Tumor Responses following Multiple Hyperthermia and X-Ray Treatments: Role of Thermotolerance at the Cellular Level

John L. Meyer, Ine Van Kersen, and George M. Hahn
Division of Radiobiology, Department of Radiology, School of Medicine, Stanford University, Stanford, California 94305

ABSTRACT

We have investigated the effect of increasing numbers of hyperthermia fractions given at 7-day intervals, with or without fractionated radiotherapy, on tumor cure, tumor growth, and cell survival after in vivo or in vitro heat. The murine RIF tumor was treated by capacitive radiofrequency hyperthermia at 44.0°C for 20 min for one to five treatments at weekly intervals (1–5 wk D). Single treatments (1 wk D) induced cure in 5% of tumors. Additional treatments (2–5 wk D) induced similar rates of cure (0–16%, P > 0.05 for 1 wk versus 2, 3, 4 or 5 wk D). 1 wk D resulted in marked growth delay compared to controls. Mean tumor diameter doubling times increased from 13.2 days to 27.5 days (P < 0.01). 2–5 wk D induced little additional growth delay (doubling times, 27.8–32.3 days, P > 0.05 for 1 wk versus 2, 3, 4 or 5 wk D). Fractionated radiotherapy of 3200 rads (400 rads given twice each week) significantly prolonged mean tumor doubling time to 26.2 days. The addition of one hyperthermia session to the fractionated radiotherapy (1 wk D + XRT) further increased doubling time to 34.2 days (P < 0.01). Additional treatments (2–5 wk D + XRT) only modestly increased doubling times (36.0–39.5 days, P > 0.05 for 1 wk versus 2, 3, 4 or 5 wk D). In vitro assay of cells dissociated from tumors 5, 10, or 15 days after 3 wk D showed increased survival to 44°C compared to previously untreated controls, and this cellular thermoresistance proved to be transient and noninheritable (i.e., thermotolerance). These results indicate that tumors can develop a prolonged thermal resistance after multiple weekly treatments which significantly modifies the response to subsequent treatment and which is associated with cellular thermotolerance.

INTRODUCTION

The number of hyperthermia fractions needed to achieve a substantial antitumor effect, with or without concurrent irradiation, is as yet poorly defined in human tumors (1). Biological investigations show that the cytotoxic effect of sequential treatments can be altered by the induction of thermotolerance (2), the selection of thermally resistant cells (3, 4), or environmental changes affecting tumor response to later hyperthermia (5). Studies on cells in culture indicate that thermotolerance usually reaches its maximum level 12–48 h after a thermal exposure and then decreases (2, 6). While differences may exist between the responses of cells in culture and tumors or normal tissues in situ (7), a similar kinetic pattern of thermotolerance development has been reported in some murine tumors (6, 8, 9), and in some normal tissues (8, 10). Tumor studies have shown a level of thermotolerance for as long as 5 days following treatment (9). We have examined the response of the RIF tumor to hyperthermia given repeatedly every 7 days and demonstrate the development of a longer thermal resistance. For sequentially increasing numbers of weekly hyperthermia exposures, alone or in combination with irradiation, single hyperthermia treatments were approximately as efficacious as multiple treatments in curing tumors or delaying their growth. To assess the basis for this, cells were dissociated from tumors 5 to 15 days after single or multiple treatments. Diminished cellular sensitivity to hyperthermia was identified which was transient and noninheritable.

MATERIALS AND METHODS

C3H mice from the Stanford pathogen free colonies were used and were 11–15 weeks old and weighed 30–40 g at the time of tumor implantation. The RIF tumor was used because of its low immunogenicity (verified in our system periodically by 50% tumor producing dose studies). The tumor has been characterized in detail elsewhere (11). Tumors were implanted into the flanks of animals by intradermal inoculation of 10⁶ tumor cells and were allowed to grow for 9–12 days until the mean tumor diameter reached 5–6 mm. Animals, 6 or more per treatment group, were anesthetized with sodium pentobarbital, 0.7 mg/g, and were subjected to local treatment with hyperthermia or irradiation.

The capacitive radiofrequency heating method used has been described previously (12). Temperatures were monitored constantly at the tumor center and were manually maintained ±0.2°C of the quoted value by frequent power adjustments. Warmup periods were less than 1 min; timings were begun after achieving the target temperature; after treatment, temperatures fell to <40°C within 1 min after the power was shut off. In a subset of animals, temperatures were also monitored at the most lateral tumor edge and both were recorded every 2 min; Fig. 1 shows that the lateral tumor temperatures were within 0.6°C of core tumor temperatures after 1 min and within 0.2°C after 5 min. To assess their responsiveness to hyperthermia, tumors were treated at 45°C for 30 min, 44°C for 30 min, and 44°C for 20 min. The rates of tumor cure at 30 days are shown in Table 1. For all subsequent experimental groups, 44°C for 20 min was used, since the exposures of higher temperature or longer duration produced high rates of cure.

Radiation therapy was given by placing tumors into bolus filled plastic jigs, which lightly held tumors at a fixed distance on the head of a 250-kVp orthovoltage unit. All tumors were treated to a midplane dose of 400 rads twice per week. To assess the tumor responsiveness to this fractionation, groups of animals were treated for 2, 4, 6, 7, or 8 weeks (1600, 3200, 4800, 5600, or 6400 rads); the growth profiles of these groups are shown in Fig. 2. No tumors were cured by irradiation alone. For all subsequent experimental groups, 4 weeks of treatment (3200 rads) were used.

Hyperthermia Experiments. Groups of animals received hyperthermia on day 1 of each week for 1, 2, 3, 4, or 5 weeks (1–5 wk D). Animals then were examined 2–3 times per week; caliper measurements of the tumors were taken in 3 dimensions and their mean diameters were calculated. Animals were followed for 60 days following treatment, or until death. Controls included tumors that received no hyperthermia (Control A) or received a treatment of 44°C for 20 min to a non-tumor bearing portion of flank skin (Control B); the tumor growth profiles of the two groups were not different (Fig. 3). Mean tumor diameters were plotted as ratios to their initial mean tumor diameter, and the time point to reach twice the value of the initial diameter was noted. The frequency of tumor cures was also recorded.

To assay the cellular sensitivity to hyperthermia, tumors from the 1-wk group were excised 7 days after D, and those from the 3-wk group were excised 5, 10, and 15 days after. Tumors were weighed, minced, and suspended in Hanks' solution containing 0.03% Pronase, 0.007% DNase, and 0.02% collagenase. Cell suspensions were filtered, centrifuged, resuspended in Waymouth's media containing 15% fetal calf serum, recentrifuged, and then resuspended in Waymouth's media. Cell counts were obtained with a hemocytometer, and graded dilutions were...
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Fig. 1. Tumor temperatures induced by the radiofrequency method used. ○, temperatures at center of tumor, which were maintained at 44.0 ± 0.2°C; •, temperatures at most peripheral aspect of tumor. Points, mean; bars, SD.

Table 1 Rate of tumor cures for single and multiple hyperthermia exposures

<table>
<thead>
<tr>
<th>Thermal exposure</th>
<th>No. of tumors</th>
<th>No. of cures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45°C for 30 min</td>
<td>15</td>
<td>7 (47)*</td>
</tr>
<tr>
<td>44°C for 30 min</td>
<td>7</td>
<td>2 (29)*</td>
</tr>
<tr>
<td>44°C for 20 min (D,)</td>
<td>21</td>
<td>1 (5)*</td>
</tr>
<tr>
<td>2 wk</td>
<td>6</td>
<td>0 (0)*</td>
</tr>
<tr>
<td>3 wk</td>
<td>39</td>
<td>4 (10)*</td>
</tr>
<tr>
<td>4 wk</td>
<td>9</td>
<td>1 (11)*</td>
</tr>
<tr>
<td>5 wk</td>
<td>49</td>
<td>8 (16)*</td>
</tr>
</tbody>
</table>

* P < 0.01, 45°C for 30 min versus 1 wk D,.
+ P < 0.05, 44°C for 30 min versus 1 wk D,.
# P > 0.05, 1 wk versus 2, 3, 4 or 5 wk D,.

The growth profiles of tumors not cured after receiving from 1 to 5 weekly D, exposures are shown in Fig. 4. The 1-wk D, group had marked growth delay compared to controls. The 2–5-wk D, groups had little additional delay in growth. The mean tumor diameter doubling times are shown in Fig. 5. Time was increased from 13.2 to 27.5 days (208%) in the 1-wk group (P < 0.01). The 2–5-wk groups had little additional growth delay (P > 0.05 for 1 wk versus 2, 3, 4 or 5 wk D,).

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Cells from tumors excised 10 days after 3-wk D, were also maintained in culture and the D, was delayed for 14 days (24 days after completing 3 wk D,); or they were reimplanted into animals after 18 days, reexcised after 12 days of growth, regrown in culture for 7 days, and given D, in vitro (47 days after D,; see Fig. 6). After incubation at 37°C for 9–11 days, clones were fixed, stained, and counted. Controls included cells that received no initial D, but the identical sequence of subsequent processing. Surviving fractions (the cloning efficiency of cells from treated tumors divided by the cloning efficiency of cells from control tumors) were calculated. The cloning efficiencies for control tumors were typically between 40 and 80%. D, values were calculated from the survival curve for each treatment group, and thermal tolerance ratios (TTR) were calculated as

$$TTR = \frac{D_0 \text{ (pretreated)}}{D_0 \text{ (control)}}$$

Hyperthermia and Radiation Experiments. In this series, all groups were treated with fractionated radiotherapy as described above (3200 rad, XRT); the 400-rad fractions were given on days 1 and 3 of each week. Hyperthermia was given on day 5 for 1, 2, 3, 4, or 5 weeks (1–5 wk D, + XRT). Tumors were measured as described 2–3 times per week for 60 days following treatment, or until death. Controls included animals receiving no treatment or fractionated radiotherapy alone. Mean tumor doubling times were calculated as described.

RESULTS

Tumors receiving 45°C for 30 min were cured in 47% of animals, and those receiving 44°C for 30 min were cured in 29% (Table 1). In contrast, only 16% or fewer were cured by multiple weekly exposures at 44°C for 20 min, indicating that their cumulative effect was significantly less curative than a single exposure of slightly longer duration or higher temperature [P < 0.01 for 45°C for 30 min, P < 0.05 for 44°C for 30 min, both versus 44°C for 20 min (D,)]. The differences in cure rates in groups 1–5 wk D, were not significant (P > 0.05 for 1 wk versus 2, 3, 4 or 5 wk D,).

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Cells that received graded D, exposures after 1 wk and 3 wk D, had increased cell survival compared to controls, indicating thermotolerance. Cells from the 1-wk group had a thermotolerance ratio of 1.57 7 days after D, The survival curve of cells 10 days after 3-wk D, is shown in Fig. 6. Cells that received D, 5 or 15 days after 3 wk D, had similar levels of thermotolerance.
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Fig. 4. Growth profiles of RIF tumors receiving hyperthermia (Di) of 44.0°C for 20 min every 7 days for 1 to 5 wk. Day 0 is first treatment day for all groups. Points, mean tumor diameter (MTD) divided by initial mean diameter for each animal; bars SE. Points are slightly offset to improve clarity. One wk Di induced marked growth delay compared to controls. Additional weekly Di induced little additional delay.

Fig. 5. Days to doubling of mean tumor diameter for groups receiving Di of 1 to 5 wk. Data are same as shown in Fig. 4. Points, mean; bars, SE.

(Fig. 7). Cells from the 10 day interval were put into culture for 14 days prior to Di; they had no such tolerance, confirming that it was transient. When reimplanted into animals, these cells also showed lack of tolerance when given Di 47 days after 3 wk Di.

Tumors that were treated with 1 wk Di in combination with 3200 rads, with hyperthermia and radiotherapy separated by 48–72 h, had delay in mean tumor diameter doubling time compared to controls receiving the same radiotherapy alone (Fig. 8) (P < 0.01). Tumors that were treated with 2–5 wk Di plus XRT had little additional delay in tumor doubling time (P > .05 for 1 wk versus 2, 3, 4 or 5 wk Di).

DISCUSSION

Our studies indicate that a murine tumor receiving multiple exposures of 44°C for 20 min at 7-day intervals develops significant levels of thermal resistance. Tumor cures increased only minimally and mean tumor diameter doubling times remained nearly constant for repeated weekly treatments; after 3 weekly treatments, in vitro survival of tumor cells to 44°C was increased for 5 to 15 days.

The addition of a fractionated moderate dose of irradiation (3200 rads) to the same sequence of hyperthermia treatments significantly increased the mean tumor diameter doubling times. However, the tumor doubling times remained nearly constant with increasing numbers of weekly hyperthermic exposures, corroborating the results using hyperthermia alone. In these investigations, the hyperthermia treatments were separated from the irradiation by 48–72 h to minimize interaction between the two modalities; it has been suggested that this separation may improve therapeutic gain by avoiding heat-

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induced sensitization of irradiation effects on normal tissues (13, 14).

Previously, we showed that the RIF tumor develops high levels of thermotolerance, maximal at 6–24 hr, after single treatments of 41°C for 15, 30, or 60 min (8). We have also observed high levels of thermotolerance in this tumor 24 to 48 h after 43°C for 30 min and after 45° for 10 min.2 Both of these investigations used the same tumor growth and cell assay techniques described in the present study. Treating this same tumor system but using an earlier heating device, Faria and Hahn (15) observed a tapering of the growth delay induced by increasingly prolonged single treatments at 44°C, which suggested them variation of response within the tumor.

Other investigators have evaluated the development of thermal resistance in murine tumors, often using single priming hyperthermia exposures. Kamura et al. (16) evaluated the response of a C3H mammary carcinoma to 43.5°C water bath heating for 30 min. Thermotolerance (evaluated by tumor growth delay) reached maximum levels after 16–24 h, with tolerance ratios of 5.2–3.8, and thereafter decayed to control levels by 120 h.

Urano et al. (9) evaluated the response of a C3Hf mammary carcinoma to up to 11 equal treatments of 43.5°C water bath given daily, up to 6 treatments given every 2 days, and up to 3 treatments given every 5 days. The level of thermal resistance observed in the 5-day group was far less than that in the daily and 2-day groups, as assayed by 50% tumor control dose. These investigators also evaluated the response of the FSA-I tumor to daily hyperthermia at 43.5°C, and noted that the 50% tumor control dose gradually increased from 83 min for 1 fraction to 367 min for 10 fractions, indicating an accumulation of thermal resistance and/or daily repair of damage. Also evaluating the response of the FSA-I tumor to D1 of 45.5°C for 10 min and variable D2 doses at the same temperature, they noted delay of tumor growth indicating thermal resistance 5 days after the D1.

Our investigations are similar in design to those of Kamura and Urano. Our results differ from theirs in the longer duration of the thermal resistance and its greater effect on growth delay at 7 days. Of note, the duration of thermotolerance reported by Kamura also differed from that reported by Urano. Overall, the results indicate that various tumors differ in the duration and magnitude of their thermotolerance development and emphasize the difficulty in developing general rules regarding the kinetics of thermotolerance in vivo. The findings are also in parallel with those of Rofstad et al., who showed variations in the magnitude of thermotolerance between different melanoma cell lines treated identically (17). The clinical implication is that some tumors, when treated with hyperthermia on a once- or twice-per-week basis, may have significantly reduced response because of persistent thermotolerance after the first treatment.

Our experiments using a D1 in vitro show that the thermal resistance measured by growth delay and tumor cure is also expressed at the cellular level, indicating thermotolerance. It is possible that other factors contributed to the thermal resistance in addition. Repeated thermal exposures have previously been shown to select thermally resistant cell populations (3, 4). It cannot be ruled out that some selection of thermally resistant cells occurred and that the redevelopement of base-line thermal sensitivity in our cell lines in culture represented overgrowth by thermally sensitive cells.

Thermotolerance has been shown to be a major influence on the response of tumor cells in culture, tumors in situ, and normal tissues in virtually all of the biological systems studied to date. While thermotolerance appears to be a universal phenomenon, differences exist in its degree of development and time course under various conditions (6, 18) and in differing tumor cells and normal tissues (8, 17). The use of repeated hyperthermia with several days between treatments has not been studied extensively, although it models current clinical practice. Our results indicate that tumor thermal resistance may persist over 7-day intervals and significantly alter the response of tumors to subsequent treatment. The factors of thermotolerance, selection of thermally resistant cells, and induction of tumor environmental changes have been demonstrated in prior studies and all may be contributing to the thermal resistance shown in this study. Because of the clinical importance of tumor thermal resistance after multiple exposures, the relative role of these factors and their possible modification remain important issues for further study in this and in other tumor models.

Overall our data suggest that maximum (or near-maximum) benefit from hyperthermia might be reached after only a limited number of heatings, perhaps only one or two. We are currently examining this possibility in the clinic in a randomized versus six heat treatment schedule. Preliminary results suggest no difference between the schedules (19), consistent with the murine data presented here.

REFERENCES

9. Urano, M., Rice, L. C., and Montoya, V. Studies on fractionated hyperthermia in experimental animal systems II. Response of murine tumors to two


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