Development of integrin αvβ3-targeted microbubbles based on clinically available ultrasound contrast agent

Kentaro Otani

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National Cerebral and Cardiovascular Center Research Institute, Suita, Japan

Background and Aim

Phagocytosis of apoptotic cells is carried out through bridging of phosphatidylserine (PS)-expressing apoptotic cells and integrin αvβ3-expressing phagocytes with lactadherin. 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 **(Figure 1)**.

Methods

PS-containing perfluorobutane-filled microbubbles (Sonazoid) 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 cell (human umbilical vein endothelial cell; HUVEC) was also examined by FACS analysis. Finally, the adhesion of Sonazoid 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

By increasing the dose of lactadherin, the number of PE-positive Sonazoid bubbles became larger. It was noteworthy that the mean diameter of Sonazoid bubbles did not change even after conjugating with lactadherin (2.90±0.04 vs. 2.81±0.02 μm). Furthermore, the acoustic property of Sonazoid bubbles was not influenced by the conjugation with lactadherin **(Figure 2)**. The binding between lactadherin and HUVEC was also confirmed by the FACS analysis. The parallel plate flow chamber study revealed that the number of Sonazoid bubbles adherent to HUVEC was increased about five times by the intermediation of lactadherin (12.1±6.0 to 58.7±33.1 bubbles) **(Figure 3)**.

Conclusion

Our study demonstrated that the development of integrin αvβ3-targeted Sonazoid bubbles could be feasible by the intermediation of lactadherin. Because integrin αvβ3 is well-known to play a key role in angiogenesis, the lactadherin-bearing Sonazoid bubbles has feasibility as a clinically translatable targeted ultrasound contrast agent for angiogenesis.

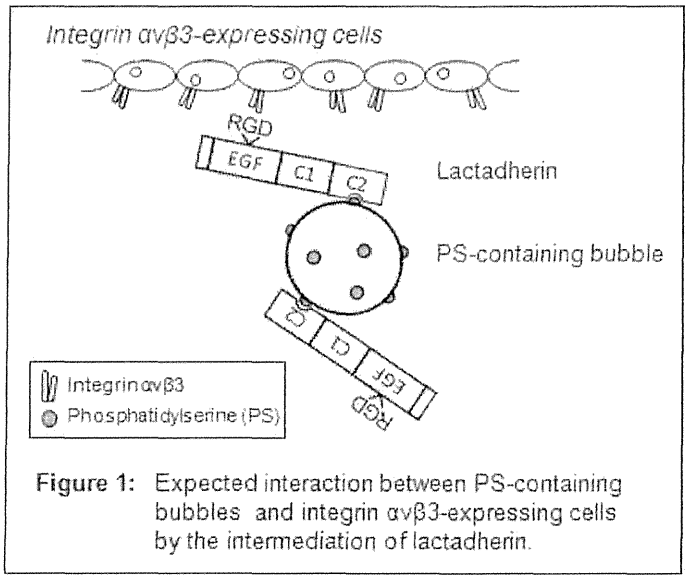

Figure 1: Expected interaction between PS-containing bubbles and integrin αvβ3-expressing cells by the intermediation of lactadherin.

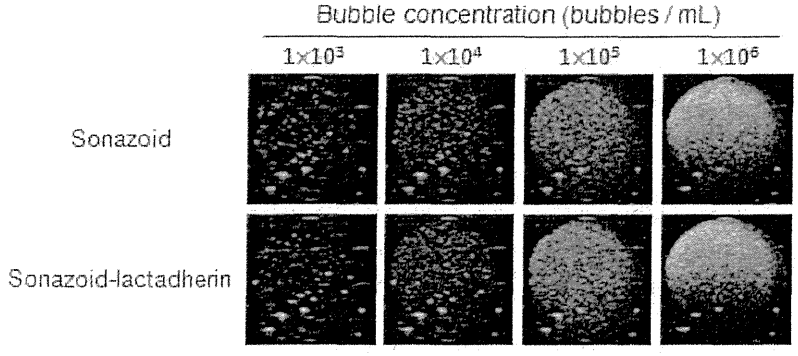

Figure 2: Comparison of acoustic property of Sonazoid with or without lactadherin conjugation.

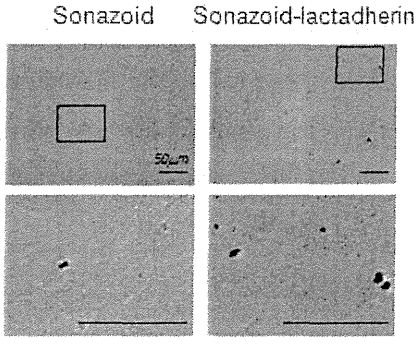

Figure 3: Adhesion of Sonazoid bubbles to HUVEC under shear flow with or without the intermediation of lactadherin. The boxed areas in upper panels are shown at higher magnification in lower panels.

Development of integrin αvβ3-targeted microbubbles based on clinically available ultrasound contrast agent

Kentaro Otani

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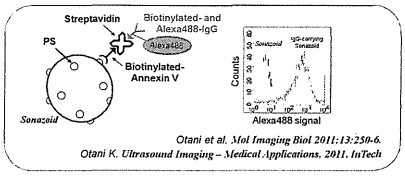
Background

- Contrast ultrasound imaging with molecular-targeted bubbles enables the noninvasive visualization of molecular dynamics in situ.

< Clinically available bubbles in Japan >

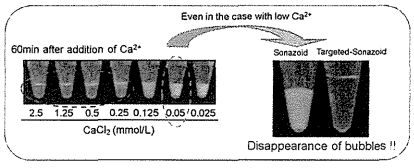
Sonazoid® *(Daiichi-Sankyo Co., Ltd.)*
- is one of 2nd generation contrast agents.
- consists of perfluorobutane gas microbubbles stabilized by a membrane of hydrogenated egg phosphatidylserine (PS).

- We reported previously the feasibility of antibody-carrying microbubbles preparation based on Sonazoid by using annexin V and biotin-avidin complex formation.



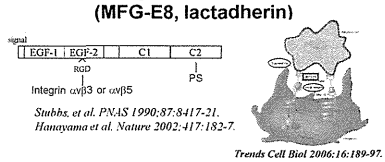
Otani et al. Mol Imaging Biol 2011;13:250-6.
Otani K. Ultrasound Imaging – Medical Applications. 2011. InTech

- However, the necessity of Ca^{2+} for achieving the binding between PS and annexin V markedly reduced the number of bubbles due to the significant aggregation.



- Additionally, the usage of biotin-avidin complex formation is a barrier for the clinical translation of molecular targeted-bubbles, because of the immunogenicity of streptavidin.

Milk fat globule EGF factor 8 (MFG-E8, lactadherin)



Stubbs, et al. PNAS 1990;87:8417-21.
Hanayama et al. Nature 2002;417:182-7.

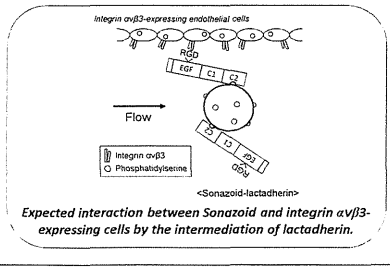
Trends Cell Biol 2006;16:189-97.

- a protein secreted from macrophages.
- accelerate the englobement of apoptotic cells.

Binding between PS and lactadherin is Ca^{2+}-independent.

Aim

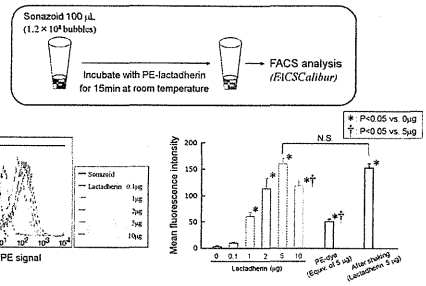
- We hypothesized that lactadherin has the potential to be a mediator between PS-containing bubbles and integrin αvβ3-expressing cells.
- The aim of this study was to examine whether microbubbles targeted to integrin αvβ3 could be produced by conjugating Sonazoid with lactadherin.



Expected interaction between Sonazoid and integrin αvβ3-expressing cells by the intermediation of lactadherin.

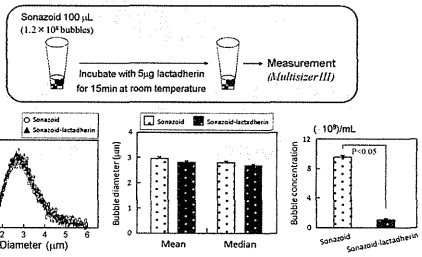
Methods and Results

1) Binding between Sonazoid and lactadherin



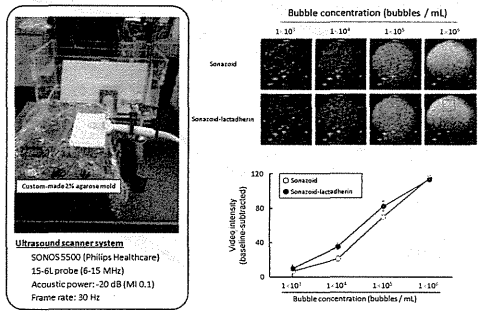
By incubating for 15 minutes at room temperature, the lactadherin is attached robustly with PS on the surface of Sonazoid bubbles.

2) Size distribution and concentration of Sonazoid before and after conjugation with lactadherin



Although the bubble concentration was reduced after the conjugation with lactadherin (due to dilution), the size of lactadherin-bearing Sonazoid was almost the same with that of naked Sonazoid.

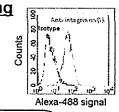
3) Acoustic property of Sonazoid with or without lactadherin conjugation

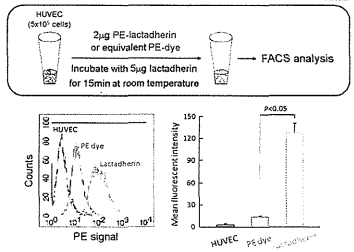


The acoustic property of Sonazoid was not impaired even after incubation with lactadherin.

4) Binding between integrin αvβ3-expressing cells and lactadherin

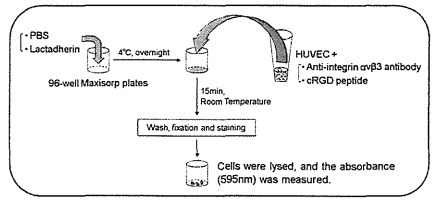
Integrin αvβ3-expressing cells:
Human Umbilical Vein Endothelial cells (HUVEC)

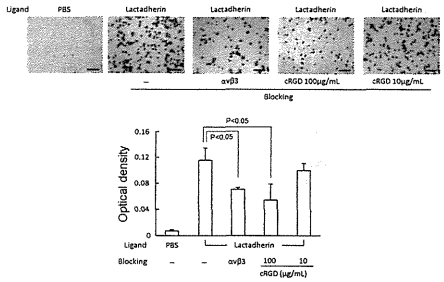




Binding between lactadherin and integrin αvβ3-expressing HUVEC was also confirmed by the FACS analysis.

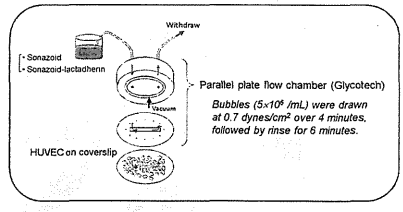
5) Specificity of binding between lactadherian and HUVEC

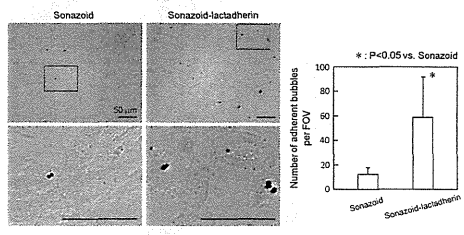




Although the adherent HUVEC were hardly detected in the absence of lactadherin, a lot of HUVEC adhered to lactadherin-coated well. However, the number of adherent HUVEC to lactadherin was significantly reduced by pre-incubating with anti-integrin αvβ3 or cRGD peptide.

6) Attachment of Sonazoid with HUVEC under shear flow





The number of attached Sonazoid bubbles to HUVEC was significantly augmented by conjugating with lactadherin (Sonazoid: 12.1±6.0 vs. Sonazoid-lacatahderin: 58.7±33.1 bubbles).

Otani et al. Mol Imaging Biol 2013;15:534-41.

Summary and Conclusion

- Bubbles size of Sonazoid was not altered even after conjugating with lactadherin.
- Attachment of Sonazoid to integrin αvβ3-expressing cells were augmented by the intermediation of lactadherin.
- Because integrin αvβ3 is well known to play a key role in angiogenesis, the lactadherin-bearing Sonazoid has feasibility as a clinically translatable targeted ultrasound contrast agent for angiogenesis.

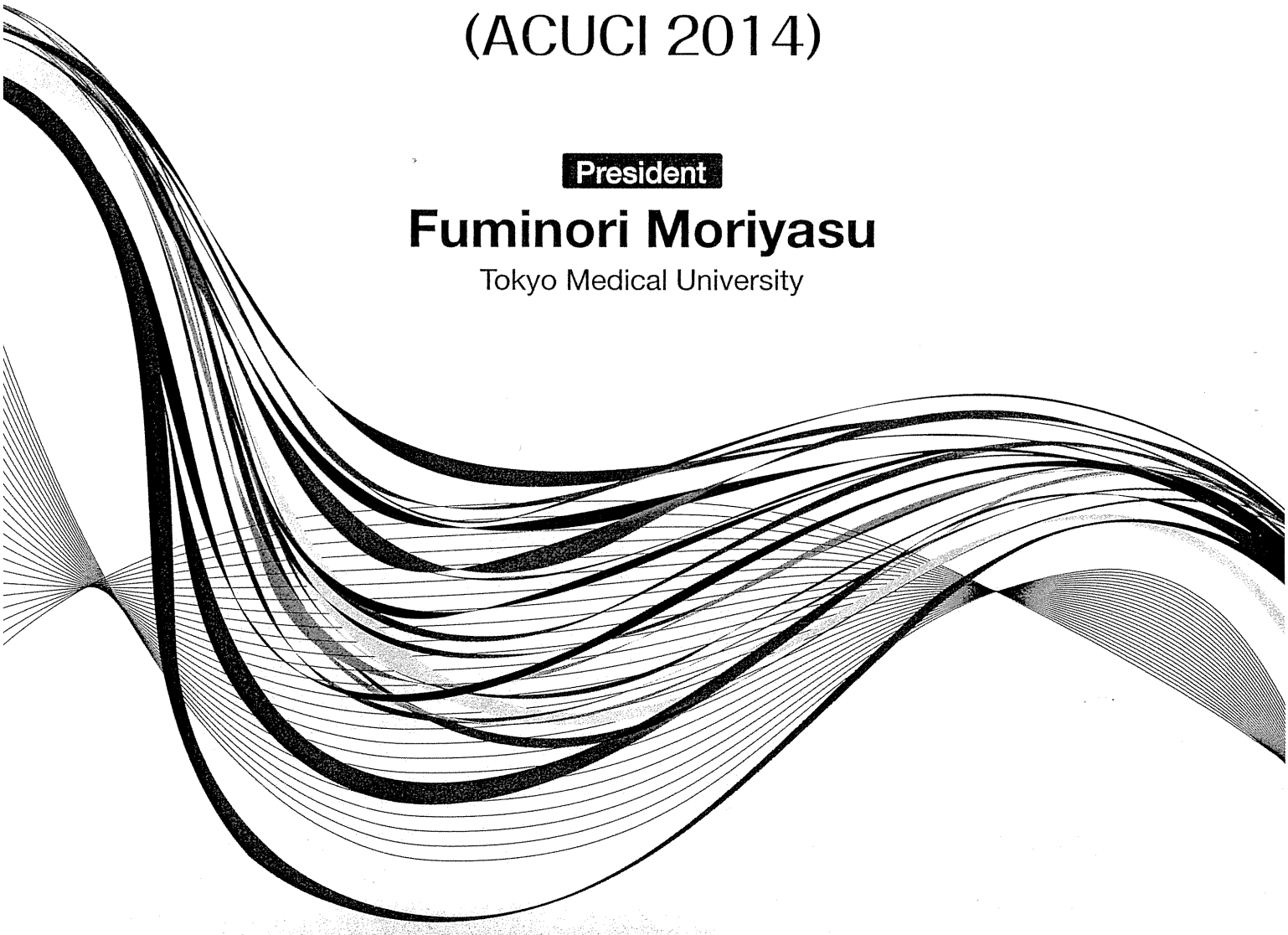
Disclosure information

I have no relationships to disclose.

第6回アジア造影超音波会議

The 6th Asian Conference on Ultrasound Contrast Imaging

(ACUCI 2014)



President

Fuminori Moriyasu

Tokyo Medical University

May 10, 2014 / Pacifico Yokohama

P-7 Development of molecular targeted-bubbles based on Sonazoid

Kentaro Otani

National Cerebral and Cardiovascular Center Research Institute

Ultrasound molecular imaging with molecular-targeted bubbles enables the noninvasive visualization of molecular dynamics in situ. Although some ultrasound contrast agents have been clinically applied for the vascular/Kupffer imaging, however the clinically translatable molecular-targeted bubble has not been developed until now. The aim of this study was to examine the feasibility of molecular-targeted bubbles preparation based on Sonazoid, a clinically available ultrasound contrast agent in Japan. As Sonazoid is stabilized by a membrane of hydrogenated egg phosphatidylserine (PS), we planned to utilize the PS as a scaffold for attaching IgGs and proteins onto Sonazoid. For detecting PS in Sonazoid, annexin V and lactadherin were utilized. By using biotin-avidin complex formation and annexin X, the attachment of IgG onto the surface of Sonazoid was feasible. However, majority of bubbles were disappeared during the bubbles preparation due to the addition of Ca^{2+} for maintaining the binding between PS and annexin V. On the other hand, lactadherin was superior to annexin V, because Ca^{2+} is unnecessary for the binding between PS and lactadherin. Furthermore, the lactadherin-bearing Sonazoid bubbles have an ability to bind with integrin $\alpha v \beta 3$-expressing endothelial cells (*Figure*). Because integrin $\alpha v \beta 3$ is well-known to play a key role in angiogenesis, the lactadherin-bearing Sonazoid might have feasibility as a clinically translatable targeted ultrasound contrast agent for angiogenesis.

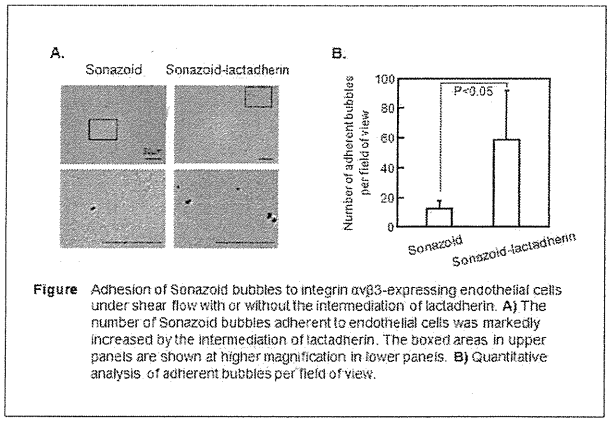


Figure Adhesion of Sonazoid bubbles to integrin αvβ3-expressing endothelial cells under shear flow with or without the intermediation of lactadherin. **A)** The number of Sonazoid bubbles adherent to endothelial cells was markedly increased by the intermediation of lactadherin. The boxed areas in upper panels are shown at higher magnification in lower panels. **B)** Quantitative analysis of adherent bubbles per field of view.

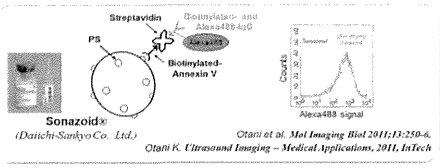
Development of molecular targeted-bubbles based on Sonazoid

Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, Suita, Japan

Kentaro Otani

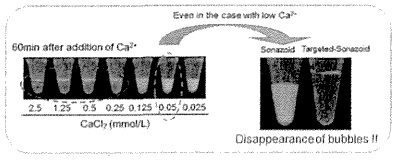
Background and Aim

- Contrast ultrasound imaging with molecular-targeted bubbles enables the noninvasive visualization of molecular dynamics in situ.
- We reported previously the feasibility of antibody-carrying microbubbles preparation based on **Sonazoid**, which consists of perfluorobutane gas microbubbles stabilized by a membrane of hydrogenated egg phosphatidylserine (PS), by using annexin V and biotin-avidin complex formation.



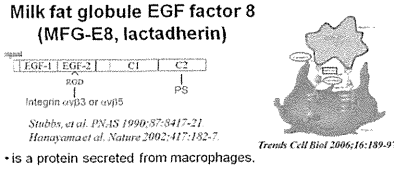
Otani et al. *Mol Imaging Biol 2011;13:250-6.*
Otani K. *Ultrasound Imaging – Medical Applications, 2011, InTech*

- However, the necessity of Ca^{2+} for achieving the binding between PS and annexin V markedly reduced the number of bubbles due to the significant aggregation.



- Additionally, the usage of biotin-avidin complex formation is a barrier for the clinical translation of molecular targeted-bubbles, because of the immunogenicity of streptavidin.

Milk fat globule EGF factor 8 (MFG-E8, lactadherin)



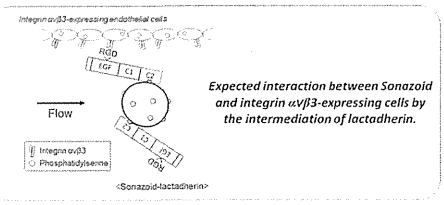
Stubbs, et al. PNAS 1990;87:8417-21
Hanayama et al. Nature 2002;417:182-7.

Trends Cell Biol 2006;16:189-97.

- is a protein secreted from macrophages.
- accelerates the englobement of apoptotic cells.

Binding between PS and lactadherin is Ca^{2+}-independent.

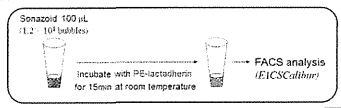
- So, we hypothesized that lactadherin has the potential to be a mediator between PS-containing bubbles and integrin αvβ3-expressing cells.
- The aim of this study was to examine whether microbubbles targeted to integrin αvβ3 could be produced by conjugating Sonazoid with lactadherin.

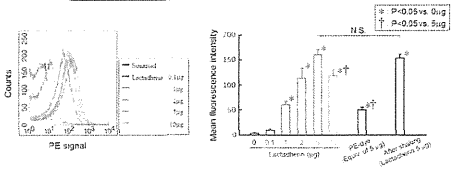


Expected interaction between Sonazoid and integrin αvβ3-expressing cells by the intermediation of lactadherin.

Methods and Results

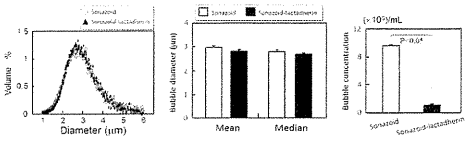
1) Binding between Sonazoid and lactadherin





By incubating for 15 minutes at room temperature, the lactadherin is attached robustly with PS on the surface of Sonazoid bubbles.

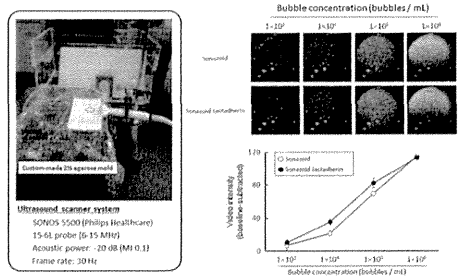
2) Size distribution and concentration of Sonazoid before and after conjugation with 5μg lactadherin



(Measured with Multisizer III)

Although the bubble concentration was reduced after the conjugation with lactadherin (due to dilution), the size of lactadherin-bearing Sonazoid was almost the same with that of naked Sonazoid.

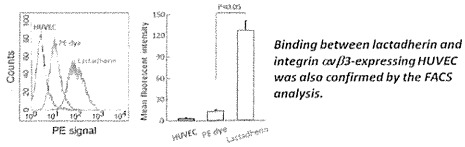
3) Acoustic property of Sonazoid with or without lactadherin conjugation



The acoustic property of Sonazoid was not impaired even after incubation with lactadherin.

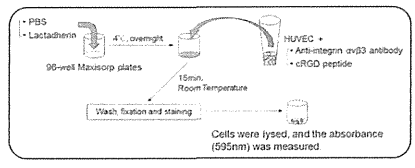
4) Binding between integrin αvβ3-expressing cells and lactadherin

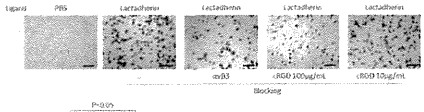
Integrin αvβ3-expressing cells:
Human Umbilical Vein Endothelial cells (HUVEC)

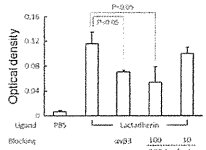


Binding between lactadherin and integrin αvβ3-expressing HUVEC was also confirmed by the FACS analysis.

5) Specificity of binding between lactadherian and HUVEC







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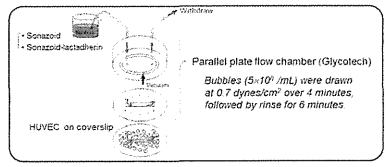
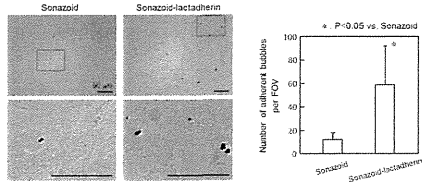
However, the number of adherent HUVEC to lactadherin was significantly reduced by pre-incubating with anti-integrin αv63 or cRGD peptide.

6) Attachment of Sonazoid with HUVEC under shear flow





The number of attached Sonazoid bubbles to HUVEC was significantly augmented by conjugating with lactadherin (Sonazoid: 12.1±6.0 vs. Sonazoid-lacatahderin: 58.7±33.1 bubbles).

Otani et al. *Mol Imaging Biol 2013;15:534-41.*

Summary and Conclusion

- Bubbles size of Sonazoid was not altered even after conjugating with lactadherin.
- Attachment of Sonazoid to integrin αvβ3-expressing cells were augmented by the intermediation of lactadherin.
- Because integrin αvβ3 is well known to play a key role in angiogenesis, the lactadherin-bearing Sonazoid has feasibility as a clinically translatable targeted ultrasound contrast agent for angiogenesis.

Disclosure information

I have no relationships to disclose.

III. 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

IV. 研究成果の刊行物・別刷

MOLECULAR IMAGING AND BIOLOGY

Mol Imaging Biol (2013) 15:534–541
DOI: 10.1007/s11307-013-0630-2

Published Online: 29 March 2013

World Molecular Imaging Society

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.

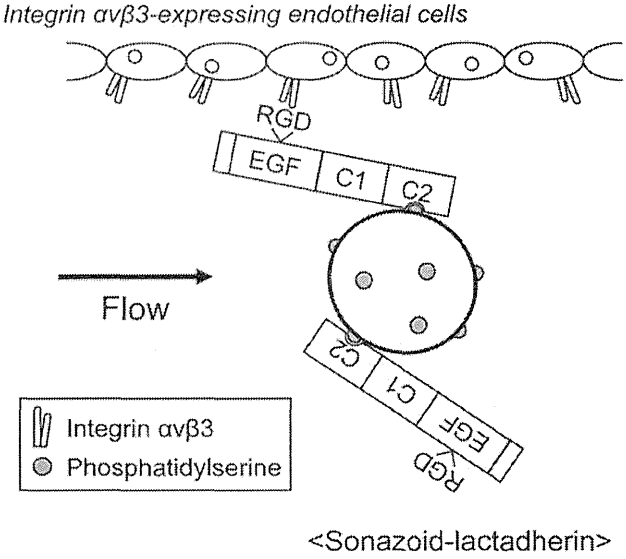

Fig. 1. Expected interaction between lactadherin-bearing Sonazoid bubbles and integrin αvβ3-expressing endothelial cells under flow condition. Epidermal growth factor-like domain (*EGF*), factor VIII-homologous domains (*C1* and *C2*), arginine–glycine–aspartic acid (*RGD*).

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).

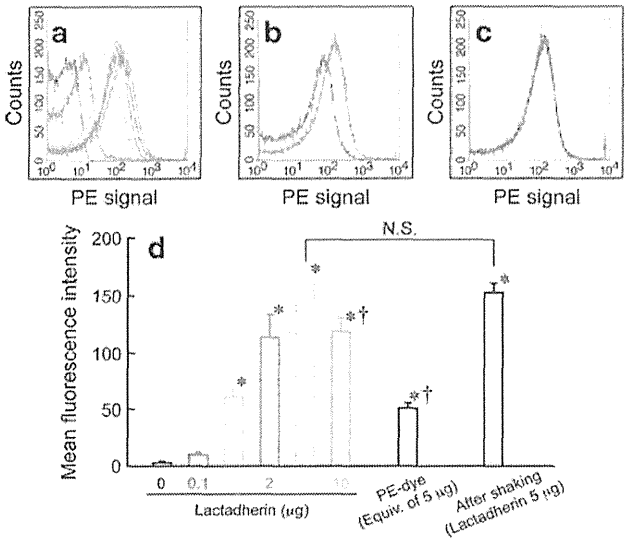

Fig. 2. Binding between Sonazoid bubbles and lactadherin. **a** PE signals derived from Sonazoid bubbles increased concomitantly with the added dose of PE-lactadherin; 0 μg, *black*; 0.1 μg, *red*; 1 μg, *green*; 2 μg, *pink*; 5 μg, *blue*; 10 μg, *orange line*. **b** Compared to incubation with PE dye, 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.

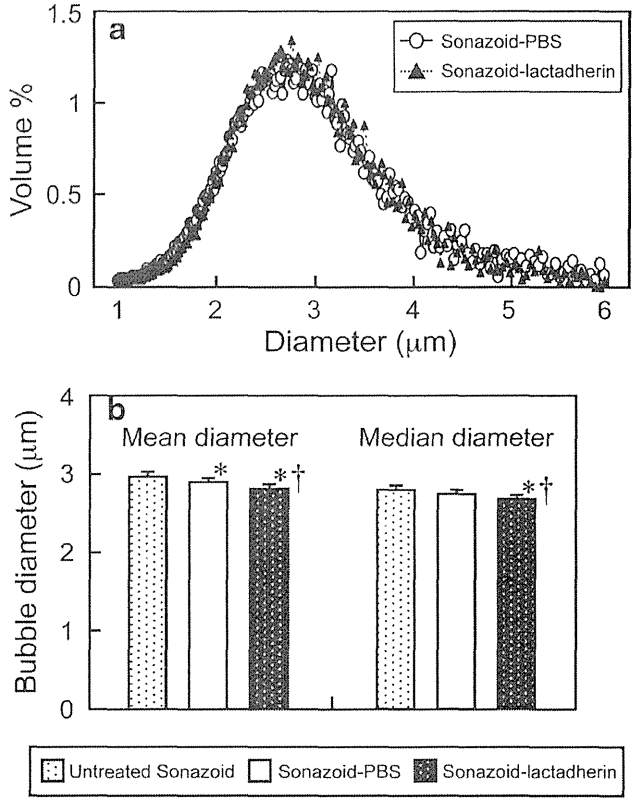

Fig. 3. Size distribution and diameters of Sonazoid bubbles. **a** The histogram of bubble size distribution was almost the same even after conjugation with lactadherin. **b** Even after conjugation with lactadherin, the mean and median diameters of Sonazoid bubbles were not increased. **P*<0.05 vs.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

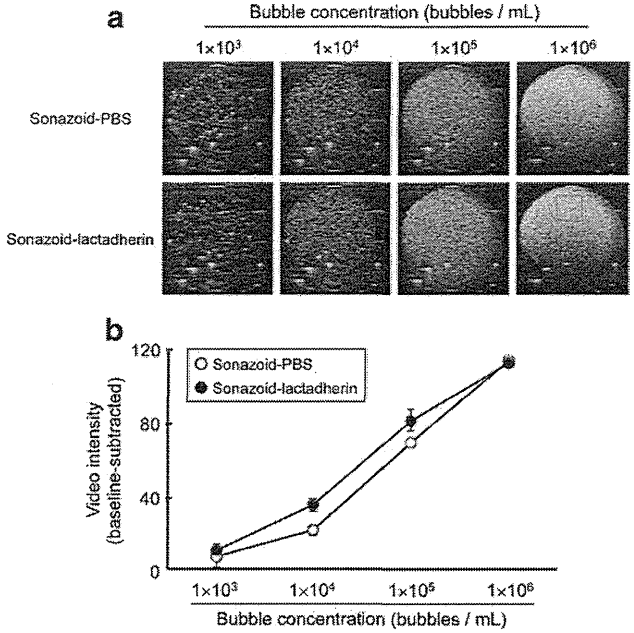

Fig. 4. Comparison of acoustic property of Sonazoid with or without lactadherin conjugation. **a** Representative ultrasound images at each bubble concentration. The region of interest is shown in a *red square*. **b** Baseline-subtracted video intensity at each experimental setting. The acoustic property 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.

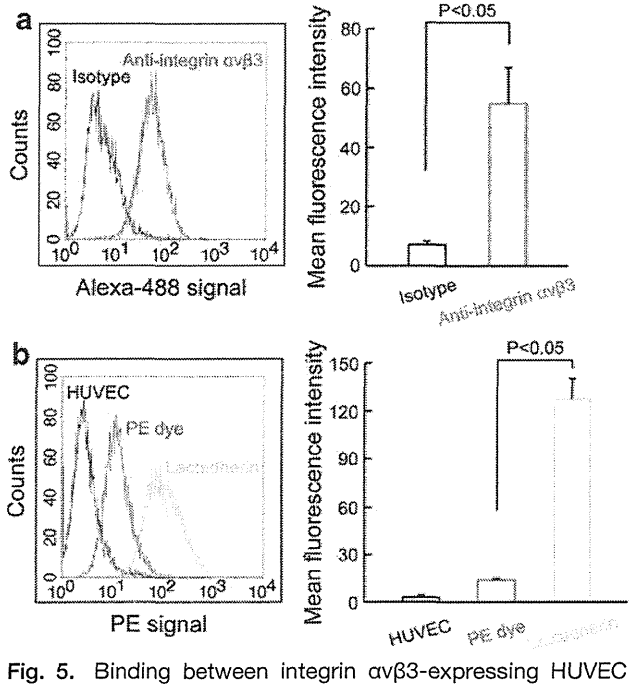

Fig. 5. Binding between integrin αvβ3-expressing HUVEC and lactadherin. **a** The fluorescence intensity derived from HUVEC after incubation with anti-integrin αvβ3 antibody was significantly higher than that after incubation with isotype control (55.0±12.2 vs. 7.1±0.7) (n=3). **b** A significant increase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

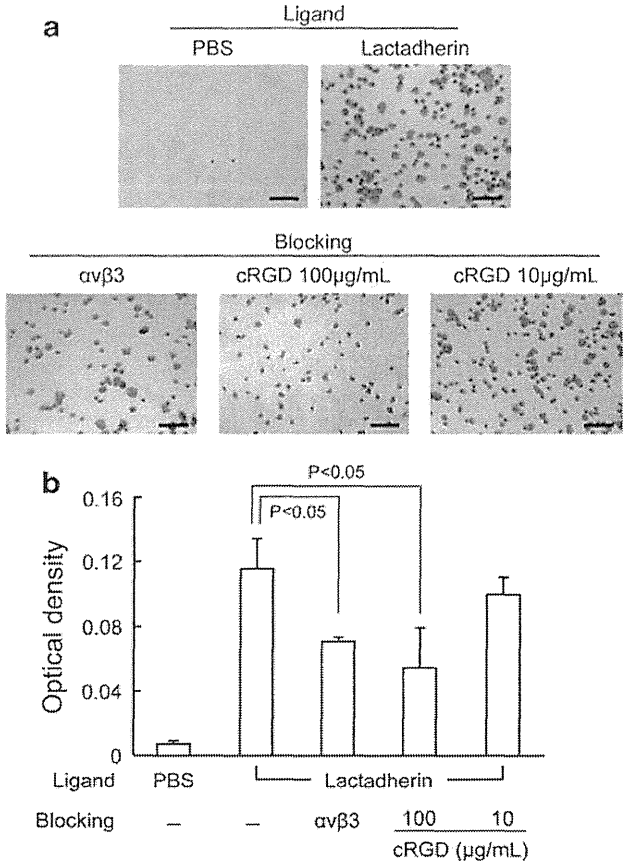

Fig. 6. Adhesion of HUVEC to the lactadherin-coated plates. **a** Representative photographs of adherent HUVEC at each experimental setting. Scale bars = 100 μm. **b** Optical density was significantly decreased by pre-incubating HUVEC with anti-integrin αvβ3 antibody or cRGD peptide.

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)

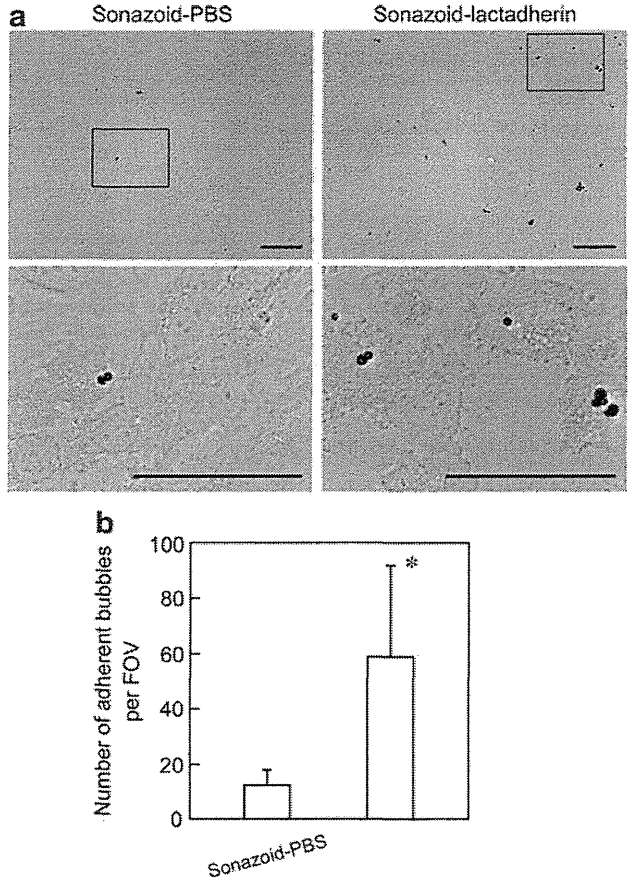

Fig. 7. Adhesion of Sonazoid bubbles to HUVEC under shear flow with or without the intermediation of lactadherin. **a** The number of Sonazoid bubbles adherent to HUVEC was increased by the intermediation of lactadherin. The *boxed areas* in *upper panels* are shown at higher magnification in *lower 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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