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The Role of Active Oxygen Species and Lipid Peroxidation in the Antitumor Effect of Hyperthermia

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ABSTRACT

The role of active oxygen species and lipid peroxidation in the antitumor effect of hyperthermia was studied in an experimental rabbit model. VX2 tumors were transplanted into rabbit hind legs, and the effect of hyperthermia on tumor growth was measured at 7 and 14 days after heating. As an index of lipid peroxidation, thiobarbituric acid-reactive substances in the tumor tissue were measured prior to hyperthermia and 3, 6, 12, and 24 h after hyperthermia. Tumor growth in rabbits treated with hyperthermia was significantly reduced, and thiobarbituric acid-reactive substances in the tumor tissue treated with hyperthermia were significantly increased until 6 h after hyperthermia. In addition, α-tocopherol in the tumor tissue was significantly decreased after hyperthermia. The antitumor effect of hyperthermia and the increase of thiobarbituric acid-reactive substances in the tumor tissue treated with hyperthermia were significantly inhibited by the administration of superoxide dismutase and catalase or dimethyl sulfoxide. These results suggest that lipid peroxidation mediated by active oxygen species plays an important role in the antitumor effect of hyperthermia.

INTRODUCTION

Recently, clinical interest in hyperthermia as a cancer treatment modality has prompted many *in vitro* and *in vivo* basic science studies designed to elucidate the mechanisms responsible for the antitumor effect of exposure to elevated temperatures. Cellular death owing to heat varies with the treatment temperature and increases abruptly at 42–43°C (1). Clarification of the mechanism of this thermal cell death is being pursued both at the cellular and at the tumor level. At the cellular level, degeneration of nucleic acid and proteins and breakdown of the cellular membrane or lysosomes may account for the mechanism (2–4). Factors at the level of the tumor may also contribute to cytotoxicity, and effects on the microvasculature have drawn particular attention (5). A major mechanism of cell toxicity after exposure to radiation or some anticancer drugs involves DNA damage and lipid peroxidation mediated by superoxide radicals (6, 7). Furthermore, accelerated *in vivo* lipid peroxidation in various organs has been demonstrated in rats and mice placed in nonphysiological environments such as high heat (8). Consequently, we conducted a study to clarify the role of active oxygen species and lipid peroxidation in the mechanism of the antitumor effect of hyperthermia.

MATERIALS AND METHODS

Experimental Animals and Tumors

The animals used in this experiment were Japanese white male rabbits weighing 2.5–3.0 kg. The implantation tumors were rabbit VX2 tumors, a rabbit carcinoma derived from virus-induced papillomas. Rabbit VX2 tumors that had been successively transplanted in our laboratory were excised, sliced thin, filtered through a mesh, adjusted to a cancer cell count of 1×10^8/ml with Hanks' balanced salt solution, and implanted (1×10^7 cells/suspension) into the muscle tissue of the lower right leg. Rabbits were used for the study 2 weeks after implantation, after confirming the major axis of the tumors had grown to 1.5–2.0 cm by ultrasonic tomography.

Animal-Use Radiofrequency Dielectric Heater

The RF[2] dielectric heater was a Thermotron RF I.V. (RF I.V. Yamamoto Vinyter Co., Osaka, Japan). The operating principle uses an 8-MHz dielectric heating system and is intended for rabbits and rats.

Heating Method and Establishment of Heating Conditions

The VX2 tumors implanted into the muscle tissue of the rabbits were heated locally using the RF I.V. Intravenous sodium pentobarbiturate (20 mg/kg) was administered. Under general anesthesia the right legs were shaved, an 18-gauge needle was inserted into the center of the tumor as a mantle to retain the temperature sensor, and the legs were heated with continuous temperature measurement. The RF output was initially 50 W, and after reaching 42.5°C it was adjusted to maintain a constant temperature. Heating continued for 20 min.

Determinations

Tumor Diameter and Growth Rate. Immediately before and 7 and 14 days after hyperthermia, the major and minor axes of the tumors were measured by ultrasonic tomography, and the volume was calculated by the following formula:

$$V = \frac{a \times b^2}{2}$$

where V is the tumor volume, a is the major axis, and b is the minor axis.

The growth rate was defined as tumor volume divided by the tumor volume immediately prior to hyperthermia.

Thiobarbituric Acid-reactive Substance. TBARS in the blood was measured by the method of Yagi *et al.* (9), and TBARS in the tumor tissue was measured by the method of Ohkawa *et al.* (10) as an index of lipid peroxidation.

α-Tocopherol. α-Tocopherol in the tumor tissue was measured by the method of Abe *et al.* (11).

Administration of Free Radical Scavengers

A cutdown of the left femoral artery was performed under i.v. sodium pentobarbiturate (20 mg/kg) anesthesia. A guidewire (SF18; Cook Company, Bloomington, IN) was inserted, and a 3 Fr. diameter polyethylene catheter (Cook Company) was retained in the cephalic branch of the abdominal aorta. Free radical scavengers were then continually administered intraarterially via the catheter during hyperthermia. CuZnSOD (10,000 units/kg; Nippon Kayaku Co., Tokyo, Japan), 10,000 units/kg catalase (Sigma Chemical Co., St. Louis, MO), and 1100 mg/kg DMSO (Wako Pure Chemical Industry, Osaka, Japan) were dissolved in physiological saline and injected intraarterially during hyperthermia. Control group 1 was given injections with heat-inactivated (90°C for 60 min) CuZnSOD and catalase, and control group 2 was given injections of physiological saline intraarterially during hyperthermia. Tumor diameter was measured 14 days after hyperthermia, TBARS in the tumor tissue were measured immediately after hyperthermia, and α-tocopherol in the tumor tissue was measured 3 h after hyperthermia in all seven groups (untreated

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[2] The abbreviations used are: RF, radiofrequency; TBARS, thiobarbituric acid-reactive substance; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase.

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group, control group 1, control group 2, CuZnSOD group, catalase group, CuZnSOD and catalase combined group, and the DMSO group).

Statistical Analysis

Results are presented as means ± SE from 4–9 rabbits/group. The Kruskal-Wallis analysis was used to determine variances. The nonparametric Mann-Whitney test was used to compare differences between the hyperthermia group *versus* the untreated group. The two-tailed nonparametric Dunnett's test was used to compare groups treated with CuZnSOD and/or catalase *versus* control group 1, and groups treated with DMSO *versus* control group 2. Time course study samples were compared by the Duncan test. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Antitumor Effect

Relative Tumor Volume Owing to Heating. Seven days after hyperthermia the tumor volume was 1.72 ± 0.20 times the pretreatment volume, and 14 days after treatment it had grown to 3.63 ± 0.30 times the volume. In contrast, after 7 days the untreated control tumor was 4.20 ± 0.47 times the pretreatment volume, and at 14 days it had grown to 11.8 ± 1.51 times the pretreatment volume (Fig. 1). Hyperthermia significantly decreased the growth of the tumors.

Administration of Free Radical Scavengers. Fourteen days after hyperthermia, the tumor volumes in control groups 1 and 2 were 3.55 ± 0.33 and 3.19 ± 0.45 times the pretreatment volume. Tumor volume in the untreated group was 12.3 ± 1.71 times the pretreatment volume. This significant decrease due to hyperthermia was suppressed significantly in the CuZnSOD and catalase combined group (tumor volume 8.30 ± 0.99 times the pretreatment volume) and in the DMSO group (tumor volume 8.97 ± 1.20 times the pretreatment volume) (Fig. 2).

TBARS

Time-Course Changes. TBARS were measured prior to hyperthermia and at 3, 6, 12, and 24 h after hyperthermia. There was 0.20 ± 0.01 nmol/mg protein of TBARS in the tumor tissue prior to hyperthermia, 0.86 ± 0.10 nmol/mg protein immediately after hyperthermia, 0.45 ± 0.06 nmol/mg protein 3 h after hyperthermia, and 0.49 ± 0.07 nmol/mg protein 6 h after hyperthermia. Thus there was a significant increase in TBARS in the tumor tissue after hyperthermia (Fig. 3). In contrast, a significant change was not observed in serum TBARS before and after hyperthermia (data not shown).

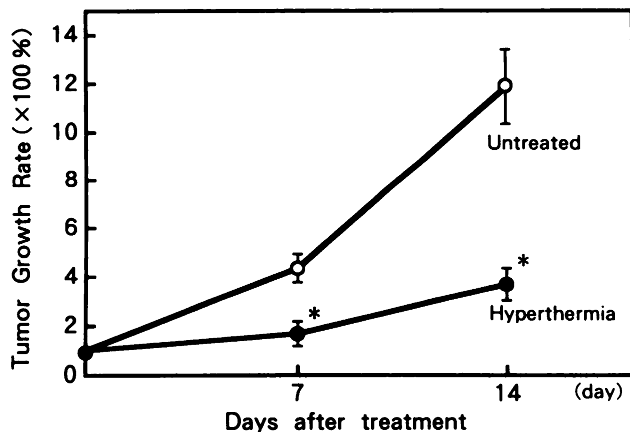

Fig. 1. Antitumor effect of hyperthermia on VX2 tumors in rabbits. Mean tumor volume in comparison to pretreatment control for groups of 9 rabbits (untreated group and hyperthermia group) are shown. Results are expressed as mean ± SE. *, $P < 0.001$ for difference from untreated group.

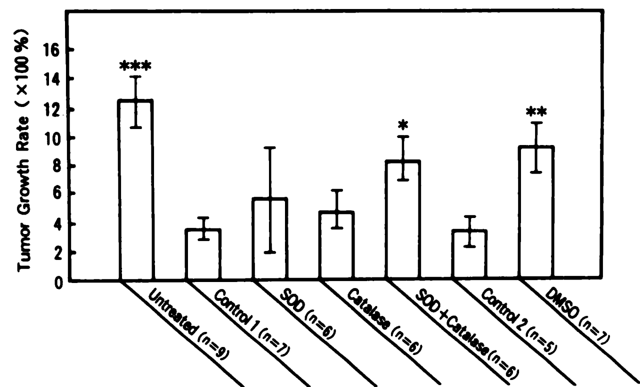

Fig. 2. Effects of CuZnSOD and/or catalase and DMSO on the antitumor effect of hyperthermia. Control group 1 was given injections of inactivated CuZnSOD and catalase as a control of the CuZnSOD and/or catalase group; control group 2 was given injections of physiological saline intraarterially during hyperthermia as a control for the DMSO group. CuZnSOD and/or catalase and DMSO dissolved in physiological saline were injected intraarterially during hyperthermia. Results are expressed as mean ± SE. *, $P < 0.01$ for difference from the value of control 1; **, $P < 0.01$ for difference from the value of control 2; ***, $P < 0.001$ for difference from the values of control 1 and control 2.

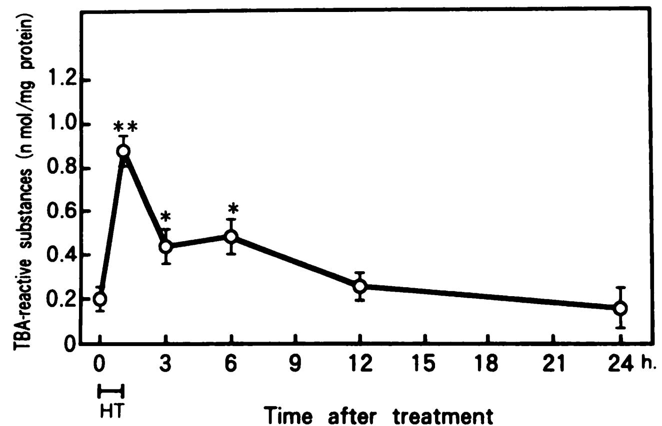

Fig. 3. Time-course changes in TBARS in the tumor tissue after hyperthermia. Each point indicates the mean ± SE of 4 rabbits. Significance between the value at 0 hour and each value after hyperthermia is shown. *, $P < 0.05$; **, $P < 0.01$.

Administration of Free Radical Scavengers. Compared to the untreated group (0.20 ± 0.01 nmol/mg protein), a significant increase was observed in the TBARS in the tumor tissue immediately after hyperthermia in control group 1 (0.87 ± 0.03 nmol/mg protein) and control group 2 (0.81 ± 0.08 nmol/mg protein). This increase was significantly lower in the CuZnSOD and catalase combined group (0.60 ± 0.02 nmol/mg protein) and the DMSO group (0.50 ± 0.08 nmol/mg protein) (Fig. 4).

α-Tocopherol

Time-Course Changes. The amount of α-tocopherol was measured prior to hyperthermia and at 3, 6, 12, and 24 h after hyperthermia. In contrast to the amount of α-tocopherol (0.109 ± 0.015 μg/mg protein) in the tumor tissue prior to hyperthermia, there was less (0.096 ± 0.016 μg/mg protein) immediately after hyperthermia. At 3, 6, 12, and 24 h after hyperthermia there were significantly lower amounts of α-tocopherol (0.059 ± 0.011, 0.070 ± 0.012, 0.063 ± 0.010, and 0.053 ± 0.017 ug/mg protein, respectively) (Fig. 5).

Administration of Free Radical Scavengers. Compared to the untreated group, a significant decrease was observed in the amount of α-tocopherol in the tumor tissue 3 h after hyperthermia in control groups 1 and 2. This decrease was not inhibited in the CuZnSOD group, the catalase group, the CuZnSOD and catalase combined group, or the DMSO group (Table 1).

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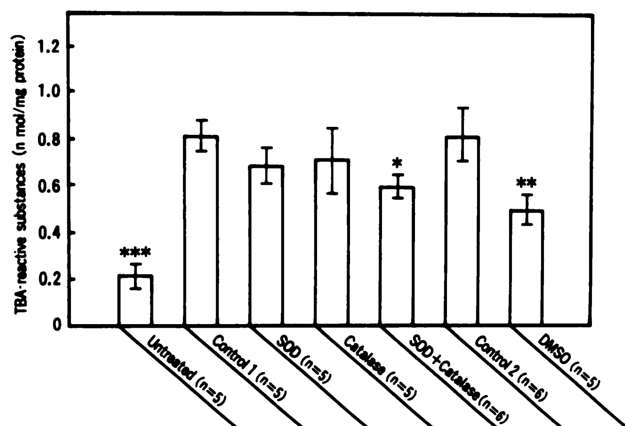

Fig. 4. Effects of CuZnSOD and/or catalase and DMSO on TBARS in the tumor tissue after hyperthermia. Results are expressed as mean ± SE. *, $P < 0.01$ for difference from the value of control 1; **, $P < 0.02$ for difference from the value of control 2; ***, $P < 0.001$ for difference from the values of control 1 and control 2.

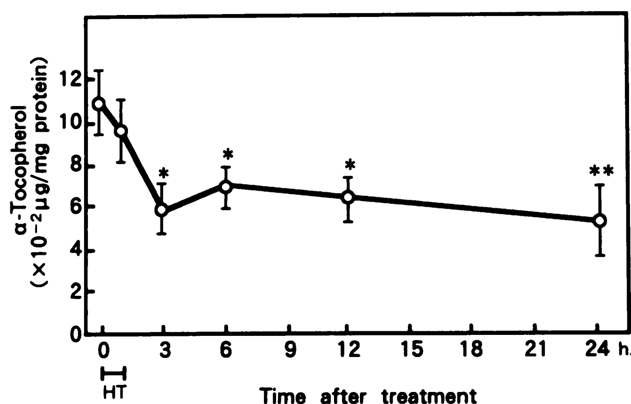

Fig. 5. Time-course changes in α-tocopherol in the tumor tissue after hyperthermia. Each point indicates the mean ± SE of 4 rabbits. Significance between the value at 0 hour and each value after hyperthermia is shown. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

In recent years it has become clear that lipid peroxidation due to free radicals is involved in a variety of different types of cell injury (12–14) and that the effective use of cell injury can be applied to cancer therapy. With regard to the relationship between high temperatures and lipid peroxidation in a living organism, Shepelev (8) has observed markedly accelerated lipid peroxidation in the brain and other tissues of rat and dogs reared at 44–45°C and simultaneously confirmed that antioxidant functions decreased and the amount of polyunsaturated fatty acids in the tissues decreased at elevated temperatures. Consequently, we used a rabbit model to investigate the role of various radical scavengers and the degree of lipid peroxidation in the tumor tissues during the antitumor effect of hyperthermia. In addition we measured changes in an important *in vivo* antioxidant, α-tocopherol. A significant antitumor effect of local heat was observed 7 and 14 days after hyperthermia in the rabbit VX2 tumor model. Furthermore, TBARS in the tumor tissue, which are the index of lipid peroxidation, increased immediately after hyperthermia and remained until 6 h later. On the other hand, α-tocopherol in the tumor tissue decreased immediately after hyperthermia until 24 h later. This suggests that injury to the cell membrane and intracellular organelle membrane due to lipid peroxidation may be involved in the antitumor effect of hyperthermia. In addition, α-tocopherol may have been consumed in order to protect the unsaturated fatty acids that form the biomembrane from peroxidation. In general, the activation of lipids and oxygen molecules is necessary to start the radical chain reaction that leads to lipid peroxide formation. Consequently, we investigated the role of active oxygen species as an initiating factor in lipid peroxidation. By administering the enzyme CuZnSOD which catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen, the antitumor effect of hyperthermia was not inhibited, but by administering the hydroxyl radical scavenger DMSO or the combined administration of the enzyme CuZnSOD with the enzyme catalase, which catalyzes the dismutation of hydrogen peroxide to water and oxygen, both the antitumor effect of hyperthermia and lipid peroxidation in the tumor tissue were inhibited significantly. This suggests that active oxygen species produced by local heating were an important factor in the antitumor effect of hyperthermia. The antitumor effect of hyperthermia at the cellular level may be attributed to the degeneration or compound inhibition of nucleic acid and proteins or cellular membrane and lysosome breakdown (3, 4), but the present study suggests that active oxygen species produced by local heating may act as an initiating factor of lipid peroxidation in the tumor cell membrane or organelle membrane. To what degree the active oxygen species produced during hyperthermia is involved in the degeneration or inhibition of synthesis of nucleic acids and proteins should be investigated in the future. Skibba *et al.* (15) carried out hyperthermic perfusion (42.5°C) of the liver in clinical cases of hepatic cancer and observed a significant increase in lipid peroxides in the liver perfusion solution 30 min later. The mechanism appeared to have been the generation of hydroxyl radical in the presence of an Fe^{3+} catalyst by superoxide produced by a hypoxanthine-xanthine oxidase reaction. The hydroxyl radical acts as an initiator, leading to membrane lipid peroxidation. Specifically, hyperthermia results in decreased blood flow to the tumor tissue and an accumulation of ADP. The subsequent production of hypoxanthine and its reaction with xanthine oxidase and oxygen produces active oxygen species. Superoxide derived from activated neutrophils is well known as a source of active oxygen species *in vivo*, but the degree to which this superoxide is involved in the carcinostatic mechanism of hyperthermia has yet to be clarified. However, in decreased blood flow, neutrophils in the margins of the microcirculation roll, stick, and directly contact the vascular endothelium, which reportedly produces an acceleration of active oxygen generation from local neutrophils (16). In addition, our studies demonstrating the relationship between neutrophil superoxide generation and temperature indicate that the superoxide generation of neutrophils isolated from the peripheral blood and peritoneum of rats rose significantly at 42°C compared to 37°C (17). Consequently, neutrophils in

Table 1 *Effects of CuZnSOD and/or catalase and DMSO on α-tocopherol in the tumor tissue after hyperthermia*

Group[a]	No. of rats	α-Tocopherol (µ/mg protein)[b]
Untreated	7	0.144 ± 0.026
Control 1[c]	6	0.079 ± 0.027[d]
SOD[e]	6	0.103 ± 0.014
Catalase[e]	6	0.084 ± 0.015
SOD + Catalase[e]	7	0.097 ± 0.013
Control 2[f]	7	0.082 ± 0.016[d]
DMSO[g]	7	0.113 ± 0.029

[a] Rats were sacrificed 3 h after hyperthermia.
[b] Mean ± SE.
[c] Control group 1 was given injections of inactivated CuZnSOD and catalase intraarterially during hyperthermia as a control for the CuZnSOD and/or catalase group.
[d] Different from untreated group ($P < 0.05$).
[e] CuZnSOD and/or catalase dissolved in physiological saline were injected intraarterially during hyperthermia.
[f] Control group 2 was given injections of physiological saline intraarterially during hyperthermia as a control for the DMSO group.
[g] DMSO dissolved in physiological saline was injected intraarterially during hyperthermia.

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the tumor microcirculation may be affected by hyperthermia and generate active oxygen species. Furthermore, clinically we have encountered acute gastric mucosal injury such as hemorrhagic erosion when performing hyperthermia of the upper abdominal region. We reported that lipid peroxidation and active oxygen species were involved in the mechanism of producing these lesions and that the involvement of the neutrophil was more important than the involvement of the hypoxanthine-xanthine oxidase system as a source of superoxide (17). If the mechanism of injury to the tumor tissues of hyperthermia is similar to the mechanism that produces gastric mucosal injury, neutrophil-derived active oxygen species may be important to antitumor effects as well. In addition, the generation of active oxygen species within cancer cells is a subject that should be investigated, and direct *in vivo* evidence of active oxygen produced during hyperthermia is a requisite topic. In summary, the present study strongly supports the conclusion that lipid peroxidation mediated by active oxygen species plays an important role in the antitumor effect of hyperthermia.

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