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厚生労働科学研究費補助金

医薬品・医療機器等レギュラトリーサイエンス総合研究事業

簡便に調製可能な分子標的気泡を用いた超音波分子イメージングの開発

—臨床用超音波造影剤の適応拡大の可能性の検討—

平成２５年度　総括研究報告書

研究代表者　　大谷　健太郎

平成２６（２０１４）年　３月

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I. 総括研究報告

厚生労働科学研究費補助金（医薬品・医療機器等レギュラトリーサイエンス総合研究事業）
総括研究報告書

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—臨床用超音波造影剤の適応拡大の可能性の検討—

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研究要旨

超音波分子イメージングは、超音波造影剤である微小気泡を生体内分子に特異的に集積させ(分子標的気泡)、それを画像化する事で炎症性血管病変・動脈硬化巣・新生血管等を非侵襲的に評価する方法として近年注目されている。本年度は、肝腫瘤性及び乳房腫瘤性病変の診断目的で既に臨床使用されている超音波造影剤Sonazoidと、アポトーシス細胞の貪食に関わる生体内タンパクであるLactadherinとの複合体(Sonazoid-Lactadherin複合体)が、新生血管及び新鮮血栓に対する分子標的気泡となり得るか否かを評価するための基礎検討として、担癌モデルマウス及び頸動脈血栓モデルマウスの作成と、その病態評価系の確立を目的に検討を行った。ヌードマウスにヒト卵巣腺腫細胞(SK-OV-3)を皮下移植することにより、造影超音波法が施行可能な大きさの腫瘍を安定的に得ることが可能であり、また造影超音波法により明瞭な微小循環造影像を得ることができた。また、作成した腫瘍組織内には、Sonazoid-Lactadherin複合体の標的分子である新生血管が豊富に含まれていた。一方、塩化鉄(III)塗布による頸動脈血栓モデルはマウスにおいても作成可能であり、頸動脈血栓に標的分子であるGPIIb/IIIaが発現していることも併せて確認した。次年度以降は、本年度の結果を基にして、Sonazoid-Lactadherin複合体の医学的有用性について検討を行う予定である。

A. 研究目的

近年、超音波造影剤である微小気泡の表面に生体内抗原に特異的な抗体やペプチド、タンパクを結合させ、生体内の特定の部位に気泡を集積させることにより（分子標的気泡）、炎症性血管病変・新生血管・動脈硬化巣などを標的とした造影超音波法による分子イメージングの開発が試みられている。

その一方、超音波照射により微小気泡が崩壊する際に発せられるマイクロジェット(物理エネルギー)を利用し、微小気泡の近傍に存在する細胞膜に一時的に穴を生じさせ、細胞内へ核酸や薬剤を導入する、超音波と微小気泡を併用した細胞内への遺伝子・薬剤導入法が確立されつつある。

また、それらと並行して、薬剤を内包した微小気泡や、気泡殻に薬剤や核酸分子を付与した微小気泡の開発も進められている。

近い将来、これらの方法を統合的に組み合わせることで、体内の特定部位に薬剤内包・核酸修飾気泡を集積させ、超音波照射で局所選択的に微小気泡を破壊し、内包された薬剤の局所放出あるいは核酸分子を細胞内に導入するといった、今までにない全く新しいDrug Delivery SystemやGene Delivery System の開発が行われる可能性がある。

我々はこれまでに、本邦で臨床使用可能な超音波造影剤 Sonazoid から分子標的気泡が作成可能であることを報告してきた(**Mol Imaging Biol** 2011, **Ultrasound Imaging-Medical Applications** 2011)。先般、生体内タンパク MFG-E8 (Lactadherin)と Sonazoid の混合することで、表面に RGD 配列を有する分子標的気泡が作成可能であることを見出した。RGD 配列は、細胞表面タンパク質であるインテグリンαvβ3や種々の糖タンパクと特異的に結合するため、Sonazoid-Lactadherin 複合体は(癌および血管新生療法による)血管新生及び血栓に対する分子標的気泡となり得る可能性を有していると考えられる。

本研究の目的は、Sonazoid-Lactadherin 複合体が腫瘍血管及び新鮮血栓に対する分子標的気泡として有用か否かについて、in vitro 及び in vivo の実験系を用いて評価することである。

B. 研究方法

<u>腫瘍血管に対する分子標的性の検討</u>

インテグリンαvβ3は血管新生において重要な因子であり、腫瘍血管などの新生血管で発現が認められる。そこで、Sonazoid-Lactadherin 複合体の新生血管に対する分子標的性を担癌モデルマウスを用いて評価する。本年度は、超音波分子イメージングを施行する至適時期を決定するため、腫瘍サイズ及び病理組織像の経時的観察を行った。担癌モデルマウスは北山ラベス株式会社に委託して作成した。4 週齢の雌性ヌードマウスの右腹部皮下に、ヒト卵巣線種細胞(SK-OV-3)を 5.0×10^6 個移植した。腫瘍径を SK-OV-3 細胞移植後 7(Day7)、10(Day10)、14 日目(Day14)に計測するとともに、Day14 に腫瘍組織を摘出し、組織切片を作成した。腫瘍体積は次の計算式により算出した。

体積(mm^3) = (π/6)×短径^2×長径

腫瘍組織内の新生血管数を、抗マウス CD31 抗体を用いた免疫染色で評価した。

また予備的検討として、Day7 において腫瘍組織内の微小循環が造影超音波法にて評価可能か否かについて検討を行った。頸静脈よりカテーテルを挿入し、Sonazoid (1.2×10^6 bubbles)を投与した。超音波装置は東芝社製 Aplio を用い、1202S プローブにて撮像を行った。音圧は Mechanical index 0.4 (AP 9%)とし、焦点位置は腫瘍組織よりも深部に設定した。

<u>新鮮血栓に対する分子標的性の検討</u>

糖タンパク GPIIb/IIIa は血栓形成において重要な因子であり、近年 RGD ペプチドを用いた血栓の分子イメージングの標的として注目されている。そこで、Sonazoid-Lactadheerin 複合体の新鮮血栓に対する分子標的性について、ヒト及びマウス血液から作製した血餅を用いた in vitro の実験系にて検討を行う。本年度は、血餅作成の至適条件の検討と、作製したヒト血餅におけ

る糖タンパク GPIIb/IIIa(CD41)の発現を免疫染色により評価した。ヒト血液を凝固促進用シリカ微粒子が添加された採血管に入れ、室温で2時間凝固させた。その後切片を作成し、抗CD41抗体を用いて免疫染色を行った。

また予備的検討として、ラットの頸動脈血栓モデルの作成を試みた。ペントバルビタールにて麻酔後、仰臥位に固定し、頸動脈を剥離した。頸動脈の下にパラフィルム及びろ紙を潜らせた。ろ紙に40%塩化鉄(III)溶液を10μL染み込ませ、15分間放置した。その後、灌流固定し、頸動脈を摘出・組織切片を作成した。頸動脈内血栓をH&E染色により組織学的に評価した。

次に、マウスにおける予備的検討も行った。剥離した頸動脈の下にパラフィルム及びろ紙を潜らせた後、ろ紙に6%塩化鉄(III)溶液を4μL染み込ませた。3分間反応させた後、ろ紙を回収し、5、10、15分後に灌流し、頸動脈を摘出・組織切片を作成した。血栓内のCD41の発現を免疫染色により評価した。

【倫理面への配慮】

本研究では、分子標的気泡を用いた超音波分子イメージングの医学的有用性及び生物学的安全性について、病態モデルマウスを用いた動物実験により評価する。そのため、実験動物に対する倫理的配慮として、動物実験は国立循環器病研究センターの動物実験指針を遵守し施行した。また、動物愛護上の配慮として、モデル作成・病態評価の際に極力苦痛を与えないように努めた。

C. 研究結果

腫瘍血管に対する分子標的性の検討

実験に用いた全てのヌードマウスにおいて、腹部にSK-OV-3細胞由来の腫瘍の形成が認められた(図1A)。また、腫瘍体積はDay7からDay14にかけて、次第に縮小していくことが明らかとなった (Day7: 168.5±21.1 mm^3 vs. Day10: 101.3±14.8 mm^3 vs. Day14: 58.3±6.6 mm^3) (図1B)。

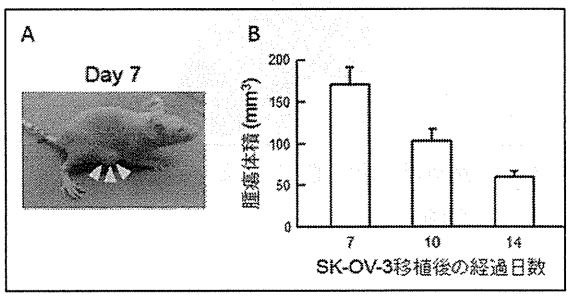

図1. 腫瘍の外観と腫瘍体積の経時変化

腫瘍内の新生血管について抗CD31抗体を用いた免疫染色で検討したところ、SK-OV-3細胞移植7日後及び14日後において、数多くのCD31陽性の血管が形成されていることが明らかとなった(図2)。

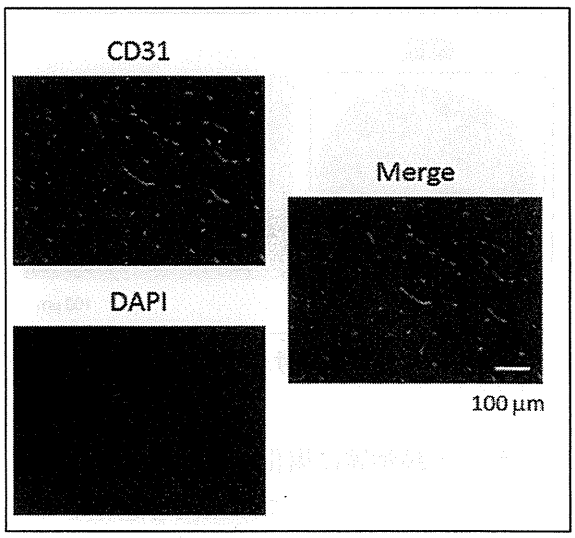

図2. 腫瘍組織内の新生血管の評価 (Day14での検討)

Sonazoid を用いた造影超音波法により、SK-OV-3 細胞移植 7 日後の腫瘍組織において、癌微小循環を非侵襲的に評価することが可能であった(図 3)。

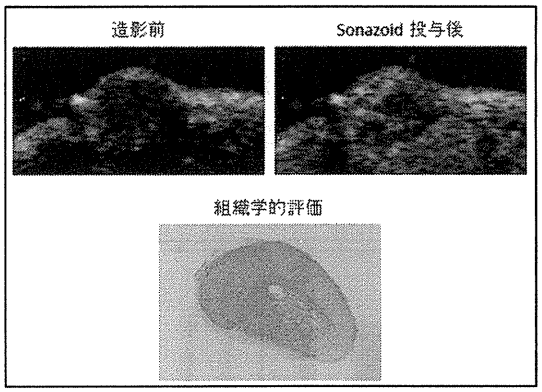

図 3. 造影超音波法による癌微小循環の評価

新鮮血栓に対する分子標的性の検討

免疫染色の結果、ヒト血液から作成した血餅に CD41 が多く発現していることが明らかとなった(図 4)。また、血液を室温よりも 37℃で凝固させることで、より強固な血餅が作成可能であることが明らかとなった(データ未掲載)。

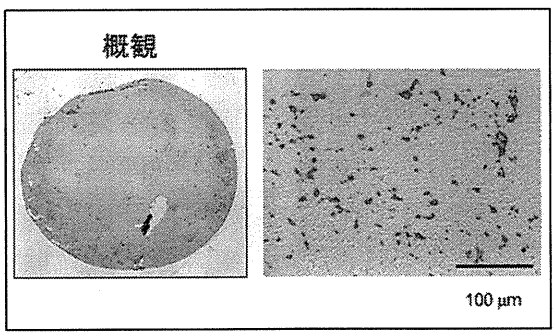

図 4. ヒト血餅における GPIIb/IIIa の発現

ラット頸動脈に塩化鉄(III)を 15 分間反応させることにより、ほぼ血流を途絶させるほどに巨大な血栓を作製することが可能であった(データ未掲載)。また、塩化鉄(III)による血栓モデルはマウスにも応用可能であり、マウスでは塩化鉄(III)との反応後 15 分の時点において、ほぼ血流を途絶するほどの大きな血栓が作製可能であった(図 5A)。また、作製された血栓において GPIIb/IIIa(CD41)の発現を免疫染色により確認することができた(図 5B)。

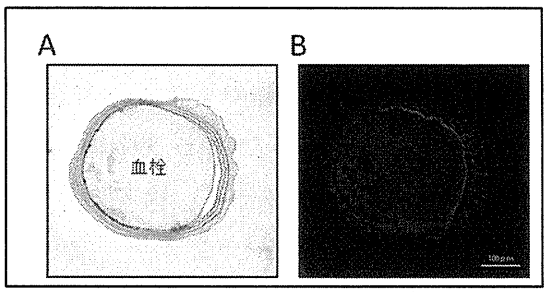

図 5. マウス頸動脈血栓モデルの作製

D. 考察

本年後の研究の結果、Sonazoid-Lactadherin 複合体の新生血管及び新鮮血栓に対する分子標的性を検証するための実験動物モデルを確立することができた。また、担癌モデルマウスにおいては、造影超音波法で癌微小循環の可視化が可能であることも同時に確認できた。分子標的気泡を用いた超音波分子イメージングでは、標的分子と気泡とが反応する時間が必要であることから、気泡投与後しばらく経ってから撮像を開始する事が一般的である。理想的には、この撮像開始時に標的分子に結合しなかった気泡は血液中に残存していないことが望ましい。しかし、標的分子への気泡の集積率を向上させるためには、多くの分子標的気泡を投与することが必要となり、この際には標的分子に結合しなかった気泡の存在が問題となってくる。本年度の基礎検討では、若干多めの 1.2×10^6 bubbles の Sonazoid を投与することで癌微小循環の可視化が可能

であった。Sonazoid 投与 10 分後の超音波画像を同時に評価したところ、この時点では腫瘍内にほとんど気泡の残存を認めなかったことから、腫瘍血管に対する Sonazoid-Lactadherin 複合体の分子標的性の検討には、10^6 オーダーの気泡量でも十分評価可能だと思われた。

担癌モデルマウスにおいては、造影超音波法が問題なく施行できたが、頸動脈血栓モデルマウスでも同様に造影超音波法が施行できるか否かについては検証が必要である。マウス頸動脈は外径が 1mm 程度と細いため、高周波数の超音波での映像化が必須である。しかし、周波数が高くなると、気泡の共振周波数との乖離が大きくなるため、十分な造影効果が得られない可能性がある。他方、造影超音波法に有利な低周波数で画像化を行うと、十分な空間分解能を有する画像が得られないという懸念がある。この点については、早急に検討を行い、最適な超音波装置の条件を見極める必要がある。

C. 結論

本年度の検討により、造影超音波法を用いた超音波分子イメージングの医学的有用性を検証する in vitro 及び in vivo の実験系を確立することができた。今後、これらの実験系を用いて Sonazoid-Lactadherin 複合体の新生血管及び新鮮血栓に対する分子標的性について検討を進めて行く予定である。

D. 健康危険情報

該当なし。

E. 研究発表

1. 論文発表

Otani K, Yamahara K. Feasibility of lactadherin-bearing clinically available microbubbles as ultrasound contrast agent for angiogenesis. **Mol Imaging Biol** 2013;15(5):534-541.

2. 学会発表

【シンポジウム】

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F. 知的財産権の出願・登録状況

1. 特許取得

該当なし。

2. 実用新案登録

該当なし。

3. その他

研究協力者　なし

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Otani K, Yamahara K.	Feasibility of lactadherin-bearing clinically available microbubbles as ultrasound contrast agent for angiogenesis	**Mol Imaging Biol**	15(5)	534-541	2013

III. 研究成果の刊行物・別刷

MOLECULAR IMAGING AND BIOLOGY

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RESEARCH ARTICLE

Feasibility of Lactadherin-Bearing Clinically Available Microbubbles as Ultrasound Contrast Agent for Angiogenesis

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Abstract

Objectives: Phagocytosis of apoptotic cells is carried out through bridging of phosphatidylserine (PS)-expressing apoptotic cells and integrin αvβ3-expressing phagocytes with lactadherin. The objective of this study was to examine whether microbubbles targeted to integrin αvβ3 could be produced by conjugating a PS-containing clinically available ultrasound contrast agent with lactadherin.

Materials and Methods: PS-containing perfluorobutane-filled microbubbles were incubated with R-phycoerythrin (PE)-labeled lactadherin, and the presence of PE-positive bubbles was examined by FACS analysis. Secondly, the attachment of lactadherin to integrin αvβ3-expressing cells (human umbilical vein endothelial cells (HUVEC)) was also examined by FACS analysis. Finally, the adhesion of PS-containing bubbles to HUVEC was examined using a parallel plate flow chamber. The number of adherent bubbles with or without the intermediation of lactadherin was compared.

Results: The more lactadherin was added to the bubble suspension, the more PE-positive bubbles were detected. The size of bubbles was not increased even after conjugation with lactadherin (2.90±0.04 vs. 2.81±0.02 μm). Binding between lactadherin and HUVEC was also confirmed by FACS analysis. The parallel plate flow chamber study revealed that the number of PS-containing bubbles adherent to HUVEC was increased about five times by the intermediation of lactadherin (12.1±6.0 to 58.7±33.1 bubbles).

Conclusion: Because integrin αvβ3 is well-known to play a key role in angiogenesis, the complex of PS-containing bubbles and lactadherin has feasibility as a clinically translatable targeted ultrasound contrast agent for angiogenesis.

Key words: Microbubble, Ultrasound molecular imaging, Sonazoid, Integrin αvβ3, Angiogenesis

Introduction

Ultrasound molecular imaging, which utilizes molecular-targeted bubbles, is a powerful tool for the noninvasive understanding of molecular dynamics *in situ*. The usefulness of ultrasound molecular imaging has been demonstrated in animal models of vascular disease and angiogenesis [1–4]. Although a lot of molecular-targeted bubbles have been developed for animal studies, the clinical translation of these targeted bubbles is still challenging.

Sonazoid (Daiichi-Sankyo Pharmaceuticals, Tokyo, Japan), perfluorobutane gas microbubbles stabilized by a membrane of hydrogenated egg phosphatidylserine (PS), is clinically available in Japan [5]. In 2000, Lindner et al. reported that PS-

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containing bubbles could be labeled with annexin V [6]. Based on their result, we recently demonstrated the feasibility of preparation of antibody-carrying bubbles based on Sonazoid through annexin V and biotin-avidin complex formation [7]. Because annexin V binds with PS in a Ca^{2+}-dependent manner, the conjugation of antibodies was performed in the presence of Ca^{2+}. However, significant aggregation and disappearance of Sonazoid bubbles were observed after the addition of Ca^{2+} [7]. Additionally, the binding between Sonazoid bubbles and annexin V was quite fragile [7, 8]. Therefore, an alternative molecule that does not require Ca^{2+} for the detection of PS in Sonazoid is desirable in the preparation of targeted bubbles based on Sonazoid.

Milk fat globule epidermal growth factor 8 (MFG-E8)/lactadherin is a secreted glycoprotein which was originally identified as a component of milk fat globules [9]. Lactadherin contains a PS-binding C-domain and an RGD (arginine–glycine–aspartic acid) motif residing in the epidermal growth factor domain and has the feature of forming a bridge between PS on apoptotic cells and integrin αvβ3 on phagocytes [10–12]. It is noteworthy that the binding between PS and lactadherin is Ca^{2+}-independent [13, 14]. Therefore, we hypothesized that lactadherin has the potential to be a mediator between PS-containing bubbles and integrin αvβ3-expressing cells. In other words, the complex of PS-containing bubbles and lactadherin has the potential to be a novel integrin αvβ3-targeted ultrasound contrast agent (Fig. 1). The aim of this study was to examine whether microbubbles targeted to integrin αvβ3 could be produced by conjugating a PS-containing clinically available ultrasound contrast agent with lactadherin.

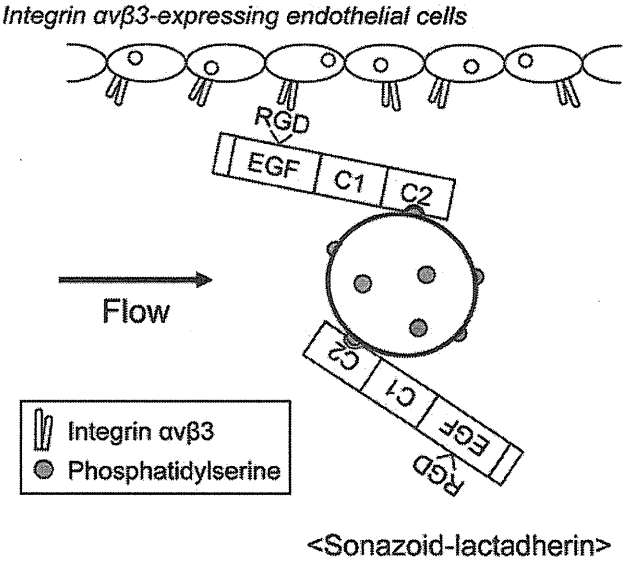

Fig. 1. Expected interaction between lactadherin-bearing Sonazoid bubbles and integrin αvβ3-expressing endothelial cells under flow condition. Epidermal growth factor-like domain (*EGF*), factor VIII-homologous domains (*C1* and *C2*), arginine–glycine–aspartic acid (*RGD*).

Materials and Methods

Preparation of Lactadherin-Bearing Sonazoid Bubbles

Sonazoid bubbles (1.2×10^8 bubbles/100 μl) were incubated with 0, 0.1, 1, 2, 5, or 10 μg phycoerythrin (PE)-labeled recombinant human MFG-E8/lactadherin (2767-MF, R&D systems, Inc., Minneapolis, MN) in microtubes for 15 min at room temperature. PE-labeling of lactadherin was performed using an R-phycoerythrin labeling kit (LK23, Dojindo Laboratories, Kumamoto, Japan). The concentration of lactadherin after PE-labeling was 0.1 mg/ml, and the added volume of lactadherin was set at 100 μl. After incubation with PE-lactadherin, the bubble suspension was washed with sterile water, and centrifuged (100×*g*, 1 min). The washing process was repeated. Then, Sonazoid bubbles were assessed using a FACSCalibur (BD Bioscience, San Jose, CA) with 50,000 counts. Mean fluorescence intensity was calculated from the fluorescence histogram.

Secondly, Sonazoid bubbles incubated only with 100 μl PE-dye (equivalent of 5 μg PE-lactadherin) were also assessed to examine the nonspecific binding of PE dye with Sonazoid bubbles. Finally, FACS analysis was repeated after violent shaking to examine the stability of binding between Sonazoid bubbles and PE-lactadherin. All FACS studies were performed three times.

Effect of Conjugation with Lactadherin on Size of Sonazoid Bubbles

The size distribution of Sonazoid bubbles that were incubated with 5 μg lactadherin (Sonazoid–lactadherin) was determined using the electrozone sensing method (Multisizer III, Beckman Coulter, Inc., Fullerton, CA). Prior to measurement, bubbles were diluted 1,000-fold with Coulter Isoton II diluent (Beckman Coulter). The mean and median diameters were calculated from the histogram of the volume-weighted size distribution of 50,000 bubbles. As a control, the size distribution of Sonazoid bubbles incubated with 100 μl saline (Sonazoid–phosphate-buffered saline (PBS)) was also determined.

Acoustic Property of Sonazoid–Lactadherin

To compare the acoustic property of Sonazoid–lactadherin with Sonazoid–PBS, *in vitro* experiments were performed, as previously reported [15]. Briefly, bubble suspension (1 ml; 1×10^3, 1×10^4, 1×10^5, and 1×10^6 bubbles/ml) was added to the sample wells of a custom-made 2 % (*w/v*) agarose mold, then real-time (frame rate, 30 Hz) ultrasound images were acquired with a clinical ultrasound scanner system (SONOS 5500, Philips Healthcare, Bothell, WA) equipped with a 15–6-l probe (6–15 MHz). The acoustic power was set at −20 dB, which corresponds to a mechanical index of 0.1. The image depth was set at 4 cm. The position of focus was set at the center of the agarose mold, and the overall gain setting was optimized at the beginning of the experiment and kept constant throughout the experiment. Acquired contrast images were transferred to an off-line computer (QLab, Philips Healthcare) to measure the baseline-subtracted video intensity at each experimental setting.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, MD) and utilized as positive control cells that express integrin αvβ3 on their surface. HUVEC were cultured in EBM-2 medium supplemented with an EGM-2 bullet kit (Lonza). All studies were examined at passage 3 or 4. For the parallel plate flow chamber study, HUVEC were seeded on 33-mmΦ glass cover slips.

Flow Cytometry

Firstly, the expression of integrin αvβ3 on the surface of HUVEC was confirmed. Anti-human integrin αvβ3 (MAB1976Z; Millipore Co., Billerica, MA) or isotype control mouse IgG_1 (CBL600; Millipore) antibodies were labeled using a Zenon® Alexa Fluor® 488 mouse IgG_1 labeling kit (Z25002; Invitrogen). After harvesting with cell dissociation buffer (13150–016; Invitrogen) with collagenase (032–10534; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 5×10^5 HUVEC were incubated with 2 μg integrin αvβ3 or isotope control antibodies for 30 min at room temperature. After washing and centrifugation twice, HUVEC were labeled with 7AAD (BD Biosciences, San Jose, CA), and 10,000 viable cells were analyzed using a FACSCalibur.

Secondly, we examined whether lactadherin is able to attach to integrin αvβ3 on HUVEC. Dispersed HUVEC were incubated with 2 μg PE-labeled lactadherin for 15 min at room temperature. After washing and centrifugation twice, HUVEC labeled with 7AAD were analyzed by FACSCalibur. To examine the nonspecific binding of PE dye with HUVEC, HUVEC incubated only with an equal amount of PE dye (equivalent of 2 μg PE-lactadherin) were also analyzed.

Specificity of Binding Between Lactadherin and HUVEC

To clarify the specificity of binding between lactadherin and HUVEC, experiments with solid-phase ELISA were performed [16]. The 96-well Maxisorp plates (439454, Thermo Fisher Scientific, Inc., Waltham, MA) were coated with lactadherin (10 μg/ml in PBS) by incubating them overnight at 4 °C with 50 μl/well. The wells were blocked with 7.5 % bovine serum albumin (BSA, A9418, Sigma-Aldrich Co., St. Louis, MO) for 4 h at 4 °C. Detached HUVEC was suspended in EBM-2 medium containing 1 % BSA, and then incubated with anti-integrin αvβ3 antibody (10 μg/ml) or cRGD peptide (10 and 100 μg/ml, Bachem AG, Bubendorf, Switzerland) for 30 min at room temperature. After washing, cells were resuspended at 4×10^5 cells/ml in EBM-2 medium containing 1 % BSA, then 100 μl cell suspension was added to each well. The plates were incubated for 15 min at room temperature. After washing with PBS to remove the unattached cells, the attached cells were fixed with 1 % glutalaldehyde for 10 min, stained with 0.1 % crystal violet for 20 min at room temperature. The cells were lysed with 50 μl of Triton X-100 (0.5 %), and the absorbance (595 nm) was measured by microplate reader (model 680, Bio-Rad Laboratories, Hercules, CA). Additionally, the adherent HUVEC was visualized microscopically after staining with crystal violet. Binding assay was carried out three times in triplicate.

Parallel Plate Flow Chamber Assay

The adhesion of Sonazoid bubbles to HUVEC was assessed using a parallel plate flow chamber system (Glycotech, Gaithersburg, MD) [17]. The silicon gasket used in this study has a width of 2.5 mm and a height of 0.254 mm (Gasket B). Cover slips of 33 mmΦ were mounted in the chamber and placed in an inverted position to maximize the interaction between HUVEC and bubbles. Bubble dilution and washing of the flow chamber were performed with fetal bovine serum-reduced EGM-2 medium (0.5 %). After 2 min of washing of the flow chamber system, 5×10^6/ml of bubbles were drawn through the chamber at a shear stress of 0.7 dynes/cm^2 over 4 min, followed by rinsing for 6 min. The number of bubbles that adhered in 15 fields of view (FOV; 273 × 362 μm) was determined under a microscope (Biorevo BZ-9000; Keyence Co., Osaka, Japan) equipped with a ×40 objective lens. Flow chamber studies were performed three times.

Statistical Analysis

All data were expressed as mean ± standard deviation. For comparison between two groups, Student's unpaired *t* test was applied. Comparison among the three groups was performed by analysis of variance followed by post hoc Tukey–Kramer test. A *p* value less than 0.05 was considered to indicate statistical significance for all comparisons.

Results

Representative FACS histograms of Sonazoid bubbles after incubation with PE-lactadherin are shown in Fig. 2a. Higher mean fluorescence intensity was observed by increasing the dose of lactadherin (0 μg [3.1±0.1] vs. 0.1 μg [10.4±1.1] vs. 1 μg [60.1±7.3] vs. 2 μg [113.8±19.2] vs. 5 μg [159.9±10.1] (Fig. 2d). However, mean fluorescence intensity was significantly decreased by increasing the lactadherin dose to 10 μg [118.9± 10.9] (Fig. 2d). PE-positive Sonazoid bubbles were detected even after incubation only with PE dye (Fig. 2b). However, the fluorescence intensity of PE dye-bearing Sonazoid bubbles was significantly lower than that of PE-lactadherin-bearing Sonazoid bubbles (51.3±4.2 vs. 159.9±10.1) (Fig. 2d). Because the binding between Sonazoid bubbles and lactadherin was robust, the histogram of FACS analysis and mean fluorescence intensity did not change even after violent shaking (before shaking; 159.9± 10.1 vs. after shaking; 152.7±8.1) (Fig. 2c, d)

Representative histograms of Sonazoid–PBS and Sonazoid–lactadherin are shown in Fig. 3a. The size distribution of Sonazoid bubbles was not altered even after conjugation with lactadherin. In quantitative analysis, the size of Sonazoid bubbles slightly decreased during the process of washing and centrifugation (untreated Sonazoid, 2.97±0.01 μm vs. Sonazoid–PBS, 2.90±0.04 μm mean diameter, untreated Sonazoid, 2.80±0.01 μm vs. Sonazoid–PBS, 2.75±0.03 μm median diameter). However, the mean and median diameters of Sonazoid bubbles were not increased even after conjugation with lactadherin (Sonazoid–lactadherin, 2.81±0.02 μm mean diameter and 2.69±0.02 μm median diameter) (Fig. 3b).

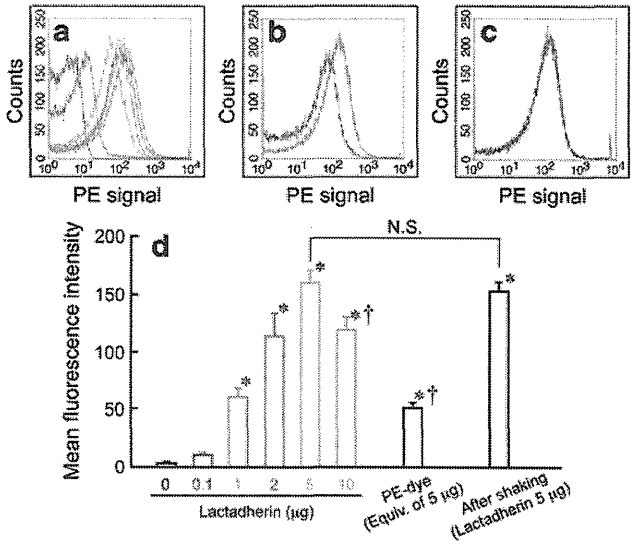

Fig. 2. Binding between Sonazoid bubbles and lactadherin. **a** PE signals derived from Sonazoid bubbles increased concomitantly with the added dose of PE-lactadherin; 0 μg, *black*; 0.1 μg, *red*; 1 μg, *green*; 2 μg, *pink*; 5 μg, *blue*; 10 μg, *orange line*. **b** Compared to incubation with PE dye, a higher PE signal was observed after incubation with PE-lactadherin. *Black line*, PE dye; *red line*, PE-lactadherin. **c** Binding between lactadherin and Sonazoid bubbles was maintained even after violent shaking. *Black line*, before shaking; *red line*, after shaking. **d** Quantitative analysis of fluorescence intensity. $^*P<0.05$ vs. 0 μg lactadherin, †$P<0.05$ vs. 5 μg lactadherin. *N.S.* not significant.

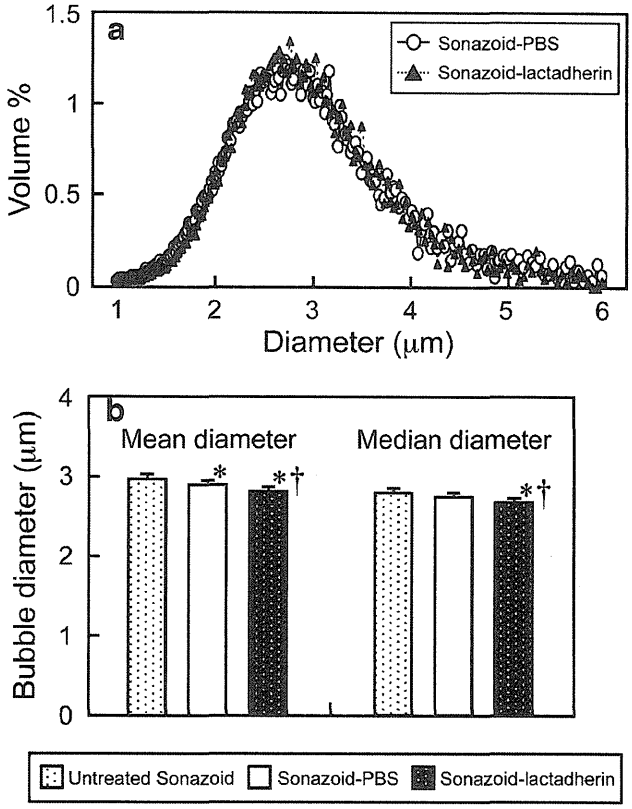

Fig. 3. Size distribution and diameters of Sonazoid bubbles. **a** The histogram of bubble size distribution was almost the same even after conjugation with lactadherin. **b** Even after conjugation with lactadherin, the mean and median diameters of Sonazoid bubbles were not increased. $^*P<0.05$ vs. Untreated Sonazoid, †$P<0.05$ vs. Sonazoid–PBS.

Representative ultrasound images of Sonazoid–PBS and Sonazoid–lactadherin are shown in Fig. 4a. The video intensity was increased concomitant with the dose of bubbles, irrespective of the incubation with lactadherin (Sonazoid–PBS, 6.9±5.8 vs. 21.5±3.1 vs. 69.8±2.0 vs. 115.0±2.0; Sonazoid–lactadherin, 10.2±3.9 vs. 35.5±3.6 vs. 82.3±5.8 vs. 113.4±1.4) (Fig. 4b). It was noteworthy that the acoustic property of Sonazoid bubbles was not impaired even after incubation with lactadherin.

The expression of integrin αvβ3 on HUVEC was supported by the significantly higher fluorescence intensity after incubation with anti-integrin αvβ3 antibody (Fig. 5a). Binding between HUVEC and PE-lactadherin was also examined by FACS analysis. Although a slight increase in the PE signal was observed even after conjugating HUVEC with PE dye, the mean fluorescence intensity after incubation with PE-lactadherin was significantly higher than that after incubation with PE dye (HUVEC; 3.2±0.3 vs. PE dye; 13.6±1.2 vs. lactadherin; 127.4±12.9) (Fig. 5b). This result indicates that binding between lactadherin and integrin αvβ3-expressing HUVEC is feasible.

Representative photographs of adherent HUVEC to lactadherin are summarized in Fig. 6a. Although the adherent HUVEC were hardly detected in the absence of lactadherin, a lot of HUVEC adhered to lactadherin-coated well (optical density; 0.007±0.001 vs. 0.115±0.019) (Fig. 6b). However, the number of adherent HUVEC to lactadherin was significantly decreased by pre-incubating with anti-integrin αvβ3 antibody or cRGD peptide (0.071±0.003 in anti-integrin αvβ3, 0.054±0.025 in 100 μg/ml cRGD, respectively).

Figure 7a shows representative images of HUVEC perfused with Sonazoid–PBS or Sonazoid–lactadherin under shear flow. Compared to perfusion with Sonazoid–PBS, a large number of adherent bubbles were observed on HUVEC after perfusion with Sonazoid–lactadherin. In quantitative analysis, the number of adherent bubbles was increased about fivefold with the intermediation of lactadherin (12.1±6.0 vs. 58.7±33.1 bubbles/FOV) (Fig. 7b).

Discussion

PS is well-known as an important molecule for the clearance of apoptotic cells, and several kinds of proteins that bind with PS have been discovered [18, 19]. Among them, it has been established that lactadherin binds with PS in a Ca^{2+}-independent manner [13, 14]. As we expected, the synthesis of lactadherin-bearing Sonazoid bubbles was feasible without requiring the addition of Ca^{2+} (Fig. 2a), and the binding between PS in Sonazoid and lactadherin was robust even after violent shaking (Fig. 2c) [8]. It was noteworthy that the

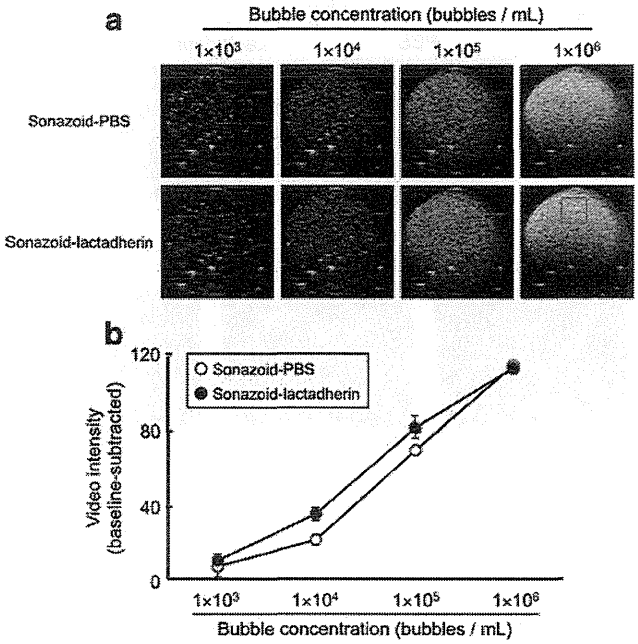

Fig. 4. Comparison of acoustic property of Sonazoid with or without lactadherin conjugation. **a** Representative ultrasound images at each bubble concentration. The region of interest is shown in a *red square*. **b** Baseline-subtracted video intensity at each experimental setting. The acoustic property of Sonazoid was not impaired even after incubation with lactadherin.

size of bubbles was not increased even after incubation with lactadherin (Fig. 3a, b). Additionally, the acoustic property of Sonazoid–lactadhrin was comparable to that of Sonazoid–PBS (Fig. 4). These results imply that an intravenous infusion of lactadherin-bearing Sonazoid bubbles would have little risk of embolization and yield sufficient contrast enhancement. As shown in Fig. 7, lactadherin augmented the number of Sonazoid bubbles adherent to integrin αvβ3-expressing endothelial cells. Furthermore, the specificity of binding between lactadherin and integrin αvβ3 on HUVEC was also confirmed (Fig. 6). Taken together, our results imply that lactadherin-bearing Sonazoid bubbles have potential as a novel and easy-to-prepare ultrasound contrast agent for detecting integrin αvβ3-expressing cells in ultrasound molecular imaging.

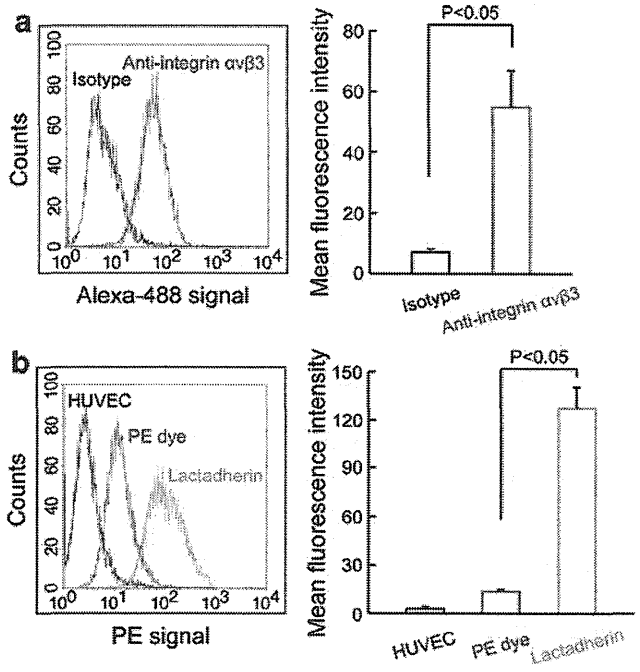

Fig. 5. Binding between integrin αvβ3-expressing HUVEC and lactadherin. **a** The fluorescence intensity derived from HUVEC after incubation with anti-integrin αvβ3 antibody was significantly higher than that after incubation with isotype control (55.0±12.2 vs. 7.1±0.7) (n=3). **b** A significant increase in mean fluorescence intensity was observed by conjugating HUVEC with PE-lactadherin (n=6).

In our previous study, we demonstrated the feasibility of antibody-carrying bubbles preparation based on Sonazoid via annexin V and biotin–avidin complex formation [7]. However, the addition of Ca^{2+}, which is necessary for binding between annexin V and PS, resulted in the obvious bubble aggregation and bubble loss. Additionally, the binding between annexin V and PS was quite fragile. Fortunately, this was not the case with lactadherin because lactadherin binds with PS in a Ca^{2+}-independent manner (Fig. 2c). In this regard, lactadherin is superior to annexin V as a mediator for detecting PS in Sonazoid.

As well as vascular endothelial growth factor (VEGF) and VEGF receptors, integrins have been identified as target molecules for imaging of angiogenesis [20, 21]. Integrin αvβ3, one of the integrin families, has been considered a useful molecule for detecting tumor angiogenesis because integrin αvβ3 is known to play a key role in angiogenesis [22]. In ultrasound molecular imaging, several studies of integrin-targeted imaging have been performed by conjugating echistatin, RGD peptides, and antibodies on the surface of bubbles [23–26]. The majority of targeted bubbles have utilized streptavidin as a

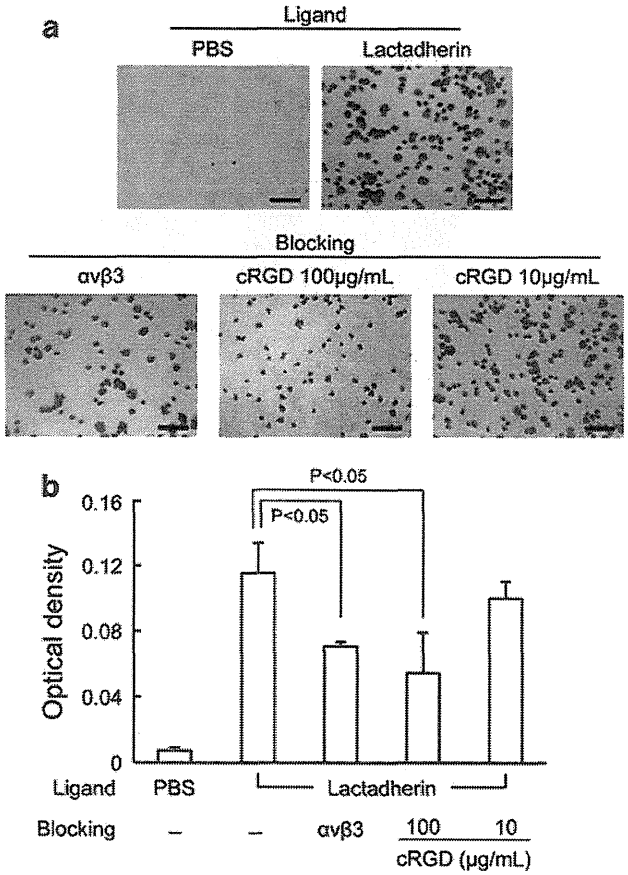

Fig. 6. Adhesion of HUVEC to the lactadherin-coated plates. **a** Representative photographs of adherent HUVEC at each experimental setting. Scale bars = 100 μm. **b** Optical density was significantly decreased by pre-incubating HUVEC with anti-integrin αvβ3 antibody or cRGD peptide.

mediator for conjugating antibodies or peptides onto the surface of bubbles. However, the clinical application of streptavidin-conjugated microbubbles would be difficult due to immunogenicity [27]. To overcome this issue, clinically translatable bubbles that do not contain streptavidin have been developed recently [28–31]. In addition to the nonuse of streptavidin for targeted bubble preparation, the utilization of a clinically available ultrasound contrast agent is the prime advantage of our approach. In this regard, lactadherin-bearing Sonazoid bubbles might lower the barrier for the clinical translation of targeted bubbles.

Weaknesses of Lactadherin-Bearing Sonazoid Bubbles

To improve the flexibility, hydrophilicity, and targetability of targeted bubbles, the majority of recently developed targeted bubbles project antibodies or peptides away from the surface of the bubble shell by means of a polyethylene glycol arm [3, 32–34]. In contrast, lactadherin binds directly with PS in the shell of Sonazoid bubbles in our approach (Fig. 1). As shown in Fig. 5, a larger number of Sonazoid bubbles were adherent to HUVEC by the intermediation of lactadherin in the flow chamber study. However, the flexibility and targetability of lactadherin-bearing Sonazoid bubbles might be lower than those of other conventional targeted bubbles. Additionally, the surface density of lactadherin (i.e., RGD motif) might also be low. Considering these issues, further study examining an alternative approach that utilizes lactadherin as a mediator to attach antibodies or peptides on the surface of Sonazoid bubbles would be beneficial.

With regard to the administration of lactadherin in an *in vivo* study, Asano et al. reported the possibility of autoantibody production by administration of an excess amount of lactadherin in mice [35]. Therefore, the optimal preparation method with minimum use of lactadherin should be established in a future study for the *in vivo* and clinical translation of lactadherin-bearing Sonazoid bubbles.

Study Limitation

In addition to the bubble size determination, the concentration of bubbles after the preparation of targeted Sonazoid bubbles was also determined using Multisizer III (n=3). As a result, the bubble concentration was decreased to one fifth due to dilution of the bubble suspension (untreated Sonazoid, $9.5\pm0.2 \times 10^8$ bubbles/ml vs. Sonazoid–PBS, $2.0\pm0.1 \times 10^8$ bubbles/ml)

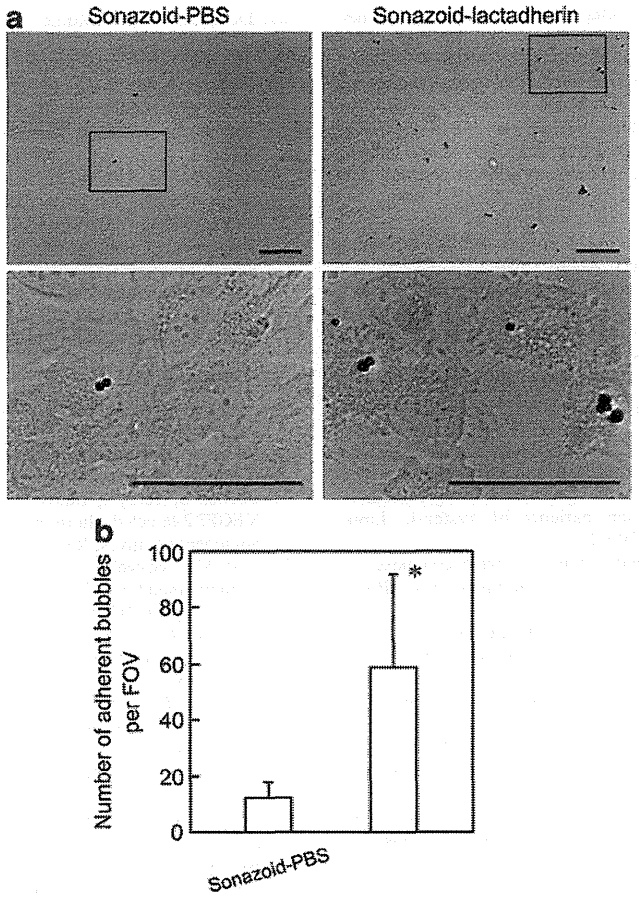

Fig. 7. Adhesion of Sonazoid bubbles to HUVEC under shear flow with or without the intermediation of lactadherin. **a** The number of Sonazoid bubbles adherent to HUVEC was increased by the intermediation of lactadherin. The *boxed areas* in *upper panels* are shown at higher magnification in *lower panels*. Scale bars = 50 μm. **b** Quantitative analysis of adherent bubbles per field of view. *FOV* field of view. *$P<0.05$ vs. Sonazoid–PBS.

(Supplementary Fig. 1a). Additionally, the decrease in bubble concentration was further enhanced by incubation with lactadherin (Sonazoid–lactadherin, $1.1\pm0.1 \times 10^8$ bubbles/ml) due to adhesion of bubbles to the wall of microtubes (Supplementary Fig. 1b). However, this loss of bubbles could partly be avoidable by coating the surface of the microtubes with 2-methacryloyloxyethyl phosphorylcholine polymer (NOF Corp., Tokyo, Japan) to suppress protein adsorption (data not shown). Furthermore, the use of glass tubes for targeted bubble preparation might further avoid the loss of bubbles. Ideally, omission of the washing process would be desirable for easy preparation of targeted bubbles. Therefore, further study regarding the development of lactadherin-bearing Sonazoid bubbles without the washing process would be beneficial.

Although the feasibility of lactadherin-bearing Sonazoid bubbles as targeted contrast agent for integrin αvβ3 was demonstrated, the potential of lactadherin-bearing Sonazoid bubbles to visualize the neovascularization *in vivo* is still unknown. Therefore, further study examining the diagnostic utility of lactadherin-bearing Sonazoid bubbles in animal models of tumor or therapeutic angiogenesis should be required.

Conclusion

In the present study, we demonstrated that adhesion of Sonazoid bubbles to integrin αvβ3-expressing endothelial cells was augmented through the intermediation of lactadherin. Lactadherin-bearing Sonazoid bubbles might be a useful contrast agent for tumors or therapeutic angiogenesis in both basic and clinical ultrasound molecular imaging in the near future.

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Conflict of interest. The authors declare that they have no conflict of interest.

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