

results (dotted line) exhibit the trend of frequency-related decreases. The cyclic fluctuation is scarcely observed on the dotted line. The significant differences in Fig. 10A and B indicate the importance of the substantial cyclic fluctuation of ultrasound transmittance at least for sonothrombolytic therapy through the temporal window.

We obtained our results in experiments using a bone-phantom plate and model calculations based on multi-layers. Since human skull bone has a complex geometrical structure with inhomogeneous density degrees, fluctuations may be less than those obtained in this work. Evaluation of fluctuations with human skulls and temporal bone merits further attention, and both this type of evaluation and fluctuation-reduction technologies are very important for clinical designs of safe and effective sonothrombolytic therapies.

In this paper, we showed that modulation of ultrasound frequency reduced fluctuations. Because transmittance significantly fluctuated with frequency (Fig. 2), we tried (successfully) to equalize the transmittance fluctuations by using the PSRF method of frequency modulation. As shown in Fig. 9, the broader a frequency deviation, the greater the equalization effect. If the medical community were to further broaden frequency deviation even more so, the equalization of transmittance might improve. However, broader frequency deviation is not always preferred. A frequency far from the center frequency of a transducer will not effectively convert into ultrasound, and this outcome can generate heat harmful to living bodies will be generated. Taking into account the above-mentioned facts, we should identify an optimal balance between equalization and transducer design.

We experimentally verified that ultrasound transmittance changed according to skin-thickness, frequency, and bone-thickness. Since frequency dependence is different from individual to individual, we cannot choose a single frequency suitable for every individual. In the absence of a single optimal frequency, the equalization of the transmittance through ultrasound modulation is a highly effective and important methodology for sonothrombolytic therapy.

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## References

- [1] The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N. Engl. J. Med.* 333 (1995) 1581–1587.
- [2] T. Yamaguchi, E. Mori, K. Minematsu, J. Nakagawara, K. Hashi, I. Saito, Y. Shinohara, Japan Alteplase Clinical Trial (J-ACT) Group, Alteplase at 0.6 mg/kg for acute ischemic stroke within 3 hours of onset: Japan Alteplase Clinical Trial (J-ACT). *Stroke* 37 (2006) 1810–1815.
- [3] W. Hacke, M. Kaste, E. Bluhmki, M. Brozman, A. Dávalos, D. Guidetti, V. Larrue, K.R. Lees, Z. Medeghri, T. Machnig, D. Schneider, R. von Kummer, N. Wahlgren, D. Toni, ECASS investigators, thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N. Engl. J. Med.* 359 (2008) 1317–1329.
- [4] A.V. Alexandrov, C.A. Molina, J.C. Grotta, Z. Garami, S.R. Ford, J. Alvarez-Sabin, J. Montaner, M. Saqqur, A.M. Demchuk, L.A. Moyé, M.D. Hill, A.W. Wojner, CLOTBUST investigators, ultrasound-enhanced systemic thrombolysis for acute ischemic stroke. *N. Engl. J. Med.* 351 (2004) 2170–2178.
- [5] K. Barlinn, G. Tsvigoulis, C.A. Molina, D.A. Alexandrov, M.E. Schafer, J. Alleman, A.V. Alexandrov, TUCSON investigators, exploratory analysis of estimated acoustic peak rarefaction pressure, recanalization, and outcome in the transcranial ultrasound in clinical sonothrombolysis trial. *J. Clin. Ultrasound* 41 (2013) 354–360.
- [6] J. Eggers, I.R. König, B. Koch, G. Händler, G. Siedel, Sonothrombolysis with transcranial color-coded sonography and recombinant tissue-type plasminogen activator in acute middle cerebral artery main stem occlusion. Results from a randomized study. *Stroke* 39 (2008) 1470–1475.
- [7] M. Daffertshofer, A. Gass, P. Ringleb, M. Sitzer, U. Sliwka, T. Els, O. Sedlaczek, W.J. Koroshetz, M.G. Hennerici, Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: Increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator – results of a phase II clinical trial. *Stroke* 36 (2005) 1441–1446.
- [8] Z. Wang, M.A. Moehring, A.H. Voie, H. Furuhashi. In vitro evaluation of dual mode ultrasonic thrombolysis method for transcranial application with an occlusive thrombosis model. *Ultrasound Med. Biol.* 34 (2008) 96–102.
- [9] J. Shimizu, T. Fukuda, T. Abe, M. Ogihara, J. Kubota, A. Sasaki, T. Azuma, K. Sasaki, K. Shimizu, T. Oishi, S. Umemura, H. Furuhashi, Ultrasound safety with midfrequency transcranial sonothrombolysis: preliminary study on normal Macaca monkey brain. *Ultrasound Med. Biol.* 38 (2012) 1040–1050.
- [10] T. Azuma, M. Ogihara, J. Kubota, A. Sasaki, S. Umemura, H. Furuhashi, Dual-frequency ultrasound imaging and therapeutic bilaminar array using frequency selective isolation layer. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* 57 (2010) 1211–1224.
- [11] K. Hynynen, N. McDannold, G. Clement, F.A. Jolesz, E. Zadicario, R. Killiany, T. Moore, D. Rosen, Preclinical testing of a phased array ultrasound system for MRI-guided noninvasive surgery of the brain – a primate study. *Eur. J. Radiol.* 59 (2006) 149–156.
- [12] K. Hynynen, G. Clement, Clinical applications of focused ultrasound – the brain. *Int. J. Hyperther.* 23 (2007) 193–202.
- [13] C. Yiallouras, C. Damianou, Review of MRI positioning devices for guiding focused ultrasound systems. *Int. J. Med. Robot (in press)*, <http://dx.doi.org/10.1002/rjcs.1601>.
- [14] M.E. Shafer, J. Alleman, A. Alexandrov, K. Barlinn, Development of an operator independent ultrasound therapeutic device for stroke treatment. in: 2012 IEEE International Ultrason. Symposium Proceedings, 2012, pp. 1948–1951.
- [15] A.Y. Amm, T.D. Mast, I.H. Huang, T.A. Abruzzo, C.C. Coussios, G.J. Shaw, C.K. Holland, Characterization of ultrasound propagation through ex-vivo human temporal bone. *Ultrasound Med. Biol.* 34 (2008) 1578–1589.
- [16] G. Bouchoux, R. Shivashankar, T.A. Abruzzo, C.K. Holland, In silico study of low-frequency transcranial ultrasound fields in acute ischemic stroke patients. *Ultrasound Med. Biol.* 40 (2014) 1154–1166.
- [17] F.J. Fry, J.E. Barger, Acoustical properties of the human skull. *J. Acoust. Soc. Am.* 64 (1978) 423–457.
- [18] S. Pichardo, V.W. Sin, K. Hynynen, Multi-frequency characterization of the speed of sound and attenuation coefficient for longitudinal transmission of freshly excised human skulls. *Phys. Med. Biol.* 56 (2011) 219–250.
- [19] P.J. White, G.T. Clement, K. Hynynen, Local frequency dependence in transcranial ultrasound transmission. *Phys. Med. Biol.* 51 (2006) 2293–2305.
- [20] J. Aarnio, G.T. Clement, K. Hynynen, A new ultrasound method for determining the acoustic phase shifts caused by the skull bone. *Ultrasound Med. Biol.* 31 (2005) 771–780.
- [21] M. Hayner, K.J. Hynynen, Numerical analysis of ultrasonic transmission and absorption of oblique plane waves through the human skull. *J. Acoust. Soc. Am.* 110 (2001) 3319–3330.
- [22] G.T. Clement, J. Sun, K. Hynynen, The role of internal reflection in transskull phase distortion. *Ultrasonics* 39 (2001) 109–113.
- [23] H. Furuhashi, O. Saito, Comparative study of standing wave reduction methods using random modulation for transcranial ultrasonication. *Ultrasound Med. Biol.* 39 (2013) 1440–1450.
- [24] S.C. Tang, G.T. Clement, Acoustic standing wave suppression using randomized phase-shift-keying excitations. *J. Acoust. Soc. Am.* 126 (2009) 1667–1670.
- [25] S.C. Tang, G.T. Clement, Standing wave suppression for transcranial ultrasound by random-modulation. *IEEE Trans. Biomed. Eng.* 57 (2010) 203–205.
- [26] C. Baron, J.F. Aubry, M. Tanter, S. Meairs, M. Fink, Simulation of intracranial acoustic fields in clinical trials of sonothrombolysis. *Ultrasound Med. Biol.* 35 (2009) 1148–1158.
- [27] J. Song, A. Pulkkinen, Y. Huang, K. Hynynen, Investigation of standing wave formation in a human skull for a clinical prototype of a large-aperture, transcranial MR-guided focused ultrasound (MRgFUS) phased array: an experimental and simulation study. *IEEE Trans. Biomed. Eng.* 59 (2012) 435–444.
- [28] A.D. Wijnhoud, M. Franckena, A. van der Lugt, P.J. Koudstaal, E.D. Dippel, Inadequate acoustical temporal bone window in patients with a transient ischemic attack or minor stroke: role of skull thickness and bone density. *Ultrasound Med. Biol.* 34 (2008) 923–929.
- [29] T.F. Hueter, Ultrasonic absorption measurements in human skull bone and their dependence upon frequency. *Naturwiss* 39 (1952) 21–22.
- [30] F. Babiloni, C. Babiloni, F. Carducci, M. Del Gaudio, P. Onorati, A. Urbano, A high resolution EEG method based on the correction of the surface Laplacian estimate for the subject's variable scalp thickness. *Electroencephalogr. Clin. Neurophysiol.* 103 (1997) 486–492.



## rt-PA 静注による閉塞血管早期再開通率の検討：虚血性脳卒中に対する新規超音波血栓溶解装置開発の対照データ

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## Early recanalization rates following intravenous recombinant tissue plasminogen activator (rt-PA) therapy in acute ischemic stroke

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**Purpose:** The objective of this study was to assess early recanalization rates following intravenous recombinant tissue plasminogen activator (rt-PA) therapy by magnetic resonance angiography (MRA) or digital subtraction angiography (DSA) in patients with acute ischemic stroke in order to plan a clinical investigation for a newly developed sonothrombolysis system.

**Methods:** We retrospectively enrolled consecutive patients with acute ischemic stroke who were treated with intravenous rt-PA. Early recanalization within 2 hours and 24 hours after the initiation of rt-PA was evaluated by modified Mori grade on follow-up MRA or Thrombolysis in Cerebral Infarction (TICI) score on follow-up DSA.

**Results:** A total of 384 patients were enrolled (243 men, age  $74 \pm 13$  years) in the study. Patients were subdivided into groups based upon arterial location as follows: 63 patients in the internal carotid artery (ICA), 181 in the middle cerebral artery (MCA [M1 and M2 segments]), 5 in the anterior cerebral artery (ACA), and 14 in the posterior cerebral artery (PCA). Among patients with major artery occlusion (ICA, MCA, ACA, or PCA), the rates of recanalization were 37.2% within 2 hours and 57.4% within 24 hours; 8 of 232 patients (3.4%) had symptomatic intracranial hemorrhage within the initial 36 hours, and 76 of 225 patients (33.8%) had a favorable functional outcome (modified Rankin Scale (mRS) 0-1) at 3 months.

**Conclusions:** We assessed early recanalization rates and clinical outcome following intravenous rt-PA therapy.

**Keywords:** sonothrombolysis, acute ischemic stroke, intravenous recombinant tissue plasminogen activator therapy, recanalization

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### 背景と目的

急性期虚血性脳卒中患者に遺伝子組み換え組織プラス

ミノゲン・アクティベータ (recombinant tissue-type plasminogen activator: rt-PA) を静注投与し、同時に血管閉塞部位に向けて 2MHz の経頭蓋ドプラで超音波連

### Reprint request

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続照射を加えると完全再開通率が高まることが Alexandrov ら<sup>1)</sup>により報告された。Eggers ら<sup>2)</sup>は、rt-PA 投与に 1.8MHz の経頭蓋カラードブラによる閉塞血管のモニターを併用すると、rt-PA 投与のみの群に比べて 1 時間後の再開通率が改善し、3 カ月後の転帰良好を認めたと報告した。一方、Daffertshofer ら<sup>3)</sup>は、300kHz の低周波バースト波の超音波を用いた超音波併用療法 (transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia; TRUMBI trial) を実施したが、超音波併用群に脳出血合併と死亡とが多くなり、研究は途中で中止となった。わが国では、東京慈恵会医科大学、国立循環器病研究センター、株式会社カネカが共同で、500kHz 前後の変調超音波の安全性と有効性を確認する前臨床研究を実施中であり、さらに本技術を搭載した新規超音波血栓溶解装置の臨床開発を計画している。

今回、開発の際に参照するデータの収集を目的として、急性期虚血性脳卒中に対する rt-PA 静注療法例において、超音波照射を併用しない場合の早期再開通率、症候性頭蓋内出血率と 3 カ月後転帰を調べた。

## 対象と方法

本研究計画は、国立循環器病研究センター倫理委員会の承認を受けた。

対象は 2005 年 10 月から 2013 年 4 月までに当院で rt-PA 静注療法を施行した 384 例である。前向きに収集したデータベースを用いて、後ろ向きに検討した。検討項目は、患者背景、脳梗塞病型、MRA もしくは脳血管造影検査 (digital subtraction angiography; DSA) による血管閉塞部位を、内頸動脈 (internal carotid artery; ICA)、中大脳動脈 (middle cerebral artery; MCA)、前大脳動脈 (anterior cerebral artery; ACA)、後大脳動脈 (posterior cerebral artery; PCA)、椎骨脳底動脈 (vertebro-basilar artery; VB) 系、閉塞血管なし、評価不能に分類して評価した。主幹動脈 (ICA, MCA, ACA, PCA) に閉塞があり、発症 2 時間以内に MRA もしくは DSA で再開通の評価を行った症例の rt-PA 投与 2 時間以内、24 時間以内の再開通の有無を調べた。2 時間以内に再開通の評価を行ったが、24 時間以内には評価しなかった症例は、2 時間以内の再開通の有無で 24 時間以内の再開通の有無に代用した。発症 36 時間以内の National Institutes of Health Stroke Scale (NIHSS) スコア 4 以上の増悪を伴う頭蓋内出血を症候性頭蓋内出血と定義した。転帰は 3 カ月後の modified Rankin Scale (mRS) スコアで評価し、mRS 0-1 を転帰良好、mRS 0-2 を日常生活自立とした。なお、rt-PA 投与後 24 時間以内に別の部位に脳梗塞を再発した症例は 24 時間以内の再開通の評価から除外した。また、rt-PA 静注療法後に閉塞血

管に対して経皮経管的脳血栓回収用機器 (Merci リトリバー、PENUMBRA システム) を使用した症例は、24 時間以内の再開通の評価と症候性頭蓋内出血、3 カ月後の転帰の評価から除外した。

再開通の評価は MRA では modified Mori grade を用い<sup>4)</sup>、DSA では Thrombolysis in Cerebral Infarction 分類 (TICI) grade を用いた<sup>5)</sup>。MRA と DSA とを併用した評価は、Yoshimura ら<sup>6)</sup>の報告を参考に、grade 0: MRA で modified Mori grade 0、DSA で TICI 0、grade 1: MRA で modified Mori grade 1、DSA で TICI 1、grade 2: MRA で modified Mori grade 2、DSA で TICI 2A、grade 3: MRA で modified Mori grade 3、DSA で TICI 2B、3 という分類を作成し使用した。modified Mori grade 2 以上、もしくは、TICI 2A 以上、MRA と DSA を併用した評価では grade 2 以上を再開通ありとした。

主幹動脈閉塞患者の閉塞血管部位 (ICA, M1, M2) と MRA, DSA の併用による再開通率、症候性頭蓋内出血、転帰の関係についてカイ 2 乗検定を用いて検討した。

## 結果

384 例中、男性は 243 例 (63.2%)、年齢は  $74 \pm 13$  歳であった。臨床病型は、心原性脳塞栓症 248 例 (64.6%)、アテローム血栓性脳梗塞 48 例 (12.5%)、ラクナ梗塞 4 例 (1.0%) であった。危険因子として、高血圧 259 例 (67.5%)、心房細動 204 例 (53.1%)、脂質異常症 136 例 (35.4%)、糖尿病を 78 例 (20.3%) に認めた。治療前の閉塞血管は、ICA 63 例 (16.4%)、MCA 水平部 (M1) 120 例 (31.3%)、島部 (M2) 61 例 (15.9%)、ACA 5 例 (1.3%)、PCA 14 例 (3.6%)、VB 系 12 例 (3.1%)、閉塞血管なし 78 例 (20.3%)、評価不能 27 例 (7.0%)、その他 4 例 (1.0%) であった。血管内治療が施行されたのは 37 例であり、閉塞血管は ICA 15 例、M1 14 例、M2 2 例、椎骨動脈 (vertebral artery; VA) 1 例、脳底動脈 (basilar artery; BA) 5 例であった。

rt-PA 投与前に MRA により閉塞血管を評価した症例は 352 例で、発症から MRA までの時間は  $103 \pm 40$  分であった。そのうち、主幹動脈 (ICA, MCA, ACA, PCA) 閉塞を認めた 263 例を対象として検討したところ、rt-PA 投与後、2 時間以内に MRA で再開通の有無を確認した症例は 71 例で、rt-PA 投与からフォローアップの MRA までの時間は  $68 \pm 21$  分であった。rt-PA 投与後、2 時間以内に DSA で再開通の有無を確認した症例は 31 例で、rt-PA から DSA の穿刺までの時間は  $82 \pm 30$  分であった。16 例が 2 時間以内に MRA, DSA とともに施行されており、発症 2 時間以内に MRA もしくは、DSA で再開通の有無が確認できた症例は合計 86 例であった。

**Table 1 Recanalization rates within 2 hours after the initiation of rt-PA**

MRA (modified Mori grade)	Patients(%)				
	0	1	2	3	Total
ICA	9(56.3)	3(19.8)	3(18.8)	1(6.3)	16
M1	20(52.6)	1(2.6)	12(31.6)	5(13.2)	38
M2	4(30.8)	0(0)	5(38.5)	4(30.8)	13
ACA	1(100.0)	0(0)	0(0)	0(0)	1
PCA	2(66.7)	1(33.3)	0(0)	0(0)	3
Total	36	5	20	10	71

MRA,DSA (modified Mori grade, TIC1)	Patients(%)				
	0	1	2	3	Total
ICA	17(65.4)	3(11.5)	5(19.2)	1(3.8)	26
M1	24(55.8)	2(4.7)	10(23.3)	7(16.3)	43
M2	4(30.8)	0(0)	5(38.5)	4(30.8)	13
ACA	1(100.0)	0(0)	0(0)	0(0)	1
PCA	2(66.7)	1(33.3)	0(0)	0(0)	3
Total	48	6	20	12	86

24 時間以内の評価は、血管内治療を施行した 31 例と脳梗塞を再発した M2 閉塞の 1 例を除外した 54 例で行った。54 例中 rt-PA 投与後 24 時間以内に MRA の評価を行った症例は 7 例で、rt-PA から MRA までの時間の中央値は 21 時間 57 分、DSA で評価を行った症例は 3 例で、rt-PA 投与から DSA 穿刺までの時間の中央値 24 時間 58 分であった。24 時間以内の MRA の評価の 47 例、MRA と DSA を併用した評価の 44 例は、2 時間以内の再開通の有無で 24 時間以内の再開通の有無を代用した。

MRA, MRA と DSA の併用による 2 時間以内の再開通を Table 1 に示す。MRA を用いた評価では、ICA 閉塞の再開通率は 25.0%, M1 閉塞 44.7%, M2 閉塞 69.2%, ACA 閉塞 0%, PCA 閉塞 0%, MCA (M1 + M2) 閉塞 51.0%, 主幹動脈 (ICA, ACA, MCA, PCA) 閉塞 42.3% であった。MRA と DSA を併用した評価では、それぞれ、23.1%, 39.5%, 69.2%, 0%, 0%, 46.4%, 37.2% であった。閉塞部位毎に再開通率を比較すると、MRA と DSA を併用した評価では、ICA の再開通率に比べて、M1 の再開通率は有意差なく ( $p=0.160$ ), M2 の再開通率が高かった ( $p=0.005$ )。

24 時間以内の再開通を Table 2 に示す。MRA を用いた評価では、ICA 閉塞の再開通率は 36.4%, M1 閉塞 55.2%, M2 閉塞 90.0%, ACA 閉塞 0%, PCA 閉塞 0%, MCA (M1 + M2) 閉塞 64.1%, 主幹動脈 (ICA, ACA, MCA, PCA) 閉塞 53.7% であった。MRA と DSA を併用した評価では、それぞれ、45.5%, 58.6%, 90.0%, 0%, 0%, 66.7%, 57.4% であった。閉塞部位毎に再開通率を比較すると、MRA と DSA を併用した

**Table 2 Recanalization rates within 24 hours after the initiation of rt-PA**

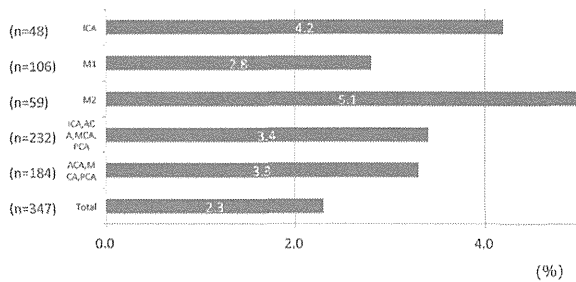
MRA (modified Mori grade)	Patients(%)				
	0	1	2	3	Total
ICA	3(27.3)	4(36.4)	2(18.2)	2(18.2)	11
M1	13(44.8)	0(0)	9(31.0)	7(24.1)	29
M2	1(10.0)	0(0)	5(50.0)	4(40.0)	10
ACA	1(100.0)	0(0)	0(0)	0(0)	1
PCA	2(66.7)	1(33.3)	0(0)	0(0)	3
Total	20	5	16	13	54

MRA,DSA (modified Mori grade, TIC1)	Patients(%)				
	0	1	2	3	Total
ICA	2(18.2)	4(36.4)	3(27.3)	2(18.2)	11
M1	12(41.1)	0(0)	8(27.6)	9(31.0)	29
M2	1(10.0)	0(0)	5(50.0)	4(40.0)	10
ACA	1(100.0)	0(0)	0(0)	0(0)	1
PCA	2(66.7)	1(33.3)	0(0)	0(0)	3
Total	18	5	16	15	54

評価では、ICA の再開通率に比べ、M1 の再開通率は有意差なく ( $p=0.455$ ), M2 の再開通率が高かった ( $p=0.031$ )。

症候性頭蓋内出血は血管内治療を施行された 37 例を除外した 347 例で検討した (Fig.1)。全体では 8 例 (2.3%) であり、ICA 閉塞で 2 例、M1 閉塞で 3 例、M2 閉塞で 3 例認めた。ICA 閉塞の 4.2% (2 例 / 48 例), M1 閉塞の 2.8% (3 例 / 106 例), M2 閉塞の 5.1% (3 例 / 59 例), ICA, ACA, MCA, PCA 閉塞患者の 3.4% (8 例 / 232 例), ACA, MCA, PCA 閉塞患者の 3.3% (6 例 / 184 例) であった。ICA, M1, M2 の閉塞血管別では、症候性頭蓋内出血の発生率に有意差は認めなかった (ICA と比較した, M1, M2 の発生率は各々  $p=0.647, p=1.000$ )。

3 カ月後の mRS を、血管内治療を施行された 37 例と、転帰を確認できなかった 10 例を除外した 337 例で検討した。転帰良好 (mRS 0-1) は全体の 38.6% (130 例 / 337 例), ICA, ACA, MCA, PCA 閉塞患者の 33.8% (76 例 / 225 例), ACA, MCA, PCA 閉塞患者の 38.3% (69 例 / 180 例), 日常生活自立 (mRS 0-2) は全体の 50.1% (169 例 / 337 例), ICA, ACA, MCA, PCA 閉塞患者の 43.6% (98 例 / 225 例), ACA, MCA, PCA 閉塞患者の 48.9% (88 例 / 180 例) であった (Fig.2)。閉塞血管別では、転帰良好 (mRS 0-1) は ICA 15.6% (7 例 / 45 例), M1 34.0% (35 例 / 103 例), M2 40.7% (24 例 / 59 例), 閉塞血管なし 53.3% (40 例 / 75 例) であり、ICA 閉塞はそれ以外に比較して転帰良好が少なかった ( $p<0.001$ ), 日常生活自立 (mRS 0-2) は ICA 22.2% (10 例 / 45 例), M1 44.7% (46 例 / 103 例), M2 49.2% (29



**Fig.1 Symptomatic intracerebral hemorrhage within the initial 36 hours**

a deterioration in NIHSS score  $\geq 4$  from baseline

例 /59 例), 閉塞血管なし 69.3% (52 例 /75 例) であり, ICA 閉塞はそれ以外に比較して日常生活自立が少なかった ( $p < 0.001$ ). 死亡 (mRS 6) は, 全体の 6.2% (21 例 /337 例), ICA, ACA, MCA, PCA 閉塞患者の 8.9% (20 例 /225 例), ACA, MCA, PCA 閉塞患者の 6.7% (12 例 /180 例) であった. 閉塞血管別では, ICA 17.8% (8 例 /45 例), M1 5.8% (6 例 /103 例), M2 10.2% (6 例 /59 例) であり, ICA 閉塞はそれ以外に比較して死亡が多かった ( $p < 0.001$ ).

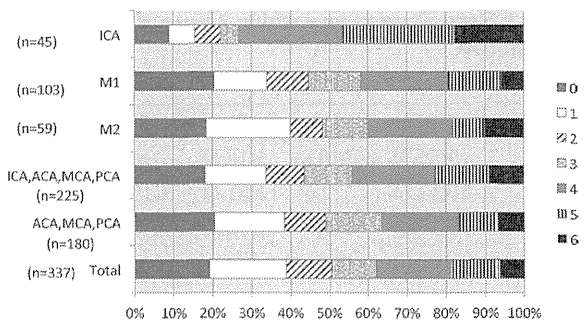
## 考察

新規超音波血栓溶解装置の臨床開発に参照するデータの収集を行った.

現在までの rt-PA 静注療法後の再開通の評価は, アルテプラゼ投与量は 0.6mg/kg, 0.9mg/kg の 2 種類で報告があり, また再開通の評価は経頭蓋超音波ドブラ法 (transcranial Doppler ultrasonography; TCD), CTA, MRA, DSA といった多様な手段が使用され, 評価のタイミングもさまざまであった<sup>7-12)</sup>. 今回われわれは, 0.6mg/kg の rt-PA 静注療法を施行された患者を対象に, MRA, DSA の所見のみを用いて検討を行った. TCD や経頭蓋カラードブラ法 (transcranial color-coded sonography; TCCS) を除外した理由は, 日本人では TCD や TCCS に必要な側頭骨窓が良好な症例が少なく, 血流が流れていても頭蓋内血管が描出されない可能性が高いためである<sup>13)</sup>. 本研究は, 低侵襲的低周波超音波装置による急性期血栓溶解療法開発のための参照データの収集が目的であり, 本装置の治療対象となると予測される ICA, MCA, ACA, PCA 閉塞もしくは MCA, ACA, PCA 閉塞をまとめた検討を追加した.

現在までに報告されている, rt-PA 投与後の閉塞血管の再開通率についての結果を示す.

Bhatia ら<sup>8)</sup>は, CTA で ICA, M1, M2 閉塞が示された 388 例を対象に, rt-PA (0.9mg/kg) 静注療法開始後 120 分までに施行した TCD, もしくは, 血管内治療目



**Fig.2 Modified Rankin Scale at 3 months by different sites of occlusion**

的で施行した DSA で再開通の評価を行った. 再開通を TCD で thrombolysis in brain ischemia (TIBI) 3-5, DSA で thrombolysis in myocardial ischemia (TIMI) 2-3 と定義したとき, ICA 終末部の再開通率は 4.4% (1 例 /24 例) であり, M1 は 32.3% (21 例 /65 例), M2 は 30.8% (4 例 /13 例), BA は 4.0% (1 例 /25 例) であった. Lee ら<sup>9)</sup>は, アルテプラゼ (0.9mg/kg) 投与後, 中央値 120 分 (60-365 分) に DSA を行い, TICI 2 以上を再開通ありとして評価している. 入院時に CTA で主幹動脈閉塞を認めた 31 例のうち, ICA もしくは, M1 閉塞の再開通は 12.5% (2 例 /16 例), M2 以遠閉塞 27.3% (3 例 /11 例), BA 閉塞 50.0% (2 例 /4 例) であった. われわれの研究とこれらの報告を比べると, アルテプラゼ 0.9mg/kg を使用している点, CTA を用いて入院時の閉塞血管の評価をしている点, 一部 TCD で再開通率の評価をしている点が異なっていた. アルテプラゼの投与量が違うため, 再開通率を今回の結果と直接比較することは困難であるが, 近位側脳主幹動脈の再開通率が低い点は今回の結果と同様であった.

MRA を用いて MCA (M1, 41 例; M2, 17 例) 閉塞患者のアルテプラゼ (0.6mg/kg) 投与後の再開通を前向きに観察した Japan Alteplase Clinical Trial II (J-ACT II)<sup>4)</sup>の報告では, MCA 閉塞患者の 6 時間後の再開通率は 51.7% であり, 24 時間後の再開通率は 69.0% であった. Kimura ら<sup>10)</sup>は, ICA, MCA 閉塞のある 64 例の患者にアルテプラゼ (0.6mg/kg) 投与後 MRA を用いて再開通の評価を行い, 42 例の MCA 閉塞 (M1, 30 例; M2, 12 例) のうち, 1 時間後の早期再開通は 52.3% (完全再開通 19.0%, 部分再開通 33.3%), 24 時間後では 80.9% (各々 47.6%, 33.3%), ICA 閉塞の 22 例では 1 時間後 31.8% (4.5%, 27.3%), 24 時間後 51.9% (14.3%, 47.6%) に再開通を認め, ICA で再開通率が低いことを示している. J-ACT II, Kimura ら<sup>9)</sup>の報告とわれわれの研究を比較すると, われわれの研究では画像の評価に MRA に加えて DSA も用いている点,

少数ではあるが ACA や PCA の閉塞症例に関して検討している点が異なっている。今回の研究に、ACA や PCA 閉塞患者も含めたのは、CT のみで rt-PA 静注療法を施行する施設も多く、閉塞血管の評価なしに新規超音波血栓溶解装置を使用することも想定したためである。今回の検討では、ACA や PCA 閉塞患者は、24 時間以内に MRA や DSA を施行する症例が少なく、合計で 4 例が検討できたのみであり、4 例とも 24 時間後まで再開通は認めないという結果であった。ACA、PCA 閉塞の検討に関しては、症例数が少なかつたため、更なる症例の蓄積と検討が必要であろう。また、われわれの研究では、rt-PA 投与後 2 回目の再開通評価は、24 時間後には全例では行われておらず、24 時間後に再開通の評価しなかつた症例は、2 時間後の再開通の有無で 24 時間後の再開通の有無を代用している。

われわれの結果のうち M1 と M2 を合わせた MRA での 24 時間後の再開通率 64.1% (25 例/39 例) を、本邦から報告されている J-ACT II<sup>4)</sup> の再開通率である 69.0% (40 例/58 例) や Kimura ら<sup>10)</sup> の再開通率 80.9% (34 例/42 例) と比較すると、24 時間後の再開通率がやや低かつた。これらの国内報告と今回の結果とを合わせると、M1、M2 閉塞患者の 24 時間後の再開通率は 71.2% と計算された。

症候性頭蓋内出血は欧州の市販後調査である SITS-MOST の 1.7%<sup>14)</sup>、本邦の市販後調査である J-MARS 研究の 3.5%<sup>15)</sup> と同様の結果であった。3 カ月後の転帰良好も、SITS-MOST の 38.9%、J-MARS の 33.1%、SAMURAI rt-PA 登録研究の 33.2% と同様であった。

われわれの研究にはいくつかの問題点がある。1 つ目は後ろ向き観察研究であること、2 つ目は MRA や DSA の検査のタイミングにバラツキがあり、症例によって検査の施行時間に差があつたことがあげられる。また、24 時間後の再開通の評価と症候性頭蓋内出血、3 カ月後の転帰の評価において、rt-PA 静注療法後に閉塞血管に対して経皮経管の脳血栓回収用機器を使用した症例は除外した。このため症例選択にバイアスが生じ、rt-PA 静注療法がもたらす再開通率や転帰が過大評価された可能性がある。

## 結論

急性期脳梗塞に対する rt-PA 静注療法時に、MRA、DSA による血管閉塞や再開通など頭蓋内血管の経時的観察を行い、超音波血栓溶解療法を併用しない場合の閉塞血管再開通頻度を評価した。これらのデータを新規超音波血栓溶解装置開発の際の歴史対照として役立てたい。

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## ●文献

- Alexandrov AV, Molina CA, Grotta JC, et al.: Ultrasound-enhanced systemic thrombolysis for acute ischemic stroke. *N Engl J Med* 2004; 351: 2170-2178.
- Eggers J, König IR, Koch B, et al.: Sonothrombolysis with transcranial color-coded sonography and recombinant tissue-type plasminogen activator in acute middle cerebral artery main stem occlusion: Results from a randomized study. *Stroke* 2008; 39: 1470-1475.
- Daffertshofer M, Gass A, Ringler P, et al.: Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: Increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator: Results of a phase ii clinical trial. *Stroke* 2005; 36: 1441-1446.
- Mori E, Minematsu K, Nakagawara J, et al.: Effects of 0.6 mg/kg intravenous alteplase on vascular and clinical outcomes in middle cerebral artery occlusion: Japan alteplase clinical trial II (J-ACT II). *Stroke* 2010; 41: 461-465.
- Tomsick T, Broderick J, Carrozella J, et al.: Revascularization results in the interventional management of stroke II trial. *AJNR Am J Neuroradiol* 2008; 29: 582-587.
- Yoshimura S, Sakai N, Okada Y, et al.: Efficacy of endovascular treatment for acute cerebral large-vessel occlusion: Analysis of nationwide prospective registry. *J stroke Cerebrovasc Dis* 2014; 23: 1183-1190.
- Saqqur M, Uchino K, Demchuk AM, et al.: Site of arterial occlusion identified by transcranial doppler predicts the response to intravenous thrombolysis for stroke. *Stroke* 2007; 38: 948-954.
- Bhatia R, Hill MD, Shobha N, et al.: Low rates of acute recanalization with intravenous recombinant tissue plasminogen activator in ischemic stroke: Real-world experience and a call for action. *Stroke* 2010; 41: 2254-2258.
- Lee KY, Han SW, Kim SH, et al.: Early recanalization after intravenous administration of recombinant tissue plasminogen activator as assessed by pre- and post-thrombolytic angiography in acute ischemic stroke patients. *Stroke* 2007; 38: 192-193.
- Kimura K, Iguchi Y, Shibazaki K, et al.: Early recanalization rate of major occluded brain arteries after intravenous tissue plasminogen activator therapy using serial magnetic resonance angiography studies. *Eur Neurol* 2009; 62: 287-292.
- Kimura K, Iguchi Y, Yamashita S, et al.: Atrial fibrillation as an independent predictor for no early recanalization after IV-t-PA in acute ischemic stroke. *J Neurol Sci* 2008; 267: 57-61.
- 青木淳哉, 井口保之, 小林和人, 他. 経静脈的血栓溶解療法中の経頭蓋超音波検査をもちいた連続モニタリング. *臨神経* 2010; 50: 547-555.
- Suzuki R, Koga M, Mori M, et al.: Visibility of the lesser sphenoid wing is an important indicator for detecting the middle cerebral artery on transcranial color-coded sonography. *Cerebrovasc Dis* 2012; 33: 272-279.
- Wahlgren N, Ahmed N, Davalos A, et al.: Thrombolysis with alteplase for acute ischaemic stroke in the safe implementation of thrombolysis in stroke-monitoring study (SITS-MOST): An observational study. *Lancet* 2007; 369: 275-282.
- Nakagawara J, Minematsu K, Okada Y, et al.: Thrombolysis with 0.6 mg/kg intravenous alteplase for acute ischemic stroke in routine clinical practice: The Japan post-marketing alteplase registration study (J-MARS). *Stroke* 2010; 41: 1984-1989.

## ORIGINAL ARTICLE

# Thrombus-targeted perfluorocarbon-containing liposomal bubbles for enhancement of ultrasonic thrombolysis: *in vitro* and *in vivo* study

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**Summary.** *Background:* External low-frequency ultrasound (USD) in combination with microbubbles has been reported to recanalize thrombotically occluded arteries in animal models. *Objective:* The purpose of this study was to examine the enhancing effect of thrombus-targeted bubble liposomes (BLs) developed for fresh thrombus imaging during ultrasonic thrombolysis. *Methods:* *In vitro:* after the administration of thrombus-targeted BLs or non-targeted BLs, the clot was exposed to low-frequency (27 kHz) USD for 5 min. *In vivo:* Rabbit iliofemoral arteries were thrombotically occluded, and an intravenous injection of either targeted BLs ( $n = 22$ ) or non-targeted BLs ( $n = 22$ ) was delivered. External low-frequency USD (low intensity,  $1.4 \text{ W cm}^{-2}$ , to 12 arteries, and high intensity,  $4.0 \text{ W cm}^{-2}$ , to 10 arteries, for both the targeted BL group and the non-targeted BL group) was applied to the thrombotically occluded arteries for 60 min. In another 10 rabbits, recombinant tissue-type plasminogen activator (rt-PA) was intravenously administered. *Results:* *In vitro:* the weight reduction rate of the clot with targeted BLs was significantly higher than that of the clot with non-targeted BLs. *In vivo:* TIMI grade 3 flow was present in a significantly higher number of rabbits with USD and targeted BLs than rabbits with USD and non-targeted BLs, or with rt-PA monotherapy. High-

intensity USD exposure with targeted BLs achieved arterial recanalization in 90% of arteries, and the time to reperfusion was shorter than with rt-PA treatment (targeted BLs,  $16.7 \pm 5.0$  min; rt-PA,  $41.3 \pm 14.4$  min). *Conclusions:* Thrombus-targeted BLs developed for USD thrombus imaging enhance ultrasonic disruption of thrombus both *in vitro* and *in vivo*.

**Keywords:** drug targeting, liposomes, RGD peptide, thrombolytic therapy, ultrasound.

## Introduction

Most life-threatening cardiovascular events, including acute coronary syndrome and ischemic stroke, are caused by arterial thrombosis. Acute ST-elevation myocardial infarction (STEMI) is characterized by atherosclerotic plaque rupture and occlusive thrombus formation associated with platelet aggregation [1,2]. Percutaneous coronary intervention (PCI) and fibrinolysis are the standard therapeutic strategies for recanalizing thrombotically occluded arteries in patients with STEMI [3]. Primary PCI is performed in most of the STEMI patients presenting to a PCI-capable facility, with a cardiac catheterization laboratory, an interventional cardiologist, and the appropriate specialized staff and equipment to perform acute PCI. Enzymatic fibrinolysis for the treatment of STEMI is less invasive and logistically more convenient; however, this option gives a lower initial recanalization rate, and a higher incidence of coronary reocclusion and life-threatening systemic bleeding, and may result in worse short-term and long-term clinical outcomes than direct PCI [4,5]. For ischemic stroke treatment, fibrinolysis is recommended only for selected patients who can be

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treated within 4.5 h of onset, owing to the substantial risk of intracranial hemorrhage [6–8].

To overcome the limitations of conventional fibrinolytic therapy, the cavitation and non-cavitation effects of ultrasound (USD) have been studied and tested in conjunction with thrombolytic agents to facilitate thrombus disruption [9–16]. Treatment without the use of a thrombolytic agent, but with the combination of echo contrast microbubbles and USD, has been found to be effective *in vitro* and *in vivo*. This has been theorized to be attributable to a lower cavitation threshold and enhanced microstreaming phenomena when microbubbles in conjunction with USD are used [17–23]. *In vivo*, transcutaneous USD in combination with microbubbles has been reported to recanalize thrombotically occluded iliofemoral, coronary and ascending pharyngeal arteries [18,20–22], reduce infarction size [23] and improve microvascular recovery [20] in animal models, without significant side effects. Clinical trials using transcutaneous USD with microbubbles in the setting of ischemic stroke have not been conducted; instead, a combination of USD, microbubbles and thrombolytic agents has been examined. This combination strategy improves recanalization rates and preserves brain function as compared with USD and thrombolytic agents without microbubbles [24–27]. However, an increased number of intracranial hemorrhages has also been reported [27].

In order to non-invasively detect thrombus location, we manufactured thrombus-targeted liposomal bubbles (bubble liposomes [BLs]), which we also expected to enhance ultrasonic clot disruption. These BLs may avoid the need for invasive angiography to identify the thrombotically occluded site prior to the application of therapeutic USD in *in vivo* studies [18,20–22]. The BL was composed of perfluorocarbon gas-containing nanosized liposomes with Arg-Gly-Asp (RGD) sequence peptides on their surface lipid layer, which attach glycoprotein IIb–IIIa complex on activated platelets and enhance the visualization of fresh thrombus by conventional diagnostic ultrasound examination [28]. We hypothesized that these thrombus-targeted BLs could also enhance disruption with the use of therapeutic external USD, and could be used to develop a fully non-invasive diagnostic and therapeutic system for the treatment of thrombotic vessel occlusion. The aim of this study was to examine the enhancing effect of the newly developed thrombus-targeted liposomal bubbles on ultrasonic disruption of the thrombus *in vitro* and *in vivo*.

## Materials and methods

### Preparation of thrombus-targeted BLs

Liposome-based perfluorocarbon-containing BLs were composed of 126 mg of 1,2-distearoyl-*sn*-glycero-phosphocholine (Avanti, Alabaster, AL, USA), 51 mg of 1,2-

distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*m*-polyethylene glycol 2000 with maleimide (Avanti), 30 mg of cholesterol (Sigma-Aldrich, Tokyo, Japan), CGGGRGDF peptide (Operon Biotechnologies, Tokyo, Japan), and perfluoropropane gas (Takachiho, Tokyo, Japan). We previously reported the manufacture of these liposome-based perfluorocarbon-containing BLs [28]. In brief, a mixture of all reagents except for CGGGRGDF peptide and perfluoropropane gas was dissolved in 2.0 mL of chloroform, and mixed with the same amount of di-isopropyl ether and normal saline. The mixture was sonicated, with a probe-type 19.5-kHz ultrasound device at 550 W (XL-2020 Sonicator; Misonix, Farmingdale, NY, USA), and then evaporated at 65 °C with a rotary evaporating system (Tokyo Rika, Tokyo, Japan). After the chemical solvent had been completely removed, the size of liposomes was adjusted to < 0.2 µm with extruding equipment and a membrane filter (Northern Lipids, Vancouver, Canada) with sizing filters. To the liposome liquid, 1 mg of linear octapeptide with the sequence CGGGRGDF (Operon Biotechnologies) was added, and allowed to conjugate to the maleimide on the liposomal surface via thio-ether covalent coupling at room temperature for 2 h. Gel filtration was then used to remove unreacted peptide fragments. The lipid concentration was measured with the Wako Phospholipid C test (Wako Pure Chemical Industries, Osaka, Japan) and the RGD-liposomes were diluted to a final concentration of 20 mg mL<sup>-1</sup>. The RGD-liposomes were sealed in a 5-mL vial, and air was exchanged with perfluoropropane gas (Takachiho); this was followed by 20-kHz USD treatment with a bath-type sonicating system (Model 3510; Branson, Emerson, CT, USA) for 5 min to generate the RGD-BLs [28]. Sterile filtration (0.45 µm) was then performed to remove the expanded and oversized BLs. Non-targeted BLs were prepared with the same methods but without the addition of RGD peptide. The amount of perfluoropropane gas trapped in the BLs was estimated to be ~ 10 µL mg<sup>-1</sup> lipid, and the diameter of each BL was 180 ± 44 nm as measured by dynamic laser light-scattering measurements with an ELS-800 particle analyzer (Otsuka Photonics, Tokyo, Japan).

### Therapeutic USD system

For both *in vitro* and *in vivo* studies, two different USD systems (low intensity or high intensity) were used. For the low-intensity USD study, the Timi3 system was used (Timi3 Systems, Santa Clara, CA, USA). This device consisted of a low-frequency USD generator (maximum intensity, 1.4 W cm<sup>-2</sup>) and a transducer that delivered 27 kHz of USD at a pulse rate of 25 Hz (acoustic pressure, 0.145 MPa; mechanical index, 1.4). For the high-intensity study, the therapeutic USD system was composed of a sine wave pulse generator (MG-422A; Anritsu, Tokyo, Japan), a radiofrequency power amplifier



(2100L; ENI, Rochester, NY, USA), and a prototype piezoelectric transducer (Fuji Ceramics, Shizuoka, Japan). The transducer consisted of 10 PZT disks (thickness, 4 mm) tightly bonded together. It was operated in a continuous-wave mode at a frequency of 27 kHz (acoustic pressure, 0.346 MPa; mechanical index, 3.2) and an intensity of  $4.0 \text{ W cm}^{-2}$  as measured by the calorimetric method [29].

#### Protocol for *in vitro* study on human thrombi

The following *in vitro* investigation conforms with the principles outlined in the Declaration of Helsinki, and the protocol was approved by the Ethical Committee of the National Defense Medical College. In total, 60 thrombi were used in this *in vitro* study. For the preparation of each thrombus, 9 mL of whole blood was collected in a test tube from a healthy volunteer, placed on a seesaw-type shaker, and allowed to coagulate at room temperature while being shaken and rotated at a speed of 60 r.p.m. for 1 h. Targeted BLs or non-targeted BLs ( $100 \mu\text{L}$ ,  $20 \text{ mg mL}^{-1}$  lipid concentration,  $\sim 1.1\%$  v/v) were added to the test tube 10 min after the initiation of coagulation. The formed thrombus was washed with normal saline, cut into small pieces, weighed on an electronic balance, and placed in a plastic test tube containing 2 mL of human plasma. Before the therapeutic USD exposure, attachment of the BLs on the clot was confirmed by conventional USD imaging, as well as by scanning electron microscopy [28]. The test tube was placed at 1 cm from the therapeutic USD transducer in a bath filled with degassed water. Thirty thrombi were exposed to low-intensity USD: 10 without BLs as controls, 10 with non-targeted BLs, and 10 with targeted BLs. Similarly, 30 thrombi were exposed to high-intensity USD: 10 without BLs as controls, 10 with non-targeted BLs, and 10 with targeted BLs. The water temperature was maintained at  $37^\circ\text{C}$ . Each thrombus was exposed to low-intensity USD ( $27 \text{ kHz}$ ,  $1.4 \text{ W cm}^{-2}$ ) or high-intensity USD ( $27 \text{ kHz}$ ,  $4.0 \text{ W cm}^{-2}$ ) for 5 min. After USD exposure, the clot was weighed again. The thrombus weight reduction rate ( $[\text{pre-treatment weight} - \text{post-treatment weight}]/\text{pre-treatment weight} \times 100 [\%])$  was calculated as an index of the thrombolytic effect.

#### *In vivo* study protocol in an acute thrombotic occlusion model of a rabbit iliofemoral artery

The animal protocol was approved by the Animal Care and Use Committee of the National Defense Medical College, and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

A total of 54 New Zealand white rabbits ( $\sim 2.4 \text{ kg}$ ) were used: 24 for the low-intensity USD study, 20 for the

high-intensity USD study, and 10 for the fibrinolysis study. Each rabbit was anesthetized with 50 mg of ketamine and 20 mg of xylazine injected intramuscularly, and anesthesia was maintained with pentobarbital ( $15 \text{ mg kg}^{-1}$ ) delivered via a marginal ear vein. The adequacy of anesthesia was monitored by the loss of the ear pinch reflex. Anesthetized rabbits were placed on a warming plate to maintain the body temperature at  $37^\circ\text{C}$ . Aseptic techniques were used for all surgical procedures. A 5Fr sheath was inserted into the right carotid artery, a balloon catheter was advanced to the right iliofemoral artery, and the intima was injured by balloon inflation and scratching. The balloon catheter was then pulled back, a 0.014-inch guide wire was positioned at the injured site, and electrical stimulation (3-V battery) was applied between the guide wire and skin electrode [18,21,28,30]. Thirty minutes later, the right iliofemoral artery was thrombotically occluded, and the arterial occlusion was confirmed by angiography. The thrombus was also imaged with a 9-MHz linear transducer and a conventional USD machine 1 min after BL injection (UF-750XT; Fukuda Denshi, Tokyo, Japan) (frame rate, 24–30/s; mechanical index, 0.3) (Fig. 1).

To determine the thrombolytic effect of targeted BLs and USD, we applied the low-intensity or high-intensity USD transcutaneously over the site of the rabbit iliofemoral arterial thrombus in combination with an intravenous injection of non-targeted BLs or targeted BLs. A total of 44 New Zealand white rabbits with iliofemoral

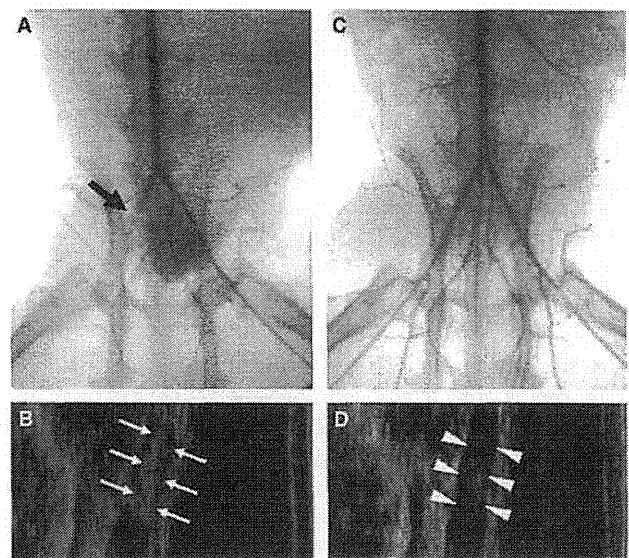


Fig. 1. (A) Angiographic image of thrombotically occluded rabbit iliac artery (black arrow) after balloon inflation and electrical (3-V battery) stimulation. (B) In sonographic images, the targeted bubble liposomes (BLs) accumulated on the thrombus (white arrows). (C, D) After combination therapy with low-frequency ultrasound (USD) and thrombus-targeted BLs, TIMI grade 3 flow was achieved (C), and the high echogenic area within the iliac artery almost disappeared (white arrowheads) (D).

arterial occlusions were divided into two groups: 24 for low-intensity USD, and 20 for high-intensity USD. In both groups, non-targeted BLs were intravenously administered in half (12 or 10, respectively) of the rabbits, and targeted BLs were used in the other half (12 or 10, respectively) of the rabbits.

Before the USD exposure, 200 IU kg<sup>-1</sup> heparin was injected intravenously. Angiography was performed every 15 min for a total of 90 min, and the TIMI blood flow grade was assessed [18,31]. Non-targeted or targeted BLs (1 mL, ~1.1% v/v) were intravenously injected every 15 min after the angiography. Subsequently, transcutaneous USD was applied externally over the site of the thrombus for 12 min. In the targeted BL and USD group, thrombus location was also confirmed with a diagnostic USD machine (UF-750XT; Fukuda Denshi) and with angiography (Fig. 1). BL injection and subsequent USD exposure were repeated four times for a total of 60 min. The vessel was observed for acute thrombotic re-occlusion for the next 30 min. The recanalization (TIMI grade 3 flow) rate was calculated, and the reperfusion time was measured only in cases in which reperfusion was achieved. All of the angiographic assessments represented the consensus of two expert cardiologists (T.N. and B.T.) blinded to group allocation.

In 10 New Zealand white rabbits with iliofemoral arterial occlusions, 27 500 IU kg<sup>-1</sup> recombinant tissue plasminogen activator (rt-PA) (Monteplase, Eisai, Tokyo, Japan) was intravenously administered within a period of 1 min immediately after intravenous injection of 200 IU kg<sup>-1</sup> heparin. Angiography was repeated every 15 min for a total of 90 min, and the TIMI blood flow grade was assessed [18,31]. At the end of the experiment, all rabbits were killed with an overdose of pentobarbital (75 mg kg<sup>-1</sup>) injected via a marginal ear vein.

#### Statistical analysis

Results are given as mean value  $\pm$  1 standard deviation. In the *in vitro* study, clot weight reduction rates were compared by use of ANOVA. *In vivo*, the reperfusion rates were compared by use of a 2  $\times$  2 or a 2  $\times$  3 chi-square test, and the reperfusion times among the three groups were compared by use of the Kruskal-Wallis test. A *P*-value of < 0.05 was considered to be statistically significant.

## Results

#### *In vitro* study

The clot weight reduction rate achieved with low-intensity USD with targeted BLs was significantly higher than that with the non-targeted BLs and in the controls without BLs (25%  $\pm$  11% vs. 14%  $\pm$  9% and 9%  $\pm$  3%, respectively; *P* < 0.01). Non-targeted BLs gave no significant

enhancement of clot weight reduction (Fig. 2). Similarly, the clot weight reduction rate achieved with high-intensity USD in combination with targeted BLs was significantly higher than that with non-targeted BLs and in the controls (65%  $\pm$  21% vs. 21%  $\pm$  9% and 21%  $\pm$  14%, respectively; *P* < 0.01). No significant enhancement of clot weight reduction was observed when non-targeted-BLs were used.

On comparison of the effects of low-intensity USD and high-intensity USD, the clot weight reduction rate was significantly higher in the high-intensity USD group than in the low-intensity USD group when USD was applied without BLs (21%  $\pm$  9% vs. 9%  $\pm$  3%) and with targeted BLs (65%  $\pm$  21% vs. 25%  $\pm$  11%).

In both groups, echo signal enhancement of all thrombi by targeted BLs declined to control and non-targeted BL levels after USD exposure.

#### *In vivo* low-intensity USD study

Rabbit iliofemoral arterial thrombus was clearly recognized with a conventional USD system with the targeted BLs, and also confirmed by angiography (Fig. 1). The Doppler signal enhancement of the iliofemoral arterial flow by both targeted and non-targeted BLs was observed even at 12 min after therapeutic USD irradiation. TIMI grade 3 flow was achieved in eight of 12 rabbits (67%) with targeted BL and USD exposure; however, TIMI grade 3 flow was achieved in only one of 12 rabbits (8%) in the non-targeted BL and USD group (Table 1; Fig. 3). These differences were statistically significant (targeted BLs, 67%; non-targeted BLs, 8%; *P* = 0.003). The time to reperfusion was ~ 40 min in the group with targeted

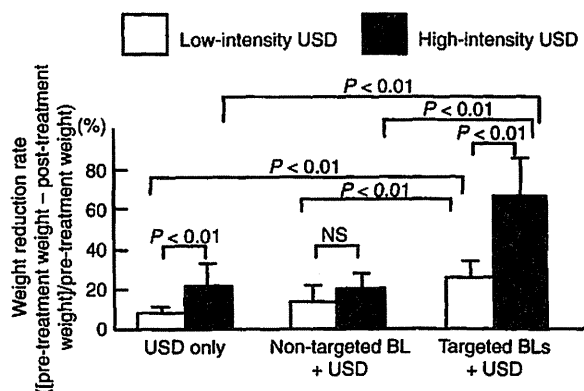


Fig. 2. The *in vitro* thrombus weight reduction rate ((pre-treatment weight - post-treatment weight)/pre-treatment weight  $\times$  100 [%]) obtained with low-frequency ultrasound (USD): high-intensity USD exposure for 5 min with the targeted bubble liposomes (BLs), with the non-targeted BLs, or without BLs (*P* < 0.01, *n* = 10, ANOVA); low-intensity USD exposure for 10 min with the targeted BLs, with the non-targeted BLs, or without BLs (*P* < 0.01, *n* = 10, ANOVA). The clot weight reduction rate increased with therapeutic USD with targeted BLs in each intensity study. NS, not significant.

BL and USD exposure (Table 1). There were three cases of blood flow reduction (TIMI grade 3 to 2) during the observation period in the targeted BL group (Fig. 3).

#### *In vivo high-intensity USD study and fibrinolysis study*

After exposure to low-frequency high-intensity transcutaneous USD, TIMI grade 3 flow was achieved in nine of 10 rabbits (90%) with targeted BLs. On the other hand, recanalization was achieved in only two of 10 rabbits (20%) with USD and non-targeted BLs, and four of 10 arteries (40%) were recanalized with rt-PA monotherapy (Table 2; Fig. 4). These differences were statistically significant (targeted BLs, 90%; non-targeted BLs, 20%; rt-PA, 40%;  $P = 0.004$ ). Moreover, the average reperfusion time for rabbits to achieve TIMI grade 3 flow was significantly shorter for those cases with high-intensity USD thrombolysis with targeted BLs than for those with high-intensity USD with non-targeted BLs or for those with

**Table 1** Frequency of TIMI grade 3 flow (A) and time to each TIMI grade 3 flow (B) achieved with a combination of transcutaneous low-intensity ultrasound (USD) and targeted bubble liposomes (BLs) or non-targeted BLs

	Targeted BLs + USD	Non-targeted BLs + USD	<i>P</i> -value
(A)			
Frequency, no. (%)	8/12 (67)	1/12 (8)	0.003
(B)			
Mean time (min)	43.1 ± 20.3	60.0 ± 0	NA

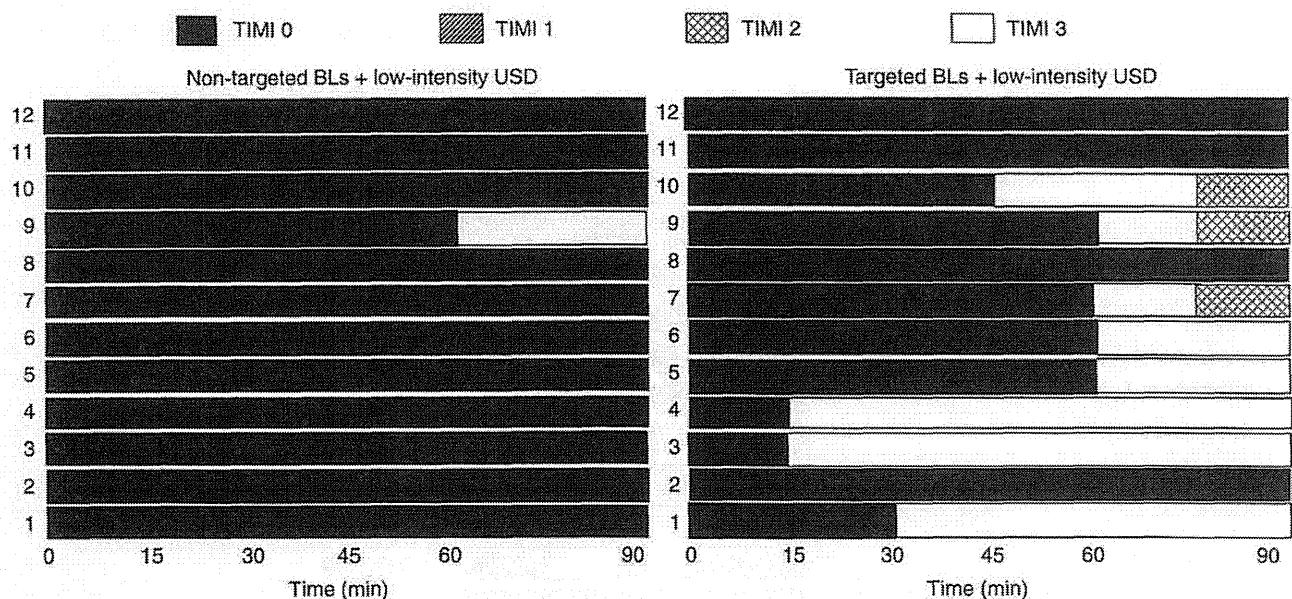
NA, not applicable.

ordinary thrombolysis with rt-PA (targeted BLs,  $16.7 \pm 5.0$  min; non-targeted BLs,  $60.0 \pm 0$  min; rt-PA,  $41.3 \pm 14.4$  min;  $P = 0.007$ ;  $n = 10$ ) (Table 2). There were no cases of acute reocclusion, and all recanalized arteries maintained TIMI grade 3 flow, whereas two cases of acute reocclusion occurred during the procedure and the observation period in the non-targeted BL group (Fig. 4).

#### Discussion

This study demonstrated significant enhancement of ultrasonic thrombus disruption by the thrombus-targeted BLs and external low-frequency USD system *in vitro* and *in vivo*, and the possibility of a completely non-invasive treatment that combines the identification of thrombus position with rapid clot disruption. This study validates the use of the low-frequency USD system with targeted BLs for rapid, effective and comprehensive thrombolytic treatment. The combination of USD and targeted BLs played a primary role in this study in both the diagnosis and treatment of thrombotic vascular occlusions.

In many of the previous studies dealing with *in vivo* USD clot disruption and using microbubbles to reinforce the USD energy, invasive angiography was used to identify the precise location of the thrombus, and to guide the manipulation of the therapeutic USD probe [9,10,18,21]. Otherwise, transcranial Doppler was used to monitor deteriorated blood flow, or a large transducer was used to cover the area of vessel occlusion [25–27,32]. Recently, we developed thrombus-targeted liposomal bubbles for USD imaging of fresh thrombus. This *in vitro* and *in vivo*



**Fig. 3.** Schematic presentation showing TIMI flow grades of the arteries treated with low-intensity ultrasound (USD) with non-targeted bubble liposomes (BLs) and targeted BLs. TIMI grade 3 flow was achieved in 67% of arteries with targeted BLs and in only 8% of arteries with non-targeted BLs ( $P = 0.003$ ,  $2 \times 2$  chi-square test).

study showed that the USD image of thrombus could be identified at a glance with a conventional diagnostic USD machine with the targeted BLs, as also found in our previous study [28]. This enhanced thrombus imaging facilitated successful USD thrombolysis without invasive angiography.

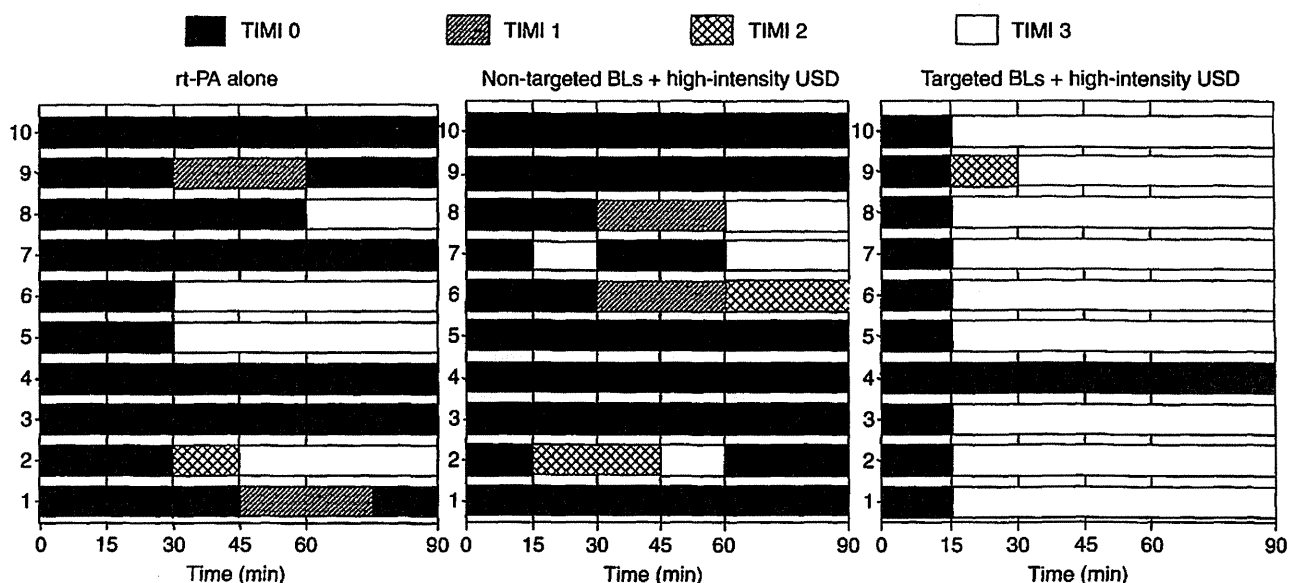
The targeted BLs significantly enhanced ultrasonic clot disruption *in vitro* and *in vivo* when used with both low-intensity and high-intensity USD. In particular, with high-intensity USD exposure with targeted BLs *in vivo*, arterial recanalization was achieved in 90% of acute thrombotically occluded rabbit iliofemoral arteries within 20 min from the beginning of the diagnostic procedure. This speed of treatment potentially surpasses that of the PCI procedures, which, on average, require at least 90 min to achieve reperfusion from arrival at the hospital

**Table 2** Frequency of TIMI grade 3 flow (A) and time to each TIMI grade 3 flow (B) achieved with a combination of transcutaneous high-intensity ultrasound (USD) and targeted bubble liposomes (BLs) or non-targeted BLs, or recombinant tissue-type plasminogen activator (rt-PA) alone

	Targeted BLs + USD	Non-targeted BLs + USD	rt-PA	P-value
(A)				
Frequency, no. (%)	9/10 (90)	2/10 (20)	4/10 (40)	0.004
(B)				
Mean time (min)	16.7 ± 5.0	60.0 ± 0	41.3 ± 14.4	0.007

[3]. This rapid and non-invasive therapy shows promise in acute cardiovascular medicine, as diagnostic and therapeutic USD equipment is compact in size, inexpensive, and does not require dedicated laboratory space and specialized PCI staff.

These results were equivalent to those of the sonothrombolysis study by Culp *et al.*, in which a combination of 2-MHz USD and eptifibatide-tagged microbubbles opened acute intracranial thrombotic occlusions in six of eight pigs without the use of a thrombolytic agent [22]. Recently, Alonso *et al.* reported that diagnostic 2-MHz USD in combination with abciximab immunobubbles induced thrombolysis (increased plasma D-dimer levels) without lytic agents in rats [33]. However, the arterial recanalization was not assessed, as a partial thrombotic occlusion model of the rat carotid artery was used. Xie *et al.* also reported that diagnostic USD (1.5 MHz) treatment with platelet-targeted microbubbles in combination with half-dose recombinant prourokinase gave a 53% coronary arterial recanalization rate at 30 min in pigs [20]. These studies demonstrate that clinically used diagnostic USD frequencies can be applied to thrombus dissolution with thrombus-targeted microbubbles. However, their thrombolytic effect was relatively limited, except for the intracranial model [22], presumably because the cavitation energy generated by high-frequency USD (MHz) and microbubbles was relatively low in the absence of a closed space such as a skull, where USD energy could be enhanced by standing wave formation [34]. To overcome this limitation, we used low-frequency (kHz) USD as a therapeutic device to achieve a higher recanalization rate.



**Fig. 4.** Schematic presentation showing TIMI flow grades of the arteries treated with recombinant tissue-type plasminogen activator (rt-PA) alone, with high-intensity ultrasound (USD) with non-targeted bubble liposomes (BLs), and with high-intensity USD with targeted BLs. TIMI grade 3 flow was achieved in 90% of arteries treated with USD with targeted BLs, in only 20% of arteries treated with USD with non-targeted BLs, and in 40% of arteries treated with rt-PA monotherapy ( $P = 0.02$ ,  $2 \times 3$  chi-square test).

Low-frequency USD is advantageous, because it penetrates deeper into tissue and has less thermal effect than high-frequency USD [35]. Low-frequency USD has been reported to recanalize canine iliofemoral and coronary arteries without tissue damage [9,10,21,36], and has been safely applied in combination with thrombolytic agent in humans with STEMI [13], with a USD intensity as low as that used in our low-intensity USD study. However, there are still safety concerns regarding the application of high-intensity, low-frequency USD with microbubbles, and these should be clarified in a future study.

Nano-sized BLs could have some potential disadvantages. It is assumed that BLs release less energy than larger microbubbles when they collapse. In fact, the non-targeted BLs showed negligible enhancement of USD clot disruption *in vitro*. Moreover, only 8–20% cases of thrombosed rabbit iliofemoral arteries recanalized *in vivo* with non-targeted BLs. Theoretically, each BL itself is too small to reflect USD waves with the frequency used in this study. However, we previously demonstrated that the targeted BLs are highly concentrated around and within the thrombus, by using scanning electron microscopy, and that they markedly enhance ultrasonic thrombus imaging [28]. Consequently, as shown in this *in vitro* and *in vivo* study, we found marked enhancement of the thrombolytic effect by attaching the thrombus-targeting ligands on the same BL structure. The small size of the BLs, with a mean diameter of 180 nm, could also have some advantageous effects. A BL size of <1  $\mu\text{m}$  ensures both a long *in vivo* circulation time and deep penetration into thrombi. The longer the circulation time, the more opportunity the targeted BL has to attach to the activated thrombus. Deep penetration into thrombi through the fibrin network allows for greater accumulation of targeted BLs within thrombi. These two features of small BLs were favorable, when USD energy was applied, for disruption and reduction of the culprit thrombus.

Liposomes are usually considered to be non-toxic, unless they are administered at very high doses [37]. Polyethylene glycol is also considered to be non-toxic, and is excreted unmetabolized in the urine [38]. The RGD peptide is an octapeptide, and is considered to be non-toxic and non-immunogenic [39,40]. Perfluoropropane is an inert gas, used as a constituent of commercially available echo contrast agents such as Optison and Definity [41], and is exhaled from the lungs [42]. Therefore, this echo contrast agent is generally considered to be non-toxic, although safety in humans remains to be demonstrated.

In a clinical setting, lower-intensity USD has some advantages over high-intensity USD in terms of safety. However, low-intensity USD exposure with targeted BLs achieved only a modest thrombolytic effect in this study. When low-intensity USD is used, an alternative approach is necessary to enhance the resonance phenomenon caused by the interaction between USD and the BLs. As the RGD peptide is not an ideal targeting ligand, because

of its broad cross-reactivity with a number of integrins, one possible option is to achieve a higher concentration of the BLs on the thrombus by using a more effective targeting ligand. The other option is to use larger BLs, which generate a higher amount of cavitation energy during collapse. However, the BLs should be small enough to penetrate into the thrombus. Therefore, the most effective size of BLs remains to be determined. Another option is to combine targeted BL-enhanced USD thrombolysis with conventional fibrinolytic therapy. The dose of the fibrinolytic agent, such as tissue-type plasminogen activator, could be reduced with this strategy. Further study is needed to elucidate this issue.

The targeted BLs enhance imaging of the culprit thrombus and enable manipulation of the therapeutic USD probe, targeting and directing it towards the culprit thrombus. Furthermore, the combination treatment with targeted BLs and high-intensity, low-frequency USD achieved a 90% recanalization rate, which is markedly higher than that with rt-PA monotherapy. This method has the potential to be a reperfusion strategy that could be more rapid than any other method, including PCI. The absence of acute reocclusion with this therapeutic approach might be attributable to minimal mural thrombus being left in the culprit lesion. Further study is needed to identify the most suitable targeting ligand and BL size for generating the maximum thrombus disruption and achieving the most effective thrombolysis with low-intensity USD.

#### Limitations

There are some technical limitations regarding thrombus formation in both the *in vitro* study and the *in vivo* study. We prepared all *in vitro* clots from the blood of a single individual, to examine the effects of USD and targeted BLs on clots with homogeneous lytic activities. However, this could simultaneously be a limitation of this study, because individual lytic response can differ as a function of the different levels of inhibitory enzymes and/or varying concentrations of plasminogen. Moreover, *in vivo* hyperacute thrombi could be more fragile than those in clinical culprit lesions.

Reocclusion of the culprit artery was not observed after successful recanalization with low-frequency USD and targeted BLs. However, the observation period after recanalization in this study might not be long enough to exclude the possibility of reocclusion, which may occur later. There are some safety limitations. It is known from the simulation study of intracranial sonothrombolysis [34] that USD sometimes causes standing wave formation and unnecessary acoustic cavitation, especially in brain tissue, even outside the targeted clot. Regarding the coronary and peripheral arteries, low-frequency, high-intensity USD energy can be delivered transcutaneously for clot disruption without concomitant tissue damage in animal models,

especially when coupled with the use of a cooling system to prevent thermal injury [9,10,21,36]. However, low-frequency USD could cause unexpected non-linear cavitation effects, and its safety has to be clarified in combination with microbubbles before clinical application.

## Conclusions

Perfluorocarbon gas-containing liposomal nanobubbles with activated thrombus-targeting RGD peptides developed for USD thrombus imaging are novel echo contrast agents that can markedly enhance USD thrombolysis both *in vitro* and *in vivo*. The combination of USD and thrombus-targeted BLs could be used as an effective and completely non-invasive recanalization therapy that does not require angiography to detect acute thrombotic vessel occlusion or therapeutic thrombus dissolution.

## Addendum

K. Hagsiwa, T. Nishioka, and R. J. Siegel: conception and design; K. Hagsiwa, T. Nishioka, R. Suzuki, B. Takase, K. Iida, and H. Luo: acquisition of data; K. Hagsiwa, T. Nishioka, and A. Kurita: analysis and interpretation of data; K. Hagsiwa, T. Nishioka, and R. J. Siegel: drafting of the manuscript; K. Maruyama, M. Ishihara, N. Yoshimoto, and Y. Nishida: supervision.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

## References

- Mizuno K, Miyamoto A, Satomura K, Kurita A, Arai T, Sakurada M, Yanagida S, Nakamura H. Angioscopic coronary macro-morphology in patients with acute coronary disorders. *Lancet* 1991; 337: 809–12.
- Fuster V, Fayad ZA, Badimon JJ. Acute coronary syndromes: biology. *Lancet*. 1999; 353: S115–9.
- Antman EM, Anbe DT, Armstrong PW, Bates ER, Green LA, Hand M, Hochman JS, Krumholz HM, Kushner FG, Lamas GA, Mullany CJ, Ornato JP, Pearle DL, Sloan MA, Smith SC Jr, Alpert JS, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, et al. ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction – executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 1999 Guidelines for the Management of Patients With Acute Myocardial Infarction). *Circulation* 2004; 110: 588–636.
- Stone GW, Grines CL, Rothbaum D, Browne KF, O’Keefe J, Overlie PA, Donohue BC, Chelliah N, Vlietstra R, Catlin T, O’Neill WW. Analysis of the relative costs and effectiveness of primary angioplasty versus tissue-type plasminogen activator: the Primary Angioplasty in Myocardial Infarction (PAMI) trial. The PAMI Trial Investigators. *J Am Coll Cardiol* 1997; 29: 901–7.
- Schomig A, Kastrati A, Dirschinger J, Mehilli J, Schricke U, Pache J, Martinoff S, Neumann FJ, Schwaiger M. Coronary stenting plus platelet glycoprotein IIb/IIIa blockade compared with tissue plasminogen activator in acute myocardial infarction. Stent versus Thrombolysis for Occluded Coronary Arteries in Patients with Acute Myocardial Infarction Study Investigators. *N Engl J Med* 2000; 343: 385–91.
- Sandercock P, Wardlaw JM, Lindley RI, Dennis M, Cohen G, Murray G, Innes K, Venables G, Czlonkowska A, Kobayashi A, Ricci S, Murray V, Berge E, Slot KB, Hankey GJ, Correia M, Peeters A, Matz K, Lyrer P, Gubitz G, et al. The benefits and harms of intravenous thrombolysis with recombinant tissue plasminogen activator within 6 h of acute ischaemic stroke (the Third International Stroke Trial [IST-3]): a randomised controlled trial [published correction appears in *Lancet*. 2012; 380: 730]. *Lancet* 2012; 379: 2352–63.
- Jauch EC, Saver JL, Adams HP Jr, Bruno A, Connors JJ, Dem-aerschalk BM, Khatri P, McMullan PW Jr, Qureshi AI, Rosenfield K, Scott PA, Summers DR, Wang DZ, Wintermark M, Yonas H; American Heart Association Stroke Council; Council on Cardiovascular Nursing; Council on Peripheral Vascular Disease; Council on Clinical Cardiology. Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* 2013; 44: 870–947.
- Wahlgren N, Ahmed N, Dávalos A, Hacke W, Millán M, Muir K, Roine RO, Toni D, Lees KR; SITS investigators. Thrombolysis with alteplase 3–4.5 h after acute ischaemic stroke (SITS-ISTR): an observational study. *Lancet* 2008; 372: 1303–9.
- Luo H, Birnbaum Y, Fishbein MC, Peterson TM, Nagai T, Nishioka T, Siegel RJ. Enhancement of thrombolysis *in vivo* without skin and soft tissue damage by transcutaneous ultrasound. *Thromb Res* 1998; 89: 171–7.
- Siegel RJ, Atar S, Fishbein MC, Brasch AV, Peterson TM, Nagai T, Pal D, Nishioka T, Chae JS, Birnbaum Y, Zanelli C, Luo H. Noninvasive, transthoracic, low-frequency ultrasound augments thrombolysis in a canine model of acute myocardial infarction. *Circulation* 2000; 101: 2026–9.
- Suchkova V, Carstensen EL, Francis CW. Ultrasound enhancement of fibrinolysis at frequencies of 27 to 100 kHz. *Ultrasound Med Biol* 2002; 28: 377–82.
- Laing ST, Moody M, Smulevitz B, Kim H, Kee P, Huang S, Holland CK, McPherson DD. Ultrasound-enhanced thrombolytic effect of tissue plasminogen activator-loaded echogenic liposomes in an *in vivo* rabbit aorta thrombus model – brief report. *Arterioscler Thromb Vasc Biol* 2011; 31: 1357–9.
- Hudson M, Greenbaum A, Brenton L, Gibson CM, Siegel RJ, Reeves LR, Sala MF, McKendall G, Bluguermann J, Echt D, Ohman EM, Weaver WD. Adjunctive transcutaneous ultrasound with thrombolysis: results of the PLUS (Perfusion by Thrombolytic and UltraSound) trial. *JACC Cardiovasc Interv* 2010; 3: 352–9.
- Nedelmann M, Eicke BM, Lierke EG, Heimann A, Kempfski O, Hopf HC. Low-frequency ultrasound induces nonenzymatic thrombolysis *in vitro*. *J Ultrasound Med* 2002; 21: 649–56.
- Alexandrov AV, Molina CA, Grotta JC, Garami Z, Ford SR, Alvarez-Sabin J, Montaner J, Saqqur M, Demchuk AM, Moyé LA, Hill MD, Wojner AW; CLOTBUST Investigators. Ultrasound enhanced systemic thrombolysis for acute ischemic stroke. *N Engl J Med* 2004; 351: 2170–8.

- 16 Tsvigoulis G, Eggers J, Ribo M, Perren F, Saqqur M, Rubiera M, Sergentanis TN, Vadikolias K, Larrue V, Molina CA, Alexandrov AV. Safety and efficacy of ultrasound-enhanced thrombolysis: a comprehensive review and meta-analysis of randomized and nonrandomized studies. *Stroke* 2010; 41: 280–7.
- 17 Porter TR, Le Veen RF, Fox R, Kricsfeld A, Xie F. Thrombolytic enhancement with perfluorocarbon-exposed sonicated dextrose albumin microbubbles. *Am Heart J* 1996; 132: 964–8.
- 18 Nishioka T, Luo H, Fishbein MC, Cercek B, Forrester JS, Kim CJ, Berglund H, Siegel RJ. Dissolution of thrombotic arterial occlusion by high intensity, low frequency ultrasound and dodecafluoropentane emulsion: an in vitro and in vivo study. *J Am Coll Cardiol* 1997; 30: 561–8.
- 19 Wu Y, Unger EC, McCreery TP, Sweitzer RH, Shen D, Wu G, Vielhauer MD. Binding and lysing of blood clots using MRX-408. *Invest Radiol* 1998; 33: 880–5.
- 20 Xie F, Lof J, Matsunaga T, Zutshi R, Porter TR. Diagnostic ultrasound combined with glycoprotein IIb/IIIa-targeted microbubbles improves microvascular recovery after acute coronary thrombotic occlusions. *Circulation* 2009; 119: 1378–85.
- 21 Birnbaum Y, Luo H, Nagai T, Fishbein MC, Peterson TM, Li S, Kricsfeld D, Porter TR, Siegel RJ. Noninvasive in vivo clot dissolution without a thrombolytic drug: recanalization of thrombosed iliofemoral arteries by transcutaneous ultrasound combined with intravenous infusion of microbubbles. *Circulation* 1998; 97: 130–4.
- 22 Culp WC, Porter TR, Lowery J, Xie F, Roberson PK, Marky L. Intracranial clot lysis with intravenous microbubbles and transcranial ultrasound in swine. *Stroke* 2004; 35: 2407–11.
- 23 Culp WC, Flores R, Brown AT, Lowery JD, Roberson PK, Hennings LJ, Woods SD, Hatton JH, Culp BC, Skinner RD, Borrelli MJ. Successful microbubble sonothrombolysis without tissue-type plasminogen activator in a rabbit model of acute ischemic stroke. *Stroke* 2011; 42: 2280–5.
- 24 Porter TR, Xie F. Can transcranial ultrasound and microbubble therapy ever enter the mainstream in acute stroke therapy? *Expert Rev Cardiovasc Ther* 2012; 10: 549–51.
- 25 Alexandrov AV, Mikulik R, Ribo M, Sharma VK, Lao AY, Tsvigoulis G, Sugg RM, Barreto A, Sierzenski P, Malkoff MD, Grotta JC. A pilot randomized clinical safety study of sonothrombolysis augmentation with ultrasound-activated perflutren-lipid microspheres for acute ischemic stroke. *Stroke* 2008; 39: 1464–9.
- 26 Molina CA, Ribo M, Rubiera M, Montaner J, Santamarina E, Delgado-Mederos R, Arenillas JF, Huertas R, Purroy F, Delgado P, Alvarez-Sabín J. 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.
- 27 Molina CA, Barreto AD, Tsvigoulis G, Sierzenski P, Malkoff MD, Rubiera M, Gonzales N, Mikulik R, Pate G, Ostrem J, Singleton W, Manvelian G, Unger EC, Grotta JC, Schellinger PD, Alexandrov AV. Transcranial ultrasound in clinical sonothrombolysis (TUCSON) trial. *Ann Neurol* 2009; 66: 28–38.
- 28 Hagiwara K, Nishioka T, Suzuki R, Takizawa T, Maruyama K, Takase B, Ishihara M, Kurita A, Yoshimoto N, Ohsuzu F, Kikuchi M. Enhancement of ultrasonic thrombus imaging using bubble liposomes containing perfluorocarbon targeted to activated platelet glycoprotein IIb/IIIa complex in a rabbit iliac artery. *Int J Cardiol* 2011; 152: 202–6.
- 29 *Instruction Manual of AC Calorimetric Thermal Constant Measuring System*. Yokohama, Japan: Sinku-Riko Inc., 1992.
- 30 Salazar AE. Experimental myocardial infarction. Induction of coronary thrombosis in the intact closed-chest dog. *Circ Res* 1961; 9: 1351–6.
- 31 TIMI Study Group. The Thrombolysis in Myocardial Infarction (TIMI) trial: Phase I findings. *N Engl J Med* 1985; 312: 932–6.
- 32 Daffertshofer M, Gass A, Ringleb P, Sitzler M, Sliwka U, Els T, Sedlacek O, Koroshetz WJ, Hennerici MG. Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator: results of a phase II clinical trial. *Stroke* 2005; 36: 1441–6.
- 33 Alonso A, Dempfle CE, Della Martina A, Stroick M, Fatar M, Zohsel K, Allémann E, Hennerici MG, Meairs S. In vivo clot lysis of human thrombus with intravenous abciximab immunobubbles and ultrasound. *Thromb Res* 2009; 124: 70–4.
- 34 Baron C, Aubry JF, Tanter M, Meairs S, Fink M. Simulation of intracranial acoustic fields in clinical trials of sonothrombolysis. *Ultrasound Med Biol* 2009; 35: 1148–58.
- 35 Ahmadi F, McLoughlin IV, Chauhan S, ter-Haar G. Bio-effects and safety of low-intensity, low-frequency ultrasonic exposure. *Prog Biophys Mol Biol* 2012; 108: 119–38.
- 36 Luo H, Fishbein MC, Bar-Cohen Y, Nishioka T, Berglund H, Kim CJ, Nagai T, Birnbaum Y, Siegel RJ. Cooling system permits effective transcutaneous ultrasound clot lysis in vivo without skin damage. *J Thromb Thrombolysis* 1998; 6: 125–31.
- 37 Storm G, Oussoren C, Peelers PAM, Barenholz Y. Tolerability of liposomes in vivo. In: Gregoriadis G, ed. *Liposome Technology*. Boca Raton, FL: CRC Press, 1993: 345–83.
- 38 Carpenter CP, Woodside MD, Kinkead ER, King JM, Sullivan LJ. Response of dogs to repeated intravenous injection of polyethylene glycol 4000 with notes on excretion and sensitization. *Toxicol Appl Pharmacol* 1971; 18: 35–40.
- 39 Lee TY, Lin CT, Kuo SY, Chang DK, Wu HC. Peptide-mediated targeting to tumor blood vessels of lung cancer for drug delivery. *Cancer Res* 2007; 67: 10958–65.
- 40 Chang DK, Lin CT, Wu CH, Wu HC. A novel peptide enhances therapeutic efficacy of liposomal anticancer drugs in mice models of human lung cancer. *PLoS ONE* 2009; 4: e4171.
- 41 Wei K, Mulvagh SL, Carson L, Davidoff R, Gabriel R, Grimm RA, Wilson S, Fane L, Herzog CA, Zoghbi WA, Taylor R, Farrar M, Chaudhry FA, Porter TR, Irani W, Lang RM. The safety of Definity and Optison for ultrasound image enhancement: a retrospective analysis of 78,383 administered contrast doses. *J Am Soc Echocardiogr* 2008; 11: 1202–6.
- 42 Hutter JC, Luu HM, Mehlhaff PM, Killam AL, Dittrich HC. Physiologically based pharmacokinetic model for fluorocarbon elimination after the administration of an octafluoropropane-albumin microsphere sonographic contrast agent. *J Ultrasound Med* 1999; 18: 1–11.

## Combination of Bubble Liposomes and High-Intensity Focused Ultrasound (HIFU) Enhanced Antitumor Effect by Tumor Ablation

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Ultrasound (US) is used in the clinical setting not only for diagnosis but also for therapy. As a therapeutic US technique, high-intensity focused ultrasound (HIFU) can be applied to treat cancer in a clinical setting. Microbubbles increased temperature and improved the low therapeutic efficiency under HIFU; however, microbubbles have room for improvement in size, stability, and targeting ability. To solve these issues, we reported that “Bubble liposomes” (BLs) containing the US imaging gas (perfluoropropane gas) liposomes were suitable for ultrasound imaging and gene delivery. In this study, we examined whether BLs and HIFU could enhance the ablation area of the tumor and the antitumor effect. First, we histologically analyzed the tumor after BLs and HIFU. The ablation area of the treatment of BLs and HIFU was broader than that of HIFU alone. Next, we monitored the temperature of the tumor, and examined the antitumor effect. The temperature increase with BLs and HIFU treatment was faster and higher than that with HIFU alone. Moreover, treatment with BLs and HIFU enhanced the antitumor effect, which was better than with HIFU alone. Thus, the combination of BLs and HIFU could be efficacious for cancer therapy.

**Key words** high-intensity focused ultrasound; tumor ablation; bubble liposome; cancer therapy

Ultrasound (US) imaging is a widely used diagnostic technique that allows for real-time imaging and combines the advantages of noninvasiveness with easy access by the public and low cost. In addition, US is used in the clinical setting not only for diagnosis but also for therapy. Recently, as a therapeutic US technique, high-intensity focused ultrasound (HIFU) has been applied for the treatment of prostate cancer and liver cancer in a clinical setting.<sup>1)</sup> HIFU can induce thermal elevation and lead to ablation and coagulative necrosis for targeted tissue. Although the concept of HIFU can be ideal for cancer therapy, HIFU has some disadvantages. Because the ablation area of HIFU exposure is narrow, HIFU requires a long time for cancer therapy. Microbubbles increase heat generation mainly by increasing inertial cavitation and improve the therapeutic efficiency under HIFU exposure<sup>2)</sup>; however, microbubbles have room for improvement in size, stability, and targeting ability. To solve these issues, we previously developed “Bubble liposomes” (BLs; approximately 500 nm). These are polyethylene glycol (PEG)-modified liposomes that contain echo-contrast gas, which can function as a gene and short interfering RNA (siRNA) delivery tool with US exposure *in vitro* and *in vivo*.<sup>3,4)</sup> However, it is not yet known whether BLs increase the temperature of tumor tissue and enhance the ablation area and antitumor effect under HIFU exposure as microbubbles. In this study, we examined whether BLs and HIFU could enhance the ablation area of tumors and the antitumor effect.

## MATERIALS AND METHODS

**Preparation of Liposomes and BLs** To prepare liposomes for BLs, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-polyethyleneglycol (DSPE-PEG<sub>2000</sub>-OMe) were mixed at a molar ratio of 94:6. The liposomes were prepared by a reverse-phase evaporation method, as described previously.<sup>4)</sup> In brief, all the reagents were dissolved in 1:1 (v/v) chloroform–diisopropylether. Phosphate-buffered saline was added to the lipid solution, and the mixture was sonicated and then evaporated at 47°C. The organic solvent was completely removed, and the size of the liposomes was adjusted to less than 200 nm using extrusion equipment and a sizing filter (Nuclepore Track-Etch Membrane, 200 nm pore size, Whatman plc, U.K.). After being sized, the liposomes were passed through a sterile 0.45- $\mu$ m syringe filter (Asahi Techno Glass Co., Chiba, Japan) for sterilization. The lipid concentration was measured using the Phospholipid C test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachiho Chemical Inc., Co., Ltd., Tokyo, Japan). First, 5 mL sterilized vials containing 2 mL of liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressurized with 7.5 mL of perfluoropropane gas. The vials were placed in a bath-type sonicator (42 kHz, 100 W, Branson 2510J-DTH, Branson Ultrasonics Co., Danbury, CT, U.S.A.) for 5 min to form BLs. The mean size of the BLs was determined using light-scattering with a zeta potential/particle sizer (Nicom 380ZLS, Santa Barbara, CA, U.S.A.).

**Animals and Tumor Models** Male BALB/c mice (6 weeks old) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). All animal use and rel-

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evant experimental procedures were approved by the Tokyo University School of Pharmacy and Life Science Committee on the Care and Use of Laboratory Animals. Colon26 cells ( $1 \times 10^6$  cells/mouse) and Matrigel 50  $\mu\text{L}$  (BD Biosciences, San Diego, CA, U.S.A.) were inoculated subcutaneously in the flank of mice. *In vivo* histological and antitumor studies were performed when the tumors were 100–200  $\text{mm}^3$ . *In vivo* temperature rise test was performed when the tumors were approximately 300  $\text{mm}^3$ .

**Histological Analysis** Tumor-bearing mice were treated with intratumoral injection of BLs (50  $\mu\text{g}/50 \mu\text{L}$ ) and HIFU exposure (Frequency: 3 MHz, Duty: 100%, Intensity: 1.5  $\text{kW}/\text{cm}^2$ , Time: 60 s). Next day, each tumor dissected, and fixed with 4% paraformaldehyde substituted with 20% sucrose and then embedded in optimal cutting temperature compound (Sakura Finetech, Co., Ltd., Tokyo, Japan) and frozen at  $-80^\circ\text{C}$ . Each tumor section was prepared with a width of 6  $\mu\text{m}$  and mounted on a poly-L-lysine coated slide. The tumor section were processed by both hematoxylin and eosin (H&E) and nicotinamide adenine dinucleotide reduced (NADH) tetrazolium reductase (NADH-TR) staining.<sup>5)</sup> The tissue sections were incubated at  $37^\circ\text{C}$  for 1 h in 150 mL of Tris-HCl buffer (pH 7.4) containing 150 mg of nitro blue tetrazolium (WAKO, Japan) and 120 mg of  $\beta$ -NADH (WAKO, Japan). The tissue sections were washed with distilled water, various con-

centrations of acetone (30, 60, 90, 60, and 30%), and finally mounted with glycerol gelatin (Sigma-Aldrich). H&E staining was also performed on the tumor sections using the standard technique. The ablated area seen by NADH-TR staining was measured using ImageJ software.

***In Vivo* Antitumor Efficacy** Tumor-bearing mice were treated with intratumoral injection of BLs (50  $\mu\text{g}/50 \mu\text{L}$ ) and HIFU exposure (Frequency: 3 MHz, Duty: 100%, Intensity: 1.5  $\text{kW}/\text{cm}^2$ , Time: 60 s), next day tumor volume was monitored. Then, this cycle was repeated three times.

## RESULTS AND DISCUSSION

First, we performed histological analysis of tumor tissue after BLs and HIFU exposure. BLs (lipid volume: 50  $\mu\text{g}/50 \mu\text{L}$ ) were administered using an intratumoral injection ( $n=5$ ). Then, tumor-bearing mice were exposed to HIFU (frequency: 3 MHz, rate: 100%, intensity: 1.5  $\text{kW}/\text{cm}^2$ , time: 60 s). Tumor sections were prepared and processed by both H&E staining and NADH-TR staining. To identify tumor ablation area, NADH-TR staining was used to differentiate the viable (blue regions) and nonviable (white/clear regions) tumor tissue. The ablation area of the treatment of BLs and HIFU was ten times more than treatment with HIFU alone (Fig. 1). When tumor-bearing mice were also exposed to HIFU, outward damage

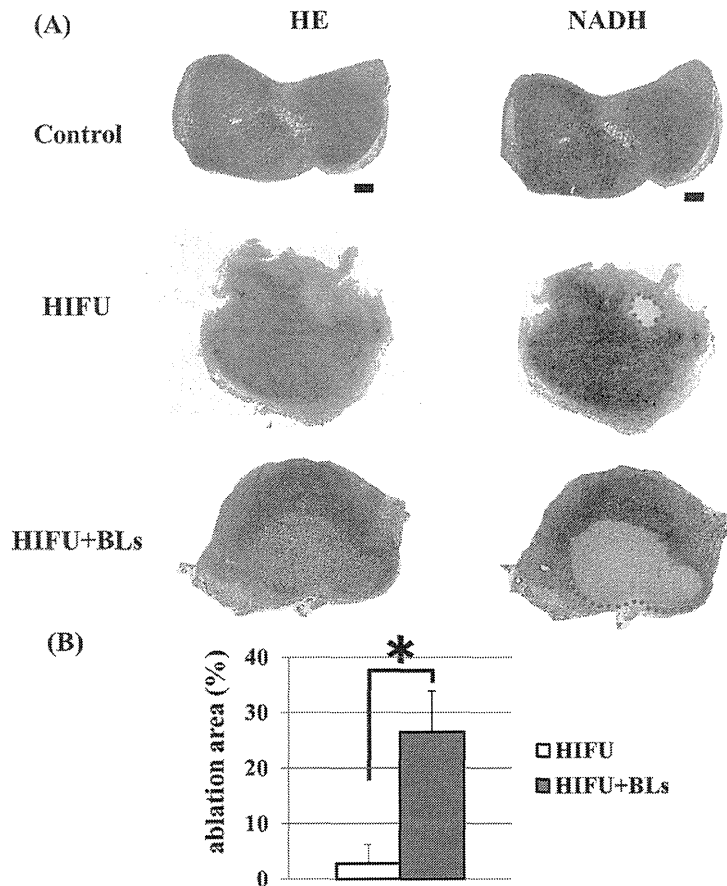


Fig. 1. Histological Analysis of Tumor after Treatment with HIFU Exposure

Mice were implanted s.c. with  $1 \times 10^6$  of colon26 cells into the flank. After 5–6 d of implantation, they were treated with intratumoral injection of BLs and HIFU exposure (frequency: 3 MHz, rate: 100%, intensity: 1.5  $\text{kW}/\text{cm}^2$ , time: 60 s). Each tumor was dissected, and the tumor section were sliced at 6  $\mu\text{m}$  ( $n=5$ ). (A) Each section underwent H&E staining (left panel) and NADH-TR staining (right panel). Scale bars represent 500  $\mu\text{m}$ . NADH-TR staining show viable tissue (blue) and nonviable tissue (clear/white). (B) The ablation area (red circle) of the tumor was measured by Image J. \* $p < 0.005$ .

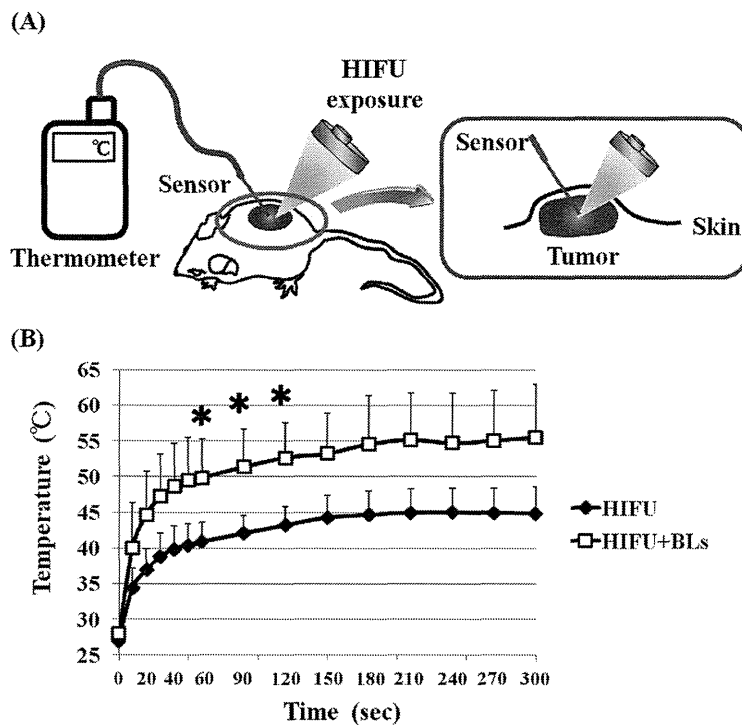


Fig. 2. Temperature Increase in Tumor Tissue Treated with BLs and HIFU Exposure

Tumor-bearing mice were treated with BLs and HIFU exposure (frequency: 3MHz, rate: 100%, intensity: 1.5kW/cm<sup>2</sup>, time: 300s, BLs: 50 μg/50 μL), and the tumor temperature monitored. Each value is the average and S.D. (n=7). \*p<0.005 compared with HIFU. (A) Scheme of tumor temperature measurement; (B) Temperature increase in tumor treated with HIFU alone or BLs and HIFU.

(e.g., ablation, necrosis) to normal tissue (e.g., liver, kidney, and spleen) was not observed (data not shown). From these results, in order to verify that the ablation of tumor was due to thermal elevation by the treatment of BLs and HIFU exposure, we monitored temperature of tumor tissue (n=7) in time-course by thermometer (TM-300, IK-300, AS ONE, Osaka, Japan) (Fig. 2). As shown in Fig. 2, the temperature of the tumor tissue rose to 45°C with continuous exposure to HIFU for 3 min, while the temperature of tumor tissue by treatment with BLs and HIFU reached 45°C in a mere 20s, rose to 50°C in 1 min, and finally reached 55°C in 3 min. Generally, tissue heated to over 55°C by an ablation technique such as HIFU has been reported as “ablative hyperthermia,” which has been used as a definitive treatment of accessible solid tumor, whereas tissue heated to 40–45°C has been reported as “mild hyperthermia,” which has been shown to be effective as an adjuvant for both radiotherapy and chemotherapy.<sup>6,7</sup> These results suggested that the ablation area of the tumor in this study was due to high temperature (50–55°C).

Next, as treatment with BLs and HIFU showed that the ablation area of the tumor was broader than that with HIFU alone by increasing temperature, the antitumor effect of BLs and HIFU treatment was evaluated in tumor-bearing mice. Tumor-bearing mice were treated with intratumoral injection of BLs and HIFU exposure, and tumor volume was monitored the following day. This cycle was repeated three times. As shown in Fig. 3, the BLs and HIFU group had significantly suppressed tumor growth compared with the control group (p<0.01). In addition, the tumor growth inhibition efficacy of the BLs and HIFU group was better than that of the HIFU alone group (p<0.05). It has been reported that local high-

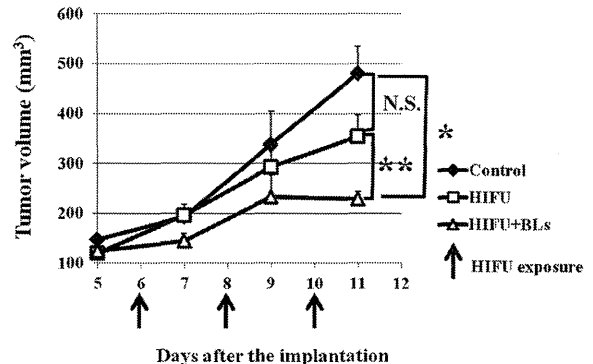


Fig. 3. Antitumor Effect after Treatment with BLs and HIFU Exposure

Tumor-bearing mice were treated with intratumoral injection of BLs and HIFU exposure (frequency: 3MHz, rate: 100%, intensity: 1.5kW/cm<sup>2</sup>, time: 60s, BLs: 50 μg/50 μL). Tumor volume of tumor-bearing mice was monitored. Each value is the average and S.D. (n=4). \*p<0.01, \*\*p<0.05.

temperature hyperthermia (50–55°C) enhanced the indices of the T helper 1 (Th1) immune response, such as interleukin-2 (IL-2), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α)<sup>8</sup>; therefore, it could be suggested that the antitumor effect of BLs and HIFU exposure was due to the ablation effect and immune response.

In this study, we observed that treatment with BLs and HIFU could enhance the ablation area of the tumor and the antitumor effect. The ablation area with BLs and HIFU treatment was broader than with HIFU alone. We next monitored the temperature of tumor tissue with BLs and HIFU treatment. In this experiment, the temperature increase with BLs and HIFU treatment was faster and higher than with HIFU

alone. Moreover, BLs and HIFU treatment enhanced the anti-tumor effect, which was better than with HIFU alone. Thus, the combination of BLs and HIFU could be efficacious treatment for cancer therapy.

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## REFERENCES

- 1) Dubinsky TJ, Cuevas C, Dighe MK, Kolokythas O, Hwang JH. High-intensity focused ultrasound: current potential and oncologic applications. *AJR Am. J. Roentgenol.*, **190**, 191–199 (2008).
- 2) Kaneko Y, Maruyama T, Takegami K, Watanabe T, Mitsui H, Hanajiri K, Nagawa H, Matsumoto Y. Use of a microbubble agent to increase the effects of high intensity focused ultrasound on liver tissue. *Eur. Radiol.*, **15**, 1415–1420 (2005).
- 3) Suzuki R, Takizawa T, Negishi Y, Hagiwara K, Tanaka K, Sawamura K, Utoguchi N, Nishioka T, Maruyama K. Gene delivery by combination of novel liposomal bubbles with perfluoropropane and ultrasound. *J. Control. Release*, **117**, 130–136 (2007).
- 4) Negishi Y, Endo Y, Fukuyama T, Suzuki R, Takizawa T, Omata D, Maruyama K, Aramaki Y. Delivery of siRNA into the cytoplasm by liposomal bubbles and ultrasound. *J. Control. Release*, **132**, 124–130 (2008).
- 5) Anderson JK, Baker M, Jaffers O, Pearle MS, Lindberg GL, Cadeddu JA. Time course of nicotinamide adenine dinucleotide diaphorase staining after renal radiofrequency ablation influences viability assessment. *J. Endourol.*, **21**, 223–227 (2007).
- 6) van der Zee J, González González D, van Rhoon GC, van Dijk JD, van Putten WL, Hart AA. Comparison of radiotherapy alone with radiotherapy plus hyperthermia in locally advanced pelvic tumours: a prospective, randomised, multicentre trial. Dutch Deep Hyperthermia Group. *Lancet*, **355**, 1119–1125 (2000).
- 7) Tachibana K. Emerging technologies in therapeutic ultrasound: thermal ablation to gene delivery. *Hum. Cell*, **17**, 7–15 (2004).
- 8) Li DY, Tang YP, Zhao LY, Geng CY, Tang JT. Antitumor effect and immune response induced by local hyperthermia in B16 murine melanoma: Effect of thermal dose. *Oncol. Lett.*, **4**, 711–718 (2012).

## Evaluation of Active Control of Bubble Liposomes in a Bifurcated Flow under Various Ultrasound Conditions

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**Abstract** Bubble liposomes (BLs), which are gas-encapsulated liposomes several hundred nanometers in diameter, are expected to be developed as a novel tool for gene and drug delivery using ultrasound acoustic radiation force. However, since BLs are several hundred nanometers in diameter, difficulties exist in controlling their behaviors in blood flow under ultrasound exposure, since acoustic radiation forces have less effect on these small bubbles. In this study, we investigated the feasibility of active control of BLs in an artificial blood vessel under ultrasound exposure and attempted to evaluate the controllability. Then, we investigated the appropriate ultrasound conditions for active path selection of BLs in a bifurcated flow by applying acoustic radiation force. We prepared a single transducer to orient BLs toward one desired path. Two other transducers were targeted at the two paths after the bifurcation. We evaluated the areas of trapped BLs in the two paths after the bifurcation, to determine which path had increased BLs. The result showed a significant increase in area of trapped BLs in the desired path compared to the other path. Then, we defined the induction index of BLs by evaluating the area of trapped BLs, and changed the ultrasound conditions for active path selection of BLs by varying the sound pressure and frequency. We found that more BLs could be oriented to a desired path at higher sound pressure. For further study, we are aiming at active control of BLs *in vivo*.

**Keywords :** Bubble liposome, active control, acoustic radiation force.

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### 1. Introduction

Many studies of drug delivery system have used microbubbles (MBs) as a drug carrier in the human body. The presence of bubbles improves the effects of ultrasound therapy by accelerating the temperature increase in thermal therapy [1, 2] and inducing sonoporation to allow uptake of larger molecules into cells in physical drug delivery [3–5]. We have previously reported our attempt to propel microbubbles in flow [6, 7] by utilizing aggregate formation of bubbles, which is effective to propel bubbles before entering an ultrasound field to be exposed to greater acoustic radiation force. We have elucidated the conditions of ultrasound and flow velocity for active path selection of bubble aggregates in an artificial blood vessel. However, we used MBs that mimicked ultrasound contrast agent, and they were developed for industrial purpose and not for medical use.

In other studies, we used another type of MBs Sonazoid to realize trapping of MBs in flow [8] and artificial embolization in capillary model [9]. Although Sonazoid is commercially available for contrast enhancement of echography, it is difficult to modify the surface of the membrane. On the other hand, the recently developed Bubble liposomes (BLs) have been found to be safe *in vivo*, with easily modified targeting ligand. We expect that BLs had the potential to become a drug delivery tool using ultrasound.

Considering that BLs are several hundred nanometers in diameter, difficulties exist in controlling their behaviors in blood flow under ultrasound exposure, since acoustic radiation forces have less effect on these small bubbles. And, it is difficult to detect the behavior of BLs in brightness because the suspension is diluted from the original BL preparation because of the diffusion in the human body.

In this study, we attempted active control of BLs in an artificial blood vessel. For active path selection of BLs, we prepared a single transducer to orient BLs toward one desired path. To evaluate controllability of BLs quantitatively, two other transducers were targeted at the two paths after the bifurcation. We evaluated the areas of trapped BLs in the two paths after the bifurcation to determine which path had increased BLs. Then, we investigated the optimal ultrasound conditions for active path selection of BLs by varying sound pressure and frequency.

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