[27] F. Berditchevski, Complexes of tetraspanins with integrins: more than meets the eye, J. Cell Sci. 114 (2001) 4143–4151.
[28] B.G. Galvez, S. Matias-Roman, M. Yanez-Mo, M. Vicente-Manzanares, F. Sanchez-Madrid, A.G. Arroyo, Caveolae are a novel pathway for membrane-type 1 matrix metalloproteinase traffic in human endothelial cells, Mol. Biol. Cell 15 (2004) 678–687.
[29] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, A.R. Horwitz, Cell migration: integrating signals from front to back, Science 302 (2003) 1704–1709.
[30] T. Kamba, D.M. McDonald, Mechanisms of adverse effects of anti-VEGF therapy for cancer, Br. J. Cancer 96 (2007) 1788–1795.

RESEARCH PAPER

Cancer Biology & Therapy 12:4, 1-8; August 15, 2011; © 2011 Landes Bioscience

Combination of ultrasound and bubble liposome enhance the effect of doxorubicin and inhibit murine osteosarcoma growth

Yoshinori Ueno,[1,†] Shozo Sonoda,[2,†] Ryo Suzuki,[3] Masahiro Yokouchi,[1,*] Yasuomi Kawasoe,[1] Katsuro Tachibana,[4] Kazuo Maruyama,[3] Taiji Sakamoto[2] and Setsuro Komiya[1]

[1]Department of Orthopaedic Surgery; [2]Department of Ophthalmology; Graduate School of Medical and Dental Sciences; Kagoshima University; Kagoshima; [3]Department of Biopharmaceutics; School of Pharmaceutical Sciences; Teikyo University; Kanagawa; [4]Department of Anatomy; Fukuoka University School of Medicine; Fukuoka, Japan

[†]These authors equally contributed to this work.

Keywords: bubble liposome (BL), ultrasound, doxorubicin, sonoporation, cavitation, osteosarcoma, drug delivery system (DDS)

If ultrasound (US) is applied to cells, permeability across the cell membrane temporarily increases, making it easier for drugs to be taken into the cells from around the cell membrane. Moreover, when used in combination with Bubble liposome (BL: liposomes which entrap an ultrasound imaging gas), even low-power ultrasound can facilitate drug delivery into cells.

In the present study, we constructed a new drug delivery system (DDS) involving concomitant use of US and BL with doxorubicin (DOX), a key drug in the chemotherapy of osteosarcoma, and demonstrated both in vitro and in vivo that it markedly inhibited the proliferation of osteosarcoma cells. Furthermore, this system achieved an equivalent antitumor effect at about 1/5 the dose of antitumor agent employed in monotherapy with DOX. These findings suggest the possibility of reduction of adverse events.

In this experiment, US and liposomes were tested, both of which are already in use in clinical practice. US and liposomes are both very safe in the body. The DDS composed of these elements we designed can be applied in simple and site-specific fashion and is therefore promising as a new, clinically feasible method of treatment.

Introduction

Osteosarcoma is the most frequent primary malignant bone tumor. Because of early pulmonary spread, this tumor had a miserable prognosis prior to the availability of chemotherapy. Currently, among the available cytostatic drugs, the four agents methotrexate, ifosfamide, cisplatinum and doxorubicin (DOX, adriamycin) are considered most effective.[1-3] Thus, the identification of effective neoadjuvant chemotherapy in combination with surgery for osteosarcoma patients has led to a significant improvement in outcome in recent decades.

However, there are still a certain number of patients who do not benefit from these improvements. Despite advancements in multimodality treatment consisting of aggressive chemotherapy, metastasis develops in more than one-third of patients, 90% of whom rarely respond to salvage treatment. Chemotherapeutic regimens require high dosages of agents to successfully eliminate tumor, adversely affecting healthy tissues in the host. The side effects of many antineoplastic agents include cardiotoxicity, immune suppression, nephrotoxicity and others.[4,5] It is therefore believed that alternative strategies for the treatment of osteosarcoma are necessary.

Microbubbles (MB), which are contrast agents for medical ultrasound (US) imaging, improve the efficiency of transfection through US-induced cavitation.[6-11] Microbubbles are, however, generally unstable, and their mean diameter of about 1–6 μm is too large for extravascular applications.[12] It is difficult to add modified products such as functional ligaments to the surface of MB. Therefore, MB should generally be smaller than red blood cells and stable after injection into the blood circulation, and ultimately their surface should be easily modifiable with functional molecules for targeting.

Liposomes have certain advantages as drug, antigen or gene delivery carriers.[13-18] Their size is easily varied, and they can be modified to add a targeting function. Based on liposome technology, we have developed novel liposomal bubbles (Bubble liposome (BL) which were liposomes containing the US imaging gas perfluoropropane).[19] In our system, DOX and BL were simply mixed rather than sealing DOX inside of liposomes. And we reported that the combination of BL and US irradiation could be

*Correspondence to: Masahiro Yokouchi; Email: masahiro@m3.kufm.kagoshima-u.ac.jp
Submitted: 12/09/10; Revised: 04/04/11; Accepted: 05/03/11
DOI:

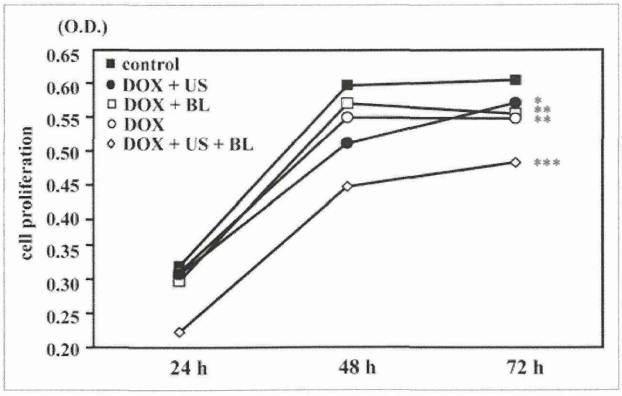

Figure 1. BL and US, used separately or in combination, facilitate drug uptake by cells. The effect of combined use of BL and US was investigated by assessing DOX-induced suppression of LM8 cell proliferation. LM8 cell proliferation was determined using MTT assay. Cell viability decreased significantly followed application of BL + US + DOX ($p < 0.001$).

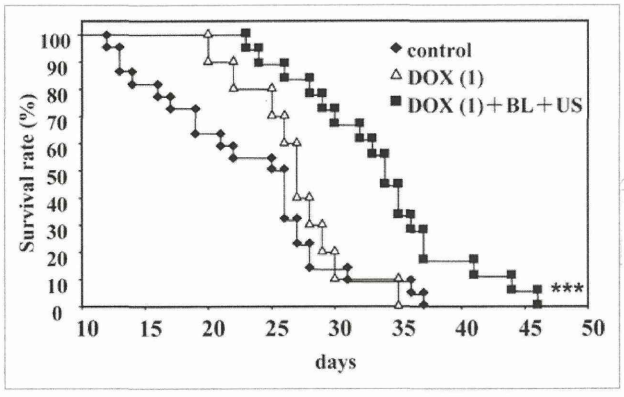

Figure 2. Kaplan-Meier survival curves show the effects of BL and US on the survival of mice bearing osteosarcoma. The DOX + BL + US group (n = 18) exhibited significant differences in survival compared with the DOX-alone group (n = 10) and control group (n = 22) ($p < 0.001$).

utilized as gene (plasmid DNA and siRNA) and antigen delivery systems.[20-24]

We used DOX in this study and designed a new drug delivery system (DDS) using BL for the development of a new and alternative approach to the treatment of osteosarcoma. In the present study, the anti-tumor effects of the new DDS using DOX in murine osteosarcoma cells in vivo and in vitro were investigated.

Results

Cytotoxicity of BL and US to LM8 cells. In the group that received no treatment (control), optical density increased to about 0.6 at 48–72 h. The value in the group receiving DOX with US irradiation was significantly different from the control ($p < 0.05$). In the groups receiving DOX with BL, receiving DOX alone, values were significantly different from the control ($p < 0.01$). In the groups receiving DOX combined with BL and US irradiation, values were significantly different from the control ($p < 0.001$) (**Fig. 1**). It was confirmed that the effect of DOX was markedly enhanced by combining it with BL and US.

Tumor growth delay. We examined whether a tumor suppressive effect could be observed with the combination of BL and US in vivo as in vitro. In the control group, the tumors increased rapidly and liver metastasis, renal metastasis and ascites were noted on day 21, and 50% of individuals died by day 25 (**Fig. 2**). In the control group, normalized tumor volume rapidly increased throughout the experiment (42.4 ± 6.3) (**Fig. 3A and B**). In the groups receiving US irradiation or BL treatment alone or US with BL treatment, normalized tumor volume was suppressed throughout the experiment (US: 22.7 ± 6.9, BL: 29.6 ± 7.5, US + BL: 21.7 ± 4.5), though without significant differences from control group ($p > 0.05$) (**Fig. 3A**). In the groups receiving DOX treatment alone at a concentration of 1.0 mg/kg with or without US irradiation, tumor volume was suppressed compared with the control group (DOX (1): 22.4 ± 2.8, DOX (1) + US: 27.8 ± 1.9), though not to a significant extent compared with control group ($p > 0.05$). With BL combined with DOX and US treatment, there was clear tumor suppression even at low concentrations of 1.0 mg/kg (DOX (1) + BL + US: 8.3 ± 2.8) ($p < 0.001$) (**Fig. 3B and C**), with effectiveness of suppression equivalent to that with five times the dose of DOX (DOX (5): 7.8 ± 1.4) (**Fig. 3B**). The survival rate of mice treated with DOX (1) + BL + US was higher than that of mice treated with the same concentration of DOX (1) alone (**Fig. 2**). Thus, as in previous studies of MB, drug uptake was increased with use of BL combined with US irradiation and a tumor suppressive effect was confirmed.

Evaluation of side effect. **Table 1** shows blood counts and liver function parameters in mice. There were no significant differences among the groups in WBC, RBC, GOT or GPT. The Hb value of mice receiving DOX (5 mg/kg) alone was statistically different from the control value. Mice receiving DOX alone at concentrations of 1.0 mg/kg or 5.0 mg/kg had Plt values significantly different from the control group. In contrast, mice receiving DOX (1 mg/kg) combined with BL and US exhibited no significant differences from the control group in blood counts and liver function.

Assay for intratumoral DOX content. Previous studies suggested that the effect of treatment with BL combined with US was due to induction of permeability of the cell membrane by US irradiation. To demonstrate this, we examined intratumoral DOX content. Mean concentration of DOX + BL + US tumors showed a significant differences compared with DOX alone group and showed a 57% (1.95 $\mu g\ g^{-1}$/1.24 $\mu g\ g^{-1}$) increase in DOX alone group ($p < 0.05$) (**Fig. 4A**). DOX uptake by cells was thus increased by the combination of BL and US.

Discussion

In the present study, we constructed a new DDS by concomitant use of US and BL with DOX, a key drug in the chemotherapy of osteosarcoma and demonstrated both in vitro and in vivo that this DDS markedly inhibited the proliferation of osteosarcoma cells. Furthermore, this system achieved an equivalent antitumor

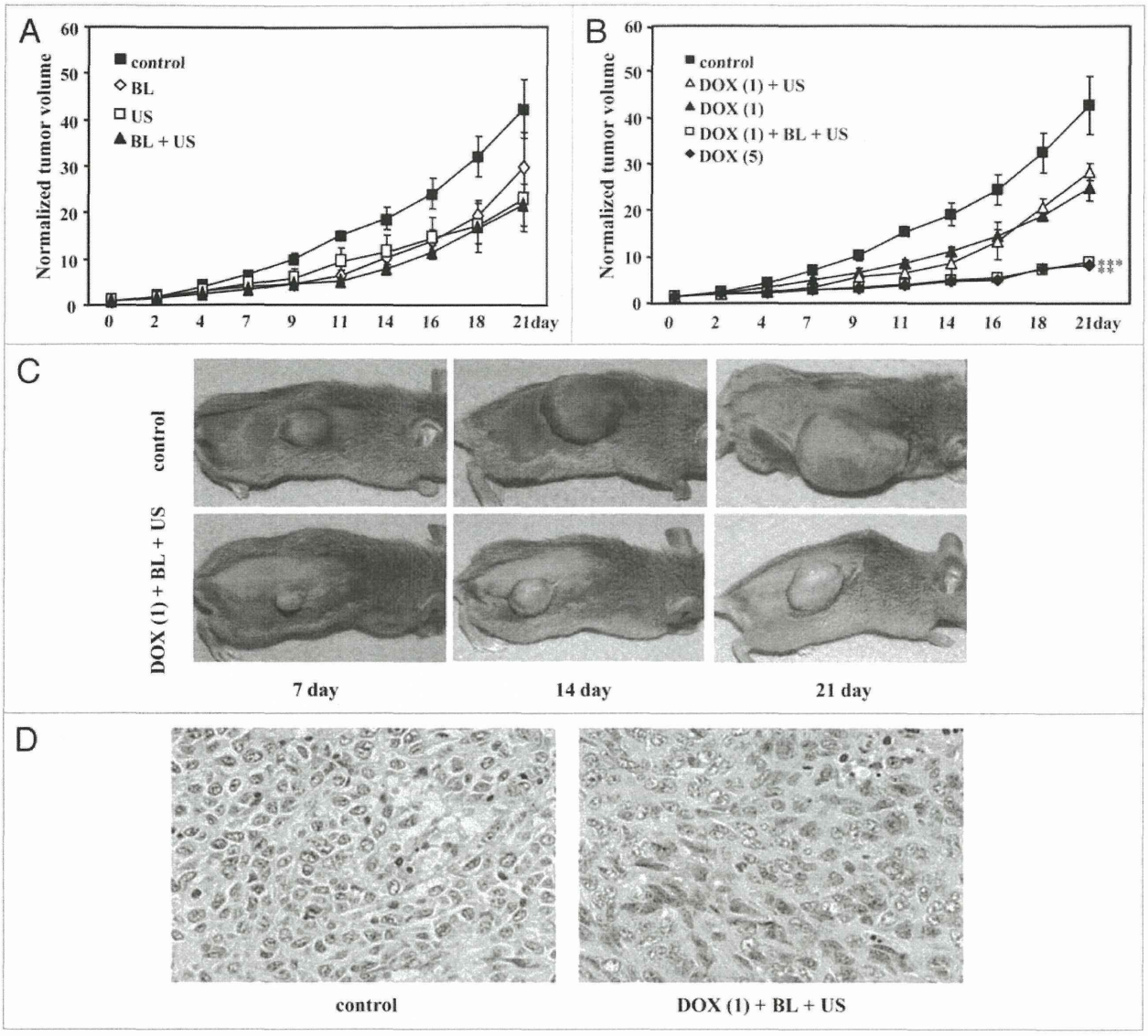

Figure 3. Tumor growth delay. (A) The anti-tumor effects of DOX were assessed by comparing the normalized tumor volume of (■) controls with (◇) the group administered BL alone, (■) the group administered US alone and (◆) the group administered BL in combination with US. There were no significant differences among the groups. (B) The anti-tumor effects of DOX were assessed by comparing the normalized tumor volume of (■) controls with (△) the group administered DOX (1) in combination with US, (▲) the group administered DOX (1) alone, (■) the group that received DOX (1) in combination with BL and US, and (◆) the group administered DOX (5) alone. The DOX (1) + BL + US group exhibited a significant difference in tumor volume from the control group ($p < 0.001$). (C) Examples of visual observation of tumor growth in the mouse model. A representative animal from each group is shown. The upper part shows control. The lower part shows the group treated with DOX (1) in combination with BL and US. The left part shows the mice at day 7, the middle part at day 14 and the right part at day 21. (D) H&E staining of LM8 tumor cells excised on day 21. H&E staining of tumor samples from (left part) the control group and (right part) the group treated with DOX, BL and US. There were no marked differences between the two groups in cell morphology. Magnification, x400. DOX (1) = received DOX at 1 mg/kg body weight, DOX (5) = received DOX at 5 mg/kg body weight.

effect at about 1/5 the dose of antitumor agent employed in monotherapy with DOX. These findings suggest the possibility of reduction of adverse events in the host.

The greatest advantage of use of a liposome preparation for drug delivery application is that the liposome itself is a drug whose safety has been established. Furthermore, it is easy to add modified products such as functional ligands to the surface of liposomes. However, problems with liposomes have included the instability of crude liposomes in the body and their uptake by the reticular endothelial system (RES) including the liver, spleen after intravenous administration. To solve these problems, which have hampered the clinical use of liposomes,

Table 1. Complete blood count and liver function enzyme assay

	WBC	RBC	Hb	plt	GOT	GPT
contorol	4260 ± 901	731 ± 46	12.86 ± 0.55	74.76 ± 12.82	59.0 ± 15.79	27.0 ± 4.06
DOX (1)	5020 ± 766	630 ± 133	11.18 ± 2.23	50.02 ± 2.92*	68.2 ± 17.69	33.4 ± 5.45
DOX (1) + BL + US	4460 ± 867	654 ± 123	11.84 ± 1.58	63.14 ± 8.40	61.8 ± 11.56	31.6 ± 4.82
DOX (5)	3740 ± 1504	588 ± 164	9.92 ± 2.48**	33.08 ± 13.37**	73.6 ± 21.51	37.6 ± 18.79

Hb level decreased in the uncombined DOX (5) treatment group ($p < 0.05$). Plt count decreased significantly in the uncombined DOX (1) treatment group ($p < 0.05$) and the uncombined DOX (5) treatment group ($p < 0.01$). No significant difference was noted in any parameter between the BL + US group and the control group.

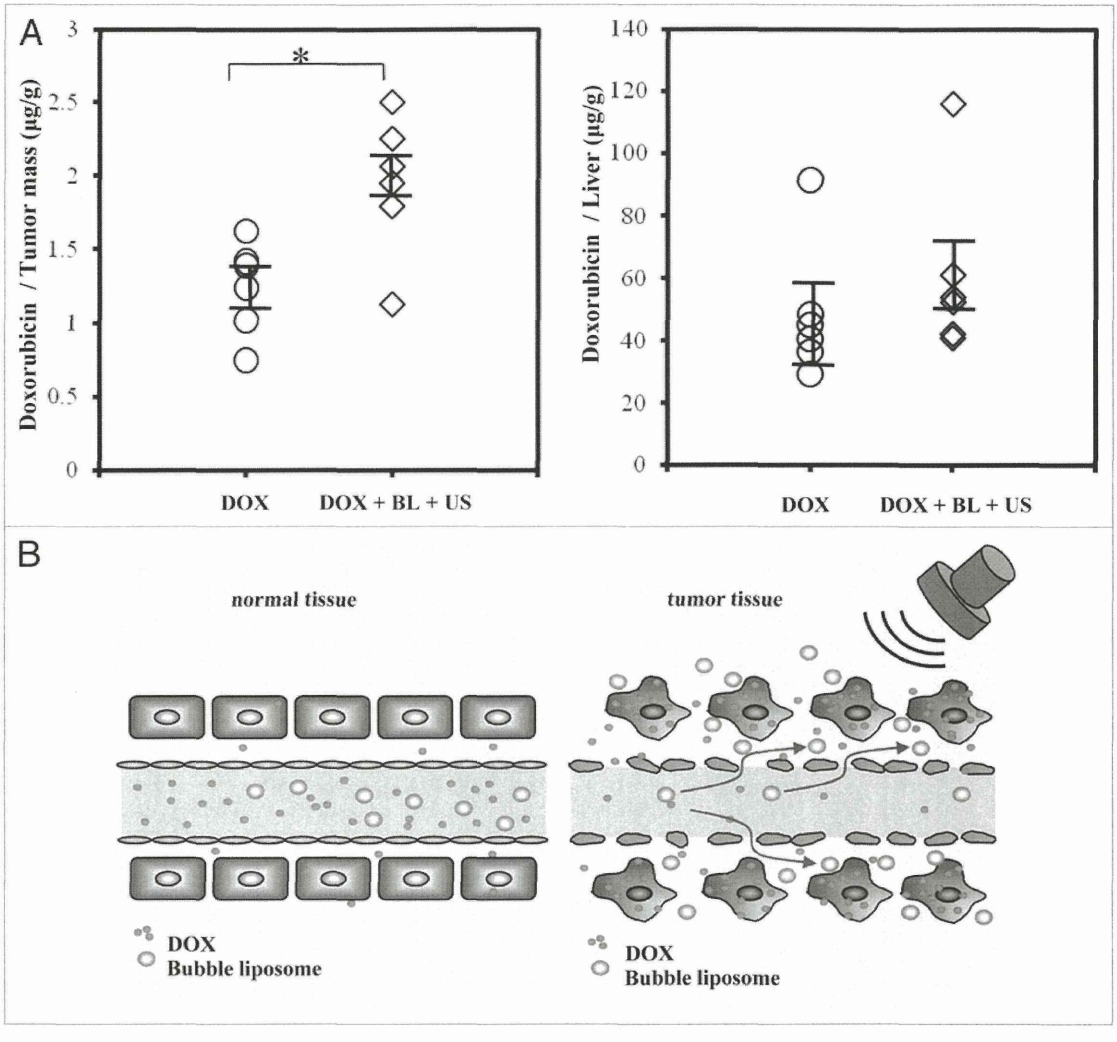

Figure 4. (A) Assay for DOX Content. Intratumoral DOX level (left part) and liver tissue DOX level (right part) were compared between mice that received injection of DOX alone and mice that received DOX in combination with BL and US. Intratumoral DOX level was significantly higher in the DOX + BL + US group than in the uncombined DOX injection group ($p < 0.05$). Liver tissue DOX level did not differ significantly between the two groups. (B) *EPR effect.* According to the anatomical and pathophysiological abnormalities of tumor tissues, biocompatible macromolecules and lipids will spontaneously and preferentially leak from tumor vessels into tumor tissues due to increased microvessel permeability and be retained in the tumor for extended periods of time due to poor lymph drainage.

polyethylene glycol (PEG) liposomes that could evade the RES as a result of modification of their surface with PEG were developed.[25-27] Because of its persistent enhanced permeability and retention (EPR) effect, the long-term retention-type liposome with PEG has been demonstrated to yield higher accumulation in tumors.[28,29] This type of targeting is called "passive

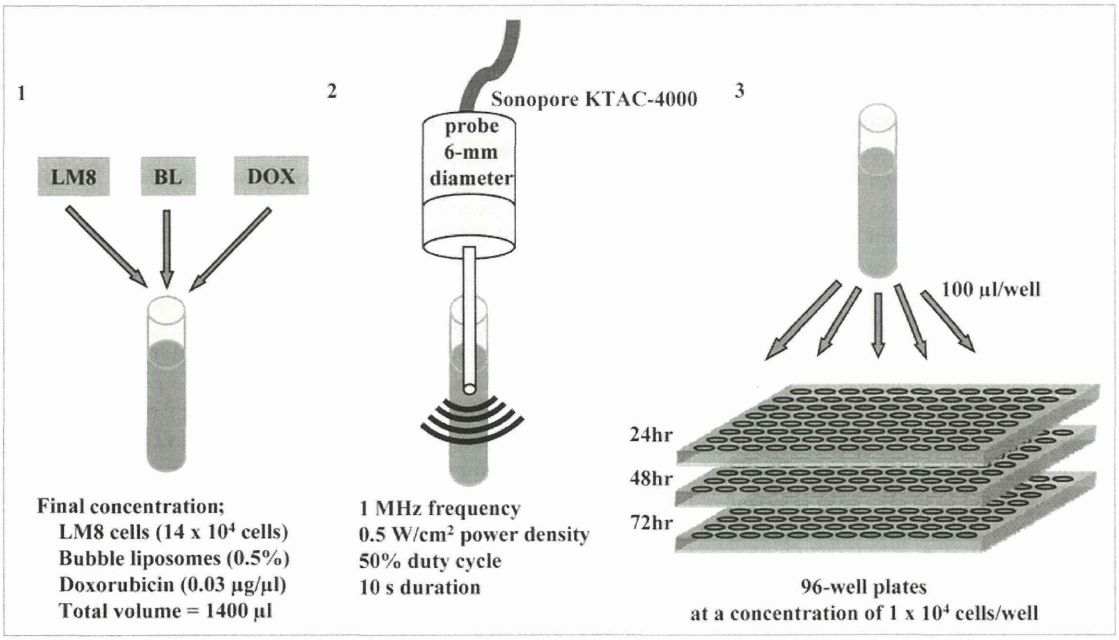

Figure 5. Method of in vitro experiment for evaluation of the effectiveness of BL and US in LM8 cells. (1) LM8 cells, BL and DOX are combined, followed by filling to a total volume of 1,400 μl (final concentration: LM8 cells (14 x 10^4 cells), BLs (0.5%), DOX (0.03 μg/μl)). (2) US is immediately applied to the mixture. (3) A 96-well plate is inoculated and cell viability is measured by MTT assay 24, 48 and 72 h later.

targeting" and serves as the basis of treatment strategies using liposomes. With the progress in research on targeting, such as passive targeting as described above, research on active targeting has also proceeded. The latter aims at targeting by liposomes to which functional ligands such as antibodies are bound and at the enhancement of drug uptake using additive application.[30-32] Concerning active targeting using DOX, there are reports on tumor inhibitory effects obtained through the concurrent use of thermosensitive liposomal DOX and hyperthermia,[32] and tumor inhibition through the use of small magnetic liposomes containing DOX and externally applied electromagnetic force.[31] At present, active targeting is considered necessary for the efficient uptake of drug in the tumor cells in addition to the EPR effect by passive targeting.

The combination of MB and US is one of the methods recently attracting attention in the field of gene introduction and drug delivery.[33-36] Through ultrasonic irradiation, MB tentatively change cell membrane transmission by sonoporation, enabling the uptake of extracellular high-molecular-weight substances without causing cell injury.[7-11,33-36] The cavitation effect caused by the collapse of bubbles is considered the key mechanism of drug delivery into cells. This technique has been experimented upon both in vitro and in vivo as a site-specific method of drug delivery.[7,12] However, MB have had the problem of difficulty in functional ligand attachment to the surface for targeting. We prepared BL from liposomes by a new method to solve this problem and succeeded in sealing perfluoropropane gas, which serves as a nucleus to cause cavitation in BL[20,37] and employed this new type of BL in this study.

Our in vitro results suggest that it is possible to significantly inhibit osteosarcoma cell growth by adding BL and US to DOX. In vitro setting, the EPR effect that observed in the tumor tissue of the living body is unlikely expected. Thus, this action mechanism is assumed to be realized purely with the drug uptake increase induced by sonoporation effect and cavitation. Tumor targeting by EPR effect can be expected in vivo in addition to the sonoporation and cavitation effects observed in vitro. In fact, the new DDS we have constructed achieved growth inhibition almost equivalent to that observed with monotherapy with DOX, though at about 1/5 the dose used in monotherapy. In the intratumoral concentration assay, DOX concentration increased to 1.5 times that in the control. This finding indicated that our new DDS could induce high concentrations of DOX and BL specifically in tumor by EPR effect, enabling the uptake of high-concentration DOX in tumor tissue via the cavitation and sonoporation induced by US irradiation to the tumor site (**Fig. 4B**). Though there is a report on another experiment using liposomal DOX of a significant increase in hepatic tissue concentration,[27] no significant difference was observed in hepatic tissue concentration in our new DDS in comparison with the DOX monotherapy group. In our system, DOX and BL were simply mixed rather than sealing DOX inside of liposomes. This is probably a reason for the decreased uptake of DOX by the RES. In our system, DOX was not sealed in liposomes. Therefore, compared with sealed DOX, the amount of delivery into RES with BL of the remaining DOX not delivered into the tumor may be small.

Reduction of adverse events is a large advantage of our new DDS. As regards side effects, no significant difference was

observed between the group treated with DOX at 1 mg/kg in combination with BL and US and the control group, while Hb and Plt values decreased in the DOX monotherapy group (at 5 mg/kg). Thus, concomitant treatment with DOX at 1 mg in combination with BL and US could achieve a tumor inhibitory effect equivalent to that observed with monotherapy with DOX at 5 mg and could also reduce side effects. By employing this method, which enables treatment at lower doses, the number of drop-outs from chemotherapy may be reduced, with improvement of prognosis.

Since this study was performed in an osteosarcoma model prepared in the back of mice, it was possible to directly irradiate tumors with US. However, the problem with clinical application of this method is how US irradiation is to be performed when osteosarcoma cells are present in the bone. According to the reports made up to present, when the US is applied for intracranial monitoring of cerebral blood flow after t-PA (tissue plasminogen activator) administration in acute ischemic stroke case, a thrombus dissolution effect reached 50% due to the ultrasonic action.[38,39] This finding suggests that US penetrated through cranial bone. Compared with transcranial Doppler (TED),[40] which is already in clinical use, the US used in the present study differs in frequency, strength and other factors. However, it should be possible to cause cavitation in the tissues of bones in the extremities with further research on US conditions. In the treatment of malignant tumor in the soft part of bone, externally infiltrating tumors are a serious problem. Since the tumors are soft part tumors, which are resistant to chemotherapy, our system seems to be a very effective option in the current treatment option of such tumor.

The 5-year survival rate of osteosarcoma has been stagnant in the past 10 years or more. A breakthrough is thus needed to drastically improve the results of treatment. The efficacy of anticancer drugs still holds the key to survival in current medical care. In this regard, the problems of lack of drug response and drug resistance need to be solved. Since powerful regimens prepared with combinations of existing anticancer drugs are still unable to markedly improve the results of treatment, it is necessary to develop a molecular targeting treatment whose mechanism is completely different from that of conventional anticancer drugs, and to create a method to increase the potency of existing anticancer drugs and at the same time reduce side effects by targeting of cancer cells as we have reported here. It is important that materials used in this method have been used safely in clinical treatment. The balance of risk and benefit is always the key issue when applying any treatment. BL and US are safe for the body, and it is possible with our method to perform location-specific treatment using a simple device. Although BL and US were used for treatment of osteorsarcoma in the present study, they are potentially applicable to treatment of some other malignant tumors as well. Accordingly, the method we have devised can be expected to be clinically useful.

Materials and Methods

Cell lines. The murine osteosarcoma cell line LM8 was obtained from RIKEN BioResource Center (Ibaraki, Japan). LM8, established from Dunn osteosarcoma, has high metastatic potential in the lung.[41] LM8 cells were cultured in the same fashion as reported previously in reference 41.

Preparation of liposomes and bubble liposomes. BL were prepared by the reverse-phase evaporation method in the same fashion as reported previously in reference 19. We prepared BL from liposomes by a new method to solve MB problem of difficulty in functional ligand attachment to the surface for targeting and succeeded in sealing perfluoropropane gas, which serves as a nucleus to cause cavitation in BL and employed this new type of BL in this study.

Regents. Doxorubicin (Doxorubicin hydrochloride) was purchased Sigma (St. Louis, MO).

Animal. C3H female mice (age, 4 weeks; weight, 16–20 g) were purchased from CLEA Co., Inc., (Tokyo, Japan). All mice were housed under specific pathogen-free conditions with a 12 h light/dark cycle. The housing care rules and experimental protocol were approved by the Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University.

In vitro assay. *Cytotoxicity of BL and US to LM8 cells.* LM8 cells were collected with trypsin (Sigma-Aldrich) and washed twice with PBS. LM8 cells (14 x 10^4 cells), DOX (0.03 μg/μl) and BL (0.5%) mixed with 1,400 μl of culture medium in 2 ml polypropylene tubes (SUMITOMO BAKELITE, Tokyo, Japan) were exposed to US. A 6-mm diameter Sonopore KTAC-4000 probe (NEPA GENE CO., LTD., Chiba, Japan) was used for ultrasonic irradiation. The probe was inserted directly into the tubes and secured 3 mm above the bottom. US irradiation was performed at 1 MHz frequency using the following conditions: 0.5 w/cm^2 power density, 50% duty cycle, 10 sec duration. Cell suspensions (100 μl) were subsequently seeded onto flat-bottomed 96-well plates at a concentration of 1 x 10^4 cells/well and incubated for 24 h, 48 h and 72 h (**Fig. 5**). Cell viability was assayed using MTT [3-(4,5 sec-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] as described by Mosmann with minor modifications.[42]

In vivo assay. *Animal model.* Before starting all experiments, osteosarcoma-bearing mice were divided into eight groups and subjected to different modalities of administration. Before implantation of tumor cells, mice were anesthetized with diethyl ether (Nacalai Tesque, Kyoto, Japan) and shaved unilaterally on the back. Cell suspensions (200 μl) in PBS were injected subcutaneously in the back of mice with a 27-gauge needle, with 1 x 10^6 LM8 cells delivered. The inoculated mice were monitored every other day and experiments were initiated approximately 7–10 days after inoculation when tumors reached 5–7 mm in size. This day was considered day 0 and C3H mice were anesthetized with a combination of ketamine HCl (Ketalal, 50 mg/kg, i.p.) and medetomidine HCl (Domitor, 0.3 mg/kg, i.p.). Mice were then divided into eight groups and subjected to different modalities of administration as follows: Group 1, control (no treatment); Group 2, DOX (1 mg/kg); Group 3, DOX (1 mg/kg) + BL + US; Group 4, DOX (5 mg/kg); Group 5, DOX (1 mg/kg) + US; Group 6, BL; Group 7, US; Group 8, BL + US. DOX and BL preparations were administered i.v. via the tail vein and the total volume of DOX and BL was fixed at 10 ml/kg

body weight. Immediately after injection, in the sonically treated groups (Group 3, 5, 7 and 8), a 6-mm US probe was placed directly on the tumor surface and US was generated (power, 2 W/cm^2; frequency, 2 MHz; duty cycle, 50%; burst rate, 2 Hz; duration, 60 sec). This treatment was repeated three times, on days 0, 2 and 4. After treatment, mice were monitored every day. These animal models were used to examine tumor growth delay, for assay of pulmonary metastasis, evaluation of side effects and determination of survival rates.

Tumor growth delay. Mice of groups 1–8 were used in this study. Tumor growth was monitored every 2 days by measuring tumor volume with a digital caliper (repeated three times). It was estimated by measuring longitudinal cross-section diameter (L) and diameter in a transverse section (W) and using the following formula:[43] $TV = L \times W^2/2$.

The mice were humanely euthanized on day 21. Tumor tissues were collected for histopathologic examination. Tumor growth was normalized by dividing tumor volume on day X by the tumor volume measured on day 0. These mice were used for examination of antitumor effects (primary tumor growth).

Evaluation of side effect. In clinical use of DOX, almost all patients suffer myelosuppression or liver dysfunction. We therefore collected blood to evaluate side effects. Group 1–4 mice were used in this study. On day 9, for measurements of red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), platelets (Plt), glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT), mice were anesthetized and 800 µl samples of blood were obtained from the inferior vena cava. At the time of blood collection, the liver was collected to confirm nonexistence of liver metastasis.

Assay for intratumoral DOX content. For further investigation, we estimated intratumoral DOX content. Mice with LM8 tumors of about 10 mm in diameter were used for this study. Either DOX (10 mg/kg) or DOX (10 mg/kg) + BL + US was administered as described above. At 5 min after drug administration, mice were sacrificed just prior to processing of tumors. The tumors of each of the mice were removed, after were after storage in liquid nitrogen homogenized with an ultrasonic disruptor. The homogenates were mixed with chloroform and isopropanol (1:1, 5 ml) and then centrifuged at 3,000 rpm for 5 min at 4°C and the supernatants were collected. Collected supernatants were centrifuged at 10,000 rpm for 5 min at 4°C again and the supernatants were collected as a sample. The level of fluorescence of each of the samples was obtained from the supernatants by fluorescence spectrophotometry and converted to DOX content. The DOX concentration of each sample was calculated according to the mass of the corresponding tumor.

Histology. In the tumor growth delay study, tumors from treated animals and from untreated animals as a control were dissected at the times specified in the Results, fixed with 10% buffered neutral formalin solution, and then embedded in paraffin as usual. Prepared sections were stained with H&E.

Statistical analysis. All values were expressed as means ± SEM. The Mann-Whitney U-test was used to determine the significance of differences in the tumor growth delay study. In the side effect study and intratumoral DOX concentration assay, Student's t-test was used to examine differences between experimental groups. The survival data were analyzed by Kaplan-Meier methods and survival periods were compared by the log-rank test. Findings of $p < 0.05$ were considered significant.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research (KAKENHI: 22591662).

References

1. Arndt CA, Crist WM. Common musculoskeletal tumors of childhood and adolescence. N Engl J Med 1999; 341:342-52; DOI: 10.1056/NEJM199907293410507.
2. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 2002; 20:776-90; DOI: 10.1200/JCO.20.3.776.
3. Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 2004; 9:422-41; DOI: 10.1634/theoncologist.9-4-422.
4. Awada A, Piccart M. Strategies offering protection from the toxic effects of anticancer treatments with a focus on chemoprotective agents. Curr Opin Oncol 2000; 12:289-96; DOI: 10.1097/00001622-200007000-00003.
5. Meinardi MT, Gietema JA, van Veldhuisen DJ, van der Graaf WT, de Vries EG, Sleijfer DT. Long-term chemotherapy-related cardiovascular morbidity. Cancer Treat Rev 2000; 26:429-47; DOI: 10.1053/ctrv.2000.0175.
6. Greenleaf WJ, Bolander ME, Sarkar G, Goldring MB, Greenleaf JF. Artificial cavitation nuclei significantly enhance acoustically induced cell transfection. Ultrasound Med Biol 1998; 24:587-95; DOI: 10.1016/S0301-5629(98)00003-9.
7. Li T, Tachibana K, Kuroki M, Kuroki M. Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison and Levovist in mice-initial results. Radiology 2003; 229:423-8; DOI: 10.1148/radiol.2292020500.
8. Shohet RV, Chen S, Zhou YT, Wang Z, Meidell RS, Unger RH, et al. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. Circulation 2000; 101:2554-6.
9. Sonoda S, Tachibana K, Uchino E, Okubo A, Yamamoto M, Sakoda K, et al. Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. Invest Ophthalmol Vis Sci 2006; 47:558-64; DOI: 10.1167/iovs.05-0889.
10. Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, et al. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther 2002; 9:372-80; DOI: 10.1038/sj.gt.3301678.
11. Taniyama Y, Tachibana K, Hiraoka K, Namba T, Yamasaki K, Hashiya N, et al. Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation 2002; 105:1233-9; DOI: 10.1161/hc1002.105228.
12. Lindner JR. Microbubbles in medical imaging: current applications and future directions. Nat Rev Drug Discov 2004; 3:527-32; DOI: 10.1038/nrd1417.
13. Harata M, Soda Y, Tani K, Ooi J, Takizawa T, Chen M, et al. CD19-targeting liposomes containing imatinib efficiently kill Philadelphia chromosome-positive acute lymphoblastic leukemia cells. Blood 2004; 104:1442-9; DOI: 10.1182/blood-2004-02-0588.
14. Kawamura K, Kadowaki N, Suzuki R, Udagawa S, Kasaoka S, Utoguchi N, et al. Dendritic cells that endocytosed antigen-containing IgG-liposomes elicit effective antitumor immunity. J Immunother 2006; 29:165-74; DOI: 10.1097/01.cji.0000190169.61416.f5.
15. Kogure K, Moriguchi R, Sasaki K, Ueno M, Futaki S, Harashima H. Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. J Control Release 2004; 98:317-23; DOI: 10.1016/j.jconrel.2004.04.024.
16. Maruyama K, Ishida O, Kasaoka S, Takizawa T, Utoguchi N, Shinohara A, et al. Intracellular targeting of sodium mercaptoundecahydrododecaborate (BSH) to solid tumors by transferrin-PEG liposomes, for boron neutron-capture therapy (BNCT). J Control Release 2004; 98:195-207; DOI: 10.1016/j.jconrel.2004.04.018.
17. Mizoue T, Horibe T, Maruyama K, Takizawa T, Iwatsuru M, Kono K, et al. Targetability and intracellular delivery of anti-BCG antibody-modified, pH-sensitive fusogenic immunoliposomes to tumor cells. Int J Pharm 2002; 237:129-37; DOI: 10.1016/S0378-5173(02)00044-3.
18. Suzuki R, Takizawa T, Kuwata Y, Mutoh M, Ishiguro N, Utoguchi N, et al. Effective anti-tumor activity of oxaliplatin encapsulated in transferrin-PEG-liposome. Int J Pharm 2008; 346:143-50; DOI: 10.1016/j.ijpharm.2007.06.010.
19. Suzuki R, Takizawa T, Negishi Y, Hagisawa K, Tanaka K, Sawamura K, et al. Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound. J Control Release 2007; 117:130-6; DOI: 10.1016/j.jconrel.2006.09.008.

20. Suzuki R, Takizawa T, Negishi Y, Utoguchi N, Sawamura K, Tanaka K, et al. Tumor specific ultrasound enhanced gene transfer in vivo with novel liposomal bubbles. J Control Release 2008; 125:137-44; DOI: 10.1016/j.jconrel.2007.08.025.
21. Suzuki R, Oda Y, Utoguchi N, Namai E, Taira Y, Okada N, et al. A novel strategy utilizing ultrasound for antigen delivery in dendritic cell-based cancer immunotherapy. J Control Release 2009; 133:198-205.
22. Negishi Y, Endo Y, Fukuyama T, Suzuki R, Takizawa T, Omata D, et al. Delivery of siRNA into the cytoplasm by liposomal bubbles and ultrasound. J Control Release 2008; 132:124-30; DOI: 10.1016/j.jconrel.2008.08.019.
23. Negishi Y, Omata D, Iijima H, Takabayashi Y, Suzuki K, Endo Y, et al. Enhanced laminin-derived peptide AG73-mediated liposomal gene transfer by bubble liposomes and ultrasound. Mol Pharm 2010; 7:217-26; DOI: 10.1021/mp900214s.
24. Suzuki R, Namai E, Oda Y, Nishiie N, Otake S, Koshima R, et al. Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure. J Control Release 2010; 142:245-50; DOI: 10.1016/j.jconrel.2009.10.027.
25. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim Biophys Acta 1991; 1066:29-36; DOI: 10.1016/0005-2736(91)90246-5.
26. Blume G, Cevc G. Liposomes for the sustained drug release in vivo. Biochim Biophys Acta 1990; 1029:91-7; DOI: 10.1016/0005-2736(90)90440-Y.
27. Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 1990; 268:235-7; DOI: 10.1016/0014-5793(90)81016-H.
28. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 1986; 46:6387-92.
29. Muggia FM. Doxorubicin-polymer conjugates: further demonstration of the concept of enhanced permeability and retention. Clin Cancer Res 1999; 5:7-8.
30. Hoving S, Seynhaeve AL, van Tiel ST, Eggermont AM, ten Hagen TL. Addition of low-dose tumor necrosis factor-alpha to systemic treatment with STEALTH liposomal doxorubicin (Doxil) improved anti-tumor activity in osteosarcoma-bearing rats. Anticancer Drugs 2005; 16:667-74; DOI: 10.1097/00001813-200507000-00012.
31. Nobuto H, Sugita T, Kubo T, Shimose S, Yasunaga Y, Murakami T, et al. Evaluation of systemic chemotherapy with magnetic liposomal doxorubicin and a dipole external electromagnet. Int J Cancer 2004; 109:627-35; DOI: 10.1002/ijc.20035.
32. Shimose S, Sugita T, Nitta Y, Kubo T, Ikuta Y, Murakami T. Effect of thermosensitive liposomal doxorubicin with hyperthermia on primary tumor and lung metastases in hamster osteosarcoma. Int J Oncol 2001; 19:585-9.
33. Gordon AN, Fleagle JT, Guthrie D, Parkin DE, Gore ME, Lacave AJ. Recurrent epithelial ovarian carcinoma: a randomized phase III study of pegylated liposomal doxorubicin versus topotecan. J Clin Oncol 2001; 19:3312-22.
34. Sonoda S, Tachibana K, Uchino E, Yamashita T, Sakoda K, Sonoda KH, et al. Inhibition of melanoma by ultrasound-microbubble-aided drug delivery suggests membrane permeabilization. Cancer Biol Ther 2007; 6:1276-83; DOI: 10.4161/cbt.6.8.4485.
35. Kinoshita M, Hynynen K. A novel method for the intracellular delivery of siRNA using microbubble-enhanced focused ultrasound. Biochem Biophys Res Commun 2005; 335:393-9; DOI: 10.1016/j.bbrc.2005.07.101.
36. Kinoshita M, Hynynen K. Intracellular delivery of Bak BH3 peptide by microbubble-enhanced ultrasound. Pharm Res 2005; 22:716-20; DOI: 10.1007/s11095-005-2586-7.
37. Yamashita T, Sonoda S, Suzuki R, Arimura N, Tachibana K, Maruyama K, et al. A novel bubble liposome and ultrasound-mediated gene transfer to ocular surface: RC-1 cells in vitro and conjunctiva in vivo. Exp Eye Res 2007; 85:741-8; DOI: 10.1016/j.exer.2007.08.006.
38. Alexandrov AV, Molina CA, Grotta JC, Garami Z, Ford SR, Alvarez-Sabin J, et al. Ultrasound-enhanced systemic thrombolysis for acute ischemic stroke. N Engl J Med 2004; 351:2170-8; DOI: 10.1056/NEJMoa041175.
39. Molina CA, Ribo M, Rubiera M, Montaner J, Santamarina E, Delgado-Mederos R, et al. Microbubble administration accelerates clot lysis during continuous 2-MHz ultrasound monitoring in stroke patients treated with intravenous tissue plasminogen activator. Stroke 2006; 37:425-9; DOI: 10.1161/01.STR.0000199064.94588.39.
40. Aaslid R, Markwalder TM, Nornes H. Noninvasive transcranial Doppler ultrasound recording of flow velocity in basal cerebral arteries. J Neurosurg 1982; 57:769-74; DOI: 10.3171/jns.1982.57.6.0769.
41. Asai T, Ueda T, Itoh K, Yoshioka K, Aoki Y, Mori S, et al. Establishment and characterization of a murine osteosarcoma cell line (LM8) with high metastatic potential to the lung. Int J Cancer 1998; 76:418-22; DOI: 10.1002/(SICI)1097-0215(19980504)76:3<418::AID-IJC21>3.0.CO;2-5.
42. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55-63; DOI: 10.1016/0022-1759(83)90303-4.
43. Martin F, Caignard A, Jeannin JF, Leclerc A, Martin M. Selection by trypsin of two sublines of rat colon cancer cells forming progressive or regressive tumors. Int J Cancer 1983; 32:623-7; DOI: 10.1002/ijc.2910320517.

Published OnlineFirst August 4, 2011; DOI:10.1158/1078-0432.CCR-11-1477

Clinical Cancer Research

microRNA-10b: A New Marker or the Marker of Pancreatic Ductal Adenocarcinoma?

Tetsuro Setoyama, Xinna Zhang, Shoji Natsugoe, et al.

Clin Cancer Res 2011;17:5527-5529. Published OnlineFirst August 4, 2011.