ABSTRACT

This study was designed to test the effect of localized ultrasound-induced hyperthermia on experimental mouse tumors. Transducers operating at 5.17 MHz with relatively uniform output over an area 1 cm in diameter were used to heat EMT6 and KHJJ tumors in 407 BALB/cKa mice. Treatments were at 43, 43.5, 44, and 44.5°C. At each temperature level, treatments were applied for 15, 30, or 45 min. Temperature profiles measured in tumors treated by ultrasound hyperthermia indicated that heating was not completely uniform. In general, both rate of tumor eradication and growth delay increased as temperature and/or time of exposure increased. The EMT6 and KHJJ tumors had comparable rates of eradication for the same temperatures and times of exposure. Cell survival studies indicated that there was considerable variation in cell killing between individual EMT6 tumors exposed to the same hyperthermic dose. In addition, cell death appeared to be progressive over a period 2 to 48 hr after hyperthermic exposure. The mechanism of this delayed cell death is not known but may be important in eradicating the tumors. Ultrasound was a relatively safe and effective method of heating tumor volumes up to 44°C, and hyperthermia alone resulted in high rates of tumor eradication in the EMT6 and KHJJ systems.

INTRODUCTION

Clinical observations dating back to the turn of the century have suggested that in some cases tumors regressed after fever. More recent laboratory data in tissue culture and in experimental tumors have confirmed the impressions that heat may be effective in causing tumor regression (3, 11, 13, 16). In addition, hyperthermia has been shown to interact with both radiation and some chemotherapeutic agents to increase the effectiveness of these agents (1, 3, 4, 9, 17, 18). Several methods of inducing localized hyperthermia are available, including hot water baths, perfusion of an extremity with heated blood, shortwave diathermy, RF3 induced currents, microwaves, and ultrasound.

There are several advantages to ultrasound as a method of producing local hyperthermia (5, 8, 12). The technology for producing relatively uniform high-intensity ultrasound fields in most parts of the body is available. Unlike electromagnetic techniques, ultrasound does not interfere with standard methods of measuring temperature (20). It can be focused. In addition, multiple transducers to produce overlapping fields can be manufactured.

In this study, we have utilized ultrasound to heat 2 transplantable mouse tumors. Both tumors could be eradicated by ultrasound-induced hyperthermia. Cell survival in tumors after curative doses of hyperthermia was higher than that reported after curative doses of X-irradiation. Cells from tumors excised hours or days after 44°C ultrasound exposure had decreased survival. This delayed cell death after hyperthermic exposure may play a role in achieving tumor eradication.

MATERIALS AND METHODS

Ultrasound Heating Equipment. The device for ultrasound heating of murine tumors is depicted in block diagram in Chart 1. It consisted of a piezoelectric ceramic crystal transducer, operating at 5.17 MHz with a circular field 1.5 cm in diameter. The piezoelectric crystal was driven by a RF amplifier which was, in turn, driven by a function generator, operating in the sine wave mode. Power output of the amplifier was regulated by a feedback in a closed loop by intratumor temperature measurements. The operating frequency was monitored by an electronic frequency counter. The transducer housing in which the piezoelectric crystal was mounted provided a water path coupling ("cuff") of the ultrasonic energy between the crystal and the tumor. A clear flexible membrane of Mylar or latex covered the water cuff and was coupled directly to the tumor surface, using ultrasound gel. The mouse was restrained on a movable micrometer stage. The ultrasound field could be centered directly over the tumor by moving the stage so that a mark on the center of the tumor was directly beneath a mark in the center of the clear membrane. The water in the cuff was continually exchanged with a temperature-controlled reservoir to allow cooling of the skin over the tumor. The temperature of the water cuff was selected to provide linear heating of the tumor. Fig. 1 shows the transducer treating a transplanted tumor in the flank of a BALB/c mouse.

Ultrasound Field and Temperature Measurement. Relative ultrasound field intensity measurements were done in a circulating temperature-controlled water bath, using a silicone-covered 26-gauge thermocouple probe attached to a micrometer stage. The probe was moved across the ultrasound field 3 cm from the transducer. Relative intensity at each location was recorded by a chart recorder. The corresponding thermal field was mapped in tumors by inserting a 26-gauge thermistor needle (Yellow Springs Instrument Company, Yellow Springs, Ohio) into the center of a tumor and recording temperature readings as the tumor was moved across the ultrasound beam from left to right (x axis), and front to back (y axis). During treatments, temperatures were monitored continuously by 2 thermocouple needles (only one of which operated the feedback device).

Tumor Treatment. The transplanted tumors studied were the KHJJ mammary carcinosarcoma and the EMT6 tumor, both syngeneic in BALB/cKa mice. The EMT6 is the tissue culture-
Effects of Ultrasound Heating

Chart 1. Block diagram of the ultrasound device used to heat flank tumors. The diameter of the piezoelectric crystal was 1.5 cm, and its operating frequency was 5.17 MHz. Output of the amplifier was modulated to maintain the desired temperature by feedback from one thermocouple needle (Temperature feedback control). During treatments, routinely 2 thermocouple needles were inserted in the tumor, but the second readout temperature passively and did not contribute to the feedback. The temperature of the coupling water was 41°C.

Fig. 1. Transducer in use heating a flank tumor in a BALB/cKa mouse. A, transducer housing; B, circulating water cuff (Plexiglas casing); C, RF input to transducer; D, inflow tube for circulating water in cuff (outflow tube hidden by transducer); E, thermocouple needles inserted into anterior and posterior edge of tumor.

Adapted derivative of the KHJJ. Radiobiological and immunological characteristics of both tumors have been described previously (19). Recent studies indicate that both tumors are immunogenic (4). Tumors were planted in the flanks of BALB/cKa mice by intradermal inoculation of 10⁶ viable tumor cells and were used for treatment approximately 10 to 12 days later, when tumor volume was approximately 100 cu mm (MTD, 5 to 6 mm). Treatment was limited to tumors less than 7 mm in longest diameter, so all parts of the tumor were well within the ultrasound field.

Prior to treatment, animals were anesthetized with Diabutal (50 to 60 mg/kg). Tumors were shaved and ultrasound gel was applied. The mice were restrained on the micrometer stage with the tumor up, and 24-gauge thermistor probes were inserted into the anterior and posterior edges of the tumor. The ultrasound transducer and housing were then visually centered over the tumor. The position of the transducer in relation to the tumor was adjusted by moving the micrometer stage so that temperature readings from the 2 thermocouples were the same. Temperatures were monitored continuously throughout the treatment, and power output of the transducer was adjusted automatically to keep temperature at the desired level (Chart 1). Each animal received only one hyperthermic treatment. The average incident ultrasound powers and ranges (watts/sq cm) used to achieve each temperature were as follows: 43°C, 0.096 (0.061 to 0.102); 43.5°C, 0.098 (0.092 to 0.104); and 44°C, 0.106 (0.094 to 0.125). Control animals were anesthetized, had thermocouples inserted, and were sham "heated."

Following treatment, animals had their tumors measured twice weekly and were checked for burning and change in body weight. MTD was calculated as the geometric mean of 3 orthogonal caliper measurements. "Cure" or "eradication" was defined as complete disappearance of visible and palpable tumor for more than 45 days. No recurrences were noted after 30 days, although many animals were followed for more than 100 days. In a separate experiment, some animals were autopsied 45 to 100 days after tumor eradication, and no histological evidence of tumor was found. Untreated tumor-bearing animals died with local tumor growth. Mean time of death was 33 days after intradermal implantation of 10⁶ tumor cells (S.E., 1.3 days).

In Vitro Cell Survival Assay. Following treatment, tumors were excised, weighed, and minced, and a single-cell suspension was obtained by agitation for 30 min at 37°C in Hanks' balanced salt solution containing 0.03% pronase (B grade, 45 proteinase units Kappen/mg; Calbiochem, San Diego, Calif.), 0.007% DNase I (1760 Kunitz units/mg; Sigma Chemical Company, St. Louis, Mo.), and 0.15% collagenase I (140 units/mg, Sigma Chemical Company). The number of cells obtained

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4 C. Nager, J. B. Marmor, and G. M. Hahn. Tumor cell rejection following tumor cure by local hyperthermia, X-irradiation, or surgery, manuscript submitted for publication.

5 Adapted from J. M. Brown, personal communication.
from untreated tumors by this method averaged $1.7 \times 10^6$ cells/tumor and $2.3 \times 10^7$ cells/g of tumor tissue. Tumor cell viability was assayed by cloning in 60-mm Petri dishes, as described previously (11, 19). Clones were stained and counted after 8 days. Control plating efficiencies averaged 50%. "Clonogenic cells per tumor" was defined as the plating efficiency times the number of cells harvested per tumor. Values were expressed as percentage of control tumors.

**RESULTS**

**Ultrasound and Thermal Fields.** The ultrasonic and thermal fields produced by the 1.5-cm transducer, operating at 5.17 MHz, are shown in Chart 2. Intensity was relatively uniform around the perimeter of the ultrasound field mapped *in vitro* but was lower in the center (Chart 2a). A similar map was done in tumor tissue by sweeping the transducer in 1-mm increments across a tumor, with a thermistor needle implanted centrally and recording temperature measurements (Chart 2b). Temperature was uniform within ±0.5°C across a 1-cm diameter in both the X and Y axes but decreased rapidly outside this field. No central "cool" area was observed. At different depths within tumor, there was also some variation in temperature. A similar map done with a cuff temperature of 41°C and probes 2 and 4 mm deep in tumor showed about 1°C difference in temperature, with the deep probe registering the lower temperature (Chart 2c).

**Tumor Treatment Experiments.** Rates of tumor eradication for 15-, 30-, and 45-min exposures at temperatures between 43 and 44.5°C for the EMT6 and KHJJ tumors are shown in Tables 1 and 2. The rate of tumor eradication increased significantly between 43 and 43.5°C. In general, cure rates increased with longer exposure times at the same temperature. Eradication of 100% of tumors was not seen with ultrasound heating, as had been seen with RF heating (11). Although at most exposures, cure rates for the KHJJ tumor were slightly less than for EMT6, these differences were not statistically significant.

Exposure at 43°C resulted in no discernible host toxicity. Of 162 animals treated at 43.5°C, there was one death (0.6%); of 90 animals treated at 44°C, 2 died (2.2%); 44.5°C was more toxic, producing 5 of 30 (17%) deaths in animals treated for 45 min at this temperature. Autopsies on animals which died showed erythema, swelling, and perforation of bowel underlying the tumor, undoubtedly due to accidental ultrasound heating of the bowel wall and fecal contents. One animal had paralysis of both hind limbs following heating, probably due to unintentional heating of the spinal cord.

MTD doubling times were calculated for EMT6 and KHJJ tumors which had not been cured (Tables 1 and 2). Doubling time was from the treatment size (MTD, 5 to 6 mm). Values shown are the means ± S.E. for uncured tumors. For 43°C, the values were only slightly outside the control range, except for exposures of 45 min; 43.5 and 44°C gave longer growth delays at each time point. Longer heating times produced longer growth delays at each temperature, except for those exposures which resulted in a large proportion of cures (45 min at 43.5 and 44°C). For these exposures, the doubling time did not accurately reflect the effect on tumor growth, since eradicated tumors were not included in this figure.

**Tumor Cell Survival.** EMT6 tumors were excised following treatment *in situ* by ultrasound for 30 min at 43.5 or 44°C. A single-cell suspension was obtained, and tumor cell survival was assayed by cloning. Immediately after ultrasound exposure, recovery of morphologically intact tumor cells was about 30% of control (Chart 3). These cells were able to exclude trypan blue, but plating efficiencies were only 4 to 6% of control, indicating that most of these cells were not able to produce clones. Over the next few hr, the number of cells
Table 1
Cure rates and MTD doubling times for EMT6 tumors in BALB/c mice
Tumors were locally heated by ultrasound for varying temperatures and exposure times.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Exposure time (min)</th>
<th>N</th>
<th>Tumor eradicated &gt;45 days</th>
<th>Cure rate</th>
<th>Deaths</th>
<th>MTD doubling time* (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°</td>
<td>15-45</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12.9 ± 0.47*</td>
</tr>
<tr>
<td>43°</td>
<td>15</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14.1 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14.8 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>20</td>
<td>4</td>
<td>0.20 ± 0.09</td>
<td>0</td>
<td>15.9 ± 1.2</td>
</tr>
<tr>
<td>43.5°</td>
<td>15</td>
<td>30</td>
<td>8</td>
<td>0.27 ± 0.08</td>
<td>0</td>
<td>15.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>30</td>
<td>9</td>
<td>0.30 ± 0.08</td>
<td>0</td>
<td>19.0 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>30</td>
<td>18</td>
<td>0.60 ± 0.09</td>
<td>0</td>
<td>16.5 ± 1.3*</td>
</tr>
<tr>
<td>44°</td>
<td>15</td>
<td>10</td>
<td>2</td>
<td>0.20 ± 0.13</td>
<td>0</td>
<td>15.2 ± 0.77</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>11</td>
<td>3</td>
<td>0.30 ± 0.14</td>
<td>1</td>
<td>19.0 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>10</td>
<td>7</td>
<td>0.70 ± 0.14</td>
<td>0</td>
<td>19.3 ± 2.0°</td>
</tr>
<tr>
<td>44.5°</td>
<td>45</td>
<td>10</td>
<td>6</td>
<td>0.86 ± 0.13</td>
<td>3</td>
<td>25°</td>
</tr>
</tbody>
</table>

* Based on animals that had tumor recurrence. Tumors eradicated for >45 days did not recur up to 180 days and were considered cured. They were not included in this determination.

Table 2
Cure rates and MTD doubling times for KHJJ tumors in BALB/c mice
Tumors were locally heated by ultrasound for varying temperatures and exposure times.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Exposure time (min)</th>
<th>N</th>
<th>Tumor eradicated &gt;45 days</th>
<th>Cure rate</th>
<th>Deaths</th>
<th>MTD doubling time* (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°</td>
<td>15-45</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10.8 ± 0.32*</td>
</tr>
<tr>
<td>43°</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12.4 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>24</td>
<td>4</td>
<td>0.17 ± 0.08</td>
<td>0</td>
<td>13.2 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>26</td>
<td>4</td>
<td>0.15 ± 0.07</td>
<td>0</td>
<td>20.0 ± 1.6</td>
</tr>
<tr>
<td>43.5°</td>
<td>15</td>
<td>23</td>
<td>3</td>
<td>0.13 ± 0.07</td>
<td>1</td>
<td>14.0 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>26</td>
<td>4</td>
<td>0.15 ± 0.07</td>
<td>0</td>
<td>19.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>23</td>
<td>11</td>
<td>0.48 ± 0.11</td>
<td>0</td>
<td>16.4 ± 0.64*</td>
</tr>
<tr>
<td>44°</td>
<td>15</td>
<td>19</td>
<td>2</td>
<td>0.11 ± 0.07</td>
<td>0</td>
<td>12.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>21</td>
<td>4</td>
<td>0.20 ± 0.09</td>
<td>1</td>
<td>17.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>19</td>
<td>13</td>
<td>0.68 ± 0.11</td>
<td>0</td>
<td>22.7 ± 4.4°</td>
</tr>
<tr>
<td>44.5°</td>
<td>30</td>
<td>10</td>
<td>3</td>
<td>0.30 ± 0.14</td>
<td>0</td>
<td>21.7 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>10</td>
<td>8</td>
<td>1.0</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Based on animals that had tumor recurrence. Tumors eradicated for >45 days did not recur up to 180 days and were considered cured. They were not included in this determination.

DISCUSSION
This study has 2 aspects. The first is the utility of ultrasound as a method of producing local hyperthermia in tissues. The second is the effect of the ultrasound-induced hyperthermia on experimental tumors.

The study indicates that, for tumor exposures less than 44.5°, ultrasound was a safe method of heating murine flank tumors. Toxicity at higher temperatures was due to heating of the underlying bowel. Absorption of ultrasound by the feetal...
contents which are not cooled by circulation could have contributed to this toxicity, since it is possible that fecal contents became hotter than 44.5°. Necrosis of small areas of kidney or liver was only rarely seen, and never to an extent that would cause death.8

The major limitation to the use of ultrasound is that heating is not totally uniform, as shown on Chart 2c. Nonuniformity of heating has also been observed during ultrasound treatment of spontaneous tumors in dogs and cats (12). Tumor cure rates are undoubtedly influenced by the temperature of each microvolume of tumor. It is, therefore, likely that the nonuniformity of heating influenced the cure rates that we observed. Cure rates for transplanted EMT6 and KHJJ tumors treated by ultrasound hyperthermia were lower than those for the same tumors treated by RF-induced hyperthermia at the same temperature and exposure time (11). Nonuniformity of heating could also account, in part, for the large differences in cell survival between individual EMT6 tumors treated identically (Chart 4), although it is likely that other factors also play a role in this variability (see below).

Another aspect of the use of ultrasound to heat tumors is the question of nonthermal effects of the ultrasound. Effects on tumors by ultrasound have been reported in the past (2, 6, 7, 22). In some early studies, temperature measurements were omitted, and the role of hyperthermia induced by the ultrasound, if any, was not adequately documented (6, 22). Others have attributed ultrasonic effects on tumors to induced heat alone (2, 7). Nonthermal effects of ultrasound at elevated temperatures have been described by Li et al. (10). However, several lines of evidence suggest that the effects on the tumors in this study were due to induced hyperthermia alone. First, the intensity necessary to produce nonthermal cell killing in the study by Li et al. was greater (1.5 to 2 watts/sq cm) than the intensities used in this study (usually less than 0.1 watt/sq cm). Second, the average ultrasound intensity needed to produce 43° hyperthermia did not differ significantly from that to produce 44°, although cure rates and growth delay varied widely between these 2 temperatures. Finally, cell survival studies showed no significant difference in cell killing between tumors heated by water bath, ultrasound, or RF, provided that temperature and time of exposure were the same.

Cell survival assayed immediately after ultrasound-induced hyperthermia at 43.5 or 44° was 1 to 2%. In contrast, for X-ray or chemotherapeutic exposures, cell survival of less than 0.1% is required before comparable numbers of cures are observed in the EMT6 tumor system (19). However, if tumors were left in situ 2 to 48 hr after heat exposure, significant additional cell death apparently occurred in some tumors, and survival decreased to levels consistent with the observed cures. After 44°, survival decreased in a large enough proportion of tumors that mean cell survival also fell significantly. After 43.5°, although mean survival does not decrease significantly, survival in some tumors decreases to near 0.1% (Chart 4). The reasons for this marked variation in delayed cell death between tumors treated identically are unclear. Delayed cell death after RF heating has also been observed, suggesting that it is due to heat and not to ultrasound effects per se.6

Delayed cell death after heating has been noted by others, but the mechanisms are not known. Crile noted a similar phenomenon in heated Sarcoma 180 tumors in SWR mice (3). If the tumors were removed, minced, and transplanted immediately after heat exposure, they grew normally; if, however, removal was delayed by 4 hr, tumors appeared 1 to 2 weeks later, and if removal was 8 or 24 hr after heating, only 3% of the tumors grew. He suggested that the tumors were not destroyed by the heat itself but by an inflammatory response which occurred after heating. Overgaard (15), in careful morphological studies, showed evidence for increased lysosomal activity following heating of a C3H mammary tumor, and suggested that tumor cells were lysed by release of hydrolyases. Others have suggested that heating tumors may increase their immunogenicity (14). Both the Sarcoma 180 used by Crile and the EMT6 are immunogenic (Sarcoma 180 has a 15% spontaneous remission rate) (3). However, studies of systemic im-

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8 L. F. Fajardo, J. B. Marmor, and G. M. Hahn, unpublished data.
6 L. F. Fajardo, J. B. Marmor, and G. M. Hahn, unpublished data.

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mune responses failed to show any evidence of a heightened systemic immune response in animals cured by heat, compared to those cured by X-ray (21). These studies did not rule out increased local immune responses. A recent morphological study showed extensive damage to EMT6 tumor vasculature by hyperthermic treatment. In addition, infiltration with inflammatory cells was seen. Vascular damage due to hyperthermia may, in part, account for the large differences in cell survival between individual tumors. There may be a critical level of vascular damage required to eradicate a tumor; vascular damage less than that level may result in regrowth. Other factors may play a role in these large differences, such as irregularity of heating, or immunogenicity of the EMT6 tumor.

We conclude that ultrasound can be utilized as a safe and effective method of heating tumor volumes. Ultrasound heating was effective both in causing tumor regression and in eradicating EMT6 and KHJJ tumors. Although there was some variability, cure rates and tumor growth delay, in general, increased with temperature and time of exposure. Immediate cell killing by ultrasound could not account for the cure rates observed; however, delayed cell death appeared to occur in some tumors if left in situ 2 hr or more after heating. This delayed cell death may contribute to tumor eradication. The mechanism of the delayed cell death is unknown.

ACKNOWLEDGMENTS

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Tumor Eradication and Cell Survival after Localized Hyperthermia Induced by Ultrasound

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