Published in final edited form as:

Mol Pharm. 2017 March 06; 14(3): 781–790. doi:10.1021/acs.molpharmaceut.6b01033.

Ultrasound Molecular Imaging of Angiogenesis Using Vascular Endothelial Growth Factor-Conjugated Microbubbles

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Abstract

Imaging of angiogenesis receptors could provide a sensitive and clinically useful method for detecting neovascularization such as occurs in malignant tumors, and responses to anti-angiogenic therapies for such tumors. We tested the hypothesis that microbubbles (MB) tagged with human VEGF₁₂₁ (MB_{VEGF}) bind to the kinase insert domain receptor (KDR) *in vitro* and angiogenic endothelium *in vivo*, and that this specific binding can be imaged on a clinical ultrasound system. In this work, targeted adhesion of MB_{VEGF} was evaluated *in vitro* using a parallel plate flow system containing adsorbed recombinant human KDR. There was more adhesion of MB_{VEGF} to KDR-coated plates when the amount of VEGF₁₂₁ on each MB or KDR density on the plate was increased. MB_{VEGF} adhesion to KDR-coated plates decreased with increasing wall shear rate. On intravital microscopic imaging of bFGF-stimulated rat cremaster muscle, there was greater microvascular adhesion of MB_{VEGF} compared to that of isotype IgG-conjugated control MB (MB_{CTL}). To determine if MB_{VEGF} could be used to ultrasonically image angiogenesis, ultrasound imaging was performed in mice bearing squamous cell carcinoma after intravenous injection of MB_{VEGF}. Ultrasound videointensity enhancement in tumor was significantly higher for MB_{VEGF} (17.3±9.7 dB) compared to MB_{CTL} (3.8±4.4 dB, *n*=6, *p*<0.05). This work demonstrates the feasibility of targeted ultrasound imaging of an angiogenic marker using MB_{VEGF}. This approach offers a non-invasive bedside method for detecting tumor angiogenesis and could be extended to other applications such as molecular monitoring of therapeutic angiogenesis or anti-angiogenic therapies in cardiovascular disease or cancer.

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Writing, review, and/or revision of the manuscript: JW, BQ, XC, FSV

The authors declare no competing financial interest.

Keywords

molecular imaging; angiogenesis; ultrasound imaging; microbubble contrast agents; VEGF; cancer; inflammation

1. INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing vessels, occurs in both physiological and pathologic processes, such as wound healing, inflammation, ischemia, malignancy, age related macular degeneration, and diabetic retinopathy.¹⁻³ Angiogenesis is a hallmark of malignant transformation: it has been well documented that for a tumor to grow beyond 2 mm in diameter, it must recruit new blood vessels for nutrient supply, waste removal and gas exchange.⁴ To this end, tumor cells secrete angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which bind to specific receptors on tumor endothelial cells and activate signaling pathways that enhance endothelial cell growth and migration from pre-existing blood vessels.^{5, 6} Due to the crucial role of angiogenesis in the growth and persistence of tumors and metastases, inhibitors targeted at tumor angiogenesis have been developed for anti-cancer therapy,^{7, 8} creating a need for imaging techniques to specifically identify angiogenic tumor endothelium and its responses to such treatments.

The kinase insert domain responsive receptor (KDR), also known as VEGF receptor 2 (VEGFR2), is a VEGF receptor that is a potential target for molecular imaging of angiogenesis due to its overexpression in tumor endothelial cells.⁹⁻¹² VEGF₁₂₁, one of the natural ligands of KDR, is the non-heparin binding isoform of VEGF and a potent inducer of angiogenesis. VEGF₁₂₁ binds specifically to VEGF receptors Flt1 and KDR expressed in response to hypoxia and angiogenesis.⁸ Such properties render VEGF₁₂₁ a potential targeting ligand for imaging angiogenic receptors. Radiolabeled VEGF₁₂₁ has been developed for early tumor detection using positron emission tomography.^{8, 13} We have previously shown that VEGF₁₂₁ can home to ischemic tissue undergoing angiogenesis: In a rabbit model of unilateral hindlimb ischemia, radiolabeled recombinant human VEGF₁₂₁ selectively accumulated in the ischemic hindlimb.¹⁴

Contrast ultrasound imaging using gas-filled microbubbles has been widely used in the clinical practice of echocardiography for left ventricular opacification and endocardial delineation. Beyond this clinical indication, contrast ultrasound is attractive for molecular imaging because it is non-invasive, low cost, portable, and does not utilize radioactive isotopes.¹⁵ We and others have previously demonstrated that ultrasound contrast microbubbles can be designed to bind specifically to endothelial markers of neovascularization via peptides or antibodies on the microbubble surface.¹⁶⁻²¹ Recently, a contrast agent covalently coupled to a recombinant single-chain vascular endothelial growth factor construct with a cysteine-containing tag (scVEGF) has been used to target VEGFR2.²² In this paper, we tested the hypothesis that ultrasound contrast microbubbles designed to adhere to angiogenic receptors can be used to detect tumor angiogenesis. Specifically, we first sought to prove the concept that microbubble binding to VEGF

receptors can be achieved by using VEGF₁₂₁ as the targeting ligand in an *in vitro* parallel plate flow system. Subsequently, we tested the hypothesis that microbubbles bearing VEGF₁₂₁ on their surface (MB_{VEGF}) bind to angiogenic endothelium using an intravital microscopy model of bFGF-stimulated cremaster muscle, and be ultrasonically imaged *in vivo* using a murine model of squamous cell carcinoma (SCC).

2. METHODS

2.1. Preparation of biotin-labeled VEGF₁₂₁

Recombinant VEGF₁₂₁ protein with or without biotin and/or FITC was provided by Scios Inc. The recombinant VEGF₁₂₁ homodimer was prepared using the yeast *P. pastoris* expression system and lyophilized as described previously.²³ The VEGF₁₂₁ protein was biotinylated with biotin-N-hydroxysuccinamide (ThermoFisher Scientific, Waltham, MA) using a 2.6 M excess over protein. Briefly, protein was dissolved and diluted into 0.1 M sodium bicarbonate buffer at pH 8.5, the biotinylation agent was added, and 2 hours later the reaction was stopped by dilution in PBS, followed by dialysis against PBS. Biotinylation of VEGF₁₂₁ was confirmed with time-of-flight mass spectrometry indicating an average of 2 biotins per VEGF molecule. For the flow cytometric studies described below, VEGF₁₂₁ was labeled with both biotin and FITC, and mass spectrometry indicated 3 FITC molecules per VEGF₁₂₁ molecule.

2.2. Microbubble preparation

Phospholipid-based perfluorocarbon gas-filled microbubbles (MBs) were prepared and VEGF₁₂₁ homodimer were conjugated to the microbubble surface using biotin-avidin bridging chemistry as described previously.²⁴ Briefly, a mixture of 1,2-distearoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids Inc, Alabaster, AL), polyethylene glycol stearate (Sigma-Aldrich Corp, St. Louis, MO), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (Avanti Polar Lipids, Inc) was hydrated in saline solution and sonicated in the presence of perfluorobutane gas using a probe type sonicator (XL2020, Misonix, Farmingdale, NY), resulting in a perfluorobutane microbubble encapsulated by a lipid monolayer bearing biotin on its surface. The biotinylated MBs (2×10^8 in 0.2 mL) were incubated with streptavidin (100 μ g, Molecular Probes, ThermoFisher Scientific) for 30 min, and then washed to remove excess streptavidin. The functionalized MBs were then made by adding biotinylated formulations of VEGF₁₂₁ homodimer or non-specific IgG isotype (BD Biosciences, San Jose, CA) to the MB suspensions using the same procedure described above for the streptavidin conjugation. The diameter of the MBs was 2.8 ± 1.5 μ m, as measured using electrozone sensing analysis (Multisizer II, Beckman-Coulter, Brea, CA).

2.3 Flow cytometric quantification of streptavidin and VEGF₁₂₁ density on the MB surface

After the MBs were made with biotin on the surface, either streptavidin labeled with known amounts of PE (1 streptavidin molecule per VEGF₁₂₁) (BD Biosciences, San Jose, CA) or VEGF₁₂₁ labeled with FITC (3 FITC molecules per VEGF₁₂₁) were coated onto the MB surface using the procedure described above. The fluorescence signal was detected with a flow cytometer (FACScan, Becton-Dickinson, Franklin Lakes, NJ) using 10,000 events per

reading. Standard 7–9 μm beads with a known amount of PE (Quantum R-PE, Bangs Labs, Fishers, IN) or FITC (Quantum FITC, Bangs Labs) were similarly analyzed to build a calibration curve that allowed calculation of the absolute amount of PE or FITC on the MB surface and hence the number of streptavidin or VEGF₁₂₁ molecules per MB.

2.4. Immobilization of KDR on glass coverslip

To confirm VEGF-conjugated microbubble (MB_{VEGF}) binding to VEGF receptors under precisely controlled experimental conditions, *in vitro* parallel plate studies of KDR-coated surfaces perfused with MB_{VEGF} were first performed.^{18–20, 25} Furthermore, we developed a method for quantifying KDR on the coated surfaces as we were interested in simulating varying degrees of receptor expression *in vitro*. To prepare KDR coated surfaces, glass coverslips were incubated overnight with 1 mL of goat anti-human IgG (GAMMA, 3 $\mu\text{g}/\text{mL}$, R&D Systems, Minneapolis, MN), and washed with PBS. The IgG coated coverslip was then blocked with 1% BSA for 2 hours, washed with PBS, further incubated with 1 mL of human KDR-Fc chimera (0.05 $\mu\text{g}/\text{mL}$, R&D Systems, Minneapolis, MN) for 2 hours at room temperature, and followed by another wash with PBS to eliminate unbound KDR-Fc. The density of KDR on the coverslip was determined using an enzyme-linked immunosorbent assay (ELISA) based method with modification.²⁶ Briefly, the same coating procedure was used to coat KDR-Fc onto a 96 well plate (Nunc Products, ThermoFisher Scientific). A calibration curve encompassing the range 0.1–40 ng KDR-Fc per well was built using a human KDR Elisa kit (R&D Systems) and the amount of KDR-Fc on the coverslip was determined by comparing the coverslip absorbency at 450 nm to the calibration curve.

2.5. *In vitro* microbubble perfusion assay

The KDR coated coverslip was mounted onto a parallel plate flow chamber with the KDR side facing the interior of the chamber.^{18–20, 25} The chamber has inflow and outflow ports through which MBs were infused using a syringe pump (PHD2000, Harvard Apparatus, Holliston, MA) which could be set at varying flow rates to achieve target wall shear rates. Due to the buoyant nature of the MBs the flow chamber was mounted on an inverted microscope (TE200, Nikon Inc, Melville, NY), with the KDR coated side of the coverslip facing downwards to maximize physical interaction between the receptors and MBs. The flow chamber was perfused with 6×10^6 MBs followed by a 3 mL perfusion of MB-free PBS at a pre-determined wall shear rate, to remove unbound MB from the chamber. For each coverslip, 20 microscopic fields were imaged at 40 $\times$ using a CCD camera (Orca, Hamamatsu, Middlesex, NJ) and the number of adhered microbubbles was counted normalized to the total area covered in the 20 views.

Separate perfusion experiments were conducted to determine the univariate effects of 3 controllable variables on targeted MB adhesion: VEGF₁₂₁ density on the MB (5 densities varying from 0 to 1.3×10^5 VEGF₁₂₁ molecules per MB); KDR receptor density (4 densities varying from 0 to 153 KDR receptors/ μm^2); and wall shear rate (4 wall shear rates varying from 100 to 400 sec^{-1}), were individually varied in separate perfusion studies, while keeping the other 2 parameters constant. The number of adherent MBs was counted as described above and plotted against the values of each manipulated variable to generate dose-response

curves for each parameter tested. To further prove the specificity of MB adhesions, the KDR coated surface was pre-treated with 1 $\mu\text{g}/\text{mL}$ VEGF₁₂₁ to block available receptors prior to MB_{VEGF} injection in additional experiments.

2.6. *In vivo* experimental protocols

The animal experimental protocols were approved by the IACUC (Institutional Animal Care and Use Committee) at the University of Pittsburgh and adhered to the guidelines for Humane Use of Animals in Laboratory Research.

2.6.1. Intravital microscopy of rat cremaster muscle—Intravital microscopy of angiogenic rat cremaster muscle was used to directly visualize *in vivo* binding of the MB_{VEGF} to endothelium. Wistar rats (120–200 g, Harlan Sprague Dawley Inc, Indianapolis, IN) were anesthetized with IP injection of sodium pentobarbital (5 mg/kg) and were then injected intrascrotally with 0.2 mL of heparin coated agarose beads pre-incubated with bFGF (3.2 μg bFGF per 6.5 mg beads),²⁷ and recovered. Five days later, the animals were re-anesthetized, an internal jugular vein was cannulated for contrast injection, and a cremaster muscle was exposed as previously described,²⁸ and mounted on the stage of an inverted microscope (TE200, Nikon) coupled with a digital camera (ORCA 285, Hamamatsu). Fluorescent MB_{VEGF} or MB_{CTL} (non-specific rat IgG) suspended in 0.2 mL saline were injected into the jugular vein. After 3 minutes to allow circulation and adhesion, 20 microscopic fields of the cremaster muscle were imaged. The number of adherent microbubbles per field was counted offline by an observer blinded to experimental conditions.

2.6.2. Mouse squamous cell carcinoma—For *in vivo* imaging of angiogenesis receptors, a mouse tumor model known to overexpress VEGF receptors was used.²⁹ Mouse squamous cell carcinoma cells SCC-VII were cultured in RPMI 1640 medium supplemented with 12.5% fetal bovine serum, 100 mg/mL streptomycin and 100 IU/mL penicillin (Gibco, ThermoFisher Scientific). C3H/NeJ female mice ($n=6$) were administered 5×10^5 squamous cell carcinoma cells subcutaneously.²⁰ After 2 weeks of tumor growth, mice were anesthetized with Avertin and the right internal jugular vein was cannulated for contrast injection.

2.6.3. Ultrasound imaging of tumor tissue—Each tumor-bearing mouse received separate intravenous injections of MB_{VEGF} and MB_{CTL} (5×10^6 MB in 0.05 mL saline), with the injection sequence alternating between mice. A previously described approach to detection of adhered MB was used.²⁸ Briefly, four and half minutes after injection, when freely circulating MB should be largely washed out, time-triggered (time interval 0.2 s) low mechanical index (MI=0.2) ultrasound imaging at 7 MHz transmit frequency was performed in the long axis plane of the tumor using Contrast Pulse Sequence (CPS), a contrast-specific modality (Acuson Sequoia, Siemens, Mountain View, CA) to acquire 2–3 frames. Because of the time elapsed from injection, videointensity resulting from these low MI images was interpreted to represent predominantly signal from adhered microbubbles and lesser signal from residual circulating bubbles. Immediately after image acquisition, 5 frames of high MI pulses (MI=1.9) were delivered to rupture the MBs in the image plane. Thirty seconds later

(approximately 5 minutes after MB injection), low MI imaging ($MI=0.2$) was repeated, with the videointensity from the 5 minute frame representing background signal from any residual freely circulating bubbles re-populating the image plane within 30 seconds of the destruction sequence. The difference between the 4.5 min and 5 min frames were attributed to adhered MBs. After completion of imaging, the mice were euthanized, and the tumor was excised for immunohistochemical analysis of VEGF receptor expression. As a control, imaging was also performed in the hearts of normal mice after injection with MB_{VEGF} or MB_{CTL} (5×10^6 MB in 0.05 mL saline) ($n=5$).

2.7. Statistical Analysis

Data were expressed as the mean $\pm$ standard deviation (SD). The difference between two groups was determined by Student's t-test (two-tailed), with $p < 0.05$ being considered statistically significant. Statistical comparisons among more than two groups were performed using one-way ANOVA, with significance defined as $p < 0.05$. If ANOVA demonstrate a significant difference among the groups, post-hoc Tukey's test was performed to examine whether the difference between two groups is statistically significant.

3. RESULTS

3.1. Conjugation of VEGF₁₂₁ to MB surface

To conjugate VEGF₁₂₁ to MB surface, biotinylated MBs were first coated with streptavidin. Figure 1A shows the relationship between the amount of streptavidin used in the conjugation and the resulting streptavidin MB surface density based on quantitative flow cytometry. As the amount of streptavidin added to the microbubbles increased, there was an increase in the amount of streptavidin bound to the microbubble surface, with saturation reached at a streptavidin concentration of about 0.07 mg/mL, corresponding to approximately 8×10^5 streptavidin molecules per MB. Based on this, we synthesized maximally coated MBs and used an excess streptavidin concentration of 0.25 mg/mL to ensure streptavidin saturation of the MB surface in subsequent experiments in which other variables were manipulated. Next, the biotinylated VEGF₁₂₁ was conjugated with MBs maximally coated with streptavidin and the number of bound VEGF₁₂₁ molecules on the MB surface was measured using quantitative flow cytometry. Based on the spectrometric measurement of 3 FITC molecules per VEGF₁₂₁ molecule, the number of VEGF₁₂₁ molecules on the bubble surface was calculated. When incubated with increasing amount of VEGF₁₂₁, the MBs were saturated with VEGF at approximately 0.03 mg/mL of VEGF₁₂₁ concentration and this corresponds to a saturation density of about 1.3×10^5 VEGF₁₂₁ molecules per MB (Figure 1B).

3.2. *In vitro* targeted MB adhesion to KDR

By changing the coating concentration of KDR-Fc chimera, the density of KDR adsorbed on the coverslip surface of the parallel plate perfusion chamber could be controlled, with saturation occurring at a KDR-Fc concentration of $\sim 2 \mu\text{g/mL}$ (Figure 2A) and highest density achieved 153 receptors/ μm^2 . Figure 2B illustrates the extent of adhesion of maximally coated MB_{VEGF} (1.3×10^5 VEGF molecules/MB) to 4 different densities of simulated KDR expression, after perfusion at a wall shear rate of 100 sec^{-1} ($n=3$ perfusions per experimental condition). Zero receptor expression was simulated by blocking the KDR-

coated surface with VEGF₁₂₁ prior to perfusion of the coverslips by MB_{VEGF}. With the increase of the KDR density, the number of adherent VEGF-bubbles increased. There was up to a 6 fold increase in bubble adhesion at maximal KDR density relative to minimal KDR density (one-way ANOVA: $p<0.05$; post-hoc Tukey's test, $p<0.05$).

Figure 2C demonstrate the effect of the density of VEGF₁₂₁ on the MB surface, ranging from no VEGF₁₂₁ to 1.3×10^5 VEGF molecules per MB, on MB adhesion to a KDR surface maximally coated with 153 receptors per μm^2 and perfused at a wall shear rate of 100 sec^{-1} ($n=3$ perfusions per experimental condition). There was an increase in the extent of MB adhesion as the number of VEGF₁₂₁ molecules on the MB surface increased. At maximal VEGF₁₂₁ MB coverage, MB adhesion was about 26 times higher compared to that with MBs coated with streptavidin alone (one-way ANOVA: $p<0.05$; post-hoc Tukey's test: $p<0.01$) (Figure 2C). The effect of wall shear rate on maximally coated MB_{VEGF} adhesion to maximally KDR-coated surfaces is shown in Figure 2D. MB_{VEGF} adhesion varied with wall shear rates between 100 sec^{-1} and 300 sec^{-1} or 400 sec^{-1} (one-way ANOVA: $p<0.05$; post-hoc Tukey's test: $p<0.05$), with a greater number of adhered MBs as wall shear rate decreased.

3.3. Microscopic visualization of MB_{VEGF} binding *in vivo*

Intravital microscopy of rat cremaster muscle lacking prior treatment with bFGF showed that neither MB_{VEGF} nor MB_{CTL} adhered to the microcirculation after intravenous MB injection (data not shown). However, there was significantly greater adhesion of MB_{VEGF} compared to MB_{CTL} in the bFGF-treated microcirculation (Figure 3) (4 ± 3 MB per 20 fields vs 23 ± 10 MB per 20 fields, $n=8$, $p<0.01$).

3.4. Targeted ultrasound imaging of tumor angiogenesis

An example of the CPS imaging of tumor tissue is shown in Figure 4, which depicts signal from adhered MB by digital subtraction of the image frames at 5 minutes from the 4.5 minute post MB injection. The contrast enhancement is color-coded as previously described,²⁸ whereby hues of red, progressing to shades of orange, yellow, and white, represent increasing videointensity change. After intravenous injection of MB_{CTL}, there was minimal contrast persistence in the tumor (Figure 4A), whereas there was strong persistent signal in the tumor after injection of MB_{VEGF} (Figure 4B). There was no significant contrast persistence in control non-tumor tissue (myocardium) after injection of either MB type (image data not shown). Overall, after MB_{VEGF} injection, ultrasound image videointensity enhancement in the tumor (17.3 ± 9.7 dB) was significantly higher than that after MB_{CTL} injection (3.8 ± 4.4 dB, $p<0.05$), and higher than that in normal myocardium after MB_{VEGF} injection (5.7 ± 6.1 dB, $p<0.05$). There was no significant difference in videointensity enhancement in normal myocardium, which was low, after injection of MB_{VEGF} compared to MB_{CTL} (5.7 ± 6.1 dB vs 5.0 ± 5.4 dB, $p=0.98$) (Figure 5, $n=6$ for each conditions).

4. DISCUSSION

The main finding of this study is that *in vivo* ultrasound imaging of tumor angiogenesis is possible using microbubbles targeted to a VEGF receptor using the naturally occurring

ligand, VEGF₁₂₁. Because VEGF₁₂₁ is the non-heparin binding isoform of VEGF, it exhibits specific affinity for KDR and Flt-1 receptors which are upregulated by endothelial cells in the setting of angiogenesis. As such, VEGF₁₂₁ is an attractive targeting ligand for an imaging probe that remains within the intravascular space, such as a microbubble. Furthermore, because the human form of VEGF₁₂₁ can be recombinantly synthesized, this protein offers advantages for human application, including non-immunogenicity (unlike monoclonal antibodies) and possibilities for large scale production. This study is distinct from other investigations in that the endogenous, naturally occurring ligand was used as the targeting moiety for the ultrasound contrast agent.

4.1 Binding characteristics of MB_{VEGF}

Conjugation of VEGF₁₂₁ to our microbubbles was achieved using biotin-avidin bridging chemistry, a simple but convenient method for a proof of concept study. We functionalized our microbubble design with great precision in terms of targeting ligand density. We first used quantitative flow cytometry to determine the saturation behavior of streptavidin because in principle, microbubble saturation by streptavidin should allow us to achieve the highest VEGF₁₂₁ density on the microbubble surface. Subsequent quantitative flow cytometry using VEGF₁₂₁ tagged with 3 FITC molecules per VEGF molecule permitted measurement of the maximum number of VEGF₁₂₁ molecules ($1.3 \times 10^5/\text{MB}$) that can be attached to the microbubbles at a saturating concentration of streptavidin, and conferred the ability to regulate VEGF₁₂₁ microbubble surface coverage. In addition, we controlled the *in vitro* receptor coating on the parallel plate system to modulate varying degrees of KDR expression and have thus developed a method for attaching, quantifying, and modulating KDR “expression” on glass coverslips for *in vitro* perfusion testing.

The *in vitro* experiments utilizing a parallel plate perfusion chamber bearing KDR and perfused with MB_{VEGF} proved a causal relationship between the presence of VEGF₁₂₁ on the MB and the binding of the MB to KDR coated surfaces: there was a “dose response” effect in that progressively greater VEGF₁₂₁ density on the MB resulted in progressively higher numbers of adherent microbubbles (Figure 2B). Specificity was further demonstrated by the fact that microbubbles coated with monomer VEGF (data not shown) or streptavidin alone did not bind to KDR surfaces, and antecedent exposure of the KDR surfaces to VEGF₁₂₁ abolished binding of the MB_{VEGF} (Figure 2B and 2C).

An important *in vitro* finding was that binding of MB_{VEGF} was impacted by the density of KDR receptors, with more MB_{VEGF} adhesion as KDR density increased (Figure 2B). This suggests that not only can MB_{VEGF} detect the presence of angiogenic receptors, but also the magnitude of receptor expression. It should be noted that the maximum KDR density achievable in our *in vitro* preparation, 153 receptors/ μm^2 , is less than that previously noted *in vivo*, with 250,000 receptors per hypoxic endothelial cell reported in the literature,³⁰ suggesting that *in vivo*, there could be even more opportunities for MB_{VEGF} to bind than was simulated in our flow chamber studies.

We also modulated flow through the perfusion chamber, resulting in variations in the wall shear rate under which MB_{VEGF} would be interacting with endothelium. As expected, there was a relationship between wall shear rate and the extent of binding, with fewer targeted

bubbles binding as wall shear rate increased. Our findings are consistent with data previously reported by our group for using microbubbles targeted to the leukocyte adhesion molecule ICAM1 using a monoclonal antibody, in which the extent of targeted microbubble adhesion was influenced by MB antibody and surface ICAM1 density and wall shear rate.²⁴ Our data are also directionally consistent with predictions from computational models that simulate particle binding probabilities based on parameters such as ligand and receptor density and wall shear rate.^{31, 32}

As would be predicted by our *in vitro* data, maximally targeted MB_{VEGF} also bound to microcirculation subjected to angiogenic stimulation, as optically visualized in our intravital microscopy studies of bFGF-injected cremaster muscle. MB_{VEGF} bound to the bFGF-stimulated cremaster microcirculation significantly more than MB_{CTL} did (Figure 3), and MB_{VEGF} did not bind to “normal” cremaster muscle microcirculation not previously exposed to bFGF (data not shown).

4.2. *In vivo* ultrasound imaging of VEGF receptors

To determine if the directly visualized MB_{VEGF} binding events could be detected *in vivo* using a clinical ultrasound scanner, we used a previously described mouse model of squamous cell carcinoma known to be highly angiogenic.³³ Using a microbubble-specific imaging approach that utilizes the non-linearity of microbubble behavior in an appropriately tuned ultrasound field, we found a strong signal from the tumors after MB_{VEGF} injection that persisted even after most freely circulating MBs had left the circulation, consistent with MB adhesion (Figures 4 and 5). Such signal persistence was not seen in the tumors after injection of MB_{CTL} or in normal myocardium after injection of either microbubble type (control or VEGF-coated), suggesting that signal persistence was due to a specific receptor binding event.

The effect of VEGF₁₂₁ MB on tumor angiogenesis or tumor growth was not investigated in this study. Mohamedali *et al* reported that treatment with a fusion protein composed of VEGF₁₂₁ and plant toxin gelonin (rGel) result in 60% tumor growth inhibition and an average increase of 34.4% of Flk1/KDR level in mice tumor, compared to controls.³⁴ In another study, Okunieff *et al* reported that intratumoral administration of exogenous VEGF to KHT murine fibrosarcoma tumor model with one or six daily dose did not significantly change tumor weight and size, compared to saline control. However, the VEGF treatment slightly decreased the mRNA expression of tumor tissue chemokine MCP-1, interleukins (IL-1beta, IL-6, and IL-18), and increased NFκB binding without altering Ap-1 binding of IκB protein expression.³⁵ It is not clear if the VEGF₁₂₁ MBs can induce angiogenesis in tumor; while the literature suggests that exogenous VEGF may elicit signaling and by extension, that VEGF₁₂₁ MBs may cause some subtle changes in molecular level, it is very unlikely that it will promote tumor growth. In our studies, the number of bound MB_{VEGF} is relatively low, suggesting that the actual number of VEGF-KDR binding events is relatively low and unlikely to cause significant angiogenesis. Indeed, even therapeutic clinical trials of VEGF₁₆₅ delivery at pharmacologic doses in peripheral vascular or coronary disease failed to significantly increase angiogenesis,³⁶ further suggesting that the relatively sparser MB_{VEGF}-KDR interaction is very unlikely to result in significant angiogenesis.

4.3. Comparison with previous studies

Other approaches to molecular imaging of angiogenesis have also targeted the integrin $\alpha_v\beta_3$ which is upregulated in angiogenesis. These approaches have utilized either antibodies,^{37–40} or peptides with affinity for integrin $\alpha_v\beta_3$ on the microbubble shell.^{38, 41, 42} Because of concerns over the immunogenicity of antibodies in general, peptides have drawn interest as targeting agents. We have previously reported on an MB bearing a cyclic tripeptide sequence biopanned using *E. coli* peptide display library against tumor derived endothelial cells, capable of imaging neovascularization in a variety of tumor models.^{22, 24} Lipid microbubbles with the integrin $\alpha_v\beta_3$ -binding peptide echistatin on the surface have been used to ultrasonically image neovascularization in rodent models of hindlimb ischemia⁴² and brain tumors.³⁶ Recently, a lipid microbubble bearing a heterodimer peptide with VEGFR2 specificity was reported to bind *in vitro* to cultured cells expressing VEGFR2 and to accumulate in tumors in rodent orthotopic models of breast and prostate adenocarcinoma.^{43, 44} A novel engineered cystine knot (knottin) peptide with low nanomolar affinity to integrin $\alpha_v\beta_3$ has been attached to lipid microbubbles and been used for targeted imaging of integrins in tumor vasculature in mice with xenografted human ovarian adenocarcinoma.⁴⁵ However, an altered VEGF molecule with a systine tag (scVEGF) has been previously used to target VEGF surface^{16–20, 46}

Ours is the first study successfully utilizing the dimer VEGF₁₂₁ attached to MBs to ultrasonically detect VEGF receptors on angiogenic tumor endothelium. Prior to the synthesis of VEGF coated MBs, we previously examined the ability of VEGF₁₂₁ to bind to angiogenic vasculature using Indium-111-labeled VEGF₁₂₁ in a rabbit model of unilateral hindlimb ischemia,¹⁴ and noted selective localization of the Indium-111 label in the ischemic hindlimb undergoing compensatory angiogenesis. These studies formed the rationale for the examination of a VEGF₁₂₁-coated microbubble in the present study.

The KDR receptor for VEGF is a sensible target for an angiogenesis imaging probe, and others have reported studies in which VEGF receptor-targeted MBs were used for molecular imaging of various tumor types in mice.^{47–51} These studies utilized VEGF receptor monoclonal antibodies as the targeting moiety, which cannot be easily extended to the clinical arena due to the immunogenicity of animal-derived monoclonal antibodies. Current modern humanized monoclonal antibodies have raised promise for antibody approaches that are less immunogenic than in the past. Our study is unique in that we used the naturally occurring ligand for VEGF receptors, VEGF₁₂₁ itself, as the targeting agent. Because we used human VEGF₁₂₁, unlike studies using monoclonal antibodies, our data have more direct extension to imaging angiogenesis in humans, although this remains to be verified.

Our study is also unique in its comprehensive scope, wherein extensive *in vitro* studies demonstrated dose-response relationships between VEGF-MB adhesion and parameters such as targeting ligand density, receptor density, and wall shear rate, and intravital microscopy proved microvascular binding to neovessels *in vivo*. Such data not only unequivocally confirmed that the microbubbles adhered due to the VEGF-VEGFR interaction, but also suggested that the extent of receptor expression could be quantified using targeted MB as well. Also, unlike some previous studies using monoclonal antibodies, in which the amount of streptavidin or antibody on the microbubble was not precisely

measured, we quantified and modulated the targeting ligand density on the microbubble, resulting in insights into dose response relationships which should be very helpful in clinical translation of this technology. In addition, the image signal from the targeted agents used in previous studies is considerably lower than that reported in our study. Likely this is due to our use of a microbubble-sensitive imaging strategy based on non-linear acoustic behaviors of microbubbles, compared to imaging methods using fundamental frequency imaging at high frequency.^{52, 53} Thus, overall, the use of the naturally occurring ligand in conjunction with an imaging system with non-linear detection of microbubble signals in a clinically relevant frequency range, represent a significant advance towards clinical translation.

4.4. Limitations

Our *in vitro* studies inherently cannot fully recapitulate physiologic conditions. Perfusions in the flow chamber were not performed using blood as the perfusion media; components in blood may unfavorably affect the availability of VEGF on the MB surface. Furthermore, the KDR surfaces that were created to simulate cells overexpressing VEGF receptor are of uncertain relevance to *in vivo* expression, with evidence suggesting that our surfaces underestimated maximal *in vivo* expression.³⁰ The findings of our intravital microscopy studies and *in vivo* ultrasound imaging studies, however, suggest that binding of the MB_{VEGF} occurs under physiologic conditions as well.

We used normal myocardium as a control tissue demonstrating no significant VEGF overexpression at baseline (Figure 5), as tumors are inherently angiogenic and we are not aware of tumor models lacking VEGF receptor up-regulation. Tumor tissue may have altered permeability but the possibility of MBs extravasation is minimal as the fenestration in the tumor tissue is generally much smaller than the size of MBs used for this study.

We used the well-established method of biotin/avidin conjugation for this proof of concept study. However, this protein based approach could influence the pharmacokinetics and other properties of the MBs. Other conjugation strategies, such as thiol/maleimide chemistry, should be investigated in future studies.

The rationale for selecting VEGF₁₂₁ for a targeting ligand is to develop an angiogenesis imaging agent which can be readily extended into a human application. An approach using the naturally occurring ligand has appeal from the standpoint of immunogenicity. Further study will be required to determine if such an approach yields acceptable image quality, is cost effective, and safe.

5. CONCLUSION

VEGF receptor imaging is a potentially power approach for localizing and quantifying angiogenesis across a spectrum of pathophysiologic states including angiogenesis in malignant tumors, ischemic heart disease, and peripheral vascular disease. The ability to identify angiogenesis using a molecular imaging approach not only has useful diagnostic applications, but also has potentially important utility in the evaluation of prognosis and responses to therapy.⁵⁴ Ultrasound molecular imaging of angiogenesis is a portable, non-invasive technique that can be performed serially and without exposure to ionizing radiation

that limits other molecular imaging methods. Clinical translation of ultrasound molecular imaging will be facilitated by the identification of targeting ligands that have specificity, bind with high affinity, are non-immunogenic, and can be synthesized readily. Our study suggests that human VEGF₁₂₁ confers these properties, potentially offering a clinically usable approach to molecular imaging of angiogenesis. Furthermore, our study shows that this human VEGF₁₂₁ which is designed to bind to human tissue, also binds to rat and mouse models, thus providing the opportunity to perform further pre-clinical validation studies in rodent models.

Acknowledgments

We thank Linda Lavery, Xiaoping Leng, and Ruth Modzelewski for technical help. We would like to express our special thanks to Ute Schellenberger of Scios Inc. for providing the VEGF used in this study. This work was partially funded by a grant from the National Institutes of Health [R01HL077534].

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Figure 1.

(A) The average number of streptavidin molecules on the MB surface as a function of streptavidin incubation concentration. The saturation concentration at 0.25 mg/mL was used for maximum conjugation, corresponding to about 8×10^5 streptavidin molecules per MB. (B) The average density of VEGF₁₂₁ molecules on the MB surface as a function of VEGF₁₂₁ incubation concentration. At the saturation concentration for VEGF₁₂₁ conjugation, there were 1.3×10^5 VEGF₁₂₁ molecules per MB. Dashed lines are mono-exponential fits of the data.

Figure 2.

(A) KDR density on coverslips as a function of KDR conjugation concentration. Dashed line is a mono-exponential fit of the data. The maximum KDR density reached was 153 KDR molecules/ μm^2 ($n=2$). (B) The effect of KDR density on the adhesion of maximally VEGF₁₂₁-coated MBs at a wall shear rate of 100 sec^{-1} ($n=3$). Adhesion of MB was abolished by blocking KDR with VEGF₁₂₁ prior to MB perfusion. (C) Adhesion of MB with varying VEGF₁₂₁ density on the MB surface ($n=3$), constant KDR density (153 molecules/ μm^2) on the coverslip, and wall shear rate of 100 sec^{-1} . (D) The number of adhered MB as at different wall shear rates. Data were obtained using MB bearing maximal VEGF₁₂₁ surface density perfused over maximally coated KDR coverslips ($n=3$ perfusions per experimental condition).

Figure 3. Number of adhered MB (20 fields) in rat cremaster muscle microcirculation ($n=8$) pre-treated with bFGF to stimulate angiogenesis. MB_{VEGF} adhesion was significantly higher than that of MB_{CTL} ($p<0.01$).

Figure 4. Targeted ultrasound imaging of SCC tumor angiogenesis 14 days after tumor cell inoculation. There is greater videointensity persistence within the tumor 4.5 minutes after MB_{VEGF} injection compared to MB_{CTL} . Color-coded images represent the net adhered MB.

Figure 5. Videointensity enhancement in SCC tumor ($n=6$) and normal myocardium ($n=5$) after injection of MB_{CTL} or MB_{VEGF}. $p<0.05$ for MB_{VEGF} in tumor versus MB_{VEGF} in normal myocardium and MB_{CTL} in either tumor or normal myocardium. For MB_{CTL}, there was no significant difference in videointensity enhancement between tumor and normal myocardium (5.7 ± 6.1 dB vs 5.0 ± 5.4 dB, $p=0.98$).