

Effects of High-Intensity Focused Ultrasound in the Treatment of Experimental Neuroblastoma

By Rong Yang, Clarence R. Reilly, Frederick J. Rescorla, Narendra T. Sanghvi, Francis J. Fry, Thomas D. Franklin, Jr, and Jay L. Grosfeld
Indianapolis, Indiana

● This report evaluates the effect of high-intensity focused ultrasound (HIFU) on subcutaneous murine neuroblastoma C1300. HIFU treatment was administered with a focused 4-MHz quartz transducer with a peak intensity of 550 W/cm². In experiment 1, 60 animals with tumor were divided into four groups. Group I (n = 15) were controls; group II (n = 15) received adriamycin, 5 mg/kg intraperitoneally; group III (n = 15) received HIFU; and group IV (n = 15) received both adriamycin and HIFU. All the animals in groups I and II died of tumor by 35 days. Fifty-three percent (8/15) of mice in group III and 80% (12/15) in group IV were cured with no evidence of tumor (NET) at 200 days. Log-rank statistics showed significant prolongation of survival in the groups III and IV as compared with groups I or II ($P < .05$). In experiment 2, 45 animals with tumor were divided into three groups. Group I (n = 15) were controls; group II (n = 15) received HIFU; and group III (n = 15) received repeated HIFU. The results showed 47% (7/15) of mice in group II and 67% (10/15) in group III were NET at 200 days. Significant survival prolongation was achieved in groups II and III in comparison with group I ($P < .05$). In experiment 3, 90 mice received either tumor (n = 60) or saline (n = 30) inoculation in the left flank. On day 5, 45 mice with tumor were treated with HIFU (group I), while the other 15 mice with tumor (group II) had a sham procedure. Nineteen mice in group I were cured with NET (group IA) and 26 had persistent tumor (group IB). The 30 mice receiving saline (without tumor) were treated with either HIFU (group III, n = 15) or a sham procedure (group IV, n = 15). On day 26, all the animals received a second tumor challenge in the right flank. Reduced tumor growth following a second tumor challenge was demonstrated in group IA as compared with other groups ($P < .001$), implying a stimulation of host tumor immunity following curative HIFU treatment. The data suggest that HIFU may be an alternative modality for the treatment of unresectable neuroblastoma.
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From the Section of Pediatric Surgery, Department of Surgery, Indiana University School of Medicine, and the Indianapolis Center for Advanced Research, Inc, Indianapolis, IN.

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Address reprint requests to Jay L. Grosfeld, MD, Department of Surgery, J.W. Riley Hospital for Children K-21, 702 Barnhill Dr, Indianapolis, IN 46223.

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NEUROBLASTOMA is one of the most common malignant solid tumors in infancy and childhood. Although significant progress has been made in the treatment of many pediatric solid tumors during the past two decades, the prognosis of neuroblastoma has remained dismal.^{1,2} This is related to the fact that most patients have advanced disease (unresectable or metastatic) at diagnosis and the effects of chemotherapy and radiotherapy are both variable and transient.³ New therapeutic approaches for unresectable tumors must be explored in an attempt to achieve prompt local control and reduce the risk of disease progression.

The development of the technology of high-intensity focused ultrasound (HIFU) offers a potentially new approach to in situ ablation of neuroblastoma. This series of experiments was performed to evaluate the effect of HIFU treatment on tumor response, survival, and host tumor immunity in mice with subcutaneous C1300 neuroblastoma.

MATERIALS AND METHODS

Animal and Tumor

Male Ajax (A/J) inbred mice weighing 20 to 22 g (Jackson Laboratories, Bar Harbor, ME) were used. All procedures were carried out under anesthesia with ketamine cocktail (ketamine 100 mg, promazine 2.2 mg, and atropine 0.5 mg/mL), 0.02 mL per mouse intramuscularly. A single cell suspension of C1300 neuroblastoma was prepared aseptically by mincing 2-week-old subcutaneous tumor in normal saline followed by filtration through 425- μ m and 63- μ m wire mesh, respectively. Cell viability was assessed by trypan blue exclusion. Viable tumor cells 1×10^5 were injected subcutaneously into the mouse flank. The tumor was allowed to grow for 5 days, at which time the tumor was approximately 4 to 5 mm in diameter and treatment was initiated. All experimental groups were randomly selected.

HIFU Treatment

The mice were anesthetized and the skin over the subcutaneous tumor was depilated. The animal was fastened on a holder with the flank bearing tumor retracted away from the trunk with two sutures to avoid ultrasound injury to the abdomen (Fig 1). Animals were partially immersed to the forelegs in a tank filled with degassed mammalian Ringer's solution (37°C) completely submerging the tumor. The HIFU system (described in detail previously⁴) is composed of a 5.5-cm diameter, focused 4-MHz quartz transducer that creates a sonic beam with half-intensity axial and lateral dimensions of 7 mm and 0.6 mm, respectively, at an 8-cm focal

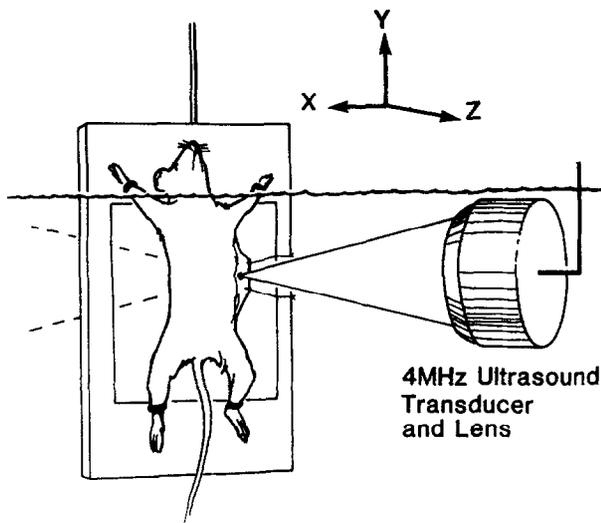


Fig 1. Schematic experimental design.

distance. A movable pointer was used to position the focal point on the skin surface over the tumor. A motorized three-dimensional coordinate system moved the mouse in 0.5-mm increments along the vertical (Y) and horizontal (X) plane during insonation to create an insonation matrix covering the tumor and 2 mm of surrounding normal tissue. Each matrix point was exposed to ultrasound for 5 seconds with an intensity of 550 W/cm² (spatial-peak temporal-peak, SPTP), followed by 5 seconds off time.

Experiment 1: Combination Therapy With HIFU and Adriamycin

Sixty mice with tumor were divided into four treatment groups as outlined in Table 1. The Adriamycin (ADM) (Adria Labs, Columbus, OH) dose of 5 mg/kg was established as the maximal tolerable dose for a single injection in a preliminary study concerning drug toxicity. The tumor growth and the length of survival of each animal was recorded.

Experiment 2: Repeat HIFU (Re-HIFU) Treatment

Forty-five animals with tumor were randomly divided into three treatment groups as outlined in Table 2. The second HIFU treatment was administered on day 13 to allow temporary skin irritation resulting from the first insonation to heal. Tumor growth and animal survival were evaluated.

Experiment 3: HIFU and Tumor Immunity

The effect of HIFU on host tumor immunity was evaluated using "immunization-excision-challenge assays" outlined in Fig 2. Ninety

mice received either tumor (n = 60) or saline (n = 30) inoculation in the left flank on day 0. Sixty mice bearing tumor were assigned to either HIFU treatment of the tumor (group I, n = 45) or a sham procedure (group II, n = 15) on day 5. On day 26, 19 (42%) mice in group I were considered cured with no evidence of tumor (group IA) and the other 26 (58%) mice had macroscopic residual tumor (group IB). The tumor in group II animals (sham procedure) grew progressively. Thirty mice without tumor inoculation also received either HIFU insonation of the left flank (group III, n = 15) or a sham procedure (group IV, n = 15).

On day 26, all the mice were reinoculated with 1 × 10⁵ C1300 neuroblastoma cells into the right flank (ie, opposite flank of the primary inoculation). The growth of primary and second tumor challenge were charted 10 days later (day 35).

Statistics

The animals were followed up to 200 days after treatment, and survival compared between the experimental groups using the log-rank method. Tumor cure rates were compared by χ^2 analysis. The tumor volumes were presented as the mean ± standard deviation and the differences between any two groups assessed with the Student's *t* test.

RESULTS

Experiment 1: Combination Therapy With HIFU and ADM

In the control and ADM groups, tumor growth was progressive and all animals died by 35 days (median survival of 28 and 30 days, respectively). The animals treated with HIFU or ADM+HIFU had a tumor remission (Fig 3). However, tumor recurrence was evident in 3 of the ADM+HIFU and 7 of the HIFU group by 21 days. Eight of 15 (53%) mice in the HIFU group and 12 of 15 (80%) in the ADM+HIFU group were cured with no evidence of tumor. There was no significant difference between the effect of HIFU and ADM+HIFU treatment, although both demonstrated significantly prolonged survival when compared with the control and ADM groups (*P* < .05; Fig 4).

Experiment 2: Repeat HIFU Treatment

Re-HIFU and HIFU treatment resulted in 67% (10/15) and 47% (7/15) cure rates, respectively (Fig 5). Survival was significantly increased in HIFU and Re-HIFU groups as compared with controls (*P* < .05).

Table 1. Experimental Design of Combination Therapy With HIFU and ADM

Group	No.	Day 5		Day 6		
		ADM IP, 2.5 mL/kg at 2.5 mg/mL	Normal saline, IP 2.5 mL/kg	Tumor Retraction	Water immersion	HIFU
ADM + HIFU	15	+	—	+	+	+
HIFU	15	—	+	+	+	+
ADM	15	+	—	+	+	—
Control	15	—	+	+	+	—

Abbreviations: ADM, Adriamycin; HIFU, high-intensity focused ultrasound; IP, intraperitoneally.

Table 2. Experimental Design of Repeated HIFU Treatment

Group	No.	Day 5			Day 14		
		Tumor Retraction	Water Immersion	HIFU	Tumor Retraction	Water Immersion	HIFU
Re-HIFU	15	+	+	+	+	+	+
HIFU	15	+	+	+	+	+	—
Control	15	+	+	—	+	+	—

Abbreviation: HIFU, high-intensity focused ultrasound.

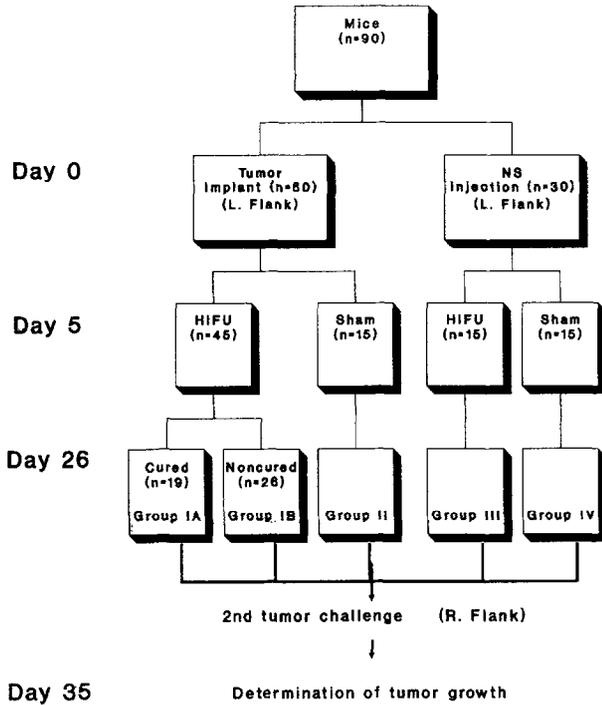


Fig 2. Flow diagram of the experimental design of HIFU effect on host antitumor immunity.

However, the differences in the length of survival and the cure rates between the former two groups are not statistically significant.

Experiment 3: HIFU on Antitumor Immunity

Two (8%) mice in group IB (the primary tumor treated palliatively with HIFU) and 7 (47%) in group II (the primary tumor untreated) died of primary (left flank) tumor before day 35 ($P < .05$). The primary tumor volumes were $4,175.6 \pm 2,164.3$ and $8,437.2 \pm 2,764.3$ mm³ in groups IB and II, respectively ($P < .001$). Tumor take after second tumor challenge was observed in all the animals on day 35 with a mean tumor volume of 197.5 ± 81.8 , 387.8 ± 192.7 , 394.1 ± 184.3 , 513.6 ± 201.4 , and 405.8 ± 151.2 mm³, in groups IA, IB, II, III, and IV, respectively (Fig 6). A significant reduction in lesion size following second tumor challenge was observed in group IA as compared with all other groups ($P < .001$).

DISCUSSION

These data demonstrate that HIFU treatment can effectively inhibit tumor growth and significantly prolong animal survival in mice with C1300 neuroblastoma. The mechanism of tumor destruction with HIFU is not yet fully understood. Although nonther-

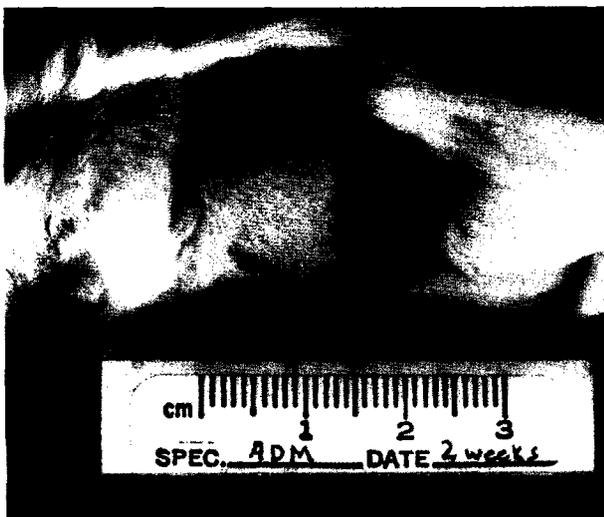


Fig 3. Subcutaneous C1300 neuroblastoma. (A) Tumor (T) 14 days after ADM treatment. (B) Complete remission of the tumor 14 days after HIFU treatment with only a skin scar (arrow) left.

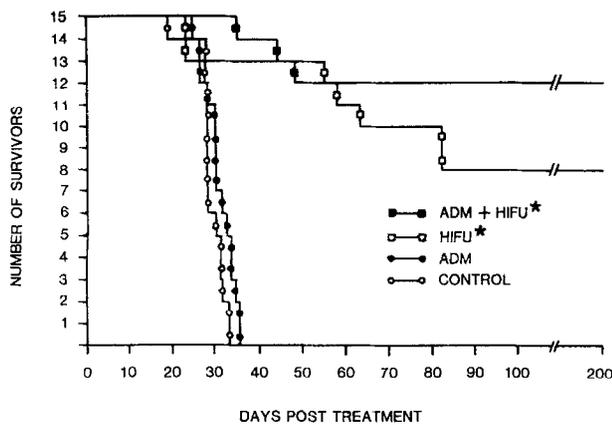


Fig 4. Kaplan-Meier survival curves of various treatment groups. $\star P < .05$ in comparison with the control or ADM groups.

mal effects such as cavitation⁵ have been suggested, we believe that thermal damage is the predominant factor. We previously reported that local tissue temperature at the sonic focus point was up to 100°C during the 5-second HIFU insonation and can cause irreversible tissue damage.⁶ However, tumor recurrence after HIFU treatment was observed in some animals. Failure to achieve local control of the tumor may be attributed to HIFU geographically missing or sublethally damaging some part of the tumor mass due to the animal movement during insonation or a system problem. Although there was a trend for combination therapy with ADM and repeated HIFU treatment to improve the cure rate and length of survival, statistical significance was not achieved. The optimal scheme for combination therapy with chemotherapeutic agents or repeated application of HIFU to maximize therapeutic effects needs further study.

In the third experiment of this study, tumor growth following a second tumor challenge in previously HIFU cured animals (group IA) was significantly reduced. Similar phenomena have been observed by

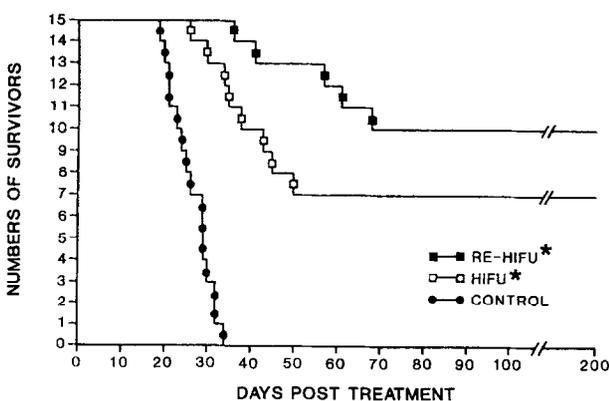


Fig 5. Kaplan-Meier survival curves of various treatment groups. $\star P < .05$ in comparison with the control group.

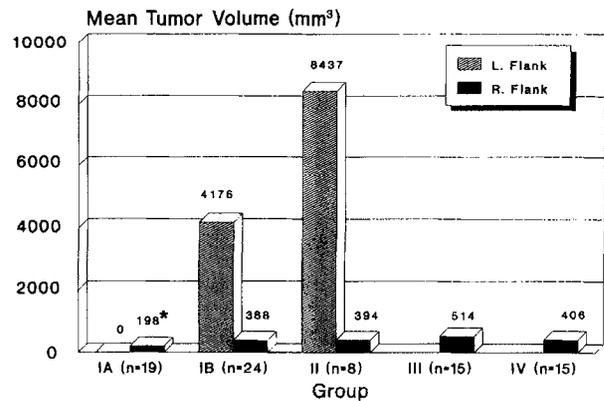


Fig 6. Mean volumes of primary tumor (left flank) and second tumor challenge (right flank). $\star P < .001$ in comparison with any other groups.

Wagai et al.⁷ They reported that after HIFU (1,000 W/cm²) treatment of Horie's sarcoma, the cured rats developed a high resistance to subsequent tumor challenge. Burov and Andreevskaya applied HIFU (350 W/cm²) to treat Brown-Pierce rabbit tumor transplanted in the testes and reported elimination of the locally treated tumor as well as noninsonated metastatic nodules.⁸ These responses after HIFU treatment may be immune-related. Tumor breakdown products after HIFU treatment may improve the tumor immunogenicity and augment host immunity. Oka et al reported that murine glioma insonated in vitro with HIFU (1,000 W/cm²) was rejected after inoculation in mice.⁹ These immune animals also resisted subsequent tumor challenge with noninsonated tumor tissue. The concept of immunostimulation by tumor breakdown products has also been advocated by other investigators using electrocoagulation,¹⁰ radiation,¹¹ and laser¹² in the treatment of C1300 neuroblastoma. Both tumor-specific and tumor-nonspecific immune responses may be involved in this phenomena. In the present study, HIFU insonation of the nontumor-bearing animals (group III) did not inhibit tumor growth following a second tumor challenge, which suggests that augmentation of tumor-specific immunity plays a major role in these observations. However, the potentiated antitumor immunity can be overcome by challenge with a large tumor volume. In the cured animals (group IA), tumor growth following a second tumor challenge tumor was not completely prevented; however, a significant reduction of tumor growth was achieved. In the palliatively treated animals (group IB), the immunostimulation effect of HIFU treatment (if any) may be suppressed by the progressively growing primary tumor.

This observation suggests that one might consider HIFU as a potential treatment modality in the man-

agement of unresectable neuroblastoma. The most common site of primary tumor in children with neuroblastoma is the retroperitoneum (adrenal, paraspinal tissues). Tumor is frequently adherent to or encases the inferior vena cava and abdominal aorta, often preventing complete tumor resection.¹³ In a recent experiment in our laboratory we investigated the response of HIFU (4-MHz, 1,500 W/cm², 5 seconds) on the inferior vena cava, abdominal aorta, and surrounding lumbar muscle in rabbits.¹⁴ The insonated lumbar muscle around the vessels was destroyed while the vessel sustained only negligible damage. This observation suggests that HIFU might be considered a relatively safe and effective modality

of therapy for tumors such as neuroblastoma that are often located adjacent to vital vessels where attempts at operative excision may be contraindicated or hazardous. The benefit of immunostimulation following local control of tumor using HIFU may also improve tumor regression and the patient's ultimate prognosis. Further investigation of this promising therapeutic technique is warranted.

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Discussion

M. Ziegler (Cincinnati, OH): This paper is an extension of work that the Indianapolis group has done using HIFU, first reporting it in a liver tumor model. It really is also an application of another kind of energy application to tumors, particularly neuroblastoma, something that we have reported first using electrocoagulation and subsequently utilizing the CO₂ laser. All three of these energy applications destroy tumor cells and in conventional tumors that would render them more immunizing or more immunogenic. Dr Yang has reported here today two very practical and hopefully fundamentally important things for the future therapy of neuroblastoma. The

first is that this form of energy can directly destroy the tumor, and the second, a somewhat more speculative observation, is that there may be an augmentation of host antitumor immune responsiveness. I must point out that if you had a third group of analysis, that is, if you compared HIFU with direct surgical excision, you would see a still further increased cure rate. In other words, one can excise C1300 neuroblastoma at that tumor size and probably cure 100% of animals. Nevertheless, for an energy application cure about 50% of animals alone is really a quite remarkable cure rate. My questions are two. The study design used adjuvant therapy in the form of chemotherapy

and used an agent that is not particularly known to be an immunomodulator. What I would ask is have you considered treating the animals with some sort of immunotherapy in addition to the focused ultrasound? Second, are you utilizing, as have we, an artificial model, namely a C1300 tumor which is immunizing? The human neuroblastoma suffers unfortunately from the fact that it is not a good expressor of class I or class II antigen nor does it express tumor antigen very well. Therefore, one would speculate that the same efficacy of an immune effect of this therapy may not occur in human neuroblastoma. Do you have any suggestion as to how we might take advantage of that? This is a very stimulating new idea.

It's obviously taken a number of years to work out and I think it should be an exciting thing to follow.

R. Yang (response): We are currently designing a study of HIFU treatment in combination with immunotherapy with τ -interferon and interleukin-2 in C1300 neuroblastoma. It is difficult to investigate the effect of HIFU on MHC antigen expression on tumor cells. This is because HIFU treatment with an extremely high temperature (which is different from conventional hyperthermia) can promptly denature the biological materials including the antigens on cell membrane. In addition, few viable tumor cells left after HIFU treatment would make cellular antigen study with flow cytometry unsuitable.