Ultrasound in Med. & Biol., Vol. ■■, No. ■■, pp. ■■–■■, 2018

0301-5629/$ - see front matter

https://doi.org/10.1016/j.ultrasmedbio.2018.01.017

● *Original Contribution*

SIMPLIFIED PREPARATION OF $\alpha_v\beta_3$ INTEGRIN-TARGETED MICROBUBBLES BASED ON A CLINICALLY AVAILABLE ULTRASOUND CONTRAST AGENT: VALIDATION IN A TUMOR-BEARING MOUSE MODEL

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(*Received* 22 *June* 2017; *revised* 14 *January* 2018; *in final form* 20 *January* 2018)

Abstract—The usefulness of ultrasound molecular imaging with $\alpha_v\beta_3$ integrin-targeted microbubbles for detecting tumor angiogenesis has been demonstrated. Recently, we developed $\alpha_v\beta_3$ integrin-targeted microbubbles by modifying clinically available microbubbles (Sonazoid, Daiichi-Sankyo Pharmaceuticals, Tokyo, Japan) with a secreted glycoprotein (lactadherin). The aims of our present study were to simplify the preparation of lactadherin-bearing Sonazoid and to examine the diagnostic utility of lactadherin-bearing Sonazoid for $\alpha_v\beta_3$ integrin-expressing tumor vessels by using SK-OV-3-tumor–bearing mice. By incubating 1.2×10^7 Sonazoid microbubbles with 1.0 μg lactadherin, the complicated washing and centrifugation steps during the microbubble preparation could be omitted with no significant reduction in labeling ratio of lactadherin-bearing Sonazoid. In addition, the number of Sonazoid microbubbles accumulated in the SK-OV-3 tumor was significantly increased by modifying Sonazoid with lactadherin. Our data suggest that the lactadherin-bearing Sonazoid is an easily prepared and potentially clinically translatable targeted microbubble for $\alpha_v\beta_3$ integrin-expressing vessels. (E-mail: otani@ncvc.go.jp)

Key Words: **Microbubbles, Ultrasound molecular imaging, Sonazoid, Lactadherin, Tumor angiogenesis.**

INTRODUCTION

The diagnostic utility of ultrasound molecular imaging with targeted microbubbles has been demonstrated in numerous animal models of cardiovascular diseases and tumors (Abou-Elkacem et al. 2015; Kiessling et al. 2012). In the majority of studies, biotin–avidin bridging was used to attach the antibodies or peptides to the surface of microbubbles. In addition, an excess of antibodies or peptides have been removed by the washing and centrifugation processes during the preparation of targeted microbubbles. However, the immunogenicity of streptavidin is a barrier to the clinical translation of these kinds of targeted microbubbles (Chinol et al. 1998). Furthermore, the complicated washing and centrifugation process would make it difficult to keep targeted microbubbles in a sterilized state. Considering the clinical translation of targeted microbubbles, therefore a simplified preparation method without the use of streptavidin or washing and centrifugation process is desirable.

Sonazoid (perfluorobutane gas microbubbles stabilized by a membrane of hydrogenated egg phosphatidylserine [PS]), is an ultrasound contrast agent approved in several countries (Sontum 2008). We previously demonstrated that the incorporation of biotinylated IgG onto the surface of Sonazoid was feasible by using annexin V, a protein that has a high affinity for PS, and biotin–avidin binding (Otani and Yamahara 2011). In addition, we (Otani and Yamahara 2013) recently developed streptavidin-free, arginine-glycine-aspartate (RGD) motif-bearing Sonazoid microbubbles by conjugating Sonazoid with an endogenous secreted glycoprotein, lactadherin (milk fat globule epidermal growth factor 8 [MFG-E8]), which contains both a PS-binding C-domain and an RGD motif (Hanayama et al. 2002). Because the RGD motif binds preferentially to $\alpha_v\beta_3$ integrin, which is known to play a key role in angiogenesis (Friedlander et al. 1995), lactadherin-bearing Sonazoid binds to $\alpha_v\beta_3$ integrin-expressing cells (Otani and Yamahara 2013). However, the targetability of

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lactadherin-bearing Sonazoid to the $\alpha_v\beta_3$ integrin-expressing neovasculature is still unknown.

Because lactadherin attaches directly to the surface of Sonazoid, we hypothesized that a simplified preparation of lactadherin-bearing Sonazoid, one without a washing and centrifugation process, might be achievable if the optimal mixing ratio of Sonazoid and lactadherin were determined.

The aim of this study was (i) to elucidate the optimal mixing ratio of Sonazoid and lactadherin to prepare lactadherin-bearing Sonazoid without washing and centrifugation steps; and (ii) to examine the feasibility of lactadherin-bearing Sonazoid as targeted microbubbles for $\alpha_v\beta_3$ integrin-expressing neovasculature in a tumor-bearing mouse model.

MATERIALS AND METHODS

Specificity of binding between lactadherin and Sonazoid

First, the specificity of binding between lactadherin and Sonazoid (Daiichi-Sankyo Pharmaceuticals, Tokyo, Japan) was examined. As a control protein, we used the recombinant human angiopoietin-like protein 5 (ANGPTL5) (Oike et al. 2004; Zeng et al. 2003) because it has a molecular weight similar to that of lactadherin. The predicted molecular weight of lactadherin and ANGPTL5 is 41.6 kDa and 42.3 kDa, respectively. In addition, an isoelectric point of lactadherin and ANGPTL5 is 8.23 and 6.51, respectively. The predicted molecular weight and isoelectric point of each protein was calculated based on its amino acid sequence, using the ExPASy calculator (https://web.expasy.org/protparam/). Recombinant human MFG-E8 (lactadherin; 2767-MF, R&D Systems, Minneapolis, MN, USA) and ANGPTL5 (6675-AN, R&D Systems) were dissolved in phosphate-buffered saline (500 μg/mL) and then labeled with an R-phycoerythrin labeling kit (LK23, Dojindo Laboratories, Kumamoto, Japan). The incubation of phycoerythrin (PE)-lactadherin or PE-ANGPTL5 with Sonazoid was as reported previously (Otani and Yamahara 2013). In brief, Sonazoid was reconstituted according to the manufacturer's instructions (1.2×10^9 microbubbles/mL) (Sontum 2008), and 100 μL of Sonazoid (1.2×10^8 microbubbles) was incubated with 5 μg (100 μL) of PE-lactadherin or PE-ANGPTL5 for 15 min at room temperature. During the incubation period, the microbubble suspension was mixed by gently tapping every 5 min to increase the reaction efficiency. Thereafter, Sonazoid microbubbles were diluted with 200 μL of Milli-Q water and centrifuged ($100 \times g$) for 1 min at room temperature, using a Himac CF16 RX centrifuge (T15 A33 rotor; Hitachi Koki Co., Ltd., Tokyo, Japan). The accumulated microbubbles on the surface of microbubble suspension were dispersed by gently tapping, and the supernatant was transferred to another tube. These procedures were repeated once more. After a washing and centrifugation process, the microbubble suspension was assessed by using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with 50,000 counts. Because PE-lactadherin and PE-ANGPTL5 contain unreacted PE dyes, Sonazoid was also incubated with PE dye (equivalent of 5 μg PE-lactadherin or PE-ANGPTL5) to examine the nonspecific binding between Sonazoid and PE dye. Fluorescence-activated cell sorting (FACS) experiments were performed using four independent microbubble suspensions, and FACS data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Alexa Fluor 488-labeling of lactadherin

Because the exclusion of unreacted PE dye from PE-labeled lactadherin solution was technically difficult because of the similarity in the molecular weight, we examined the non-specific binding between Sonazoid and PE dye as a control (Otani and Yamahara 2013). However, residual unreacted fluorescent dye is a barrier to determining the optimal mixing ratio of lactadherin and Sonazoid and developing a simplified preparation method of lactadherin-bearing Sonazoid without a washing and centrifugation process. We therefore labeled lactadherin with Alexa Fluor 488, which can be easily excluded by using commercially available centrifugal device. Recombinant human MFG-E8 (2767-MF, R&D Systems) was dissolved in 50 mmol/L $NaHCO_3$ (pH = 8.3) to obtain 0.2 mg/mL solution and was incubated for 1 h at room temperature with 5 mg/mL (in DMSO) Alexa Fluor 488 carboxylic acid, succinimidyl ester (A20000; Thermo Fisher Scientific Inc., Waltham, MA, USA) at a protein/dye ratio of 1:100 (mol/mol). After the incubation, the mixture was washed five times with 50 mmol/L $NaHCO_3$ (pH = 8.3) in a Nanosep centrifugal device with Omega membrane (10 K; Pall Corp., Port Washington, NY, USA). After the final washing process, Alexa Fluor 488–labeled lactadherin was collected in 50 mmol/L $NaHCO_3$ (pH = 8.3). The Alexa Fluor 488:protein ratio was determined by measuring the absorbance of the protein solution at 280 nm and 500 nm with an Implen NanoPhotometer (Waken B Tech Co., Ltd., Kyoto, Japan). In addition, the concentration of Alexa Fluor 488–lactadherin was calculated using the following formula: protein concentration (mg/mL) = A_{280} / E_{280}, where A_{280} and E_{280} is the absorbance at 280 nm and the absorbance coefficient, respectively.

Determination of optimal mixing ratio between Sonazoid and lactadherin

The incubation setting of Sonazoid and lactadherin was down-scaled to determine the optimal mixing ratio between Sonazoid and lactadherin. Sonazoid (10 μL; 1.2×10^7 microbubbles) was incubated with 0, 0.1, 1.0 or 10 μg of Alexa Fluor 488–labeled lactadherin (35–