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ANTI-TUMOR CELL-MEDIATED IMMUNE REACTION FOLLOWING LOCAL HYPERTHERMIA COMBINED WITH RADIATION IN MICE

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Abstract The immunological activity of host mice following local hyperthermia combined with radiation was examined and compared with results obtained by determining the specific anti-tumor cell-mediated immunity in host mice induced and/or enhanced by local irradiation of transplanted tumor. MM 46 tumor cells were inoculated into the left thigh of C3H/He mice (2×10^6 cells each) and then treated with a single dose of X-ray irradiation and/or local hyperthermia 7 days after inoculation. Suppression of tumor growth in the group of mice treated with local hyperthermia combined with radiation was greater than that in the group of mice treated with irradiation alone. Spleen cells from these treated mice inhibited the growth of tumor cells *in vitro* when assessed by ^{3}H-TdR incorporation by tumor cells (cytostatic activity). The suppressive activity of spleen cells from mice treated by local hyperthermia with irradiation were clearly reduced. These results suggest that local hyperthermia combined with irradiation induce the anti-tumor immunity more effectively than irradiation alone.

Key words: Local hyperthermia, Local irradiation, Anti-tumor immunity, MM 46

INTRODUCTION

Combined therapy by local hyperthermia with irradiation has been in widespread use in the clinical radiotherapy of advanced tumors, but biological effects of hyperthermia have not been completely investigated. The role of the immune response to tumor cells is poorly understood in hyperthermia either alone or in combination with radiotherapy. Dickson *et al.*[1], Harris[2], Yerushalmi[3], and Walker *et al.*[4] have reported that local or whole body hyperthermia supppresses the host immune response, thus leading to proliferation of cancer cells or to metastasis. We previously reported that anti-tumor cell-mediated immunity in host mice could be induced and/or enhanced by local irradiation of the tumor, and enhancement of specific anti-tumor cell-mediated immunity depended on the cooperation of non-killer T cells and macrophages[5,6]. The present study was carried out to clarify whether the anti-tumor immunity of the host was more effectively induced by the combined therapy of local irradiation with local hyperthermia than by simple local irradiation.

MATERIALS AND METHODS

Animals and tumor cells

C3H/He female, 10-12 week old mice were used. The mice inoculated with tumor cells were kept in a 24±2 °C airconditioned clean room and fed commercial pellet chow and tap water *ad libitum*.

MM 46 tumor cells originating from a spontaneous mammary tumor in C3H/He strain mice were used for *in vivo* experiments. For *in vitro* experiments, MAC tumor cells were newly established by the authors from the mam-

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mary tumor MM 46 line[5]. The MAC cells were able to grow in ascites and solid tumors in C3H/He mice, in suspension culture, and to form colonies on soft agar. Both tumor cells are nearly the same in character. The MM 46 tumor was weekly passaged as ascites in syngeneic C3H/He mice. The MAC tumor cells were maintained in suspension culture in a complete culture medium, RPMI 1640 medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% FCS (same supplier), penicillin (100 units/m*l*) and streptmycin (100 μg/m*l*), for *in vitro* experiments.

Hyperthermic exposure and X-irradiation

Suspension cultured MAC cells or tumors in the left leg of C3H/He mice were heated in a water bath (Coolnics thermobath CTE-21 and R. K. I. No 1832 A) regulated to 43$\pm$0.1°C, determined by a thermometer (Model MGA-3, Shibaura electronics. Co. Ltd., Tokyo). The X-irradiation of the tumor cells was done at room temperature using a Toshiba EXS-300-4 X-ray machine (Toshiba Co. Ltd., Tokyo). The X-irradiation factors were 250 kVp, 12 mA, 0.5 mm Cu+1.0 mm Al filter, 50 cm FSD and a dose-rate of 0.47 Gy/min.

Cell survival assay

Immediately after treatment, the cell suspension was further diluted with 2$\times$RPMI1640 containing 20% FCS, penicillin (200 units/m*l*) and streptmycin (200 μg/m*l*), mixed with an equal volume of 0.6% agar medium, and overlaid on a basal layer of 0.5% agar in 60-mm Falcon plastic dishes. The cells were incubated for 10 days at 37°C under a humidified atmosphere of 5% CO_2 in air; and the colonies were then counted.

Inoculation of tumor-bearing mice and treatment of tumor

The MM 46 cells from ascites were washed in PBS and subcutaneously injected into the left thigh at 2×10^6 cells/mouse in 0.05 m*l*. One week after the inoculation, for some experiments, the tumors were treated by irradiation and/or hyperthermia. The diameter of the tumors was about 1 cm when the tumors were treated. Mice were anesthetized with sodium pentobarbital (50 mg/kg). Local irradiation was administered by 15 Gy of X-ray. The whole body except the left leg was shielded by a box of 5 mm lead plate. Local hyperthermia (43°C) was administered with a water bath within 30 min. after the irradiation. The tumor-bearing leg was immersed in the water bath through a hole in a platform, so that the tumor was completely submerged.

Tumor measurements

Two perpendicular diameters of tumors in each group of mice were measured with vernier calipers in millimeters twice a week after inoculation. The size was expressed as the product of the length of the major and minor axes of the tumor. The relative sizes of tumors after treatments were expressed by the ratio of pre- and post-treatment sizes. For each assay, 5 to 7 mice were used. The effects of treatments were evaluated from the relative tumor size.

Assays of cell-mediated immune response of spleen cells in vitro

1) Cytostatic assay: The protocols for determination of growth inhibition activity of spleen cells from tumor treated mice have been described elsewhere[5]. Briefly, spleens were aseptically removed from normal or treated mice. The spleen cells were gently dissociated and were used after lysing red blood cells by ammonium chloride solution for assay. The spleen cells were resuspended in a complete culture medium at a concentration of 10^6 cells/m*l*. The mixtures of 1×10^5 normal or treated spleen cells and 2×10^3 tumor cells (MAC cells) in 0.2 m*l* of complete culture medium (E/T=50/1) were allowed to undergo cell-mediated immune reaction in wells of round-bottomed microtiter culture plates at 37°C for 48 hr in 5% CO_2 and 95% air. For the final 24 hours, 3.7 kBq (0.1 μCi) of tritiated thymidine (^{3}H-TdR) was added to each well. The cells were collected on a glass filter and the radioactivity of labeled cells on the filter was counted in a liquid scintillation counter with no treatment for reduction of the acid soluble fraction. The cytostatic activity of spleen cells was expressed as percentage of inhibition of DNA synthesis by tumor cells calculated from the formula:

Percent cytostatic activity= [1$-$(cpm of tumor cells plus spleen cells from treated

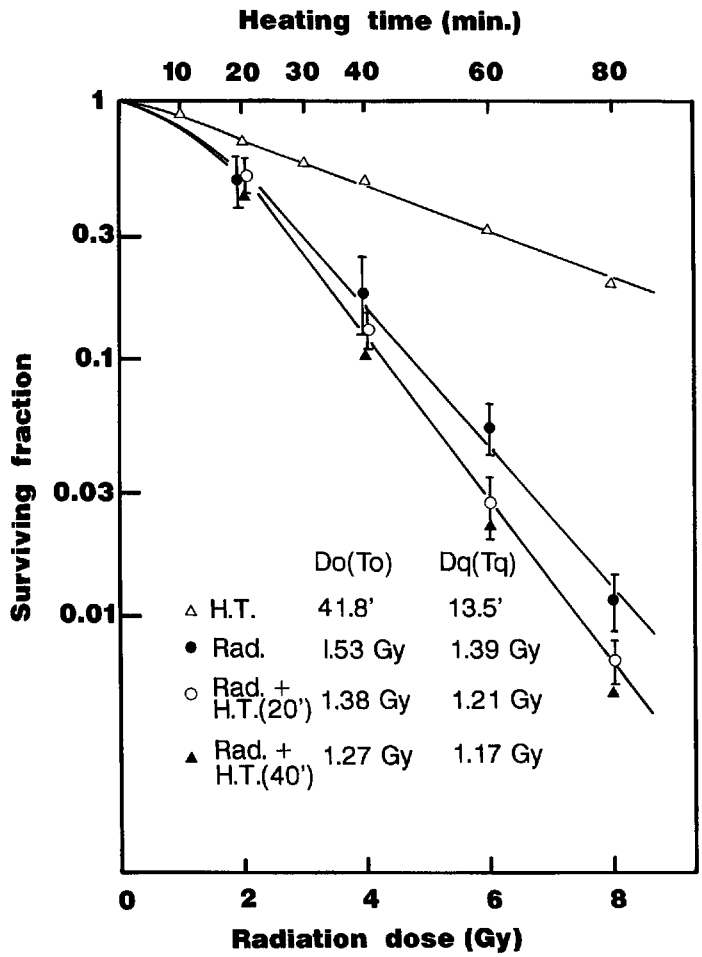

Fig. 1. Survival curves of MAC tumor cells exposed to X-irradiation and/or water bath hyperthermia in *in vitro* experiments.

mice − cpm of spleen cells alone from treated mice) / (cpm of tumor cells plus normal spleen cells − cpm of normal spleen cells alone)] × 100

2) *Suppressor cell assay*: The suppressive activity of spleen cells from treated mice was assessed by testing the capacity of these cells to inhibit the T-lymphocyte blastogenic response of normal spleen cells. Prior to being added to responder spleen cells, the spleen cells from treated mice were X-irradiated (20 Gy). This dose was chosen because preliminary studies showed that the "suppressive" activity assayed by the Concanavalin A (Con A) response, was intact after irradiation, although intake of ^{3}H-TdR into spleen cells was inhibited. Suppressor cells were added to responder spleen cells in a 1: 1 ratio in a final volume of 0.2 m*l* with Con A at 5 μg/m*l* final concentration. These cells were incubated in a 96-well, round-bottom plate for 3 days in a 37°C, 5% CO_2 atmosphere. For the final 16 hours, 18.5 kBq (0.5 μCi) of ^{3}H-TdR was added to each well, then the labeled cells were collected and counted by the same method used for the cytostatic assay.

RESULTS

Cell killing effect of combined treatment

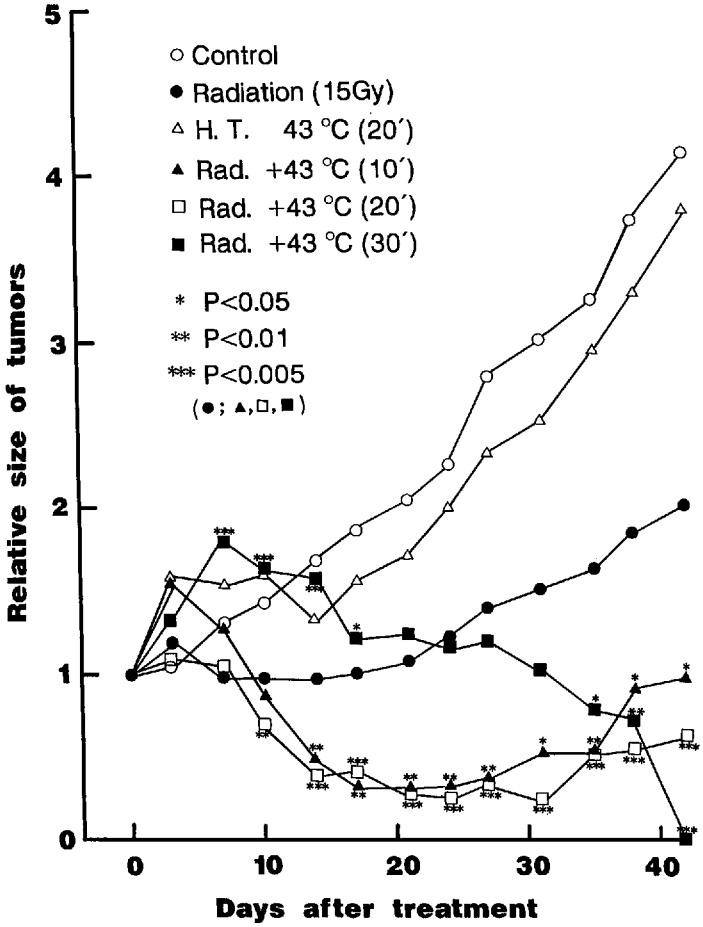

Fig. 2. Growth curves of MM 46 tumors treated with 15 Gy X-irradiation and/or water bath hyperthermia at 43 °C. Five to seven mice were used in each group. The results were compared using Student's two tailed *t* test.

with X-irradiation and 43°C hyperthermia

Hyperthermia was lethal to exponentially growing MAC tumor cells, the survival curve being characterized by a broad shoulder and a T_0 of 41.8 minutes (Fig. 1). The survival curves for cells irradiated *in vitro* are shown in Fig. 1 with a D_0 value of 1.53 Gy and a D_q value of 1.39 Gy. Hyperthermia treatment immediately after irradiation slightly reduced the colony forming ability compared to irradiation alone, but this treatment just slightly radiosensitized MAC tumor cells.

Anti tumor effects of X-irradiation and hyperthermia on solid MM 46 tumor growth

The effect of local irradiation and hyperthermia on the growth of MM 46 tumors in syngeneic C3H/He mice are shown in Fig. 2. Hyperthermia alone had no effect on tumor growth. There were no tumor-free mice in either the hyperthermia only group nor the irradiation only group, on the 42nd day. On the other hand, combined treatment of irradiation plus hyperthermia significantly inhibited tumor growth except for initial swelling. Hyperthermia for 30 minutes after irradiation resulted in completely tumor free mice on the 42nd day. Irradiation followed by hyperthermia induced long dose related survival (Fig. 3).

	CR (on day 42)	Mean Survival days (on day 115)
○ Control	0/7	71.0 ± 8.7
● Radiation (15Gy)	0/7	>97.7 ± 14.0
△ H. T. 43 °C (20′)	0/7	77.3 ± 9.5
▲ Rad. +43 °C (10′)	2/7	>104.7 ± 11.0
□ Rad. +43 °C (20′)	3/7	>106.0 ± 12.4
■ Rad. +43 °C (30′)	5/5	>115.0

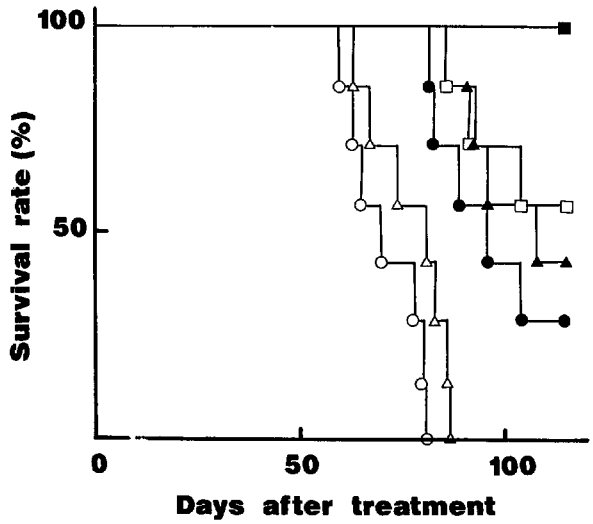

Fig. 3. Cure rate on day 42, and mean survival days on day 115 after the various treatments described for Fig. 2. CR is complete response which shows the rate of tumor disappearance on day 42.

Cytostatic activity of spleen cells from mice treated with irradiation and hyperthermia in suspension culture

The spleen cells from five groups of mice were used: (1) spleen cells from normal mice: (2) spleen cells from mice with unirradiated control tumors: (3) spleen cells from mice with tumors irradiated locally by 15 Gy X-rays: (4) spleen cells from mice treated with local hyperthermia (43°C, 30′) alone: (5) spleen cells from mice with tumors treated with local irradiation by 15 Gy X-rays and local hyperthermia (43°C, 30′).

Spleen cells from mice with irradiated tumors (the third group) markedly inhibited the growth of tumor cells for six days after irradiation, but during the next two weeks the cytostatic activity diminished and by the fourth week the activity had recovered, as shown in Table 1. Spleen cells from mice treated with hyperthermia alone (the fourth group) retained their activity as well as those from the control mice (the second group). Spleen cells from mice treated with irradiation plus hyperthermia (the fifth group) markedly inhibited the growth of tumor cells for more than 4 weeks.

Reduction of splenic suppressor cells by treatment with irradiation and hyperthermia

Table 1. Cytostatic activity of spleen cells of mice treated with irradiation and hyperthermia

Treatment	Days after treatment				
	1	6	13	20	27
Control (tumor)	28.5	8.0	8.1	22.0	28.1
Radiation	7.7	22.5	−5.1	2.5	25.2
H. T. alone	27.0	17.1	10.0	9.0	15.3
Rad. +H. T.	25.7	19.9	20.4	21.8	31.4

The mean c.p.m. of tumor cells alone and tumor cells with normal spleen cells are 11323.4±1531.5 and 10538.3±1260.2, respectively (mean±S. D. in each experiment).
The data are mean of the twice experiments except the day 27.

Table 2. Reduction of splenic suppressor cells by treatment with irradiation and hyperthermia: Effect on the Con A blastogenesis response of normal spleen cells

Cells added[a]	Normal	Control tumor	Radiation	H. T.	Rad.+H.T.
	47050.0±837.8[b] (100)	22059.4±1039.7 (46.9)	43142.6±1724.8 (91.7)	30438.1±1488.8 (64.7)	57090.9±999.5 (121.3)
		16919.6± 895.5 (36.0)	36870.4±1487.7 (78.4)	19274.9± 988.7 (41.0)	50947.7±779.2 (108.3)
		41.5[c]	85.1	52.9	114.8

[a] Added cells were obtained from mice on day 30.
Added cells were X-irradiated with 20 Gy to inhibit incorporation of [^{3}H] thymidine.
[b] Uptake of [^{3}H] thymidine (cpm±S. D.) for triplicated wells of each mouse.
Percentage of the response of normal spleen cells with irradiated normal spleen cells is in parentheses.
[c] Mean percentage of the response of two mice.

On the thirtieth day after treatment the spleens were removed. Suppressor cell activity in the spleen cells was defined as the capacity of cells from treated mice to inhibit the Con A blastogenic response of normal spleen cells (Table 2). There were significant differences in suppressor cell activity in each group by one-way analysis of variance ($p<0.01$). The spleen cells obtained from the non-treated control mice and those treated by local hyperthermia alone significantly suppressed the Con A response to 41.5% and 52.9%, respectively. Spleen cells obtained from locally irradiated mice were slightly suppressive. There was no suppressor activity of the spleen cells obtained after local irradiation plus hyperthermia.

DISCUSSION

This study was carried out to clarify whether the immune activity of the host is more effectively induced by local hyperthermia plus irradiation. Available evidence on the effects of hyperthermia on the host immune response is contradictory. It has been reported that hyperthermia was accompanied by enhanced metastasis; possibly by depression of the immune defense mechanisms of the host[1-4]. In contrast, there is some evidence consistent with hyperthermia stimulation of host anti-tumor immunity[7-10]. It has been shown in hamsters bearing bilateral tumors that impaired growth of the untreated tumor was related to heat treatment of the contralateral tumor[7]. Other studies have demonstrated that whole-body hyperthermia could be beneficial in preventing the spread of Lewis Lung Carcinoma in mice, possibly via a mechanism involving natural killer cells[8]. In the present study, we showed that hyperthermia treatment immediately after X-irradiation gave little radiosensitization of MAC tumor cells in an *in vitro* study. However, local hyperthermia after irradiation of solid tumors significantly reduced tumor growth except for some initial swelling. In a cytostatic study, anti-tumor cell-mediated

activity of spleen cells from mice treated by irradiation plus hyperthermia was greater than that in groups of control mice or mice treated by irradiation alone or by hyperthermia alone. Reduction of splenic suppressor cells in this group was also observed. Therefore, it is possible that the marked reduction of tumor growth *in vivo* by hyperthermia and irradiation is attributable, at least in part, to the host's immunological responses.

We previously reported that anti-tumor cell-mediated immunity in host mice was induced or enhanced by local irradiation of the tumor. That activity depended on non-killer and cytostatic T cells, and the effector T cells had no inhibitory activity by themselves, but required the involvement of macrophages to show inhibition in *in vitro* cytostatic tests[5,6]. In histological studies, Hirota *et al.* reported that the lymphocytes infiltrating into tumor tissue after local irradiation were analyzed using monoclonal antibodies and MM 46 tumors[11], and Lyt 1 T cells and L3T4 T cells are a major group of tumor-infiltrating lymphocytes after irradiation. Other studies have demonstrated marked infiltration of mononuclear cells, particularly macrophages, into tumor tissue after hyperthermia by the same method and in the same tumor system[12]. Alfieri *et al.* reported local hyperthermia-induced tumor eradication mediated by an activated macrophage-antigen-T-cell interaction[13]. Activated macrophages are likely to be important in host defense against tumors. In some tumor models, T cells recognize internally processed tumor antigens and the tumor antigens presented by macrophages. These T cells have the potential to produce MAF (macrophage activating factor), and non-specific macrophages are then activated as the ultimate tumoricidal effector cells[14,15]. The results of this study and these findings suggest that local hyperthermia with local irradiation induced and/or enhanced anti-tumor cell-mediated immunity by activating the lymphocytes and macrophages that infiltrated into the tumor tissue. Generation of immunosuppressor cells has been reported in tumor-bearing animals[16,17]. Recently, several investigators reported that low-dose chemotherapy with cyclophosphamide was curative via inhibition of the development or activity of suppressor T cells in mice bearing advanced tumors[18–20]. In the present study, suppressor cell activity was examined in treated mice and was found to be significantly suppressed in the animals treated with local irradiation plus hyperthermia. Cytostatic activity of the spleen cells from mice treated with irradiation plus hyperthermia markedly inhibited the growth of tumor cells for a long time.

These findings suggest that local irradiation plus hyperthermia induces alteration of suppressor-effector T cell ratios, infiltration of mononuclear cells, and the activity of macrophages to induce and/or enhance anti-tumor cell-mediated immunity. However, further studies are needed to confirm our conclusion.

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要旨：マウス移植腫瘍を用いて局所放射線温熱療法時の宿主免疫機能について検討した．C3H/He マウス由来の自然発生乳癌を 2×10^6 個大腿皮下に移植し 1 週間後 15 Gy の局所放射線照射あるいは恒温水槽による 43°C 温熱処理を行った．その結果温熱処理 (20 分) 単独群ではコントロール群とほぼ同様の腫瘍増殖を示した．局所照射 15 Gy 処理後，温熱処理を 10, 20, 30 分間併用するといずれの群でも腫瘍の増殖抑制効果は照射単独に比し有意に増強され，30 分間併用群では全例腫瘍消失を認めた．各処理マウス脾細胞の in vitro での抗腫瘍活性を検討したところ (cytostatic assay)，局所照射・温熱併用群で終始高い抗腫瘍活性を認めた．また同群のマウスでは脾細胞中のサプレッサー細胞活性の消失を認めた．これらの結果は温熱療法が局所照射の効果を増感するだけでなく宿主の免疫機能の増強を誘導する可能性を示唆した．