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研究課題名（和文）　収束超音波による軟部肉腫の治療

研究課題名（英文）　An experimental treatment of soft tissue sarcoma by HIFU (high-intensity focused ultrasound)

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研究成果の概要：平成 19,20 年度に収束超音波による抗腫瘍作用の解析、特に腫瘍免疫学的効果をマウス固形肉腫を対象に以下のように行った。具体的には１．細胞の継代と腫瘍細胞浮遊液の調整、２．超音波照射装置の作成（日立中央研究所との共同開発)、３．腫瘍中心部のみへの収束超音波の単回照射をおこない、残存した腫瘍内、及びその周囲での反応を観察し、以下の結果を得た。経時的に腫瘍最大径を観察すると、対照では徐々に腫瘍径が増大していたが、照射群では増大は軽度で対照群に比べ有意に小さかった。壊死部周辺では腫瘍細胞の旺盛なアポトーシスが観察され、その頻度は照射群で有意に多かった。さらに対照群に比べ照射群では腫瘍周囲を中心として TRAP 陽性で多核のマクロファージの浸潤が認められ、腫瘍内でも CD4, CD8 陽性のリンパ球の有意な集族性の浸潤が観察された 。またマウス生存率は照射群では対照群に比べ有意に生存率が良好であった。以上から収束超音波の単回照射により、前腫瘍の絵師が生じなくても、抗腫瘍免疫の活性化により、残存腫瘍に対しても抗腫瘍効果が得られると考察した。

交付額

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2007 年度	1,700,000	510,000	2,210,000
2008 年度	1,500,000	450,000	1,950,000
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年度			
総　計	3,200,000	960,000	4,160,000

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１．研究開始当初の背景

Musculoskeletal sarcoma is a relatively rare tumor that represents fewer than 1% of all adult malignancies (1); more than 50% of these tumors occur in the extremities. Surgical resection of a malignant tumor must include a margin of healthy tissue to prevent local recurrence, and surgical therapy is frequently combined with chemotherapy and/or radiotherapy. Although newer chemotherapy and radiotherapy regimens have changed the nature and scope of surgical management of primary sarcomas, they are not always effective. Recent studies have shown that approximately one-third of patients with primary sarcoma develop recurrent disease.

Ultrasound antitumor therapies, which include sonodynamic therapy and high-intensity focused ultrasound (HIFU) therapy, offer great promise for the treatment of cancer. Sonodynamic therapy depends on the synergistic effect of drugs and ultrasound. When a liquid is irradiated with ultrasound, cavitation occurs. Cavitation results in oxidation, luminescence, destructive dispersion, and stirring. Hematoporphyrin and acridine orange have been used as photodynamic compounds in cancer treatment; however, hematoporphyrin can cause photodermatitis, and is not generally used in clinical practice.

HIFU is a less invasive technique for tumor ablation. In HIFU, a focused beam passes through the skin over a wide area, and the tissue at a focal point is selectively ablated. HIFU destroys tumors by inducing direct thermal necrosis, which inhibits tumor growth . In addition, experimental data suggest that antitumor responses in the tumor-bearing host are stimulated after HIFU irradiation (7-10). However, there have been few studies of immunological response after HIFU treatment for soft tissue sarcoma. If chemotherapy or radiotherapy for musculoskeletal sarcoma are not effective, HIFU treatment may be a suitable alternative. In this study, we examined histological changes, including anti-tumor immunological response, in soft tissue sarcoma after HIFU treatment.

２．研究の目的

To evaluate antitumor effect, especially immunological responses of HIFU in the mouse soft tissue sarcoma model.

３．研究の方法

The protocols for the animal experiments described in this paper were previously approved by the Animal Research Committee, Akita University School of Medicine; all subsequent animal experiments adhered to the “Guidelines for Animal Experimentation” of the university.

Preparation of tumor cells. Ascitic sarcoma 180 cells (Medical Cell Resource Center, Tohoku University Gerontology Research Institute, Sendai, Japan) were used for induction of the experimental tumor. One ml of a suspension of sarcoma 180 (3.0-4.0 x 10^5/ml) was injected intraperitoneally into ICR (Institute for

Cancer Research) male mice (Japan SLC Inc., Shizuoka, Japan), and 3.0 to 4.0 ml of ascitic fluid, collected approximately 10 to 14 days later, was diluted in phosphate-buffered saline (PBS) so that the final number of cells was 3.0 x 10^5 / ml. The survival rate of tumor cells was evaluated using the trypan blue dye exclusion method with a hemocytometer (Kayagaki, Tokyo, Japan) under an optical microscope (Olympus BH-210, Tokyo, Japan x400). Viability before treatment was always over 98%.

Ultrasonic transducer and generator. The HIFU transducer (Hitachi Central Research Laboratory, Tokyo, Japan) had a resonant frequency of 3.303 MHz, an aperture diameter of 28 mm, a focal spot of 1 mm^2, and a focal length of 30 mm. The elements were mounted in an aluminum housing that also contained a small imaging probe (SSD-2200, ALOKA Co., Ltd., Tokyo, Japan) operating at 7.5 MHz . The position of the imaging probe was adjusted in calibration experiments before conducting the animal experiments.

The ultrasonic generator used in this study was manufactured at the Department of Electronic Engineering, Akita University Mining College, Akita, Japan. The system included a high power generator (DX-801, ALINCO, Osaka, Japan) with a frequency range of 1.6 to 30 MHz and an output power of 0 to 100 W, and a power meter (NT-636 Network Tuner, KURANISHI, Hiroshima, Japan) with a frequency range of 1.8 to 54 MHz and a power measurement range of 0 to 200 W.

Tumor implantation. Ten-week-old ICR male mice were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg), and their right back was shaved. Two ml of sarcoma 180 suspension (3.0 x 10^5/ml cells) was injected subcutaneously into the right back with a 26-gauge needle. Two weeks after implantation, mice that had tumors with a maximum diameter of 15 ± 2 mm were included in the subsequent experiments.

Experiment protocol. The anesthetized mice (n=25) were placed on a specially designed holder in a degassed 37° C water bath, to facilitate proper acoustic coupling. The head of the mouse was held above water to prevent drowning. The transducer was positioned in the water bath by using ultrasound imaging and a 26-mm high conical, plastic base. The base was removed during the HIFU treatment (Fig 1-C). A single shot of HIFU (10 W/cm^2 at a frequency of 3 MHz for 10 seconds) was administered to the center of the tumor. The control group comprised tumor-bearing mice that did not undergo HIFU treatment (n=25).

A caliper (SM-7, Nakamura Mfg. Co., Ltd., Tokyo, Japan) was used to measure maximum tumor diameter at 1, 3, 7, 14, 21, and 28 days after irradiation. Survival rate was observed for 8 weeks (n=9 for each group). Treated and control mice were sacrificed at 1, 3, 7, and 14 days after irradiation (n=16 for each group). To assess histological changes, we used hematoxylin-eosin stain (HE stain), TUNEL stain by ApopTag (Chemicon International

Inc., California, USA), tartrate-resistant acid phosphatase (TRAP) stain to detect macrophages and dendritic cells (Okada et al. 1999), and immunohistochemical stains for CD4 and CD8 (Nichirei Bioscience Inc., Tokyo, Japan). After TUNEL staining, the number of positive tumor cells were counted. After TRAP and immunohistochemical staining, the number of positive cells within the remnant tumor tissue adjacent to the area of necrosis were counted. Averages were calculated based on counts for 20 randomly selected high-power fields.

Statistical analysis. Tumor size was analyzed by using the ANOVA test, and the number of positive cells in each stain were analyzed using the Student t test. The survival rate was analyzed with the Kaplan-Meier method, and differences between groups were analyzed by using the log-rank test. Differences between groups were considered significant if $P < 0.05$.

４．研究成果

Tumor size and the survival rate. At 1, 3, and 7 days after treatment, tumor size in the HIFU group did not significantly differ from that in the control group. However, at 14 days after treatment, tumor size in the HIFU group was significantly smaller than that in the control (14.5 ± 3.4 mm [mean ± SD] vs. 24.0 ± 6.5 mm, p=0.016). Similarly, at 21 and 28 days after treatment, tumor size in the HIFU group was significantly smaller than in the controls (16.2 ± 6.1 mm vs. 33.8 ± 9.0 mm, p=0.006; 18.8 ± 7.3 mm vs. 43.5 ± 12.2 mm, p=0.004, respectively, Fig. 2). The survival rate in the HIFU group was significantly higher than that of the control group (at 8 weeks, 67% vs. 23%, p=0.016, log-rank test).

Skin condition. The tumors were clearly visible before irradiation. The skin overlying the tumor was investigated macroscopically for 8 weeks after irradiation; there were no apparent changes such as necrosis, redness, or swelling.

Histological changes. In HE staining, the ablation area displayed necrotic changes, and remnant tumor cells adjacent to the area of necrosis appeared normal. In TUNEL staining, positive tumor cells were observed within the tumor, especially around the area of necrosis (Fig. 5A). In the HIFU group, the number of TUNEL-positive cells was 3.58 ± 2.02, 10.55 ± 3.86, 11.48 ± 3.77, and 4.00 ± 1.70 at 1, 3, 7, and 14 days, respectively. In the control group, the number of positive cells in the tumor was 3.03 ± 1.62, 3.80 ± 1.73, 5.25 ± 2.95, and 3.35 ± 1.46 at 1, 3, 7, and 14 days, respectively. At 3 and 7 days, the number of positive cells in the HIFU group was significantly higher than in the control group (p<0.001).

In the HIFU group, multinucleated cells within the tumor were TRAP-positive. The number of TRAP-positive cells in the tumor was 2.42 ± 1.10, 4.56 ± 1.80, 4.78 ± 2.28,

and 1.97 ± 1.27 at 1, 3, 7, and 14 days, respectively (Fig. 6B). In the control group, the number of positive cells in the tumor was 0.07 ± 0.25, 0.03 ± 0.18, and 0.09 ± 0.29 at 1, 3, 7 days; no TRAP-positive cells were observed at 14 days. At 1, 3, and 7 days, the number of TRAP-positive cells in the HIFU group was significantly higher than that of the control group (p<0.001).

Nodular or scattered infiltration of lymphocytes was observed in the HIFU group. These cells were positive for CD4 or CD8. The number of CD4-positive cells in the HIFU group were significantly higher than control at 1, 3, 7, and 14 days (4.59 ± 7.12 vs. 0.13 ± 0.43, 3.13 ± 2.51 vs. 0.15 ± 0.76, 4.38 ± 3.64 vs. 0.05 ± 0.22, and 1.65 ± 1.82 vs. 0.10 ± 0.39, p<0.001, respectively). The number of CD8-positive cells in the HIFU groups were significantly higher than control at 1, 3, 7, and 14 days (1.59 ± 4.58 vs. 0.03 ± 0.18, 5.50 ± 6.84 vs. 0.26 ± 0.86, 6.60 ± 5.96 vs. 0.10 ± 0.35, and 1.98 ± 2.68 vs. 0.03 ± 0.16, p<0.001, respectively).

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔学会発表〕（計３件）
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