Anti-GD2 antibody for radiopharmaceutical imaging of osteosarcoma

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Abstract

Purpose: Osteosarcoma (OS) is the most frequently diagnosed bone cancer in children with little improvement in overall survival in the past decades. The high surface expression of disialoganglioside GD2 on OS tumors and restricted expression in normal tissues makes it an ideal target for anti-OS radiopharmaceuticals. Since human and canine OS share many biological and molecular features, spontaneously occurring OS in canines has been an ideal model for testing new imaging and treatment modalities for human translation. In this study, we evaluated a humanized anti-GD2 antibody, hu3F8, as a potential delivery vector for targeted radiopharmaceutical imaging of human and canine OS.

Methods: The cross reactivity of hu3F8 with human and canine OS cells and tumors was examined by immunohistochemistry and flow cytometry. The hu3F8 was radiolabeled with Indium-111 and the biodistribution of [^{111}In]In-hu3F8 was assessed in tumor xenograft-bearing mice. The targeting ability of [^{111}In]In-hu3F8 to metastatic OS was tested in spontaneous OS canines.

Results: The hu3F8 cross reacts with human and canine OS cells and canine OS tumors with high binding affinity. Biodistribution studies revealed selective uptake of [^{111}In]In-hu3F8 in tumor tissue. SPECT/CT imaging of spontaneous OS canines demonstrated avid uptake of [^{111}In]In-hu3F8 in all metastatic lesions. Immunohistochemistry confirmed the extensive binding of radiolabeled hu3F8 within both osseous and soft lesions.

Conclusion: This study demonstrates the feasibility of targeting GD2 on OS cells and spontaneous OS canine tumors using hu3F8-based radiopharmaceutical imaging. Its ability to deliver an imaging payload in a targeted manner suggests that labeling hu3F8 with a therapeutic radionuclide may yield a potent radiopharmaceutical agent against OS.

Introduction

Osteosarcoma (OS) is the most common primary bone malignancy diagnosed in children and young adults [1]. Despite aggressive surgery and combination chemotherapy, long-term disease-free survival of patients with metastatic or recurrent OS has remained poor with the median 5-year survival rate being < 30% [2]. Progress in developing new effective treatments has stagnated over the past three decades [3, 4]. Therefore, new treatment modalities that can improve the outcomes are urgently needed.

The disialoganglioside GD2 is a tumor-associated antigen widely expressed by pediatric solid tumors, including OS [5, 6] and neuroblastomas [7, 8]. In metastatic OS, GD2 expression is maintained [6, 9] or upregulated [10]. The intimate involvement of GD2 in tumor progression and restricted expression in normal tissues make GD2 an ideal target for immunotherapy and imaging [9, 11]. Murine 3F8, a monoclonal antibody that selectively binds to GD2 and activates human complement and antibody-dependent cell-mediated cytotoxicity [5], has been used for patients with high-risk neuroblastoma and achieved long-term remissions without notable toxicities [12]. Humanized 3F8 (hu3F8) was constructed

to circumvent the immunogenicity of its murine counterpart [13]. The recent FDA approval of hu3F8 for treating high-risk neuroblastoma in the bone or bone marrow [14] and its demonstrated 10 times higher binding affinity to GD2 [13] highlight the potential of hu3F8 as a promising targeting vector for treating metastatic OS.

OS is also the most common bone tumor found in dogs, with the majority of dogs succumbing to the disease with or without a treatment [15]. Both canine and human OS are likely to be high-grade histologically, with rapidly metastasizing tumors primarily to the lungs [16]. At the molecular level, canine and human OS present overlapping transcriptional profiles and shared DNA copy number aberrations [15]. The higher prevalence of OS in dogs and the more rapid disease progression, renders spontaneously occurring OS in dogs an ideal model for human OS safety and efficacy studies. In addition, canine OS occurs predominantly in large breed dogs, thus the imaging and pharmacokinetic studies that are essential to implementation of a new therapy in humans can be directly translated.

Radiopharmaceutical therapy (RPT) is emerging as a promising therapeutic modality for a variety of cancers, including metastatic OS, by targeted delivery of high dose of radiation in the form of alpha- or beta-particle emitting radionuclides to tumor-associated targets [17–21]. Prior to disease treatment, especially in the early stages, it is essential to develop targeted radiopharmaceutical imaging probes that could be used to visualize primary and metastatic lesions with high sensitivity.

The purpose of this study is to evaluate the potential of a humanized anti-GD2 antibody, hu3F8, that was developed for neuroblastoma therapy, as a potential delivery vector for targeted radiopharmaceutical imaging of human and canine metastatic OS. We developed the methodology for efficient radiolabeling of hu3F8 with ^{111}In without compromising its immunoreactivity and investigated the biodistribution of [^{111}In]In-labeled hu3F8 in canine OS and human neuroblastoma xenografts. Furthermore, the ability of radiolabeled hu3F8 to target metastatic OS lesions was examined in spontaneously occurring OS dogs to facilitate future translation to human OS patients.

Materials And Methods

Reagents, cell lines, and Canine osteosarcoma tissue samples

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. S-2-(4-Isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid (p-SCN-Bn-DTPA) and ^{111}In ([^{111}In] $InCl_3$) were purchased from Macrocyclics (Plano, TX, USA) and BWXT ITG Canada, respectively. The hu3F8 antibody was provided by Dr. Nai-Kong Cheung in the Memorial Solan Kettering Cancer Center. Canine OS cells, OSCA78 (Kerafast, Boston, MA, USA), human OS cells (U2OS), human neuroblastoma cells (IMR32 and SK-N-SH, ATCC, Manassas, VA, USA) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 µg/ml Primocin.

Canine OS tissue was acquired from the Center for Image-Guided Animal Therapy at the Johns Hopkins Hospital. OS tissue was either freshly frozen and embedded in OCT compound or was fixed with 10% formalin followed by paraffin embedding.

Antibody radiolabeling

The [^{111}In]In-labeled hu3F8 and Rituximab were prepared as previously described [22, 23]. Briefly, the antibody was conjugated to p-SCN-Bn-DTPA (molar ratio: 1:7–9 (hu3F8), 1:1 (Rituximab)) at 37°C for 1 hour, and the conjugate was purified by size-exclusion centrifugation. The resulting antibody conjugate was then added to 40.7-129.5 MBq of [^{111}In]In-$InCl_3$, 0.5 mL of 0.2 M HCl, and 0.06 mL of 3 M NH_4OAc, pH 4. The resulting mixture was incubated at 37°C for 30–60 minutes and then purified by a PD-10 column. The radiochemical purity was determined by radio-thin layer chromatography and the protein concentration was determined by spectrophotometry (Nanodrop, Wilmington, DE, USA).

In vitro **studies**

Immunocytochemical and immunohistochemical Staining: The cross-species reactivity of hu3F8 with human (U2OS) and canine (OSCA78) OS cells and spontaneously occurring canine OS tumor tissue was first evaluated by immunocytochemistry (ICC) and immunohistochemistry (IHC), respectively. Cells grown in 8-well chambers were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. After washing with PBS, cells were blocked for 30 min by serum-free antigen blocking solution (Dako Agilent, Carpinteria, CA, USA), followed by overnight incubation with hu3F8 (1:200) at 4°C. Cells were then washed and incubated with FITC-labeled anti-human Fc secondary antibody (1:1000, ThermoFisher Scientific, Waltham, MA, USA) for 1 hour. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). For IHC of canine OS tissue, six micron-thick sections were deparaffinized. Endogenous peroxidase was quenched by 0.3% H_2O_2. The samples were incubated in an antigen retrieval solution (Dako) and pressure cooked for 3 minutes. The samples were blocked and incubated with hu3F8 (1:200) for overnight. After washing with PBS, OS samples were incubated with a biotinylated anti-human Fc secondary antibody (1:800 dilution, ThermoFisher Scientific), followed by incubation with streptavidin-horseradish peroxidase complex and developed with 3,3'-diaminobenzidine peroxidase substrate kit (Vector Laboratories). The OS sections were then counterstained with hematoxylin. Human IMR32 cells known to express GD2 were used as a positive control, and SK-N-SH cells and dog spleen tissue were used as GD2 negative controls. Images were captured at 200× magnification using an upright microscope (Eclipse Ti, Nikon Instruments Inc., Melville, NY, USA).

Flow cytometry

Human IMR32, U2OS, canine OSCA78 cells, and canine white blood cells (negative control) (1 x 10^6) were collected and fixed with 4% PFA. The cells were then incubated with hu3F8 (5 μg/mL) for 30 min at 4°C. After washing with PBS, cells were incubated with Alexa Fluor 647-labeled goat anti-human IgG (Southern

Biotech, Birmingham, AL, USA) for 30 min, washed with PBS, and analyzed on a FACS flow cytometer (BD Biosciences).

Receptor binding assay

The binding affinity of [^{111}In]In-DTPA-hu3F8 was determined in IMR32 and OSCA78 cells as described previously with modifications [23, 24]. In brief, cells were seeded in 24-well plates at 1.5 x 10^5 cells/well 24 hours prior to the experiment. After 24 hours, the cells were washed twice with PBS and treated with a serial dilution of [^{111}In]In-DTPA-hu3F8 (0.05–100 nmol/L) for 4 hours at 4°C. Non-specific binding was assessed by pretreating cells with 10 µg/well of unlabeled hu3F8 for 30 minutes at 4 ℃ followed by [^{111}In]In-DTPA-hu3F8 incubation. Each assay was conducted in triplicate. After 4 hours of incubation, cells were washed twice with ice cold PBS, dissolved in 0.5% sodium dodecyl sulfate solution and counted on a γ-well counter (PerkinElmer 2470 WIZARD2®). The protein concentration of the cell lysates was determined with a BCA protein assay kit (Pierce). The measured activity was normalized to the number of cells (sites/cell). The Michaelis-Menten equation was fitted to the binding curve to determine the dissociation constant (K_d) and maximum number of binding sites (B_{max}) using Prism 9 (GraphPad, La Jolla, CA, USA).

Immunoreactivity

The immunoreactivity of [^{111}In]In-labeled antibodies was determined as previously described [24] by antibody adherence to IMR32 and OSCA78 cells. Two sets of tubes (1 x 10^7 cells/tube) were prepared for each cell line and 0.1µg of [^{111}In]In-DTPA-hu3F8 or [^{111}In]In-DTPA-Rituximab was used for immunoreactivity assessment. Immunoreactivity (%) = (Bound activity in cells / Total activity) x 100.

In vivo mouse studies

All animal studies were approved by the Animal Care and Use Committee of the Johns Hopkins University, School of Medicine. Six- to 8-week-old healthy female Nu/Nu mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice received subcutaneous injections of either 1 × 10^7 IMR32 or 3 x 10^6 of OSCA78 in Matrigel in the right flank for biodistribution studies or both flanks for SPECT imaging.

Biodistribution

Biodistribution studies were carried out as previously described [23]. Following a growth period of approximately 8 weeks, IMR32 and OSCA78 tumor-bearing mice (n = 3–4) were injected intravenously with 100 µL of [^{111}In]In-DTPA-hu3F8 (3.03 MBq/10 µg). Mice were euthanized with isoflurane at either 24 or 72 hours after injection. The major organs, including blood, heart, lungs, liver, kidneys, spleen, stomach (with content), intestine (with content), femur, muscle, and tumors were harvested, weighed and measured in an automatic γ- well counter. The percentage of injected activity per gram (%IA/g) was calculated by comparison with a weighed, diluted standard. In addition, to examine the specific accumulation of

radiolabeled hu3F8 in OS tumors, additional biodistribution studies were performed in OSCA78 tumor-bearing mice using an [^{111}In]In-labeled irrelevant antibody, [^{111}In]In-DTPA-Rituximab (1.74 MBq/10 µg) (n = 3). These mice were sacrificed at 72 hours after injection and their organs were harvested and processed, as described above. After counting, fresh OSCA78 tumors were embedded in OCT compound and sectioned for IHC staining to detect radiolabeled hu3F8 using an anti-human Fc antibody.

SPECT Imaging

Mice with established OSCA78 tumors in both flanks were injected intravenously with either [^{111}In]In-DTPA-hu3F8 (3.03 MBq, 10 µg) or [^{111}In]In-DTPA-Rituximab (1.74 MBq, 10 µg). IMR32 xenografted mice received intravenous injection of [^{111}In]In-DTPA-hu3F8 (3.03 MBq, 10 µg). At 24-, 48-, and 96-hours post-injection, SPECT/CT images were acquired with a NanoSPECT/CT system (Bioscan Inc., Washington, DC, USA) using multiplexed multipinhole gamma detectors and high-resolution collimators. Images were acquired for 60 min at each time point and were reconstructed at voxel size of 0.6 mm^3 isotropic using the vendor-supplied iterative algorithm. SPECT images were co-registered with CT images for an anatomic reference using PMOD, version 3.7(PMOD Technologies LLC, Zurich Switzerland), and the voxel intensity was calibrated using images of a standard with known activity and volume.

In Vivo **dog studies**

Tumor-free dogs: Three healthy research dogs (13.6–17.0 kg) received intravenous injection of either the unlabeled hu3F8 (100 µg; n = 2) or [^{111}In]In-DTPA-hu3F8 (17.0 MBq/100 µg; n = 1) to assess the tolerance of the antibody. Fifteen minutes prior to injection, the dogs were fasted and received intravenous analgesia and antihistamine prophylaxis with 0.05 mg/kg hydromorphane and 1 mg/kg of diphenhydramine, respectively. The dog that received [^{111}In]In-DTPA-hu3F8 was sedated with 0.005 mg/kg fentanyl and induced with 0.25 mg/kg midazolam and 4 mg/kg propofol, intubated, and placed under isoflurane anesthesia with mechanical ventilation. Whole body SPECT/CT imaging with two bed positions (SPECT: voxel size = 2.4 mm^3, final matrix = 256 x 256 x 337; CT: matrix size = 512 x 512, pixel size = 0.9766 x 0.9766 mm^2, slice thickness = 1.0 mm, tube voltage = 130 kVp, tube current = 26 mA) was performed at 4 and 24 hours after injection on the Symbia T16 Series scanner (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) using a medium energy collimator. SPECT images were reconstructed using vendor's Flash3D OSEM algorithm with compensation for attenuation and resolution. The attenuation map was generated from CT images. Volume of interests (VOIs) were drawn for the liver, spleen, kidneys, and heart on the first 4-hour CT images, and were then transposed to the 24-hour time point after deformable registration of the scans had been performed using Velocity software (version 3.1, Varian Medical Systems, Palo Alto, CA, USA). One syringe containing 18.35 MBq [^{111}In]In was imaged for 5 min to determine the calibration factor (MBq/CPM) of the system. VOI for urinary bladder was draw on both 4- and 24-hour CT images due to the change of the bladder size with time.

Spontaneous OS Dogs: Two companion dogs (A: 30.0 kg and B: 32.1 kg) were diagnosed with spontaneously occurring OS. Metastatic recurrence of OS after amputation and chemotherapy was

confirmed by fine needle aspirate at 5 weeks in dog A and ^{18}F-fluorodeoxyglucose-positron emission tomography ([^{18}F]F-FDG-PET) at 12 months in dog B. Dog A received 100 MBq of [^{111}In]In-DTPA-hu3F8 (0.35 mg) intravenously while dog B received 36 MBq of [^{111}In]In-DTPA-hu3F8. Whole body SPECT/CT imaging was performed 48 hours post-injection using similar parameters to those for tumor-free dogs. Dog A was euthanized after pain palliation failed, four months after [^{111}In]In-DTPA-hu3F8 injection. Necropsy was performed by a board-certified pathologist. The shoulder and lung metastatic tissues were obtained and processed for histological analysis as described above.

Statistical analysis

Statistical analysis was performed using Prism 9. All data are presented as mean ± standard deviation (SD). Normality was tested using the Shapiro-Wilk test. Biodistribution groups were compared using one-way ANOVA. Values were considered significant at a P value of < 0.05.

Results

Cross Reactivity

We first validated by immunostaining (Fig. 1) and flow cytometry analysis (Fig. 2) that human and canine OS cells and canine OS tumor tissues highly reacted with hu3F8. GD2 expression was seen in a characteristic, uniform distribution on the surface of human U2OS and canine OSCA78 cells, similar to that on human IMR32, a neuroblastoma cell line that is known to express GD2. Canine OS tumor tissue showed positive reactivity to hu3F8 with heterogenous, moderate staining. In contrast, SK-N-SH cells and tumor-free dog spleen tissue showed no evidence of GD2 expression (Fig. 1). Flow cytometry analysis revealed high GD2 expression in human IMR32 (81%), human U2OS (85%), and canine OSCA78 (93%) cells with mean fluorescence intensity of 2807, 2589, and 1386, respectively (Fig. 2).

Radiolabeling and immunoreactivity

When starting with a molar ratio of hu3F8 to DTPA at 1:7–9, the [^{111}In]In-DTPA-hu3F8 was radiolabeled at an average specific activity of 27.75 MBq/nmol and high yield ($> 80\%$) and purity ($> 97\%$). The immunoreactivity of [^{111}In]In-DTPA-hu3F8 was 35–40% for OSCA78 cells and 44–60% for IMR32 cells. The immunoreactivities toward OSCA78 and IMR32 cells were markedly reduced as the amount of chelator (DTPA) in the reaction increased. Similarly, the irrelevant antibody, Rituximab, was radiolabeled at an average specific activity of 20.5 MBq/nmol and $> 95\%$ yield. As expected, the immunoreactivity of [^{111}In]In-DTPA-Rituximab was low ($< 3\%$) for both cell lines.

Saturation binding assay

The saturation binding assay demonstrated that [^{111}In]In-DTPA-hu3F8 binds to GD2 with high affinity, having a K_d of 7.4 ± 1.0 and 6.2 ± 1.9 nM, and a B_{max} of 51,900 and 288,300 sites/cell for OSCA78 and IMR32, respectively (Fig. 3).

Biodistribution and SPEC/CT imaging of mice

The 24-hour biodistribution of [^{111}In]In-DTPA-hu3F8 in mice bearing flank tumors of either OSCA78 or IMR32, demonstrated the highest uptake in tumors, followed by the blood, spleen, lung and kidneys (Fig. 4A). The tumor uptakes were 12.0 ± 1.4 and 15.0 ± 7.6% IA/g, tumor-to-muscle ratios 10.6 and 21.1, and tumor-to-blood ratios 1.1 and 2.4 for OSCA78 and IMR32 tumors, respectively. Normal tissue uptakes were similar between tumor-bearing and tumor-free mice. The uptake in OSCA78 tumors was significantly different from all the other tissues ($p < 0.0001$) except for the blood. Likewise, the IMR32 tumor had significantly higher uptake than all other tissues except for the spleen (10.7 ± 4.6% IA/g) (Fig. 4A). It is worth noting that one IMR32 tumor was significantly bigger and had a relatively lower uptake (6.3% IA/g) as compared to other IMR32 tumors (Fig. 4B).

At 72 hours the highest uptake of [^{111}In]In -DTPA-hu3F8 was observed in both OSCA78 (28.0 ± 3.6% IA/g) and IMR32 (51.6% IA/g) tumors, with a tumor-to-muscle ratio of 93.3 and 206.6, and a tumor-to-blood ratio of 6.7 and 8.4, respectively. Conversely, the radiolabeled irrelevant antibody, showed minimal uptake across all the tissues except for the kidneys (15.9 ± 3.0% IA/g) (Fig. 4C). Immunohistochemical staining of retrieved OSCA78 tumors confirmed the specific accumulation of [^{111}In]In-DTPA-hu3F8 within the tumors (Fig. 4D).

The maximum intensity projection SPECT/CT images of the tumor-bearing mice with [^{111}In]In-DTPA-hu3F8 are shown in Fig. 5. For [^{111}In]In-DTPA-hu3F8, at 24 hours post-injection, most of the activity had accumulated in the tumor with a slight uptake in the liver and heart. At 48 hours, most of the delivered activity cleared from the circulation, while OSCA78 and IMR32 tumors retained a high uptake, which was clearly shown in both flanks of the animals. In contrast, [^{111}In]In-DTPA-Rituximab injected OSCA78 tumor-bearing mice only showed high contrast to the background in the kidneys, which was consistent with the biodistribution data. By 96 hours, normal tissue uptakes of [^{111}In]In-DTPA-hu3F8 including the liver, spleen, and kidneys were not distinguishable from the background and only OSCA78 and IMR32 tumors still showed high contrast to the background. The OSCA78 tumor-bearing mice treated with [^{111}In]In-DTPA-Rituximab demonstrated very low tumor and normal tissue accumulation, which was not discernable above background (Fig. 5).

SPECT/CT imaging of dogs

The measured activities for the major organs of the tumor-free dog, post-injection of 17.0 MBq of [^{111}In]In-DTPA-hu3F8, from the SPECT images are shown in Table 1. The primary uptake was seen in vascular organs, with the highest in the liver, followed by the heart and the spleen at both 4 h and 24 h after injection (Fig. 6).

Table 1

Uptake of [^{111}In]In-DTPA-hu3F8 (of 17.0 MBq) in a tumor-free dog's organs at 4 and 24 hours after administration from the SPECT/CT images

Time after injection	Liver (%IA)	Heart (%IA)	Spleen (%IA)	Left kidney (%IA)	Right kidney (%IA)	Urinary bladder content (%IA)
4 hours	19.3	11.3	4.0	1.4	1.9	1.2
24 hours	23.2	8.1	3.0	1.3	1.8	2.1

Subsequently, the targeting ability of [^{111}In]In-DTPA-hu3F8 to OS lesions was investigated in client-owned dogs with metastatic OS. In dog B, [^{18}F]F-FDG-PET/CT revealed three metastatic OS lesions, two in the ribs and one in the fourth lumbar vertebra (Fig. 7A and supplementary video S1). SPECT/CT imaging of this dog showed avid uptake of the radiolabeled hu3F8 in all three metastatic lesions 48 hours after injection (Fig. 7B and supplementary video S2). In dog A, [^{111}In]In-DTPA-hu3F8 selectively bound to metastatic OS lesions in the right shoulder area 48 hours post-injection (Fig. 7C). No appreciable uptake in the lungs was observed. Dog A was euthanized per owner's request four months after [^{111}In]In-DTPA-hu3F8 injection. Necropsy evaluation revealed metastatic spread in both the right shoulder and the lungs. Immunohistochemical staining of these tissues demonstrated extensive distribution of the radiolabeled hu3F8 throughout the shoulder and lung lesions (Fig. 7D), indicating its ability to target both metastatic bone and soft lesions.

Discussion

Primary and metastatic OS is known to stably express cell surface disialogangliosides, particularly GD2 [5–7, 10, 25]. With the clinical success of GD2-targeted immunotherapy in high-risk neuroblastoma [8, 12], there is strong interest in repurposing anti-GD2 antibodies for radiopharmaceutical imaging and therapy of OS [10], in which GD2 expression is nearly ubiquitous but may be lower and more heterogeneous than in neuroblastoma [26]. In this study, we present preclinical assessment of a humanized anti-GD2 antibody, hu3F8, as a targeted delivery vector for radiopharmaceutical imaging of OS in both a tumor xenograft murine model and a spontaneous OS canine model and demonstrate the selective binding of [^{111}In]In-DTPA-hu3F8 to GD2-positive tumors. The detection of radiolabeled hu3F8 in canine skeletal and pulmonary metastatic tissues suggests its ability to target both osseous and soft tissue OS lesions.

The majority of preclinical testing of cancer radiopharmaceutical imaging and therapy largely relies on genetically identical, inbred, tumor-bearing mouse models [18, 24, 27–29], which often underrepresents the heterogeneity of naturally occurring tumors in humans and the complex interplay of the immune response to such therapy [30]. The canine OS model provides an important link between murine models and human studies to directly address the safety and therapeutic efficacy of RPT in the setting of an intact immune system. The immunohistochemistry and flow cytometry studies demonstrated that hu3F8

was strongly reactive to canine OS cells and tumors, paving the way to use spontaneous OS canines for RPT evaluation.

Preferential uptake of [^{111}In]In-DTPA-hu3F8 in GD2-positive tumors was demonstrated in biodistribution studies and was further validated by SPECT imaging. Consistent with the slow clearance of the full-length anti-GD2 antibody from the blood ($t_{1/2}$ α = 1.7 days) [31], we observed similar uptake of [^{111}In]In-DTPA-hu3F8 in OSCA78 tumors (12.0 ± 1.4% IA/g) to the blood (10.5 ± 0.8% IA/g) at 24 hours after injection. However, the significantly higher uptake in OSCA78 tumors than other blood rich organs, such as heart, liver, spleen, and kidneys suggested a blood-independent uptake of [^{111}In]In-DTPA-hu3F8 in OSCA78 tumors. The improved uptake of [^{111}In]In-DTPA-hu3F8 in tumors at 72 hours as compared to 24 hours was supportive of selective binding of [^{111}In]In-DTPA-hu3F8 to GD2-expressing tumors. This kinetics were also favorable for a therapeutic radionuclide with longer half-life, such as Actinium-225 ($t_{1/2}$ = 9.92d), because it decays and delivers potent alpha-particle radiation within the time window when the uptake in tumor is high, thereby minimizing the radiation damage to normal tissues.

Both the saturation binding assay and flow cytometry revealed lower expression of GD2 on OSCA78 than on IMR32 cells. Nevertheless, the highly specific uptake of [^{111}In]In-DTPA-hu3F8 within both types of tumors as demonstrated earlier indicates that even tumors with low GD2 expression could be effectively targeted with hu3F8-based, alpha-emitted labeled radiopharmaceuticals.

Using spontaneous OS canine as a model, we demonstrated the specific targeting of [^{111}In]In-DTPA-hu3F8 in metastatic OS lesions that are more closely mirroring human OS. In both dogs with naturally occurring OS, avid uptake of [^{111}In]In-DTPA-hu3F8 in OS lesions was observed, which was correlated well to the findings of the standard [^{18}F]F-FDG-PET (Fig. 7). In dog B, SPECT imaging revealed an extra lesion in the chest that was absent from [^{18}F]F-FDG-PET imaging, this could be attributed to either motion artifact during SPECT imaging, the presence of metabolically non-reactive lesions, or extension of disease due to time between the PET/CT and SPECT/CT. In OS dog A, the pulmonary uptake of [^{111}In]In-DTPA-hu3F8 48 hours after injection was not readily distinguishable on SPECT/CT images, despite the fact that metastatic spread to the lungs was identified postmortem. This discrepancy could arise from the fact that the metastatic lesions in the lungs at the time of imaging were too small to be detected by the SPECT system, due to its relatively poor spatial resolution (~ 14 mm^3). This suggests that treatment decision based on imaging alone may lead to a missed opportunity for early interventions in some patients whose tumor lesions are not appreciable during screening. Nevertheless, immunohistochemistry of postmortem canine OS tissues showed the accumulation of radiolabeled hu3F8 within both osteoblastic and soft lesions, suggesting the potential of targeting high-risk OS with hu3F8-based RPT.

Conclusion

Our findings demonstrate that the potential of anti-GD2 antibody as a targeted delivery vector for radiopharmaceutical imaging of human and canine OS. The cross-species reactivity and high binding

affinity of anti-GD2 antibody to canine OS cells/tissue and its ability to selectively deliver an imaging payload (Indium-111) to OS tumors suggest that conjugating anti-GD2 antibodies with a therapeutic radionuclide, such as alpha-emitter, Actinium-225, may provide a potent radiopharmaceutical agent for treating human and canine OS.

Declarations

Ethics approval

Animal protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University, School of Medicine. Client-owned dogs with confirmed metastatic OS was recruited from owners with written informed consent.

Availability of data and material

All data associated with this study are present in the paper or the Supplementary Materials.

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Authors' Contributions

YF, JY, and IL designed and performed all the experiments, collected, and analyzed the data. YF and JY wrote the original manuscript and all authors reviewed and edited the manuscript.

YD, AJ and JN collected, analyzed, and interpreted data from SPECT/CT images.

HR and JB collected and analyzed flow cytometry data.

DLK and GS acquired funding, directed the research, and supervised in the design and interpretation of the experiments and the writing of the manuscript.

Competing Interests

The authors report no relevant conflicts of interest.

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Figures

Figure 1

Hu3F8 cross reacts with human and canine osteosarcoma cells and canine osteosarcoma tumor tissue. Immunofluorescence staining showed surface GD2 expression for both human (U2OS) and canine (OSCA78) osteosarcoma cells, similar to the very high GD2-expressing human neuroblastoma line, IMR32. Canine osteosarcoma tissue showed heterogenous GD2 expression. Canine spleen and human neuroblastoma cells, SK-N-SH, were negative to hu3F8 immunostaining. Yellow bars represent 25 μm and black bars represent 100 μm

Figure 2

Flow cytometry analysis showed all human IMR32 cells (81%), human U2OS cells (85%), and canine OSCA78 cells (93%) express GD2 while canine white blood cells (WBC) were negative to hu3F8

Figure 3

Binding affinity of [^{111}In]In-DTPA-hu3F8 to GD2 determined by saturation binding assay in IMR32 and OSCA78 osteosarcoma cells. The K_d is 7.4 ± 1.0 nM for OSCA78, and 6.2 ± 1.9 nM for IMR32. B_{max} is 51,900 sites/cell for OSCA78 and 288,300 sites/cell for IMR32. Specific binding = Total bound - Non-specific uptake

Figure 4

Biodistribution of [^{111}In]In-DTPA-hu3F8. (A) Biodistribution of [^{111}In]In-DTPA-hu3F8 in IMR32 (n = 3) and OSCA78 tumor-bearing mice (n = 4) or tumor-free (n = 3) mice 24 hours after i.v. injection (3.03 MBq/10

µg). Each symbol on the plot represents a mouse. The mean and standard deviation (SD) correspond to the height of the bar and the indicated error bars. (B) Digital photograph of retrieved OSCA78 and IMR32 tumors. (C) Biodistribution of [^{111}In]In-DTPA-hu3F8 (3.03 MBq/10 µg) and [^{111}In]In-DTPA-Rituximab (1.74 MBq/10 µg) at 72 hours in mice bearing OSCA78 or IMR32 tumors. (D) Immunohistochemical staining of retrieved OSCA78 tumors 72 hours after [^{111}In]In-DTPA-hu3F8 injection was positive for radiolabeled-hu3F8 (brown staining within the tumor tissue). Bars represent 1 cm in (B) and 25 µm in (D)

Figure 5

Maximum intensity projection (MIP) images of whole-body SPECT/CT of the mice bearing subcutaneous flank IMR32 or OSCA78 tumor xenografts imaged at 24 h, 48 h, and 96 h after i.v. injection of [^{111}In]In-DTPA-hu3F8 (3.03 MBq/10 µg) or [^{111}In]In-DTPA-Rituximab (1.74 MBq/10 µg). Voxel intensity (%IA/g) was calibrated from SPECT image of a phantom with known activity and volume

Figure 6

Fusion SPECT/CT images of a tumor-free dog at 4 and 24 hours after administration of [^{111}In]In-DTPA-hu3F8 (17.0 MBq/100 µg)

Figure 7

Imaging of spontaneously occurring osteosarcoma-bearing dogs. (**A**) FDG-PET/CT image of OS-bearing dog B shows three metastatic tumors with two in the ribs and one in the fourth lumbar vertebra (arrows). SPECT/CT image of the same dog shows avid uptake of [^{111}In]In-DTPA-hu3F8 (36 MBq) in all OS lesions (arrows) 48 hours after injection. An additional lesion (arrow head) not seen on FDG-PET/CT was detected by SPECT/CT imaging. (**B**) SPECT/CT images of one OS lesion from dog B. (**C**) SPECT/CT images of OS-bearing dog A show avid uptake of [^{111}In]In-DTPA-hu3F8 (100 MBq) in metastatic lesions in the right thoracic limb (arrow). (**D**) Immunohistochemical staining of postmortem shoulder and pulmonary metastatic tissues shows the detection of [^{111}In]In-DTPA-hu3F8 (brown) within both lesions of OS-bearing dog A . Bar represents 100 µm

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