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A Molecular Imaging Paradigm to Rapidly Profile Response to Angiogenesis-directed Therapy in Small Animals

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Abstract

Purpose—The development of novel angiogenesis-directed therapeutics is hampered by the lack of non-invasive imaging metrics capable of assessing treatment response. We report the development and validation of a novel molecular imaging paradigm to rapidly assess response to angiogenesis-directed therapeutics in preclinical animal models.

Procedures—A monoclonal antibody-based optical imaging probe targeting vascular endothelial growth factor receptor-2 (VEGFR2) expression was synthesized and evaluated *in vitro* and *in vivo* via multispectral fluorescence imaging.

Results—The optical imaging agent demonstrated specificity for the target receptor in cultured endothelial cells and *in vivo*. The agent exhibited significant accumulation within 4T1 xenograft tumors. Mice bearing 4T1 xenografts and treated with sunitinib exhibited both tumor growth arrest and decreased accumulation of NIR800- α VEGFR2ab compared to untreated cohorts ($p=0.0021$).

Conclusions—Molecular imaging of VEGFR2 expression is a promising non-invasive biomarker for assessing angiogenesis and evaluating the efficacy of angiogenesis-directed therapies.

Keywords

Molecular imaging; Biomarker; VEGFR2; Sunitinib; Angiogenesis; Optical imaging; Multispectral fluorescence; Near-infrared fluorescence; Tyrosine kinase inhibitor; VEGF

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Introduction

Angiogenesis, or the formation and recruitment of new vasculature, is a highly orchestrated biological process that is primarily confined to wound healing and reproduction in healthy individuals. Dysregulated angiogenesis is a pathological condition and characteristic of a number of common diseases including diabetes, psoriasis, rheumatoid arthritis, and cancer [1]. Angiogenesis plays a central role in the development and progression of tumors, as neovascularization is required to supply oxygen and nutrients to rapidly growing tumor cells and in turn facilitates the spread of metastases [2].

Appreciation of the critical role angiogenesis plays in cancer progression has spurred development of a variety of angiogenesis-directed therapeutic agents, several of which are now clinically approved for use in various cancers [3]. Tumor-induced angiogenesis is predominately driven by paracrine vascular endothelial growth factor (VEGF) signaling between tumor and/or stromal cells, which can secrete a variety of soluble VEGF ligands, and endothelial cells, which express tyrosine kinase VEGF receptors [4-6]. Clinically employed approaches to inhibit VEGF signaling include therapeutic monoclonal antibodies (mAbs) such as bevacizumab (Avastin™) that aim to neutralize and sequester soluble VEGF ligands as well as VEGF receptor-targeted small molecule tyrosine kinase inhibitors (TKIs) that inhibit receptor activation and signaling [7-11]. While these and other angiogenesis-directed therapies appear promising and are rapidly entering into mainstream clinical use, a lack of objective, non-invasive biomarkers of response to this class of pharmaceuticals hinders the development and evaluation of these agents in the preclinical setting as well as the prediction of patients that are likely to benefit from these drugs clinically.

Non-invasive imaging modalities that directly assess molecular targets show considerable promise for monitoring numerous biological processes and therapeutic responses. The VEGF family of receptors is an attractive class of imaging targets as they are primarily expressed on the surface of endothelial cells [12] which enables facile delivery of imaging compounds throughout the bloodstream. Molecular imaging of angiogenesis via VEGF receptor expression requires the use of sensitive imaging modalities because the receptors are predominately expressed on tumor-related vasculature, rather than tumor cells themselves, and therefore comprise only a minor fraction of a given tumor volume [13]. Nuclear imaging methods, such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), as well as optical imaging methods, such as fluorescence and bioluminescence techniques, possess the requisite sensitivity and are suitable modalities for studying angiogenesis [14]. To this end, various VEGF receptor ligands have been labeled for PET/SPECT [15-23] and near-infrared (NIR) fluorescence imaging [24]. In many cases, imaging probes of this type have shown promise but may be limited by their reactivity with more than one VEGF receptor subtype, notably VEGFR1 and VEGFR2. VEGFR2 expression is most relevant to tumor angiogenesis, as VEGFR2 mediates the major angiogenic effects of VEGF. The significance of VEGFR1 is not fully understood in cancer but it appears that a possible role is to sequester VEGF [25]. An additional drawback of ligand-based probes is that they can be prohibitively expensive to produce in sufficient quantity to support large, statistically meaningful studies in small animals or humans.

In this study, we capitalize upon the strengths of optical molecular imaging to demonstrate a rapid, low-cost, non-invasive approach to assess VEGFR2 expression in small animal models. We report the synthesis, validation, and capability of a novel optical imaging probe, NIR800- α VEGFR2ab, to measure tumor angiogenesis and profile response to angiogenesis-directed therapy in a preclinical mouse model of breast cancer. Our approach employs a VEGFR2-specific monoclonal antibody as a targeting vector, thereby minimizing potential cross reactivity with other VEGFR subtypes [26]. We anticipate that this strategy may provide further

insight into the role angiogenesis plays in tumor progression and enable non-invasive, longitudinal monitoring of novel angiogenesis-directed therapies in preclinical models of cancer.

Materials and Methods

Imaging Agent Synthesis

Biotin-conjugated antibodies to mouse VEGFR2 and mouse isotypic IgG2a were acquired from eBioscience (San Diego, CA, USA). Prior to conjugation, antibodies were dialyzed against 1× phosphate-buffered saline (PBS, pH 7.4, 0.2 g/l) using a 5,000 molecular weight cutoff dialysis cartridge (Pierce, Rockford, IL, USA) and concentrated using a Centricon tube (Millipore, Billerica, MA, USA) centrifuged at 3,000 rpm for 30 min. The N-hydroxy succinimide ester of near-infrared 800CW reactive dye (Licor, Lincoln, NE, USA) was added to the antibody in ice-cold PBS with dye to protein stoichiometry 10:1. The antibody–dye mixture was gently agitated in a light-protected vessel for 3 h at room temperature. Unconjugated dye was removed from the solution by dialysis against 1× PBS at 4°C using a 7,000 molecular weight cutoff dialysis cartridge (Pierce). Dialysis was continued for approximately 42 h with buffer changes every 14 h. Gel filtration liquid chromatography was used to assess conjugate identity and resultant purity. Chromatography was conducted using an Amersham Superdex 200HR column eluting with 1× phosphate-buffered saline flowing at 0.4 ml/min at room temperature. Under these conditions, the antibody product eluted at approximately 20.3 min and free, non-conjugated dye eluted at 54.5 min.

Spectroscopic Analysis

Spectroscopic analysis of VEGFR2 antibody conjugated to the NIR dye (NIR800- α VEGFR2ab) was performed in aqueous media at room temperature. Absorbance was measured using an Agilent UV/Vis spectrophotometer (Santa Clara, CA, USA) and fluorescence was determined using a PTI NIR fluorometer (Birmingham, NJ, USA).

The dye to protein ratio (D/P) was determined using methods outlined in the Licor 800CW product literature. Briefly, the absorbance at 780 nm (corresponding to absorbance by the NIR dye) was divided by the molar extinction coefficient of the dye (240,000 l/mol cm) to yield the dye concentration. The absorbance at 280 nm (corresponding to the absorbance of the antibody) minus a 3% correction factor accounting for NIR dye absorbance at 280 nm was divided by the molar extinction coefficient of the antibody (203,000 l/mol cm) to determine the protein concentration. D/P was determined by dividing these two quantities.

Immunoblotting

Protein lysates were extracted from cultured bEnd3 and 4T1 cells with RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) at 1×10^7 cells/ml and supplemented with a protease inhibitor mixture (Sigma-Aldrich Co., St Louis, MO, USA). Lysates were clarified by centrifugation ($15,000 \times g$) for 15 min at 4°C. The whole cell protein extract was denatured by boiling 5 min in Laemmli buffer (62.5 mM Tris–HCl, 2% SDS, 1 mM EDTA, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, pH 6.8). Twenty micrograms of each protein sample was then separated by 7.5% SDS-polyacrylamide gel electrophoresis. After separation, the proteins were transferred to PVDF (polyvinylidene difluoride) membranes (Millipore) in 25 mM Tris/200 mM glycine (pH 8.3) buffer containing 20% methanol at 4°C for 2 h at a constant current of 100 mA. The membrane was blocked with 5% fat-free dry milk in Tris-buffered saline–Tween 20 (TBST, 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 1 h, and then incubated with primary antibodies (1:500 dilution for VEGFR2 antibody, and 1:5,000 for actin antibody) for 2 h at room temperature. The primary antibody against VEGFR2 was the same one used in the

imaging probe and the actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). After washing three times for 10 min each with TBST buffer, the membrane was incubated for 30 min at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000; Sigma-Aldrich), followed by three 10-min washes with TBST and one final wash with TBS. The protein bands were detected by ECL plus (Enhanced chemiluminescence Plus, Amersham Biosciences, Pittsburgh, PA, USA) by following the manufacturer's recommended procedure. The predicted molecular weight of mouse VEGFR2 is 150 kDa and was consistent with our blotting observations (observed 180 kDa).

In Vitro Binding Assay

Mouse brain-derived endothelial cells (bEnd3) cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum under standard culture conditions in a humidified incubator maintained at 5% CO₂/95% room air at 37°C. Cells were plated at a density of 4×10^5 cells per well in a 96-well optical bottom plate. Cells were incubated with 2% BSA and CD16/CD32 antibodies to block non-specific Fc bindings. Analogous to previous studies [27], cells were incubated with near-infrared dye labeled VEGFR2 antibody (NIR800- α VEGFR2ab) or near-infrared dye labeled non-specific isotypic IgG (NIR800-IgG) in serial dilutions for 30 min and washed three times with 1 $\times$ PBS to remove unbound imaging probe. For the blocking experiment, cells were incubated with 40 nM NIR800- α VEGFR2ab, 40 nM NIR800-IgG, or pre-incubated with 400 nM unlabeled VEGFR2 antibody for 30 min prior to incubation with 40 nM NIR800- α VEGFR2ab. Fluorescence was imaged using an Odyssey plate reader (Licor). The fluorescence emission from each well was quantified by summing photon emission from a region of interest (ROI) encompassing each well. Analysis was performed in triplicate.

Animal Model

All experiments involving animals were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Athymic nude mice (5–6 weeks old, Harlan, Indianapolis, IN, USA) were inoculated with 5×10^6 4T1 mouse breast cancer cells suspended in PBS subcutaneously in the right hind limb. Nude mice were used in order to minimize NIR light attenuation. Tumors were allowed to grow to a consistent, palpable size (longest dimension as assessed by caliper measurement approximately 1.0 ± 0.3 cm) for 2 weeks and subsequently imaged.

In vivo Imaging

Mice were injected intravenously via the retro-orbital sinus with 0.5×10^{-9} mol (diluted in sterile saline to a total injection volume of 100 μ l) of NIR800- α VEGFR2ab imaging agent or an equimolar amount of NIR800-IgG. Fluorescence images were taken using a MaestroTM imaging system (CRI, Woburn, MA, USA). Imaging was performed longitudinally up to 40 h after agent administration, with optimal tumor binding determined to occur 24 h after injection. Images were taken with a 1 s exposure time. Spectral un-mixing was performed on fluorescence images to remove auto fluorescence. Briefly, two distinct fluorescence spectral libraries were created: one of the NIR800 dye and another consisting of auto fluorescence from the animal skin and food. Non-linear least squares fitting was employed to distinguish the proportion of fluorescence from each of these two spectra at each pixel. Total fluorescence intensity from the NIR800 dye was quantified by photon counting measurements within a region of interest (ROI) encompassing the tumor normalized by measurements of an equal area ROI over the contralateral hind limb.

Sunitinib Treatment

Sunitinib was synthesized by the Vanderbilt Institute of Chemical Biology Core Synthesis facility using a five-step method previously described [28]. For use, the drug was reconstituted in 75% phosphate-buffered saline (PBS) and 25% dimethyl sulfoxide. Treated mice were injected daily with 40 mg/kg of sunitinib via the intraperitoneal cavity for four consecutive days. Control animals were injected daily with the same volume of vehicle. Tumor size (longest dimension) was measured by caliper measurement immediately prior to treatment and post treatment (5 days later).

Histological Evaluation

Animals were sacrificed <1 h following final imaging. Formalin-fixed paraffin-embedded tissues were sectioned at 5 μ m for IHC studies. The tissues were immunoassayed with a rabbit monoclonal VEGF Receptor 2 antibody (Cell Signaling, Beverly, MA, USA; 1:100 primary dilution) and a mouse monoclonal CD34 (Dako Cytomation, Carpinteria, CA, USA; 1:100 primary dilution). The slides were deparaffinized, rehydrated, and an antigen retrieval step was performed using an EDTA (pH 9.0) solution applied for 15 min at 120°C followed by a 10-min bench cool down. The samples were then treated with 0.03% H₂O₂ with sodium azide to eliminate endogenous peroxidase activity. A subsequent serum-free protein block was applied for 60 min prior to primary incubation. The VEGFR2 and CD34 samples were incubated for 90 and 30 min, respectively, at the noted dilutions. Both primary incubations were done at room temperature. Subsequent detection was accomplished utilizing the Envision+ System-HRP, peroxidase-labeled polymer staining method for 30 min (Dako Cytomation). The stain was completed with a DAB substrate–chromogen solution. For hematoxylin and eosin staining, slides were deparaffinized and rehydrated, stained in hematoxylin (Richard Allan Scientific, Kalamazoo, MI, USA) for 5 min, rinsed and cleared in acetic acid for 90 s, and then placed in bluing agent for 30 s. After rinsing, slides were stained in eosin (Richard Allan Scientific) for 80 s, dehydrated in increasing concentrations of ethanol, and coverslipped. Digital images of each slide were taken with a 20 $\times$ objective. Morphometric analysis was performed on VEGFR2 and CD34 stained slides. Tumors from 26 mice (17 vehicle and nine sunitinib-treated mice) were analyzed for VEGFR2 expression; tumors from ten mice (five vehicle and five sunitinib-treated mice) were analyzed for CD34 expression. The amount of VEGFR2 or CD34 staining in each of three fields per slide was determined with computer-aided morphometric analysis using Maestro™ software (CRI).

Statistics

An unpaired *t* test was used to compare experimental results using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego, CA, USA). Data are expressed as means $\pm$ SEM. Values of *p* $\leq$ 0.05 were considered significant.

Results

Spectroscopic Characterization of NIR800- α VEGFR2ab Imaging Probe

Aqueous-phase spectroscopic characterization of NIR800- α VEGFR2ab demonstrated peak absorbance at 777 nm and fluorescence emission peak at 798 nm (Fig. 1). The dye-to-protein ratio of the purified conjugate was determined to be approximately 1:1 and was maintained through subsequent imaging probe syntheses. Gel filtration liquid chromatography characterization revealed a single labeled product species that eluted at 20.3 min with no detected free dye (RT=54.5 min).

Immunoblot of bEnd3 Cells Reveals Endothelial VEGFR2 Expression

As previously described [29], mouse brain-derived endothelial (bEnd3) cells were found to express large amounts of VEGFR2 (Fig. 2a), making them suitable for characterization of the imaging probe *in vitro*. Conversely, 4T1 breast tumor cells, which were subsequently used for *in vivo* imaging experiments, expressed minimal amounts of VEGFR2 (Fig. 2a). These observations suggest that *in vivo* accumulation of the imaging probe in xenograft tumors prepared from this cell line would primarily stem from recruited or co-opted endothelial cells rather than the tumor cells.

In Vitro NIR Fluorescence of Imaging Probe Binding to Endothelial Cells

We compared the uptake properties of the VEGFR2-targeted NIR800- α VEGFR2ab and non-targeted IgG-based imaging probes in live endothelial cells. Murine endothelial VEGFR2-expressing (bEnd3) cells were found to uptake the NIR800- α VEGFR2ab imaging probe. Furthermore, we found significantly reduced, yet detectable, retention of a non-specific IgG-based probe (Fig. 2b). Specificity of NIR800- α VEGFR2ab was further illustrated in bEnd3 cells that were pre-incubated with excess unlabeled VEGFR2 antibody for 30 min. As shown, pretreatment with unlabeled (cold) VEGFR2 antibody effectively reduced the specific binding of NIR800- α VEGFR2ab to levels of uptake comparable to the non-specific control IgG-based probe (Fig. 2c).

In vivo NIR Fluorescence Imaging of NIR800- α VEGFR2ab

Immediately following intravenous administration of the NIR800- α VEGFR2ab imaging probe, fluorescence was uniformly visible throughout the mouse indicating systemic circulation of the probe. Twenty-four hours after injection, the dye had accumulated primarily in the liver, kidney, and tumor region. Significant accumulation of NIR800- α VEGFR2ab was observed in the anatomical location of the hind limb tumor (Fig. 3a). Importantly, a non-specific imaging probe of similar size (NIR800-IgG) showed similar biodistribution but did not display accumulation in the tumor over the same time course, indicating the specificity of NIR800- α VEGFR2ab (Fig. 3b). Fluorescence of NIR800- α VEGFR2ab in the tumor region was on average 1.691 ± 0.080 times higher than the contralateral hind limb. Fluorescence of the non-specific NIR800-IgG was found to be lower (average 0.925 ± 0.074 tumor-to-contralateral ratio) than the contralateral limb, indicating a lack of any binding or pooling within the tumor region at 24 h. Relative fluorescence (tumor vs. contralateral hind limb) of NIR800- α VEGFR2ab probe was significantly ($p < 0.0001$) higher than that of the non-specific NIR800-IgG probe (Fig. 3c).

Imaging probes that elicit biological responses are non-ideal for repetitive, longitudinal imaging. To determine whether imaging with the NIR800- α VEGFR2ab probe resulted in a therapeutic response, we carefully examined NIR800- α VEGFR2ab binding and tumor size measurements in two cohorts of mice: one receiving an imaging dose of NIR800- α VEGFR2ab 5 days prior and one naïve to NIR800- α VEGFR2ab injection. We found no significant difference in NIR800- α VEGFR2ab fluorescence or tumor growth rates between each set of animals, indicating that an imaging dose of NIR800- α VEGFR2ab probe had no detectable therapeutic effect on the 4T1 tumors (Fig. 1 of the Electronic Supplementary Material).

Assessment of Response to Sunitinib Treatment

Control, tumor-bearing animals receiving vehicle consistently displayed tumor growth over the course of the experimental regimen, with tumor sizes on average $28 \pm 4\%$ larger after 5 days (Fig. 4a). In contrast, tumor-bearing animals treated daily with sunitinib demonstrated significantly reduced tumor growth over the 5-day regimen ($p = 0.0007$) (Fig. 4a). Fluorescence imaging of NIR800- α VEGFR2ab retention in the tumor was similarly reduced in sunitinib-

treated animals ($p=0.0021$) (Fig. 4b). Fluorescence imaging of NIR800- α VEGFR2ab retention in the tumors of untreated animals rose to 2.356 ± 0.074 ($n=10$) higher than the contralateral limb while for sunitinib-treated animals this ratio was 1.832 ± 0.1284 ($n=9$). As total NIR800- α VEGFR2ab uptake within the tumor region reflects the total quantity of VEGFR2 expression, which may depend on the size of the vascular bed, a correlation was observed between tumor size and NIR800- α VEGFR2ab imaging results in vehicle-treated mice (Fig. 2a of the Electronic Supplementary Material).

Histological Evaluation

Post-mortem immunohistochemical analysis of VEGFR2 expression was performed on tumor sections immediately following imaging. Tumor specimens collected from vehicle-treated animals exhibited robust staining for VEGFR2 expression that appeared to be primarily confined to endothelial cells (Fig. 5a). Alternately, staining of VEGFR2 in tumor specimens collected from sunitinib-treated animals was significantly reduced compared to untreated cohorts (Fig. 5b), corroborating the imaging observations. Assessment of vessel density via immunohistochemical staining for CD34 (Fig. 5c, d), a transmembrane glycoprotein constitutively expressed on endothelial cells, as well as typical hematoxylin and eosin (Fig. 5e,f) was found to be somewhat reduced in sunitinib-treated animals, although the difference was not statistically significant. Quantitative morphometric analysis of VEGFR2 expression in vehicle- and sunitinib-treated animals revealed significantly reduced ($p=0.0266$) VEGFR2 staining in sunitinib-treated animals (Fig. 5g). Furthermore, morphometric analysis of VEGFR2 expression correlated with NIR800- α VEGFR2ab retention (Fig. 5h), indicating that imaging results reflect expression of the target receptor. In contrast, the density of VEGFR2+ vessels did not correlate with tumor size, indicating that caliper measurements are not capable of reflecting expression of VEGFR2 (Fig. 2b of the Electronic Supplementary Material). CD34 + vessels were somewhat reduced in sunitinib-treated animals compared to vehicle-treated animals but the difference was not significant ($p=0.1483$) (Fig. 5g).

Discussion

Objective, quantitative, non-invasive biomarkers of response to angiogenesis-directed therapies are increasingly important as the role angiogenesis plays in tumor initiation and progression is further appreciated. The development of improved tools that enable evaluation of novel therapeutic agents in preclinical animal models is essential. Within this setting, technologies are sought that facilitate rapid, cost-effective screening of large groups of animals [14]. Imaging strategies commonly employed to assess angiogenesis include dynamic contrast-enhanced magnetic resonance imaging [30], ultrasound [31], computed tomography [32], and fluorescence perfusion imaging [33]. In many cases, these techniques are well-established surrogates of angiogenesis and have proven utility [34,35], but they are limited by their non-specific nature and low throughput [36-38]. Since structural alterations resulting from inhibition of the VEGF pathway lag the molecular event, early assessment of treatment response with these anatomical techniques is limited [39], underscoring a key advantage of our described molecular imaging approach. Furthermore, optical imaging methodology as demonstrated here is reasonably high throughput; in contrast to nuclear methods such as PET or SPECT, screening rates of 40–60 animals/h can be easily achieved.

In this study, we report an optical molecular imaging approach to non-invasively monitor expression of the proangiogenic vascular endothelial growth factor receptor 2 (NIR800- α VEGFR2ab). Assessment of this specific molecular event (VEGFR2 expression) is highly relevant to cancer imaging and evaluation of treatment response due to VEGFR2's fundamental role in the promotion of angiogenesis and vascular permeability [25]. Except in the case of specialized transgenic species, in the majority of mouse models, recruited tumor-associated

endothelium is of murine origin. We therefore assembled our imaging probe using a mouse-derived mAb for increased specificity, in contrast with previous studies using a human-derived VEGFR2 antibody [16].

To illustrate the utility of our imaging probe to indicate anti-angiogenic treatment response in a common mouse model of breast cancer, we imaged 4T1 xenograft-bearing mice with NIR800- α VEGFR2ab that had either been treated with the VEGFR tyrosine kinase inhibitor sunitinib or a saline vehicle. Sunitinib is a pan-kinase inhibitor that is known to effectively inhibit VEGFR2 expression as well as other angiogenesis-related receptors and has demonstrated efficacy in phase 2 trials of breast cancer [40]. We found that mice bearing 4T1 xenografts that had been treated with sunitinib for 5 days displayed reduced NIR800- α VEGFR2ab retention when compared to animals treated with vehicle alone. This data suggests that the presented imaging paradigm is capable of reporting response to anti-angiogenic treatment. Corroborating the efficacy of the drug in this model, we noted reduced tumor growth in treated animals compared to untreated cohorts. In post-mortem tissue analysis, we found significantly reduced endothelial staining of VEGFR2 within the tumors of treated mice compared to untreated cohorts following just 5 days of treatment, validating that changes observed by molecular imaging were brought on by down-regulation of endothelial cell VEGFR2 expression. Importantly, within the relatively short treatment window employed by these investigations, we did not observe statistically significant changes in vessel density by anti-CD34 staining, suggesting that the presented molecular imaging paradigm is suitable for assessment of early responses to anti-angiogenic therapies that may precede disruption of the tumor vascular bed. Importantly, imaging assessment of VEGFR2 expression as illustrated here offers a significant advantage over traditional measures of response such as tumor regression or histology. Accumulation of NIR800- α VEGFR2ab is reflective of total tumoral VEGFR2 expression, incorporating aspects of both VEGFR2+ vessel density as well as the size of the vascular bed. Though not the primary focus of these investigations, we envision that this imaging approach could be utilized as both a correlative biomarker that facilitates quantitative assessment of experimental therapeutic regimens as well as a biomarker capable of predicting treatment outcome in more sophisticated preclinical mouse models of cancer that more accurately reflect human disease.

Conclusion

The optical imaging paradigm reported here enables rapid, non-invasive assessment of VEGFR2 expression in tumor-associated vasculature. We have demonstrated the suitability of this imaging method to assess the efficacy of angiogenesis-directed therapy in preclinical animal models. In addition to measuring the effects of angiogenesis-directed therapy directly, we envision that this imaging method could aid optimization of more complicated therapeutic regimens which may emphasize blockade of multiple molecular pathways such as inhibition of HER1 (EGFR) or HER2 in concert with the VEGF signaling axis [41]. Assessment of therapeutic response using NIR800- α VEGFR2ab can be correlated with other markers of treatment such as a recently described imaging biomarker for VEGF-targeted pharmaceutical response [42]. Finally, an analogous imaging approach could be applied to clinical studies by combining a humanized mAb or active fragment targeting VEGFR2 with a radioactive label such as ^{99m}Tc for SPECT imaging or ^{64}Cu for PET imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Spectroscopic characterization NIR800- α VEGFR2ab imaging probe in aqueous medium revealed peak absorbance at 777 nm and peak fluorescence emission at 798 nm.

Fig. 2.

a Immunoblot for VEGFR2 expression and actin control in cultured endothelial (*bEnd3*) and breast tumor (*4T1*) cells. **b** Endothelial (*bEnd3*) cells were incubated with serial dilutions of NIR800-αVEGFR2ab probe and non-specific NIR800-IgG probe, washed, and imaged for fluorescence. **c** Fluorescence intensity was assessed from *bEnd3* cells incubated with NIR800-αVEGFR2ab, *bEnd3* cells blocked with excess unlabeled VEGFR2 antibody followed by incubation with NIR800-αVEGFR2ab, and *bEnd3* cells incubated with non-specific NIR800-IgG.

Fig. 3.

a Representative fluorescence image of a mouse bearing a 4T1 xenograft tumor on the right hind limb. Image was collected 24 h after administration of NIR800-αVEGFR2ab demonstrating significant accumulation of the imaging probe within the tumor region. Single channel unmixed image of NIR800-αVEGFR2ab uptake (*left*) as well as multi-channel fluorescence composite image of autofluorescence (*white*) and NIR800-αVEGFR2ab uptake (*red*) are shown (*right*). **b** Representative fluorescence image of a mouse bearing a similar 4T1 xenograft tumor 24 h after administration of non-specific NIR800-IgG probe. Retention of the non-specific probe was not observed within the tumor region. Single channel unmixed image of NIR800-IgG uptake (*left*) as well as composite image of autofluorescence (*white*) and

NIR800-IgG (*red*) are shown (*right*). **c** Quantified fluorescence intensity of the tumor region normalized to the contralateral flank for mice injected with the NIR800- α VEGFR2ab probe ($n=20$) and the non-specific NIR800-IgG probe ($n=6$).

Fig. 4.

a Tumor size, as assessed by caliper measurement of the longest dimension, for animals treated with sunitinib ($n=9$) and treated with vehicle ($n=10$) before and after the treatment period (5 days). **b** Fluorescence intensity from NIR800- α VEGFR2ab accumulation in the tumor normalized to the contralateral hind limb for sunitinib-treated animals and animals receiving vehicle before and after the treatment period (5 days).

Fig. 5.

a Immunohistochemical staining for VEGFR2 expression (*brown*) within the tumors of vehicle-treated animals (**a**) and sunitinib-treated animals (**b**). Immunohistochemical staining for CD34 (*brown*) within the tumors of vehicle-treated animals (**c**) and sunitinib-treated animals (**d**). Immunohistochemical staining with hematoxylin and eosin within the tumors of vehicle-treated animals (**e**) and sunitinib-treated animals (**f**). **g** Morphometric analysis of CD34 and VEGFR2 expression in vehicle- and sunitinib-treated mice. **h** Morphometric analysis of VEGFR2 expression *versus* fluorescence intensity from NIR800- α VEGFR2ab accumulation in the tumor normalized to the contralateral hind limb.