Changes in Thermosensitivity of Mouse Mammary Carcinoma following Hyperthermia in Vivo

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ABSTRACT

The time course of change in thermosensitivity of SCK tumor cells in vivo was investigated following preheating at 43.5° for 30 min. At varying times after the preheating with water bath, tumors were subjected to graded doses of second heating at 43.5° in vivo, and cell survival was determined by using in vitro cloning method. Thermosensitivity of the tumor cells in vivo [duration of heating for exponential inactivation of cells to 1/e (Do): 15.5 min] gradually increased after the preheating, reaching a maximum increase at 5 hr (Do: 7.5 min), and then decreased thereafter, due probably to a development of thermotolerance. Maximum thermotolerance was observed at 12 hr after the preheating, leading to a 3-fold increase in Do (48 min). The thermotolerance gradually decayed for several days thereafter. The initial increase in thermosensitivity might be attributed to the acidic and nutritionally deprived intratumor environment as a result of vascular damage in heated tumor. It appears that thermotolerance gradually develops as time elapses and that it eventually overcomes the thermal sensitization of tumor cells in vivo.

INTRODUCTION

A considerable amount of experimental data on the treatment of cancer with hyperthermia used alone or in combination with drug or radiation have been accumulated during the last decade (4-6, 10, 14, 18, 24, 27, 33, 37-40). Most of these studies have been performed on the effect of a single heat, and our knowledge on the effect of heat in multiple doses is still sparse, despite the fact that fractionated heating will be the common protocol in clinical hyperthermia (1, 9, 17, 20, 34, 35, 41).

It has been reported that biological systems both in vitro (11, 12, 19, 21, 25, 31) and in vivo (5, 22, 26) acquire thermotolerance either during a prolonged heating at mild temperatures (40-42°) or some time after an acute heating at higher temperatures (43-45°). Such a phenomenon would render the fractionation protocol of clinical hyperthermia more complicated. Among a number of factors which may modify thermotolerance, acidity appears to play the cardinal role; acidic environment significantly suppresses the development of thermotolerance (13, 15, 31, 32). Interestingly, intratumor environment is intrinsically acidic (2, 7, 8, 16, 30), and it becomes further acidic when tumors are heated (3, 36). It is conceivable that the development of thermotolerance of tumor cells in vivo might be suppressed due to acidic environment in heated tumor. In the present study, we investigated this possibility by quantitating the cell survival using in vitro cloning method following split heatings of mouse mammary carcinoma of A/J mice in vivo.

MATERIALS AND METHODS

Tumor Model. The SCK tumor, a mammary carcinoma of A/J mouse, was used in the present study. This tumor arose spontaneously in 1974 and was adapted to grow both in vivo and in vitro in our laboratory. The cells of the 38th generation (one generation consists of altered growth in vivo and in vitro for 10 to 14 days each) were preserved in liquid nitrogen. The frozen cells were thawed, cultured in vitro, and injected into animals for in vivo study. After about 12 passages in vitro, we discarded the cells and activated other frozen cells for further experiments. Detailed information on the characteristics of the tumor are described elsewhere (23).

For the present study, the cells in exponential growth phase in culture were trypsinized, and 1.6 x 10⁶ cells in 0.025 ml of medium without serum were inoculated i.m. into the thighs of male A/J mice. The depth of inoculation was controlled to obtain the overlying muscle layer of less than 0.5 mm. Tumors 8 to 10 mm in diameter, obtained about 10 days after inoculation, were subjected to hyperthermia.

Hyperthermia of Tumors. Mice were fixed on a specially designed heating jig. The tumor-bearing legs were anchored to supporters, and the legs were submerged in a preheated water bath (Thermomix 1480, B. Braun Apparatebau, Melsungen, W. Germany). The temperature of the water bath was 43.5 ± 0.01° (S.E.). The tumor temperature, measured by a 29-gauge needle thermocouple (type MT-3; Bailey Instruments Co., Saddle Brook, N. J.), equilibrated at 43.2 and 42.8° in the shallow part and in the deepest part of the tumor, respectively, within 2 to 3 min of heating at 43.5°. The animals were anesthetized with ether for fixation onto the heating jig, but they were not anesthetized during heating. The animals were released from the heating jig and left at room temperature in animal cages during the interval of the 2 heatings.

Cell Survival Studies. Control or heated tumors were excised and weighed. The tumors were then minced and treated with 0.25% trypsin solution containing 0.00125% of DNase in Roswell Park Memorial Institute Culture Medium 1640 for 20 min at room temperature with continuous stirring. The cell suspensions were centrifuged, and the cells were washed once with medium containing 20% calf serum. The cells were passed through 2 layers of gauze and suspended again in medium with 10% calf serum.

The number of cells which were able to exclude trypan blue was counted with a hemocytometer, and the cell number in unit weight of tumor was calculated. PE3 of the recovered cells was obtained by seeding an appropriate number of cells in plastic culture flasks (Falcon, Model 3013) with Roswell Park Memorial Institute Culture Medium 1640 supplemented with 20% calf serum and antibiotics. At least 4 replicated cultures were set up for each cell preparation. After incubation for 8 to 10 days at 37° under a humidified mixture of 5% CO2-95% air, clones were fixed with 95% ethanol and stained with 1% crystal violet. The number of clones containing more than 50 cells

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were counted, and the PE was determined. By multiplying the number of recovered cells per unit weight of tumor and the PE of these cells, the clonogenic cell number in unit weight of tumor was calculated.

RESULTS

Kinetics of Cell Death in Heated Tumors. The changes in cell survival in tumors at various times after heating at 43.5° for 30 min are shown in Chart 1. The number of trypan blue-excluding cells recovered from the unheated control tumors was 9.85 ± 0.85 × 10^7/g. It decreased slightly to 8.83 ± 0.63 × 10^7/g immediately after heating. The number of recoverable cells further decreased up to 12 hr after heating and then increased thereafter. At 48 hr after heating, the number of recoverable cells was about 67% of that in control tumors (Chart 1, top).

The PE of the recoverable cells from control tumors was 50.0 ± 2.3%. The recoverable cells from tumors immediately after heating showed a PE of 10.51 ± 1.57%. The PE remained at this level for about 5 hr after heating and then recovered to near the control level at 12 hr after heating (Chart 1, top). It should be pointed out that PE started to increase while recoverable cells were still decreasing. A similar phenomenon of increase in PE without concomitant increase in recoverable cell number was observed also by other investigators (29). When lethally damaged but morphologically intact cells in a cell population undergo lysis, the PE of the cell population may inversely increase. However, such an increase in PE may be masked or cancelled when further damage occurs in heated tumor, and thus further decrease in PE takes place at the same time. It appears that these 2 processes, an increase in PE as a result of lysis of already damaged cells and a decrease in PE due to further damage of the cells in heated tumors, occur 0 to 5 hr after heating, resulting in a relatively constant PE during this period (Chart 1). The rise in PE from 5 hr after heating may be attributed to disintegration of already damaged cells without further damage of cells in the tumor.

The clonogenic cell number in each tumor at different times after heating was calculated from the number of recoverable cells and the PE of these cells, and is shown in Chart 1, bottom. The clonogenic cell number of unheated control tumor was 4.93 ± 0.48 × 10^7/g. The clonogenic cell number decreased to 9.28 ± 1.54 × 10^6/g when measured immediately after heating. This initial decrease in clonogenic cell number was due mainly to the decrease in PE during heating. When the tumors were left in situ after heating, the clonogenic cell number continuously decreased because of a subsequent and progressive damage of cells and of a lysis of already damaged cells in heated tumor, reaching a minimum level, 7% of control, at 3 to 5 hr after heating. The clonogenic cell number started to recover thereafter, but at 48 hr after heating it was still smaller than that in control tumors. The above results with i.m. tumors are qualitatively similar to that in s.c. tumors observed previously in our laboratory.

Thermosensitivity of Heated Tumor. The response of heated tumor cells to subsequent heating in vivo was studied. At varying times after preheating at 43.5° for 30 min, the preheated tumors were subjected to graded doses of second heating at 43.5° in vivo and the cell survival was determined at the end of second heatings using in vitro cloning method. Chart 2 shows the changes in clonogenic cell number in unit weight of tumor subjected to graded doses of second heatings at 1 to 48 hr after the preheating. The solid lines are the best-fitting lines of regression, indicating an exponential cell killing during the course of the second heating. It can be seen that the clonogenic cell numbers at the beginning of second heatings are different because the clonogenic or surviving cell number in the tumors significantly changes with time in situ after preheating as shown in Chart 1, bottom.

For a clear comparison of the thermosensitivity of tumor cells at different times after preheating, we normalized the cell survival at the beginning of the second heating to 100% survival. Chart 3 shows that the response of preheated tumor cells in vivo to the second heating varies, depending upon the length of the time interval between the preheating and the second heating. The slopes of the survival curves are significantly different from each other at the level of 95% confidence, except between the slopes of the survival curves for 1 and 3 hr after preheating. The survival curves for the second heatings applied 1, 3, and 5 hr after preheating were far steeper than the survival curve for the single heating, i.e., no time interval between preheating and second heating. This fact indicated that heating in 2 fractionations with intervals of 1 to 5 hr was significantly more effective for tumor cell killing in vivo than was the single heat exposure. However, the second heatings at 12, 24, and 48 hr after the preheating were far less effective than was the single heating.

D_0 of each survival curve in Chart 3 was calculated from the slope of the best-fitting line of regression. The D_0 is shown with standard error in Chart 4. D_0s were significantly different from each other at the level of 95% confidence, except between the
$D_0$s for 1 and 3 hr after preheating. Also shown in Chart 4 is the $D_0$ of SCK tumor cells in vitro subjected to a single heating at 43.0° (23). It can be seen that $D_0$s for a single heating in vitro and in vivo are 55.5 and 15.5 min, respectively. The changes in $D_0$ for the second heating in vivo showed a discreet turning point between 5 and 12 hr after preheating. The $D_0$ decreased maximally at 5 hr after preheating with a factor of 2 and increased maximally at 12 hr after preheating with a factor of 3. This demonstrated that the SCK tumor cells in vivo were maximally heat sensitive at 5 hr after preheating and heat resistant 12 hr after preheating, which is probably due to a development of thermotolerance. Although the $D_0$ at 48 hr after preheating was smaller than that at 12 hr, it was greater than that for the single heating, indicating thermotolerance still existed 48 hr after preheating.

**DISCUSSION**

To our knowledge, the present study is the first attempt to use the in vivo-in vitro assay method to quantitate the changes in thermosensitivity of tumors in vivo following heating. The SCK tumor cells heated in vivo exhibited a pronounced increase in the response to subsequent heating applied within 5 hr after the preheating at 43.5° for 30 min in a water bath. After reaching a maximal thermosensitivity 5 hr after the preheating, the heated cells rapidly became thermally resistant. In fact, 12 hr after the preheating, the cells were far more thermotolerant than were the unheated cells. The heated cells then exhibited a gradual recovery of thermosensitivity, but the acquired thermotolerance was still evident 48 hr after the preheating.

It has been a well-known fact that intratumor environment is intrinsically acidic with a pH of about 7.0 as opposed to the normal tissue pH of about 7.4 (2, 7, 8, 16, 30). It has also been reported that the intratumor environment becomes further acidic upon heating (3, 36). For example, the intratumor pH in SCK tumor prior to heating was 6.8 to 7.1 and it decreased to 6.5 to 6.8 when the tumors were heated at 43.5° (36), and the heat-induced acidity lasted for 12 hr after heating. We found recently that the lactic acid content in SCK tumors increased by more than 2-fold following heating at 43.5° for 30 min, suggesting that the decrease in pH in heated tumors was the result of an increase in lactic acid content. It has been known that tumor blood vessels are quite vulnerable to hyperthermia (7, 42). The functional intravascular volume was reduced to almost 10% of the control value in SCK tumors when heated at 43.5° for 30 min, indicating that severe vascular stasis or occlusion was induced by the heating (23). Undoubtedly, a depletion of nutrients, including oxygen, may ensue from the vascular occlusion. The acidic environment together with a prolonged deprivation of nutrients in heated tumors appear to

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heating were incriminated for the enhanced response of tumors to fractionated heating with a time interval of 2 hr. In this study, however, increasing the time interval longer than 6 hr between 2 heatings significantly reduced the effectiveness of heating, probably because of the development of thermotolerance. These observations are in general agreement with our results in the present study.

If the results observed in the present study are general phenomena in human tumors, the efficacy of fractionated hyperthermic treatment would be greatly influenced by fractionation schedule. It may be possible to take advantage of the heat-induced thermal sensitization in heated tumors, provided that such an increase in thermosensitivity occurs preferentially in tumors. The question as to whether the magnitude and kinetics of the increase in thermosensitivity and subsequent development of thermotolerance in tumors and normal tissues are different remain to be investigated.

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REFERENCES


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