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Optimizing Radiolabeled Engineered Anti-p185^{HER2} Antibody Fragments for *In vivo* Imaging

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Abstract

We have recently described the *in vivo* properties of an iodinated anti-p185^{HER2} engineered antibody fragment [minibody (scFv-C_H3)₂; 80 kDa], made from the internalizing 10H8 monoclonal antibody. Although the 10H8 minibody showed excellent binding to the target *in vitro*, only modest tumor uptake [$5.6 \pm 1.7\%$ injected dose per gram (ID/g) of tissue] was achieved in nude mice bearing MCF7/HER2 breast cancer tumors. Here, in an attempt to improve targeting, the 10H8 minibody was conjugated to 1,4,7,10-tetraazacyclododecane-*N*, *N'*, *N''*, *N'''*-tetraacetic acid (DOTA), radio metal labeled, and evaluated *in vivo*. The tumor uptake of ¹¹¹In-DOTA 10H8 minibody was $5.7 \pm 0.1\%$ ID/g, similar to the radioiodinated 10H8 minibody. However, in addition to the expected liver clearance, the kidneys had unexpectedly high activity ($34.0 \pm 4.0\%$ ID/g). A minibody derived from a second anti-p185^{HER2} antibody (trastuzumab; hu4D5v8) was also made. Tumor uptakes, evaluated by quantitative microPET using ⁶⁴Cu-DOTA hu4D5v8 minibody, were $4.2 \pm 0.5\%$ ID/g. Furthermore, in non-tumor-bearing mice, ¹¹¹In-DOTA hu4D5v8

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Note: Supplementary data (tables on biodistribution of ¹¹¹In-labeled DOTA-10H8 mAb and MX-DTPA-trastuzumab in nude mice bearing MCF7/HER2/neu xenografts) for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

minibody exhibited similar elevated uptake in the kidneys ($28.4 \pm 6.5\%$ ID/g). Immunohistochemical staining of kidneys from non-tumor-bearing mice showed strong specific staining of the proximal tubules, and Western blot analysis of kidney lysate confirmed the presence of cross-reactive antigen. To further improve tumor uptake and normal tissue distribution, a larger hu4D5v8 fragment [(scFv-C_H2-C_H3)₂; 105 kDa] was made, engineered to exhibit rapid clearance kinetics. This fragment, when evaluated by microPET, exhibited improved tumor targeting ($12.2 \pm 2.4\%$ ID/g) and reduced kidney uptake ($13.1 \pm 1.5\%$ ID/g). Thus, by manipulating the size and format of anti-p185 antibody fragments, the kidney activity was reduced and high or low expression of p185^{HER2} in xenografts could be distinguished by microPET imaging.

Introduction

Antibody-based targeted delivery of radioisotopes to malignant tissues is a promising approach in cancer diagnostics. However, intact antibody molecules are large glycoproteins (150 kDa) that have limited application in molecular imaging due to their relatively slow clearance from the circulation leading to a high background signal. However, the sensitivity can be increased with enzymatically produced Fab fragments (55 kDa) and engineered antibody fragments such as single chain Fv (scFv; 25 kDa), which consists of variable light (V_L) and heavy (V_H) chains connected by a linker, and diabody (noncovalent scFv dimer; 55 kDa), which is formed by shortening the linker between the variable domains in the scFv (1). It has been shown that diabodies show higher tumor retention and higher tumor to blood ratios over both scFv and Fab in various animal tumor models (reviewed in ref. 2). This superiority has been attributed to increased avidity rather than size as monovalent Fab and scFv have similar tumor retention (3,4).

The principal determinants of the rate with which immunoglobulins are cleared from the circulation are their molecular size and the presence of the immunoglobulin Fc portion (C_H2-C_H3 region). Both diabodies and scFv fragments clear rapidly through the kidneys due to their low molecular weights. Increasing the size to above 60 kDa will bypass the kidneys and result in a slower clearance via the liver. One approach to produce larger, stable, and multivalent scFv fragments is the addition of COOH-terminal multimerization domains. Others and we have used individual constant immunoglobulin domains from human immunoglobulin G1 (C_H3; refs. 5–7) and human immunoglobulin E (C_H4; ref. 8) as dimerization domains to express intermediate-sized, bivalent scFv fragments of about 80 kDa in size. Our radiolabeled anti-carcinoembryonic antigen (CEA) T84.66 minibody (scFv-C_H3 dimers; Fig. 1A) has shown excellent tumor uptake (21.4–32.9% ID/g at 6 hours) *in vivo* (2, 5, 9). In addition, high-resolution microPET images of xenografts in nude mice were achieved when this fragment was radiolabeled with the positron emitters ⁶⁴Cu (*T*_{1/2} = 12.7 hours; ref. 10) and ¹²⁴I (*T*_{1/2} = 4.2 days; ref. 11). Recently, in a pilot clinical study involving 10 patients with colorectal cancer, tumor imaging was observed with ¹²³I-labeled anti-CEA T84.66 minibody in seven patients (12).

We and others have also examined the *in vivo* properties of a slightly larger antibody fragment, scFv-Fc of 105 kDa (Fig. 1A; refs. 13–15), which seems to behave analogous to

an intact antibody due to the presence of an intact Fc region. However, certain mutations in the Fc region that mediate Fc receptor interactions will modulate the clearance kinetics of this antibody fragment (13, 16), and of particular interest for this work is a variant containing two mutations (H310A/H435Q; Kabat numbering system), which shows similar pharmacokinetics to that of the minibody (15). In addition, high-resolution microPET images are also obtained when this antibody fragment is labeled with ^{124}I (15) and ^{64}Cu positron emitters.⁹

Recently, we described the construction and characterization of a minibody specific for p185^{HER2}, a transmembrane glycoprotein of 185 kDa encoded by the HER2/neu proto-oncogene (17). The overexpression of the tyrosine kinase receptor HER2/ neu (c-erbB-2) in 20% to 30% of breast cancers and in a variety of other tumors of epithelial origin is often associated with poor prognosis (18). Herceptin (trastuzumab; Genentech, San Francisco, CA), version 8 of the humanized 4D5 monoclonal antibody (mAb; ref. 19), has been approved by Food and Drug Administration for the treatment of p185^{HER2}-positive tumors (20). However, the overall objective response rate to trastuzumab monotherapy is only 15% to 20% (21, 22), necessitating the development of additional approaches for detection and treatment p185^{HER2}-positive tumors. One such approach is to develop engineered antibody fragments as radiolabeled pharmaceuticals for diagnostic and therapeutic use.

In our previous study, a total of four variants of anti-p185^{HER2} minibodies were made (17) from the internalizing anti-p185^{HER2} 10H8 mAb (23). The 10H8 minibodies showed high, specific binding to p185^{HER2}-positive cells *in vitro*. One variant was radioiodinated and evaluated for its blood clearance, tumor targeting properties, and normal organ uptake of the radiolabel in nude mice bearing p185^{HER2}-positive xenografts (17). The anti-p185^{HER2} 10H8 mini-body showed the expected blood clearance, but the tumor activity reached a maximum of only $5.6 \pm 1.7\%$ ID/g at 12 hours, which was substantially less than that previously observed with the radioiodinated anti-CEA minibody. The relatively low tumor uptake was thought to be partially due to dehalogenation on internalization of the fragment and/or metabolism of the label. In this work, we have therefore labeled the 10H8 minibody with radiometal to improve targeting to the tumor. In addition, a second anti-p185^{HER2} minibody was created from the humanized 4D5v8 mAb (19) and compared for its tumor targeting properties to that of the 10H8 minibody. Finally, a hu4D5v8 scFv-Fc H310A/H435Q [scFv-Fc double mutant (scFv-Fc DM)], was made and evaluated in nude mice to further optimize pharmacokinetics and normal tissue distribution.

Materials and Methods

Design and gene assembly of anti-p185^{HER2} antibody fragments

Assembly and production of the 10H8 minibody have been previously described (17). To make hu4D5v8 scFv (19), splice overlap extension PCR was used to create fully synthetic variable genes, as described (24). Here, six overlapping oligonucleotides (Integrated DNA Technologies, Inc., Coralville, IA), ranging in size from 84 to 90 bases, and three splice overlap extension-PCR amplifications were required to build each variable domain gene.

⁹V.E. Kenanova et al., unpublished data

Full-length hu4D5v8 V_L and V_H chain genes were assembled into a scFv with an 18-amino-acid GlySer-rich linker (17) by another splice overlap extension-PCR amplification. The scFv fragment was inserted into the mammalian expression vector pEE12 (Lonza Biologics, Slough, United Kingdom; ref. 25) containing the anti-CEA T84.66 minibody (26) and scFv-Fc DM (15) digested with *AgeI-XhoI*.

Expression, selection, and purification

All three anti-p185^{HER2} constructs were expressed in NS0 murine myeloma cells (27) that were selected in glutamine-deficient media (JHR Biosciences, Lenexa, KS; ref. 28), and supernatants were screened for expression by ELISA and analyzed by Western blot for size as described (17). The proteins of interest were purified from 450 to 800 mL dialyzed cell culture supernatants that had been pretreated with 5% AG1-X8, 100 to 200 mesh (Bio-Rad Laboratories, Hercules, CA), for removal of phenol red and cell debris, using a BioCAD 700E chromatography system (Applied Biosystems, Foster City, CA) as described (26). For 10H8 minibody, a two-step purification scheme with anion exchange followed by ceramic hydroxyapatite chromatography was employed as described (17). This scheme resulted in poor recovery and, as a result, an alternate three-step purification scheme was developed for the hu4D5v8 minibody and scFv-Fc DM. Here, the supernatant was dialyzed against 50 mmol/L acetic acid (pH 5.0) before being loaded onto a cation exchange column (Poros HS20, Perkin-Elmer, Foster City, CA). Bound proteins were eluted with a NaCl gradient from 0 to 0.4 mol/L in the presence of 50 mmol/L acetic acid (pH 5.0). Eluted fractions, containing the desired protein, were pooled, diluted 5× in 50 mmol/L MES (pH 6.5), and loaded onto the hydroxyapatite column (Macro-Prep Type 1, 20 μm, Bio-Rad Laboratories). Bound proteins were eluted with a KPi gradient from 0 to 0.15 mol/L in the presence of 50 mmol/L MES (pH 6.5). Eluted fractions containing the hu4D5v8 minibody or scFv-Fc DM were pooled, diluted 4× with 50 mmol/L HEPES (pH 7.4), and loaded onto the anion exchange column (Source 15Q, Amersham Biosciences Corp., Piscataway, NJ). Bound proteins were eluted with a NaCl gradient from 0 to 0.4 mol/L in the presence of 50 mmol/L HEPES (pH 7.4). SDS-PAGE analysis showed that the desired protein was in the flow-through, whereas the contaminants were bound to the column. The flow-through was concentrated using a Centricon 80 (Amicon, Inc., Beverly, MA) to 1 mL. The final concentration of purified protein was determined by A₂₈₀ nm using an extinction coefficient (ε) of 1.4 mg/mL.

Characterization of purified anti-p185^{HER2} antibody fragments

Aliquots of purified proteins were analyzed by SDS-PAGE under nonreducing or reducing (1 mmol/L DTT) conditions. Samples were also subjected to size-exclusion high-pressure liquid chromatography (HPLC) on a Superdex 200 HR 10/30 column (Amersham Biosciences) using a 0.5 mL/min flow rate and 50 mmol/L Na₃PO₄/0.15 mol/L NaCl (pH 7.0) buffer. Retention time was compared with standards of intact anti-CEA cT84.66 antibody, minibody, and diabody as described (15). Binding to p185^{HER2} was assessed by ELISA and by indirect immunofluorescence on the human breast tumor cell line MCF7/HER2 (gift of Dr. Dennis J. Slamon, University of California at Los Angeles School of Medicine; ref. 29) as described (17). Relative binding affinity was assessed by competition assays carried out in triplicate in ELISA microtiter plates as described (17).

Immunohistochemistry and kidney lysate preparation

Immunohistochemical staining was done on frozen sections of MCF7/HER2 tumors as described (17), whereas staining of the kidneys was done on paraffin-embedded sections. Kidneys from a normal nude mouse were removed, and 5- μ m sections were cut and mounted. The sections were deparaffinized, rehydrated, and antigen retrieval was achieved by steaming with 0.01 mol/L EDTA-Tris (pH 8.0) for 20 minutes (30). For staining, 10H8 and hu4D5v8 minibodies were used, as well as intact trastuzumab and a polyclonal rabbit anti-c-erbB2 antibody (A485, DAKO, Carpinteria, CA). The stain was developed by an avidin-biotin complex method using biotinylated goat antihuman (H + L) or goat anti-rabbit antibodies (H + L), respectively, included in the Vectastain ABC Elite Kits (Vector Laboratories, Inc., Burlingame, CA).

Kidney lysate was prepared as described (31). Briefly, kidneys from normal mice were removed, rinsed with PBS, and homogenized in an equal volume of PBS containing a cocktail of protease inhibitors (Complete tablets, Roche/Boehringer Mannheim, Indianapolis, IN) using a Kinematica homogenizer (Brinkman Instruments, Westbury, NY). The homogenized kidney was centrifuged at 14,000 rpm in a Brinkmann Eppendorf 5415C centrifuge for 60 minutes, passed through a 0.22 μ m filter, and analyzed by Western blot for the presence of p185^{HER2}.

Conjugation and radiolabeling with ¹¹¹In

Purified proteins (except trastuzumab) were conjugated to 1,4,7,10-tetraazacyclododecane-*N*, *N'*, *N''*, *N'''*-tetraacetic acid (DOTA; Macrocyclics, Dallas, TX) by using the water-soluble *N*-hydroxysuccinimide method as described (9, 32). Trastuzumab was conjugated to *p*-isothiocyanatobenzyl-diethylenetriami-nepentaacetic acid (MX-DTPA or 1B4M-DTPA; ref. 33). The molar ratio of the conjugate to antibody used was 15:1 and the reaction occurred at pH 7.2 over 18 hours at room temperature (13, 16). Following conjugation, the protein was extensively dialyzed in 0.9% NaCl (pH 7.2) and concentrated. The extent of modification was evaluated by isoelectric focusing.

10H8 and hu4D5v8 DOTA minibodies, as well as 10H8 DOTA mAb (ranging from 200 to 400 μ g of protein), were incubated with 0.5 to 2.9 mCi of carrier-free ¹¹¹In-chloride (Mallinckrodt, Inc., Hazelwood, MO) in 0.25 mol/L NH₄OAc (pH 5.0) for 1 hour at 43 °C, whereas MX-DTPA trastuzumab was incubated with ¹¹¹In in 0.9% NaCl (pH 7.2) for 35 minutes at room temperature. The reactions were terminated and purified by HPLC size-exclusion chromatography and the labeling efficiency as well as the immunoreactivity was determined as previously described (9, 17). The 10H8 DOTA minibody was labeled twice and the labeling efficiency was 2% and 20% with its immunoreactivity being 81% and 62%, respectively. The labeling efficiency for the hu4D5v8 DOTA minibody was 48% and only 18% for the parental 10H8 DOTA mAb, and their immunoreactivities were 65% and 80%, respectively. For MX-DTPA trastuzumab, the labeling efficiency was 100% and the immunoreactivity was 77%.

Animal biodistribution studies

All animal handling was done in accordance with City of Hope Research Animal Care Committee and University of California at Los Angeles Chancellor's Animal Research Committee guidelines. The biodistribution of ^{111}In -DOTA hu4D5v8 minibody (specific activity: 1.1 $\mu\text{Ci}/\mu\text{g}$) was evaluated in normal, non-tumor-bearing female nude mice (activity administered per animal: 3 μCi), whereas ^{111}In -DOTA 10H8 minibody and antibody as well as ^{111}In -MX-DTPA trastuzumab were evaluated in tumor-bearing mice. MCF7/HER2 tumor xenografts were established as described (17). About 10 to 14 days postinoculation, mice bearing xenografts were injected with ^{111}In -DOTA 10H8 minibody (specific activity: 0.2 $\mu\text{Ci}/\mu\text{g}$), ^{111}In -DOTA 10H8 mAb (specific activity: 0.9 $\mu\text{Ci}/\mu\text{g}$), or ^{111}In -MX-DTPA trastuzumab (specific activity: 0.5 $\mu\text{Ci}/\mu\text{g}$) via the tail vein. The activity administered per animal was in the range of 1 to 4 μCi , and corresponded to 4 to 5 μg of protein. Time points for analysis of the minibodies were 0, 2, 6, 12, 24, and 48 hours whereas for the intact antibodies 0, 6, 12, 24, 48, 72 and 96 hours were used. At the selected time points, groups of five mice were euthanized and percent of injected dose per gram (% ID/g) tissue was determined as described (9). Animal blood curves were calculated using the ADAPT II software (34) and biostatistical analysis was done with two-way ANOVA and all significant testing was done at $P < 0.01$ level using SAS/STAT software (SAS, Inc., Cary, NC) as described (9). Stability of the radiolabeled minibodies *in vivo* was evaluated by size-exclusion HPLC analysis as previously described (5).

Radiolabeling of hu4D5v8 minibody and scFv-Fc DM conjugates with ^{64}Cu

The positron emitting isotope ^{64}Cu (copper chloride in 0.1 mol/L HCl; radionuclide purity, >99%) was provided by Mallinckrodt Institute of Radiology (Washington University School of Medicine, St. Louis, WA). The hu4D5v8 DOTA-conjugated minibody and scFv-Fc DM (290–440 μg) were incubated with 0.7 to 3 mCi of ^{64}Cu in 0.1 mol/L NH_4 citrate (pH 5.5) for 50 minutes at 43°C. The reaction was stopped by addition of DTPA to 1 mmol/L. Labeled minibody was purified by HPLC size-exclusion chromatography using Superdex 75 (Amersham Biosciences). Labeling efficiency was determined by HPLC and immunoreactivity was determined by cell binding assay as described above. The hu4D5v8 DOTA minibody was labeled twice with ^{64}Cu with a labeling efficiency essentially 100%, whereas the immunoreactivities were 75% and 39%. For the scFv-Fc DM, instant TLC using the monoclonal antibody instant TLC Strips Kit (Biodex Medical Systems, Shirley, NY) was used to determine the labeling efficiencies, which were 100% and 77%, with the immunoreactivities being 58% and 52% for these labelings.

MicroPET imaging

The human Burkitt lymphoma cell line Daudi (ATCC no. CLL 213) and the human breast cancer cell line MD-MBA-231 (ATCC no. HTB-26) were obtained from American Type Culture Collection (Manassas, VA) and maintained under standard conditions. MCF7/HER2 (p185^{HER2} positive) and Daudi (p185^{HER2} negative) or MD-MBA-231 (p185^{HER2} low expressing; ref. 35) xenografts were established as described above. Mice were imaged using a P4 microPET scanner (Concorde Microsystems, Inc., Knoxville, TN). Mice were injected in the tail vein with 128 to 165 μCi of ^{64}Cu -DOTA hu4D5v8 minibody (specific

activity: 5.3 $\mu\text{Ci}/\mu\text{g}$) or with 128 to 140 μCi of ^{64}Cu -DOTA hu4D5v8 scFv-Fc DM (specific activity: 1.8 $\mu\text{Ci}/\mu\text{g}$). To enable imaging, mice were anesthetized using 2% isoflurane, positioned in a prone position along the long axis of the microPET scanner and imaged. Acquisition time was 10 minutes (1 bed position), and images were reconstructed using a filtered backprojection reconstruction algorithm (36, 37). After scanning, tumors were excised and either weighed and counted in a well counter (Cobra II AutoGamma, Packard, IL) or frozen for immunohistochemical analysis. Images were displayed and regions of interest (ROI) were drawn as described (11) and quantitated using AMIDE (38). ROIs from a cylinder with known weight and radioactivity were used to determine a calibration factor ($\mu\text{Ci}/\text{voxel}$) for use in calculating %ID/g from the image ROIs.

Results

Expression, purification, and characterization of anti-p185^{HER2} antibody constructs

Three engineered anti-p185^{HER2} antibody fragments (10H8 minibody, hu4D5v8 minibody, and hu4D5v8 scFv-Fc DM) were expressed in high quantities (20–70 $\mu\text{g}/\text{mL}$ by ELISA) in terminal cultures of the mouse myeloma cell line NS0. The yields after purification were 4.4, 9.3, and 27.3 mg/L for 10H8 minibody, hu4D5v8 minibody, and scFv-Fc DM, respectively.

Analysis of the purified proteins on SDS-PAGE (Fig. 1A) showed that the minibodies and scFv-Fc DM migrated as a monomer consistent with their predicted MW of ~ 40 kDa (*lanes 2 and 4*) and 53 kDa (*lane 6*), respectively, under reducing conditions, and as a covalent dimer of ~ 80 kDa (*lanes 1 and 3*) and 105 kDa (*lane 5*) under nonreducing conditions. Size exclusion chromatography verified that the hu4D5v8 minibody and the scFv-Fc DM eluted at times corresponding to correctly folded dimers of expected molecular weights (Fig. 1B). The purity of the proteins was also determined from the size-exclusion chromatography to be above 95%.

Binding to target antigen was initially shown by ELISA and by indirect immunofluorescence using flow cytometry of MCF7/HER2 cells with crude supernatants (data not shown). Affinity of purified proteins was measured by ELISA in the presence of competitors at different concentrations. As shown in Fig. 1C, by competition assay the affinities of hu4D5v8 minibody and scFv-Fc DM are essentially the same with their relative K_D estimated to be 6.7 nmol/L, whereas the relative K_D for the intact trastuzumab antibody is estimated to be 2 nmol/L. The relative K_D for 10H8 mAb and minibody were estimated to be 1.6 and 4.2 nmol/L, respectively (17). These results suggest about a 3-fold reduction in apparent affinity when the variable genes of the parental antibodies are rearranged into these dimeric scFv fragments.

In vivo biodistribution and targeting of ^{111}In -DOTA conjugated proteins

Biodistribution studies of ^{111}In -DOTA 10H8 mAb, ^{111}In -MX-DTPA trastuzumab, and ^{111}In -DOTA 10H8 minibody were conducted in athymic mice bearing MCF7/HER2 xenografts. The intact antibodies showed excellent tumor targeting with the 10H8 mAb reaching a maximum of $39.8 \pm 9.0\%$ ID/g at 96 hours, and trastuzumab a maximum of 33.9

$\pm 5.1\%$ ID/g at 72 hours (see Supplementary data). The nonspecific accumulation of the intact antibody in normal organs (liver, spleen, kidney, and lung) was as expected for intact radiolabeled antibodies.

The ^{111}In -DOTA 10H8 minibody reached a maximum tumor uptake at $5.7 \pm 0.1\%$ ID/g at 24 hours as the uptake persisted from 6 hours ($4.5 \pm 1.3\%$ ID/g) through 48 hours ($4.7 \pm 1.5\%$ ID/g; Table 1). However, unexpectedly, the 10H8 minibody showed high localization in the kidneys, with $27.6 \pm 2.4\%$ ID/g at 2 hours and reaching a maximum of $34.0 \pm 4.0\%$ ID/g at 24 hours. We examined the biodistribution in non-tumor-bearing animals to rule out the effect of shed p185^{HER2} extracellular domain-forming complexes that could get trapped in the kidney. The ^{111}In -DOTA hu4D5v8 minibody, however, also showed elevated activity in the kidneys in non-tumor-bearing mice with the uptake being $16.9 \pm 1.8\%$ ID/g at 2 hours, which was increased to a maximum of $28.4 \pm 6.5\%$ ID/g at 24 hours (Table 1).

Blood clearance and stability *in vivo*

The intact, parental anti-p185^{HER2} antibodies showed the expected prolonged blood clearance pattern as previously observed with the ^{111}In -DOTA 4D5 mAb in the same animal model (39). The slow blood clearance resulted in tumor to blood ratio of 4:1 for 10H8 mAb and 3.4:1 for trastuzumab at 96 hours (see Supplementary data). On the other hand, the minibodies exhibited a much more rapid blood clearance, similar to that seen with the anti-CEA T84.66 minibody (Fig. 2A), with the majority clearing by 48 hours resulting in a tumor to blood ratio of 13.3:1 (Table 1). As shown in Fig 2C, the ^{111}In -DOTA 10H8 and hu4D5v8 minibodies have similar kinetics with their respective rapid kinetic half-lives ($T_{1/2\alpha}$) being 0.55 and 0.75 hours and their terminal half-lives ($T_{1/2\beta}$) being 6.3 and 7.7 hours. For comparison, the ^{111}In -DOTA anti-CEA T84.66 minibody (9) and scFv-Fc DM half-lives are also shown. According to Fig. 2A and C, the terminal half-lives seem slightly shorter for the T84.66 minibody and slightly longer for the T84.66 scFv-Fc DM, relative to the anti-p185^{HER2} minibodies. However, there is no significant difference between the three minibodies ($P = 0.01$), whereas a significant difference is observed between the minibodies and the scFv-Fc DM at each time point ($P < 0.0001$), except at 48 hours.

The *in vivo* stability of the ^{111}In -DOTA 10H8 and hu4D5v8 minibodies in the serum of the mice was also examined. As shown in Fig. 2B, the radioactivity elutes as a single peak of about 80 kDa throughout the time interval of 0 to 12 hours following injection, and shows no indication of aggregation or association with serum proteins, or presence of low MW components during the course of the experiments. Similar results were observed with the hu4D5v8 minibody (data not shown).

MicroPET imaging of ^{64}Cu -DOTA hu4D5v8 minibody and scFv-Fc DM

The tumor targeting of ^{64}Cu -DOTA hu4D5v8 minibody and scFv-Fc DM was evaluated in nude mice carrying antigen-positive (MCF7/HER2; breast cancer cells) and antigen-negative (Daudi; Burkitt lymphoma cells) or low antigen expressing (MDA-MB-231; breast cancer cells) tumors established by s.c. inoculation on the shoulders. A whole-body microPET scan was done at 3 to 4 and 18 to 21 hours. The image of ^{64}Cu -DOTA hu4D5v8 minibody at 18 hours shows uptake in the positive tumor (*arrow*) and low activity in the antigen negative

control tumor (*arrowhead*), whereas the kidney and liver regions have relatively higher activity (Fig. 3A). The positive tumor to control tumor uptake ratio deduced from quantitative microPET analysis was 1.8:1 at 18 hours, demonstrating specificity. The uptake in the tumors and normal tissues calculated from the scans is shown in Fig. 3C. The positive tumor uptake is similar to the tumor uptake observed with the ^{111}In -DOTA 10H8 minibody in the biodistribution study, being ~4.2% ID/g at both 4 and 18 hours. In this study, the animals were sacrificed at 48 hours and the activities in tissues at the time of death (Fig. 3C) resemble those observed for the ^{111}In -DOTA hu4D5v8 minibody in the non-tumor-bearing mice (Table 1).

The image of ^{64}Cu -DOTA hu4D5v8 scFv-Fc DM at 21 hours shows uptakes in both the positive tumor (*arrow*) and the low antigen expressing control tumor (*arrowhead*; Fig. 3B). The uptake in the tumors and normal tissues calculated from ROIs is shown in Fig. 3C. The mean positive tumor uptake at 21 hours was $11.8 \pm 1.0\%$ ID/g, whereas the uptake in the low antigen expressing tumor (MB-MDA-231) was $8.8 \pm 1.9\%$ ID/g, resulting in a tumor to background ratio of 4.5:1 and 3.4:1, respectively. At 21 hours the activity in the kidney was $10.8 \pm 1.7\%$ ID/g and in the liver $18.2 \pm 3.4\%$ ID/g. Again, the tumor and normal tissue uptakes quantitated by microPET were similar to those measured at the time of sacrifice (21 hours; Fig. 3C).

Anti-p185^{HER2} versus anti-carcinoembryonic antigen antibody fragments

The tumor activity of the anti-p185^{HER2} mini-bodies and scFv-Fc DM in the MCF7/HER2 tumors was compared with that observed with the anti-CEA minibody (10) and scFv-Fc DM¹⁰ in the LS174T tumors (Fig. 4A). Only about one fourth of the activity observed with the anti-CEA minibody is obtained with the anti-p185^{HER2} minibodies, whereas the anti-p185^{HER2} scFv-Fc DM shows almost equal tumor activity to that of the anti-CEA minibody and scFv-Fc DM in the different animal models at the late time. When the renal and hepatic activities of the anti-p185^{HER2} minibodies and the anti-CEA T84.66 minibody (9) are compared, the anti-p185^{HER2} minibodies exhibit a significantly higher activity in the kidneys ($P < 0.0001$; Fig. 4B), whereas the anti-CEA minibody has a significantly higher activity in the liver ($P < 0.0001$; Fig. 4C). A higher activity in the liver is expected as these molecules have a molecular mass above the threshold for renal filtration. The anti-CEA scFv-Fc DM has uptakes in the kidney and liver similar to that of the anti-CEA minibody. The intact, parental anti-p185^{HER2} antibodies have been included for comparison and behave as expected for intact antibodies, with moderate to low levels of activity in both organs.

Expression of p 185^{HER2} in MCF7/HER2 tumors and normal mouse kidneys

Tumors were excised, frozen, and analyzed for expression of p185^{HER2} by immunohistochemistry as described (17). The staining pattern of the cell membrane by the anti-p185^{HER2} minibodies was indistinguishable from that achieved with the intact trastuzumab (Fig. 5A). Unlike the tumors, the kidneys showed elevated uptake of the radiolabeled minibodies, even in non-tumor-bearing animals (Table 1). Kidneys from a

¹⁰V.E. Kenanova et al., unpublished data.

normal nude mouse were therefore excised and paraffin-embedded sections were examined by immunohistochemistry. The minibodies showed specific staining in the proximal tubules with the 10H8 minibody staining being significantly stronger than that of the hu4D5v8 minibody (Fig. 5B). Using a commercially available rabbit polyclonal anti-c-erbB2 antibody resulted in diffuse staining of both proximal and distal tubules. The anti-CEA T84.66 minibody, used as isotype control, showed no staining. A Western blot of normal kidney lysate was done (Fig. 5C), and the presence of a high molecular weight band was detected with anti-c-erbB2 antibody (*lane 1*). The same band plus a smaller molecular weight band was also detected with the parental 10H8 mAb (*lane 2*). As for the 10H8 minibody, preferential binding to the smaller band was observed (*lane 3*). Similar bands, but with much less intensity, were also detected with trastuzumab and hu4D5v8 minibody (data not shown). These results indicate expression and presence of a cross-reactive antigen in the kidneys.

Discussion

Our previous studies show that the radioiodinated minibody derived from the anti-p185^{HER2} 10H8 mAb has moderate tumor uptake *in vivo* (17). In this study, we have investigated the possible explanations for the relatively modest tumor activity and arrived at an improved format suitable for *in vivo* imaging of p185^{HER2} expression. Because 10H8 is an internalizing antibody, we originally postulated that one of the explanations for low activity might be metabolism, dehalogenation, or clearance from the tumor. The 10H8 minibody was therefore stably labeled with a radiometal nuclide and examined for its tumor targeting and normal tissue uptake in athymic nude mice carrying MCF7/HER2 xenografts. We found that the magnitude of tumor uptake was not improved and that the kidneys had elevated activity as compared with that previously observed for the anti-CEA minibody (9). However, the intact, parental 10H8 mAb, when evaluated in the same animal model, showed excellent tumor targeting and expected normal tissue distribution. Because the activity remained below expectations for the radiometal-labeled 10H8 minibody, we postulated that uptake might be due to poor accessibility by the 10H8 minibody to its epitope. Therefore, a second minibody derived from the humanized 4D5 version 8 (19) was made, which we had previously shown to recognize a different epitope on p185^{HER2} (17). The hu4D5v8 minibody was also radiometal labeled and evaluated for its tumor targeting properties *in vivo* by microPET. This minibody, however, behaved very similarly to the 10H8 minibody, exhibiting modest tumor uptake and elevated kidney activity. Hence, we concluded that the epitope specificities of these minibodies did not affect tumor uptake. Next, we considered that the tumors might be expressing low levels of antigen *in vivo*. However, immunohistochemical examination of excised tumors showed strong surface staining, ruling out this explanation.

Another explanation for the modest tumor uptake and the elevated kidney uptake could be shed antigen, which would form antigen-antibody complexes in blood that can be trapped in the kidneys, thus preventing efficient tumor targeting. However, the presence of HER2 extracellular domain in sera could not be detected by ELISA (17). Furthermore, biodistribution in non-tumor-bearing animals revealed the same elevated kidney uptake, which suggests that circulating antigen is not responsible for this uptake.

Because the minibody has a molecular weight above the renal threshold, another explanation might be that the minibody dissociates or is cleaved in the serum, resulting in molecules of lower molecular mass that are subjected to renal filtration. However, serum samples taken from mice at different times show that only the intact minibody was detected. It is known that p185^{HER2} is expressed in the kidneys (40) and that renal tumors frequently overexpress this antigen (41). The distinct staining by the minibodies in the proximal tubules in the kidneys from normal mouse and the more general staining by polyclonal anti-p185^{HER2} antibodies suggest the presence of a cross-reacting antigen. Moreover, Western blots of normal kidney lysate, probed with anti-p185^{HER2} antibodies and minibodies, resulted in detection of distinct bands, verifying the presence of a cross-reactive antigen.

Because the presence of antigen in the kidneys presents a problem for efficient tumor targeting of the minibodies and not so for intact antibodies, we hypothesized that by increasing the size of the antibody format we would also be able to increase the tumor uptake. Hence, we proceeded to generate a larger fragment in which the C_H2 domain was included to produce a scFv-Fc fragment. However, this fragment clears almost as slowly as an intact antibody, resulting in high background, thus lowering the sensitivity in imaging studies. It has been shown that one mutation in the Fc region, affecting the interaction with the neonatal Fc receptor (FcRn, Brambell receptor; refs. 42, 43), is enough to affect the half-life of the chimeric TNT-3 antibody (44). Other sites that interfere with the FcRn receptor binding have also been identified (13, 16). Based on these data, we have produced several anti-CEA scFv-Fc variants with site-specific mutations in the Fc region that show different blood clearance patterns (15). The fastest clearing variant contains two mutations (H310A and H435Q) and exhibits similar blood clearance as that of the minibody. Targeting p185^{HER2} with this double mutant fragment format did indeed result in almost 3-fold improved tumor uptake as well as reduced kidney activity. Hence, we have shown that size matters in this animal model.

The profile observed for the anti-p185^{HER2} minibodies regarding their blood clearance is statistically the same to that seen with the anti-CEA minibody, and their stability in the serum also seems very similar. The reason why the anti-p185^{HER2} minibodies behave so different from the anti-CEA minibody is puzzling as they have equal opportunity to reach the target. The explanation for this difference may be embedded in the tumor models and a reflection of the tumor physiology. Others have targeted p185^{HER2} using different tumor systems and antibody fragments than those described in this work. In one study, using a ^{99m}Tc-labeled 4D5 scFv tetramer, only modest tumor uptake (maximum, 4.3 ± 1.9% ID/g) in nude mice bearing SK-OV-3 tumors (45) was achieved, which is similar to the tumor uptake of our anti-p185^{HER2} minibodies. The low tumor uptake of the 4D5 tetramer, however, was explained to be possibly due to instability and the orientation and accessibility of the targeted epitope. A more recent study shows excellent tumor uptake with an In-DOTA trastuzumab (Fab)₂ fragment (maximum, 20.4 ± 6.8% ID/g) in nude mice bearing BT-474 tumors (46). Despite their differences in tumor uptake, the maximum activity in the kidney for both proteins was about 70% ID/g, which is 2- to 3-fold higher than that observed with our minibodies, suggesting that these proteins probably dissociate and clear via the kidneys.

Shifting to the larger scFv-Fc DM optimized the pharmacokinetics and normal tissue distribution in the MCF7/HER2 tumor model. However, the fragments described in this work may behave differently in other tumor models, considering that almost 9% ID/g of the scFv-Fc DM localized in the low expressing MB-MDA 231 tumors at 21 hours. Further work will be required to determine the relationship between signal and antigen density using other cell lines such as SK-BR-3, which has an even higher antigen density than MCF7/HER2 (35). Another difference between MCF-7/HER2 and other tumor models is that these cells have been transfected with p185^{HER2} to overexpress the receptor, whereas the others inherently overexpress the receptor. Subtle differences between these cells may affect targeting.

To conclude, we have shown that anti-p185^{HER2} minibodies preferentially localize in the kidneys in mice. Furthermore, we have shown that the kidneys express the antigen in the proximal tubules, which seem to act as a sink for the minibodies. Therefore, these fragments seem not suitable for targeting this antigen. By generating a slightly larger antibody fragment that was also tailored to clear fast, we have shown that increased tumor uptake and reduced kidney uptake can be achieved. These results suggest that the scFv-Fc format may become potentially useful as an imaging agent for HER2-positive tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Biochemical characterization of purified anti-p185^{HER2} minibody and scFv-Fc DM. **A**, schematic presentations of the minibody and the scFv-Fc DM. Both constructs assemble into covalently bound dimers through the cysteines in the hinge region. *Arrows*, location of the mutations in the scFv-Fc DM protein. V_L, variable light; V_H, variable heavy; C_H, constant heavy. SDS-PAGE of purified proteins under reducing (*lanes 2, 4, and 6*) and nonreducing (*lanes 1, 3, and 5*) conditions. *Lanes 1 and 2*, 10H8 minibody; *lanes 3 and 4*, hu4D5v8 minibody; *lanes 5 and 6*, hu4D5v8 scFv-Fc DM. **B**, size-exclusion HPLC analysis

of purified hu4D5v8 minibody and scFv-Fc DM. The major peaks eluted at retention times of 29.3 and 27.5 minutes were consistent with dimers of 80 and 105 kDa, respectively. Standards consisting of intact immunoglobulin G, minibody, and diabody with the corresponding retention times of 25.8, 28.5, and 38.2 minutes. C, competitive ELISA binding assay. Microtiter plates were coated with extracellular domain of HER2 fused to mouse immunoglobulin G2a or human immunoglobulin G1 Fc that had been expressed in NSO mouse myeloma cells and purified by Protein A affinity chromatography (Poros20; Perkin-Elmer). A fixed concentration (1 nmol/L) of biotinylated intact 10H8 mAb and trastuzumab was competed off with increasing concentrations (0–100 nmol/L) of nonbiotinylated competitors.

Figure 2.

Blood activity, stability, and half-lives of the anti-p185^{HER} 10H8 and hu4D5v8 minibodies in comparison with anti-CEA T84.66 minibody and scFv-Fc DM fragment. **A**, blood activity curves of the engineered antibody fragments *in vivo*. Percent injected dose per gram of the antibody fragments plotted against time from blood drawn from groups of five mice per time point in the biodistribution studies. **B**, stability of 10H8 minibody *in vivo*. Serum samples (100–200 μ L) were obtained from mice at the time of sacrifice in the biodistribution study and chromatographed on Superdex 200 HR 10/30 (Amersham Biosciences) and radioactivity was followed during elution. Results of analysis are shown for the time points indicated. **C**, table showing the estimated values of the blood half-lives for the engineered antibody fragments. SDs are shown in parentheses. A_1 and A_2 , amplitudes of the faster and slower clearance components. $T_{1/2}$ are related to the inverse of k_1 and k_2 by the relationship $T_{1/2} = 0.693 / k$. AUC, area under the curve. MRT, mean residence time.

Figure 3.

MicroPET scans images (0.4 mm thick slices) and estimated activity in tumors and normal tissue of ^{64}Cu -DOTA hu4D5v8 antibody fragments. **A**, transverse and coronal view of an athymic mouse given 165 μCi minibody and scanned 18 hours after injection. MCF7/HER2 breast cancer (antigen positive; *arrow*) and Daudi (Burkitt lymphoma; antigen negative; *arrowhead*) tumors were implanted s.c. on the shoulder. **B**, transverse and coronal view of an athymic mouse given 128 μCi scFv-Fc DM and scanned 21 hours after injection. MCF7/HER2 breast cancer (high antigen expression; *arrow*) and MD-MBA-231 breast cancer (low

antigen expression; *arrowhead*) tumors were implanted s.c. on the shoulder. C, estimated activities from drawn ROIs in tumors, liver, and soft tissue at 4 and 18 (21) hours. The kidney uptake was not determined from the images due to lack of anatomic reference in this region. Activities present in the tissues at the time of sacrifice (21 and 48 hours), with kidney activity included.

Figure 4.

Tumor, renal, and hepatic uptake of the anti-p185^{HER2} fragments relative to anti-CEA T84.66 antibody fragments in tumor-bearing nude mice. A, tumor uptake of ⁶⁴Cu-labeled minibody and scFv-Fc DM constructs targeting p185^{HER2} and CEA as quantitated from microPET images. Early refers to 4 to 6 hours, and late to 18 to 24 hours after injection. However, the late quantitation of the T84.66 minibody was at 12 hours. ■, hu4D5v8 Mb; □, hu4D5v8 scFv-Fc DM; ■, T84.66 Mb; ▨, T84.66 scFv-Fc DM. Uptake in kidney (B) and liver (C) over time as observed in biodistribution studies. —■—, 10H8 mAb; —□—, 10H8 Mb;

●, Trastuzumab; ○, hu4D5v8 Mb; ▲, T84.66 scFv-Fc DM; △, T84.66 Mb. The T84.66 minibody data are based on previous publication.

Figure 5.

Immunohistochemistry staining of frozen sections of MCF7/HER2 tumor (A; magnification, $\times 20$) and paraffin-embedded kidney sections (B; magnification, $\times 40$). C, Western blot of kidney lysate from normal non-tumor-bearing mice. *Lane 1*, anti-c-erbB2/c-neu (Ab-3) mAb (Oncogene Research Products, Boston, MA); *lane 2*, parental 10H8 mAb; *lane 3*, 10H8 minibody. Alkaline phosphatase-conjugated goat anti-mouse and anti-human antibodies (Fc specific) were used for developing the bands.

Table 1

Biodistribution of ¹¹¹In-DOTA 10H8 and hu4D5v8 minibodies

Organ (%ID/g)	0h	2h	6h	12 h	24 h	48 h
<i>10H8 minibody in MCF7/HER2-bearing nude mice</i>						
Tumor (T)	1.21 (0.53)	2.59 (0.54)	4.53 (1.32)	4.47 (1.24)	5.68 (0.11)	4.66 (1.53)
Blood	23.60 (2.64)	10.17 (1.18)	6.07 (1.30)	2.63 (0.89)	1.27 (0.23)	0.35 (0.07)
Liver	9.81 (0.96)	11.32 (1.27)	13.30 (2.34)	8.67 (1.93)	11.28 (1.38)	10.49 (1.42)
Spleen	7.17 (3.16)	5.12 (0.76)	6.43 (0.71)	4.99 (1.29)	6.35 (0.66)	5.76 (1.16)
Kidney	13.69 (2.12)	27.55 (2.36)	33.20 (2.97)	32.14 (5.89)	33.98 (3.99)	22.54 (2.69)
Lung	7.92 (0.97)	4.42 (0.53)	3.39 (0.72)	1.95 (0.56)	1.64 (0.29)	0.99 (0.15)
Stomach	0.98 (0.31)	1.16 (0.09)	0.92 (0.23)	0.55 (0.10)	0.75 (0.15)	0.49 (0.09)
Intestine	1.38 (0.14)	1.84 (0.12)	1.70 (0.14)	1.26 (0.23)	1.27 (0.20)	0.86 (0.11)
Bone	2.13 (0.40)	2.41 (0.32)	1.92 (0.26)	2.03 (0.34)	2.27 (0.42)	1.85 (0.34)
Ratios						
T/Blood	0.05	0.25	0.75	1.70	4.48	13.31
T/Liver	0.12	0.23	0.34	0.52	0.50	0.44
T/Kidney	0.09	0.09	0.14	0.14	0.17	0.02
Tumor						
Tumor weight	0.046 (0.045)	0.062 (0.047)	0.079 (0.047)	0.122 (0.070)	0.099 (0.032)	0.138 (0.075)
<i>Hu4D5v8 minibody in non-tumor-bearing nude mice</i>						
Blood	31.59 (4.41)	13.46 (2.20)	7.41 (1.65)	3.71 (0.94)	1.60 (0.61)	0.40 (0.08)
Liver	5.86 (0.55)	7.04 (1.54)	9.30 (0.98)	9.25 (1.77)	10.00 (2.37)	8.69 (0.98)
Spleen	5.18 (2.02)	4.11 (0.62)	3.74 (0.61)	3.53 (0.52)	3.98 (1.22)	3.51 (0.42)
Kidney	9.04 (0.72)	16.93 (1.78)	20.58 (2.16)	22.46 (2.80)	28.35 (6.54)	19.40 (3.33)
Lung	9.55 (1.31)	5.85 (0.88)	4.05 (0.33)	2.62 (0.56)	1.83 (1.02)	1.33 (0.19)
Stomach	0.86 (0.20)	1.27 (0.36)	1.50 (0.37)	0.77 (0.20)	0.94 (0.38)	0.70 (0.22)
Intestine	1.65 (0.25)	2.13 (0.32)	1.92 (0.34)	1.48 (0.28)	1.31 (0.34)	0.95 (0.03)
Bone	2.13 (0.40)	1.91 (0.24)	1.83 (0.23)	1.74 (0.29)	2.10 (0.54)	1.83 (0.25)

NOTE: Tumor and normal organ uptakes are expressed as percent injected dose per gram Tumor masses are in grams. SDs are shown in parentheses.