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Anti-tumor Effects of Hyperthermia Plus Granulocyte Colony-stimulating Factor

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The role of neutrophils in the anti-tumor effects of hyperthermia was investigated in an experimental rat model, and the efficacy of hyperthermia combined with recombinant human granulocyte colony-stimulating factor (G-CSF) was similarly investigated. AH109A carcinoma cells were transplanted into the hind legs of Donryu rats, then heated by a radio-frequency dielectric heater. In this study, because the myeloperoxidase (MPO) activity of neutrophils was not affected by heating or G-CSF, MPO activity was measured as an index of neutrophil migration into tumor tissue. After hyperthermia, MPO activity in tumor tissue increased significantly, suggesting migration of neutrophils into tumor tissue. Depletion of circulating neutrophils by the intraperitoneal injection of anti-rat neutrophil antibody decreased the anti-tumor effects of hyperthermia. Subsequently, we used hyperthermia plus intraarterial G-CSF to enhance the anti-tumor effect. Hyperthermia was induced 1 h after injection of G-CSF, a time when MPO activity in tumor tissue was maximal. A satisfactory thermal effect was noted even in cases where tissue could not be heated sufficiently. In conclusion, neutrophils have an important role in the anti-tumor effects of hyperthermia, and administration of G-CSF enhances these effects.

Key words: Neutrophil — Hyperthermia — Granulocyte colony-stimulating factor — Myeloperoxidase activity

Previous work in our laboratory has demonstrated that active oxygen species and lipid peroxidation are involved in the underlying mechanisms of hyperthermia.[1] Lipid peroxidation is believed to be an important cause of damage to cell membranes, because polyunsaturated fatty acids of the cellular membrane are degraded by lipid peroxidation.[2] Membrane peroxidation can lead to changes in membrane fluidity and permeability, and ultimately cell lysis. Neutrophils are one major source of active oxygen species. Neutrophils infiltrate extravascular spaces and can generate superoxide and hydrogen peroxide, which are important causes of cell damage.

In clinical studies of the anti-tumor effect of hyperthermia, it was evident that in cases in which the internal temperature of the tumor could not be raised to 42°C, the efficacy of hyperthermia was reduced.[3] In such cases, some type of combined therapy is needed. Consequently, because of the importance of neutrophils in hyperthermia, we investigated the efficacy of recombinant human granulocyte colony-stimulating factor (G-CSF) in combination with hyperthermia for inhibiting tumor growth.

MATERIALS AND METHODS

Experimental animals and tumors We used six-week-old male Donryu rats. Rat AH109A carcinoma was first transplanted into the peritoneum of the Donryu rats. Ascites containing 5×10^6 carcinoma cells was then subcutaneously grafted into the hind leg of the rat. Rats were treated 7 days after implantation.

Radio-frequency dielectric heater The radio-frequency (RF) dielectric heater was a Thermotron RF I.V. (Yamamoto Vinyter Co., Osaka) that uses an 8-MHz dielectric heating system.

Heating method AH109A carcinoma implanted into the hind leg was locally heated using the RF I.V. During the hyperthermia experiment, rats were anesthetized by intraperitoneal injection of 35 mg/kg of sodium pentobarbiturate. The tumor tissue temperature was monitored with a thermosensor (Yamamoto Vinyter Co.). The RF output was initially 20 W. When the temperature reached 41–43°C, the output was adjusted to maintain a constant temperature. Heating was continued for 15 min.

Evaluation of tumor growth Immediately before and 3, 5, and 7 days after hyperthermia, the major and minor axes of the tumors were measured. The volume was then calculated with the following formula[4];

$$V = (\text{longest diameter}) \times (\text{shortest diameter})^2 \times (1/2)$$

where V is the tumor volume. The growth ratio was defined as the tumor volume divided by the tumor volume immediately prior to hyperthermia.

Time course study of hyperthermia Groups of treated rats were killed by exsanguination under inhaled anesthesia 30 or 90 min after hyperthermia. Specimens were prepared by resection of the tumor and homogenization at 0°C in an equivalent volume of 0.05 *M* potassium phosphate buffer (pH 5.4), containing 0.5% hexadecyltrimethylammonium bromide and were analyzed for myeloperoxidase (MPO) activity. The samples were then

centrifuged at 20000×*g* for 15 min at 4°C. The MPO activity is an index of neutrophil migration into tumor tissues and was quantitated in the supernatant by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine.[5]

Neutrophil-depletion and hyperthermia A suspension of neutrophils isolated from a rat abdominal cavity was mixed with Freund's complete adjuvant (Difco Laboratories, Detroit, MI), and was injected subcutaneously into domestic rabbits. This immunizing procedure was repeated weekly for 4 weeks. One week after the final immunization, blood was drawn, and the serum was frozen at −80°C until use.[6] To prepare neutrophil-depleted rats, serum was injected intraperitoneally at a dose of 10 ml/kg, 18 h prior to hyperthermia.

Injection of G-CSF In some rats, G-CSF (250 μg/kg, provided by Sankyo Co., Tokyo) was injected into the femoral artery, and the peripheral blood neutrophil count and MPO activity in tumor tissue were measured at various time points. Hyperthermia was begun 1 h after injection.

Thiobarbituric acid (TBA)-reactive substances The concentration of TBA-reactive substances, an index of lipid peroxidation, was measured in serum samples by the method of Yagi,[7] and that in tissue homogenates was measured according to Ohkawa *et al.*[8] The concentration of TBA-reactive substances were expressed as nmol of malondialdehyde. Thiobarbituric acid (BDH Chemicals, Poole, England) and 1,1,3,3-trimethoxypropane (Tokyo Kasei Co., Tokyo) were used for the TBA assay, and all other chemicals were of reagent grade. Protein concentration in the tumor homogenate was measured by the method of Lowry *et al.*[9]

Statistical analysis Results are presented as mean±SEM for four to seven rats/group. The Kruskal-Wallis analysis was used to determine variances. The two-tailed non parametric Dunnett's test was used for comparison of group means. A value of $P<0.05$ was accepted as statistically significant.

RESULTS

Myeloperoxidase activity in tumor tissue after hyperthermia MPO activity was measured prior to hyperthermia and then 30 and 90 min after hyperthermia. Mean MPO activity was 6.79±1.1 mU/mg protein in tumor tissue prior to hyperthermia, 25.0±4.3 mU/mg protein 30 min after hyperthermia ($P<0.01$), and 18.3±4.1 mU/mg protein 90 min after hyperthermia ($P<0.05$) (Fig. 1).

Neutrophil-depletion and hyperthermia Following intraperitoneal administration of anti-rat neutrophil antibody, neutrophil counts were significantly decreased within 6 h after injection. The lowest neutrophil counts were observed after 18 h and were below 500/μl. The decrease persisted for up to 24 h. To study the effect of neutrophil-depletion, animals were subjected to hyperthermia (43°C for 15 min) 18 h after administration of anti-neutrophil antibody. An identical amount of normal domestic rabbit serum was administered to rats in the control group.

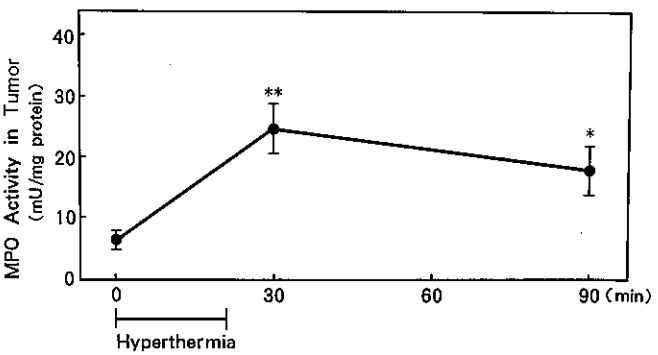

Fig. 1. Time-course of changes in MPO activity in tumor tissue after hyperthermia. Each point indicates the mean±SEM of 5 rats. The significance of differences between the value at 0 h and each value after hyperthermia is shown. * $P<0.05$, ** $P<0.01$.

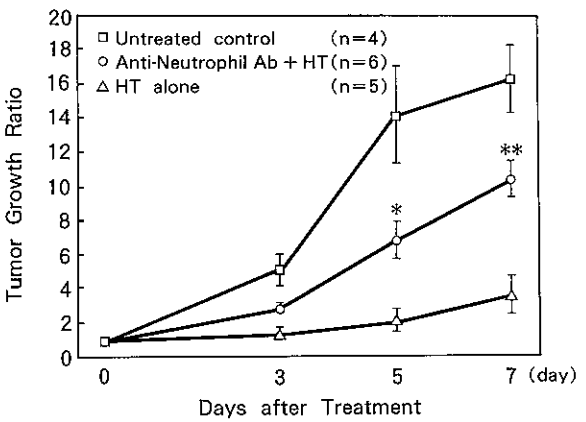

Fig. 2. Effect of neutrophil-depletion on the anti-tumor efficacy of hyperthermia. Control rats for neutrophil-depletion were injected intraperitoneally with normal rabbit serum (10 ml/kg) 18 h before hyperthermia. Anti-neutrophil antibody (10 ml/kg) was injected intraperitoneally 18 h before hyperthermia to induce neutrophil-depletion. Hyperthermia was performed at 43°C for 15 min. The tumor growth ratio for each group of four to six rats is shown. Each point indicates the mean±SEM. The significance of differences between the hyperthermia alone group and the neutrophil-depleted group is shown. * $P<0.05$, ** $P<0.02$.

Seven days after hyperthermia, the tumor growth ratio of the untreated control group was 16.08±2.05. The hyperthermia alone group had a growth ratio of 3.44± 1.16, showing that hyperthermia inhibited the growth of the tumor. When anti-neutrophil antibody was given along with hyperthermia, the growth ratio was 10.28± 1.01. Thus, inhibition of tumor growth by hyperthermia was significantly ($P<0.02$) abrogated by treatment with anti-neutrophil antibody (Fig. 2).

Changes in peripheral neutrophil count after injection of G-CSF The neutrophil count prior to injection of G-CSF was 2306±295.0/μl. The count increased significantly 1 h after injection ($P<0.02$) and peaked at 12 h (10585± 2108/μl, $P<0.01$) (Fig. 3).

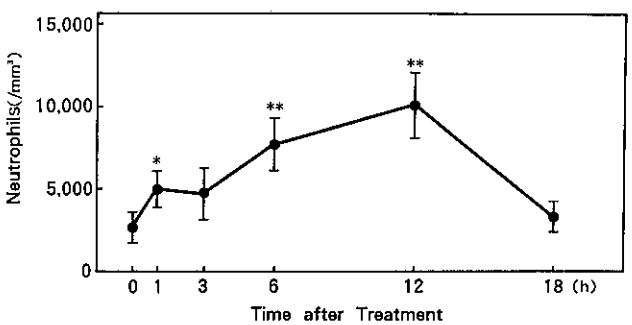

Fig. 3. Time-course of changes in peripheral neutrophil count after injection of G-CSF. Each point indicates the mean± SEM of 5 rats. The significance of differences between the value at 0 h and each value after injection of G-CSF is shown. * $P<0.02$, ** $P<0.01$.

Changes in tumor MPO activity after injection of G-CSF MPO activity in tumor tissue prior to injection of G-CSF was 5.9±1.57 mU/mg protein, and rose to 16.0±3.05 mU/mg protein 1 h after injection, a significant increase ($P<0.05$) (Fig. 4a).

Changes in tumor MPO activity after hyperthermia with or without G-CSF MPO activity of G-CSF plus hyperthermia at 4 h after the injection of G-CSF was 22.1 +4.3 mU/mg protein. It was significantly increased ($P<0.02$) to the level of the untreated control (neither G-CSF nor hyperthermia was given) group (6.69±1.3 mU/mg protein). Hyperthermia alone did not affect the activities of MPO at 3 or 24 h after hyperthermia (Fig. 4b).

Changes in lipid peroxides after hyperthermia with or without G-CSF TBA-reactive substances in tumor tissue, an index of lipid peroxidation, increased significantly from a basal concentration of 0.14±0.02 nmol/mg protein to 0.21±0.02 and 0.45±0.06 nmol/mg protein after hyperthermia alone and hyperthermia plus G-CSF, respectively ($P<0.05$, $P<0.01$). Moreover, increase of TBA-reactive substances after hyperthermia was significantly ($P<0.01$) accelerated by the injection of G-CSF. Hyperthermia alone and hyperthermia plus G-CSF did not affect the level of TBA-reactive substances in serum, however (Fig. 5).

Effect of G-CSF on the anti-tumor efficacy of hyperthermia Hyperthermia (41°C for 15 min) was performed 1 h after injection of G-CSF, a time when MPO activity was maximally enhanced. Compared to the saline group

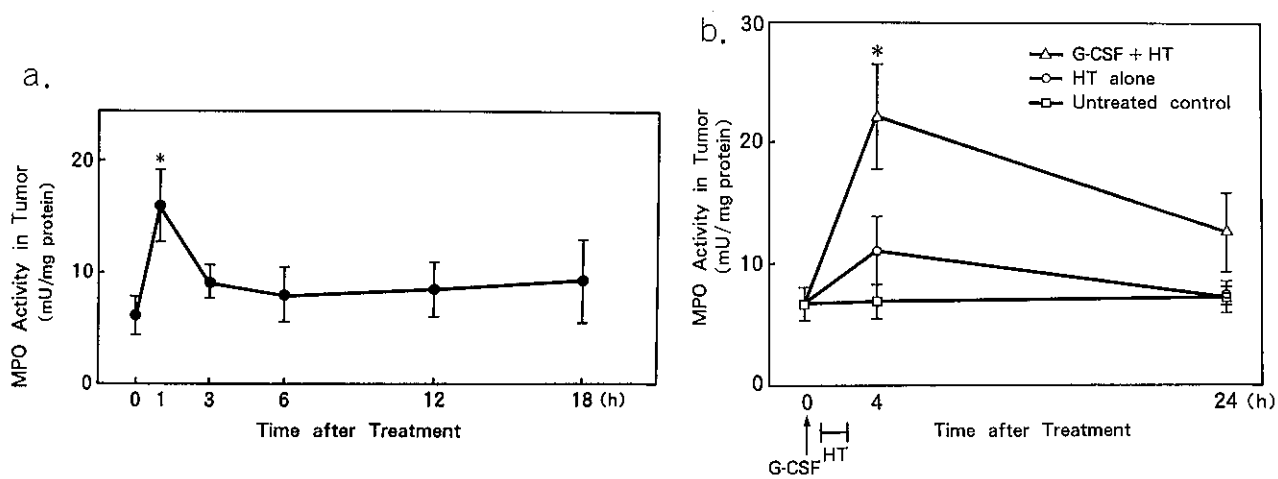

Fig. 4. a, Time-course of changes in MPO activity in tumor tissue after injection of G-CSF. Each point indicates the mean± SEM of 5 rats. The significance of differences between the value at 0 h and each value after injection of G-CSF is shown. * $P<0.05$. b, Time-course of changes in MPO activity in tumor tissue after hyperthermia with or without G-CSF. Each point indicates the mean±SEM of 7–10 rats. The significance of differences between the untreated control group and the hyperthermia plus G-CSF group at 4 h after injection of G-CSF is shown. * $P<0.02$.

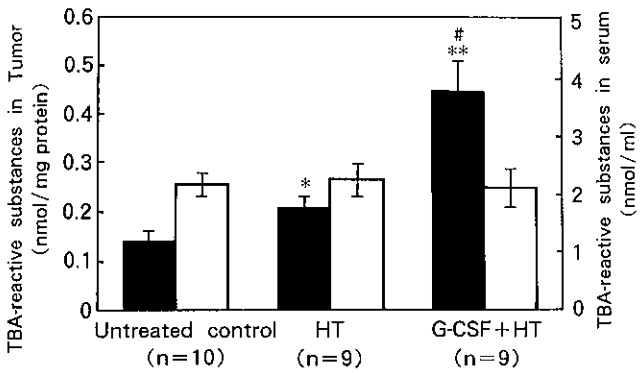

Fig. 5. Thiobarbituric acid (TBA)-reactive substances in tumor tissue and in serum 1 h after hyperthermia with or without G-CSF. Solid bars indicate the values of TBA-reactive substances in tumor tissue and blank bars indicate those in serum. Each point indicates the mean±SEM of 9 or 10 rats. * $P<0.05$ for difference from the untreated control. ** $P<0.01$ for difference from the untreated control. # $P<0.01$ for difference from the hyperthermia alone group.

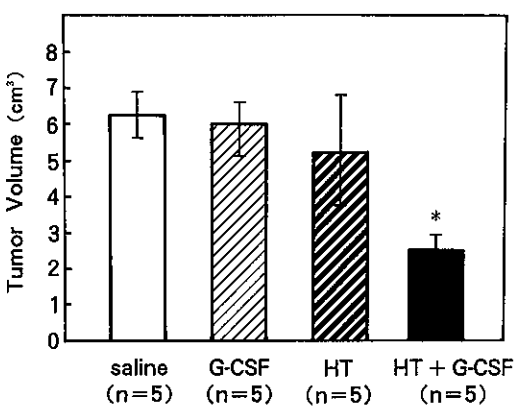

Fig. 6. Effect of G-CSF on the anti-tumor efficacy of hyperthermia. Saline group rats were injected with physiological saline (0.5 ml) into the femoral artery. Hyperthermia was performed at 41°C for 15 min. G-CSF (250 μg/kg, 0.5 ml) was injected, with or without hyperthermia. The tumor growth ratio for each group of 5 rats is shown. Each point indicates the mean±SEM. The significance of the difference between the saline group and the hyperthermia plus G-CSF group is shown. * $P<0.01$.

(6.241±0.637 cm^3), no significant difference was observed in tumor volume 7 days after treatment with G-CSF alone (5.979±0.731 cm^3) or hyperthermia alone (5.181±1.545 cm^3). However, there was a significant reduction in the G-CSF plus hyperthermia group (2.473 ±0.431 cm^3, $P<0.01$) (Fig. 6).

DISCUSSION

We have previously reported that active oxygen species are important factors in the development of gastric mucosal[10] and liver[11] damage. Cytotoxicity of free radicals has been reported in cancer therapy, and we have found that reperfusion injury can occur in tumors and can even destroy tumors.[12] Further, oxygen radicals produced by hypoxanthine and xanthine oxidase can cause an anti-tumor effect.[13] Previously, we found that active oxygen species may mediate hyperthermia-induced damage in cancer tissue.[1] Activated neutrophils have been the primary focus of research on active oxygen-induced tissue damage. Grisham *et al.*[14] investigated neutrophils in a feline intestinal ischemia model and noted inhibition of neutrophil infiltration and decreased vascular permeability by allopurinol, superoxide dismutase (SOD), and anti-neutrophil antibodies. It was speculated that active oxygen species produced by hypoxanthine-xanthine oxidase and neutrophils played a major role in this tissue damage. Furthermore, Suzuki *et al.*,[15] in a model of gastric mucosal injury in rats, emphasized the importance of neutrophil-derived active oxygen in tissue injury, and they observed increased gastric mucosa myeloperoxidase activity and enhanced production of neutrophil superoxide in gastric blood.

In our study, myeloperoxidase activity in tumor tissue was significantly enhanced by hyperthermia. Because the hyperthermic anti-tumor effect was markedly inhibited by pretreatment with anti-neutrophil antibodies, we concluded that neutrophils were required for the anti-tumor effect of hyperthermia. Therefore, we further investigated whether G-CSF, which increases peripheral counts and accelerates neutrophil function, would enhance the anti-tumor effect of hyperthermia. It has been reported that MPO activity of neutrophils was not enhanced by G-CSF.[16,17] Our preliminary experimental data also showed MPO activity of neutrophils was not affected by heating and G-CSF administration *in vitro*, and systemic administration of G-CSF did not accelerate MPO activity of transplanted tumor tissue in rat (data not shown). Because hyperthermia and G-CSF did not activate MPO of neutrophils, MPO activity was measured as an index of neutrophil migration into tumor tissue. When G-CSF was injected into the dominant tumor artery and MPO activity was measured, MPO activity rose in tissues 1 h after injection. The cell number of peripheral neutrophils peaked at 12 h after G-CSF administration, whereas MPO activity in tumor tissue peaked at 1 h. These results showed that neutrophils induced to migrate into tumor tissue by G-CSF before the peripheral neutrophils greatly increased. When hyperthermia was performed 1 h after injection of G-CSF, MPO activity increased, lipid peroxidation was accelerated, and the anti-tumor effect was

enhanced compared to hyperthermia alone. It was speculated that hyperthermia resulted in further accumulation of neutrophils, and active oxygen species produced by the neutrophils caused tumor tissue lipid peroxidation and the anti-tumor effect was enhanced. In our study, it was not proven histologically that the numbers of tumor-infiltrated neutrophils increased when G-CSF administration or hyperthermia was performed, but the augmentation of MPO activity after G-CSF administration or hyperthermia provides strong evidence of an increase of neutrophil numbers in tumor tissue. Neutrophil accumulation at the microcirculation level may occur in tumor vessels. In summary, neutrophils appear to mediate the anti-tumor effect of hyperthermia, and administration of G-CSF enhances the anti-tumor effect of hyperthermia.

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