Changes in Intratumor pH by Two Heatings

Jyh-Cherng Lin and Chang W. Song

Department of Therapeutic Radiology-Radiation Oncology, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT

It is a known fact that pH in rodent tumors decline significantly upon heating most likely due to breakdown of the tumor blood circulation. We recently observed that tumor blood vessels become thermotolerant after being heated with a sublethal thermal dose. The purpose of the present study was to reveal whether heating can reduce intratumor pH when the tumor vessels are thermotolerant. When the SCK tumors of A/J mice were heated at 42.5°C for 1 h, the tumor vessels became most thermotolerant at 18 h postheating, as measured with the ⁸⁶Rb uptake method. The intratumor pH in the control SCK tumors was 7.05 ± 0.14 (SD), and it significantly decreased to 6.70 ± 0.08 (P < 0.001) after heating at 44.5°C for 1 h. However, when the tumor vessels were thermotolerant, i.e., 18 h after heating at 42.5°C for 1 h, reheating at 44.5°C for 1 h could not reduce the intratumor pH. We concluded that such a failure to increase tumor acidity by a second heating at temperatures as high as 44.5°C was due to vascular thermotolerance developed by the first heating.

INTRODUCTION

It has been known that the pH in tumors is low relative to the pH in normal tissues probably due to the intrinsic nature of malignant cells to convert glucose to lactic acid through glycolytic pathway. Another cause of the low pH environment in tumors is believed to be the insufficient supply of oxygen due to poor blood perfusion, which in turn enhances the formation and accumulation of acidic metabolites. Such an acidic intratumor environment may be exploited in the treatment of tumors by hyperthermia or chemotherapy, since an acidic environment potentiates thermal damage and enhances the effects of some chemotherapeutic drugs (1-3).

We, as well as other investigators, have reported previously that heating at temperatures commonly used in clinical hyperthermia, e.g., 42-44°C, significantly reduces the pH in rodent tumors (4-10). We hypothesized that such an increase in intratumor acidity results from heat-induced decline in blood perfusion (4-6). Severe vascular occlusion or stasis would inevitably cause hypoxia which would in turn increase the formation of acidic metabolites from glucose and ATP (11-13). The removal of these acidic metabolites through the occluded vessels would be sluggish.

We have recently observed that the response of tumor vasculatures to a second or third heating is quite different from the response to a first heating (14-16). For example, in SCK tumors of A/J mice, a single heating at 43.5°C caused a marked reduction in blood perfusion. However, when the tumors were preheated at 42.5°C for 1 h, a reheating 18 h later at temperatures as high as 44.5°C for 1 h could not significantly reduce the tumor blood perfusion (14). Similar phenomena were observed in RIF-1 tumors of C3H mice (16). In this context, the second heatings, applied within 1-2 days after a preheating, were not as effective as a single heating in increasing the blood flow in the skin and muscle of mice (15). These observations led us to conclude that thermotolerance develops in blood vessels, as in nearly all biological systems (17-24). In light of the close relationship between blood flow and intratumor environment, a relevant question is whether or not the intratumor pH would decrease upon heating when the tumor vasculatures are thermotolerant or heat resistant. In the present study, we examined the change in intratumor pH in SCK tumors by a second heating applied when the tumor vasculature was thermotolerant.

MATERIALS AND METHODS

Animals and Tumors. The SCK mammary carcinomas grown s.c. in the legs of A/J mice were used. The characteristics of this tumor have been described previously (4, 18). The stock cells stored in liquid nitrogen were periodically thawed and cultured in RPMI 1640 medium supplemented with 10% calf serum and antibiotics. The SCK cells in exponential growth phase in culture were dispersed to single cells by treatment with 0.25% trypsin for 10 min at 37°C. The cells were first washed with medium containing 10% calf serum and then with plain medium. About 2 x 10⁵ of the single cells suspended in 0.05 ml of isotonic saline were injected s.c. into the right hind legs of 8- to 10-week-old male A/J mice. The tumors were used for the experiments when they had grown to 7-8 mm in diameter, which took about 10 days.

 Heating the Tumors. The hair over and around the tumors was clipped 1 day before heating. For the heating, the mice were mounted without anesthesia on a specially designed heating jig (15, 18). The tumor-bearing right legs were loosely anchored and immersed in circulating prewarmed water in a water bath (Thermomix 1480; B. Braun Co., Federal Republic of Germany). The water temperature varied within 0.02°C and the intratumor temperature, measured with a needle type (29-gauge) copper-constantan thermocouple, was 0.1-0.3°C lower than the water temperature. In this paper, we will refer to the water temperatures as the heating temperatures. When the tumors were heated twice, the animals were removed from the heating jigs after the first heating and kept in their cages until the second heating was applied. The tumors used for the measurement of temperature were not used for the measurement of blood perfusion or intratumor pH.

 Measurement of Blood Perfusion. The blood perfusion was measured with the ⁸⁶Rb uptake method. Although the ⁸⁶Rb uptake method does not provide an exact quantity of blood flow in tissues in terms of ml/min/g, it does indicate the fraction of cardiac output into each tissue in terms of percentage of cardiac output or percentage of injected ⁸⁶Rb (14, 15, 25). About 25 μCi of ⁸⁶RbCl dissolved in 0.1 ml of isotonic saline were injected into the tail vein of each mouse. The tumor-bearing legs were amputated with a pair of sharp scissors 90 s later, and the mice were killed by cervical dislocation. When the blood perfusion during heating was determined, ⁸⁶Rb was injected while the tumor-bearing legs were still in the heating water, and the mice were removed from the water bath 60 s later. The removal of animals from the water bath and freeing the animals from the heating jig took about 20 s, so that the leg amputation could be done 90 s after the isotope injection. Because the tumor-bearing legs had to be amputated while the animals were still alive, the mice were anesthetized with an i.p. injection of pentobarbital (60 mg/kg) about 10 min prior to the ⁸⁶RbCl injection. The tumors were dissected, weighed, and the radioactivity was counted with a well-type gamma counter (14). The tail was also removed and
the 86Rb radioactivity was counted. When the radioactivity of 86Rb in the tail of any mouse exceeded 5% of the injected activity, the injection of 86Rb was regarded as a failure and the tumor from that mouse was excluded from further analysis. Since heating causes varying degrees of edema, the percentage of injected 86Rb accumulated/g of dried tissue was calculated from the radioactivity in the tumors and by the total radioactivity of 86Rb injected. The dry weight of the tumors was obtained by drying the tumors overnight in a 100°C oven. We used 8 to 16 tumors to determine the statistical values of 86Rb uptake in tumors during and various times after heating.

Measurement of pH. The intratumor pH was determined by using glass microelectrodes. Detailed discussions on the method of fabrication and characteristics of the electrode as well as the method of pH determination have been published previously by us (6). Glass micro-capillaries with outer diameters of 50–80 µm were prepared, a small amount of melted pH-sensitive glass was sucked into the tip of the capillaries and then gently blown out so that a pH-sensitive hemisphere with an outer radius of 40–60 µm was produced. The capillaries were then filled with a mixture of 3 M KCl solution saturated with AgCl and pH 6.0 buffer at a 2:1 volume ratio. Reference electrodes were constructed by closing the tip of microcapillaries with 3% Noble agar suspended in the aforementioned filling solution and filling the capillary with the same filling solution. Into each of the pH and reference electrodes, silver/AgCl wire was inserted and connected to an amplifier. The pH electrodes were calibrated with standard buffer solutions of varying pHs in a Faraday cage.

The animals were immobilized for the measurement of pH by an i.p. injection of Inactin [sodium salt of ethyl(1-methyl)amalonitriourea] (BYK Gulden Konstanz, West Germany). Inactin has been known to minimally affect cardiovascular function (26). An i.p. injection of 180 mg/kg of Inactin anesthetizes mice for 2–3 h, long enough to make multiple determinations of pH in each tumor. The anesthetized mice were mounted on a holding jig and securely taped. The skin over the tumor was removed with a pair of sharp scissors, and the exposed tissue (5 mm in diameter) was covered with drops of isotonic saline. The mice were placed on a board in a Faraday cage and a pH electrode was inserted about 1 mm into the tumor with a micromanipulator. A reference electrode was also inserted into the edge of the tumor and the potential difference (mV) was recorded on a chart recorder. The pH electrode was then advanced about 0.5 mm and the potential difference was read again about 2 min later. By advancing the electrode about 0.5 mm/step, 3–5 pH values were determined along each of 3–5 tracks in each tumor. Thus the pH values at 10–15 different loci in each tumor were determined and a total of 249 to 341 pH values in 14 to 21 tumors were obtained for each experimental group.

Data Analysis. The Student's t test was used to determine the statistical significance of any changes observed.

RESULTS

The effect of a heating at 44.5°C for 1 h on the 86Rb uptake in the control tumors and that in the tumors heated 18 h earlier at 42.5°C for 1 h are shown in Fig. 1. The 86Rb uptake in the control tumors was 17.6% of injected dose/g of dried tumor tissue, which was equivalent to 3.1% of injected dose/g of wet tumor tissue. When the tumors were heated for the first time at 44.5°C, the 86Rb uptake declined to 11.2%/g (dry) within 30 min and further decreased to 8.1%/g (dry) at the end of 1-h heating. The 86Rb uptake continued to decrease after the heating, reaching about 2.5%/g (dry) in 1–5 h after the heating. The 86Rb uptake recovered only slightly 24 h after the heating. As previously reported, heating at 42.5°C for 1 h promptly induced vascular thermotolerance peaking 18 h later in SCK tumors (14). Fig. 1 shows that the 86Rb uptake at 18 h after the preheating at 42.5°C for 1 h was 17.3%/g (dry). The effect of heating at 44.5°C on the 86Rb uptake in the preheated tumors was quite different from that in the unconditioned tumors. In the preheated tumors, the 86Rb uptake slightly increased during the initial 30 min of reheating at 44.5°C and then began to decline thereafter. At the end of a 1-h reheating, the 86Rb uptake was 17.6%/g (dry), which was almost identical to that prior to the reheating, declining to 8.8%/g (dry) 1 h after the reheating. The 86Rb uptake 5 h after the reheating was 6.3%/g (dry) but recovered to the preheating value 24 h after the reheating.

Fig. 2 shows the histograms of pH distribution in control SCK tumors and that in the tumors heated once or twice. The intratumor pH in the control SCK tumors ranged from 6.77 to 7.42, with an average of 7.05 ± 0.14 (mean ± SD of 341 observations in 21 control tumors) when measured 18 h after a heating at 44.5°C for 1 h. With preheating, heating at 44.5°C for 1 h could not reduce the intratumor pH. The average pH in the tumors heated twice was 7.00 ± 0.10 (244 observations in 14 tumors), which was almost identical to the average pH (7.02) in experimental control tumors, i.e., tumors heated 18 h earlier.
marked decline in pH by a 44.5°C heating in the unconditioned SCK tumors, the pH did not change by a 1-h reheating at 44.5°C in the tumors in which the blood vessels were thermotolerant; the pH values before the reheating and 3 h after the reheating were 7.02 ± 0.10 and 7.00 ± 0.10 (244 observations in 14 tumors), respectively. The difference between the pH values of 7.00 ± 0.10 and 7.05 ± 0.14 in the reheated tumors and in the control tumors, respectively, were statistically insignificant.

**DISCUSSION**

The present study demonstrated that a preheating of SCK tumors at 42.5°C for 1 h prevented the decline in the intratumor pH by a second heating or reheating applied 18 h later. Circumstantial evidence suggests that there is a correlation between the lack of a decline in intratumor pH by reheating and the thermoresistance or thermotolerance in the tumor vessels after preheating. We used 86Rb uptake to measure the tumor blood circulation in the present study. As Sapirstein (25) reported, the 86Rb uptake in the tumors merely indicates the fraction of the cardiac output to the tumors. Nevertheless, we have repeatedly demonstrated that 86Rb uptake is a valid measure for the relative output to the tumors. Nevertheless, we have repeatedly demonstrated that 86Rb uptake is a valid measure for the relative output to the tumors. Nevertheless, we have repeatedly demonstrated that 86Rb uptake is a valid measure for the relative output to the tumors.

It has been reported that blood flow in human tumors does not change significantly by heating as it does in rodent tumors (29, 31), although marked histopathological damage in human tumor blood vessels after receiving hyperthermia treatment in combination with radiotherapy has been observed (32, 33). While it is quite possible that the blood vessels in human tumors are intrinsically heat resistant as compared with the blood vessels in rodent tumors, we cannot exclude the possibility that blood vessels in the human tumors studied in the aforementioned reports were initially not thermoresistant but became thermoresistant during the course of hyperthermic treatment. Likewise, the lack of a significant decline in pH in heated human tumors (8, 34) may be attributed to the development of vascular thermotolerance. A recent report appears to indicate that the pH in some human tumors declines during the first hyperthermia session (35). The effects of a first heating and subsequent heatings on the blood flow and pH in human tumors remain to be investigated.

**REFERENCES**


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