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Targeted, Activatable, *IN VIVO* Fluorescence Imaging of Prostate-specific Membrane Antigen (PSMA)-positive Tumors Using the Quenched Humanized J591 Antibody-ICG Conjugate

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Abstract

In patients with prostate cancer, a positive surgical margin is associated with an increased risk of cancer recurrence and poorer outcome, yet, margin status cannot be determined during the surgery. An *in vivo* optical imaging probe that could identify the tumor margins, during surgery, could result in improved outcomes. The design of such a probe focuses on a highly specific targeting moiety and a near infrared (NIR) fluorophore that is activated only when bound to the tumor. In this study, we successfully synthesized an activatable monoclonal antibody-fluorophore conjugate consisting of a humanized anti-prostate specific membrane antigen (PSMA) antibody (J591) linked to an indocyanine green ICG-derivative. Prior to binding to PSMA and cellular internalization, the conjugate yielded little light, however after binding an 18-fold activation was observed permitting the specific detection of PSMA+ tumors up to 10 days after injection of a low dose (0.25 mg/kg) of the reagent. This agent demonstrates promise as a method to image the extent of prostate cancer *in vivo* and could assist with real time resection of extracapsular extension of tumor.

Keywords

prostate cancer; activatable fluorescence imaging; prostate specific membrane antigen; humanized monoclonal antibody; optical surgery

INTRODUCTION

Prostate cancer is the 2nd leading cause of cancer mortality in men. Surgical resection is associated with a high cure rate in men with low risk tumors, however, the risk of recurrent disease rises with the Gleason score and clinical stage of the tumor. Nevertheless, especially in patients with locally invasive prostate cancer, long term cancer control can be achieved if adequate margins are obtained at surgery. Unfortunately, despite improving clinical imaging modalities¹⁻³, the process of achieving negative margins is retrospective and is determined *post hoc* by histopathology. A method to aid the surgeon in obtaining negative margins during the procedure would be welcomed and could be anticipated to result in better patient

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outcomes. In order to achieve this goal, a highly specific optical imaging probe must be developed that fulfills at least two criteria. The agent must be highly targeted to prostate cancer with few false positives and the fluorescent probe to which the targeting ligand is attached, must be activatable only at sites of tumor in order to achieve adequate target to background ratios (TBR) to display the tumor in real time to the surgeon. Additionally, the fluorescent probe should emit light in the near infrared to permit deeper tissue penetration than is possible with visible light.

Prostate specific membrane antigen (PSMA) is a type 2 integral membrane glycoprotein, also known as glutamate carboxypeptidase II (GCPII) ⁴ and folate hydrolase 1, found initially in prostate cancer cell lines (LNCaP) ⁵ and a few other tissues including kidney, intestine and central nervous system ⁶. It is considered the most well-established cell membrane marker of prostate cancer and therefore a plausible target for an optical probe. PSMA is expressed on tumor cells as a noncovalent homodimer ⁷. PSMA has been used in imaging as a single photon emission computed tomography (SPECT) scan in which a PSMA antibody (Capromab Pentitide, Prostascint) is labeled with radioactive indium-111 ⁸. Experimentally, small PSMA receptor binding ligands have also been tested ⁹ for imaging prostate cancer. These methods have met with variable success, in part owing to the specificity of the capromab antibody used: it recognizes an intracellular epitope that is only accessible to circulating antibody when the cell membrane is disrupted. Furthermore, while SPECT scans are potentially useful in the pre-operative or post-operative setting, they are of little value for surgical guidance or on-site tissue diagnosis because they offer low spatial and temporal resolution. Optical probes, however, could provide higher resolution and near real time imaging without the risks to the patient and surgeon, associated with exposure to ionizing radiation.

Herein, we describe our initial experience with an optical antibody-fluorophore conjugate consisting of an anti-PSMA humanized antibody (J591) coupled to an activatable near infrared (NIR) fluorophore, indocyanine green (ICG) ¹⁰. We tested this agent in PSMA expressing and non-expressing prostate cancer xenografts in mice and compared it to a comparable conjugate, trastuzumab-ICG (Tra-ICG) which targets HER2 expressing tumors.

EXPERIMENTAL PROCEDURES

Reagents

Two humanized monoclonal antibodies were used in this study; J591, a humanized PSMA-specific monoclonal antibody, was developed at Weill Cornell Medical College by one of the authors (NHB), trastuzumab (Tra), a humanized anti-HER-2 antibody, was purchased from Genentech Inc. (South San Francisco, CA). For activatable labeling of antibodies, ICG-sulfo-OSu was purchased from Dojindo Molecular Technologies (Gaithersburg, MD), IR700 was purchased from LI-COR Biosciences (Lincoln, NE). All other chemicals used were of reagent grade.

Synthesis of fluorophore conjugated antibodies

Both humanized monoclonal antibodies; J591 and Tra (1 mg, 6.2 nmol) were incubated with ICG-sulfo-OSu (50 nmol, 1:8) or IR700 (31 nmol, 1:5) in 0.1M Na₂HPO₄ (pH 8.5) at room temperature for 30 min. Then, the mixture was purified with a SephadexTM G50 column (PD-10; GE Healthcare, Piscataway, NJ). The elution fraction of 1.8 mL obtained from 2.6 ml to 4.4 mL of the elutant, was collected. The concentration of ICG or IR700 was measured by absorption with a UV-Vis system (8453 Value UV-Visible Value System; Agilent Technologies, Santa Clara, CA) to confirm the number of fluorophore molecules conjugated to each antibody molecule. The number of ICG per each antibody measured was ~1 for ICG

at the quenching state or ~3 for IR700. As a quality control of the J591-ICG conjugate, we performed SDS-PAGE and a binding assay with modified Lindmo's method¹¹ and Scatchard plot using ¹²⁵I-labeled J591 and J591-ICG, and PC3-PSMA+ cells, as previously described¹².

Validation of quenching ability *IN VITRO*

For validation of the quenching and dequenching ability of bound ICG, both J591-ICG and Tra-ICG conjugates were treated with 1% SDS to diminish hydrophobic k-k interactions and separate IgG chains. The change in fluorescence intensity for each conjugate was measured with an *in vivo* imaging system, (Pearl Imager, LI-COR Biosciences, Lincoln, NE) using 785 nm laser excitation and 820 nm emission.

Cell culture

A PSMA transfected PC3 cell line, PC3-PSMA+ (PC3pip) and a control blank-vector transfected PC3 cell line, PC3-PSMA- (PC3flu)¹³ were used for PSMA targeting studies with activatable J591-ICG conjugates. Both cells were established at the Cleveland Clinic Foundation. For HER2 targeting studies by trastuzumab conjugates, the HER2 transfected NIH3T3 (3T3/HER2) cell line and a control Balb/3T3 cell line were used¹⁴.

All cell lines were grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) (Life Technologies) and 1% Pen-Strep (Biofluids, Camarillo, CA). All cell cultures were maintained in 5% carbon dioxide (CO₂), at 37.0°C in a humidified incubator.

Flow cytometry

Fluorescence signal from PC3 cells after incubation with ICG- or IR700-conjugated J591 agents was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and CellQuest software (BD Biosciences). Briefly, for the binding experiment, 1×10^5 PC3-PSMA+ or PC3-PSMA- cells were incubated with J591-ICG or J591-IR700 at 37.0°C for 8 hours. To validate the specific binding of the antibody, excess J591 antibody (50 µg) was used to block 0.5 µg of J591-IR700 conjugates.

Fluorescence microscopy studies

PC3-PSMA+ cells (1×10^4) were plated on a covered glass-bottomed culture well and incubated for 16 h. Then, J591-ICG was added to the medium (5 µg/mL) and the cells were incubated for either 1 or 8 h. Cells were washed once with PBS, and fluorescence microscopy was performed using an Olympus BX61 microscope (Olympus America, Inc.) equipped with the following filters: excitation wavelength 672.5 to 747.5 nm, emission wavelength 765 to 855 nm. Transmitted light differential interference contrast (DIC) images were also acquired. As a control, the conjugates were also incubated with PC3-flu (PSMA negative) cells.

Animal tumor model

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the local Animal Care and Use Committee. Seven- to 8-week old, athymic nu/nu mice were used for all experiments. Both receptor positive and negative tumor cell lines were implanted in the same mice. For PSMA targeting studies, 2×10^6 PC3-PSMA+ cells suspended in PBS were injected subcutaneously in the left dorsum of the mice, and the same number of PC3-PSMA- cells were injected subcutaneously into the right dorsum in each mouse.

For HER2 targeting studies, 1×10^6 3T3/HER2 cells suspended in PBS were injected subcutaneously in the left dorsum of the mice, 2 days prior to injecting with 1×10^6 Balb/3T3 cells in PBS into the right dorsum. The imaging experiments were performed at 5-7 days after cell injection, when tumor size is approximately 0.5 cm in diameter.

IN VIVO PSMA targeted imaging studies

J591-ICG was diluted in 200 μ L PBS at the dose of 50 μ g, 25 μ g, 12.5 μ g, or 6.25 μ g was then injected via the tail vein into mice bearing both PC3-PSMA+ and PC3-PSMA- tumors. All mice were anesthetized with 2% isoflurane throughout all procedures. Then, fluorescence images were obtained using the Pearl Imager (LI-COR Biosciences) at 800 nm at the following time points: 6 hours, 1, 2, 3, 4, 6 and 10 d after injection.

Regions of interest were placed on ICG spectrum images with reference to white light to measure fluorescence intensities of PC3-PSMA+ and PC3-PSMA- tumors and body (background) at every time point up to day 6.. The software, Pearl Cam Software (LI-COR Biosciences) was used for calculating ROI signal data of each point. After acquiring images at day 10, mice were sacrificed with carbon dioxide. *Ex vivo* images of resected tumors were obtained.

Comparison of images between PSMA+/- and HER2+/- tumors

To compare the signal obtained with J591-ICG from PC3-PSMA+ tumor with HER2 targeting images injected with Tra-ICG, both PSMA targeted and HER2 targeted images were serially acquired in side by side views 6 hrs, 1, 2, 3, and 4 days after injection of J591-ICG or Tra-ICG antibodies (12.5 μ g in 200 μ L PBS) into PC3-PSMA+/- or 3T3-HER2+/- tumor bearing mice, respectively. The fluorescence intensities of tumors and body (background) were processed with the same method as above.

RESULTS

Antibody-fluorophore conjugates show high quenching capacity

Fluorescence intensity was low for both J591-ICG and Tra-ICG in their native state. After exposure to 1% SDS, a 18-fold and 15-fold increase in fluorescence signals was detected for J591-ICG and Tra-ICG, respectively (Fig. 1A).

As defined by SDS-PAGE, 70% of ICG on the J591-ICG conjugate was covalently conjugated to the antibody. The specific immunoreactivity fraction of 125 I-labeled J591-ICG (69%) was only 7% less than the 125 I-labeled J591 without ICG (76%). The dissociation constant of 125 I-labeled J591 or J591-ICG as measured by Scatchard plot analysis was 2.8 nM, similar to published values^{15, 16}. In addition, the copy number of PSMA antigen on a single PC3-PSMA+ cell was calculated to be 2.0 million.

IN VITRO cell analysis

J591-IR700, a non-activatable control conjugate, demonstrated intense signal arising from PC3-PSMA+ cells and minimal signal from PC3-PSMA- cells (Fig. 1B). Blocking experiments using 100-fold levels of unlabeled J591 showed almost complete competitive blocking of J591-IR700 uptake indicating highly specific binding of J591-IR700 to PSMA (Fig. 1C). Flow cytometry analysis using J591-ICG showed strong fluorescence from PC3-PSMA+ cells and weak signal from PC3-PSMA- cells (Fig. 1D). Since this weak binding could not be blocked with a 100-fold excess of unlabeled J591 antibody, this signal represents low level, non-specific activation of quenched ICG, which was seen only *in vitro*. *In vitro* microscopy demonstrated results similar to flow cytometry experiments namely,

specific uptake by PSMA expressing cells that could be blocked with unlabeled J591 (Fig. 1E).

***IN VIVO* imaging studies showed specific target visualization with J591-ICG even at low doses**

The time course of fluorescence intensity changes in PC3-PSMA+ and PC3-PSMA- tumors and of background signal after injection of various doses of J591-ICG are summarized in Fig. 2A. PC3-PSMA+ tumors yielded significantly higher fluorescent signal at all injected doses =1 day after injection. PC3-PSMA+ tumors were clearly depicted at all dose levels (50 μ g, 25 μ g, 12.5 μ g and 6.25 μ g) (Fig. 2B) up to 10 days after injection (Fig. 3A), although fluorescence signal from PC3-PSMA+ tumors increased with dose. All PC3-PSMA+ tumor specimen, which obtained 10 days after injection of 6.25 μ g J591-ICG, were clearly identified with ICG-specific fluorescence signal, although all PC3-PSMA- tumor specimen did not produce detectable signal (Fig. 3B). Signal intensity ratios between PC3-PSMA+ and PC3-PSMA- tumors increased with time reaching 14 at 96 hr after injection (Fig. 3C). Since fluorescence from PC3-PSMA- tumors was practically equivalent to the background, ICG signals from scattered PC3-PSMA-tumors were often measured as negative numbers and thus, were eliminated from the calculation. Therefore, the ratio of PC3-PSMA+ tumor-to- PC3-PSMA- tumor as shown in Fig. 3C is an underestimate.

J591-ICG yielded brighter fluorescence signal from PC3-PSMA+ tumor than Tra-ICG yielded from 3T3/HER2+ tumor

At the same dose injection (12.5 μ g), the fluorescence signal arising from PSMA+ tumors after J591-ICG was higher than that from HER2+ tumors using the same dose of Tra-ICG (Fig. 4).

DISCUSSION

This agent demonstrated several desirable features of a prostate cancer imaging optical probe. It appeared to be highly specific for PSMA expressing tumors, producing very bright targets within 24 hours of administration. It appeared to have a favorable efficiency in comparison to a similar HER2 targeted Tra-ICG compound¹⁰. Its ability to be activated only at the PSMA+ cells led to very high TBRs. This is related to a unique property of ICG, whereby it loses fluorescence when bound to the antibody but regains it, once it has been chemically separated from the antibody as occurs after internalization and degradation of the conjugate in the lysosome. One drawback of this agent is that it requires 2 days to be sufficiently taken up by the tumor, internalized and activated. However, once this has occurred the activation appears to last for at least 10 days. This suggests that there is a wide “window of opportunity” wherein the agent could be administered prior to a surgical procedure and still retain the desired benefits. Potential applications include detecting extracapsular extension, identifying lymph node metastasis, detecting remnant tumor after resection, and validating at the point of care that tissue obtained via optical biopsy or surgery contains cancer.

Optical imaging is one of the few types of imaging that can be “activated”^{17, 18}. Ideally, in the unbound state a targeted compound would yield no signal but after specific binding would become fluorescent. In its injected state the probe is quenched, emitting no light. After binding and internalization the ICG is separated from the antibody, allowing it to dequench and leading to high TBR. In this study, quenched J591-ICG was efficiently activated in PC3-PSMA+ tumors. This phenomenon occurred at all doses of PSMA and suggests that the injected dose for clinical applications could be minimized to 0.25 mg/kg that is comparable to the antibody doses used in radioimmunoscinigraphy. The flow

cytometry results suggest that although the numbers of PSMA and HER2 antigens on respective PC3-PSMA+ cells and 3T3Her2+ cells were similar at 2.0 and 2.2 million copies per cell, respectively, the anti-PSMA conjugate was considerably more effective as an imaging agent than the anti-HER2 conjugate. The difference in fluorescence signal could derive from differences in the delivery of the antibody and/or the internalization/activation ratio¹⁷. In any case, the low dose of antibody required for effectiveness is encouraging for clinical translation.

Although safety was not specifically studied, there is reason for optimism regarding toxicity. Both major components of J591-ICG have been safely administered to patients. In the case of J591 this testing has been limited to several hundred patients in clinical trials at considerably higher doses^{19, 20} but in the case of ICG there is more than 50 years of clinical experience, primarily in ophthalmology²¹. Of course, formal safety studies will be required prior to approval for human use but the low dose needed for both components bodes well for a favorable toxicity profile.

In order to utilize a conjugate such as J591-ICG it will be necessary to have NIR cameras in the operating room²². Such cameras could be incorporated easily into existing endoscopic cameras providing both white light and NIR imaging during the procedure. Such endoscopic cameras have already been developed experimentally²³.

In conclusion, we successfully synthesized an activatable anti-PSMA humanized monoclonal antibody, J591, conjugated with a linkable ICG derivative. The activatable antibody was able to depict PSMA+ tumors with high contrast compared to PSMA- tumors up to 10 days after injection of low dose of reagent (0.25mg/Kg). Activatable J591-ICG is a promising molecular imaging agent for detecting both primary and metastatic PSMA-positive prostate cancer based on the expression of PSMA and could be especially important in assisting surgeons performing biopsies and prostatectomies for cancer.

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Figure 1.

A. Quenched (left) and chemically dequenched (right) J591-ICG and Tra-ICG. Dequenched conjugates show increased fluorescence signal shown in green. In vitro cell experiment with flow cytometry (B-D) and microscopy (E) showing specific binding of J591 to PSMA and activation of fluorescence signal after binding. Scale bar indicates 20 μ m.

Figure 2.

A. In vivo serial fluorescence signals of PC3-PSMA+ and PC3-PSMA- tumor as well as background in the body of mice injected with 50, 25, 12.5, and 6.25 µg of J591-ICG. B. In vivo serial fluorescence images of a PC3-PSMA+ and PC3-PSMA- tumor bearing mouse injected with 6.25 µg of J591-ICG. Only PC3-PSMA+ yields ICG fluorescence signal 3 days after injection or later.

Figure 3.

A. In vivo fluorescence images of a PC3-PSMA+ and PC3-PSMA- tumor bearing mice 10 days after injection of 50, 25, 12.5, or 6.25 μg of J591-ICG are shown. Although ICG signal from PC3-PSMA+ tumors is stronger in mice injected with a larger dose, all PC3-PSMA+ tumors show ICG fluorescence signal. B. Ex vivo fluorescence images of five PC3-PSMA+ and PC3-PSMA- tumors obtained 10 days after injection of 6.25 μg J591-ICG are shown. PC3-PSMA+ tumors clearly show specific fluorescence signal of ICG. C. PC3-PSMA+ tumor-to-PC3-PSMA- tumor signal intensity ratio is plotted. All doses show a similar trend of changes on PC3-PSMA+ tumor-to-PC3-PSMA- tumor signal intensity ratio. Scattered PC3-PSMA- tumors, which showed signal below background, are eliminated from the calculation.

Figure 4. Side-by-side comparison of images (A) and serial fluorescence intensity curves (B) for PC3-PSMA+ and PC3-PSMA- tumor bearing mouse injected with 12.5 μ g of J591-ICG versus 3T3HER2+ and 3T3HER2- tumor bearing mouse injected with 12.5 μ g of Tra-ICG. Images (A) are obtained 3 days after injection of antibodies. The anti-PSMA conjugate yields higher signal than the anti-HER2 conjugate suggesting better delivery and/or more efficient internalization.