was calculated at 21 days after tumor inoculation.

**Table 3**

<table>
<thead>
<tr>
<th>Treatment^a</th>
<th>Av. grade of lung metastases^b</th>
<th>Incidence of metastasis-negative lungs/no. of animals treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.6 ± 0.63^c</td>
<td>1/25</td>
</tr>
<tr>
<td>TBH (42 °C)</td>
<td>2.4 ± 0.98^d</td>
<td>0/26</td>
</tr>
<tr>
<td>cis-DDP (0.3 mg/kg)</td>
<td>1.6 ± 0.59^e</td>
<td>0/23</td>
</tr>
<tr>
<td>cis-DDP (1.0 mg/kg)</td>
<td>2.2 ± 0.66^f</td>
<td>0/23</td>
</tr>
<tr>
<td>cis-DDP (1.0 mg/kg) + TBH</td>
<td>1.4 ± 0.71</td>
<td>2/22</td>
</tr>
<tr>
<td>cis-DDP (3.0 mg/kg) + TBH</td>
<td>2.2 ± 0.66</td>
<td>0/23</td>
</tr>
<tr>
<td>cis-DDP (3.0 mg/kg) + TBH</td>
<td>2.2 ± 0.66</td>
<td>0/23</td>
</tr>
<tr>
<td>MMC (0.1 mg/kg)</td>
<td>1.7 ± 0.66^g</td>
<td>0/24</td>
</tr>
<tr>
<td>MMC (0.1 mg/kg) + TBH</td>
<td>2.4 ± 0.60^h</td>
<td>0/19</td>
</tr>
<tr>
<td>MMC (0.3 mg/kg)</td>
<td>1.5 ± 0.65</td>
<td>0/21</td>
</tr>
<tr>
<td>MMC (0.3 mg/kg) + TBH</td>
<td>1.6 ± 0.65</td>
<td>0/21</td>
</tr>
<tr>
<td>MMC (1.0 mg/kg)</td>
<td>1.3 ± 0.63</td>
<td>0/22</td>
</tr>
<tr>
<td>MMC (1.0 mg/kg) + TBH</td>
<td>1.4 ± 0.58</td>
<td>0/21</td>
</tr>
</tbody>
</table>

a At 5 days after tumor inoculation, the mice were treated with TBH at 42 °C and the indicated anticancer drug.

b Grade was calculated at 17 days after tumor inoculation.

c Average ± SD.

d Significantly different from c at P < 0.01.

e This group was compared with f.

f Significantly different from e at P < 0.01.

g This group was compared with h.

h Significantly different from g at P < 0.05.

**DISCUSSION**

Although the mechanism underlying the antitumor effect of hyperthermia in heat-sensitive malignant cells remains obscure, in our study on tumor-bearing mice, 42 °C TBH inhibited tumor growth in 2 strains (C57BL/6 and C3H/He) (Chart 2). Hyperthermia has been used in the treatment of human cancers (1, 4, 6). Since 1980, we have induced TBH by an extracorporeal circulation apparatus in patients treated at our clinic for unresectable terminal cancer. We were able to obtain favorable results by this treatment method (8).

However, hyperthermic treatment may accelerate tumor metastasis. Suzuki (20) and Muckle and Dickson (13) studied the effect of LH induction in experimental animals. They found that, in combination with anticancer drugs, LH inhibited the growth of primary tumors; however, they observed no life prolongation. This attributed to the spread of metastases to the lungs, liver, and lymph nodes which was not inhibited by the combined treatment. Walker et al. (21) reported the promotion of distant metastasis of C3H mouse mammary carcinoma by 42–46 °C LH. On the other hand, Yerushalmi (23) noted a delay in the first appearance of lung metastases and a decrease in the number of metastatic foci when LLC-bearing mice were treated with 42.2 °C or 43.5 °C LH. Schechter et al. (18) also reported that 42.3 °C LH applied to Me-H tumors in rats resulted in the...

**Table 3**

Effects of TBH (42 °C) and drugs on the incidence of lung metastasis in LLC-bearing mice

- **Table 3**

- **Chart 3**

- **Tumor Volume (ml)**

- **Means in parentheses, number of mice per group.**

CANCER RESEARCH VOL. 45 APRIL 1985

1534
inhibition of both primary tumor growth and metastasis to retroperitoneal lymph nodes. Other workers also presented data showing that LH induction decreased rather than increased the development of tumor metastasis (10, 19). We previously reported that, at 40 °C, 42 °C, and 43 °C, LH did not decrease or inhibit the development of lung metastasis from LLC and that, at 40 °C and 42 °C, LH did not affect lymph node metastasis from MH-134 (14). Furthermore, in that study, at 43 °C, LH significantly inhibited the spread of metastasis from MH-134 into the right inguinal and lumbar lymph nodes. On the other hand, at 41.9 °C, TBH enhanced lung metastasis from LLC (23), and 41.5 °C TBH enhanced lung metastasis from sarcoma L-1 (17). Our present and earlier study (14) showed that 42 °C TBH enhanced lung metastasis from LLC and that 43 °C LH inhibited lymph node metastasis from MH-134. The other temperatures, induced by either TBH or LH, did not enhance or inhibit the development of metastases from LLC or MH-134. At 21 days after MH-134 inoculation, there was no difference in metastasis between the control and heat-treated groups. Furthermore, there was no evidence of increased lymph node metastasis in MH-134-bearing mice subjected to TBH (Table 2). These observations suggest that the effect of hyperthermia on tumor metastases may differ according to the tumor strain.

Although the possible mechanisms of tumor metastases in hyperthermia is complicated, Dickson and Ellis (2) postulated that, in heat-treated Yoshida tumor-bearing rats, tumor cell dissemination was stimulated by the heat-induced hyperdynamic state of the circulation. However, our data show that the incidence of lung metastasis was increased only in 42 °C TBH-treated LLC-bearing mice but not in those subjected to 42 °C LH (14). Therefore, the manifestation of lung metastasis may be attributable to factors other than hyperthermia. Two factors that can affect tumor metastasis in hyperthermia could be pointed out: (a) changes of the tumor-bearing host, e.g., a reduction in host immunity and morphological or functional changes which facilitate implantation of tumor cells in host’s organs; (b) changes of tumor cells, e.g., alteration in the tumor cell membrane and intravascular increase by tumor cells derived from the change in tumor blood flow volume. Actually, it has already been indicated that immunological reduction took place in tumor-bearing hosts after thermal treatment (5–7). We are now studying the effects of TBH on the lungs and on the intravascular invasion by tumor cells.

Roszkowski et al. (17) reported that immunization could prevent the spread of lung metastases due to 41.5 °C TBH. In our study, metastatic spread was inhibited by combined chemotherapy (Table 3). This inhibition may be ascribable to the inactivation of tumor cells in the blood. Furthermore, we observed that the combined use of 42 °C TBH and anticancer drugs increased the inhibition of primary tumor growth to a greater degree than did 42 °C TBH alone or anticancer drugs alone. Although we have no data relating the incidence of lung metastases and primary tumor size in our experiment, this inhibition in primary tumor growth by combined chemotherapy and TBH may have influenced the reduction in metastatic spread.

We have performed 76 combined TBH and chemotherapy treatments in 27 patients with far-advanced cancer. No increase in remote metastasis was observed in that series. We believe that the combined treatment with TBH and anticancer drugs is advisable for the prevention of metastasis and the higher exertion of antitumor effect in clinical situations.

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Effects of Total-Body Hyperthermia on Metastases from Experimental Mouse Tumors

Masayuki Oda, Shigemasa Koga and Michio Maeta


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