Infiltration by Macrophages and Lymphocytes in Transplantable Mouse Sarcoma after Irradiation with High-intensity Focused Ultrasound

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Abstract. *Backgound: High-intensity focused ultrasound (HIFU) therapy offers great promise for the treatment of cancer. The histological changes, including the antitumor immunological response, after HIFU treatment was examined in soft tissue sarcoma. Materials and Methods: Sarcoma 180 cells were injected subcutaneously in mice. Approximately 2 weeks after the injection, the tumor was irradiated by a single shot of HIFU. The tumor diameter was measured and the survival rate was observed after treatment. The tumors were resected, and stained with TUNEL stain, tartrate-resistant acid phosphatase (TRAP) stain to detect tumor-associated macrophages, and immunohistochemical stains for CD4 and CD8. Results: The tumor size in the HIFU group was significantly smaller than the control and survival rate was significantly higher. The numbers of TUNEL-, TRAP-, CD4- and CD8-positive cells infiltrating the tumor were significantly higher in the HIFU group. Conclusion: HIFU, even when administered as a single shot, induces apoptosis of tumor cells and intratumoral infiltration of macrophages and lymphocytes.*

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 (1).

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 (2). When a liquid is irradiated with ultrasound, cavitation occurs. Cavitation results in oxidation, luminescence, destructive dispersion and stirring (3). Hematoporphyrin and acridine orange have been used as photodynamic compounds to enhance cancer treatment (3), 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 (4-6). In addition, experimental data have suggested that antitumor responses in the tumor-bearing host are stimulated after HIFU irradiation (7-10). However, there have been few studies of the immunological response after HIFU treatment for soft tissue sarcoma. If chemotherapy or radiotherapy for musculoskeletal sarcoma is not effective, HIFU treatment may be a suitable alternative. In this study, the histological changes, including the antitumor immunological response was examined in soft tissue sarcoma after HIFU treatment.

Materials and Methods

The protocols for the animal experiments described in this paper had previously been approved by the Animal Research Committee, Akita University School of Medicine; all subsequent animal

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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 the induction of the experimental tumors. One ml of a suspension of sarcoma 180 ($3.0\text{-}4.0\times10^5$/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 3.0×10^5/ml. The survival rate of the 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 ×400). The 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 (Figure 1-A, B). 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 Instruments Ltd., 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 (3.0×10^5/ml cells) was injected subcutaneously into the right back with a 26-gauge needle. Two weeks after implantation, the 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 (Figure 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 the maximum tumor diameter at 1, 3, 7, 14, 21 and 28 days after irradiation. Survival 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). Tissues were fixed in neutral formalin and embedded in paraffin as usual. Three-point-five-micrometer sections were cut on glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan). Five histological slides were obtained from each paraffin block. To assess histological changes, we used hematoxylin-eosin stain (HE stain), TUNEL stain by ApopTag (Chemicon International Inc. Temecula, CA, USA) and tartrate-resistant acid phosphatase (TRAP) stain to detect macrophages and dendritic cells (18) and immunohistochemical stains for CD4 and CD8 (Nichirei Bioscience Inc., Tokyo, Japan). After TUNEL staining, the number of positive tumor cells was counted. After TRAP and immunohistochemical staining, the number of positive cells within the remnant tumor tissue adjacent to the area of necrosis was counted. The averages were calculated based on counts of 20 randomly selected high-power fields.

Statistical analysis. Tumor size was analyzed using the ANOVA test and the number of positive cells in each stain was analyzed using the Student's *t*-test. The survival rate was analyzed with the Kaplan-Meier method and differences between groups were analyzed by the log-rank test. Differences between groups were considered significant at $p<0.05$.

Results

Tumor size and survival rate. At 1, 3 and 7 days after treatment, the tumor size in the HIFU group did not significantly differ from that in the control group. However, at 14 days after treatment, the 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, the 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, Figure 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, Figure 3).

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 the remnant tumor cells adjacent to the area of necrosis appeared normal (Figure 4). In TUNEL staining, positive tumor cells were observed within the tumor, especially around the area of necrosis (Figure 5A). In the HIFU group, the mean number of TUNEL-positive cells per high power field 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, compared to 3.03±1.62, 3.80±1.73, 5.25±2.95 and 3.35±1.46 respectively in the control group. At 3 and 7 days, the number of positive cells in the HIFU group was significantly higher than in the control group ($p<0.001$, Figure 5B).

In the HIFU group, the multinucleated cells within the tumor were TRAP-positive (Figure 6A). The mean number

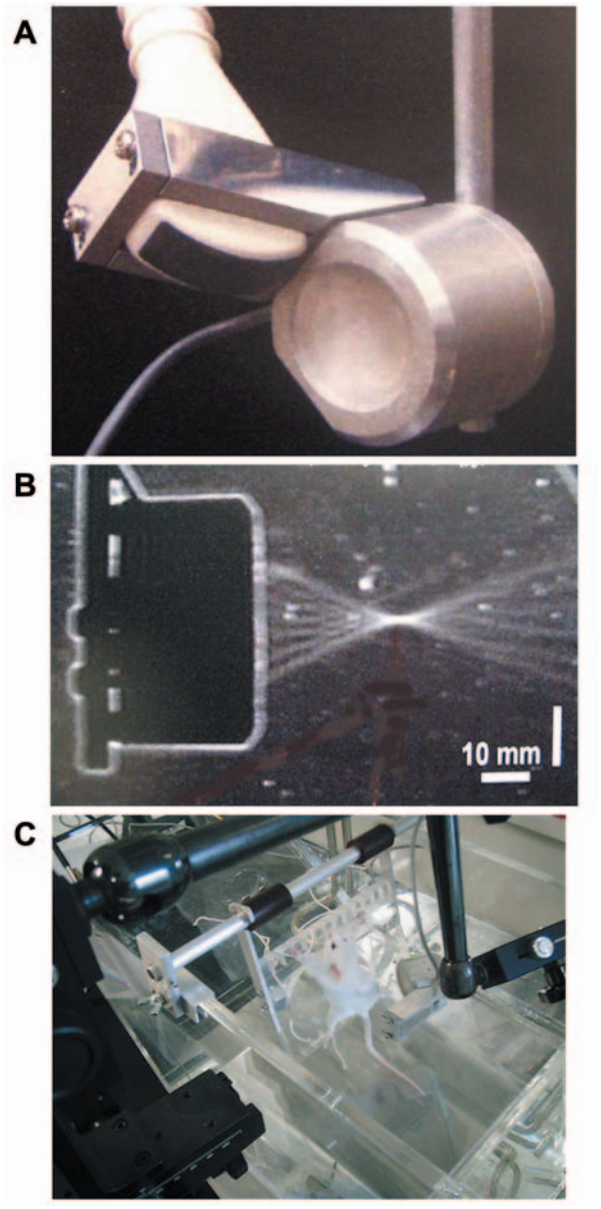

Figure 1. *The HIFU transducer and imaging probe (A). A schlieren image of the transducer (B). Operative view (C).*

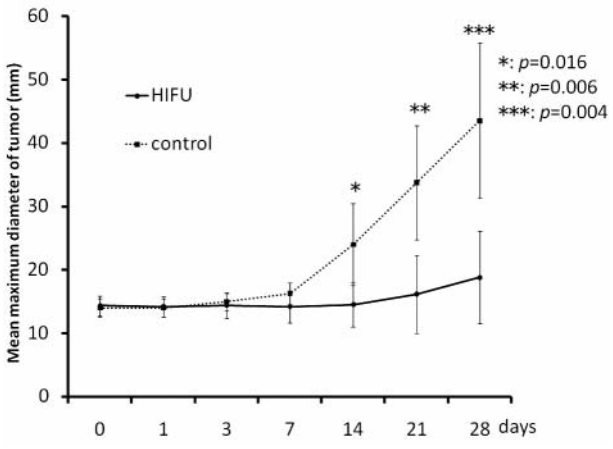

Figure 2. *Mean maximum diameter of transplanted tumors.*

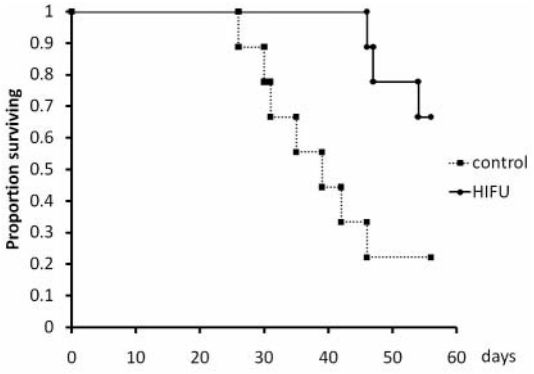

Figure 3. *Mean survival in tumor-bearing mice. (HIFU vs. control at 8 weeks, p=0.016).*

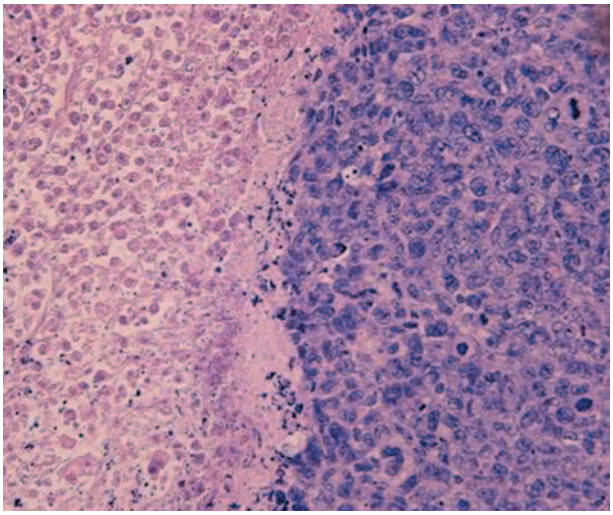

Figure 4. *Histological features in HE stain at 1 day after HIFU treatment (×200). Necrotic changes are present in the area of ablation (left side).*

of TRAP-positive cells in the tumor per high power field 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 in the HIFU group (Figure 6B). In the control group, the mean number of positive cells in the tumor was 0.07±0.25, 0.03±0.18 and 0.09±0.29 at 1, 3 and 7 days ($p<0.001$ *vs.* HIFU group); no TRAP-positive cells were observed at 14 days.

Nodular or scattered infiltration of lymphocytes was observed in the HIFU group. These cells were positive for CD4 or CD8 (Figure 7A, 8A). The mean number of CD4-

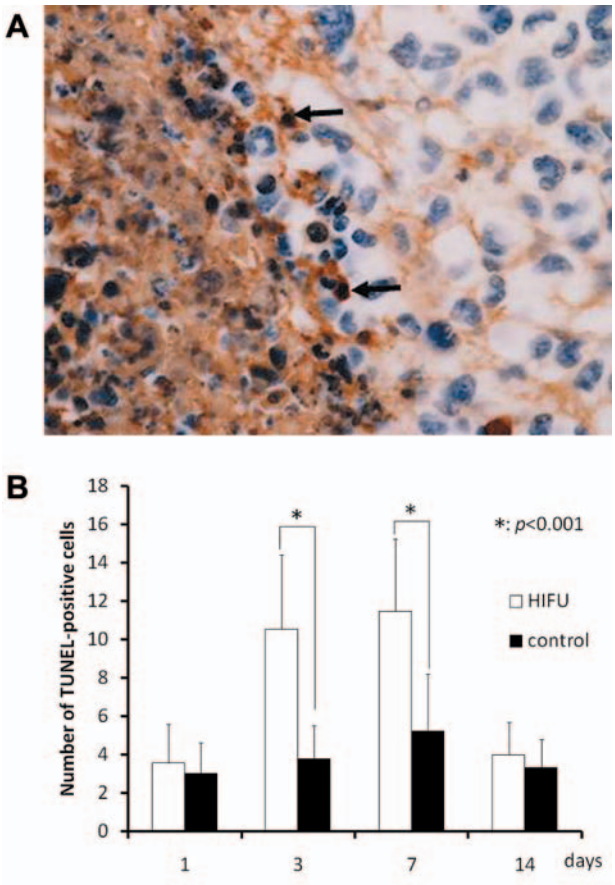

Figure 5. *TUNEL-positive cells. (A) TUNEL-positive cells (arrows) in tumor issue 7 days after HIFU treatment (Apoptag, ×400). (B) Mean number of TUNEL-positive cells in 20 random high power fields.*

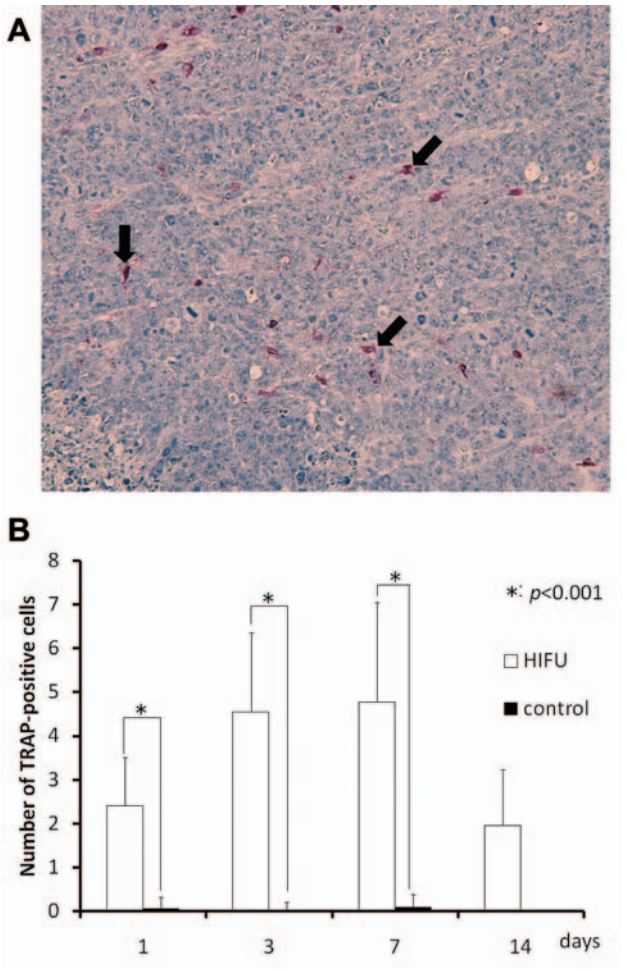

Figure 6. *TRAP-positive cells. (A) Infiltration of TRAP-positive multinucleated cells (arrows) into the tumor 3 days after HIFU treatment (×100). (B) Mean number of TRAP-positive cells in 20 random high power fields.*

positive cells in the HIFU group was significantly higher than the 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, Figure 7B). The mean number of CD8-positive cells in the HIFU group was significantly higher than the 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, Figure 8B).

Discussion

Although only partial necrosis was observed in the tumors after a single shot of HIFU, the treatment inhibited the growth of mouse transplantable sarcoma and increased survival in the tumor-bearing mice. The observed histological changes, which included necrosis, an increase in TUNEL-positive tumor cells and immunological responses such as infiltration of TRAP-positive cells and the presence of CD4- and CD8-positive lymphocytes, were evidence of the immunological antitumor effect of a single shot of HIFU.

Several reports have described the immunological effects of HIFU (8, 10, 11). Wu *et al.* showed that CD4+ lymphocytes increased in the circulation of cancer patients after HIFU treatment (10). An *in vivo* study with tumor-bearing mice confirmed that HIFU induced antitumor immunity by significantly enhancing the activity of specific cytotoxic T lymphocytes (CTL) and increasing the number of tumor-specific IFN-γ-secreting cells (8). $CD4^+$ helper T cells (Th) play critical roles in initiating, regulating and maintaining the antitumor immune response. In addition, they maintain and induce the production of $CD8^+$ cytotoxic T-lymphocytes. They also have effector functions against tumors *via* macrophage activation, cytokine production and

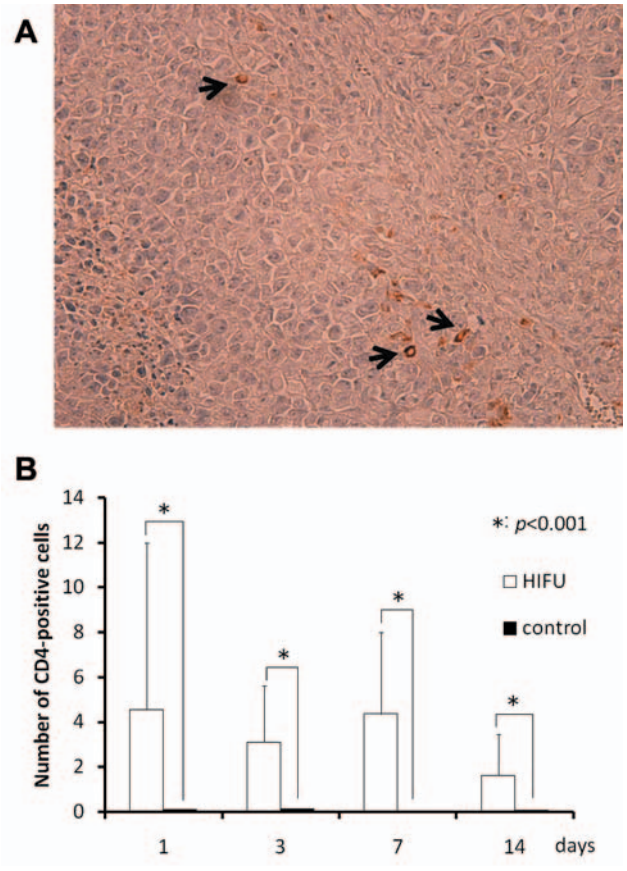

Figure 7. *Immunohistochemical stain for CD4. (A) Diffusely distributed CD4-positive cells (arrows) in tumor tissue 3 days after HIFU treatment (×200). (B) Mean number of CD4-positive cells in 20 random high power fields.*

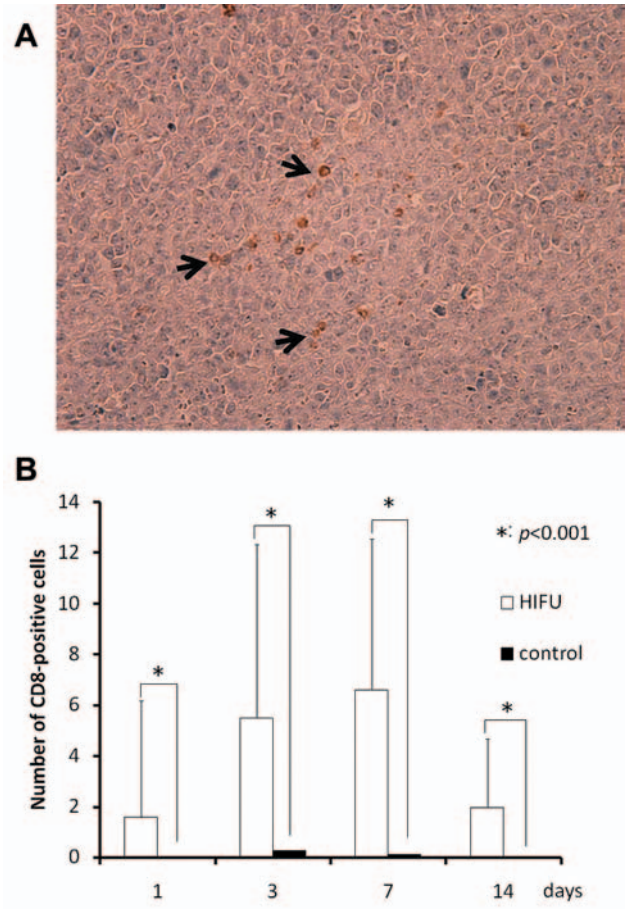

Figure 8. *Immunohistochemical stain for CD8. (A) Diffusely distributed CD8-positive cells (arrows) in tumor tissue 3 days after HIFU treatment (×200). (B) Mean number of CD8-positive cells in 20 random high power fields.*

direct killing of MHC class II-positive tumors (12-14). In the present study, the histologically observed intratumoral infiltration of CTLs (CD8-positive), Th cells, and macrophages after a single shot of HIFU, indicated that the HIFU treatment had an immunological antitumor effect.

TRAP is highly expressed by osteoclasts, activated macrophages and dendritic cells (15-17). Since the TRAP-positive cells were multinucleated, they were most likely to be macrophages. Dendritic cells capture and process antigens and present fragments to the Th cells. These helper cells coordinate the activities of the various immune cells to effectuate an immune response. Upon activation, Th1 cells cause macrophages to produce reactive oxygen intermediates and nitric oxide, stimulate the phagocytic functions of macrophages and enhance macrophage antigen presentation by up-regulating MHC class II molecules (17). Furthermore, these activated macrophages are able to specifically recognize tumor cells *in vivo* and inhibit growth of solid tumors by means of infiltration and direct cytotoxicity (18).

Apoptosis, or programmed cell death, is a single-cell phenomenon characterized by DNA fragmentation and nuclear shrinkage (19). Apoptosis allows for the rapid removal of damaged cells and is an active form of cell death, with an important role in the development and homeostasis of multicellular organisms. In contrast, necrosis is a form of cell death that results from overwhelming cellular injury: cells undergo lysis, thereby releasing their cytoplasmic contents, which can inflame surrounding cells. The TUNEL method has become the most widely used histochemical method of detecting apoptotic cells (20). In the present study, the number of TUNEL-positive tumor cells present at 3 and 7 days after HIFU treatment was significantly higher than the control. Similarly, Luo *et al*. observed that apoptotic cells began to increase in normal rabbit liver after HIFU exposure, peaking at 72 hours (21). Although the reasons underlying the induction of apoptosis after HIFU are not clear, several factors have been suggested, including heat stress stimuli and an ischemia-reperfusion mechanism (21).

Partial ablation of tumors by HIFU produces antitumor immunological effects. However, it should be obvious that partial ablation and the attendant immunological responses are insufficient to eradicate tumors completely. Keshavarzi *et al*. showed that proliferation of tumor cells occurred in areas adjacent to necrosis after treatment (22). In addition, the immunological response observed in the current study tended to be diminished at 14 days after HIFU treatment. The possibility of enhancing the immunological effect of HIFU should be a subject for future research.

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