Fluorescent Image-Guided Surgery with an Anti-Prostate Stem Cell Antigen (PSCA) Diabody Enables Targeted Resection of Mouse Prostate Cancer Xenografts in Real Time

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Abstract

Purpose: The inability to visualize cancer during prostatectomy contributes to positive margins, cancer recurrence, and surgical side effects. A molecularly targeted fluorescence probe offers the potential for real-time intraoperative imaging. The goal of this study was to develop a probe for image-guided prostate cancer surgery.

Experimental Design: An antibody fragment (cys-diabody, cDb) against prostate stem cell antigen (PSCA) was conjugated to a far-red fluorophore, Cy5. The integrity and binding of the probe to PSCA was confirmed by gel electrophoresis, size exclusion, and flow cytometry, respectively. Subcutaneous models of PSCA-expressing xenografts were used to assess the biodistribution and in vivo kinetics, whereas an invasive intramuscular model was utilized to explore the performance of Cy5-cDb-mediated fluorescence guidance in representative surgical scenarios. Finally, a prospective, randomized study comparing surgical resection with and without fluorescent guidance was performed to determine whether this probe could reduce the incidence of positive margins.

Results: Cy5-cDb demonstrated excellent purity, stability, and specific binding to PSCA. In vivo imaging showed maximal signal-to-background ratios at 6 hours. In mice carrying PSCA+ and negative (−) dual xenografts, the mean fluorescence ratio of PSCA+/− tumors was 4.4:1. In surgical resection experiments, residual tumors <1 mm that were missed on white light surgery were identified and resected using fluorescence guidance, which reduced the incidence of positive surgical margins (0/8) compared with white light surgery alone (7/7).

Conclusions: Fluorescently labeled cDb enables real-time in vivo imaging of prostate cancer xenografts in mice, and facilitates more complete tumor removal than conventional white light surgery alone. Clin Cancer Res; 22(6); 1403–12. ©2015 AACR.

See related commentary by van Leeuwen and van der Poel, p. 1304

Introduction

Radical prostatectomy remains the most common definitive treatment option for the >250,000 men newly diagnosed with localized prostate cancer each year (1), with up to 85% of all prostatectomies in the United States performed robotically (2–4). Early extracapsular extension of prostate cancer is rarely visible during prostatectomy, and proximity of the prostate to the rectum, urinary sphincter, and erectile nerves precludes wide local excision. As such, positive surgical margin rates range from 6.5% to 32% in contemporary series (5), and are directly correlated with poor cancer control (6, 7). The posterolateral prostate and prostatic apex remain the most common sites for positive surgical margins (8), which are intimately associated with the neurovascular bundle controlling erectile function and urinary sphincter, respectively. As urinary incontinence and sexual dysfunction remain major issues postoperatively (9), surgeon reluctance to remove healthy tissue in an attempt to reduce these side effects likely account for these higher rates. The ability to visualize small foci of extracapsular extension of prostate cancer at the time of surgery may reduce the incidence of positive surgical margins while reducing damage to critical adjacent structures.

Several research teams have endeavored to develop cancer specific optical imaging agents that use fluorescence to highlight cancer in real time during surgery. Intraprostatic free indocyanin green (ICG) has been utilized clinically in prostatectomy as a lymphangiographic agent in the detection of sentinel lymph nodes and for delineation of prostate by limited diffusion (9, 10). However, use of free ICG is limited by the lack of biochemical specificity to prostate or prostate cancer, and suffers from dye spillage from handling or manipulating fluorescent tissue. Cancer-specific optical agents that overcome these limitations are currently being developed by various groups in the
Translational Relevance
The ability to visualize cancer in real time using molecularly targeted fluorescent probes has the potential to transform the modern practice of cancer surgery. In the case of prostate cancer, the inability to differentiate cancer from normal surrounding structures contributes both to incomplete cancer removal and surgical side effects. Technology for fluorescence imaging in humans during robotic surgery is commercially available, but its application is limited by the lack of prostate-specific optical probes. We report the development and validation of a novel targeted optical imaging probe using an antibody fragment against a cell surface marker of prostate cancer. Such probes will be useful for maximizing resection of primary and metastatic cancers while minimizing damage to critical adjacent tissues.

Materials and Methods
Reagents
The 2B3 A2 cysteine-diabody, (cDb, 50 kDa) was developed and validated for preclinical in vivo targeting of PSMA at UCLA (Los Angeles, CA; ref. 29). It was derived by yeast affinity maturation of a humanized monoclonal anti-PSMA antibody, 2B3, and engineered to contain a C-terminal–free cysteine that forms an inter-chain disulfide bond stabilizing dimerization. Upon mild reduction this disulfide bond can be broken and free thiols are available for site-specific labeling away from the antigen-binding site using, for example, maleimide chemistry. A2 cDb was purified from mammalian cell culture supernatant using immobilized metal affinity chromatography. Protein concentrations were determined photometrically and purity was analyzed by SDS-PAGE. Detailed biodistribution data for the A2 cDb was previously determined (21). Nonspecific binding was not seen. Fluorescent signals were present in liver, kidney, and bladder due to the metabolism and urinary excretion of the probe. Cy5 Maleimide (649 nm absorbance, 670 nm emission) was purchased from GE Healthcare.

Synthesis of Cy5-cDb probe
To achieve optimal conjugation efficiencies, the diabody was first concentrated using an Amicon Ultra-0.5 mL (10 K) Centrifugal Filter Device (Millipore) to a concentration greater than 2.8 mg/mL. Then, 50 μmol/L diabody was reduced in 40-fold molar excess of TCEP for 2 hours at room temperature. A 20-fold molar excess of Cy5 maleimide dissolved in dimethylformamide was then added to the reduced diabody and the mixture was incubated for 2 hours at room temperature. After incubation, excess dye was removed using a 2 mL Zeba Desalt Spin Column (Thermo Scientific). Cy5 and diabody concentrations were then measured using a spectrophotometer at 650 and 280 nm, respectively. The ratio of Cy5 to diabody was calculated to confirm the number of fluorescent molecules conjugated to each diabody molecule.

Size exclusion
Size exclusion chromatography (SEC) was performed using a Superdex 75 HR 10/30 column (GE Healthcare Life Sciences) on an AKTA Purifier and PBS as mobile phase at a flow rate of 0.5 mL/min. Both A280 for protein detection and A550 for fluorophore detection were monitored during elution. Retention time was compared to following standard proteins: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa; Sigma-Aldrich, Saint Louis).

Cell culture
CWR22Rv1 cells that express minimal levels of endogenous PSMA were obtained from ATCC and cultured in RPMI1640 medium containing 10% FBS, 1× sodium pyruvate and 1% penicillin–streptomycin–glutamine (PSG). A PSMA-expressing lentivirus was used to transduce these cells to generate a 22Rv1-PSCA line, as previously described (30). Quantitative flow cytometry with the murine 1G8 anti-PSMA antibody previously showed little or no expression of PSMA on 22Rv1 cells, with
2.2 × 10^6 PSCA antigens on 22Rv1-PSCA^+ cells (30). LAPC-9 cells that endogenously express PSCA were passaged in vivo and explanted tumors were processed into single-cell suspension before injection into nude mice as described previously (28).

Flow cytometry analysis

Both parental and PSCA^+ 22Rv1 cells were dissociated with glucose-EDTA and stained with the Cy5-c Db probe (1 μg per 1 × 10^6 cells) on ice for 1 hour followed by 3 × washes with PBS + 2% FBS. A murine anti-human PSCA antibody, IGA8 (28), was used as positive control followed by Alexa Fluor 647-goat-anti-mouse IgG as secondary antibody (Invitrogen). Fluorescence signal was acquired and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Measurement of affinity using an Attana Cell 200 C-Fast system

The antigen, human PSCA-mouseFc fusion protein, was immobilized on an LNB-carboxyl sensor chip by amine coupling at a concentration of 50 μg/mL according to the manufacturer’s protocol, resulting in a frequency shift of 200 Hz. Binding experiments were performed with HBS-T (10 mmol/L HEPES, 150 mmol/L NaCl, 0.005% Tween20, pH 7.4) as running buffer and the temperature was controlled at 22°C. Varying concentrations of the antibody fragments (300–3 nmol/L, quadruplicates) were analyzed for binding and the chip surface was regenerated using 10 mmol/L glycine pH 2.5 between each sample. Buffer injections prior to each sample were used as internal reference and an activated/deactivated (no antigen) surface chip was used for external reference. Data were collected and analyzed using the Attana Attaché software and the binding curves were fit using a mass transport limited binding model.

Xenograft models

All procedures were approved by the UCLA Animal Research Committee. Five- to 8-week-old male athymic nude mice were used for all experiments. Mice were fed irradiated, alfalfa-free food (Harlan Laboratories) to reduce nonspecific binding (Fig. 1B). Both parental and PSCA^+ 22Rv1 or LAPC-9 cells were mixed with Cultrex (1:1, v/v, Trevigen, Inc) and injected into the bilateral flank muscles (in the preliminary surgical resection sites with reference to white light. Maximal fluorescence intensity in the ROIs was analyzed using Living Image software. In mice with 2 tumors (22Rv1 experiments), the fluorescence intensity of the PSCA^+ tumor was compared with the PSCA^- tumor. In mice with 1 tumor (LAPC-9 experiment), the fluorescence intensity of the tumor was compared with the body (background).

Surgery

For all surgery experiments, 25 μg of Cy5-c Db was injected intravenously on the day of operation. Mice were imaged in vivo on the IVIS at 5 hours to confirm fluorescence detection in the tumor. Surgery was performed at 6 hours. Animals were sacrificed immediately before surgery in accordance with our Animal Research Committee protocol to reduce unnecessary duress. Tumors were resected using a fluorescence dissecting microscope (Leica M205 FA, Leica Microsystems) at 10× magnification under white light or with excitation and emission parameters for Cy5 (excitation 620/60 nm and emission 700/75 nm). Images of the fluorescence signal were captured with a Leica DFC360 FX monochrome camera and displayed on an adjacent computer monitor. After surgery, any residual fluorescent tissue and/or tumor margins were surgically collected, fixed in formalin, and sent to the UCLA pathology core facility for hematoxylin and eosin (H&E) staining and histologic analysis in the feasibility experiment, a margin of remaining nonfluorescent tissue was also removed and processed as control.

Results

Development and characterization of Cy5-cys-Dd

The engineered anti-PSCA dDb contains a C-terminal cysteine residue that can be reduced to enable site-specific labeling (Fig. 1A; ref. 31). After reduction, the dimeric diabody is held together solely by noncovalent interchain interactions. Therefore, completely reduced diabody migrates on SDS-PAGE as a 25 kDa monomer band, whereas the nonreduced diabody migrates as a 50 kDa band (Supplementary Fig. S1A). Following reduction, the cysteine moiety is available for binding to the Cy5 dye via maleimide chemistry (Fig. 1A). We investigated a variety of different concentrations of dye to maximize site-specific conjugation. Optimal conjugation was obtained using a 15-fold molar excess of Cy5 relative to the cys-diabody (Supplementary Fig. S1B). To assess reproducibility of the diabody–Cy5 conjugation, the dye:protein ratio was checked (n = 8). The mean dye:protein ratio was 1.31 (SD = 0.04). To evaluate purity and integrity of the conjugate, a size exclusion experiment was performed. This demonstrated good purity (major peak representing a Cy5-d Db molecule) with no sign of protein aggregation and only a small amount of unbound dye (late elution peaks; Fig. 1B). Both the unconjugated diabody and the Cy5-d Db eluted with similar retention times (22.4 and 22.5 minutes, respectively) indicating

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Guided Surgery with an Anti-PSCA Diabody

fluorescence imaging of tumor and normal tissue. In vivo imaging was performed using the IVIS 200 In Vivo Imaging System (Xenogen). Cy5 was excited using a 615 to 665 nm bandpass filter and emission was measured at 695 to 770 nm. These settings are optimized for the dye Cy5,5, which has peak absorbance and emission at wavelengths approximately 25 nm longer than Cy5. Exposure time was 1 second and binning was set at medium. Prior to imaging, the probe was prepared by mixing the Cy5-c Db, 4 μL of 10% human serum albumin, and sterile normal saline to bring the injected volume to 100 μL. Twenty-five micrograms or indicated doses of diabody were used in imaging experiments. The probe was intravenously injected via the lateral tail vein. Animals were anesthetized with 2% isoflurane during imaging sessions. Imaging was performed at 6 hours or indicated time points after probe administration. When multiple time-points were used, animals were allowed to awaken after imaging, and then reanesthetized for each time point. Following the final time point for each mouse, mice were sacrificed by isoflurane overdose and cervical dislocation. The skin was then removed and the mice were again imaged with the whole-body mouse imaging system. Regions of interest (ROIs) were delineated over the tumor sites with reference to white light. Maximal fluorescence intensity in the ROIs was analyzed using Living Image software. In mice with 2 tumors (22Rv1 experiments), the fluorescence intensity of the PSCA^- tumor was compared with the PSCA^- tumor. In mice with 1 tumor (LAPC-9 experiment), the fluorescence intensity of the tumor was compared with the body (background).
that reduction of the cys-diabody and conjugation of the Cy5 did not interfere with dimer maintenance.

To determine whether the Cy5–cDb conjugate maintains its binding to PSCA in vitro, a series of flow cytometry experiments were performed using PSCA overexpressing 22Rv1 cells (Fig. 1C). Specific binding of Cy5-cDb to PSCA was demonstrated, although a low level of binding to 22Rv1-PSCA/C0 cells was also observed due to low endogenous levels of PSCA expression. We further confirmed these results in an endogenous PSCA-expressing pancreatic cell line, Capan-1 (data not shown). Furthermore, to investigate whether Cy5 labeling had affected the binding affinity quantitatively, quartz crystal microbalance (QCM) measurements were conducted using an Attana Cell 200 C-Fast system to assess the interaction of Cy5-cDb to recombinant PSCA protein, which was immobilized on an LNB carboxyl sensor chip, in comparison with the mock-treated cDb control. As shown in Fig. 1D, both Cy5-cDb (0.42 nmol/L) and mock-labeled cDb (1.96 nmol/L) had apparent affinity in the low nanomolar range confirming that reducing the C-terminal disulfide bridge and site-specific conjugation of maleimide-Cy5 did not impair binding of

Figure 1. Biochemical and functional characterization of Cy5-conjugated anti-PSCA cys-diabody. A, schematic of the conjugation reaction. Anti-PSCA cys-Diabody was first reduced by TCEP to open up the interchain disulfide bond, revealing cysteine residues that can then be conjugated to Cy5 via maleimide chemistry. B, size exclusion chromatography of purified A2_cDb and Cy5-labeled A2_cDb. Retention time of standard proteins is indicated. C, flow cytometry analysis comparing binding of Cy5-cDb to 22Rv1 cells that overexpress PSCA (left) and the parental line (right) that expresses very low level of PSCA. Strong binding of Cy5-cDb to PSCA (orange line) was evident compared with the positive control (red line). Right, a small amount of binding to the PSCA-22Rv1 cells (orange line) was also present. D, quartz crystal microbalance (QCM) measurements of the interaction between mock labeled cDb or Cy5-cDb and recombinant PSCA. The table summarizes the on and off rates and $B_{\text{max}}$ for indicated samples. Mock-treated cDb had an apparent affinity of 1.96 nmol/L and Cy5-cDb 0.42 nmol/L, both in the low nanomolar range.
the cys-diabody to PSCA. Small differences in $K_d$ might be attributed to minor inaccuracy in protein concentration due to the presence of BSA in the samples, which was required during the purification procedure to stabilize the diabody.

**In vivo tumor imaging**

To determine whether the Cy5-cDb probe binds to PSCA-expressing tumors in vivo, we first performed imaging experiments with subcutaneous xenografts. Four mice, each bearing PSCA$^+$ and PSCA$^{-}$ 22Rv1 xenografts, were imaged on the IVIS 200 (excitation 615–665 nm, emission 695–770 nm) at 6 hours after intravenous injection of 25 μg of the probe. This revealed fluorescence in the PSCA$^+$ tumor and lack of fluorescence in the non-PSCA-expressing tumor (Fig. 2A and B). Strong autofluorescence was detected from the skin and intestines at this wavelength, and fluorescence was also identified in the kidneys due to renal excretion of the probe. We repeated the experiment using the endogenously PSCA-expressing LAPC-9 xenograft to rule out any model-specific effects. We consistently observed positive fluorescent signal only in PSCA$^+$ tumors, with a mean tumor-to-muscle ratio of 4.48 (SD 0.52; Supplementary Fig. S2). Furthermore, to confirm that the positive signal observed in PSCA$^+$ tumors was attributable to Cy5-cDb and not preferential uptake of free Cy5 dye, we imaged mice bearing both PSCA$^+$ and PSCA$^{-}$ 22Rv1 xenografts with 5 μg of free Cy5. At 4 hours after injection, PSCA$^{-}$ tumors exhibited greater fluorescence than their PSCA$^+$ counterparts.
counterparts. Mean PSCA$^+$ to PSCA$^-$ ratio was 0.55 (SD 0.18; data not shown).

**Dose determination.** To determine the optimal dose for *in vivo* imaging, mice bearing PSCA$^+$ and PSCA$^-$ 22Rv1 xenografts were injected with 2.4 μg, 12 μg, and 25 μg (n = 5) of the probe and imaged at 2, 4, and 6 hours using the IVIS 200. PSCA$^+$ tumors fluoresced more than PSCA$^-$ tumors at each dose. Post mortem PSCA$^+$ to PSCA$^-$ ratios at 6 hours were 1.58 (SD 0.52), 2.43 (SD 0.58), and 4.47 (SD 1.63) for the 2.4 μg dose, 12 μg dose, and 25 μg dose, respectively (Fig. 2C). Based upon this result, subsequent imaging experiments were performed using the 25 μg dose.

**Optimal time for imaging.** To evaluate the optimal interval from probe injection to *in vivo* imaging, mice with PSCA$^+$ and PSCA$^-$ 22Rv1 xenografts were administered 25 μg of Cy5-cDb and serially imaged at 1, 2, 4, 6, 8, and 24 hours (n = 5). Maximum fluorescence intensity and ratios from PSCA$^+$ and PSCA$^-$ tumors were determined at all timepoints (Fig. 2D–F). Maximal PSCA$^+$ to negative ratio and fluorescence intensity were reached at 6 hours postinjection (Fig. 2E and F). To remove interference from skin autofluorescence, the experiment was repeated at the 2, 6, and 24 hour timepoints without skin overlying the tumor. Again, peak PSCA$^+$ to PSCA$^-$ fluorescence intensity ratio was achieved at 6 hours. The post mortem ratios were 1.94 (SD 0.66) at 2 hours, 4.04 (SD 1.52) at 6 hours, and 3.53 (SD 1.58) at 24 hours (P = 0.06; Supplementary Fig. S3).

**Surgical resection of tumors under fluorescence guidance**

To assess the feasibility and utility of optical imaging to aid complete tumor resection, we established intramuscular xenografts of PSCA$^+$ 22Rv1 (n = 7; Fig. 3 and Supplementary Fig. S4) and LAPC-9 (n = 3; Supplementary Fig. S5), in which tumors are invasive and therefore difficult to resect under white light alone. Upon tumor establishment, 25 μg of Cy5-cDb were administered intravenously and surgery was performed 6 hours later. We first made a skin incision and exposed the surface of thigh muscle (Fig. 3A). We attempted to resect the tumor completely under white light alone (Fig. 3B, left). Next, the fluorescent mode was turned on to evaluate for residual signal (Fig. 3B, right). Areas with residual fluorescence were surgically resected, fixed, and examined histologically to confirm that the fluorescence was indeed coming from cancer cells (Fig. 3C). A final fluorescent image was taken after the secondary surgery (Fig. 3D) and tissues from the tumor...
bed were collected and examined histologically to confirm the absence of residual tumor. As demonstrated in Fig. 3A, these tumors were often indistinct compared with muscle, and the extent and margins difficult to readily identify under white light. Of the 10 tumors, 8 had postresection sites (six 22Rv1 and two LAPC-9) that had detectable residual fluorescence following white light surgery, some areas as small as <1 to 2 mm. In some cases, there were multiple sites of residual fluorescence adjacent to a single tumor. Histology of residual fluorescent foci was positive for cancer in all 8 cases (exemplified by Fig. 3B and C).

Prospective comparison of surgical margins following resection with or without the aid of fluorescence

To demonstrate the ability of fluorescent surgery to reduce positive surgical margin rates in a controlled, objective manner, we next performed a prospective randomized study comparing white light versus white light and fluorescent surgery. As shown in Fig. 4A, 17 nude mice received intramuscular implantation of PSCA+ 22Rv1 xenografts on their thighs 21 to 22 days before the intravenous injection of Cy5-Cdb. Mice were then randomized into two cohorts to receive either just a white light surgery or an additional fluorescence-guided surgery. The surgeon was blinded to the groupings while performing the first-round white light surgery to insure that all 17 mice received the same unbiased operation, with the goal of removing as much tumor as possible while preserving adjacent normal tissues (akin to radical prostatectomy). As expected, the xenografts were invasive into the thigh musculature and demonstrated strong fluorescence and contrast with adjacent nerves and normal tissue (Supplementary Fig. S6). Following first stage white light resection, the fluorescent light was turned on to assess margin status; all mice (n = 17) had residual fluorescent signals (Fig. 4B). At this point, mice randomized to the fluorescence cohort underwent secondary surgery to remove residual fluorescent tissue, which was then subjected to histologic staining and analysis. Consistent with the previous pilot study, all remaining fluorescent tissues collected from the 9 animals in this cohort contained residual cancer (Fig. 4C). Finally, to determine final margin status, remaining thigh musculature was harvested and examined for the presence of prostate cancer with the aid of an expert uropathologist. Positive surgical margins were found in 8 of 8 mice in the white light only cohort but 0 of 9 mice assigned to fluorescent image–guided surgery (Fig. 4D and Supplementary Fig. S7).

Discussion

The inability to visualize cancer during surgery leads to incomplete cancer removal and surgical side effects. This is especially true in prostatectomy, where small foci of extracapsular extension of prostate cancer can easily be overlooked, leading to positive surgical margins and compromised oncologic control. This encourages surgeons to resect healthy tissue in an attempt at surgical margins and compromised oncologic control. This failure to adequately capture the complexities of radical prostatectomy, we believe our findings highlight the potential utility in identifying small foci of extracapsular extension of cancer during prostatectomy that would otherwise be missed. We envision this can play a role in the posterolateral prostate during nerve sparing, and may aid in the apical dissection, which lacks distinct capsule and surgical planes. Presence of fluorescence may prompt intraoperative frozen sections when the exact extent of cancer is unclear in vital areas such as the neurovascular bundle (32, 33).

Several other research groups have investigated similar strategies of targeted fluorescent molecular imaging, Jiang and colleagues imaged tumor xenografts that overexpress matrix metalloproteinases 2 and 9 using activatable cell penetrating peptides (ACP) that are cleaved by the MMPs, thereby activating fluorescence (11). The group injected their ACPs intravenously into mice bearing isoforms two days prior to imaging, then resected the tumors and demonstrated improved survival in mice whose tumors were removed using fluorescence guidance (12). The ability of the ACP to image prostate cancer has yet to be investigated. Nakajima and colleagues developed an activatable antibody–fluorophore conjugate made of a humanized anti-PSMA antibody (j591) linked to ICG. They used this to perform in vivo imaging of prostate cancer xenografts expressing PSMA (16). As with the study using ACPs, this method is limited by the 2 days required from administration to imaging. The use of this probe to guide surgical resection has not been published. The specifically recognizes prostate cancer xenografts expressing the cell surface glycoprotein PSMA. The far-red fluorescent dye Cy5 was chosen because of the stability and ease of conjugation of cyanine dyes, the feasibility of performing site-specific labeling of our cys-diabody, the lack of affinity change with Db-Cy5 conjugation, and the availability of equipment for whole-body and microscopic imaging during surgery. The probe enabled fluorescent imaging in real time to guide surgical resection of tumors in mice. The small size of the diabody (50 kDa, below the threshold for renal clearance) enabled administration and imaging on the same day, and persisted throughout the course of surgery.
Figure 4. Cy5-cDb–directed fluorescence-guided surgery enabled complete removal of infiltrative intramuscular prostate cancer. A, schematics of the study design. B and C, representative white light (B) and fluorescent (C) images of a tumor bed after white light only surgery revealing tumor that would otherwise have been missed. D, the residual fluorescent tissue from (B and C) proved to be cancer (*) by H&E staining (10x magnification). E, representative H&E staining of tumor margins from the white light surgery alone [top; with residual tumors (**) and the fluorescence-guided surgery cohorts (bottom; no residual tumors).
strategy of using an antibody fragment to shorten the interval from administration to imaging was employed by Oliveira and colleagues. They used an anti-EGFR antibody fragment (7D12 nanobody) labeled with the NIR dye IRDye800CW. This enabled tumor visualization as early as 30 minutes postinjection (20). Use of this probe in surgical resection has not been reported. More recently, Neuman and colleagues reported the use of a low molecular weight NIR fluorescent agent (YC-27) that targets PSMA in preclinical models of prostate cancer (13, 34). Survival surgery in a mouse subcutaneous xenograft model comparing white light to real-time fluorescence was performed 20 hours postinjection, with no recurrence in the fluorescent group (0/8). Investigators noted adequate tumor contrast with YC-27 within 6 hours of administration. Although to date clinical studies of fluorescently labeled prostate probes have not been reported, Maurer and colleagues recently described the use of a hand-held gamma camera to detect signal from a gallium-labeled PSMA PET probe in metastatic lymph nodes intra-operatively (35). One would predict that use of an optical label would improve the sensitivity of detection. Consistent with this hypothesis, we are developing dual PET/optical tracers to enable PET imaging followed by fluorescently guided surgery.

Although the results using our probe to resect tumors in mice are promising, several limitations are acknowledged. First, imaging and surgery were performed in xenograft tumor models using human prostate cancer cell lines. The humanized diabody does not recognize mouse PSCA. Therefore, evaluating the signal to background that will be achieved for cancer surgery in humans will require use of genetically engineered PSMA knock-in mouse models or an antibody fragment capable of recognizing both human and murine PSCA. Second, while this proof-of-concept study demonstrates the ability to find and resect small foci of residual cancer following white light surgery and significantly reduce positive surgical margins, no small animal model exists that recapitulates the complexities of prostatectomy, and as such the extent to which this improves surgical outcomes will have to be determined clinically (12). Third, the use of a fluorescent probe to improve detection and resection of lymph node metastasis is an exciting potential application. We have yet to investigate the feasibility of this application by evaluating lymph node imaging with our probe. Fourth, autofluorescence at the emission wavelength of Cy5 is greater than that for near-infrared (NIR) dyes (36). This is particularly apparent in skin and the gastrointestinal tract, although it can be reduced by dietary changes (37). We acknowledge that the current trend for imaging applications is towards NIR dyes. Cy5 was chosen given the ease of conjugation of cyanine dyes and the feasibility of performing site-specific binding to our cys-diabody. In addition, Cy5 is compatible with commonly available light sources and CCD cameras at our facility without significant alterations. We are currently developing ICG and IR800–labeled diabodies. Fifth, the differential fluorescence between benign human prostate and prostate cancer has yet to be evaluated.

Notwithstanding these limitations, the development of novel targeted fluorescent probes has tremendous translational potential. Optical probes are relatively easy to produce and imaging technology is relatively inexpensive (20). In fact, a fluorescent camera is already available for the Da Vinci robot (Intuitive Surgical), the surgical system used to perform the overwhelming majority of robotic prostatectomies in the world. To make use of this technology, a method to selectively deliver the fluorescent dye to cancer cells is required. The Cy5-cDb probe developed herein enables targeted detection and resection of cancers expressing PSCA using an antibody fragment that enables same day administration and imaging. Efforts to translate this probe into the clinic are ongoing.

Conclusion

We successfully synthesized a monoclonal antibody fragment–fluorophore conjugate consisting of a humanized anti-PSCA diabody linked to the fluorescent dye Cy5 that enables same day administration and in vivo imaging. This probe was used successfully to resect residual cancer fragments in mice under real-time fluorescent guidance, and demonstrated a significant reduction in positive surgical margins compared with white-light surgery alone.

Disclosure of Potential Conflicts of Interest

E.J. Lepin is an employee of ImaginAb. A.M. Wu has ownership interest (including patents) in and is a consultant/ advisory board member for ImaginAb. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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