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A 3E8.scFv.Cys-IR800 Conjugate Targeting TAG-72 in an Orthotopic Colorectal Cancer Model

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Abstract

Purpose—Optical surgical navigation (OSN) will be a potent tool to help surgeons more accurately and efficiently remove tumors. The purpose of this study was to evaluate a novel humanized 3E8 antibody (3E8 MAb) fragment site-specifically conjugated with IR800, 3E8.scFv.Cys-IR800, as a potential OSN agent to target colorectal adenocarcinoma.

Procedures—An engineered single-chain variable fragment of 3E8 MAb (targeted to TAG-72), appending a C-terminal cysteine residue (3E8.scFv.Cys), was created and reacted with IRDye800-maleimide. 3E8.scFv.Cys-IR800 identity and purity were verified by MALDI-TOF mass spectra and 800 nm detected size exclusion column HPLC. *In vitro* human colon adenocarcinoma LS-174 T cells binding and competition assay validated biological functionality. We further evaluated the imaging ability and receptor-specific binding of 3E8.scFv.Cys-IR800 in an orthotopic LS-174 T mouse model.

Results—A 1:1 dye to protein conjugate was achieved at greater than 90 % HPLC purity. A 1 nmol dose of 3E8.scFv.Cys-IR800 *via* intraperitoneal injection administration was sufficient to produce high tumor to background fluorescence contrast. Blocking competition studies both *in vitro* and *in vivo* using a different blocking protein, 3E8 CH2, demonstrated 3E8.scFv.Cys-IR800 binding specificity for TAG-72 antigen.

Conclusions—3E8.scFv.Cys-IR800 shows properties useful in a clinically viable OSN agent for colorectal cancer.

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Compliance with Ethical Standards

Conflict of Interest

TM and EM are officers of Enlyton, LLC which owns patent rights to 3E8 antibody fragments. All other authors declare no conflicts of interest.

Keywords

Optical surgical navigation; Colorectal cancer; Fluorescence; Humanized 3E8 antibody; Single-chain variable fragment 3E8; 3E8.scFv; Intraperitoneal injection

Introduction

More than 135,000 colorectal carcinomas are predicted in 2017 (USA), 96 % of which will be adenocarcinoma [1]. The overall management of these patients, whether for early stage or more advanced disease, involves surgery within the treatment plan. It is well-known that incomplete surgical clearance of the tumor and inability to locate and completely resect regional and distant metastatic disease leads to disease recurrence and poor long-term survival. Despite advances in surgery, including minimally invasive, laparoscopic [2, 3], and robotic approaches [4], and advances in diagnostic imaging [x-ray computed tomography (CT), single photon computed tomography, positron emission tomography (PET)/CT, and magnetic resonance imaging (MRI), ultrasound (US)] [5, 6], surgeons still rely heavily on visual inspection and palpation that can miss occult sites of disease, especially when these sites are not apparent on preoperative imaging. Hence, intraoperative guidance is a well-established field of inquiry [7].

Existing imaging technologies are suboptimal for intraoperative use, either not cancer specific (MRI, US, CT) or unsuitable for use in real-time (PET/CT). Intraoperative frozen section analysis by microscopy is too slow and evaluates only a small fraction of the entire specimen [8]. Radio-guided antigen-directed surgery (RADS) uses systemically delivered radiolabeled cancer-specific antibodies (Ab) detected with handheld radiation detection probes [7, 9, 10]. This technique definitively produced a long-term survival advantage for patients in whom complete resection could be accomplished [9]. But RADS is also a slow intraoperative process in most patients.

A breakthrough approach could be optical surgical navigation (OSN) using cancer-specific near-infrared fluorescence imaging (NIRF) agents [11–14] which report the presence of the NIRF molecules visually, in real time, and over a wide (*e.g.*, ~10–20 cm) field of view. Most attempts to create OSN fluorescent imaging agents targeted to colon cancer have used antibodies to promising targets, TAG-72, CEA, ILGF-1, and uPAR. While all attempts reported successful imaging of tumors in rodents, all were suboptimal in one or more respects. Three reports used suboptimal fluorescent dyes like CY5 and CY7 that were designed for microscopy and emit most of their light at <800 nm which is undetectable by clinical imagers that detect only >800 nm emission [15–17]. Two reports used dyes emitting in the clinically useful range, but the dyes were conjugated to the proteins by labeling methods that are not site specific [18, 19], which leads to well-known complications [20, 21], particularly lack of control of the label's location on the protein surface, and the number of labels attached to the protein, making poorly defined, heterogeneous immunoconjugates. To reduce the overly long circulation times of full-sized antibodies, Zou et al. successfully tested a delta CH2-deleted fragment of a TAG-72 targeted CC49 antibody [22], but this fragment also was not site specifically labeled and used a suboptimal dye.

Herein, we begin to rectify these deficiencies by engineering a 3E8.scFv antibody fragment of 28 kDa, small enough for renal excretion, to contain a C-terminal cysteine residue (3E8.scFv.Cys) to allow the site-specific labeling. We also use a NIRF dye, IRDye800, with a peak emission matched to clinically used imagers [23, 24], and test the product for binding specificity in plated cells and in an orthotopic mouse tumor model. The new dye allowed us to image the mice with a practical optical surgical imager, the Fluoptics Fluobeam™.

Materials and Methods

Human TAG-72 antigen was purchased from Sigma (Saint Louis, MO, USA). Amicon Ultra-0.5 Centrifugal filter units were purchased from Millipore (Billerica, MA, USA). IRDye800-maleimide was purchased from Li-COR, Inc. (Lincoln, NE, USA). The 3E8 CH2 was a gift from Enlyton, LLC. It was manufactured by Catalent Pharma Solution (Middleton, WI, USA), according to the method established by Slavin-Chiorini [25]. The 3E8 binding sequence for 3E8.scFv.Cys was identical to those in 3E8 Mab antibody and CH2 delete proteins [26].

Cell Line

LS-174 T cells were obtained from ATCC (Manassas, VA, USA) and grown in McCoy's 5A medium in addition to 10 % of bovine serum (FBS, Atlanta Biologicals, Awrenneville, GA, USA) and 1 % of penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA), at 37 °C in a humidified atmosphere containing 5 % CO₂. All cell incubations were done in the incubator under these conditions. Quantitative data are reported as the mean, with uncertainty as the standard deviation (SD).

Engineered 3E8.scFv.Cys Antibody Fragment

The 3E8.scFv was constructed with the intention of creating a low molecular weight protein that retained high specificity for targeting to TAG-72. 3E8.scFv.Cys was expressed in a Walker series C43 *E. coli* from the overexpression plasmid, pHLIC [26, 27]. Transformants were escalated to a larger scale of growth through standard techniques. After cells were lysed with an Emulsiflex, the supernatant was purified through a GE Healthcare HiTrap Protein L column and a cation exchange chromatography Resource S column.

A dot blot assay showed that the 3E8.scFv.Cys fragment binds to BSM (bovine submaxillary mucin, Type I-S, Sigma-Aldrich, St. Louis, MO) which contains sialy-Tn [26]. We validated the binding specificity of 3E8 CH2 to TAG-72 by an indirect ELISA (Fig. S1). Briefly, a two-step ELISA was applied to detect the absorbance using a plate reader described below. Primary antibody was first bound to the TAG-72 antigen coated 96-well microplates, and then incubated with labeled secondary antibody HRP-IgG Fcγ (Peroxidase-conjugated AffiniPure Goat Anti-Human IgG, Fcγ Fragment Specific, Jackson ImmunoResearch, Inc., West Grove, PA, USA), then absorbance was read at 490 nm. The binding constants of 3E8 CH2 and 3E8.scFv.Cys to BSM-coated plates were determined by surface plasmon resonance (SPR) using a GE Health Sciences Biacore T100 system, calculating K_D from the quotient of the measured rate constants, k_{off}/k_{on} (Fig. S2).

Synthesis of 3E8.scFv.Cys-IR800 Conjugate

The 3E8.scFv.Cys-IR800 conjugate was synthesized using an optimized maleimide conjugation protocol [28]. A total volume of 100 μ l of a PBS solution containing 200 μ g (7.14 nmol) of 3E8.scFv.Cys was prepared and reacted with 1.8 μ l of 10 mM IRDye800-maleimide (1: 2.5 3E8.scFv.Cys: IRDye800-maleimide mole ratio) that was gradually added to the 3E8.scFv.Cys solution with gentle vortexing, followed by shaking the reaction tube on an arm shaker for 3 h at room temperature in the dark. The solution was loaded onto an Amicon Ultra-0.5 centrifugal filter unit to remove excess IRDye800-maleimide and concentrate the product. The filter was washed twice with PBS. Then, the solution above the 10 kDa cutoff filter was collected. Linear mode ultrafleXtreme MALDI-TOF/TOF (Bruker, UK) was run (sinapinic acid was used as the matrix) to verify the identity of the product. Size exclusion column HPLC (LC-10ATVPT, Shimadzu, Columbia, MD; column: Agilent Bio SEC-3 column 3 μ m, 100 $\text{\AA}$, 4.6 $\times$ 30 cm) run in PBS with an 800 nm fluorescence detector (Shimadzu RF-10AXL) was used to determine purity of the product. The biological functionality of 3E8.scFv.Cys-IR800 was validated by applying the indirect ELISA method as above.

In vitro Binding Studies

The human adenocarcinoma cell line, LS-174 T, expresses and secretes TAG-72 [29]. To verify the TAG-72 binding specificity of 3E8.scFv.Cys-IR800 conjugate, we ran a blocking experiment. LS-174 T cells (1×10^5 cells per well) were cultured in a 96-well plate 16 h prior to the binding assay. The cells were incubated with 100 μ l of serum-free growth medium containing 7.5 μ M 3E8 CH2 for 3 h and then washed twice with PBS to remove the unbound 3E8 CH2. One hundred fifty picomole of 3E8.scFv.Cys-IR800 was then added to these cells in 100 μ l of serum-free growth medium. At the same time, control cells not exposed to 3E8 CH2 were treated with varying concentrations of 3E8.scFv.Cys-IR800: 1.5, 0.75, and 0.375 μ M, each in 100 μ l. All treated cells were incubated for 3 h then washed five times with 200 μ l of PBS. Sixty microliter of PBS were then added to all wells on the plate for measurement of the fluorescence intensity at (764 nm ex, 809 nm em) nanometer in a Synergy H4 hybrid multi-mode microplate reader (BioTek, Winnoski, VT, USA).

In vivo Tumor Model

The animal experiments were performed under a protocol approved by The Ohio State University Institutional Animal Care and Use Committee. All of the guidelines were followed including euthanization of animals in obvious distress. The orthotopic LS-174 T tumor model creation was patterned after a published method [30–35]. Five to six-week-old female athymic nu/nu mice (Charles River Laboratories, MA) were injected intraperitoneally (i.p.) with 6×10^6 LS-174 T cells in 600 μ l of PBS. Due to the small size of the of orthotopic tumors, palpation was not possible, and tumor progress was assessed by body weight and general physical condition changes, which were monitored three times per week. Through experimentation, we found that the implanted LS-174 T cells required 12–14 days to produce tumors clearly visible by eye at necropsy.

Pharmacokinetics Studies

Non-tumor bearing female BALB/c mice ($N=4$) were injected under isoflurane anesthesia with 2 nmol 3E8.scFv.Cys-IR800 in 100 μ l PBS *via* tail vein to perform pharmacokinetics studies. Mice remained conscious except during the blood draws. The blood was collected at 1 min, 0.5, 1, 2, 4, 8, and 24 h post-injection, 5 μ l of blood being collected from the saphenous vein, and loaded into a black wall 96-well plate containing 95 μ l PBS buffer with 0.15 % EDTA (pH 8.5) and 0.2 % BSA per well. The mouse blood volume was calculated as 78 ml/kg mouse [36]. Blood and urine samples from an uninjected mouse were used as negative controls, 0 % ID (injected dose), and 100 % ID standards. The fluorescence intensity was measured with the Synergy H4 microplate reader.

Near Infrared Fluorescence In Vivo Imaging

Imaging experiments performed in the LS-174 T tumor-bearing mice used 1 nmol of 3E8.scFv.Cys-IR800 administered i.p. into $N=4$ conscious mice, and $N=3$ mice were sham injected (PBS containing no 3E8.scFv.Cys-IR800). Six tumor bearing mice were used for the blocking experiment. Mice in the blocked imaging group ($N=3$) were injected i.p. with 10 nmol of 3E8 CH2, and then 3 h later, with 1 nmol 3E8.scFv.Cys-IR800. These were compared with $N=2$ mice injected only with 1 nmol 3E8.scFv.Cys-IR800 and $N=2$ mice that were sham injected. All animals were euthanized at 24-h post-administration, and the abdomen was opened immediately for imaging using a laser excitation Fluobeam™ 800 NIR imaging system (Fluoptics, Grenoble, France). The Fluobeam system excites with a 780-nm emission laser and records with a CCD camera with >800 nm emission filtering.

Results

Synthesis of 3E8.scFv.Cys-IR800

The 3E8.scFv.Cys fragment was created by protein engineering based on the full-length sequence of the humanized 3E8 antibody [37]. Figure 1a shows the structures of the components of the final product, 3E8.scFv.Cys-IR800. The mass spectrum confirmed the identity of 3E8.scFv.Cys-IR800 as shown in Fig. 1b. The mass spectral results of several syntheses varying the dye to protein mole ratio up to 10 indicated that the reaction of 2.5 mol of IRDye800-maleimide with 1 mol of protein produced the most 1:1 conjugate. The fluorescence detected HPLC shown in Fig. 1c showed that greater than 90 % 3E8.scFv.Cys purity of the 1:1 conjugate was achieved, with about 10 % of free IRDye-800-maleimide. The 800 nm fluorescence intensity of 3E8.scFv.Cys-IR800 was found to be 39 ± 18 % diminished compared to IR800-maleimide, using a fluorescence detected concentration series measured between 0.2 and 1.0 μ M protein.

In vitro Binding Studies

The binding constant of 3E8.scFv.Cys was 12 nM, compared to 13.6 nM for the bivalent fragment, 3E8 CH2 (Fig. S2). Figures 2 and S3 demonstrate concentration-dependent binding of 3E8.scFv.Cys-IR800 to TAG-72 on LS-174 T cells (Fig. 2) and TAG-72 coated plates (Fig. S3). Binding was proportional to the concentration of 3E8.scFv.Cys-IR800 in the incubation media, measured after equivalent durations of incubations. 3E8 CH2 binds to

the same epitope on TAG-72, so we used a fivefold excess concentration of 3E8-CH2 as a blocking reagent to confirm the specific binding of 3E8.scFv.Cys-IR800 to TAG-72 on LS-174 T cells. 7.5 μ M of non-fluorescent 3E8-CH2 blocked the binding of 1.5 μ M 3E8.scFv.Cys-IR800, as shown in Fig. 2.

In vivo Imaging

Blood concentration *vs.* time curves for the 3E8.scFv.Cys-IR800 in non-tumor bearing mice are shown in Fig. 3. Treating the blood clearance as bimodal, the first three points yielded a half-time of ~30 min for distribution ($r^2 = 0.974$), and the last three points yielded an elimination half-time of 10 h ($r^2 = 0.997$), based on linear log %ID *vs.* time fits. Urinary excretion was obvious from a brightened bladder on fluorescent images, and 26 % of the administered agent was recovered in urine after the first 3 h.

Tumor deposits in the peritoneal cavity were observed 12–14 days after i.p. inoculation, similar to a published model [38]. Most of the tumor deposits were small (1–3 mm in diameter) and located mainly in the upper abdomen in the liver hilum, the greater omentum, and adjacent to the spleen (Fig. 4a, right side). H&E staining validated the identification of the growths as tumors (Fig. 4b) [32]. Based on previous experience [22, 39] and pilot studies, we chose 24 h post administration as the imaging window in the tumor bearing mice. A pilot experiment at 1 nmol administered i.v. showed only modest fluorescence intensity in tumors, while i.p. administration of 1 nmol 3E8.scFv.Cys-IR800 showed well-highlighted tumors (Fig. S4). Figure 4a (left side) shows one of the four mice imaged with 1 nmol i.p. administration compared to a barely visible control mouse. Imaged mice always showed fluorescent signal in the gastrointestinal tract due to coprophagy, which occurs whether or not fluorescent free chow is used, and can transfer a very slight amount of fluorescence signal to co-housed control mice. To demonstrate receptor specificity, in another group of tumor bearing mice, 10 nmol of 3E8-CH2 was administered i.p. 3 h prior to i.p. administration of 1 nmol 3E8.scFv.Cys-IR800. Figure 5 shows that 3E8-CH2 successfully blocked 3E8.scFv.Cys-IR800 binding to the LS-174 T tumors, which further supports the binding specificity of 3E8.scFv.Cys-IR800.

Discussion

TAG-72 is membrane-bound glycoprotein that is expressed in 80 % of colorectal cancers with relatively little expression in normal mucosa. It is expressed at high concentrations due to secretion of TAG-72 from the cancer cells and pooling in the tumor microenvironment. It also lacks a significant circulating fraction that would create background signal for an imaging agent [7, 40–43]. A radiolabeled 125 I-antibody to TAG-72 was used clinically in a successful RADS study, demonstrating remarkably long adherence time (up to 1 month) at the antigen [9].

In initial efforts to create TAG-72 OSN agents, TAG-72 specific humanized CC49 antibody and a CH2-deleted fragment were conjugated with Cy7-NHS. We detected tumor-associated fluorescence in mice xenografts [22]. But Cy7 is suboptimally low in its peak emission (*i.e.*, <800 nm), precluding the use of thousands of surgical imagers optimized for >800 nm emission. Labeling of the proteins with the fluorescent dyes was also suboptimal,

using NHS conjugation chemistry that reacts with random amines on the protein surface, making inevitably heterogeneous mixtures of products. The significant advantage of site specific labeling of immunoconjugate imaging agents stems from the reduction of this heterogeneity, as recently reviewed [20, 21]. 3E8.scFv.Cys-IR800 was created to rectify these two deficiencies.

Incorporation of the C-terminal cysteine into 3E8.scFv yielded 3E8.scFv.Cys, allowing coupling with IRdye800-maleimide to maximize the 1:1 labeling ratio *versus* unlabeled and multi-labeled protein. We also hoped to reduce dye quenching that is commonly encountered in conjugating dyes to proteins by non-site specific labeling methods [44, 45] but were not fully successful, encountering 39 % dye quenching in the final product compared to IR800-maleimide. While it was not possible to absolutely prove that the C-terminal cysteine was where the IR800-maleimide dye reacted, the choice of the C-terminus position probably ensures that the cysteine is exposed in 3E8.scFv.Cys, based upon the structures of similarly engineered scFv.Cys proteins and Cys-linked diabodies [46, 47]. The conditions arrived at allowed a mostly 1:1 formulation to be made, and at 90 % NIRF purity. The SPR data demonstrated that very strong binding ($K_d = 12$ nM) to plated TAG-72 was maintained after the protein modification. The concentration-dependent binding of 3E8.scFv.Cys-IR800 to plated human LS174T cells, blockable by a separate protein, 3E8 CH2 demonstrated strong, target specific binding.

The 3E8.scFv.Cys-IR800 cleared the blood after intravenous administration very rapidly ($T_{1/2} \sim 0.5$ h) compared to the published Cy7-3E8 CH2 antibody fragment (120 kDa; $T_{1/2} = 3-5$ h) and Cy7-CC49 antibody (220 kDa; $T_{1/2} = 61.2$ h) [22, 48]. This is generally consistent with the behavior of radiolabeled antibodies and fragments, where lower molecular weight correlates with more rapid blood clearance [49]. The scFv-sized fragments are desirable when more rapid blood clearance and renal excretion is useful for the clinical indication, and some kidney retention is tolerable. In the case of 3E8.scFv.Cys-IR800, 26 % was excreted renally in the first 3 h. Radionuclide labeling will be required to quantitatively determine the full solid tissue distribution.

Orthotopic LS-174 T tumors have been reported [34, 50], as have biodistributions of LS-174 T targeted immunoconjugates. In a biodistribution of a radiolabeled anti-CEA antibody in this model, i.p. and i.v. administration routes were compared [32]. The i.p. route produced 25 % higher tumor uptake, but otherwise no differences in organ uptakes at 24 h post administration. We used the i.p. route for the tumor imaging, because it allowed us to use a very low (1 nmol) dose of the scarce 3E8.scFv.Cys-IR800 protein. Even with a largely 1:1 dye: protein ratio, fluorescent emissions can only be quantitatively related to agent concentration in transparent biofluids like dilute blood and urine [51]. This is due to high and variable attenuation and scatter of both the incident and emitted photons in solid tissue. Hence, we do not report quantitative NIRF data in solid tissues. Nevertheless, the positive fluorescent images of the LS-174 T orthotopic xenografts, blockable by 3E8 CH2, further confirm the binding specificity of 3E8.scFv.Cy-IR800.

The 3E8.scFv.Cys-IR800 conjugate was generated as an OSN candidate for use in the context of an overall strategy to more effectively select and treat TAG-72 operable

adenocarcinoma patients. An *in vitro* test of the biopsy specimen [41], followed by TAG-72 specific PET/CT would be used to select surgical candidates [49]. Then, simultaneously applied TAG-72 OSN plus RADS would guide potentially curative surgery on this well-selected population, avoiding the common finding of unexpected extensive disease that requires immediate termination of the surgery [9]. The combination of the highly sensitive, small FOV RADS with the less sensitive but rapid and wide field view of OSN is particularly appealing.

Conclusion

3E8.scFv.Cys-IR800 is a new OSN agent targeted to TAG-72. Site-specific labeling by the maleimide dye resulted in a discrete, 1 protein:1 dye conjugate of high purity that maintained specific target binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSM	bovine submaxillary mucin
NIRF	near infrared fluorescence
OSN	optical surgical navigation
RADS	Radioguided antigen-directed surgery

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Fig. 1.

a Structure of IRDye800-maleimide and schematic of 3E8.scFv.Cys. C-terminal cysteine in 3E8.scFv allows the protein to react with maleimide site specifically. **b** Verification of 3E8.scFv.Cys-IR800 identity by MALDI-TOF/TOF mass spectrometry. The predominant peak is at the correct mass for the protein conjugated to a single dye. **c** A greater than 90 % purity of 3E8.scFv.Cys-IR800 was achieved, as determined by size exclusion column HPLC with 800 nm fluorescence detection. Emission of the 3E8.scFv.Cys-IR800 is 39 % less intense per mole than that of the unreacted IR800-maleimide impurity.

Fig. 2.

In vitro binding of 3E8.scFv.Cys-IR800 to live LS-174 cells grown on plates. The magnitude of the bound 3E8.scFv.Cys-IR800 increased proportional to the incubation concentration. Prior incubation of cells with a fivefold excess concentration of 3E8 CH2 blocked 83 % of the 3E8.scFv.Cys-IR800 binding.

Fig. 3.

Blood clearance of 3E8.scFv.Cys-IR800 in non-tumor bearing mice showed an initial clearance half-life of ~30 min, 26 % cumulative urine accumulation after 3 h, and complete clearance from blood by 24 h post i.v. administration.

Fig. 4.

a Optical images detecting >800 nm emission (*left*) and photographs (*right*) of two of the mice bearing orthotopic LS-174 T tumors. Mice were euthanized 24 h after i.p. administrations and the abdomen opened for imaging. Prominent tumors are indicated with *arrows* when visible. The mouse labeled 3E8.scFv.Cys-IR800 was administered 1 nmol of 3E8.scFv.Cys-IR800. The control mouse was sham injected. **b** H&E stained histology confirmed tumor presence in optically enhanced locations. Figure S5 shows additional mice.

Fig. 5. Optical images detecting >800 nm emission of three mice bearing orthotopic LS-174 T tumors. Mice were euthanized 24 h after i.p. administrations and the abdomen opened for imaging. A prominent tumor is indicated with an *arrow*. The control mouse was sham injected, the mouse labeled 3E8.scFv.Cys-IR800 received 1 nmol of that agent, and the mouse labeled block received 10 nmol of 3E8 CH2 3 h prior to receiving 1 nmol of 3E8.scFv.Cys-IR800. Figure S6 shows additional mice.