Contrast-Aided Diagnostic Ultrasound Does Not Enhance Lung Metastasis in a Mouse Melanoma Tumor Model

Douglas L. Miller, PhD, Chunyan Dou, MD

Objective. The purpose of this research was to test the hypothesis that contrast-aided diagnostic ultrasound (CADUS) could exacerbate the metastatic spread of mouse melanoma tumor cells to the lungs. ***Methods.*** The melanoma cell lines B16 and B16-D5 (metastatic specifically to lung) were implanted on a hind leg of female C57/bl6 mice. Growing tumors were scanned by 1.5-MHz diagnostic ultrasound in a 37°C water bath. Four hundred image frames were triggered at a 1-Hz rate with 4 retroorbital injections of an ultrasonographic contrast agent at dosage of 10 μL/kg at 100-second intervals. Sham-treated mice received 400 frames of ultrasonography followed by the contrast agent with the ultrasound off. The primary tumor was surgically removed 1 day after ultrasound administration. Lungs were removed and evaluated blind after 2 weeks of bleaching in Fekete solution. ***Results.*** Three experiments were performed. The first experiment involved scanning sham and CADUS groups of 20 mice each with B16 tumors; B16 metastasis was not enhanced. The second experiment repeated this test with the D5 cell line; the metastasis enhancement was marginally significant for average number (0.3 and 3.2; $P = .06$) and incidence (3 and 9 of 19; $P = .08$) in mice without tumor recurrence. Finally, a third experiment was performed to clarify ambiguous results in the second experiment and consisted of 2 groups of 40 mice each. In this larger experiment, the results were essentially equal for the sham and CADUS groups. ***Conclusions.*** Overall, the results do not support the hypothesis of CADUS-enhanced metastasis. ***Key words:*** adverse effects; cancer; contrast agent; diagnostic ultrasound; metastasis.

Abbreviations
CADUS, contrast-aided diagnostic ultrasound; PRPA, peak rarefactional pressure amplitude

Received October 5, 2004, from the Department of Radiology, University of Michigan Medical Center, Ann Arbor, Michigan USA. Revision requested November 18, 2004. Revised manuscript accepted for publication November 30, 2004.

We thank Dr A. E. Chang of the University of Michigan Comprehensive Cancer Center for providing the D5 mouse melanoma cell line. This work was supported by US Public Health Service grant EB00338, awarded by the National Institutes of Health.

Address correspondence and reprint requests to Douglas L. Miller, PhD, University of Michigan Medical Center, Room 3315, Kresge III, 200 Zina Pitcher Pl, Ann Arbor, MI 48109-0553 USA.

E-mail: douglm@umich.edu

Metastasis of cancer by hematogenous or lymphatogenous spread can be augmented by perturbations of tumors, which result in the release of malignant cells. The accidental spread of malignant cells is a concern in medical procedures that disturb the tumor microvasculature, such as surgery and biopsy.[1] Diagnostic or therapeutic medical procedures using ultrasound are generally not expected to enhance metastasis because the imaging or therapy per se does not appear to have any metastatic mechanism. For example, high-intensity focused ultrasound, which is used to treat tumors by generating heat coagulation, has been shown not to induce detectable metastasis or tumor cell release.[2,3] However, ultrasound can cause mechanical perturbation of the microvasculature if the ultrasound induces acoustic cavitation. Extracorporeal shock wave lithotripsy is a high-amplitude therapeutic modality primarily for treatment of kidney stones, but

research has been conducted on therapy of other targets. Shock wave lithotripsy treatment has shown some promise in application to cancer tumor therapy.[4,5] The extremely high pressure amplitudes used by lithotripsy systems can induce acoustic cavitation in tissue,[6] leading to hemorrhage in the lung,[7] intestine,[8] and even non–gas-bearing tissues such as the kidney.[9] Most studies of shock wave tumor treatment have not reported increased metastasis; however, a direct test of this potential adverse side effect of shock wave tumor treatment showed that this modality has a tendency for an increase in metastasis from the treatment of highly malignant tumors.[10] This report was recently confirmed by a different tumor model.[11] The use of enhanced nucleation of acoustic cavitation by intratumoral injection of an ultrasonographic contrast agent yielded enhancement of metastasis, thus specifically linking the effect to in vivo cavitation.[11]

Commercial contrast agents for diagnostic ultrasonography contain gas bodies (stabilized microbubbles) to intensify the echogenicity of blood. The presently available ultrasonographic contrast agents have been approved for echocardiography but have not yet been approved for radiologic applications such as tumor detection and characterization in the United States. Research suggests a role for ultrasonographic contrast agents for tumor imaging, particularly for liver tumors.[12] The method compares favorably with computed tomography for liver lesions.[13] Contrast-aided ultrasonography is also valuable for guiding ablation procedures for liver masses[14] and for assessing the outcome of treatment.[15]

The gas bodies in ultrasonographic contrast agents have been shown to be efficient cavitation nucleation agents in vitro and in vivo.[16] The interaction of diagnostic ultrasound with contrast agent gas bodies can induce microscale bioeffects such as petechial hemorrhage.[17,18] On the basis of the demonstrated ability of shock wave ultrasound to enhance metastasis by a contrast agent–aided cavitation mechanism, there is reason to suspect that a similar process might be possible during contrast-aided diagnostic ultrasound (CADUS) examinations. This is particularly true for relatively high pressure amplitudes and low frequencies, which translate into a high mechanical index (an index of ultrasound exposure provided on most diagnostic platforms).[16] A full exploration of this concern is needed to provide guidance for development of the CADUS imaging method for radiologic applications. The goal of this study was to test the hypothesis that CADUS could exacerbate the metastatic spread of mouse melanoma tumor cells to the lungs.

Materials and Methods

All animal research was conducted with the approval of the University Committee for the Use and Care of Animals and the guidance of the Unit for Laboratory Medicine of the University of Michigan. The B16 and B16-D5 melanoma cell lines were used with female C57/bl6 mice to grow subcutaneous tumors. The D5 cells are metastatic especially to the lung and have been used in metastasis research.[19] Cells injected into the tail vein form visible lung metastases after about 18 days. Established methods were used to test for enhanced lung metastasis, which have been used for the study of metastasis resulting from therapeutic or diagnostic manipulation of tumors implanted on the hind leg.[11,20–22] A suspension of 1 million cells in 0.05 mL was injected subcutaneously on the right hind leg of each mouse under ether anesthesia. Treatment was applied after 10 days of tumor growth.

For ultrasonic scanning, mice were weighed and anesthetized with an intraperitoneal injection of ketamine (Ketaset; Aveco Co, Fort Dodge, IA) at 75 mg/kg and xylazine (Rompun; Mobay Corp, Shawnee, KA) at 15 mg/kg. The tumor area was shaved and depilated. The volume of each tumor was estimated with the use of a digital caliper to measure the 3 major axes of the tumor and calculation of the ellipsoidal volume. Average tumor volumes for each group are listed in Table 1. The mouse was then mounted on a plastic board with Velcro (Manchester, NH) strips with the tumor centered over a 2.5-cm hole in the board. A wetting agent was applied to the tumor area to minimize air entrapment. Finally, the mounting board was set up in a 37°C water bath for ultrasonic scanning. This arrangement provided essentially free field conditions for the ultrasound beam, so that the ultrasound exposure was pertinent to acoustic conditions found in clinical examinations.

The ultrasonographic contrast agent tested in this study was Definity (Bristol-Myers Squibb Medical Imaging, Inc, North Billerica, MA). This agent contains octafluoropropane gas bodies (stabilized microbubbles) at a concentration of

Table 1. Overall Outcome for the Treatment Groups.

Experiment	Group	Total	Volume, µL (Mean ± SD)	Tumor Recurrence	21-d Survival	28-d Survival
1, B16	Sham	20	50 ± 38	5	18	17
	CADUS	20	47 ± 46	3	17	16
2, D5	Sham	20	79 ± 63	1	20	19
	CADUS	20	105 ± 109	1	20	19
3, D5	Sham	40	77 ± 53	3	39	36
	CADUS	39*	71 ± 60	3	38	37

*One mouse was excluded because a visible tumor failed to grow.

12×10^9/mL with a mean diameter range of 1.1 to 3.3 µm according to the package insert. Each treatment day, a fresh vial of the agent was mixed in the VialMix shaker supplied with the agent. This agent was diluted 100:1 in saline by mixing 10 µL of the agent with saline in a 1-mL syringe. Four bolus doses of the diluted agent, each 1 mL/kg (10 µl/kg for the stock agent), were then given by retro-orbital injection with a 25-gauge needle. The doses were spaced 100 seconds apart, which was intended to maintain a roughly constant level of circulating gas bodies throughout the 400-second ultrasound exposure. The total dosage of the stock agent was then 40 µL/kg, which was 4 times the normal recommended dosage of 10 µL/kg.

A Vingmed System V unit (GE Healthcare, Cincinnati, OH) with a cardiac phased array probe (FPA2.5) was used as the diagnostic ultrasonic scanner. The same scanning (ie, exposure) conditions were used for all experiments. The imaging parameters in the octave (harmonic imaging) mode were frequency, 1.5 MHz; depth, 10 cm; focus, 5 cm; power, 0 dB; and frame rate, 30.4 frames per second. The ultrasonic field was measured by a calibrated hydrophone (model 805 polyvinylidene difluoride bilaminar membrane hydrophone; Sonora Medical Systems, Longmont, CO) positioned at the tumor location but without the mouse or mounting board. The maximum pulse during each scan had a peak rarefactional pressure amplitude (PRPA) of –2.3 MPa and a duration of 1.45 microseconds. Because attenuation through the skin would be expected to be negligible, this corresponds to an equivalent MI of 1.9 at the tumor. The pulse amplitude PRPA decreased with distance from the center of the scan plane with a –6 dB thickness of 4.6 mm perpendicular to the plane, which was sufficient to cover the small tumors.

Figure 1. Sonograms of a tumor-bearing mouse leg before (**A**) and after (**B**) injection of a dose of the contrast agent. The cardiac probe produces a pie-shaped sector scan image. Note that the small tumor region lights up with the contrast agent, which provided assurance that the contrast agent was reaching the tumor and interacting with the imaging ultrasound pulses.

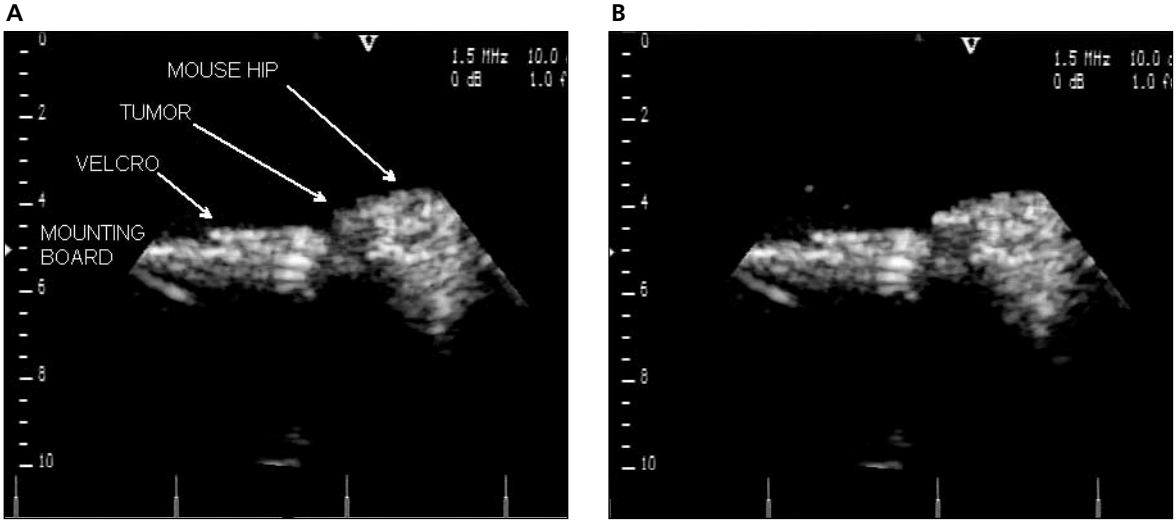
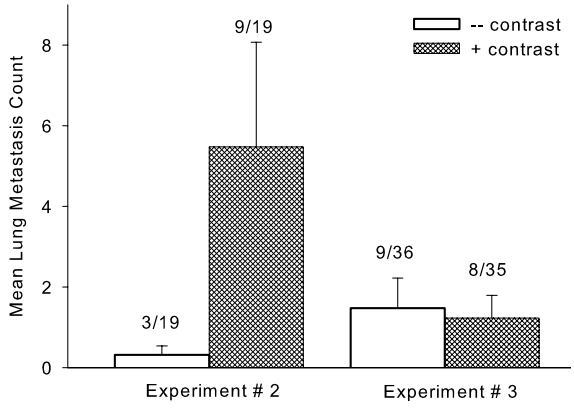


Table 2. Results for Counts and Incidence Rates of Lung Metastasis for Mice Surviving at Least 21 Days and Without Tumor Recurrence

Experiment	Group	n	Metastasis Incidence	Incidence *P*	Metastases (Mean ± SEM)	Count *P*
1, B16	Sham	15	1	.82	0.31 ± 0.22	.73
	CADUS	17	1		1.9 ± 1.1	
2, D5	Sham	19	3	.081	0.32 ± 0.22	.063
	CADUS	19	9		5.5 ± 2.6	
3, D5	Sham	36	9	.94	1.5 ± 0.7	.95
	CADUS	35	8		1.2 ± 0.6	

(In the direction parallel to the scan plane, the PRPA was approximately constant for several centimeters.) For scanning, the mice were mounted in a warmed water bath. The ultrasonic probe was clamped in the water bath and adjusted to locate the mouse tumor 4.5 cm away from the transducer face (with the mounting board ≈5 cm away). An initial real-time image was obtained at 3.6 MHz to clearly locate the tumor in the image plane, and then the ultrasonic frequency was switched to triggered 1.5-MHz imaging for CADUS. Image frames were intermittently triggered each 1 second to allow the contrast agent to refill the tumor circulation after gas body destruction by the previous frame. Each triggered frame delivered a single sweep of the pulsed beam formed by the phased array transducer (the same as each frame of a 30.4-Hz frame rate real-time image). Typical images of a tumor are shown in Figure 1 for 1.5 MHz, which provided only modest resolution of the tumors (Figure 1A). When the contrast agent was injected (Figure 1B), the tumor area quickly brightened in the image frames, and this was apparently more than the brightening of the surrounding thigh muscle tissue. This enhanced contrast provided assurance that the agent was present in the circulation at a concentration sufficient for CADUS. Sham exposure consisted of a combination of both control conditions of ultrasound alone and contrast agent alone: the tumor was scanned without injection of the contrast agent for 400 seconds, followed by injection of the contrast agent with the ultrasound off.

Figure 2. Comparison of the mean lung metastasis count for each group in experiments 2 and 3 with SE bars. The metastasis incidence in mice surviving at least 21 days without tumor recurrence is shown above each bar.



After scanning, the mice were removed from the bath, dried, and allowed to recover in warmed chambers before return to regular cages. The primary tumor was surgically removed 1 day after CADUS. Removal of the primary tumor extends survival to allow formation of visible lung metastases. After the mice were reanesthetized, this was accomplished by removal of the leg at the hip and suture of the skin over the area. Recovery was aided by intraperitoneal injection of 0.3 mL of warm saline together with buprenorphine analgesic at 0.1 mg/kg subcutaneously.

The mice were euthanized if a tumor recurred and reached 3000 µL in volume or at 28 days after CADUS. Data were collected for mice that survived at least 21 days. For evaluation, the lungs were removed and bleached in Fekete solution to bring out the pigmented lung metastases.[11] The metastasis nodules in the lungs were evaluated blind after 2 weeks in the Fekete solution by examination under a low-power stereo microscope. Experimental groups included sham and CADUS. Three experiments were conducted: B16 cells with 20 mice in each group, D5 cells with 20 mice in each group, and D5 cells with 40 mice in each group. The imaging (exposure) was identical for all experiments. Statistical comparisons of the mean metastasis counts were made by the Mann-Whitney rank sum test, and the occurrence rates were compared by the *z* test (SigmaStat 3.1; SPSS Inc, Chicago IL).

Results

The first experiment using the B16 cell line resulted in several early deaths and recurrences of the primary tumors. The overall outcomes of the groups are listed in Table 1. The metastases are compared in Table 2 for the mice surviving at least 21 days and not having a recurrence of the primary tumor. The mice with tumor recurrence were excluded from this analysis because the recurrence would have altered the expected metastasis rate and did not comply with the primary tumor "cure" protocol. There was no significant difference between sham and CADUS results in the first experiment.

The results of the second experiment using the D5 cell line are also listed in Tables 1 and 2. This test had a lower recurrence rate, with only 1 mouse of each group excluded. The results for the numbers of metastases and for the occurrence rate were both marginally statistically significant (ie, $.05 < P < .1$). The numbers of metastasis had a value of $P = .063$, and the occurrence rate had a value of $P = .081$. The results are plotted in Figure 2. Unfortunately, these results with marginal significance precluded any firm conclusion regarding the initial hypothesis. The ambiguity of this result motivated the third experiment using larger groups to obtain higher statistical power. The results of the third experiment are listed in Tables 1 and 2. The sham and CADUS groups essentially had identical outcomes. This result is also plotted in Figure 2. The results of the second and third experiments were not evaluated as pooled groups (potentially giving an initial total of 60 mice in each group) because the tumor volumes of the CADUS group were statistically significantly larger in the second than in the third experiment, and results of both experiments were separately negative ($P > .05$).

Discussion

This study tested the hypothesis that CADUS of mouse melanoma tumors could exacerbate the metastatic spread of tumor cells to the lungs. The B16 and B16-D5 (metastatic specifically to lung) melanoma cell lines were implanted on the hind legs of female C57/bl6 mice. Growing tumors were scanned in a water bath using 1.5-MHz diagnostic ultrasound at 1 frame per second with injection of the Definity ultrasonographic contrast agent at 40 µL/kg. The primary tumor was surgically cured 1 day after ultrasound administration to allow identification of lung metastases in mice surviving at least 21 days without tumor recurrence. Three experiments were performed. In the first experiment with groups of 20 mice, B16 metastasis was not enhanced. However, in the second experiment with groups of 20 mice, the metastasis of the D5 tumors was marginally enhanced in terms of both the numbers of metastases ($P = .063$) and the incidence rate ($P = .081$). The larger third experiment involving groups of 40 mice was intended to clarify the ambiguous results of the second experiment. The results were essentially equal for the sham and CADUS groups. Overall, the results show that CADUS did not increase the metastatic spread of mouse melanoma tumor cells to the lungs.

Several considerations have a bearing on this conclusion. The subcutaneous melanoma tumor model as used in this research may not be sufficiently sensitive to show the metastasis enhancement effect. A tumor model with tumors growing in the interior of organs might be more sensitive because of improved vascularity. However, the melanoma model used here was sufficiently sensitive to allow demonstration of increased metastasis with intratumoral injection of a perflutren contrast agent (Optison; Amersham Health, Princeton, NJ) and lithotripter shock wave treatment.[11] Another consideration may be the influence of tumor volume on metastasis. In the previous study,[11] tumor volumes were larger (≈300 µL) to allow for intratumoral injection of the agent. In this study, with retro-orbital injection of Definity into the circulation, tumors were smaller (≈75-100 µL) to ensure full coverage of the tumor by the ultrasound scan. The tumor volume may be important because a relatively large number of cells must be released to the circulation for successful implantation in the lungs due to metastatic inefficiency.[23] This consideration might play a role in the previous positive result[11] versus the negative result in this study. Tumor volume might also have had a role in the results of the second versus the third experiment in this study; that is, the marginally significant results in the second experiment involved significantly larger tumors than the negative third experiment. Given the importance of this question for various ultrasonographic applications of contrast agents, further research may be warranted to examine the factors of tumor type, tumor volume, contrast agent type, and the power level of CADUS needed for metastasis enhancement.

References

1. Hoorntje LE, Schipper ME, Kaya A, Verkooijen HM, Klinkenbijl JG, Rinkes IH. Tumour cell displacement after 14G breast biopsy. Eur J Surg Oncol 2004; 30:520–525.
2. Oosterhof GO, Cornel EB, Smits GA, Debruyne FM, Schalken JA. Influence of high-intensity focused ultrasound on the development of metastases. Eur Urol 1997; 32:91–95.
3. Wu F, Wang ZB, Jin CB, et al. Circulating tumor cells in patients with solid malignancy treated by high-intensity focused ultrasound. Ultrasound Med Biol 2004; 30:511–517.
4. Hoshi S, Orikasa S, Suzuki K, et al. High-energy underwater shock wave treatment for internal iliac muscle metastasis of prostatic cancer: a first clinical trial. Jpn J Cancer Res 1995; 86:424–428.
5. Gamarra F, Spelsberg F, Dellian M, Goetz AE. Complete local tumor remission after therapy with extra-corporeally applied high-energy shock waves (HESW). Int J Cancer 1993; 55:153–156.
6. Coleman AJ, Kodama T, Choi MJ, Adams T, Saunders JE. The cavitation threshold of human tissue exposed to 0.2 MHz pulsed ultrasound: preliminary measurements based on a study of clinical lithotripsy. Ultrasound Med Biol 1995; 21:405–417.
7. Hartman C, Child SZ, Mayer R, Schenk E, Carstensen EL. Lung damage from exposure to the fields of an electrohydraulic lithotripter. Ultrasound Med Biol 1990; 16:675–679.
8. Miller DL, Thomas RM. Thresholds for hemorrhages in mouse skin and intestine induced by lithotripter shock waves. Ultrasound Med Biol 1995; 21:249–257.
9. Evan AP, Willis LR, McAteer JA, et al. Kidney damage and renal functional changes are minimized by waveform control that suppresses cavitation in shock wave lithotripsy. J Urol 2002; 168:1556–1562.
10. Oosterhof GO, Cornel EB, Smits GA, Debruyne FM, Schalken JA. The influence of high energy shock waves on the development of metastases. Ultrasound Med Biol 1996; 22:339–344.
11. Miller DL, Dou C, Song J. Lithotripter shockwave-induced enhancement of mouse melanoma lung metastasis: dependence on cavitation nucleation. J Endourol 2004; 18:925–929.
12. Dietrich CF. Characterisation of focal liver lesions with contrast enhanced ultrasonography. Eur J Radiol 2004; 51(suppl):S9–S17.
13. Esteban JM, Molla MA, Tomas C, Maldonado L. Improved detection of liver metastases with contrast-enhanced wideband harmonic imaging: comparison with CT findings. Eur J Ultrasound 2002; 15:119–126.
14. Youk JH, Lee JM, Kim CS. Therapeutic response evaluation of malignant hepatic masses treated by interventional procedures with contrast-enhanced agent detection imaging. J Ultrasound Med 2003; 22:911–920.
15. Numata K, Isozaki T, Ozawa Y, et al. Percutaneous ablation therapy guided by contrast-enhanced sonography for patients with hepatocellular carcinoma. AJR Am J Roentgenol 2003; 180:143–149.
16. Nyborg WL, Carson PL, Carstensen EL, et al. Exposure Criteria for Medical Diagnostic Ultrasound, II: Criteria Based on All Known Mechanisms. Bethesda, MD: National Council on Radiation Protection and Measurements; 2002. Report 140.
17. Skyba DM, Price RJ, Linka AZ, et al. Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. Circulation 1998; 98:290–293.
18. Li P, Armstrong WR, Miller DL. Impact of myocardial contrast echocardiography on vascular permeability: comparison of three different contrast agents. Ultrasound Med Biol 2004; 30:83–91.
19. Teitz-Tennenbaum S, Li Q, Rynkiewicz S, et al. Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration. Cancer Res 2003; 63:8466–8475.
20. Kadmon D, Heston WD, Fair WR. Treatment of a metastatic prostate derived tumor with surgery and chemotherapy. J Urol 1982; 127:1238–1242.
21. Nathanson SD, Nelson L, Anaya P, Havstad S, Hetzel FW. Development of lymph node and pulmonary metastases after local irradiation and hyperthermia of foot pad melanomas. Clin Exp Metastasis 1991; 9:377–392.
22. Schreiber S, Gross S, Brandis A, et al. Local photodynamic therapy of rat C6 glioma xenografts with Pd-bacteriopheophorbide leads to decreased metastases and increase of animal cure compared with surgery. Int J Cancer 2002; 99:279–285.
23. Cameron MD, Schmidt EE, Kerkvliet N, et al. Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency. Cancer Res 2000; 60:2541–2546.