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Molecular Targeting of CEACAM6 Using Antibody Probes of Different Sizes

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Abstract

Carcinocinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is overexpressed in a number of human malignancies, especially in pancreatic cancer. It has been demonstrated that CEACAM6 is a potential target for monoclonal antibody (mAb) therapy with a safe therapeutic index. Here, we labeled three anti-CEACAM6 antibodies of different sizes, including a single-domain antibody 2A3 (16 kDa), a heavy chain antibody 2A3-mFc (80 kDa) and a full length antibody 9A6 (150 kDa), with ⁶⁴Cu to image CEACAM6 expression in a xenografted pancreatic tumor model. For positron emission tomography (PET) imaging, the tumor mice were intravenously injected with ⁶⁴Cu-DOTA-antibodies and static scans were obtained at 5 min, 0.5, 1, 2, 4, 8 and 24 h post-injection (p.i.). All three antibodies showed strong CEACAM6 binding. *Ex vivo* immunostaining on tumor sections at 24 h after Ab injection demonstrated specific tumor targeting of both 2A3-mFc and 9A6. ⁶⁴Cu-DOTA-2A3 showed fast BxPC3 tumor uptake and rapid whole-body clearance. At 24 h p.i., the tumor uptakes were 98.2 ± 6.12 %ID/g for ⁶⁴Cu-DOTA-2A3-mFc and 57.8 ± 3.73 %ID/g for ⁶⁴Cu-DOTA-9A6, respectively. Compared with the full length antibody 9A6, the heavy chain antibody 2A3-mFc showed higher tumor uptake, lower liver uptake and shorter circulation half-life. All the data supported that the heavy chain antibody 2A3-mFc is superior to the single domain antibody and the full-length antibody with regard to tumor detection and pharmacokinetics, which has great potential to be developed for CEACAM6-targeted pancreatic cancer imaging and therapy.

Keywords

CEACAM6; pancreatic cancer; heavy chain antibody; ⁶⁴Cu; positron emission tomography (PET)

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Introduction

Due to the lack of means for early diagnosis and effective treatment, pancreatic cancer causes high mortality every year and the survival of patients with pancreatic cancer has shown the least improvement over the past 30 years [1]. Once metastasized, most patients have a median overall survival of only 6 months. Pancreatic ductal adenocarcinoma (PDA) is the most common form of pancreatic cancer and several gene expression profiling studies on PDA cell lines [2, 3] and human PDA biopsy samples [4] have shown that CEACAM6 is highly overexpressed over normal pancreatic ductal epithelial cells.

CEACAM6, also known as CD66c or NCA-90, along with other 6 members of the CEACAM subgroup, belong to the human carcinoembryonic antigen (CEA) family [5]. CEACAM6 is a cell surface glycoprotein capable of both homophilic and heterophilic adhesion to other CEACAM family members [6, 7]. There is a low level of CEACAM6 expressed on granulocytes and epithelia from various organs. Proliferating cells of hyperplastic colonic polyps and adenomas also express CEACAM6 but the level is 1 to 2 orders of magnitude lower than that in malignant tumors [8]. Besides pancreatic cancer, CEACAM6 overexpression was also found in a wide variety of epithelial cancer types such as lung, breast, colorectal, and hepatocellular carcinomas [9–11].

Overexpression of CEACAM6 was found in > 90% of invasive pancreatic adenocarcinomas as well as in intraepithelial neoplastic lesions [12]. A small interfering RNA targeting CEACAM6 reversed anoikis resistance and inhibited the *in vivo* metastatic potential in a mouse xenograft model of PDA by enhancing caspase-3-mediated apoptosis [13]. It has also been found that stable transfection of CEACAM6 in Capan2 cells increased gemcitabine chemoresistance, whereas in BxPC-3 human pancreatic cancer cells, gene silencing of CEACAM6 markedly increased the sensitivity to gemcitabine-mediated cytotoxicity [14]. Thus, it appears that CEACAM6 is a valid biomarker for cancer diagnosis and therapy, especially for pancreatic cancer.

So far, antibodies have proven to be an excellent paradigm for the development of high-affinity protein-based binding reagents [15–17]. Indeed, a humanized anti-CEACAM6 single-chain variable fragment (scFv) has been designed based on a monoclonal antibody. The PEGylated scFv bound CEACAM6 with high affinity, exhibited cytotoxic activity, and induced dose-dependent poly(ADP-ribose) polymerase cleavage [18]. With CEACAM6 as the therapy target, an antibody-drug conjugate (ADC) showed efficacy against established CEACAM6-expressing tumors [12]. The biodistribution of CEACAM6 antibody was also monitored by PET scans after being labeled with ^{64}Cu , a positron emitting radioisotope with a half-life of 12.7 h [12].

The large molecular size of intact antibodies (about 150 kDa) leads to prolonged serum half-life [19]. Moreover, intratumoral diffusion of full-length IgG is limited by its size to a penetration rate of about 1 mm every 2 days, potentially resulting in heterogeneous deposition inside tumor [20, 21]. Antibody engineering has made it possible to tailor antigen-binding domains into a single polypeptide with a much smaller size than intact immunoglobulin [15]. Apart from laboratory-based efforts to reduce the size of antibodies, naturally occurring heavy-chain antibodies (HCABs) that lack the first constant domain of the heavy-chain ($\text{C}_{\text{H}}1$) and the complete light-chain [22] provide an attractive alternative as it contains a complete Fc yet is only approximately half the size of IgG. The antigen-binding fragments of such HCABs are confined in a single domain, referred to as single domain antibody (sdAb), V_{HH} or nanobody [23].

2A3, an anti-CEACAM6 sdAb with high binding affinity to CEACAM6, was previously isolated [24]. In this study, we further constructed a mammalian expressing vector of HCAB

by fusing 2A3 with a murine Fc fragment. A dimerized HcAb 2A3-mFc with a molecular size around 80 kDa was obtained after protein expression and purification. We also chose 9A6, a murine monoclonal antibody with almost identical affinity to CEACAM6 for comparison. In a BxPC3 human pancreatic cancer model known to highly express CEACAM6, the pharmacokinetics and tumor delivery of the three antibodies with different sizes were evaluated and compared by quantitative PET imaging.

Materials and Methods

Chemicals and reagents

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (*N*-hydroxysuccinimide ester) (DOTA-NHS) was purchased from Macrocyclics (Dallas, TX). Fluorescein 5(6)-isothiocyanate (FITC) and Chelex 100 resin (50–100 mesh) were purchased from Sigma-Aldrich (St. Louis, MO). Water and all buffers were passed through Chelex 100 columns (1×15 cm) before use in radiolabeling procedures to ensure that the aqueous buffers were free of heavy metals. PD-10 desalting columns were purchased from GE Healthcare (Piscataway, NJ). ^{64}Cu was produced at the cyclotron facility of the National Institutes of Health (NIH). Radio-TLC was performed on an AR-2000 Bioscan scanner (Washington DC), using silica gel plates (LK6DF, 60 Å, 200 mm, Whatman) and 1% ethylenediaminetetraacetic acid (EDTA), 5% NH_4OAc in water : methanol (1:1) as a developing solvent.

Preparation of Antibodies

Monoclonal antibody 9A6 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The 2A3 sdAb was isolated from an immune llama library, expressed in TG1 cells, and purified on immobilized metal affinity chromatography (IMAC) as described [24]. The heavy chain antibody (sdAb $\text{V}_{\text{H}}\text{H}$ fragment, fused to mouse Fc2b fragment) was synthesized by Genscript (Piscataway, NJ), and was cloned into a pTT5 vector (Figure 1A). Recombinant proteins were expressed and purified as described [25]. Briefly, plasmid DNA was purified using Qiagen maxiprep kit, and was used to transfect 293-6E cells using PEI transfection reagent (Polysciences, Warrington, PA). The transfected cells were allowed to grow for 5 days in F17 medium (Invitrogen, Carlsbad, CA). The medium was harvested by centrifugation, and then filtered using a 0.22 micron membrane to remove cell debris. Recombinant protein in the medium was purified using protein G column (GE healthcare, Piscataway, NJ) as recommended by the manufacturer.

Surface plasmon resonance (SPR)

The binding affinities of 9A6 and 2A3-mFc antibodies to CEACAM6 were determined through Surface Plasmon Resonance (SPR) measurement by using a BIAcore 3000 (GE Healthcare). A total of 2179 resonance units (RUs) of 2A3-mFc, 4438 RUs of 9A6, and 3745 RUs of Ovalbumin (reference protein), were immobilized onto a CM5 research grade sensor chip (GE HealthCare). The affinity measurements were carried out in HBS-EP running buffer comprising 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 (GE Healthcare). One hundred twenty microliters of CEACAM6 recombinant protein at concentrations in the range of 0.1 nM to 200 nM were injected over the surfaces at a flow rate of 40 $\mu\text{l min}^{-1}$. Analyses were carried out at room temperature in HBS-EP. Regeneration was performed with HBS-EP as a running buffer. The data were analyzed by using BIA evaluation software 4.1.

Cell lines and tumor models

The human head and neck squamous carcinoma cell line UM-22B was obtained from the University of Michigan and used as a CEACAM6 negative control. The BxPC3 human pancreatic cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA). Human PBMCs (peripheral blood mononuclear cells) were purchased from Genlantis (ImmunoPure™, Genlantis, CA). Two hours before immunostaining study, the PBMCs were thawed according to the manufacturer's protocol.

All animal studies were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Clinical Center, NIH. Subcutaneous BxPC3 tumor model was established in 5 to 6-week-old female athymic nude mice obtained from Harlan (Indianapolis, IN). Typically, 5×10^6 cells were suspended in 50 μ l of phosphate buffered saline (PBS) and mixed well with 50 μ l of Matrigel (BD Biosciences). Then the cells were injected subcutaneously on the right shoulders. The mice underwent small animal PET studies when the tumor volume reached 100–200 mm³ (3–4 weeks after inoculation).

Antibody labeling

Detailed procedures for FITC and DOTA conjugation of antibodies have been reported earlier with minor modification [26, 27]. The reaction ratios of DOTA to 9A6, 2A3-mFc and 2A3 were 20:1, 10:1 and 2:1, respectively. ⁶⁴CuCl₂ (74 MBq) was diluted in 300 μ l of 0.1 M sodium acetate buffer (pH 6.5) and added to 50 μ g of DOTA-antibodies. The reaction mixture was incubated for 1 h at 40 °C with constant shaking. ⁶⁴Cu-DOTA-antibodies were then purified by PD-10 column using PBS as the mobile phase. Complexation of ⁶⁴Cu with the conjugated antibodies was monitored by radio-TLC (R_f ⁶⁴Cu-DOTA-9A6 = 0.032, R_f ⁶⁴Cu-DOTA-2A3-mFc = 0.076, R_f ⁶⁴Cu-DOTA-2A3 = 0.090, R_f ⁶⁴Cu = 0.779). Radio-TLC showed incorporation of greater than 95% for 9A6 and 2A3-mFc and 50% for 2A3.

Flow cytometry

Cells were harvested and washed with PBS containing 0.5% bovine serum albumin (BSA). After being blocked by 2% BSA in PBS, the cells were incubated with FITC-2A3-mFc (10 μ g/mL in PBS containing 2% BSA) for 1 h at room temperature. After washing 5 times with PBS, the cells were analyzed using a C6 flow cytometer (BD Accuri Cytometers Inc., MI).

Cell uptake and efflux studies

Cell uptake, internalization, and efflux of ⁶⁴Cu-DOTA-2A3-mFc were performed with BxPC3 and UM-22B tumor cells. For cell uptake, the cells were seeded into 24-well plates at a density of 1×10^5 cells per well and incubated with 18.5 kBq (0.5 μ Ci, around 15 ng)/well of ⁶⁴Cu-labeled tracer at 37°C for 15, 30, 60, and 120 min. The cells were then washed three times with chilled PBS and lysed with 500 μ l of 0.1 M NaOH. For efflux studies, about 18.5 kBq (0.5 μ Ci)/well of ⁶⁴Cu-DOTA-2A3-mFc were first incubated with BxPC3 cells in 24-well plates for 2 h at 37°C. The cells were washed three times with chilled PBS and allowed to stand with fresh buffer. At various time points, the medium was removed and the cells washed three times with chilled PBS. The cells were then lysed with 500 μ l of 0.1 M NaOH. The cell lysate was collected and the remaining radioactivity was measured in a γ counter (Packard, Meriden, CT). The cell uptake and efflux were expressed as the percentage of the added dose (%AD) after decay correction. All experiments were performed with triplicate wells.

Small animal PET and image analysis

PET scans and image analysis were performed using an Inveon microPET scanner (Siemens Medical Solutions). About 3.7 MBq (100 μ Ci/ 3–6 μ g) of 64 Cu-DOTA-antibodies were administered via tail vein injection under isoflurane anesthesia. Five-minute static PET images were acquired at different time points postinjection (p.i.; $n = 4$ /group). The images were reconstructed using a two-dimensional ordered-subset expectation maximization (2D OSEM) algorithm, with no correction for attenuation or scattering. For each scan, regions of interest (ROIs) were drawn over the tumor and major organs using vendor software (ASI Pro 5.2.4.0) on decay-corrected whole-body coronal images. The radioactivity concentrations (accumulation) within the tumors, muscle, liver, and kidneys were obtained from mean pixel values within the multiple ROI volume and then converted to MBq per milliliter per minute using the calibration factor determined for the Inveon PET system. These values were then divided by the administered activity to obtain (assuming a tissue density of 1 g/ml) an image-ROI-derived percent injected dose per gram (%ID/g).

Immunofluorescence staining

Frozen tumor slices (5- μ m thickness) were fixed with cold acetone for 10 min and dried in the air for 30 min. The slices were rinsed with PBS for 2 min and blocked with 10% donkey serum for 30 min at room temperature. The slices were then incubated with 9A6 and rat anti-mouse CD31 antibody for 1 h at room temperature and visualized using Dylight 488-conjugated donkey anti-rat secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc.) and Cy3-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.). The slides were observed with an epifluorescence microscope (Olympus, X81).

For ex vivo staining, tumor samples were collected after completing the last time point PET scan, i.e., at 24 h after i.v. injection of 3.7 MBq (100 μ Ci/ around 3 μ g) of either 64 Cu-DOTA-2A3-mFc or 64 Cu-DOTA-9A6. The tumor sections were fixed and stained with Cy3-conjugated donkey anti-mouse IgG (1: 200) without any more primary antibody. The sections were also co-stained with CD31 using a procedure similar to that described above.

Around 10 μ g of FITC conjugated antibodies (2A3, 2A3-mFc, 9A6 and murine IgG) were injected to BxPC3 tumor bearing mice through tail vein. Twenty-four hours after (two hours for FICT-2A3), the tumors and normal organs were collected and sectioned. After mounted with the mounting medium containing DAPI (Vector Laboratories Inc. CA), the sections were observed with an epifluorescence microscope (Olympus, X81).

Ex vivo biodistribution

Immediately after PET imaging, the tumor-bearing mice were sacrificed and dissected. Blood, tumor, major organs, and tissues were collected and wet-weighed. The radioactivity in the wet whole tissue was measured with a γ -counter (Packard). The results were expressed as percentage of injected dose per gram of tissue (%ID/g) for a group of 4 animals. For each mouse, the radioactivity of the tissue samples was calibrated against a known aliquot of the injected radiotracer and normalized to a body mass of 20 g. Values were expressed as mean $\pm$ SD ($n = 4$ /group).

Statistical analysis

Quantitative data were expressed as mean $\pm$ SD. Means were compared using one-way analysis of variance and Student's *t* test. P value of < 0.05 was considered statistically significant.

Results

Antibody preparation and characterization

The sdAb protein (2A3) was purified using a Ni-NTA column. This one-step purification resulted in protein with over 95% purity as evidenced by SDS-PAGE. The yield was 25 mg of purified protein per liter of bacterial culture [24]. The 2A3 HCAb (2A3-mFc) was purified using protein G column with a total of 20–25 mg/l protein eluted and recovered. The protein was dialyzed against PBS, and SDS-PAGE gels showed >95% purity with a molecular weight of about 85 KD (Figure 1B).

The affinities of the monoclonal antibody, 9A6, and HCAb antibody, 2A3-mFc, to CEACAM6 N-domain were determined using SPR. The antibodies were immobilized on CM5 chips, and CEACAM6 was passed over at concentration ranging from 0.1 to 200 nM. Affinity constants (K_D) for 9A6 and 2A3-mFc were 8 nM and 13 nM, respectively (Figure 1D).

Specific binding of three antibodies to BxPC3 cells in vitro

After FITC conjugation, we performed immunostaining on both CEACAM6 negative UM-22B cells and CEACAM6 positive BxPC3 cells. BxPC3 cells showed strong fluorescence signal with all three antibodies. Most of the fluorescent signal localized on cell membrane, due to the cell surface distribution of CEACAM6 antigen. We also conjugated murine IgG with FITC as control and found that the fluorescence signal is much weaker than FITC labeled specific antibodies. Incubation with excess amount of unlabeled specific antibodies (2A3 and 2A3-mFc) blocked the binding of the corresponding FITC conjugated antibodies with BxPC3 cells, further substantiating the binding specificity of these antibodies (Figure 2A). Flow cytometry of FITC-2A3-mFc stained UM-22B and BxPC3 cells showed consistent results with that from fluorescent microscopy. The antibody showed no binding to human PBMCs (Figure 2B).

After DOTA conjugation and ^{64}Cu labeling, we performed cell uptake and efflux studies with ^{64}Cu -DOTA-2A3-mFc. BxPC3 cells showed increased accumulation of radioactivity with time. After 2 h incubation, the total uptake was 10.13 ± 0.05 % of added dose. At the same time, the cells almost showed no uptake of ^{64}Cu -DOTA-antibody when a control IgG was employed. UM-22B cells had much lower uptake of ^{64}Cu -DOTA-2A3-mFc than BxPC3 cells (Figure 2C). These results confirmed the specific binding of ^{64}Cu -DOTA-2A3-mFc to BxPC3 cells via CEACAM6 recognition. When the labeled cells were incubated in serum-free medium devoid of radioactivity, ^{64}Cu -DOTA-2A3-mFc showed very slow dissociation and efflux from the cells (Figure 2D). After 2 h incubation, more than 65% of the radioactivity was still retained in the cells.

Non-invasive PET imaging of CEACAM6

In order to evaluate the *in vivo* pharmacokinetics of the three antibodies with different sizes, we scanned BxPC3 tumor-bearing mice from 5 min to 24 h after antibody injections. Due to its smaller size, ^{64}Cu -DOTA-2A3 showed tumor accumulation at as early as 30 min p.i. Liver and kidneys had very high radioactivity, indicating both hepatic and renal-urinary clearance of this sdAb. On the contrary, ^{64}Cu -DOTA-2A3-mFc showed slower tumor accumulation. At 4 h p.i., the tumor was clearly visible and the accumulation kept increasing over time. As for the full length antibody 9A6, the tumor uptake was much more delayed than 2A3-mFc. Liver and heart were the major organs with high radioactivity accumulation besides tumor for 9A6 and 2A3-mFc. The kidneys were not visible with these two antibodies (Figure 3). We also labeled murine IgG as a control antibody, the BxPC3 tumor showed much lower uptake, representing non-specific perfusion of IgG to the tumor region.

The accumulation of ^{64}Cu -DOTA-antibodies on the tumor and major organs was quantified based on PET images (Figure 4). For 2A3, the tumor showed highest uptake at 30 min p.i. (4.22 ± 1.13 %ID/g), which decreased gradually to 3.85 ± 0.37 %ID/g at 2 h time point. The kidneys showed extremely high accumulation, reaching 102.7 ± 3.15 %ID/g at 60 min. Liver showed similar level of radioactivity as tumors. The tumor uptakes of ^{64}Cu -DOTA-2A3-mFc kept increasing with time, which were 11.8 ± 2.66 , 22.7 ± 5.90 , 43.1 ± 6.78 and 98.2 ± 6.12 %ID/g at 2, 4, 8 and 24 hr p.i., respectively. The liver uptake dropped from 23.4 ± 2.68 %ID/g at 30 min to 10.6 ± 1.14 %ID/g at 24 h p.i. ^{64}Cu -DOTA-9A6 showed similar pattern with the HcAb. At 24 h, the tumor uptake was 57.8 ± 3.73 %ID/g and liver uptake was 11.6 ± 1.53 %ID/g. The tumor uptake of ^{64}Cu -DOTA-IgG was 8.33 ± 1.66 %ID/g at 24 h, which was significant lower than that of both ^{64}Cu -DOTA-9A6 and ^{64}Cu -DOTA-2A3-mFc.

Tumor/non-tumor ratios of the three tracers were listed in Table 1. It is of no surprise to see that 2A3 showed good tumor/blood ratio at very early time points and kept increasing with time, from 3.90 ± 1.47 at 30 min to 8.51 ± 1.12 at 2 h p.i. The tumor/muscle ratio was even higher. However, the tumor showed no significant contrast over the liver. As for 2A3-mFc, the tumor/blood ratio was rather low at early time points after tracer injection. At 24 h after injection, the tumor showed excellent contrast to background with tumor/blood ratio of 9.25 ± 1.64 , tumor/liver ratio of 9.29 ± 0.43 and tumor/muscle ratio of 36.1 ± 13.9 . The clearance of 9A6 was slower than that of 2A3-mFc, with a tumor/blood ratio of 3.61 ± 0.28 , tumor/liver ratio of 5.06 ± 1.04 and tumor/muscle ratio of 29.9 ± 2.26 at 24 hr p.i.

Biodistribution assay

In order to further confirm the PET imaging quantification, the biodistribution of ^{64}Cu -DOTA-antibodies was evaluated in tumor-bearing athymic nude mice immediately after PET imaging. As shown in Figure 5, the BxPC3 tumor uptake of 2A3 was 5.65 ± 0.58 %ID/g at 2 h p.i.. Consistent with PET imaging, 2A3-mFc showed highest tumor uptake at 24 hr p.i., which was 95.4 ± 29.3 %ID/g. The tumor uptakes of 9A6 and IgG were 66.8 ± 13.7 %ID/g and 11.2 ± 0.39 %ID/g respectively at 24 h time point.

Ex vivo staining

Immediately after PET imaging, we also collected the tumor tissue for immunostaining. Since we already injected DOTA conjugated 2A3mFc or 9A6, no primary antibody was needed and the fluorescent signal reflected the microscopic distribution of antibodies inside the tumors. As shown in Figure 6, for 9A6, the fluorescent signal was primarily detected within several cell diameters of the blood vessels. While for 2A3-mFc, fluorescent signal was also limited to peri-vascular region with longer diffusive distance. Co-staining with CD31 further demonstrated limited peri-vascular localization of both 9A6 and 2A3-mFc in BxPC3 tumors. These results indicate that 2A3-mFc has a better penetration through perivascular tissues than the full length antibody 9A6. In addition, it also demonstrated that both 9A6 and 2A3-mFc retain their immunoreactivity after DOTA conjugation.

To further confirm the tumor specific targeting of the antibodies, we also performed FITC conjugation and collected tumors and normal organs at 24 hrs after tail vein injection (2 hrs for FITC-2A3). Strong cell-bound fluorescent signal was observed within BxPC3 tumors with FITC conjugated 2A3, 2A3-mFc and 9A6. Positive signal was also identified in the spleen but with a total different pattern, indicating the non-specific accumulation. No cell-bound signal was seen with FITC-IgG within the tumor.

Discussion

Emerging evidences support the important role of CEACAM6 in tumor diagnosis and treatment, especially in pancreatic cancer [11, 12, 18, 28]. We used murine Fc to construct 2A3-Fc fusion because we wanted to eliminate influence possibly caused by differential binding of human Fc to murine Fc receptors when a human Fc is employed. The three antibodies, the sdAb 2A3, the HCAb 2A3-mFc and the monoclonal antibody 9A6 were therefore evaluated in a mouse model of human pancreatic cancer. All three antibodies showed positive tumor accumulation. Moreover, the tumor/non-tumor ratios are high enough to present a very clear tumor contrast. All the imaging data supported the feasibility of pancreatic cancer imaging by targeting CEACAM6. Especially, both 2A3-mFc and 9A6 showed high tumor/liver ratio, which facilitates primary pancreatic cancer detection.

Owing to a small molecular weight of 16 kDa, SdAb 2A3 exhibited fast clearance kinetics. The tumors were clearly visible as early as 30 min after tracer injection with decent uptake of 4.22 ± 1.13 %ID/g. However, the tumors showed no apparent contrast to liver, which may inhibit tumor detection near this organ. One advantage of small-sized antibodies is to perform imaging repetitively after labeling with short-lived radioisotopes such as ^{68}Ga ($t_{1/2} = 68$ min) or ^{18}F ($t_{1/2} = 109.8$ min) [29], allowing sequential non-invasive imaging of the pharmacodynamics of the targeted drug. Like other small molecular scFv [30] or affibodies [31], CEACAM6 sdAb had predominant renal-urinary excretion with extremely high activity accumulation in the kidneys. This is why we chose 2 hr as the end-point for sdAb imaging and biodistribution studies.

With weaker binding affinity and fast clearance, the absolute dose deposition is much lower for the sdAb, compared with the full length antibody. In BxPC3 model, 9A6 showed gradual increase of tumor uptake with time. At 24 h after injection, the tumor uptake reached 57.8 ± 3.73 %ID/g, which was higher than that from anti-EGFR or HER2 antibodies in EGFR or HER2 positive tumor models [32, 33]. However, the tumor to blood ratio was only 3.61, probably resulting from its prolonged blood circulation time.

To reach a compromise between sdAb and the intact IgG, we developed an HCAb, 2A3-mFc. With the bivalent binding, it exhibits better tumor deposition than the single domain counterparts, yet clears faster from circulation than intact antibodies because of its relatively smaller molecular size. At 24 h after injection, the BxPC3 tumors showed an extremely high accumulation of 2A3-mFc (98.2 ± 6.12 %ID/g), which was significantly higher than that of 9A6 ($p < 0.01$). Besides, the tumor/non-tumor ratios were much higher than those of 9A6 too, especially tumor/blood ratio (9.25 ± 1.64 vs. 3.61 ± 0.28 , $p < 0.01$). At as early as 8 hr after tracer injection, the tumor already showed apparent contrast to the liver. However, it is worth noting that the high tumor uptake was also partially related to the tumor size, which was relatively small at the time of imaging with an average of 184 mm^3 . Roughly, 18% of total injected dose distributed to the tumor region. With a molecular size around 80 kDa, 2A3-mFc still showed much less renal-urinary excretion than sdAb 2A3.

In order to confirm the specificity of antibody distribution in the tumor region, we also performed PET imaging with a control antibody, murine IgG. The tumors showed radioactivity uptake of 8.33 ± 1.06 %ID/g at 24 h after injection, which is attributable to non-specific perfusion of the antibody [34]. Based on the dramatic difference of tumor uptake between IgG and 9A6, it is reasonable to conclude that the high tumor uptake of 9A6 mainly resulted from specific targeting of the antibody to the CEACAM6 antigen. When we further investigated the microscopic localization of 9A6 and 2A3-mFc within BxPC3 tumors, we found that 2A3-mFc showed more diffusive distribution than 9A6. Moreover, the cell membrane binding of both antibodies further confirmed specific binding of the

antibodies (Figure 6). However, 2A3-mFc still did not achieve homogenous distribution within the tumor. Consequently, we would be cautious to relate PET imaging quantification to CEACAM6 expression level in our future studies.

Although we successfully visualized CEACAM6 positive tumors using specific antibodies as imaging probes, it is of note that the murine model is not ideal for CEACAM6 study since there is no corresponding CEACAM6 orthologues in rodents [35]. Thus, the tumor accumulation and background level of imaging might be different with the presence of endogenous CEACAM6 in case of humans and primates. For example, besides malignant tissues, CEACAM6 is also expressed on granulocytes, especially on activated granulocytes during infection and inflammation [8], even though we observed almost no binding of 2A3-mFc with human PBMCs. By taking advantage of this mechanism, ^{99m}Tc -sulesomab (LeukoScan; Immunomedics Europe, Hillegom, The Netherlands), a radiolabeled murine monoclonal antibody fragment against CEACAM6 [36], has been used for imaging inflammation in Europe since 1997. However, a comparison study between human serum albumin (HSA) and sulesomab also found that sulesomab does not localize in inflammation as a result of binding to circulating granulocytes but of nonspecific clearance into inflammation via increased vascular permeability [37]. It is predictable that CEACAM6 immuno-PET will recognize both malignant lesions and inflammations after clinical translation. Nevertheless, distinguishably high tumor uptake might justify the off-label use of LeukoScan for tumor detection.

For copper labeling, DOTA is not ideal chelator since trans-chelation may present a problem, especially for high liver uptake at late time points [32, 38]. For this proof-of-principle study, we did not expect any severe problem resulting from DOTA conjugation. If necessary, other chelators suitable for more stable ^{64}Cu -labeling under mild conditions [39, 40] may be investigated in the future. Another minor issue is that the clinical utility of murine monoclonal antibodies is limited by their immunogenicity and often inefficient secondary immune functions. We chose murine Fc to better compare HCAb with the monoclonal antibody. It has been found that mouse antibodies are immunogenic and induce mouse-antibody-specific antibody response in patients receiving multiple doses. The replacement of mouse-antibody Fc with human Fc substantially reduces the immunogenicity of the antibody and that humanization might further reduce the immunogenicity [41]. Consequently, in clinical use, chimeric or humanized antibodies should be constructed to guarantee the application in repetitive imaging [42].

Conclusion

In summary, we described here the use of quantitative PET imaging with ^{64}Cu -DOTA-anti-CEACAM6 antibodies with different sizes in a human pancreatic xenograft model. The heavy chain antibody 2A3-mFc is superior to the single domain antibody and the full length antibody with regard to tumor delivery and pharmacokinetics. It has great potential to be applied for CEACAM6-targeted pancreatic cancer imaging and therapy.

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Figure 1. Purification and characterization of anti-CEACAM6 heavy chain antibody 2A3-mFc. (A) Construct of expressing cassette of 2A3-mFc; (B) SDS-PAGE of purified 2A3-mFc under both reducing (left) and non-reducing (right) conditions; (C) Structure and size of single domain Ab 2A3 (left), heavy chain Ab 2A3-mFc (middle) and full length IgG 9A6 (right). (D) SPR of 2A3-mFc and 9A6.

Figure 2.

(A) *In vitro* immunostaining of CEACAM6 negative UM-22B and CEACAM6 positive BxPC3 cells. (B) Flow cytometry after immunostaining UM-22B, BxPC3 and hPBMC cells with FITC-2A3-mFc. (C & D) Cell uptake (C) and efflux (D) assay of 64 Cu-DOTA-2A3-mFc and 64 Cu-DOTA-IgG on BxPC3 or UM-22B cells. The cell uptake and efflux was expressed as percentage of decay-corrected total input radioactivity. Data were from 2 experiments with triplicate samples and were expressed as mean $\pm$ SD.

Figure 3. microPET whole body images (coronal plane) of BxPC3 tumor-bearing mice at different time points after tail vein injection of 3.7 MBq of ^{64}Cu -DOTA-2A3, ^{64}Cu -DOTA-2A3-mFc, ^{64}Cu -DOTA-9A6, or ^{64}Cu -DOTA-IgG. Tumors were indicated by white arrows at the last time point. The displayed plane was selected to best show the tumor cross section.

Figure 4. Uptake values at different time points after tracer injection in the kidneys, liver, muscle, and BxPC3 tumor quantified from region of interest (ROI) analysis on microPET scans (n = 4/group).

Figure 5. Biodistribution of ^{64}Cu -labeled antibodies in BxPC3 tumor bearing athymic nude mice after microPET imaging at 2 h time point (2A3; n = 4) or 24 h time point (2A3-mFc, 9A6 and IgG; n = 4/group).

Figure 6.

Microscopic observation of antibody distribution. (A) Twenty-four hours after DOTA-antibody injection, tumors were harvested and tumor sections were stained with Cy3-conjugated goat anti-mouse IgG. The sections were also co-stained with CD31 for tumor vasculature visualization (Green color from Dylight 488 for CD31; red color from Cy3 for CEACAM6; blue color from DAPI for nuclei visualization). (B) Twenty-four hours after FITC-antibody injection (two hours for FITC-2A3), tumors (upper panel) and spleen (lower panel) were harvested and the sections were observed with an epifluorescent microscope.

Table 1Tumor/non-tumor ratios of ^{64}Cu -DOTA-Abs in BxPC3 tumor bearing mice (n = 4/group)

	30 min	1 hr	2 hr	4 hr	8 hr	24 hr
2A3	T/B	3.90 ± 1.47	6.94 ± 2.01	8.51 ± 1.12		
	T/L	1.07 ± 0.20	1.25 ± 0.25	1.15 ± 0.16		
	T/K	0.05 ± 0.02	0.04 ± 0.00	0.04 ± 0.01		
	T/M	5.41 ± 1.15	9.13 ± 2.21	14.8 ± 6.46		
2A3-mFc	T/B	0.12 ± 0.04	0.23 ± 0.05	0.49 ± 0.07	1.07 ± 0.20	2.48 ± 0.15
	T/L	0.15 ± 0.03	0.24 ± 0.03	0.57 ± 0.10	1.20 ± 0.22	2.53 ± 0.22
	T/M	1.90 ± 0.51	2.65 ± 0.20	4.88 ± 0.86	13.9 ± 0.62	36.1 ± 13.9
9A6	T/B	0.08 ± 0.02	0.09 ± 0.01	0.16 ± 0.04	0.39 ± 0.04	0.79 ± 0.08
	T/L	0.20 ± 0.08	0.19 ± 0.01	0.32 ± 0.07	0.73 ± 0.05	1.33 ± 0.05
	T/M	1.54 ± 0.63	1.28 ± 0.04	1.94 ± 0.56	5.03 ± 0.84	10.9 ± 3.10

The results were presented as mean ± SD (n=4). T, tumor; L, liver; K, kidneys; M, muscle.