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Experimental Brain Tumor Response to Hyperthermia Evaluated by *in vivo* ^{31}P Magnetic Resonance Spectroscopy

Shigenari Kin

Department of Neurosurgery, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan
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Abstract *In vivo* ^{31}P-MR spectroscopy was employed to measure the energy metabolism and pH of T9 gliosarcomas in the flank of Fisher rats before and serially up to 24 h after hyperthermia at various temperatures. Water bath hyperthermia was used to heat tumors at 41°C, 43°C and 45°C for 30 min. Following hyperthermia at 41°C, the ratio of β-nucleoside triphosphate to inorganic phosphate (β-NTP/Pi) significantly decreased at 1 h and 3 h ($p<0.05$), but recovered at 6 h. The β-NTP/Pi ratio after hyperthermia at 43°C and 45°C decreased significantly up to 24 h ($p<0.05$). The intracellular pH significantly decreased from the pretreatment level at 6 h after hyperthermia at 45°C. Histological comparison of tumors with and without hyperthermia 24 h posttreatment showed the loss of the endothelial lining and enlargement of the interstitial space in tumors with hyperthermia at 43°C and 45°C. Thrombosis in vessels and pyknosis were also demonstrated in tumors treated at 43°C and 45°C. But these changes were more marked in tumors with hyperthermia at 45°C. Our data suggest that *in vivo* ^{31}P-MR spectroscopy provides useful information about the therapeutic effects of hyperthermia within 6 h after the treatment.

Key words:Experimental brain tumor, Histology, Hyperthermia, Magnetic resonance spectroscopy

Introduction

Hyperthermia has been advocated as a treatment for malignant brain tumors[1,2,3,4]. There are few useful methods which provide information about the effects of hyperthermia. ^{31}P-magnetic resonance (MR) spectroscopy is a non-invasive method for continuous and repetitive monitoring the *in vivo* tumor energy metabolism[5,6,7,8]. It has been applied successfully to evaluate tumor growth and the response to therapy of several experimental tumors[9,10,11,12,13]. Changes of phosphate metabolites in response to hyperthermia, detected on ^{31}P-MR spectroscopy, have been reported in several tumor models, such as Dunn osteosarcomas[14], RIF-1 tumors[15], mammary adenocarcinomas[16], and NU-82 tumors[17,18]. However, little information is available concerning the serial metabolic changes with different doses of hyperthermia in brain tumors.

The purpose of this study is to elucidate the response of an experimental brain tumor to a different doses of hyperthermia by means of ^{31}P-MR spectroscopy. If the early changes of the energy state and pH are correlated with the late histological changes, ^{31}P-MR spectroscopy will be useful parameters for monitoring the effects of hyperthermia.

Materials and methods

1. *Animals and tumor model*

Female Fisher rats (100-200 g) were anesthetized with sodium pentobarbital (30 mg/kg i.p.) for implantation, the MR study, and hyperthermia. A gliosarcoma cell line (T9), which derived from CD Fisher rats with methylnitrosourea, was used to produce the tumor models[19]. Tumor cells (10^6-10^7) were injected subcutaneously into the flank of the rats. Tumor growth was observed and the size was determined with slide calipers.

2. *Hyperthermia*

When the average tumor size had reached 20 mm in diameter, rats were divided into 4 groups, i. e., a control group (n=7), and ones subjected to hyperthermia at 41°C (n=7), 43°C (n=7), and 45°C (n=7) for 30 min, respectively. Heat was applied locally to the tumors by immersing only the flank where tumor grew in a water bath. The target temperatures were 41°C, 43°C, and 45°C. The tumor and bath temperatures were measured with electronic thermometers (DT-300; Inter Medical Co., ltd., Japan) and Teflon-sheathed copper-constant thermocouples (DTE-40CA; Inter Medical Co., ltd., Japan). The thermocouples were inserted into the centers of the tumors. The water temperature was adjusted manually so that the tumor temperature could be maintained at a target temperature ±0.1°C. The duration of hyperthermia was timed from the moment the target temperature was reached. The rectal temperature was kept below 40°C during hyperthermia.

3. *MR measurement*

MR studies were carried out with a BEM 170/200 spectrometer (Otsuka Electronics Co., ltd., Japan), operating at 4.7 T, equipped with a 190-mm horizontal-bore superconducting magnet. The surface coil was a 2 cm diameter two-turn one, doubly tuned to ^{31}P (81.0 MHz) and ^{1}H (200.1 MHz). The diameter of the coil was matched to the tumor size to eliminate or to minimize any possible contamination from tissues around the tumor. The magnetic field (B_0) homogeneity was adjusted for each sample by shimming on the water proton (^{1}H) signal within the sample at 200.1 MHz. The surface coil was placed over a tumor and ^{31}P-MR spectra were obtained. The acquisition parameters for these studies were as follows: 35 μsec 90 degree pulse, 1024 data points per FID, repetition time of 3 s, and 512 scans. FIDs were processed with exponential line broadening of 10 Hz before Fourier transformation. ^{31}P-MRS experiments were performed before, and 1, 3, 6 and 24 h after hyperthermia.

The intracellular pH was determined from the chemical shift of the inorganic phosphate (Pi) peak from the phosphocreatine (PCr) peak using the equation of Petroff *et al.*[20]. When there was no detectable phosphocreatine (PCr) in a tumor tissue, the value of 0 ppm was assumed from the α-ATP peak, that was located at 7.5 ppm. Peak areas were calculated using the software package supplied with the instrument.

Data for each group were expressed as mean ± SEM, and statistical analysis was performed using one-way analysis of variance and the Scheffe method; differences were considered significant at $p<0.05$.

4. *Histology*

Immediately after the last NMR studies, the tumors were resected and the specimens were fixed with 70% alcohol and then embedded in paraffin. Sections were made from each tumor and stained with hematoxylin-eosin. The specimens were examined at × 400 magnification under a Nikon binocular compound microscope. Histological changes of tumor cells and the microvasculature were evaluated, and graded semiquantitatively as follows; 0: none, 1: slight (<30% affected), 2: moderate (30-70% affected), and 3: severe (>70% affected).

Results

Representative *in vivo* ^{31}P-MR spectra of T9 gliosarcomas heated at 41°C, 43°C and 45°C are shown in Fig. 1, A, B and C, respectively. The typical spectra of untreated gliosarcoma are shown in the bottom of Fig. 1, A, B and C. They are, in order of low resonant frequency: phosphomonoesters

(PME); inorganic phosphate (Pi); phosphodiesters (PDE); phosphocreatine (PCr); γ-nucleoside triphosphate (NTP) and β-nucleoside diphosphate (NDP); α-NTP and α-NDP with NAD; and β-NTP. The different heat doses resulted in different serial spectral changes. The spectra after hyperthermia at 43°C showed partial changes without higher Pi peaks than those before heating, but those after hyperthermia at 45°C changed Pi dominant pattern.

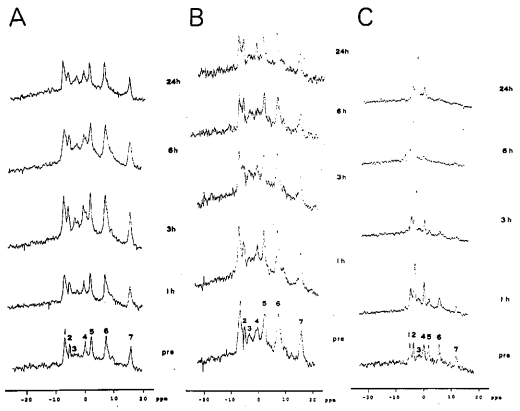

Fig. 1: Representative serial *in vivo* ^{31}P-MR spectra of T9 tumors treated at 41°C (A), 43°C (B) and 45°C (C) for 30 min. The peak assignments are as follows: 1, phosphomonoesters; 2, Pi (inorganic phosphate); 3, phosphodiesters; 4, phosphocreatine; 5, γ-NTP (nucleoside triphosphate) and β-NDP (nucleoside diphosphate); 6, α-NTP and α-NDP with NAD; 7, β-NTP.

Fig. 2 shows the β-NTP/Pi ratio as a percentage of the value before hyperthermia. The β-NTP/Pi ratio after hyperthermia at 41°C was significantly reduced to 57% and 59%, at 1 and 3 h, respectively. The β-NTP/Pi ratio after hyperthermia at 43°C was significantly reduced to 32% at 1 h, 41% at 3 hr, and 44% at 6 hr, but it recovered to 50% at 24 h. After hyperthermia at 45°C, the β-NTP/Pi ratio was significantly reduced to 24% at 1 hr, 34% at 3 hr and 23% at 6 hr, and did not recover at 24 h.

While the intracellular pH showed no change in the tumors heated at 41°C, it decreased within 6 h in the groups with hyperthermia at 43°C and 45°C (Fig. 3). The intracellular pH significantly decreased to 7.01 at 6 h after heating at 45°C.

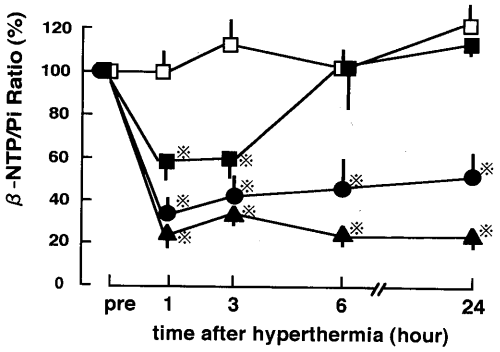

Fig. 2: The β-NTP/Pi ratio in T9 tumors as a percentage of the value before treatment, at different intervals after 30 min hyperthermia at 41°C (■), 43°C (●), 45°C (▲) and control (□). ※: Significant ($p<0.05$) difference compared with control. (n= 7)

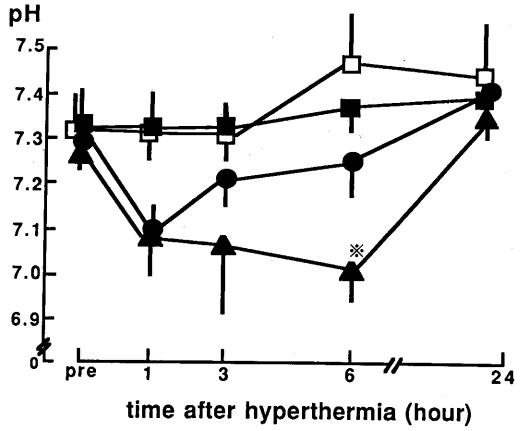

Fig. 3: Changes in the mean intracellular pH after hyperthermia for 30 min at 41°C (■), 43°C (●), 45°C (▲) and control (□). ※: Significant ($p<0.05$) difference compared with control. (n= 7)

There were no apparent histological changes after hyperthermia at 41°C compared with control (Fig. 4 A, B). But histological studies of tumors with hyperthermia at 43°C and 45°C revealed that tumor cells decreased and the interstitial space increased (Fig. 4 C, D). Tumors heated at 43°C and 45°C demonstrated thrombosis in vessels and pyknosis of tumor nuclei, these changes were more marked after hyperthermia at 45°C (Tables 1 and 2).

Microvasculature

	Loss of endothelium	Bleeding	Thrombosis
Control	0	0	0
41℃	0	0	0
43℃	2	2	1
45℃	3	3	2

0: none, 1: slight, 2: moderate, 3: severe

Table 2

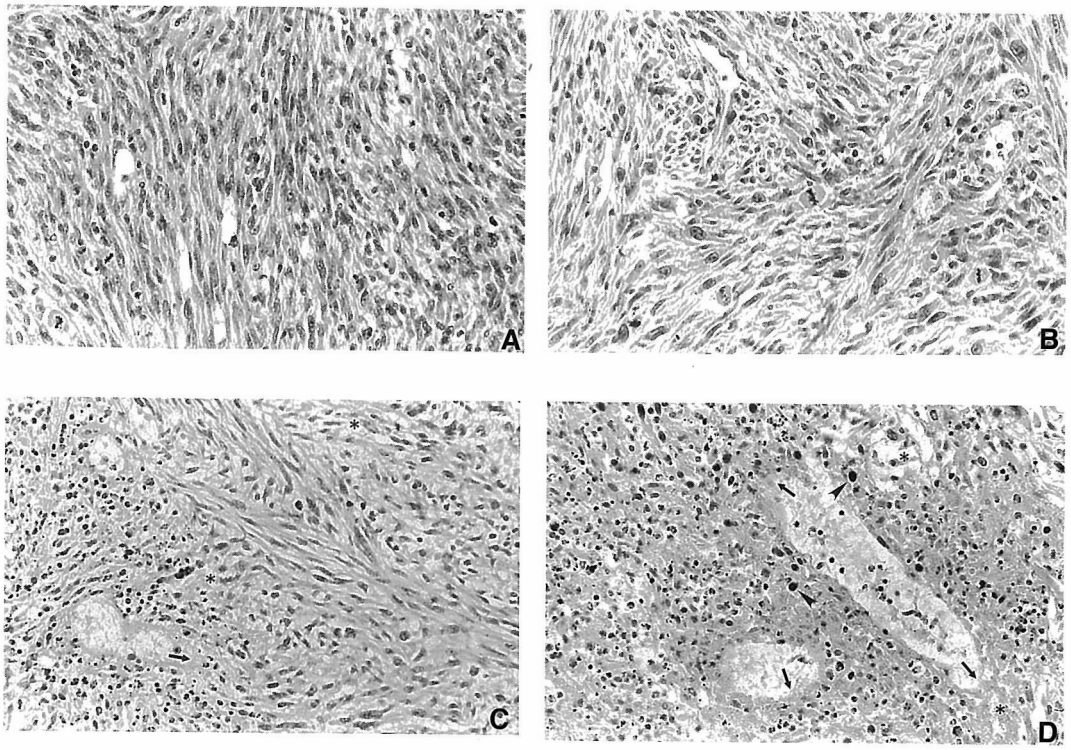

Fig. 4: Histological sections of T9 tumor: control (A), 24 h after hyperthermia for 30 min at 41°C (B), 43°C (C), and 45°C (D). Note the high density of tumor cells and the intact vessel. Many mitoses can be seen (A, B). Note the abundant interstitial space (*star*), loss of the endothelial lining (*arrow*, C, D) and focal necrosis and pyknosis (*arrowhead*, D). Hematoxylin-Eosin stain, ×400.

Histological findings

	Enlargement of interstitial space	Pyknosis	Necrosis
Control	0	0	1
41℃	0	0	1
43℃	2	1	2
45℃	3	2	2

0: none, 1: slight, 2: moderate, 3: severe

Table 1

Discussion

1. ^{31}*P-MR spectroscopy*

The present study elucidated that the higher the heat dose rose, the more the β-NTP/Pi ratio decreased in T9 gliosarcomas. Dose-related reductions in the NTP/Pi ratio after hyperthermia have been reported in

Dunn osteosarcomas[14], RIF-1 tumors[15], mammary adenocarcinomas[16] and NU-82 tumors[17,18], but not investigated in details in brain tumors. Naruse *et al.* have reported that in their rat glioma model, an increased Pi peak and decreased high energy phosphates after radiofrequency hyperthermia at a power of 5 w for 60 min.[21] In their study, the temperature in the tumors was not accurately monitored and serial changes in the NTP/Pi ratio were not mentioned.

It was reported that, in NU-82 tumors, the NTP/Pi ratio decreased to approximately 40% at 4 h, and was reduced permanently to approximately 10% after heat doses of 43°C and 45°C for 15 min, respectively[18]. In our T9 gliosarcomas, the ratio decreased to 40% 3 h after hyperthermia at 43°C for 30 min, and was reduced permanently to 20% after hyperthermia at 45°C for 30 min. The temporal profile of the NTP/Pi ratio is similar in these two tumor models, although the duration of hyperthermia is different. In contrast, Dunn osteosarcomas showed a mean decrease in the NTP/Pi ratio, to approximately 50%, after hyperthermia at 45°C for 15 min[14]. The degree of reduction of NTP in favor of Pi varies among different tumor models, even if the temperature and duration of hyperthermia are the same. This variance may be caused by the sensitivity of each tumor model. ^{31}P-MR measurement, however, exhibits sensitivity as to the detection of differences in tumor energy status following various doses of hyperthermia[22,23].

The intracellular pH (pHi) decreased during and after hyperthermia in our T9 gliosarcomas. pHi is decreased (0.18 pH units) 1 h after hyperthermia at 43°C for 30 min. Following hyperthermia at 45°C for 30 min, pHi decreased at 1 h (0.24 pH units), 3 h (0.27 pH units), and 6 h (0.32 pH units). The higher dose had a larger decrease in hyperthermia of no less than 43°C, as demonstrated in several experimental tumors. In mammary adenocarcinoma, for example, the pHi at 4 h after hyperthermia of 45°C for 2 h is lower than that after hyperthermia of 43.5°C for 30 min and 1 h[16]. Decrease of pHi during after hyperthermia are reported in other experimental tumors. Dunn osteosarcomas showed a decrease in pHi (1.5 pH units) immediately after and (0.6 pH units) 6 h after hyperthermia at 47°C for 15 min[14]. After hyperthermia at 45°C for 30 min the mean pHi value decreased (0.3 pH units)[24]. Naruse *et al.* noted a decrease of less than 0.12 pH units 3 h after hyperthermia, when spectra were of Pi dominant pattern, in their rat gliomas[21]. However, the pHi after hyperthermia has not been measured serially in their gliomas.

A fall in pHi is thought to be due to an increase in acidic metabolites and disturbance of the clearing of them following a decrease in tumor blood flow[15,25]. pHi rapidly recovered to the pretreatment level in Dunn osteosarcomas[14] and mammary adenocarcinomas[16], but the reason remains unknown. pHi in NU-82 tumors did not significantly change after microwave hyperthermia at 41-45°C for 15 min[17] or at 43 and 44°C for 30 and 60 min[18]. The discrepancy may be due to the nature of mammary carcinoma, which exhibits great buffering capacity in the cells and sufficient microcirculation to neutralize the acidic metabolites[17].

2. *Histological examinations*

The mechanisms underlying anti-tumor effects of hyperthermia have been attributed to the heat sensitivity of tumor cells, to the decreased vascularity of tumors, or to a combination of both effects[23]. Devascularized tumors showed similar changes on NMR spectroscopy and blood flow to tumors treated with hyperthermia[15]. Eddy *et al.* observed continuing stasis in vessels from the end of heating in an *in vivo* squamous cell carcinoma heated at 45°C for 30 min[22]. Other authors pointed out that vascular damage was the primary factor in producing thermal cell kill *in vivo*[15,26]. A dysfunction of tumor circulation is caused by thrombosis in vessels, loss of the endothelial lining, increased viscosity of the blood following loss of red cell membrane flexibility under acidic conditions, and increased extravascular pressure as a result of plasma leakage in the interstitial space[22,26]. Histological examinations of our T9 tumors after hyperthermia showed the loss of the endothelial lining and thrombosis resulting in insufficient blood flow. These findings suggest that cell killing by heat does not seem to be a primary factor of anti-tumor

effect of *in vivo* hyperthermia, but the necrotic changes appears to be caused by a deficiency in blood flow.

3. *Therapeutic effect*

There is a clear correlation between the temporal pattern of the NTP/Pi ratio and the occurrence of necrosis[17]. Heat treatment inducing permanent reduction of the NTP/Pi ratio does, a temporal decrease of the NTP/Pi ratio followed by complete recovery does not, cause tumor necrosis[18]. ^{31}P-MR spectroscopy, the NTP/Pi ratio in particular, is a useful method for determining the effects of hyperthermia, and can be performed noninvasively and repeatedly.

Our data showed that the metabolic changes at 24 h after hyperthermia could be predicted from the spectral changes serially up to 6 h. The hyperthermia at 41°C caused a transient reduction of NTP/Pi ratio at 1 and 3 h, which was followed by a recovery at 6 and 24 h and minimal histological changes. The hyperthermia at 43°C and 45°C resulted in persistent reduction of the NTP/Pi ratio up to 24 h and irreversible histological changes. A significant reduction of pH occurred only 6 h after hyperthermia of 45°C. From these observations, it is suggested that more than 50% reduction of the NTP/Pi ratio, together with an acidic change of pH, at 6 h after hyperthermia seem to be a useful parameter to predict the effects of the treatment.

4. *Clinical application*

^{31}P-MR spectroscopy can provide useful information for hyperthermia to brain tumor. But there are differences in magnetic field and tumor size between experimental and clinical measurement. Although 4.7 T MR spectrometer for biological test was used in this study, magnetic field of clinical apparatus is from 1.5 to 2.0 T. Low magnetic field deteriorate signal-to-noise ratio, but it does not matter. Several investigators have described the ^{31}P-MR spectra from human brain and brain tumors using generally available MR system[27,28,29,30,31]. Moreover metabolic changes of human brain tumors after chemotherapy and radiation are observed with a 1.5 T MR instrument[27]. ^{31}P-MR spectroscopy has a good chance of clinical application to assessment the effect of hyperthermia at this stage. But establishment of methodology of hyperthermia to human brain tumor is indispensable. The size of tumor and surface coil was matched to eliminate contamination from surrounding tissue. ^{31}P-MR spectroscopy of human brain is acquired from block-shaped volumes ($>3\times3\times3$cm), so at present this study is limited in tumor size.

Conclusion

In vivo ^{31}P-MR spectroscopy is a useful method for monitoring tumor energy metabolic changes after hyperthermia in an experimental brain tumor. ^{31}P-MR spectroscopy exhibits potential for clinical application. The decrease in the ratio of β-NTP to Pi is a good index of thermal cell damage, and the serial changes in the ratio up to 6 h after hyperthermia can be used to predict later changes. Decreased blood flow seemed a primary factor for the energy depletion. Histological section revealed the causes of the circulatory failure were apparently loss of the endothelial lining, thrombosis in vessels, and enlargement of extravascular space.

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