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Ultrasound molecular imaging of tumor angiogenesis with a neuropilin-1-targeted microbubble

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ABSTRACT

Ultrasound molecular imaging has great potential to impact early disease diagnosis, evaluation of disease progression and the development of target-specific therapy. In this paper, two neuropilin-1 (NRP) targeted peptides, CRPPR and ATWLPPR, were conjugated onto the surface of lipid microbubbles (MBs) to evaluate molecular imaging of tumor angiogenesis in a breast cancer model. Development of a molecular imaging agent using CRPPR has particular importance due to the previously demonstrated internalizing capability of this and similar ligands. *In vitro*, CRPPR MBs bound to an NRP-expressing cell line 2.6 and 15.6 times more than ATWLPPR MBs and non-targeted (NT) MBs, respectively, and the binding was inhibited by pretreating the cells with an NRP antibody. *In vivo*, the backscattered intensity within the tumor, relative to nearby vasculature, increased over time during the ~6 min circulation of the CRPPR-targeted contrast agents providing high contrast images of angiogenic tumors. Approximately 67% of the initial signal from CRPPR MBs remained bound after the majority of circulating MBs had cleared (8 min), 8 and 4.5 times greater than ATWLPPR and NT MBs, respectively. Finally, at 7–21 days after the first injection, we found that CRPPR MBs cleared faster from circulation and tumor accumulation was reduced likely due to a complement-mediated recognition of the targeted microbubble and a decrease in angiogenic vasculature, respectively. In summary, we find that CRPPR MBs specifically bind to NRP-expressing cells and provide an effective new agent for molecular imaging of angiogenesis.

1. Introduction

Ultrasound (US) molecular imaging combines many advantages of US imaging, including low cost, non-invasive and real-time protocols, with the opportunity to assay vascular receptor expression. To accomplish US molecular imaging, microbubble contrast agents are coated with targeting ligands and are injected into the vascular space [1–3]. Microbubble contrast agents circulate within the vasculature for minutes providing the opportunity to survey vascular surface receptors without a confounding signal from an extravasated imaging probe. Here, with two neuropilin-1 (NRP)-targeted peptides, we constructed two NRP-targeted microbubbles (MBs), established optimized US parameters for molecular imaging and compared the performance of the targeted MBs with non-targeted (NT) MBs both *in vitro* and *in vivo*.

NRP has been identified as an isoform-specific receptor for vascular endothelial growth factor [4] and its expression is up-regulated in multiple tumor types as well as on tumor vasculature [5–8]; therefore, NRP-based molecular imaging can be used as an indication of local angiogenesis. Recently, peptides containing a C-terminal arginine with a free carboxyl group (CendR) were shown to target NRP [9], and two peptides, ATWLPPR (abbreviated as ATW in this paper) [10] and CRPPR (abbreviated as CRP in this paper) [11], were reported as CendR peptides binding to NRP. Further, CendR peptides, and CRPPR in particular, have been shown to enhance the internalization of their cargo and are therefore particularly important targets for tumor drug delivery [11]. Such

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peptides are also hypothesized to be produced at the tumor site by enzymes acting upon circulating internalizing RGD (iRGD) peptides [12]. Therefore, the creation of an imaging probe capable of assaying NRP receptors that are accessible on tumor vasculature has the potential to facilitate personalized treatment based upon the iRGD or CendR strategy.

In this paper, we conjugated lipids, polyethylene glycol and the ATW or CRP peptides to form lipo-PEG-peptides that were then incorporated with other lipids into targeted MBs. A major advantage of peptide targeting and the lipo-PEG-peptide strategy employed here is a reduction in immunogenicity as compared with antibodies and biotin-avidin conjugation. However, we have previously shown that the incorporation of an exposed peptide on the relatively large surface of a microbubble has the potential to enhance the attachment of C3/C3b and production of soluble C3a anaphylotoxin [13]. Repeated imaging over a duration of several days to weeks is also often required to optimize a tumor model [14], to study biological changes with tumor growth [15] or to evaluate anti-angiogenic therapy [16]. Few studies have evaluated the effect of repeated MB injections on imaging. Therefore, we assess whether repeated microbubble administration affects the circulation or accumulation.

Our central goal is to design a strategy to use US molecular imaging to assess the local NRP concentration and detect small tumors with high sensitivity. In nuclear medicine, the relative accumulation of molecularly-targeted contrast agents is quantified and the receptor kinetics are modeled to facilitate comparisons of performance [17–19]. Ultrasound contrast agents are multi-valent constructs that rapidly accumulate on the vascular surface and are not typically characterized by classical receptor kinetics. Still, quantification of the rate of accumulation provides an effective scheme to compare molecularly-targeted microbubbles.

Blood flow has been reported to be reduced for up to 40 min within tumor vasculature after insonation of targeted, but not NT MBs, with a high frequency (5 MHz) and high peak-negative pressure (PNP) (2 or 4 MPa) [20]. With a higher PNP and lower center frequency, similar effects have been reported for non-targeted MBs [21–23]. The hypothesized mechanism was the oscillation and collapse of MBs mechanically disrupting a limited number of endothelial cells, which can expose the basement membrane and recruit platelets to the site. A reduction in blood flow is undesirable as multiple targeted MB injections are often required within a single imaging session to study short-term biological changes, to optimize system parameters or to evaluate novel MB formulations [24,25]. Therefore, we seek the ideal insonation parameters that facilitate quantitative imaging but do not result in significant biological effects in order to facilitate the incorporation of this new imaging probe into pre-clinical and clinical studies.

2. Materials and methods

All reagents, including solvents, amino acids, Fmoc-Peg27-OH and Fmoc-Arg(Pbf)-Wang resin, were purchased from EMD Biosciences (La Jolla, CA) unless otherwise specified. Lipids, disteroylphosphatidylcholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2K), were purchased from Avanti Polar Lipids (Alabaster, AL). Complement-preserved human serum was purchased from Valley Biomedical (cat. no. HC1004, Winchester, VA). Serum was thawed and/or stored on ice prior to experiments.

2.1. Lipo-Peg-peptide (LPP) synthesis

Two CendR [9] peptides, ATWLPPR [10] and CRPPR [11], were synthesized with standard Fmoc protocol [26] on a Biotage® Initiator + Microwave Synthesizer (Biotage, Charlotte, NC), with Fmoc-Arg(Pbf)-Wang resin used to produce carboxylic acid at the C-terminus. After the two Fmoc-peptides were synthesized on the resin, a small amount of Fmoc-peptide was cleaved from the resin and the mass was checked with MALDI (ABI-4700 TOF–TOF, Applied Biosystems, Foster City, CA): Fmoc-ATWLPPR, expected 1061.55, measured (H^+) 1062.68; Fmoc-CRPPR, expected 849.40, measured (H^+) 849.66. The two peptidyl resins were then coupled with PEG and lipids on resin, as described in Ref. [27]. The two LPPs were then cleaved from the resin and purified with RP-HPLC, and the molecular weights were further assessed with MALDI (ATW LPP, expected 5410.41, measured (H^+) 5412.19; CRP LPP, expected 5198.25, measured (H^+) 5194.31). The CRP LPP was further dimerized with air and the dimer was purified with RP-HPLC, as described in Ref. [27].

2.2. MB preparation

The lipids and LPPs were mixed in chloroform (Sigma–Aldrich, St. Louis, MO), and the chloroform was blow-dried under a gentle nitrogen gas stream and thoroughly vacuumed on the lyophlizer overnight. MB buffer was made by mixing 0.9% sodium chloride (B. Braun Medical Inc., Irvine, CA), propylene glycol (Sigma–Aldrich, St. Louis, MO), and glycerol (MP Biomedicals, LLC) with the ratio of 80:10:10 (v/v/v), and the pH was adjusted with sodium hydroxide to 7.4. The dried lipid mixture was re-suspended with degassed MB buffer by first warming in a water bath (60-65 °C for 15 min), and then sonicating (60-65 °C for 15 min) until all of the lipids were re-suspended in a clear solution of 2.5 mg per ml. After being cooled to room temperature, the liposome solution was aliquoted into 2 ml serum glass bottles (VWR, Visalia, CA), containing 1.0 ml per bottle. After sealing the bottle, the air in the bottle headspace was purged with 10 ml perfluorobutane C_4F_{10} (FluoroMed, L.P., Round Rock, TX), and the resulting liposome solutions were kept at 4 °C until use.

MBs were activated by shaking on a Vialmix (Lantheus Medical Imaging, North Billerica, MA), and purified by centrifugation, similar to [28], as detailed below. The activated MBs suspension was collected into a 3 ml syringe (Covidien, Mansfield, MA), which was further filled with degassed Dulbecco's phosphate-buffered saline (DPBS, Mediatech, Inc. Manassas, VA). Centrifugation (10 min, 300 relative centrifugal force (RCF)) was performed to collect the MBs into a cake resting against the syringe plunger. After removing the infranatant, the bubble cake was re-suspended with 2.5 ml DPBS. MBs larger than 10 μm were removed by centrifugation: 16 RCF for 1 min, followed by 45 RCF for 1 min, in which the infranatant was collected. MBs smaller than 1 μm were removed by 3 times centrifugation (300 RCF for 3 min), in which the infranatant was removed and the bubble cake was collected. For *in vitro* tests, the steps involved in removing MBs larger than 10 μm were omitted. The size and concentration of the MBs were measured with an Accusizer 770A (Particle Sizing Systems, Port Richey, FL), and the MBs were used within 3 h after shaking.

2.3. Cell culture

While the *in vivo* imaging target for microbubbles is the angiogenic endothelium, an NRP-expressing tumor cell line, primary prostate carcinoma-1 (PPC-1) was used to assess *in vitro* binding as the expression of NRP on this cell line is well established and endothelial expression varies between *in vitro* and *in vivo* assays [11]. The PPC-1 cell line was a generous gift from Dr. Arthur Brothman (University of Utah, School of Medicine), and was cultured and maintained using DMEM high glucose media (Invitrogen, Carlsbad, CA) supplemented with 1% Penicillin-Streptomycin (10,000 U/ml, Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Omega Scientific Inc., Tarzana, CA). For MB binding experiments, PPC-1 cells were plated onto collagen-coated 25-mm glass coverslips the day before experiments, followed by incubation at 37 °C in a humidified tissue culture incubator 95%/5% air/CO_2 to reach a confluency of 95% on the day of experiments.

2.4. In vitro MB binding and inhibition study

MB binding was tested following a procedure similar to [29], and described briefly here. A glass coverslip with a PPC-1 cell mono-layer was mounted in a stainless steel holder to provide a frame with a 2-mm deep well above the cell layer (Supplementary Fig. 1). After 1 ml of MB suspension (with 2 or 5 × 10^7 MB/ml) in DPBS was added into the well, the well was covered with a 35-mm glass coverslip to retain the liquid in the well, then inverted and maintained at 37 °C for 5 min to allow MBs to rise via buoyancy to the cell plate. The well was then flipped back to its original position, the 35-mm coverslip was removed, and the cell layer was gently rinsed with DPBS 3 times to remove unbound MBs. The cell plate was imaged on a custom upright microscope (Mikron, San Marcos, CA) with a digital Cascade 512b camera (Photometrics, Tucson, AZ) using bright field imaging with a 63 × water-immersion objective (Achroplan, Zeiss, NY) driven with SimplePCI 6 software. For each condition, 4–5 plates of cells were tested (n = 4–5). Five images were acquired randomly per plate and analyzed with ImageJ (imagej.nih.gov/ij/), and the MB area per field of view was calculated from the "Analyze Particle" function in ImageJ.

For the inhibition study, an anti-NRP antibody was generated as in Ref. [9]. Glass coverslips with PPC-1 cell monolayers were incubated with NRP antibody solution (20 μg/ml) at 37 °C for 30 min prior to the MB treatments described above.

2.5. Overview of the in vivo studies

All animal studies were conducted under a protocol approved by the University of California, Davis Animal Care and Use Committee. Female FVB mice, 5–6 weeks old, 15–25 g, were purchased from Charles River Laboratory International Inc. (Wilmington, MA). Tumors were grown by transplanting one 1 mm^3 piece of donor NDL tumor into each of the two 4th mammary fat pads, and allowing the tumors to