Relationship between Heat-induced Vascular Damage and Thermosensitivity in Four Mouse Tumors

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ABSTRACT

The relationship between heat-induced vascular damage and thermosensitivity was studied using four mouse transplantable tumors. The tumors used were spontaneous mammary carcinoma, SCC VII carcinoma, EMT6 sarcoma, and B16 melanoma. Under cultured conditions, B16 was more thermosensitive at 43°C and 44°C than SCC VII or EMT6. The in vivo tumor response to heat was evaluated by the growth delay after heating at 44°C for 30 min. Among the four tumors, SCC VII was the most thermosensitive in vivo followed by EMT6, whereas B16 and spontaneous mammary carcinoma were thermoresistant. Vascular damage was studied quantitatively up to 24 h after heating by using microangiography. The order of the four tumors in vascular damage was well correlated with the tumor response in vivo. Histologically, tumor vessels of spontaneous mammary carcinoma were supported by connective tissues, and those of B16 had dense endothelial cells, compared to sparse endothelial cells of SCC VII and EMT6. Our findings suggest that variability in heat sensitivity of tumors is related to variation in the histological structure of tumor vasculature. That is, tumor vasculature with perivascular connective tissues and/or dense endothelial cells is less heat labile than that composed only of sparse endothelial cells.

INTRODUCTION

Thermal damage in tissues is greatly affected by the changes in the tissue blood flow during heating, since temperature is dependent on blood flow rate (1–3). In addition, blood flow also controls the intratissue microenvironment, which affects the thermosensitivity of the tissues (1, 2). An acidic and nutritionally deprived environment is known to potentiate the thermal injury to mammalian cells in vitro (1, 2, 4). The intratissue acidity and the supply of nutrients are directly related to the blood perfusion to the tissues.

The heat-induced change of blood flow in tumors as well as in normal tissues of animals has been studied extensively (1, 3, 5, 6). Using microangiography and correative histological sections, we first studied the effects of hyperthermia on the microcirculation of two types of mouse tumors (7). In the present study, we compared the changes of vascular damage following hyperthermia and tumor response in vivo in 4 different types of mouse tumors. To determine the inherent heat sensitivity of the tumor cells, cell survival fractions after heating in vitro were also investigated in 3 of the 4 tumor cell lines.

MATERIALS AND METHODS

Tumors and Mice. MCa1 of the C3H/He mouse, SCC VII carcinoma of the C3H/He mouse, B16 melanoma of the C57BL mouse, and EMT6/KU sarcoma of the BALB/c mouse were used. C3H/He mice were obtained from the Animal Center of Kyoto University, and C57BL and BALB/c mice were from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Some of the biological characteristics of the tumors were described previously (7–9).

Spontaneously arisen mammary tumors of a C3H/He mouse were minced and preserved in several ampuls in liquid nitrogen. When required, the content of an ampul was thawed and transplanted to new recipients, and the tumors from this second transplanted generation were used for the experiments. The tumors were minced with scissors in Hanks’ medium supplemented with 5% fetal bovine serum. The supernatant was filtered through gauze to obtain a cell suspension.

About 2 x 10^6 viable cells were inoculated s.c. into the right thigh of 8-week-old male C3H/He mice.

The in vitro culture conditions, tumor transplantation methods, and experimental procedures were the same for SCC VII, B16, and EMT6 tumors. The 3 tumor cell lines were thawed from original frozen stocks and maintained by alternate passage in syngeneic mice and cell culture in Eagle’s minimal essential medium supplemented with 12.5% fetal bovine serum. Approximately 1 x 10^6 cells collected from monolayer cultures were inoculated s.c. into the right thigh of syngeneic 8-week-old male mice. Experiments were performed when the tumors reached 10–12 mm in the longest diameter for all 4 tumor types.

Cell Survival Fraction. The cell survival fraction after heating in vitro at 43°C and 44°C was estimated for SCC VII, B16, and EMT6 tumors. A water bath (Model ET-35P; Toyo Seisakusho Co., Ltd., Tokyo, Japan) was used for heat treatment, and the temperature was maintained at a set temperature within 0.05°C. Approximately 3 x 10^6 cells were seeded as monolayers in glass flasks (25 cm²) and cultured for 24 h. Exponentially growing cells in the flask were heated by immersing the flasks in a water bath set at heating temperature. After the treatment, cells in each flask were trypsinized to obtain a single cell suspension and the cell number was determined. After the serial dilution of cells in each flask, 1 ml from each cell suspension was seeded in plastic plates filled with fresh medium.

The cells in the plastic plates were incubated for 7–10 days to obtain macroscopic colonies composed of 50 cells or more. The colonies were stained with Giemsa solution, and the number of colonies per plate was counted. The cell survival fraction after each treatment was estimated as the ratio of number of colonies formed and the number of cells inoculated. The estimated fractions were normalized to the surviving fraction of the untreated control. Four replicate plates were used for each survival point, and the replicate experiments were repeated 3 times. The data points represent the mean of 12 plates, and the standard error of the mean is given for each survival point.

Growth Delay Assay. Hyperthermia was given by immersing the animal’s foot in a water bath. The mice were held in a specially constructed jig with the tail and left leg firmly fixed by taping them to the jig. The right tumor-bearing leg was pulled down by a special sinker (approximately 45 g), which was fixed to the skin of the toe with super glue (Aron-arufa; Konishi Co., Ltd., Osaka, Japan). The fully awake mice were then placed on a circulating water bath maintained at the desired temperature, and the extended right leg was locally heated. The mice were air-cooled during the heat treatment. All temperatures mentioned in this paper refer to the water bath temperature.

Intratumor thermometry data have been described elsewhere (7). Generally, temperatures at the tumor center were equilibrated within 3–4 min after immersion in the water bath and remained 0.2–0.3°C below the water bath temperature. The temperature difference between the tumor center and the periphery was within 0.1°C.

The tumor response to heat was evaluated by growth delay analysis.
for the 4 tumors. Three perpendicular diameters of each tumor were measured every 2 days after the treatment using a caliper. Each treatment group consisted of 11–18 mice.

Vascular Damage following Hyperthermia. Vascular damage following hyperthermia was evaluated by microangiography and correlative histological sections. The technique of microangiography was described previously (7). Briefly, a filtered barium sulfate solution (0.25 g/ml) was injected at a pressure of 150 mm Hg after flushing the circulatory system with warmed heparinized saline. When the muscular vasculature of the left normal hind limb was not opacified sufficiently in the in vivo microangiogram, the mouse was excluded from the study to avoid a poor filling artifact. After the tumor was fixed with 10% buffered formalin, contact radiographs of 1-mm-thick tumor slices were obtained. The tumor slice was cut along a sagittal direction through its center with as much surrounding normal tissues as possible. Correlative histological sections 4 μm thick were prepared for each tumor slice and stained with hematoxylin and eosin.

Microangiographic changes were analyzed quantitatively as follows (7). Opacified vascularized areas and avascular areas in a tumor were demarcated on an enlarged microangiogram (approximately ×10), and the vascularized areas (V) and the entire tumor area (T) were measured using a digital planimeter (Planix 7; Tamaya Co., Ltd., Tokyo, Japan). Thereafter, the percentage of vascularized area was calculated as V/T × 100 in each microangiogram. The mean SE of the percentage of vascularized area was obtained for 5–9 angiograms.

RESULTS

Cell Survival Fraction. Fig. 1 shows the time-survival curves of SCC VII, B16, and EMT6 cells exposed to 43°C and 44°C. B16 was most thermosensitive among the 3 cell lines when the cells were exposed to 43°C, but the difference was not clear at 44°C heating.

Growth Delay Assay. Fig. 2 shows the tumor growth curves for unheated control tumors and tumors heated at 44°C for 30 min. For all 4 tumors, the exponential part of the growth curves was parallel for the unheated and heated tumors. Table 1 shows the time required for the tumor to reach twice its initial volume (volume-doubling time). The volume-doubling time for the heated (72') and the control tumors (72) was measured. Specific growth delay ([72'−72]/72) allows comparisons to be made between the response to treatment of tumors with different rates of growth prior to treatment. The specific growth delay was 0.8 ± 0.6 (SD) for B16, 1.0 ± 0.4 for Mca, 1.4 ± 0.8 for EMT6, and 2.1 ± 0.6 for SCC VII. The specific growth delay for SCC VII was significantly longer than that for the other 3 tumors (P < 0.001 for B16 and Mca; P < 0.05 for EMT6), and EMT6 was significantly more thermosensitive than B16 (P < 0.05).

Vascular Damage following Hyperthermia. The percentage of vascularized areas of unheated control tumors were 100 ± 0% (SE) for SCC VII, 99 ± 1% for Mca, 92 ± 3% for B16, and 82 ± 4% for EMT6. Because EMT6 and B16 had a central necrotic area without vascular supply histologically, the control values were less than 100%. Therefore, the estimated percentage of vascularized areas after heating were normalized to the percentage of vascularized area of the unheated control (relative percentage of vascularized area).

Fig. 3 shows the changes in relative percentage of vascularized area for the 4 tumors from 0 to 24 h after heating at 44°C for 30 min. The relative percentage of vascularized areas 3 h after heating were 8, 25, 49, and 83% for SCC VII, EMT6, B16, and Mca, respectively. The relative percentage of vascularized area of SCC VII was significantly lower than that of Mca (P < 0.001), B16 (P < 0.05), and EMT6 (P < 0.05), and that of EMT6 was significantly lower than that of Mca (P < 0.05). Although marked avascular areas appeared on the microangiograms of SCC VII and EMT6, dilated tumor vessels were opacified on those of B16 and Mca. Avascular areas in the microangiograms were correlated to congestion or rupture of the tumor vessels in the histological sections. Six h after
heating, the relative percentage of vascularized area of MCa decreased to 50%, when rupture and congestion of the tumor vessels were noted in the histological sections of MCa.

The relative percentage of vascularized areas 24 h after heating were 5, 5, 24, and 24% for SCC VII, EMT6, B16, and MCa, respectively. The value of B16 was significantly higher than that of EMT6 or SCC VII (both \( P < 0.05 \)), although the difference between MCa and EMT6 or between MCa and SCC VII was not significant \( (P < 0.10) \). The tumor vessels of SCC VII and EMT6 showed nearly no filling in the microangiograms obtained 24 h after heating, and dilated thrombotic tumor vessels with no apparent endothelial cells were observed in the histological sections. On the other hand, some areas of the tumor vessels especially in the tumor periphery were opacified for MCa and B16 24 h after heating, although large avascular areas were noted in the tumor center. Thus, the 4 tumors were roughly divided into 2 groups according to vulnerability of the tumor vessels to heat. SCC VII and EMT6 had thermoresistant vessels, and B16 and MCa had more thermoresistant tumor vessels.

Histological examination of the unheated control tumors revealed the characteristics of the tumor vessels for the 4 tumors (Fig. 4). Tumor vessels of MCa were supported by a connective tissue band \( (7,10) \), and those of B16 had a dense endothelial cell layer. On the other hand, SCC VII and EMT6 had sparse endothelial cells, and a gap of the endothelial cells was also noted in SCC VII and EMT6.

**DISCUSSION**

Heating of the 4 different types of mouse tumors at \( 44^\circ C \) for 30 min resulted in a transient inhibition of tumor growth (Fig. 2). Specific growth delay was used as a quantitative measure of the heat response in the present study, as the volume-doubling time of unheated control tumors differed considerably among the 4 tumors \( (11) \). The 4 tumors could be divided into 2 groups according to thermosensitivity in vivo based on the specific growth delay. SCC VII was the most thermosensitive tumor followed by EMT6, whereas B16 and MCa were thermoresistant.

To investigate vascular damage following hyperthermia quantitatively, we used the percentage of vascularized area estimated by microangiography. Although it may be possible to study the changes of tumor blood flow by using isotope-trapping methods, we preferred the morphological methods because microangiographic changes can be correlated to histopathological lesions. In the previous study, the time course of percentage of vascularized area after heating at \( 44^\circ C \) for 30 min was investigated for SCC VII and MCa \( (7) \). The percentage of vascularized area was lowest 24 h after heating in both tumors and increased thereafter. Therefore, we investigated the changes of percentage of vascularized area up to 24 h after heating for the 4 tumor lines in this study. As shown in Fig. 3, vascular damage after heating continued to progress up to 24 h after the end of the treatment. Vascular damage estimated immediately or 3 h after heating may be a useful parameter for comparing vascular vulnerability to heat between the tumors. However, we consider that the vascular damage 24 h after heating is the most important, because it represents the maximum decrease of the percentage of vascularized area and angiogenesis of the tumor vessels, which is closely related to tumor regrowth, occurs thereafter \( (7) \).

The results of the present study demonstrated the close relationship between the heat-induced vascular damage and tumor response in vivo. Although many investigators have shown that blood perfusion plays an important role in the tissue damage by hyperthermia \( (1-3) \), the relationship between heat-induced vascular damage and tumor response has not been clarified. The tumor vasculature of the thermosensitive SCC VII and EMT6 tumors showed nearly complete destruction 24 h after heating at \( 44^\circ C \) for 30 min, whereas the rather thermodaistant MCa and B16 had many functioning tumor vessels 24 h after the same treatment.

Clinical investigations with hyperthermia have shown that the response to treatment may vary considerably with the tumors \( (12) \). This variability may be due to heterogeneous tumor heating or an adequate heating technique as well as to biological differences between the tumors. The measured intratumor temperature difference was within 0.1°C by our heating method. This indicates that the observed differences in heat response were mainly due to biological differences between the tumors.

There are a number of factors that could influence the thermal sensitivity biologically \( (1,2,4) \). Inherent cellular sensitivity to heat is known to vary considerably with the cell line \( (13) \). In the previous study, B16 was more thermosensitive in vitro than
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Fig. 4. Histological sections of unheated control tumors of the 4 tumors. In A, tumor vessels of mouse mammary adenocarcinoma pass through the interlobular connective tissue. The tumor vessels are filled with barium sulfate as contrast medium. In B, tumor vessels of B16 melanoma had dense endothelial cell layers without gaps between them. In C, only a few endothelial cells are visible in EMT6 sarcoma, and gaps of endothelial cell are noted (arrows). In D, tumor vessels of SCC VII carcinoma consist of only sparse endothelial cells. H & E, × 100.

SCC VII or EMT6, whereas B16 was the most thermoresistant in vivo among the tumors. Rofstad and Brustad (11), who studied human melanoma xenografts, have shown that the heat response in vivo was not positively correlated with the intrinsic heat sensitivity of the tumor cells in vitro and concluded that heat-induced cell death experiments under in vitro conditions are not likely to be of prognostic value for prediction of the heat-induced tumor response in vivo. Thus, the thermosensitivity of tumor cells in vivo is quite different from that in vitro. It is obvious that the acidic and nutritionally deprived intratumor environment is one of the causes of this difference in thermosensitivity (1, 2).

Another explanation of the difference between in vitro and in vivo thermosensitivity is delayed or secondary cell death after heating (14, 15). This intriguing phenomenon in the response of tumor cells in vitro is that cells are killed during a period lasting up to a few days after completion of hyperthermia as a consequence of ischemic changes by vascular damage. Occlusion of tumor vessels following hyperthermia has been reported in several experimental and clinical studies (16, 17). In the previous and present study, a close relationship between the vascular damage and the tumor cell degeneration of tumor cells was noted in the histological sections (7). Thus, damage to the tumor vasculature, which also controls intratumor environment, may be a major mechanism of tumor control in hyperthermia, and it is very likely that the extent of vascular damage correlates with tumor response in vivo.

Some investigators (18) have suggested that slowly growing tumors may respond better to heat treatment than rapidly growing ones, but the accumulating evidence does not support this view (11). In the present study, the volume-doubling time of the most thermoresistant SCC VII was nearly equal to that of rather thermoresistant MCa. Thus, the heat response of tumors probably does not correlate with the rate of tumor volume growth.

Although the mechanism of heat-induced vascular damage is not yet clearly understood, it is evident that the retardation of the tumor microcirculation is caused by a combination of intravascular events, including sticking of leukocytes or erythrocyte aggregation in tumor vessels and degenerative changes of the endothelial cells (1, 3, 5). Among the various elements of the heat-induced vascular change, endothelial cells may be the most important possible targets of thermal injury. There are conflicting conclusions on thermosensitivity of endothelial cells in culture. Rhee and Song (19) found the endothelial cells thermoresistant by clonogenic assay, whereas Fajardo et al. (20) found them thermosensitive. Histological examination of the present study revealed that SCC VII and EMT6, which showed...
severe vascular damage after hyperthermia, had only sparse endothelial cells with a gap of the cells, whereas B16 and MCa had dense endothelial cells (Fig. 4). Whatever the thermosensitivity of endothelial cells, it is likely that tumor vasculature with high density of endothelial cells is resistant to heat treatment.

Another interesting histological finding on tumor vasculature is the presence of fibrous connective tissue supporting the parenchyma and carrying the blood supply. Of the 4 tumors examined, only MCa, which had rather thermoreistant tumor vessels, had this perivascular structure. In the previous and present study, vascular occlusion following hyperthermia was not observed frequently in the tumor vessels of MCa surrounded by thick connective tissues. Similar observations have been made by several investigators (15, 21). It is thus suggested that tumor vessels supported by collagenous tissues are less vulnerable to heat than those without such support. We consider that tumor vasculature with such perivascular supporting structures resists mechanical or passive dilation of tumor vessels by the increased blood flow from the surround normal tissue, which occur following the heat-induced retardation of tumor blood flow due to the aforementioned mechanism.

In conclusion, we conclude that the tumor response in hyperthermia is closely related to the histological structure of tumor vessels. Our findings on vascular damage, histological examinations, and in vitro and in vivo thermosensitivity of the tumors suggest that tumors with abundant perivascular connective tissue and/or dense endothelial cells without a gap are less sensitive to heat than those with sparse endothelial cells regardless of the intrinsic cellular thermosensitivity. If the results observed in the present study are general phenomena in human tumors, the tumor response would be predicted roughly by the histological sections before heat treatment.

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