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原著

# 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

鈴木 理恵子[1)]，古賀 政利[2)]，山本 晴子[3)]，大山 賢[1)]，徳永 敬介[1)]，松木 孝之[1)]，長束 一行[4)]，豊田 一則[1)]，峰松 一夫[1)]

[1)] 国立循環器病研究センター 脳血管内科
[2)] 国立循環器病研究センター 脳卒中治療科
[3)] 国立循環器病研究センター 先進医療・治験推進部，
[4)] 国立循環器病研究センター 脳神経内科

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

**Rieko SUZUKI[1)], Masatoshi KOGA[2)], Haruko YAMAMOTO[3)], Satoshi OYAMA[1)], Keisuke TOKUNAGA[1)], Takayuki MATSUKI[1)], Kazuyuki NAGATSUKA[4)], Kazunori TOYODA[1)], Kazuo MINEMATSU[1)]**

[1)] Department of Cerebrovascular Medicine, National Cerebral and Cardiovascular Center
[2)] Division of Stroke Care Unit, National Cerebral and Cardiovascular Center
[3)] Department of Advanced Medical Technology Development in Research and Development Initiative Center, National Cerebral and Cardiovascular Center
[4)] Division of Neurology, National Cerebral and Cardiovascular Center

**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 ± 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
鈴木理恵子：〒 565-8565 大阪府吹田市藤白台 5-7-1
Rieko SUZUKI: 5-7-1 Fujishiro-dai Suita, Osaka 565-8565, Japan
E-mail: rsuzuki@ncvc.go.jp, Tel: 06-6833-5012, Fax: 06-6835-5267

続照射を加えると完全再開通率が高まることがAlexandrovら[1]により報告された．Eggersら[2]は，rt-PA投与に1.8MHzの経頭蓋カラードプ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[3]は，300kHzの低周波パ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と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（vertebrobasilar 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は行ったが，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リトリーバー，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[4]，DSAではThrombolysis in Cerebral Infarction分類（TICI）gradeを用いた[5]．MRAとDSAとを併用した評価は，Yoshimuraら[6]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 ± 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 ± 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 ± 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 ± 30分であった．16例が2時間以内にMRA，DSA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(1918.8) | 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, TICI) 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 |

**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, TICI) 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 |

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，M 1 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

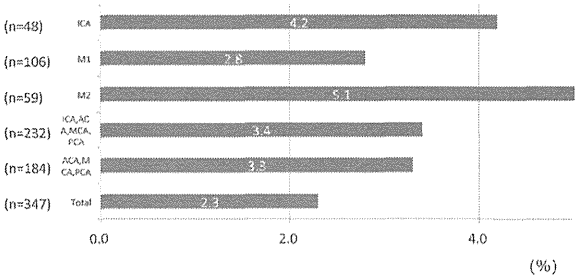


**Fig.1 Symptomatic intracerebral hemorrhage within the initial 36 hours**
a deterioration in NIHSS score ≧ 4 from baseline

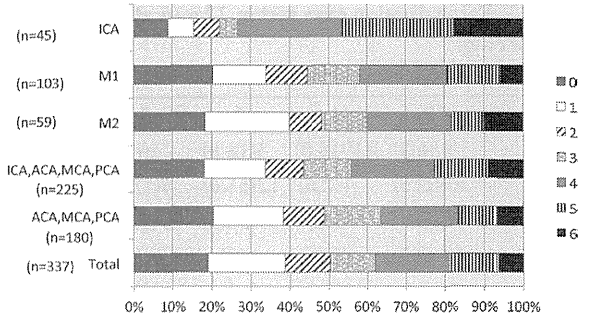


**Fig.2 Modified Rankin Scale at 3 months by different sites of occlusion**

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，アルテプラーゼ投与量は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プラ法（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[7-12]．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や経頭蓋カラードプラ法（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[13]．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[8]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[9]は，アルテプラーゼ（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，アルテプラーゼ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．アルテプラー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ーゼ（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）[4]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[10]は，ICA，MCA閉塞のある64例の患者にアルテプラーゼ（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[9]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，症例数が少なかったため，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った症例は，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[4] の再開通率である 69.0%（40 例 /58 例）や Kimura ら[10] 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った．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%[14]，本邦の市販後調査である J-MARS 研究の 3.5%[15] 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っ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的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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ORIGINAL ARTICLE

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

K. HAGISAWA,* T. NISHIOKA,† R. SUZUKI,‡ K. MARUYAMA,‡ B. TAKASE,§ M. ISHIHARA,§ A. KURITA,§ N. YOSHIMOTO,† Y. NISHIDA,* K. IIDA,¶ H. LUO¶ and R. J. SIEGEL¶

*Department of Physiology, National Defense Medical College, Tokorozawa; †Division of Cardiology, Saitama Medical Center, Saitama Medical University, Kawagoe; ‡Department of Drug Delivery System, School of Pharmaceutical Sciences, Teikyo University, Tokyo; §Division of Biomedical Engineering, National Defense Medical College Research Institute, Tokorozawa, Japan; and ¶Cardiac Non-invasive Laboratory, Heart Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA*

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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 W $cm^{-2}$, to 12 arteries, and high intensity, 4.0 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 ± 5.0 min; rt-PA, 41.3 ± 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.

Correspondence: Toshihiko Nishioka, Division of Cardiology, Saitama Medical Center, Saitama Medical University, 1981, Kamoda, 350-8550 Kawagoe, Japan.
Tel.: +81 49 228 3587; fax: +81 49 226 5274.
E-mail: nishioka@saitama-med.ac.jp

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## 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

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 cavitational and non-cavitational 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 cavitational 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-disteoyl-*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 $mL^{-1}$. 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 $mg^{-1}$ 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 $cm^{-2}$) 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 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 μL, 20 mg $mL^{-1}$ lipid concentration, ~ 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 °C. Each thrombus was exposed to low-intensity USD (27 kHz, 1.4 W $cm^{-2}$) or high-intensity USD (27 kHz, 4.0 W $cm^{-2}$) for 5 min. After USD exposure, the clot was weighed again. The thrombus weight reduction rate ([pre-treatment weight – post-treatment weight]/pre-treatment weight × 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 (~ 2.4 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 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 °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

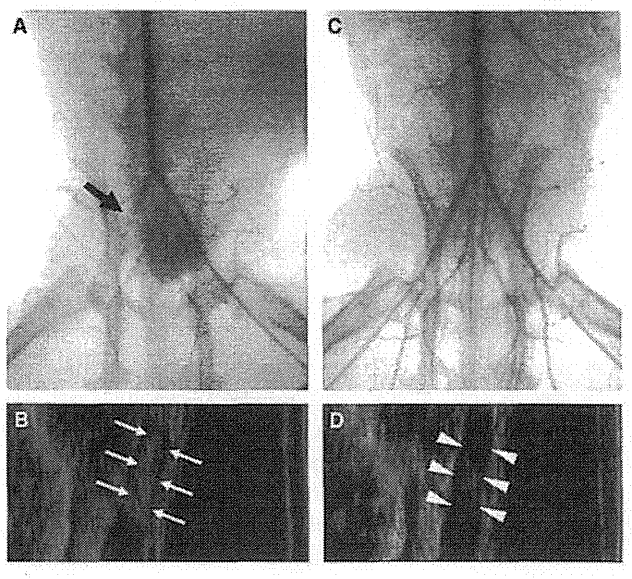


**Fig. 1 .** (A) Angiographic image of thrombotically occluded rabbit iliac artery (black arrow) after balloon inflation and electrical (3-V battery) stimulation. (B) In sonographic images, the targeted bubble liposomes (BLs) accumulated on the thrombus (white arrows). (C, D) After combination therapy 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 $kg^{-1}$ 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 reocclusion 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 $kg^{-1}$ recombinant tissue plasminogen activator (rt-PA) (Monteplase, Eizai, Tokyo, Japan) was intravenously administered within a period of 1 min immediately after intravenous injection of 200 IU $kg^{-1}$ 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 $kg^{-1}$) injected via a marginal ear vein.

### *Statistical analysis*

Results are given as mean value ± 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 × 2 or a 2 × 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% ± 11% vs. 14% ± 9% and 9% ± 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% ± 21% vs. 21% ± 9% and 21% ± 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% ± 9% vs. 9% ± 3%) and with targeted BLs (65% ± 21% vs. 25% ± 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

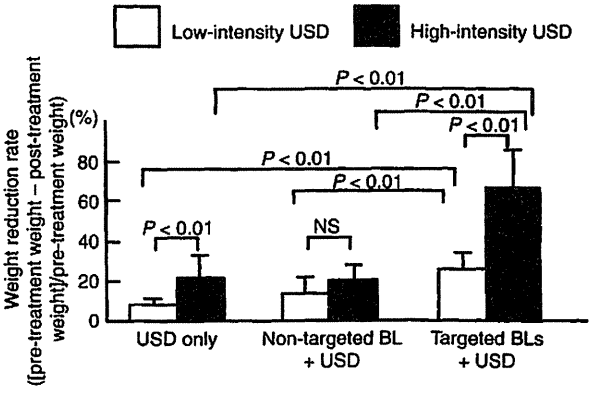


**Fig. 2.** The *in vitro* thrombus weight reduction rate ([pre-treatment weight − post-treatment weight]/pre-treatment weight × 100 [%]) obtained with low-frequency ultrasound (USD): high-intensity USD exposure for 5 min with the targeted bubble liposomes (BLs), with the non-targeted BLs, or without 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 achived 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 ordinary thrombolysis with rt-PA (targeted BLs, 16.7 ± 5.0 min; non-targeted BLs, 60.0 ± 0 min; rt-PA, 41.3 ± 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).

**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 | *P*-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.

## 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*

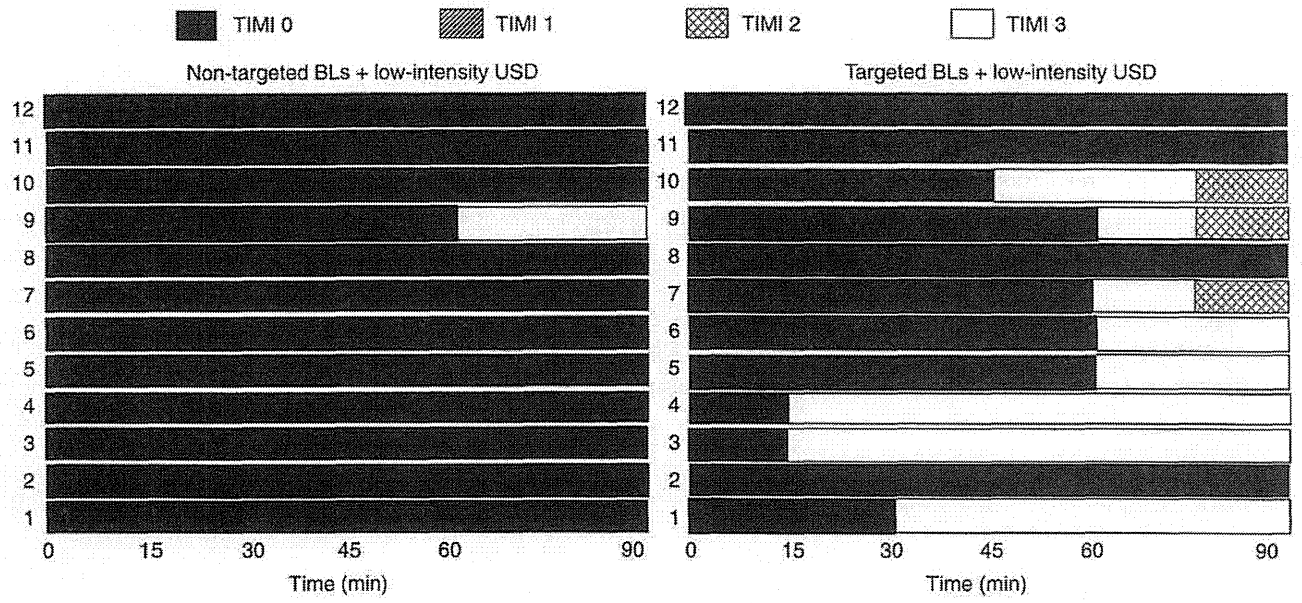


**Fig. 3.** Schematic presentation showing TIMI flow grades of the arteries treated with low-intensity ultrasound (USD) with non-targeted bubble liposomes (BLs) and targeted BLs. TIMI grade 3 flow was achieved in 67% of arteries with targeted BLs and in only 8% of arteries with non-targeted BLs ($P =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

**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 |

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 cavitational 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.

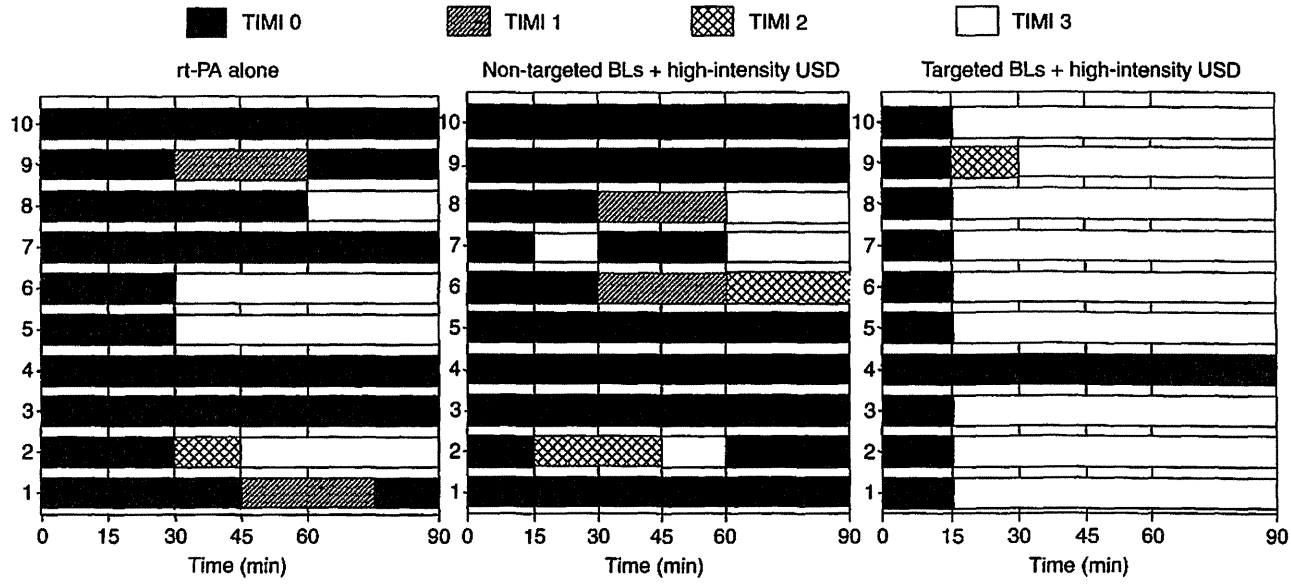


**Fig. 4.** Schematic presentation showing TIMI flow grades of the arteries treated with recombinant tissue-type plasminogen activator (rt-PA) alone, with high-intensity ultrasound (USD) with non-targeted bubble liposomes (BLs), and with high-intensity USD with targeted BLs. TIMI grade 3 flow was achieved 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 × 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 μm 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 cavitational 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 cavitational 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. Hagisawa, T. Nishioka, and R. J. Siegel: conception and design; K. Hagisawa, T. Nishioka, R. Suzuki, B. Takase, K. Iida, and H. Luo: acquisition of data; K. Hagisawa, T. Nishioka, and A. Kurita: analysis and interpretation of data; K. Hagisawa, 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.

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

Nobuhito Hamano,[a,#] Yoichi Negishi,*[,a,#] Kyohei Takatori,[a] Yoko Endo-Takahashi,[a] Ryo Suzuki,[b] Kazuo Maruyama,[b] Takuro Niidome,[c] and Yukihiko Aramaki[a]

[a] *Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan:* [b] *Laboratory of Drug and Gene Delivery Research, Faculty of Pharma-sciences, Teikyo University; 2–11–1 Kaga, Itabashi-ku, Tokyo 173–8605, Japan: and* [c] *Department of Applied Chemistry and Biochemistry, Graduate School of Science and Technology; 2–39–1 Kurokami, Chuo-ku, Kumamoto 860–8555, Japan.*

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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.[1] HIFU can induce thermal elevation and lead to ablation and coagulative necrosis for targeted tissue. Although the concept of HIFU can be ideal of 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[2]; 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*.[3,4] 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-$PEG_{2000}$-OMe) were mixed at a molar ratio of 94:6. The liposomes were prepared by a reverse-phase evaporation method, as described previously.[4] 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, Bransonic 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 (Nicomp 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-

The authors declare no conflict of interest.

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: negishi@toyaku.ac.jp

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 ($1\times10^6$ cells/mouse) and Matrigel 50 $\mu$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 $mm^3$. *In vivo* temperature rise test was performed when the tumors were approximately 300 $mm^3$.

**Histological Analysis** Tumor-bearing mice were treated with intratumoral injection of BLs (50 $\mu$g/50 $\mu$L) and HIFU exposure (Frequency: 3 MHz, Duty: 100%, Intensity: 1.5 kW/$cm^2$, 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°C. Each tumor section was prepared with a width of 6 $\mu$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.[5] The tissue sections were incubated at 37°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 concentrations 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 $\mu$g/50 $\mu$L) and HIFU exposure (Frequency: 3 MHz, Duty: 100%, Intensity: 1.5 kW/$cm^2$, 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 $\mu$g/50 $\mu$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 kW/$cm^2$, 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

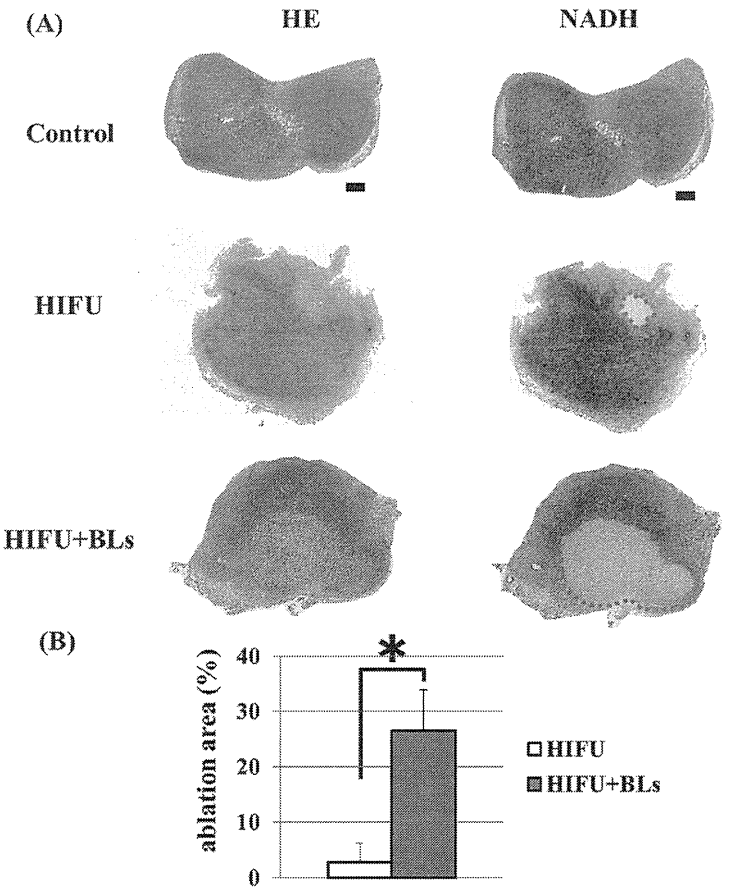


Fig. 1. Histological Analysis of Tumor after Treatment with HIFU Exposure

Mice were implanted s.c. with $1\times10^6$ 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 kW/$cm^2$, time: 60 s). Each tumor was dissected, and the tumor section were sliced at 6 $\mu$m ($n$=5). (A) Each section underwent H&E staining (left panel) and NADH-TR staining (right panel). Scale bars represent 500 $\mu$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.

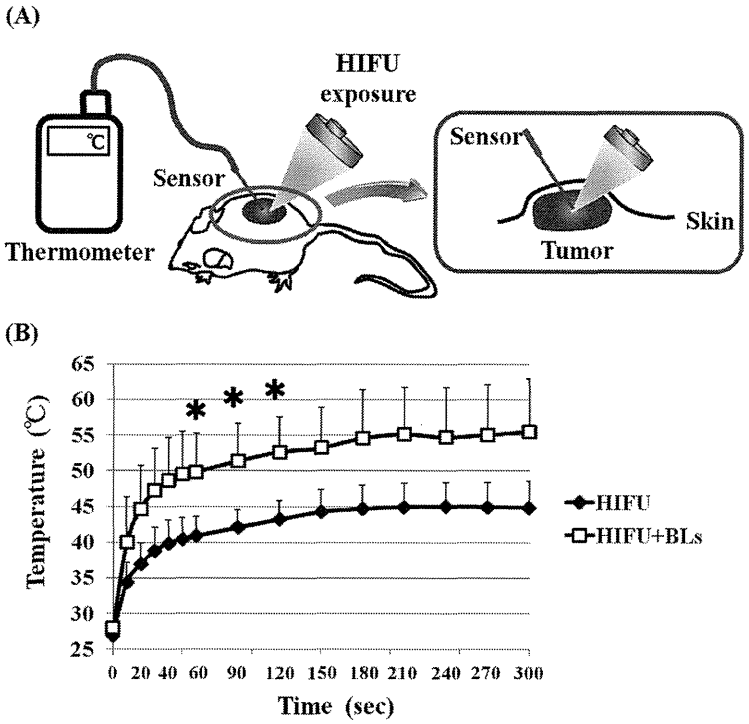


Fig. 2. Temperature Increase in Tumor Tissue Treated with BLs and HIFU Exposure

Tumor-bearing mice were treated with BLs and HIFU exposure (frequency: 3 MHz, rate: 100%, intensity: 1.5 kW/cm$^2$, time: 300 s, BLs: 50 $\mu$g/50 $\mu$L), 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 20 s, 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.[6,7] 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-

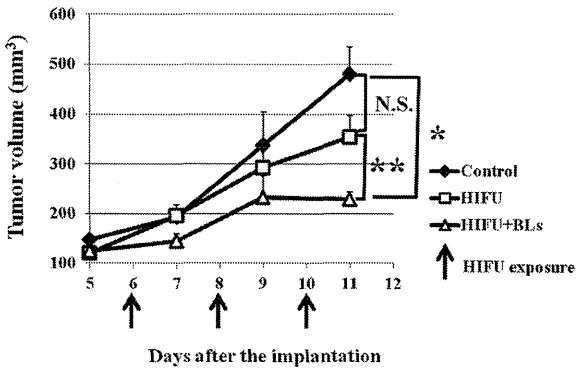


Fig. 3. Antitumor Effect after Treatment with BLs and HIFU Exposure

Tumor-bearing mice were treated with intratumoral injection of BLs and HIFU exposure (frequency: 3 MHz, rate: 100%, intensity: 1.5 kW/cm$^2$, time: 60 s, BLs: 50 $\mu$g/50 $\mu$L). 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-$\gamma$), and tumor necrosis factor-alpha (TNF-$\alpha$)[8]; 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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Original Paper

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

Ren KODA,*, # Jun KOIDO,* Naoto HOSAKA,* Shinya ONOGI,*
Takashi MOCHIZUKI,* Kohji MASUDA,* Ryo SUZUKI,** Kazuo MARUYAMA**

***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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* Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Tokyo, Japan.
** Laboratory of Drug and Gene Delivery, Faculty of Pharma-sciences, Teikyo University, Tokyo, Japan.
# 2-24, Nakacho, Koganei, Tokyo 184-8588, Japan.
E-mail: rkoda@eng.u-toyama.ac.jp

## 2. Methods

### 2.1 Bubble liposomes

We used Bubble liposomes prepared with polyethyleneglycol-modified liposomes (PEG-liposomes) and perfluoropropane gas (Takachiho Chemical Inc., Co., Ltd., Tokyo, Japan). The liposomes were prepared by a reverse-phase evaporation method, as described previously[10]. To prepare liposomes for the BLs, 1, 2-distearoyl-sn-glycero-phosphatidylcholine (DSPC) and 1, 2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-methoxypolyethyleneglycol [DSPE-PEG (2k) -OMe] were mixed at a molar ratio of 94:6. First, 5 mL sterilized vials containing 2 mL of PEG-liposome suspension (lipid concentration: 1 mg/mL) were placed in vials supercharged with 7.5 mL of perfluoropropane ($C_3F_8$) gas, then sonicated by continuous ultrasound at a frequency of 42 kHz for two or three min. During this process, gas was trapped inside the liposomes, resulting in a cloudy suspension. A bath-type ultrasound cleaner (Branson 2510) was used for sonication. **Figure 1** shows an image of BLs under a microscope equipped with a Darklite Illuminator (NEPA Gene Co. Ltd., Chiba, Japan). **Figure 2** shows the size distribution of BLs and Sonazoid measured by dynamic light scattering (ELS-Z, Otsuka Electronics Co., Ltd., Osaka, Japan). Although sub-micron sized objects are not shown in the microscopic image, **Fig. 2** indicates that the average diameters of BLs are approximately 400 to 500 nm and are smaller than the conventional MBs Sonazoid. **Table 1** shows the characteristics of BLs and another type of MBs F-04E[6, 7]. The suspension of BLs was freshly prepared before the experiment, diluted with saline to a concentration of 0.01–0.05 mg lipid/ml.

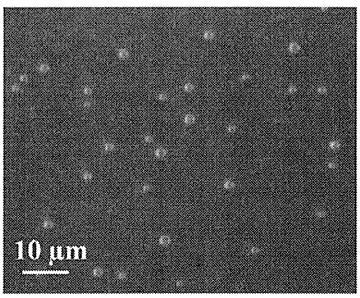


**Fig. 1** Microscopic image of BLs.

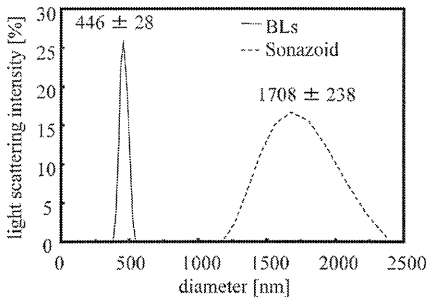


**Fig. 2** Size distribution of BLs and Sonazoid.

### 2.2 Experimental setup

We prepared an artificial blood vessel with a Y-form bifurcation structure, which was made of a mixture of wax and poly (vinyl alcohol) (PVA) [11]. The inflow path of 2 mm was repeatedly divided into two lower courses to provide artificial capillaries until the middle of the model, where the minimum path width was 0.50 mm. The path widths and the cross-sectional areas were designed to guarantee a constant flow velocity in any part of the model. The whole view, *x-y* plane view and *x-z* plane view of the experimental setup are shown in **Fig. 3a, b and c**, respectively. The artificial blood vessel with external dimensions of 180 × 70 × 8 $mm^3$, was positioned 30 mm above the bottom of a water tank, to prevent multiple reflections of ultrasound between the artificial blood vessel and the bottom of the tank. The blood vessel was divided into Paths A and B with a branch angle of 75 degrees. The path widths $w_1$ and $w_2$ corresponded to 1.4 mm and 1.0 mm, respectively. We used an optical microscope (Hirox KH-7700) to observe the area of interest in the blood vessel. The image size was 800 × 600 pixels and the optical resolution of the digitized images was 31.5 μm per pixel. The light source was located above

**Table 1** Characteristics of BLs and MBs.

| | *BLs* | *MBs (F-04E[6, 7])* |
|---|---|---|
| Mean diameter [μm] | 0.4–0.5 | 2.7 |
| Shell | Lipid (bilayer) | PVC-AN copolymer |
| Gas | $C_3F_8$ | $C_3H_8$, $C_4H_{10}$ |

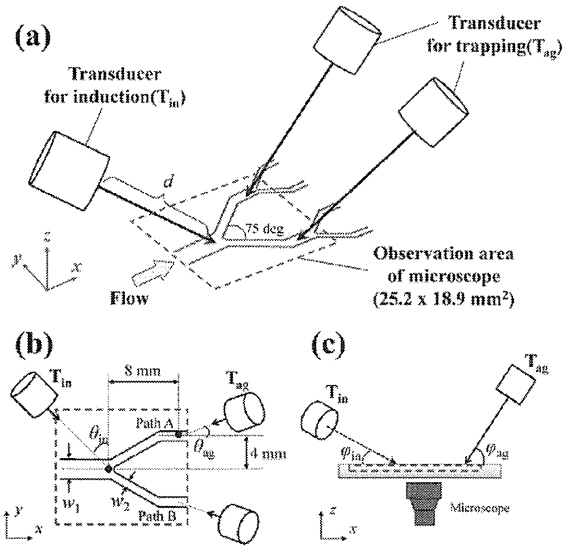


**Fig. 3** Schematic presentation of the experiment with the artificial blood vessel, the microscope and the ultrasound transducers.