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Evaluation of ^{18}F-labeled targeted perfluorocarbon-filled albumin microbubbles as a probe for microUS and microPET in tumor-bearing mice

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ABSTRACT

Objective: In this study, albumin-shelled, targeted MBs (tMBs) were first demonstrated with the expectation of visualization of biodistribution of albumin-shelled tMBs. The actual biodistribution of albumin-shelled tMBs is of vital importance either for molecular imaging or for drug delivery.
Motivation: Recently, albumin microbubbles (MBs) have been studied for drug and gene delivery in vitro and *in vivo* through cavitation. Targeted lipid-shelled MBs have been applied for ultrasound molecular imaging and conjugated with radiolabeled antibodies for whole-body biodistribution evaluations. The novelty of the work is that, in addition to the lipid tMBs, the albumin tMBs was also applied in biodistribution detection.
Methods: Multimodality albumin-shelled, ^{18}F-SFB-labeled VEGFR2 tMBs were synthesized, and their characteristics in mice bearing MDA-MB-231 human breast cancer were investigated with micro-positron-emission tomography (microPET) and high-frequency ultrasound (microUS).
Results: Albumin-shelled MBs can be labeled with ^{18}F-SFB directly and conjugated with antibodies for dual molecular imaging. The albumin-shelled tMBs show a lifetime in 30 min in the blood pool and a highly specific adherence to tumor vessels in mice bearing human breast cancer.
Conclusions: From the evaluations of whole-body biodistribution, the potential of the dual molecular imaging probe for drug or gene delivery in animal experiments with albumin shelled MBs has been investigated.

1. Introduction

Lipid-shelled microbubbles (MBs) were recently conjugated with *N*-succinimidyl-4-[^{18}F]fluorobenzoate (^{18}F-SFB)-radiolabeled antibodies for micro-positron-emission tomography (microPET) imaging [1,2]. Imaging tumor angiogenesis with contrast-enhanced ultrasound (US) has been explored with MBs targeted to vascular endothelial growth factor receptor 2 (VEGFR2) [3]. Here we developed a contrast agent for high-frequency US (microUS) and microPET multimodality targeted imaging using perfluorocarbon (C_3F_8)-gas-filled and VEGFR2 antibody-conjugated albumin with ^{18}F-SFB. The hypothesis tested in this study was that these radiolabeled, albumin-shelled, VEGFR2-targeted MBs show a lifetime in 30 min in the blood pool and a highly specific adherence to tumor vessels in mice bearing human breast cancer. The feasibility of the albumin-shell targeted MBs in ultrasound molecular imaging was investigated.

Albumin is an important proteinaceous microcapsule that may be modified for biomedical imaging purposes. Albumin-shelled MBs and a paramagnetic-labeled macromolecule, albumin-(Gd-DTPA), are used as US and MRI contrast agents, respectively [4,5]. Some studies have found that local inflammation and angiogenesis can be detected by incorporating the targeting ligands in albumin-shelled MBs [6]. Moreover, some drugs have been encapsulated in albumin microspheres [7]. These methods employ the focusing ability of US to exert selective therapeutic effects on targeted tissues. Moreover, molecular imaging using radiotracers is not only useful for tumor diagnosis but also provides better radiation dosimetry for internal radiotherapy [8].

Gas-filled echogenic MBs are clinically used as the contrast agent for US. MBs for US imaging are designed for nonspecific and passive image enhancement. MBs with an albumin shell, such

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as Optison®, can persist in the bloodstream for a sufficiently long time for imaging because the denatured albumin shell and core gas have low solubility in blood [4]. Albumin-based MBs consisting of C_3F_8 gas surrounded by a human serum albumin-based shell have been clinically used to enhance the contrast in US imaging [9]. Because their size is in the order of several micrometers, MBs remain within the vascular system after intravenous (i.v.) administration [2].

Angiogenesis (the growth of new blood vessels) is promoted in the early stage of tumor growth and plays an important role in tumor growth, invasiveness, and metastatic potential [10]. Several specific endothelial molecular markers of angiogenesis, including vascular endothelium growth factor receptor (VEGFR), are up-regulated on tumor vascular endothelial cells [11]. Overexpression of VEGFR1 and/or VEGFR2 has been associated with tumor progression and poor prognosis in several tumors, including colorectal cancer and malignant glioma [12,13]. Thus, applying noninvasive imaging techniques to specific molecular markers of angiogenesis, such as VEGFR2, may be particularly advantageous for imaging tumor angiogenesis and tracking antiangiogenic tumoricidal treatments [14]. Previous studies have shown that VEGFR2 monoclonal antibodies can be used to detect mouse VEGFR2 on tumor vessels in nude mice bearing MDA-MB-231 human breast cancer [15]. The *in vivo* tumor VEGFR2 expression level can be determined using ^{64}Cu-DOTA-VEGF$_{121}$ small-animal PET imaging [16]. Contrast-enhanced US has been conducted with lipid-shelled MBs targeted to VEGFR2, $\alpha_v\beta_3$ integrin, and endoglin [3,17,18]. However, the shells of the lipid-shelled targeted MBs (tMBs) were not labeled directly to allow evaluation of their biodistribution [2].

Imaging the tumor vasculature can be clinically useful both in diagnosis and in monitoring tumor responses to antiangiogenic therapy. MBs have also been used as drug carriers and have the potential to combine US imaging with US-mediated therapy [19]. In this study, the albumin shells of VEGFR2 tMBs were labeled with ^{18}F-SFB. An albumin-based multimodality probe, ^{18}F-SFB-labeled tMBs (^{18}F-SFB-tMBs), with a lifetime in 30 min in blood was prepared for *in vivo* targeted imaging of VEGFR2-expression tumor. The anti-VEGFR2 antibodies were attached to the MB shells using an avidin–biotin conjugation system. tMBs are well suited for imaging events within the vascular compartment, such as inflammation, thrombus formation, and angiogenesis [20]. By using site labeling to tMBs to specific molecular markers, it has recently been shown that contrast-enhanced US allows specific intravascular molecular markers of tumor angiogenesis to be detected [3]. The targeting efficiency of tMBs for microUS imaging of tumor angiogenesis in a murine tumor model was evaluated here. Moreover, we used ^{18}F-SFB-tMBs as a scintillation probe to study the pharmacokinetics of the tMBs in mice bearing MDA-MB-231 human breast cancer by microPET imaging. The purpose of this study was to elucidate the potential and advantages of albumin-shelled multifunctional MBs specifically targeted to VEGFR2 in breast cancer from imaging to therapeutic levels.

2. Materials and methods

2.1. Production of albumin-shelled tMBs

Albumin-shelled MBs were prepared according to the procedure used in our previous study [21]. The albumin MBs were filtered with a 5 μm syringe filter (Sartorius, Goettingen, Germany) and were then cross-linked by 2.5% glutaraldehyde. Glutaraldehyde is a fixative used for scanning electron microscopy (SEM). The samples were then prepared for SEM by coating with platinum. SEM images were recorded on a Hitachi S-3000 N system at an acceleration voltage of 15 kV. For tMBs, avidin-containing MBs (Av-HSA-MBs) were generated by sonicating 2.5 mL of solution containing 1.2% human serum albumin (HSA) (Octapharma, Vienna, Austria), 0.04% avidin (Pierce Biotechnology, Rockford, IL), and C_3F_8 gas in phosphate-buffered saline (PBS). The solution was sonicated by an US sonicator (Branson, USA) for 2 min. The number of MBs in the solution was measured with a MultiSizer III device (Beckman Coulter, Fullerton, CA) with a 30-μm-aperture probe whose measurement boundary was between 0.6 to 20 μm. The size distribution in a suspension were measured by dynamic light scattering (Nanoparticle Analyzer, Horiba, Kyoto, Japan). The size distribution of MBs ranges from 0.5 nm to 6 μm. The MB, avidin-MBs, and tMBs concentrations were 2.4×10^9/ml, 3.3×10^9/ml, 2.5×10^9/ml and their mean diameters (number-weighted) were 1.98, 1.55, and 1.76 μm. Before incubation with biotinylated antimouse Flk-1 VEGFR2 antibody (eBioscience, San Diego, CA), the avidin-containing MBs were centrifuged (1200 rpm, 128.7 g) (F2402 rotor, Beckman Coulter, Brea, CA) for 1 min and then washed three times to ensure that the free avidin was removed. The 20 μg biotinylated antimouse VEGFR2 antibody was then incubated with 0.5 ml avidin-containing MBs for 30 min at room temperature to produce VEGFR2-targeted tMBs. The washing procedure was performed three times to ensure that the unbound biotinylated antimouse VEGFR2 antibody was removed.

2.2. Determination of number of antibodies on tMBs

Biotin antimouse VEGFR2 antibodies were radiolabeled with ^{131}I, linked to MBs, and analyzed by a γ counter. Iodination of biotin antimouse VEGFR2 was performed using either the chloramine-T method or the IODOGEN method with similar results as described previously [22,23]. Biotin antimouse ^{131}I-VEGFR2 was separated from free iodine using size exclusion chromatography. The specific activity of biotin antimouse ^{131}I-VEGFR2 was about 1.5×10^5 cpm/ng. The average amount of biotinylated anti-VEGFR2 antibody per VEGFR2-targeted tMB was quantified as (the ratio of the radioactivity remaining in the MBs conjugated with ^{131}I-labeled biotinylated anti-VEGFR2 monoclonal antibody to the total radioactivity) × (the number of molecules of biotinylated anti-VEGFR2 monoclonal antibody)/(the surface area of avidin-containing MBs) [2].

2.3. Radiolabeling of tMBs

^{18}F-SFB was synthesized from *tert*-butyl 4-*N*,*N*,*N*-trimethylammoniumbenzoate triflate, as reported previously [24]. ^{18}F-SFB contains an active ester functionality, proteins can be tagged with ^{18}F by simple incubation of the protein in a buffer (preferably borate, pH 8–9) at room temperature for a few minutes [25]. The radiochemical purity of ^{18}F-SFB was determined using radio-thin-layer chromatography that was performed on an aluminum sheet coated with silica gel powder using MeCN/H_2O (1/1, v/v) as the developing agent. ^{18}F-SFB was obtained at decay-corrected radiochemical yields of 40–50% within 90 min after end of bombardment with a radiochemical purity of >95%. The ^{18}F-SFB was dissolved in 50 μL of dimethyl sulfoxide and incubated with MBs or tMBs in 1 mL of borate buffer (0.1 N, pH 8.5) for 30 min at room temperature. The reaction solution was then centrifuged (128.7 g) for 1 min, redissolved in PBS, and washed twice to ensure that the unconjugated ^{18}F-SFB was removed. Typical decay-corrected radiochemical yields for ^{18}F-SFB-MBs will be about 32–36%.

2.4. Cell attachment studies

Mouse endothelioma bEnd.3 cells purchased from the American Tissue Culture Collection (ATCC; Manassas, VA) were grown in Dul-