Primary and Secondary Cell Death in Human Melanoma Xenografts following Hyperthermic Treatment

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ABSTRACT

The response to hyperthermic treatment (42.5°C for 60 min) of 5 human malignant melanomas grown in athymic mice (BALB/c/nu/nu/BOM) was studied. Local hyperthermia was given by immersing the tumor-bearing leg of the mice into a thermostatically regulated water bath. Growth delay studies indicated that the melanomas were different in heat responsiveness. The differences were confirmed by measuring the fraction of clonogenic cells in the melanomas as a function of time after treatment. The latter experiment showed that some tumor cells were inactivated during the treatment, while others lost clonogenicity first after completion of the treatment. Examinations of histological sections from tumors fixed 1 h after treatment revealed considerable vascular occlusion in all 5 melanomas. This indicates that the observed delayed cell death might be due to a number of factors, e.g., insufficient supply of oxygen and nutrients, increased tumor acidity, and accumulation of toxic metabolic products. It is concluded that at least two different mechanisms govern the overall heat response of the melanoma xenografts: the primary cell death, induced during treatment, is due to direct cytotoxic effects of the heat; the secondary cell death, induced after completion of treatment, is due to heat-induced vascular damage. The differences among the melanomas in overall heat responsiveness appeared mainly to be a consequence of differences in secondary cell death. The secondary cell death was shown to be least pronounced for those melanomas in which most of the larger vessels were embedded in broad bands of connective tissue.

INTRODUCTION

Several studies have shown that hyperthermic treatments may induce changes in the vascular function of both normal tissues and experimental tumors (1, 2). The blood flow in most normal tissues is reversibly increased by treatments at temperatures commonly used for clinical hyperthermia (3–7). The changes induced in the vasculature of tumors are highly temperature dependent and vary among different tumor lines. Mild hyperthermic treatments cause either no change or a modest, reversible increase in blood flow (8–10). However, at therapeutic temperatures, hyperthermic treatments may lead to significant vascular damage and reduced blood flow in many tumors (11–16). Detrimental effects on the microcirculation usually occur after treatments at 40.5–43.5°C for 30–60 min, depending on the tumor type (1, 2).

In some tumors, heat-induced vascular damage may result in a secondary, progressive cell death; i.e., cells are killed during a period lasting up to a few days after completion of a heat treatment as a consequence of reduced perfusion (17–21). Thus, Kang et al. (18) and Song et al. (21), who studied the SCK tumor, found that the fraction of clonogenic cells was reduced from about 0.2 to about 6 × 10⁻³ within 12 h after exposure to 43.5°C for 30 min. Similarly, Fajardo et al. (17) and Marmor et al. (19) demonstrated in the EMT6 tumor that the fraction of clonogenic cells decreased from about 1 × 10⁻² to about 3 × 10⁻⁴ during a 48-h period after treatment at 44°C for 30 min. However, results from the same laboratory (22) showed that secondary cell death did not occur in the RIF tumor after the same treatment, indicating that the phenomenon may depend on the tumor type and/or the treatment temperature.

The effects of heat treatment on human tumors grown in congenitally athymic mice are currently studied at our institute. The experiments have mainly been performed on 5 melanoma xenograft lines, originally derived from different donor patients. Studies of growth delay after treatment in vivo (23) and single cell survival after treatment in vitro (24) have shown that these melanomas are highly different in heat responsiveness. However, the heat response in vivo was not correlated to that measured in vitro after treatment of single cells (23). This discrepancy might be due to secondary, delayed cell death after treatment in vivo. In fact, microangiographic studies of one of the melanoma lines revealed that heat treatment at 42.5°C for 60 min resulted in a marked deterioration of the functional vascular volume, and this effect led to secondary cell death which contributed significantly to the growth delay (20). In the present communication, we show results from studies of secondary cell death following treatment at 42.5°C for 60 min for 5 melanoma lines. The results are discussed in relation to those from studies of growth delay in vivo and cell survival in vitro. The purpose of extending our work on secondary cell death from 1 to 5 melanomas was triplicate: (a) to determine to what extent the magnitude and kinetics of secondary cell death may vary among melanomas; (b) to analyze to what extent secondary cell death may contribute to differences in overall heat response; and (c) to make an attempt to find correlations between secondary cell death and vascular parameters.

MATERIALS AND METHODS

Mice and Tumors. BALB/c/nu/nu/BOM mice of both sexes, bred at the animal department of our institute, were used. They were kept under specific pathogen-free conditions.

The melanoma xenograft lines (E. E., E. F., G. E., M. F., V. N.) were originally derived from lymph node metastases of patients admitted to The Norwegian Radium Hospital. Tumor tissue was transplanted directly into athymic mice without previous adaptation to in vitro culture conditions. Histologically, the metastases were composed of melanin-poor...
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atypical nevus cells varying greatly in size and shape. Numerous mitoses were seen.

The melanomas were grown serially in athymic mice by implanting tumor fragments, approximately 2 x 2 x 2 mm in size, s.c. into the flanks of recipient mice. Passages 25-46 of the melanomas were used in the present work. Small tumor fragments were implanted s.c. in the right hind leg (25). The tumors were heated when they attained a volume within the range 150-300 mm³. Light- and electron-microscopic examinations showed that the histological appearance of the xenografts was similar to that of the metastases in the donor patients.

**Heat Treatment.** The tumors were heated by immersing the tumor-bearing leg into a water bath. Non-anaesthetized mice were placed in Perspex mouse holders and immobilized by a piston. A hole was cut in the holders through which the leg with the tumor protruded. The leg was loosely fixed with tape below the tumor. Precautions were made to avoid impairment of the blood flow in the leg. The temperature of the water bath was thermostatically kept at 42.7°C. The temperature in the tumors, measured with a needle thermocouple probe (diameter, 0.7 mm) connected to an electric universal thermometer (Ella, type TE 3-S; Ella Instruments A/S, Copenhagen, Denmark), was 42.5 ± 0.1°C (SE) a few minutes after the tumors were immersed in the water bath and remained within this range during prolonged treatments. Experiments showed that the temperature readings did not vary significantly with the position of the probe within a tumor or among individual tumors (Chart 1). Care was taken to ensure that the temperature measurements were not influenced by thermal conduction along the thermocouple probe or by leakage of water into probe-tumors, as described previously in detail (26). The mice were air-cooled during the heat treatment. The body core temperature, measured with a rectal probe, was kept at 37°–38°C.

**Growth Delay Assay.** Growth curves for heated and unheated tumors were established on the basis of caliper measurements of tumor volume. Two perpendicular diameters (length and width) were recorded, and the tumor volumes were calculated as \( V = \frac{1}{2} ab^2 \), where \( a \) and \( b \) are the longest and the shortest diameter, respectively. Since the skin around the tumors was thin, the measured tumor diameters were not corrected for the skin thickness. The time taken for the heated \( (T_2') \) and the control tumors \( (T_2) \) to double their volume as measured immediately before treatment was recorded. Tumor growth delay was calculated as \( T_2' - T_2 \).

**Colony Assay.** The clonogenicity of the tumor cells was measured in vitro by using the soft agar colony assay developed by Courtenay (27) and Courtenay and Mills (28). Since the yield of morphologically intact cells was higher after mechanical than after enzymatic dispersion of the tumors, single-cell suspensions were prepared without the use of enzymes. The tumors were finely minced by a scalpel and a pair of tweezers in culture medium [Ham's F-12 medium with 20% fetal calf serum, penicillin (250 mg/liter), and streptomycin (50 mg/liter) (Gibco-Biocult, Glasgow, Scotland)]. The resulting suspensions were filtered through 30-μm filters. The cell concentration was determined by the use of a hemocytometer. The number of host cells in the tumors, especially macrophages, tended to increase when the tumors were left in situ for some days after heat treatment. The host cells could usually be distinguished from the melanoma cells on the basis of size. Melanoma cells having an intact and smooth outline with a bright halo were scored as morphologically intact and counted. The soft agar was prepared from powdered agar (Bacto agar; Difco, Detroit, MI) and culture medium. Erythrocytes from August rats and melanoma cells were added as described previously (29). Aliquots of 1 ml of soft agar with the appropriate concentration of melanoma cells were seeded in glass tubes. Immediately afterwards, the tubes were flushed with a gas mixture of 5% \( \text{O}_2 \), 5% \( \text{CO}_2 \), and 90% \( \text{N}_2 \) and were carefully sealed. The tubes were incubated at 37°C for 3 to 4 weeks (E. E., E. F.), 4 to 5 weeks (M. F., V. N.), or 5 to 6 weeks (G. E.). Culture medium (2 ml) was added 5 days after seeding and was later changed weekly. The tubes were flushed on each occasion and also once between each medium change. A stereomicroscope was used to count colonies. The dense colonies formed by melanoma cells could easily be distinguished from the loose colonies formed by macrophages. Melanoma cells giving rise to colonies larger than 50 cells were scored as clonogenic. The plating efficiency of untreated cells was 5 to 15% (G. E.), 15 to 35% (E. E., M. F., V. N.), or 30 to 60% (E. F.) and was independent of the number of cells seeded in the range 100 to 5000 cells/tube. Heavily irradiated "feeder" cells, up to 100,000 cells/tube, neither enhanced the plating efficiency nor changed the surviving fraction of heated cells. Consequently, such "feeder" cells were not used in the present work. Details of the experimental procedure are reported elsewhere (29).

**RESULTS**

Growth curves for heated control tumors and tumors heated at 42.5°C for 60 min are presented in Chart 2. Statistical analysis showed that the heat response was independent of the pretreatment tumor volume, which ranged from 150 to 300 mm³. Thus, mean normalized tumor volume was plotted versus time after treatment in Chart 2. Heating resulted in inhibition of tumor growth before the tumors again grew exponentially. The exponential part of the growth curves was nearly parallel for the unheated and the heated tumors. The volume-doubling time for the unheated tumors \( (T_2) \) and the growth delay \( (T_2' - T_2) \) for the heated tumors are presented in Table 1. The heat responses measured in the present work were in good agreement with those reported previously for the same melanoma xenograft lines, although the tumors were somewhat smaller than were those used in earlier studies (23).

In order to study the mechanisms behind the overall heat response of the melanomas, the fraction of clonogenic cells in the tumors was measured at different times after treatment at 42.5°C for 60 min (Chart 3). The cell yield, i.e., the number of cells obtained per unit tumor volume, was determined for both heated and unheated tumors. All tumor volumes were measured immediately before treatment. The relative cell yield, i.e., the cell yield from the heated tumors relative to that from the unheated ones, fell significantly below 1.0 at 0.5–7 days after treatment (Chart 3a). The cell yield from the unheated tumors and the
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Chart 2. Mean relative tumor volume for human melanoma xenografts as a function of time after heat treatment (42.5°C for 60 min). Each curve is based on at least 15 tumors. Bars, SE.

Table 1

<table>
<thead>
<tr>
<th>Melanoma</th>
<th>( T_2 ) (days)</th>
<th>( T'<em>{2} - T</em>{2} ) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. E.</td>
<td>3.3 ± 0.2a</td>
<td>13.9 ± 1.4</td>
</tr>
<tr>
<td>E. F.</td>
<td>10.7 ± 1.0</td>
<td>16.3 ± 2.9</td>
</tr>
<tr>
<td>G. E.</td>
<td>3.2 ± 0.3</td>
<td>13.0 ± 0.9</td>
</tr>
<tr>
<td>M. F.</td>
<td>9.5 ± 1.2</td>
<td>29.5 ± 3.7</td>
</tr>
<tr>
<td>V. N.</td>
<td>3.5 ± 0.2</td>
<td>9.1 ± 0.8</td>
</tr>
</tbody>
</table>

\[ a \text{ Mean ± SE.}\]

tumors excised immediately after treatment were not significantly different. The relative plating efficiency, i.e., the plating efficiency of the morphologically intact cells from the heated tumors relative to that from the unheated ones, is shown as a function of time after treatment in Chart 3b. This chart demonstrates that the clonogenicity of the morphologically intact cells decreased when the tumors were left in situ for hours to days after treatment. The relative cell yield (Chart 3e) and the relative plating efficiency (Chart 3b) were multiplied to obtain the fraction of clonogenic cells, i.e., the number of clonogenic cells per unit tumor volume relative to that for unheated tumors (Chart 3c). The fraction of clonogenic cells decreased after treatment and reached a minimum within 0.5–3 days. This decrease indicates that a considerable fraction of the tumor cells was killed subsequent to completion of the heat treatment. For some of the melanomas the fraction of clonogenic cells increased beyond the third day after treatment, indicating that the total number of repopulating cells was larger than the total number of dying cells.

Cell survival following treatment in vitro at 42.5°C for 60 min has been reported previously for cells from the present melanoma xenografts (30). Chart 4 shows the surviving fraction after treatment in vivo, i.e., the fraction of clonogenic cells measured immediately after treatment (from Chart 3c), as a function of the surviving fraction measured after treatment in vitro. The survival levels measured after treatment in vivo were positively correlated with those measured after treatment of single cells in vitro, but they tended to be somewhat lower than those of the latter.

Examinations of histological sections from tumors fixed 1 h after treatment at 42.5°C for 60 min revealed that many vessels, capillaries as well as larger vessels, were occluded (Fig. 1). The corpuscles often appeared rigid and deformed and were densely packed in the lumen of the vessels. Vascular occlusions were not observed so frequently in vessels surrounded by bands of connective tissue as in vessels lacking such bands. Most of the larger vessels in the E. F. and V. N. melanomas were embedded in connective tissue, whereas in the E. E., G. E., and M. F. melanomas only sparse bands of connective tissue were seen (Fig. 2). The amount of connective tissue in tumors may be quantified by the use of stereological techniques, but this was not attempted in the present work, since the melanomas could easily be divided into two distinct groups on the basis of ordinary light microscopic examinations.

DISCUSSION

The volume-doubling times for the E. E., G. E., and V. N. melanomas were not significantly different, whereas the growth delay by heat was found to be shorter for the V. N. melanoma than for the E. E. and G. E. melanomas (Table 1). Similarly, the E. F. melanoma showed a shorter growth delay than did the M. F. melanoma, while the volume-doubling times for these two melanomas were comparable (Table 1). The curves for the
Chart 3. Cell yield per unit tumor volume (a), plating efficiency (b), and number of clonogenic cells per unit tumor volume (c) for human melanoma xenografts as a function of time after heat treatment (42.5°C for 60 min). Tumor volumes were measured immediately before treatment. All values are expressed as fractions of the values for unheated control tumors. The fraction of clonogenic cells was obtained by multiplying the relative cell yield and the relative plating efficiency for each tumor. Each point is based on 4–6 tumors. The relative plating efficiency for each tumor was calculated from the number of morphologically intact cells seeded and the mean number of colonies in 4–5 tubes with cells from heated tumors and 4–5 tubes with cells from unheated tumors. Bars, SE.

Fraction of clonogenic cells in the melanomas (Chart 3c) are compared in Chart 5. Chart 5a refers to the most rapidly growing melanomas (E. E., G. E., V. N.), and Chart 5b refers to the most slowly growing ones (E. F., M. F.). The shape of the curves is determined by several, partly competing processes, e.g., mitotic delay, primary cell death, secondary cell death, and repopulation.
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During the first 12–24 h after treatment the fraction of clonogenic cells decreased, i.e., the total number of dying cells was larger than the total number of repopulating cells. On the other hand, beyond the third day after treatment for the rapidly growing melanomas and somewhat later for the slowly growing ones, the fraction of clonogenic cells increased, indicating that the repopulating cells were in the majority. Because of this masking effect due to the repopulation, the true surviving fractions were lower than was indicated by the nadir of the curves in Chart 5, i.e., lower than about $5 \times 10^{-3}$. Consequently, the heat sensitivity of the melanomas cannot be assessed from Chart 5. However, Chart 5 shows that the fraction of clonogenic cells began to increase earlier in the V. N. melanoma than in the E. E. and G. E. melanomas and earlier in the E. F. melanoma than in the M. F. melanoma. The curves in Chart 5 are therefore in good agreement with the growth delay data, indicating that the V. N. melanoma was more heat resistant than were the E. E. and G. E. melanomas and that the E. F. melanoma was more heat resistant than was the M. F. melanoma.

A positive correlation was found between the fraction of clonogenic cells in the melanomas measured immediately after treatment in vivo and the surviving fraction measured after treatment of single cells in vitro (Chart 4). This finding indicates that during the treatment in vivo, tumor cells were inactivated mainly because of direct cytotoxic effects of the elevated temperature. The fraction of clonogenic cells in the melanomas decreased further during the following hours or days after the treatment was completed. Histological sections prepared from tumors fixed 1 h after treatment showed that a large fraction of the vessels was occluded in all melanomas (Fig. 1). It seems, therefore, that the secondary cell death was caused by a variety of factors, e.g., protracted hypoxia, increased acidity, nutrient deficiency, and accumulation of toxic metabolic products. Consequently, at least two different mechanisms are involved in the heat-induced cell inactivation in the xenografts: firstly, the primary cell death, induced during treatment, is due to direct cytotoxic effects of the heat; and secondly, the indirect, secondary cell death, induced after completion of treatment, is due to a transient or a permanent heat-induced collapse of parts of the vascular system.

As discussed above, the melanoma xenografts were different with respect to overall heat response (Table 1). The fraction of clonogenic cells measured immediately after treatment differed within a factor of about 2 among the rapidly growing melanomas as well as among the slowly growing melanomas (Chart 5). It can be shown that tumor growth delay for the rapidly growing melanomas does not correlate with the fraction of clonogenic cells as measured immediately after treatment. Furthermore, the large difference in growth delay between the E. F. and M. F. melanomas cannot be explained from the relatively small difference in the primary cell death. Thus, although the cell death due to the direct cytotoxic effects of the heat varied among the melanomas, this primary cell death did not give the main contribution to the differences in overall heat response. Consequently, the difference in heat response among the melanomas were primarily due to differences in the kinetics and the magnitude of the secondary cell death.

The following question then arises: why did the secondary cell...
death vary so much among the different melanomas? Secondary cell death is probably a complex phenomenon depending on several biological characteristics of tumors. Firstly, it may depend on the requirements of the tumor cells for oxygen and nutrients. These requirements, and hence the secondary cell death, may have varied among the melanomas because of a difference in the growth fraction and the cell cycle time (31, 32). Secondly, secondary cell death may depend on the pH distribution in tumors, since it has been shown that the degree of heat-induced vascular damage, as well as the ability of cells to survive under hypoxic conditions, may be pH dependent (33, 34). Thirdly, the secondary cell death may have varied among the melanomas due to differences in the architecture of the vascular network. The extent of heat-induced vascular damage in tumors may depend on the frequency of elongated capillaries, redundant bending capillaries, and lacuna-like sinusoids. In fact, detailed studies have shown that the present melanoma xenografts exhibit individual and characteristic vascular morphologies (35).

Secondary cell death following heat-induced vascular damage has been reported previously for the SCK (18, 21) and EMT6 (17, 19) murine tumors. The present work demonstrates the phenomenon in 5 human melanoma xenografts. It should be noted that the vascular system and the supporting stromal elements of the melanoma xenografts were of murine origin. Secondary cell death of similar magnitude as observed for the xenografts may therefore occur in tumors in patients only if human tumor blood vessels are as vulnerable to heat as are tumor vessels of murine origin. However, if this is so, severe vascular damage may occur so frequently in tumors during hyperthermperative treatments that it should be taken into consideration in the design of clinical treatment protocols involving hyperthermia.

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REFERENCES

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Fig. 1. Light micrographs (100x (a) and 400x (b)) of the G. E. melanoma fixed 1 h after heat treatment (42.5°C for 60 min). The micrographs illustrate that many vessels are occluded.

Fig. 2. Light micrographs (400x) of the V. N. (a) and the E. E. (b) melanoma. The vessel in the V. N. melanoma is embedded in a band of connective tissue but that in the E. E. melanoma is not.
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