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***In Vivo*, Dual-Modality OCT/LIF Imaging Using a Novel VEGF Receptor-Targeted NIR Fluorescent Probe in the AOM-Treated Mouse Model**

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Abstract

Purpose—Increased vascular endothelial growth factor (VEGF) receptor expression has been found at the sites of angiogenesis, particularly in tumor growth areas, as compared with quiescent vasculature. An increase in VEGF receptor-2 is associated with colon cancer progression. The *in vivo* detection of VEGF receptor is of interest for the purposes of studying basic mechanisms of carcinogenesis, making clinical diagnoses, and monitoring the efficacy of chemopreventive and therapeutic agents. In this study, a novel single chain (sc)VEGF-based molecular probe is utilized in the azoxymethane (AOM)-treated mouse model of colorectal cancer to study delivery route and specificity for disease.

Procedures—The probe was constructed by site-specific conjugation of a near-infrared fluorescent dye, Cy5.5, to scVEGF and detected *in vivo* with a dual-modality optical coherence tomography/laser-induced fluorescence (OCT/LIF) endoscopic system. A probe inactivated via excessive biotinylation was utilized as a control for nonreceptor-mediated binding. The LIF excitation source was a 633-nm He:Ne laser, and red/near-infrared fluorescence was detected with a spectrometer. OCT was used to obtain two-dimensional longitudinal tomograms at eight rotations in the distal colon. Fluorescence emission levels were correlated with OCT-detected disease *in vivo*. OCT-detected disease was verified with hematoxylin and eosin stained histology slides *ex vivo*.

Results—High fluorescence emission intensity from the targeted probe was correlated with tumor presence as detected using OCT *in vivo* and VEGFR-2 immunostaining on histological sections *ex vivo*. The inactivated probe accumulated preferentially on the surface of tumor lesions and in lymphoid aggregate tissue and was less selective for VEGFR-2.

Conclusion—The scVEGF/Cy probe delivered via colonic lavage reaches tumor vasculature and selectively accumulates in VEGFR-2-positive areas, resulting in high sensitivity and specificity

for tumor detection. The combination of OCT and LIF imaging modalities may allow the simultaneous study of tumor morphology and protein expression for the development of diagnostic and therapeutic methods for colorectal cancer.

Keywords

Angiogenesis; Colon cancer; Laser-induced fluorescence; Molecular imaging; Optical coherence tomography; Vascular endothelial growth factor receptor

Introduction

Role of VEGF in Carcinogenesis

Angiogenesis is an essential component of tumor development and metastasis. Vascular endothelial growth factor (VEGF) is produced in tumor cells in response to increasing metabolic needs. VEGF binds to VEGF receptors (VEGFR), primarily VEGFR-2 (kinase-insert domain receptor/fetal liver kinase (Flk)-1), on the surface of vascular endothelial cells, inducing the growth and survival of endothelial cells as well as increasing the permeability of tumor vasculature [1].

Many drugs and combination treatment regimens, clinical and preclinical, target angiogenesis with the idea of “starving” the tumor, as first presented by Folkman in 1971 [2]. Anti-angiogenic drugs, such as Avastin (Genentech, San Francisco, CA, USA), are now used clinically in some cases, in conjunction with chemotherapy, and have been shown to increase life expectancy in cancer patients [3]. Research performed in the Jain laboratory has also shown that the high concentration of VEGF around tumor areas results in disorganized vasculature and heightened permeability of vessels in the region [3]. The tortuosity of the vasculature and the heightened permeability (up to ten times the permeability in normal blood vessels) causes intravenous (IV) drug treatments to be delivered unevenly in the tumor. Some areas of the tumor may not receive treatment at all, increasing the risk of tumor regrowth.

Due to these findings and other research, the prevalence of VEGFR is considered a predictor for clinical outcome and can be impacted by seemingly unrelated cancer therapies, including photodynamic therapy, radiotherapy, and chemotherapy [4–7]. Therefore, monitoring VEGFR during disease progression and therapy is essential to improving our understanding of cancer and ultimately bolstering the efficacy of current treatments [8].

Molecular Imaging

The paradigm for detecting, stratifying, and monitoring tumors is shifting from purely structural imaging to include molecular imaging. Molecular imaging facilitates monitoring biomarkers, such as molecular signals for angiogenesis that are critical to cancer development.

Monitoring these processes *in vivo* can expedite drug development and facilitate personalized treatment [9,10]. For example, a drug inhibiting angiogenesis, such as a VEGFR inhibitor like pazopanib, can be evaluated based on the prevalence of angiogenesis biomarkers, such as VEGFR. Recent preclinical studies indicate that imaging biomarkers may predict therapy outcome earlier than more downstream effects like tumor size [11,12]. Molecular imaging in the clinic is expected to guide decision making regarding treatment, allowing doctors to select the optimal combination of anti-angiogenic and chemotherapy and to achieve precise, evidence-based treatment scheduling. Fine-tuning treatment based on

feedback from molecular imaging may increase the chances of successful tumor elimination [3].

In vivo molecular imaging is a two-fold process, involving first the development of a stable, specific molecular probe and second the development of a noninvasive or minimally invasive imaging system and protocol for detecting that probe. In this paper, we describe using a VEGFR-targeted near-infrared fluorescent probe [13] and a minimally invasive imaging method for locating premetastatic tumors in mice *in vivo*.

Optical Imaging—a Dual-Modality Approach

In this paper, we report a dual-modality, optical imaging system for minimally invasive tumor identification of VEGFR expression in tumor vasculature. Optical imaging methods are a relatively inexpensive and simple means to monitor cancer development *in vivo*, particularly in settings that allow endoscopic procedures. In this study, optical coherence tomography (OCT) is used to acquire depth-resolved, structural images of tumors and laser-induced fluorescence (LIF) is used to monitor VEGFR expression with a novel fluorescent VEGFR-targeted probe. These imaging modalities are combined and miniaturized in a 2-mm-diameter endoscope for minimally invasive imaging in the mouse colon *in vivo*.

OCT is rapidly acquiring acceptance for several biomedical applications. The leading clinical application is in assessing retinal diseases of the eye [14]. Preclinically, OCT is being studied as a means of “optical biopsy” in human patients, e.g., in diagnosing Barrett’s esophagus and ovarian cancer [15,16]. It is also being studied as a research tool for longitudinal animal studies, toward ultimately expediting drug development. Previous work in our group has shown the ability of OCT to detect early stage tumors *in vivo* in a mouse model of colorectal cancer [17]. In that study, a blinded panel detected 95% of benign tumors (adenomas) and only misclassified one normal location as adenoma using OCT images, out of 38 total adenomas identified and correlated with histology.

LIF provides biochemical information about the sample, complementary information to the structure provided by OCT. In previous studies, LIF images based on autofluorescence at 325 nm were co-registered with OCT images of Apc^{min/+} mouse colon *in vivo* [18]. Decreased autofluorescence at 390 nm, associated with type I collagen, and 450 nm, associated with NADH, correlated with adenomas. Decreased 390 and 450 nm autofluorescence may result from mucosal thickening with the growth of abnormal cells and leaky vasculature, which attenuates fluorescence from the deeper collagen layers. However, the ratio of NADH to collagen autofluorescence increases, strongly suggesting increasing metabolic needs associated with tumor development. While autofluorescence has been shown to be proficient at identifying colonic adenomas based on mucosal thickening, increasing metabolism, and bleeding, targeted contrast agents can enhance the functionality of LIF imaging by enabling specific molecular imaging. In this respect, a near-infrared fluorescent probe single chain (sc)VEGF/Cy5.5 has demonstrated its utility for VEGFR imaging in angiogenic vasculature in tumor and inflammation models [12,13,19–21]. In one study, luciferase-expressing adenocarcinoma cells were implanted subcutaneously in a mouse. Bioluminescence imaging overlaid with near-infrared fluorescence imaging confirmed the selective uptake of the VEGF-based probe in the bioluminescent tumor. Furthermore, immunofluorescent slides showed that dye fluorescence co-localized with VEGFR-2 immunostaining, confirming *in vitro* that the tracer is internalized via VEGFR-2-mediated endocytosis [13].

This study examines the *in vivo* utility of this VEGFR-targeted near-infrared fluorescent probe in the azoxymethane (AOM)-treated mouse model. AOM is a carcinogen that induces aberrant crypt foci in the colon, a condition that has been observed in humans prior to

developing cancer [22]. In mice, this condition precedes the development of tumors, called adenomas [23,24]. Mice treated with AOM develop colonic tumors randomly, similarly to how the disease progresses in humans.

Our imaging method utilizes a dual-modality OCT/LIF system to observe the structural and molecular changes that accompany tumor development. Using criteria developed by Hariri *et al.* [25] for identifying adenomas using OCT, we were able to map adenomas in the colon and compare these data with co-registered fluorescence data from scVEGF/Cy5.5. In addition to reporting on the performance of scVEGF/Cy5.5, this study demonstrates that a dual-modality system may allow tumors to be evaluated based on morphology, size, and VEGFR expression.

Materials and Methods

scVEGF/Cy5.5 Synthesis

Fluorescent tracer scVEGF/Cy5.5 (SibTech, Inc.) is an engineered scVEGF that combines two 3–110 aa fragments of human VEGF₁₂₁ expressed with cysteine-containing Cys-tag that was used for site-specific conjugation of a fluorescent dye, Cy5.5-maleimide (GE Healthcare), as described in Backer *et al.* [13]. An inactivated probe, inVEGF/Cy5.5 (SibTech, Inc.), was generated by excessive biotinylation of scVEGF/Cy5.5 via NHS chemistry, resulting in the complete loss of VEGFR-binding ability of the probe [13].

Dual-Modality OCT/LIF Imaging

The endoscopic OCT/LIF system is shown schematically in Fig. 1. A similar system has been described in detail by our group previously [26]. Briefly, the OCT channel utilizes a 1,300-nm center wavelength and a 70-nm bandwidth superluminescent diode. The reference arm modulates the signal at 100 kHz, which is detected using a lock-in amplifier. Two-millimeter A-scans (depth scans) are collected over a length of 30 mm. The LIF channel utilizes a He:Ne laser at 633 nm for exciting Cy5.5. Both OCT and LIF fibers are packaged in a 2-mm-diameter endoscope. The OCT channel is focused with a gradient index lens, resulting in 18 μ m lateral resolution. The axial resolution is determined by the light source bandwidth and is 11 μ m in air and 8 μ m in tissue (assuming a refractive index in tissue of 1.4). The LIF channel is unfocused and produces a 1.25-mm-diameter spot on the tissue. Fluorescence is detected by two fibers on either side of the LIF excitation fiber. The collection fibers are relayed to a CCD-based spectrometer.

Imaging Protocol

OCT and LIF images were collected from 11 female AOM-treated A/J mice and four saline-treated (control) A/J mice. The AOM-treated mice were injected with 10 mg/kg of azoxymethane purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) dissolved in sterile saline subcutaneously once a week for 5 weeks, starting at 6 weeks of age. Control mice were given subcutaneous saline injections of equivalent volume following the same schedule. Prior to imaging, mice were anesthetized with 2.5% Avertin, delivered intraperitoneally. The targeted scVEGF/Cy5.5 and inactivated inVEGF/Cy5.5 probes were produced in a 20-mM Tris–HCl solution by SibTech, Inc. We diluted this solution 3:1 in phosphate-buffered saline. Each mouse was treated with 0.2 mL of the diluted solution, containing 20 μ g (0.67 nmol) of the probe. This solution was delivered to AOM-treated mice ($n=7$ for targeted, $n=4$ for inactivated) and control (tumor-free) mice ($n=2$ for targeted, $n=2$ for inactivated) via colon lavage 4 h prior to imaging. The colon was rinsed with saline immediately prior to imaging, to remove unbound and/or degraded probes. This procedure was established as being preferable to IV injection in a previous study comparing both

methods, the drawback to IV injection being significant nonspecific uptake in the mammary fat pad, bladder, and small intestines [27].

After administering the fluorescent probe, the endoscope was lubricated with a water-based lubricant and inserted 30 mm inside the colon. OCT/LIF images were taken in eight rotations, 45° apart, around the colon. Each OCT image was 30 mm lateral×1.5 mm axial (in tissue). LIF spectra were collected approximately every 200 µm.

Histology

After OCT/LIF imaging, animals were sacrificed and the distal 40 mm of the colon was excised. The colons were sliced longitudinally, flattened, and fixed in HistoChoice (AMRESCO, Solon, OH, USA). Samples were paraffin-embedded, sliced longitudinally for comparison with longitudinal OCT images, and stained with hematoxylin and eosin. Approximately 16 histology slides were prepared per colon.

A map of adenomas in each colon was prepared based on the histology slides. Adenoma size was estimated from maximal diameter in each histology slide and number of slides in which the adenoma was identified. Location was defined as distance from the anus and angular position relative to the ventral side of the mouse. Matching adenomas were identified in the OCT image sets. Because some shrinkage and distortion of the colon during histological processing was unavoidable, tolerances of ±3 mm longitudinally, ±22.5° rotationally, and 0.5 mm in diameter were allowed when matching adenoma. Since adenomas were discrete, this tolerance did not confound matching. Since OCT and LIF spectra were acquired simultaneously, the OCT image was used to identify adenoma-associated LIF spectra.

Data Analysis

LIF spectra were processed to separate Cy5.5 fluorescence from autofluorescence. Both Cy5.5 fluorescence in colon tissue and the autofluorescence of colon tissue were characterized *in vivo* in colon tissue using the OCT/LIF system. The Cy5.5 spectrum was measured in a control (tumor-free) mouse injected with scVEGF/Cy5.5. The mouse had no appreciable autofluorescence prior to probe injection. The injection method was utilized because it provided sufficient nonspecific uptake of scVEGF/Cy5.5, as reported in previous work [27]. Otherwise, control mice with colon lavage of scVEGF/Cy5.5 exhibited fluorescence too weak to characterize. The autofluorescence spectrum was measured in an AOM-treated mouse with no administered Cy5.5. In both cases, all the spectra collected from each mouse were averaged to produce the final Cy5.5 and autofluorescence spectra, shown in Fig. 2. Use of a mouse injected with contrast agent for defining Cy5.5 fluorescence was considered superior to simply using the spectrum of Cy5.5 dissolved in saline *in vitro* because the *in vivo* environment and presence of the dye in highly scattering tissue can alter the detected emission spectrum.

To separate the two sources of fluorescence, two points on each acquired LIF spectrum were considered: the value at 706 and 670 nm. At 670 nm, fluorescence from Cy5.5 was very low; therefore, this value was used to initially approximate the fluorescence due to tissue autofluorescence. By knowing the value at 670 nm and using the autofluorescence spectrum characterized as described above, the autofluorescence at 706 nm could be estimated. This value was then used to estimate the fluorescence due to Cy5.5 at 706 nm, by subtracting it from the measured spectral value at 706 nm. This procedure was iterated to obtain incrementally better estimates of both autofluorescence and Cy5.5 fluorescence as follows. The estimated value of fluorescence due to Cy5.5 at 706 nm, together with the Cy5.5 fluorescence spectrum characterized as described above, was used to compute the fluorescence value of Cy5.5 at 670 nm. This value was then subtracted from the measured

value at 670 nm to obtain a better estimate for the value of autofluorescence at 670 nm. Using this more accurate estimate of autofluorescence at 670 nm, the level of autofluorescence at 706 nm was computed. This value was again subtracted from the measured value at 706 nm to obtain a better estimate of the level of Cy5.5 fluorescence at 706 nm. This procedure for separating autofluorescence and Cy5.5 fluorescence was iterated ten times, at which point the value converged to less than 1% changes. For the remainder of the analysis, the final value of Cy5.5 fluorescence at 706 nm was used to quantitatively represent Cy5.5 fluorescence.

To study the fluorescence in normal *versus* adenoma regions in the colon, OCT and LIF data were subdivided into 5 mm lateral segments, the approximate size of the observed adenomas. The maximum value of Cy5.5 fluorescence emission at 706 nm was found for each segment. OCT was used to classify a region as normal or adenoma, using criteria developed by Hariri *et al.* [25]. Using OCT-detected adenomas correlated with histology as the gold standard, the sensitivity and specificity of scVEGF/Cy5.5 and inVEGF/Cy5.5 were calculated for a range of thresholds for maximum Cy5.5 fluorescence. Sensitivity was calculated as the number of true positives divided by the total number of adenoma segments detected using OCT. An adenoma segment was considered a true positive when the fluorescence value exceeded threshold in that segment or an immediately adjacent segment. Specificity was calculated as the number of true negatives divided by the total number of normal segments as detected using OCT. A normal segment was considered a true negative when the fluorescence value was below threshold.

Immunofluorescence Staining and Ex Vivo Microscopy

A short follow-up study with six AOM-treated mice was performed *ex vivo* to compare scVEGF/Cy5.5 and inVEGF/Cy5.5 accumulation using fluorescence microscopy. Each mouse was treated with either scVEGF/Cy5.5 ($n=3$) or inVEGF/Cy5.5 ($n=3$) *in vivo* using the lavage procedure described in “Imaging Protocol”. Mice were sacrificed and up to 40 mm of the distal colon was excised. Tissue was frozen using isopentane precooled in liquid nitrogen and embedded in optimal cutting temperature compound in cryomolds. Cryosectioning was performed, producing 5- μ m-thick tissue sections. For each mouse, sections were collected from eight to ten sites spaced 500 μ m apart circumferentially. Two sections, spaced 5 μ m, were collected per site. One section per site was treated with an immunofluorescent antibody for VEGFR-2 (Flk-1), as described previously [13]. Imaging was performed on an Olympus Macro Zoom Fluorescence Microscope (Olympus MVX10 MacroView). A green fluorescence protein (GFP) filter cube with Mercury lamp was used to view immunofluorescence. A Cy5.5 filter cube with Mercury lamp was used to view Cy5.5 fluorescence.

VEGFR-positive regions were identified on sections treated with immunofluorescent antibody and viewed with the GFP filter cube. These regions were compared to their adjacent sections (without immunofluorescent antibody) and viewed with the Cy5.5 filter cube to identify scVEGF/Cy5.5 or inVEGF/Cy5.5. For both scVEGF/Cy5.5 and inVEGF/Cy5.5 sections, we identified Cy5.5 regions co-localized with VEGFR-2-positive regions (true positives, TP), Cy5.5 regions not co-localized with VEGFR-2-positive regions (false positives, FP), and VEGFR-2-positive regions without fluorescence from Cy5.5 (false negatives, FN). Using these values, we computed the positive predictive value (PPV) and sensitivity of each agent for identifying VEGFR-2-positive regions. The PPV indicates the percentage of incidences in which Cy5.5 fluorescence indicates a VEGFR-2-positive region. PPV is computed by taking the number of co-localized regions (TP) and dividing by the total number of regions with Cy5.5 fluorescence (TP+FP). The sensitivity indicates the percentage of VEGFR-2-positive regions correctly identified by Cy5.5 fluorescence. The

sensitivity is computed by taking the number of co-localized regions (TP) and dividing by the total number of VEGFR-2-positive regions (TP+FN).

Results

Correlating OCT and histology revealed 110 adenoma and 185 normal segments in mice treated with targeted probe and 96 adenoma and 78 normal segments in mice treated with inactivated probe. Both the targeted and inactivated probes resulted in high sensitivity and specificity for disease *in vivo*. Plots of sensitivity and specificity for a range of thresholds are shown in Fig. 3 for both targeted (scVEGF/Cy5.5) and inactivated (inVEGF/Cy5.5) probes. The values were similar with the targeted probe achieving 83% sensitivity and 84% specificity at one threshold (0.060 arb. units) and the inactive probe performing slightly better with 87% sensitivity and 84% specificity at a higher threshold (0.125 arb. units).

The controls exhibited very weak fluorescence in comparison to the AOM-treated mice. All fluorescence values in the four control mice treated with either scVEGF/Cy5.5 or inVEGF/Cy5.5 were below 0.020 arb. units, averaging under 0.010 arb. units, compared to the example thresholds 0.060 and 0.125 for achieving over 80% sensitivity and specificity for both contrast agents. Histological examination of the normal areas in the control mice *versus* the AOM-treated mice showed that the AOM-treated mice tended to have elevated mucosal thickening throughout their colons, indicating that the areas classified as normal in AOM-treated mice were exhibiting abnormalities. Therefore, we consider the control mice to be a better indicator of how the agents will respond to normal areas in a human colon. Using a fluorescence threshold of 0.020 arb. units, which results in no false positives or 100% specificity in the control mice, scVEGF/Cy5.5 and inVEGF/Cy5.5 are 93% and 99% sensitive for adenomas, respectively.

The fluorescence from the targeted probe was less intense than that from the inactivated probe, resulting in a lower sensitivity with the targeted probe. Figs. 4 and 5 show sample OCT/LIF images obtained with targeted and inactivated probes. Note fluorescence enhancement in both figures of adenoma regions. Some adenomas show a dark center in the fluorescence images, which was not uncommon in the dataset. This effect is especially evident in the adenoma labeled number 2 in Fig. 4 and adenoma number 1 in Fig. 5. Note that these adenomas exhibit marked mucosal thickening as well as hypointense regions corresponding to blood vessels. Both mucosal thickening and blood vessels contribute to light attenuation, resulting in the dark centers as compared to the edges of the adenomas. Significant variability in tumor fluorescence was observed with LIF imaging and *in situ* fluorescence microscopy of scVEGF/Cy5.5, shown in Fig. 4, believed to correlate with differences in VEGFR expression. *In situ* fluorescence microscope images shown in Figs. 4 and 5 also verify the difference in fluorescence intensity between colons treated with targeted and inactivated probes. Note also the difference in probe accumulation. The targeted probe appears dotted in areas, whereas the inactivated probe appears to delineate folds and creases in the adenomas.

While specificity for adenomas was expected using targeted probe due to VEGFR binding [12,13,19–21], the highly specific uptake of the inactivated probe in tumor regions was a surprising finding. This finding warranted further investigation into the selectivity of scVEGF/Cy5.5 for VEGFR using lavage administration. A short study of six AOM-treated mice was conducted *ex vivo* to gain insight into how targeted and inactivated probes are distributed in the colon. Fresh frozen sections from mice treated with either scVEGF/Cy5.5 or inVEGF/Cy5.5 were immunostained for VEGFR-2 in order to determine localization of Cy5.5 fluorescence relative to VEGFR-2. Figs. 6 and 7 show representative fluorescent images of scVEGF/Cy5.5 and inVEGF/Cy5.5 sections, respectively. These figures show

Cy5.5 fluorescence in the left-most images, fluorescence from VEGFR-2 immunostain in the center images, and a merge of the two in the right-most images. The yellow arrows in the rightmost images indicate TP, the red arrows indicate FP, and the green circles indicate FN. For the targeted probe, scVEGF/Cy5.5, the a PPV was 82% out of 49 Cy5.5-positive regions, and the sensitivity was 57% out of 70 VEGFR-2-positive regions. For the inactivated probe, inVEGF/Cy5.5, the PPV was 69% out of 81 positive Cy5.5 regions and the sensitivity was 63% out of 89 positive VEGFR-2 regions. We observed both probes on the colon surface as well as inside colonic crypts (Figs. 6 and 7, red arrows). The inactivated probe appeared more prevalent in these areas and additionally appeared within lymphoid aggregate tissue (data not shown).

Discussion

In this paper, we report on the performance of a new targeted fluorescence agent, scVEGF/Cy5.5, applied via lavage, for detection of colorectal tumors and describe a method and an endoscopic tool for simultaneously studying tumor morphology and VEGFR expression in small animal models. VEGF receptors are overexpressed in colorectal tumor vasculature and are therapeutic targets in FDA-approved anti-angiogenic therapy regimens in colorectal cancers. Thus, early imaging of these receptors or imaging in the course of therapy might facilitate early diagnostic and treatment management. In previous work, we demonstrated an imaging system for gathering morphological and biochemical information simultaneously [18,26,28]. In this study, we used this tool and a targeted scVEGF/Cy5.5 probe for VEGFR imaging to explore mice with AOM-induced colorectal lesions. Our goal was to correlate the presence of tumors with fluorescence from a targeted agent, scVEGF/Cy5.5 that binds to and is internalized by VEGF receptors, primarily VEGFR-2 [13]. Using OCT-detected tumors confirmed by histology as the gold standard, we established high sensitivity and specificity of scVEGF/Cy5.5-based tumor detection, reaching 83/84% for one threshold.

Interestingly, while VEGFR expression and therefore fluorescence from scVEGF/Cy5.5 are particularly prominent in tumors, we also discovered higher levels of fluorescence in normal areas of AOM-treated mice than in the control mice. Histology from the AOM-treated mice showed that the areas classified as normal exhibited mucosal thickening, indicating early stages of disease development. Furthermore, immunostaining of normal areas in the AOM-treated mouse colon using a VEGFR antibody showed significant positive staining in some cases, suggesting that the enhanced scVEGF/Cy5.5 uptake might be used as indication of the very early stages of malignant development.

An inactivated probe, inVEGF/Cy5.5, created by overbiotinylation of scVEGF/Cy5.5, performed as well for identifying adenomas in our study. inVEGF/Cy5.5 and other overbiotinylated scVEGF-based probes have not bound to VEGF receptors in previous studies and have been successfully used as controls for nonspecific accumulation of imaging tracers in several preclinical tumor and inflammation models [12,19–21].

To understand the difference between uptake of scVEGF/Cy5.5 and inVEGF/Cy5.5, we examined *in situ* fluorescence microscopy images of the colon as well as *ex vivo* slides. *In situ* fluorescence images showed brighter fluorescence from the inactivated probe, consistent with *in vivo* LIF measurements, as well as difference in accumulation that could not be resolved with LIF imaging. Fluorescence microscopy on *ex vivo* slides allowed us to examine these effects more closely. Some nonspecific uptake of scVEGF/Cy5.5 was observed on the colon surface that may be due to impaired transport through epithelial surface in abnormal lesions, as described by Wang *et al.* [29]. However, a significant fraction of scVEGF/Cy5.5 was highly selective for VEGFR-2 with a PPV of 82%. As in other tumor models [13], Cy5.5 fluorescence co-localized only with some VEGFR-2-

positive regions, suggesting significant variability in the access of probe to VEGFR-expressing cells in tumor vasculature. Future improvements for this system are now in progress to achieve a higher-resolution LIF imaging to better visualize Cy5.5 distribution *in vivo*.

As expected, the inactivated probe, inVEGF/Cy5.5, was less selective for VEGFR-2 with a PPV of 69%. The sensitivity of this probe was 63%. This value is slightly higher than the sensitivity of the targeted probe (57%) which may be coincidental due to the increased amount of inVEGF/Cy5.5 observed in the tissue overall. Fluorescence microscopy images from *ex vivo* slides also exhibited brighter fluorescence from inVEGF/Cy5.5-positive regions, evident in LIF images and *in situ* fluorescence microscopy images. A significant fraction of Cy5.5 fluorescence was observed on the colon surface, as well as in lymphoid aggregate tissue. Further study is necessary to establish if the presence of multiple biotin residues (12–13 residues per scVEGF) enhances the affinity of inVEGF/Cy5.5 to the surface of tumor lesions and stimulates its uptake in lymphoid aggregate tissue, not evident for scVEGF/Cy5.5. However, it is evident from this experiment that inVEGF/Cy5.5 is not an appropriate control for nonspecific binding of scVEGF/Cy5.5 delivered to colon tumor vasculature via lavage. We are exploring alternative approaches to eliminating the binding capacity of scVEGF-based tracers in order to create a better control (inVEGF) for future experiments.

Conclusions

The main finding of this work is that a significant fraction of scVEGF/Cy5.5 probe delivered via lavage transverse colonic epithelium and selectively co-localizes with VEGF receptors in tumor lesions in AOM-treated mice. The resulting appreciable yet variable Cy5.5 fluorescence signal detected with LIF provides information which is complementary to anatomical findings visualized using OCT. We expect that dual-modality OCT/LIF imaging with scVEGF/Cy5.5 provides co-registered structural and molecular information relevant for the study of new drugs and therapies for colorectal cancer.

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Fig. 1. OCT/LIF system diagram. OCT source is a superluminescent diode with 1,300-nm center wavelength and 70-nm bandwidth. A 50:50 coupler splits the light into the reference and sample arms. The sample arm contains a 2-mm-diameter endoscope for mouse colon imaging. The LIF excitation source is a He:Ne laser at 633 nm. The light is fiber coupled into the endoscope. Two collection fibers detect the fluorescence emission, which is relayed to a CCD-based spectrometer. The OCT and LIF excitation illuminate adjacent sections of tissue. This shift is accounted for in post-processing to achieve co-registration of OCT and LIF imaging.

Fig. 2.
Spectra of autofluorescence from a diseased mouse colon and Cy5.5 from a control mouse colon *in vivo* with 633 nm excitation.

Fig. 3. Sensitivity *versus* (1-specificity) is plotted for a range of fluorescence value thresholds for both the targeted (scVEGF/Cy5.5; *left*) and inactivated (inVEGF/Cy5.5) probes (*right*).

Fig. 4.

Example OCT/LIF images from five radial locations spaced 45° apart in one AOM-treated mouse colon treated with scVEGF/Cy5.5. On the *left*, the five OCT images are stacked vertically. Each OCT image is 15×1.5 mm. On the *right*, five co-registered LIF images are stacked vertically. The gray-scale value corresponds to the fluorescence emission intensity of Cy5.5 at 706 nm at each lateral position. Beneath is shown an *ex vivo* fluorescence microscope image. Adenomas in the images are outlined by a *white box* and numbered for comparison between OCT, LIF, and *ex vivo* fluorescence. Adenomas 2 and 3 show a sharp decrease in fluorescence at the center of the adenoma. In the data set, the center of the adenoma often appeared darker than the edges, which may be due to mucosal thickening or increased blood absorption.

Fig. 5.

Example OCT/LIF images from three radial locations spaced 45° apart in one AOM-treated mouse colon treated with inVEGF/Cy5.5. At the *top*, three OCT images are stacked vertically. Each OCT image shown is 30×1.5 mm. In the *middle*, three LIF images are stacked vertically. The gray-scale value corresponds to the fluorescence emission intensity of Cy5.5 at 706 nm at each lateral position. Beneath is shown an *ex vivo* fluorescence microscope image. Adenomas in the images are outlined by a *white box* and numbered for comparison between OCT, LIF, and *ex vivo* fluorescence.

Fig. 6.

Fluorescence microscopy images of two *ex vivo* sections (labeled 1 and 2) from one AOM-treated mouse colon are shown. *Section 1* is a normal (tumor-free) area, and *section 2* contains a developing tumor. The mouse was treated with scVEGF/Cy5.5 *in vivo* and fresh frozen sections from that mouse were treated with an immunofluorescent probe for VEGFR-2 (Flk-1) *ex vivo*. The *left-most images* (1a and 2a) show Cy5.5 fluorescence in *red*. The *middle images* (1b and 2b) show VEGFR-2 immunofluorescence in *green*. The *right-most images* (1c and 2c) show a merge of the two, with *yellow* indicating co-localized fluorescence. The *red arrows* indicate nonspecific Cy5.5 fluorescence at the tissue surface and in some colonic crypts. The *yellow arrows* indicate co-localized Cy5.5 and VEGFR-2 immunofluorescence. Heterogeneous binding is evident in one location of *section 2*, as shown by the *green circle*, in which weak VEGFR-2 immunofluorescence occurs but no Cy5.5.

Fig. 7.

Fluorescence microscopy images of two *ex vivo* sections (labeled 1 and 2) from an AOM-treated mouse colon are shown. Both sections contain developing tumors. The mouse was treated with inVEGF/Cy5.5 *in vivo* and fresh frozen sections from that mouse were treated with an immunofluorescent probe for VEGFR-2 (Flk-1) *ex vivo*. The *left-most images* (1a and 2a) show Cy5.5 fluorescence in *red*. The *middle images* (1b and 2b) shows VEGFR-2 immunofluorescence in *green*. The *right-most images* (1c and 2c) show a merge of the two, with *yellow* indicating co-localized fluorescence. Note the absence of co-localized Cy5.5 and VEGFR-2 immunofluorescence. Punctate VEGFR-2 immunofluorescence is evident in the merged images (*green circles*) as well as nonspecific Cy5.5 fluorescence at the tissue surface and in some colonic crypts (*red arrows*).