Nuclear Medicine and Biology 42 (2015) 945–957

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

Development and characterization of an $\alpha_v\beta_6$-specific diabody and a disulfide-stabilized $\alpha_v\beta_6$-specific cys-diabody

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ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form 25 July 2015
Accepted 31 July 2015

Keywords:
Diabody
Fluorine-18
Copper-64
$\alpha_v\beta_6$ integrin
Site-specific conjugation
Radiolabeling

ABSTRACT

Introduction: This work describes the development and characterization of two antibody fragments that specifically target the $\alpha_v\beta_6$ integrin, a non-covalent diabody and a disulfide-stabilized cys-diabody. The diabodies were analyzed for their ability to bind both immobilized and cell surface-bound $\alpha_v\beta_6$. Radiolabeling was done using non-site-specific and site-specific conjugation approaches with *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]-SFB) and the bifunctional chelator 1,4,7-triazacyclononane-triacetic acid maleimide (NOTA-maleimide) and copper-64 ([^{64}Cu]), respectively. The affects of each radiolabeling method on RCY, RCP, and immunoreactivity were analyzed for the [^{18}F]-FB-$\alpha_v\beta_6$ diabody, [^{18}F]-FB-$\alpha_v\beta_6$ cys-diabody, and the [^{64}Cu]-NOTA-$\alpha_v\beta_6$ cys-diabody.
Methods: Diabodies were constructed from the variable domains of the humanized 6.3G9 anti-$\alpha_v\beta_6$ intact antibody. The anti-$\alpha_v\beta_6$ cys-diabody was engineered with C-terminal cysteines to enable covalent dimerization and site-specific modification. Biochemical characterization included SDS-PAGE, Western blot, and electrospray ionization to confirm MW, and flow cytometry and ELISA experiments were used to determine binding affinity and specificity to $\alpha_v\beta_6$. The diabodies were radiolabeled with [^{18}F]-SFB and in addition, the anti-$\alpha_v\beta_6$ cys-diabody was also radiolabeled site-specifically using NOTA-maleimide and [^{64}Cu]. Immunoreactivities were confirmed using *in vitro* cell binding to DX3Puroβ_6 ($\alpha_v\beta_6$+) and DX3Puro ($\alpha_v\beta_6$−) cell lines.
Results: The diabodies were purified from cell culture supernatants with purities >98%. Subnanomolar binding affinity towards $\alpha_v\beta_6$ was confirmed by ELISA (diabody IC_{50} = 0.8 nM, cys-diabody IC_{50} = 0.6 nM) and flow cytometry revealed high specificity only to the DX3Puroβ_6 cell line for both diabodies. RCYs were 22.6% ± 3.6% for the [^{18}F]-FB-$\alpha_v\beta_6$ diabody, 8.3% ± 1.7% for the [^{18}F]-FB-$\alpha_v\beta_6$ cys-diabody and 43.5% ± 5.5% for the [^{64}Cu]-NOTA-$\alpha_v\beta_6$ cys-diabody. *In vitro* cell binding assays revealed excellent specificity and retention of immunoreactivity ([^{18}F]-FB-$\alpha_v\beta_6$ diabody = 58.7% ± 6.7%, [^{18}F]-FB-$\alpha_v\beta_6$ cys-diabody = 80.4% ± 4.4%, [^{64}Cu]-NOTA-$\alpha_v\beta_6$ cys-diabody = 59.4% ± 0.6%) regardless of the radiolabeling method used.
Conclusions: Two novel diabodies with excellent binding affinity and specificity for the $\alpha_v\beta_6$ integrin *in vitro* were developed. Radiolabeling of the diabodies with fluorine-18 ([^{18}F]) and [^{64}Cu] revealed advantages and disadvantages with regards to methodologies and RCYs, however immunoreactivities were well preserved regardless of radiolabeling approach.

1. Introduction

The ability of monoclonal antibodies (mAbs) to recognize antigens with high specificity has paved the way towards the discovery of a plethora of new biomarkers for disease detection and treatment, particularly for cancer. The commercial success of therapeutic mAbs has resulted in significant investment in research and development, however the clinical translation of mAbs into molecular imaging agents has been met with limited success. There are currently only four antibody-based diagnostic agents approved by the FDA for cancer detection including [^{111}In]-satumomab pendetide (OncoScint), [^{111}In]-capromab pendetide (ProtaScint), [^{99m}Tc]-nofetumomab nerpentan (Verluma), and [^{99m}Tc]-arcitumomab (CEA-Scan), with only ProtaScint

Abbreviations: NOTA-maleimide, 1,4,7-triazacyclononane-1,4,7-triacetic acid maleimide; Ni-NTA, nickel nitrilotriacetic acid; RCY, radiochemical yield; RCP, radiochemical purity; mAbs, monoclonal antibodies; immuno-PET, immuno-positron emission tomography; kDa, kilodaltons; V_H, variable heavy domain; V_L, variable light domain; CEA, carcinoembryonic antigen; HER2, human epidermal growth factor receptor 2; ALCAM, activated leukocyte cell adhesion molecule; PSCA, prostate stem cell antigen; CDR, complementary determining region; scFv, single chain Fv; OSCC, oral squamous cell carcinoma; MMP, matrix-metalloproteinase; ECM, extracellular matrix; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; ELISA, enzyme-linked immunosorbant assay; HPLC, high performance liquid chromatography; SFB, *N*-succinimidyl 4-fluorobenzoate; [^{18}F]-SFB, *N*-succinimidyl-4-[^{18}F]-fluorobenzoate; iTLC, instant thin-layer chromatography.

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http://dx.doi.org/10.1016/j.nucmedbio.2015.07.014
0969-8051/

currently still on the market in the U.S. [1]. Recently, there has been a revival of interest in combining the high specificity of mAbs with the sensitive quantitative imaging of positron emission tomography (PET) to generate immuno-PET diagnostic imaging agents [1–3]. Consolidation of these technologies has the potential to enable clinicians to quantify antigen expression, assess therapeutic efficacy, and ultimately contribute to a more personalized approach to the diagnosis and treatment of disease [2].

The therapeutic impact that intact mAbs have made in the clinic is profound, however the ability to rapidly generate high-contrast tumor images with intact antibodies is often hindered by their large size (~150 kDa) and slow blood clearance properties [4]. Diabodies represent an intermediate-sized (~55 kDa) genre of engineered antibody fragments comprised of V_H and V_L domains connected by a short, flexible linker to induce formation of the scFv dimer [5–7]. The diabody is the smallest antibody fragment that retains the bivalent binding properties of the intact antibody. These engineered fragments exhibit rapid tumor targeting and blood clearance, qualities that afford high-contrast tumor images in as little as 2–4 h post-injection (p.i.) [2,8]. Imaging figure of merit (IFOM) values have shown that when diabodies are radiolabeled with positron emitters such as [^{18}F] ($t_{1/2}$ = 109.7 min), optimal imaging occurs at 2 h p.i. [9]. This concept was further examined with the T84.66 anti-carcinoembryonic antigen (CEA) diabody, which produced high-contrast PET images of LS174T human colon carcinoma xenografts in mice within 1 h after injection [10].

Several studies have successfully demonstrated the conversion of anti-CEA, anti-HER2, anti-PSCA, and anti-ALCAM diabodies into PET imaging agents through the conjugation of fluorine-18 ([^{18}F] $t_{1/2}$ = 109.7 min) and copper-64 ([^{64}Cu] $t_{1/2}$ = 12.7 h) on exposed lysine residues [10–13]. These methods rely on the chemical modification of lysines using activated esters such as *N*-hydroxysuccinimide (NHS). This technique allows random conjugation of the radiolabel to lysines but does not enable tight control over the location of conjugation on the diabody. This is problematic when lysine residues present within the CDRs become altered, which can potentially have adverse affects on antigen recognition.

An alternative to the global introduction of the radiolabel is to perform site-specific conjugation in which the radiolabel is introduced at specific locations on the diabody distal to the CDR regions. Engineered cysteine residues have been appended onto the C-terminus of anti-CEA, anti-HER2, and anti-ALCAM diabodies to generate covalently bound dimers termed cys-diabodies [13–15]. The engineered disulfide bond creates a more stable assembly between the two scFvs in the cys-diabody versus the non-covalent diabody, and also provides a chemical group that after mild reduction can be used for site-specific modification via thiol chemistry [14]. This concept has been successfully applied to site-specifically radiolabel anti-HER2 and anti-ALCAM cys-diabodies with [^{64}Cu] and yielded excellent preservation of immunoreactivity [13,16].

2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]-FDG), approved by the FDA in 2004, is the most commonly used radiotracer to detect tumors and monitor therapeutic response in patients [17]. However, the quantification of glucose metabolism for tumor detection is not without its limitations. The most prominent of these is the generation of false positives due to [^{18}F]-FDG uptake in inflamed tissue [17]. Therefore the need exists to develop targeted molecular imaging agents that are disease-specific in order to achieve more accurate diagnoses and enhance the development of improved therapies. The integrins are a large family of cell surface receptors that have recently garnered increased scrutiny as targets for molecular imaging [18]. Specifically, the $\alpha_v\beta_6$ integrin is expressed at nominal levels in healthy epithelia, but in aggressive cancers such as oral squamous cell carcinoma (OSCC), pancreatic, ovarian, and cervical cancer, this integrin is highly overexpressed particularly at the leading edge of invasion [19–22]. This integrin has also been implicated as a modulator of MMPs, enzymes that act to degrade and remodel the ECM to promote tumor invasiveness and metastasis [23]. The activation of transforming growth factor-β (TGF-β), a cytokine suspected of promoting tumor cell survival and metastasis, is also mediated through $\alpha_v\beta_6$ binding to latency-activated peptide (LAP) [24]. Recent studies have shown that function-blocking antibodies against $\alpha_v\beta_6$ can successfully inhibit tumor growth via blockade of $\alpha_v\beta_6$-mediated TGF-β activation [24,25]. Moreover, increased expression of the $\alpha_v\beta_6$ integrin has been correlated with advancement in tumor grade and reduced survival, indicating that this integrin could serve as a potential prognostic indicator and further solidifying evidence that $\alpha_v\beta_6$ may be a valuable target for diagnosis and treatment [22,25,26].

Research to develop $\alpha_v\beta_6$-specific molecular imaging agents has resulted in the characterization of peptides and antibody fragments as PET and SPECT imaging agents, respectively [27–29]. Hausner et al. revealed rapid tumor targeting of the A20FMDFV2 peptide in $\alpha_v\beta_6$-expressing tumors *in vivo* however the peptide was prone to rapid degradation *in vivo*, which ultimately led to lower tumor uptake [27]. Efforts to improve peptide stability have included peglyation and the development of cysteine-knot peptides with improvements in radiotracer uptake observed in BxPC-3 ($\alpha_v\beta_6$+) tumor models [28,30]. Kogelberg at al. successfully used CDR grafting to develop an anti-$\alpha_v\beta_6$ diabody that when radiolabeled with [^{99m}Tc], enabled visualization of A375β_6 ($\alpha_v\beta_6$+) tumors with single photon emission tomography (SPECT) [29]. Although SPECT imaging reveals a plethora of information, arguably the biggest drawbacks of this modality are the lower sensitivity and less accurate quantification when compared to PET.

Taking these recent advances into consideration, we describe the production and characterization of two humanized diabody formats: a non-covalent anti-$\alpha_v\beta_6$ diabody and a covalent disulfide-stabilized anti-$\alpha_v\beta_6$ cys-diabody designed for site-specific radiolabeling (Fig. 1). The intact 6.3G9 antibody was selected as the basis for the engineered diabodies because it exhibited superior binding affinity and function-blocking capabilities against $\alpha_v\beta_6$ over the commercially available 10D5 antibody (anti-$\alpha_v\beta_6$). Further, 6.3G9 was previously humanized whereas 10D5 is of murine origin, reducing the possibility of the diabodies eliciting an immune response [24,31]. Biochemical characterization using SDS-PAGE, Western blot, electrospray ionization (ESI), and size-exclusion chromatography (SEC) confirmed MW and purity of each diabody. *In vitro* characterization was performed using competitive binding ELISA and flow cytometry to confirm binding affinity and specificity towards $\alpha_v\beta_6$, respectively. The diabodies were converted into PET imaging agents using two approaches. First, the diabody and cys-diabody were radiolabeled non-site-specifically on exposed lysine residues using [^{18}F]-SFB. Second, the cys-diabody was radiolabeled site-specifically using NOTA-maleimide and [^{64}Cu]. *In vitro* cell binding assays were conducted on the [^{18}F]-FB-$\alpha_v\beta_6$ diabody, the [^{18}F]-FB-$\alpha_v\beta_6$ cys-diabody, and the [^{64}Cu]-NOTA-$\alpha_v\beta_6$ cys-diabody and we demonstrated that regardless of the radiolabeling technique used, immunoreactivity was well preserved for each of these PET imaging agents. These results lay the groundwork for future *in vivo* evaluation of these PET imaging agents in a preclinical setting.

2. Materials and methods

2.1. Cell lines and reagents

The 293-F cell line (Invitrogen) was maintained as described by the vendor. Stable cell lines expressing the diabodies were cultured in selective growth media with DMEM, 10% FBS, 1% NEAA, 1% PSG, and 300 μg/mL Zeocin (Invitrogen). Production media consisted of Opti-MEM (Invitrogen) supplemented with 1% NEAA, 1% PSG, 100 μg/mL Zeocin. The DX3Puro and DX3Puroβ_6 cell lines (a generous gift from Dr. J.F. Marshall, Barts Cancer Institute) were cultured as previously described [32]. All cell lines were cultured at 37 °C in a humidified incubator with 5% CO_2. All chemicals were of reagent grade and used without further purification.

2.2. Design and construction of anti-$\alpha_v\beta_6$ diabody and anti-$\alpha_v\beta_6$ cys-diabody

To generate the anti-$\alpha_v\beta_6$ diabody, the sequences from the variable domains of the intact humanized hu6.3G9 antibody were separated by a