Highly Specific in Vivo Tumor Targeting by Monovalent and Divalent Forms of 741F8 Anti-c-erbB-2 Single-Chain Fv


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ABSTRACT

The in vivo properties of monovalent and divalent single-chain Fv (sFv)-based molecules with the specificity of the anti-c-erbB-2 monoclonal antibody 741F8 were examined in scid mice bearing SK-OV-3 tumor xenografts. 741F8 sFv monomers exhibited rapid, biphasic clearance from blood, while a slightly slower clearance was observed with the divalent 741F8 (sFv)2 comprising a pair of 741F8 sFv’ with a C-terminal Gly4Cys joined by a disulfide bond. Following i.v. injection, the 741F8 sFv monomer was selectively retained in c-erbB-2-overexpressing SK-OV-3 tumor, with excellent tumor:normal organ ratios uniformly exceeding 10:1 by 24 h. The specificity of this effect was demonstrated by the lack of retention of the anti-digoxin 26-10 sFv monomer, as evaluated by biodistribution studies, gamma camera imaging, and cymacrouautoradiography studies. The specificity index (741F8 sFv retention/26-10 sFv retention) of 741F8 monomer binding, measured by the percentage of injected dose per g of tissue, was 13.2:1 for tumor, and 0.8 to 2.1 for all tested normal organs, with specificity indices for tumor:organ ratios ranging from 7.0 (kidneys) to 16.7 (intestines). Comparing divalent 741F8 (sFv)2 with the 26-10 (sFv)2, similar patterns emerged, with specificity indices for retention in tumor of 16.9 for the Gly4Cys-linked (sFv)2. These data demonstrate that, following their i.v. administration, both monovalent and divalent forms of 741F8 sFv are specifically retained by SK-OV-3 tumors. This antigen-specific binding, in conjunction with the 26-10 sFv controls, precludes the possibility that passive diffusion and pooling in the tumor interstitium contributes significantly to long-term tumor localization. 741F8 (sFv)2 species with peptide spacers exhibited divalent binding and increased retention in tumors as compared with 741F8 sFv monomers. Since the blood retention of the (sFv)2 is slightly more prolonged than that of the monomer, it was necessary to demonstrate that the increased tumor localization of the peptide-linked (sFv)2 was due to its divalent nature. The significantly greater localization of the divalent bismaleimidohexane-linked 741F8 (sFv)2 as compared with a monovalent 741F8 Fab fragment of approximately the same size suggests that the increased avidity of the (sFv)2 is a factor in its improved tumor retention. This is the first report of successful specific in vivo targeting of tumors by divalent forms of sFv molecules. The improved retention of specific divalent (sFv)2 by tumors may have important consequences for targeted diagnostic or therapeutic strategies.

INTRODUCTION

The development of murine MAb and their proteolytic fragments has resulted in significant interest in antigen-specific targeting of tumors for diagnostic or therapeutic purposes. However, successful targeting of radionuclides, drugs, or toxins with MAb has been limited by the distribution of these proteins to normal organs (1). Furthermore, the quantitative delivery of IgG molecules and their fragments to tumors has been quite low, because of such factors as heterogeneity of tumor antigen expression (2) and poor diffusion of MAb from the vasculature into the tumor (3). The recent development of recombinant sFv molecules has provided agents which may alleviate some of these limitations to the use of MAb in the targeting of neoplasms (4, 5). Radioiodinated sFv administered to tumor-bearing mice exhibit faster plasma clearance rates and higher tumor:normal tissue ratios than corresponding IgG or Fab (6, 7). The penetration of sFv into tumor from the vasculature, as determined by microscopic autoradiography, is superior to that of the corresponding IgG, F(ab′)2, or Fab′ (8).

This investigation addresses the relative impact of binding avidity, molecular mass, and rapidity of clearance from the blood pool on sFv targeting of tumors. We have produced tumor-specific sFv species directed against the c-erbB-2 antigen and compared them to control sFv species specific for the drug digoxin. Conventional sFv molecules, sFv’ molecules, and divalent disulfide-bonded (sFv’)2 molecules have been prepared, with the sFv’ molecules having carboxy-terminal cysteine residues to facilitate site-specific dimerization. We have obtained pharmacokinetic and biodistribution profiles, as well as scintigraphic and macroautoradiographic images of sFv and (sFv’)2 administered i.v. to scid mice bearing human tumor xenografts expressing c-erbB-2. These results differentiate between the specific binding of 741F8 anti-c-erbB-2 sFv and the nonspecific interstitial pooling of irrelevant 26-10 sFv in tumors and permit the examination of the contributions of sFv valency and size to tumor targeting.

MATERIALS AND METHODS

In these experiments, results with sFv and sFv’ monomers of the same specificity did not significantly differ. Accordingly, for the purpose of clarity, both sFv and sFv’ monomers will be referred to as sFv’ monomers.

Preparation of sFv Molecules.

The sFv species utilized in these experiments were produced at Creative BioMolecules, Inc. (Hopkinton, MA), as described elsewhere (Ref. 9; Footnote 4). All of the sFv and sFv’ species were produced in Escherichia coli and were devoid of leaders except for the 741F8 sFv’ with a Gly4Cys tail, which had a 9-residue N-terminal extension (ADNK-FNKDP) to promote high expression levels. Refolding of the M, 27,000 sFv’ analogues used a slight modification of the 3 m urea/glutathione oxidation reduction refolding procedure of Tai et al. (10). Anti-digoxin 26-10 and anti-c-erbB-2 741F8 sFv’ species were refolded to yield stable monomers with the C-terminal cysteine in a mixed disulfide with glutathione. Blocked 741F8 sFv’ was purified by anion/cation exchange followed by size exclusion chromatography and then converted to (sFv’)2 homodimers. Blocked 26-10 sFv’ was purified by ouabain-Sepharose affinity chromatography, as described previously for 26-10 sFv (4). Monovalent sFv’ was deblockd with mild reduction, and (sFv’)2 dimers were formed through disulfide bonds by oxidation (for fused tails comprising either -SerCys or -Gly4Cys), by MCA-peptide bridging (MCA-GlySer,Gly2Ser3Lys-MCA), or through thioether bonds by BMH.

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2 To whom requests for reprints should be addressed, at Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111.

3 The abbreviations used are: MAb, monoclonal antibody; sFv, single-chain Fv fragment; MCA, N,N,N-bismaleimidoacryloyl amino acid; BMH, bismaleimidohexane; ECD, extracellular domain; CHO, Chinese hamster ovary; HMP, hydroxymethylphenoxycetic acid; DIC, diisopropylcarbodiimide; DMAF, dimethylaminopyridine; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; %ID/g, percentage of injected dose per g of tissue; T/O ratio, tumor:normal organ ratio; IgG, immunoglobulin G.

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(Fruehwald, Rockford, IL). The MCA-peptide was assembled on a polystyrene resin modified with HMP to allow formation of a free acid at the C-terminus (Pierce, Rockford, IL). The MCA-peptide was assembled on a polystyrene and DIC/hydroxybenzotriazole activation. Coupling time was 90 min, and subsequent amino acids were added as the N-a-C-fluorenylmethyloxycarbon-

resin by DIC/DMAP activation to give a resin substitution of 0.6 iriM/g. was allowed to react at room temperature overnight. The peptide was purified by reverse-phase HPLC on a Vydac 1 x 25-cm column using acetonitrile/ water/TFA buffers. The c-erbB-2 ECD was expressed by baculovirus cells (Cetus Oncology) or from CHO cells5 (Creative BioMolecules) transfected in a manner similar to that described by Hudziak and Ullrich (11). The ECD binding activity of 741F8 sFv' or (sFv')2 was assessed by analytical ultracentrifugation and affinity chromatography using ECD produced in CHO cells.

Association Constant Determination. Proteins were immobilized on the surface of plasmon resonance chips for analysis using the BIAcore instrument (Pharmacia, Brussels, Belgium). The proteins were immobilized using N-ethyl-N-3-(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide methodology as described in the directions provided by the manufacturer. Rabbit anti-mouse Fe was immobilized to capture 741F8 IgG. 741F8 (sFv')2 was immobilized directly onto the chip. Various concentrations of c-erbB-2 were passed over the chip, and the rates of association and dissociation were determined. The thermodynamic association constant was derived by dividing the rate of dissociation into the rate of association.

Sedimentation Analysis. Demonstration of divalent binding of soluble c-erbB-2 ECD by 741F8 (sFv')2 was achieved through sedimentation analysis. Sedimentation equilibrium experiments with 741F8 (sFv')2, CHO cell-produced c-erbB-2 ECD, or equimolar amounts of 741F8 (sFv')2 and c-erbB-2 ECD were carried out on an Analytical Ultracentrifuge (Model-E; Beckman Instruments, San Ramon, CA) equipped with a real-time video-based data acquisition system and Rayleigh optics (12). The video-based system automatically converted each digitized Rayleigh pattern into a computer disk file of fringe displacement versus radius. The camera lens was focused at the 95 plane of the cell. The cells were equipped with sapphire windows and 12-mm, 6-channel external loading centerpieces (13). Other details and methods of data analysis and curve fitting were described previously (14). All protein samples were dialyzed against their respective buffers, and the diameters of 100 to 200 mg, Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Three days later, the mice were used in pharmacokinetic, biodistribution, imaging or autoradiography studies.

Biodistribution Studies. The radiolabeled sFv and Fab preparations were diluted in PBS (pH 7.2) to a concentration of 200 µg to 1 mg/ml. Each mouse was given 100 µl of radiopharmaceutical by tail vein injection, containing 20 to 100 µg of protein. In any given experiment, all mice received identical protein doses. The total injected dose was determined by counting each animal on a Series 30 multichannel analyzer/probe system (Probe Model 2007; Canaberra, Meridian, CT). Blood samples and whole-body counts of the mice were obtained at regular intervals. Groups of 3 to 6 mice were sacrificed at various times after injection, and the tumors and organs were removed, weighed, and counted in a gamma counter to determine the %ID/g (23, 24). The mean and SEM for each group of data were calculated, and T:O ratios were derived. Specificity indices were derived by dividing the mean %ID/g by T:O ratios of the 125I-741F8 sFv by the corresponding mean %ID/g or T:O ratios of the 125I-741F8 sFv for imaging studies. At 2 or 24 h after injection, the mice were immobilized by treatment with chloral hydrate (0.1 ml/10 g of body weight), and images were acquired on a gamma camera (Picker Dynacamera, Series-5; Cincinnati, OH) using a high-energy collimator. Preset imaging periods of 200 s were used for the 2-h images, and 600 s for the 24-h images, in order to compensate for the rapid whole-body clearance of the sFv. Radioimmunomaging. Mice with tumors ranging from 100 to 200 mg were given injections i.v. with 100 µg of either 125I-741F8 sFv' or 125I-26-10 sFv' for imaging studies. At 2 or 24 h after injection, the mice were immobilized by treatment with chloral hydrate (0.1 ml/10 g of body weight), and images were acquired on a gamma camera (Picker Dynacamera, Series-5; Cincinnati, OH) using a high-energy collimator. Preset imaging periods of 200 s were used for the 2-h images, and 600 s for the 24-h images, in order to compensate for the rapid whole-body clearance of the sFv. Autoradiography. The specificity of each radiopharmaceutical in mice was also evaluated by whole-body sagittal section autoradiographs. Female C:B17/ls-cscid mice (2 to 3 per group) bearing SK-OV-3 xenografts on their hips were given injections i.v. with 100 µg of either 125I-741F8 sFv', 125I-741F8 (sFv')2, or 125I-26-10 sFv' (about 100 µCi per mouse). The mice were sacrificed by i.p. injection of chloral hydrate (0.1 ml/10 g of body weight) and

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RESULTS

Purity, Stability and Binding Characteristics. The 741F8 and 26-10 sFv' monomers were ≥95% pure by reduced SDS-PAGE analysis. After oxidation to homodimers, the corresponding purified (sFv')2 proteins were monitored on nonreducing SDS-PAGE and found to be stable in the pure state and in vivo following i.v. administration, where 4-h plasma samples were examined by SDS-PAGE and migration of the radiolabeled protein was determined by autoradiography. The native properties of the 741F8 (sFv')2 were monitored on a BIAcore system and by analytical ultracentrifugation, making use of c-erbB-2 ECD produced in CHO cells. The kinetics of association and dissociation of immobilized 741F8 IgG and soluble c-erbB-2 ECD, determined on the BIAcore, was fitted by a single reaction component with an overall association constant of about 0.5 × 10^8 M−1. The association of c-erbB-2 ECD and the divalent (sFv')2 was also described by a single reaction component, but the dissociation kinetics was biphasic (about 5- to 10-fold difference). The source of this heterogeneity is being investigated and could be associated with the immobilization chemistry and/or the small size of the divalent (sFv')2 molecule. Using the slower dissociation rate constant, which was similar to that of the parent IgG, the overall association constant was 0.5 × 10^8 M−1. The results of both velocity and equilibrium sedimentation indicated that neither 741F8 (sFv')2 nor ECD underwent significant self-association. Velocity sedimentation analysis showed that, when there was a slight excess of 741F8 (sFv')2 [3.5 S (sedimentation coefficient) over ECD (4.3 S), essentially all of the ECD formed bivalent complexes (6.8 S) (Fig. IA). The 741F8 (sFv')2 sedimentation coefficient of about 3.5 S is similar to the value commonly observed for Fab fragments. These sedimentation velocity files were consistent with an association constant on the order of 10^7 M−1 (data not shown). Equilibrium sedimentation analysis showed that the stoichiometry of association was one molecule of 741F8 (sFv')2 to two molecules of ECD with essentially no free ECD remaining (Fig. 1B). The c-erbB-2 ECD protein mass is about 70 kDa, which is additionally glycosylated such that ECD from CHO cells exhibits a molecular weight on SDS-PAGE of about 105,000 and a minimum weight-average of about 85,000 by analytical ultracentrifugation under native conditions. It is not unusual for SDS-PAGE results to deviate from solution thermodynamic measurements of native molecular weights for highly glycosylated proteins. Pharmacokinetics. The whole-body and blood clearances of 125I-741F8 sFv' from the tumor-bearing scid mice were extremely rapid and biphasic (Fig. 2). The initial blood distribution phase half-life (t1/2b) was 0.2 h for the 125I-741F8 sFv', while the terminal blood elimination phase half-life (t1/2e) was 3.9 h. The cumulative blood retention over the course of the study, expressed as blood level under the curve, was 51.4 μg/ml. Total-body half-life (K1/2b) was 4.4 h. The whole-body and blood clearances of the 125I-26-10 sFv' (data not shown) did not significantly differ from those of the 125I-741F8 sFv'. Consistent with its larger size, at 24 h after injection, the blood retention of the 125I-741F8 sFv' (0.17% injected dose/ml) was 3 times greater than that of the monomer (0.05% of injected dose/ml). Biodistribution Studies: 741F8 sFv' Monomer. Groups of 5 to 6 scid mice bearing 200 to 300 mg of subcutaneous SK-OV-3 tumors were sacrificed at various times following the i.v. administration of

monomeric 125I-741F8 sFv'. In an experiment representative of five such studies, shown in Table 1, the %ID/g retained in tumor was 2.9% at 4 h following the injection. Twenty-four h following the injection, approximately 1%ID/g remained in tumor, while considerably lower %ID/g values were noted in normal organs. Tumor:normal organ ratios were greatest at 24 and 48 h. This resulted from the kinetics of the first 24 h, during which the clearance of the sFv' from tumor was slower than from blood and antigen-negative normal tissues. Although the T:O ratios were slightly higher at 48 h, most of the comparative biodistribution studies which follow were performed at 24 h since this earlier time point was associated with higher levels of sFv' in tumor.

Antigen Specificity of Tumor Localization. The above findings do not distinguish antigen-specific sFv' localization from blood pool-
TUMOR TARGETING BY ANTIC-erbB-2 sFv' AND (sFv')2

Divalent 741F8 sFv’. Addition of a carboxyl-terminal Ser-Cys sequence to the sFv allowed disulfide bond formation between the sFv’ monomers, creating (sFv’)2 dimers. The biodistributions of glutathionyl-blocked 741F8 sFv’ monomers, divalent 741F8 (sFv’)2, and divalent 26-10 (sFv’)2 were compared in scid mice bearing SK-OV-3 tumors. One %ID/g of the blocked monomer was retained in tumor at 24 h, and the distribution to normal organs was similar to that seen with the unmodified 741F8 sFv’ monomer. The 741F8 (sFv’)2 (produced by the Ser-Cys linkage) exhibited identical tumor localization, but with significant accumulation in liver, lung, and spleen. The 26-10 (sFv’)2 showed less tumor localization (0.22%ID/g) at 24 h after injection ($P < 0.05$) and low normal tissue accumulation (Table 3).

To introduce more steric flexibility into the divalent 741F8 (sFv’)2 designs, the following longer spacers were used to join the monovalent sFv’ units: Gly4Cys2Gly4 (designated Gly4Cys); MCA-Gly-Ser3Gly2Ser3Lys-MCA (designated MCA); and the homobifunctional cross-linker bismalimidohexane (designated BMH). The biodistribution of these peptide-linked (sFv’)2 molecules was studied in scid mice bearing subcutaneous SK-OV-3 xenografts (Table 3). The three 741F8 (sFv’)2 species created with long spacers exhibited superior tumor localization when compared with the 741F8 sFv’ monomer, without loss of specificity. A separate comparison study was performed with 125I-741F8:sFv’ monomer; Fab; Gly4Cys-linked (sFv’)2; MCA-linked (sFv’)2; and BMH-linked (sFv’)2. In a pooled analysis, the tumor localization of the divalent peptide-linked 741F8 (sFv’)2 preparations was significantly greater than that observed with the Fab ($P < 0.05$), the sFv’ monomer ($P < 0.00001$), and the 26-10 (sFv’)2 ($P < 0.00001$) (Fig. 5). The tumor retention of the BMH-linked 741F8 (sFv’)2 species significantly exceeded that of the 741F8 Fab ($P < 0.05$). The average 24-h tumor retention of the BMH and both peptide linked 125I-741F8 (sFv’)2 molecules, incorporating values pooled from five separate studies, was 1.84 ± 0.09%ID/g and was significantly greater than the 0.89 ± 0.07%ID/g observed in five pooled 125I-741F8 sFv’ monomer studies ($P < 0.00001$).

Macror autoradiography. Whole-body sagittal sections of mice bearing SK-OV-3 tumor xenografts, treated with 125I-741F8 sFv’, Gly4Cys-linked divalent (sFv’)2 or 125I-26-10 sFv’, showed further evidence of specificity of targeting of the 741F8 sFv’ preparations distributed diffusely throughout the tumor, while the kidney contained significantly less activity. With the 125I-26-10 sFv’, only the kidney could be visualized as a result of the injected dose; in the figure shown, the

Table 1: Comparison of biodistributions of 125I-741F8 sFv’ and 125I-26-10 sFv’

One hundred µg of either 125I-741F8 sFv’ (specific for c-erbB-2) or the control, 125I-26-10 sFv’ (specific for digoxin), were injected i.v. into scid mice bearing s.c. SK-OV-3 tumors expressing c-erbB-2. The average %ID/g and the SEM ≤ 20% of the value, unless indicated) were determined.

<table>
<thead>
<tr>
<th>Organs</th>
<th>741F8 sFv</th>
<th>26-10 sFv</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Tumor</td>
<td>5.64</td>
<td>2.89</td>
</tr>
<tr>
<td>Liver</td>
<td>4.05</td>
<td>1.05</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.61</td>
<td>1.44</td>
</tr>
<tr>
<td>Kidney</td>
<td>27.48</td>
<td>2.05</td>
</tr>
<tr>
<td>Lung</td>
<td>7.20</td>
<td>1.40</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.87</td>
<td>0.51</td>
</tr>
<tr>
<td>Heart</td>
<td>4.06</td>
<td>1.01</td>
</tr>
<tr>
<td>Stomach</td>
<td>18.98</td>
<td>4.09</td>
</tr>
<tr>
<td>Intestine</td>
<td>3.28</td>
<td>1.53</td>
</tr>
<tr>
<td>Bone</td>
<td>2.17</td>
<td>0.45</td>
</tr>
<tr>
<td>Blood</td>
<td>7.80</td>
<td>1.10</td>
</tr>
</tbody>
</table>

*SEM ≤ 40% of the value shown.

**SEM ≤ 30% of the value shown.

***SEM ≤ 20% of the value shown.

* Represented as %ID/ml.
Table 2 Comparison of tumor: organ ratios of 125I-741F8 sFv' and 125I-26-10 sFv'

One hundred μg of 125I-sFv' were injected i.v. into C.B17/ICr-scid mice bearing SK-OV-3 ovarian carcinoma xenografts. Groups of 4 to 6 mice were sacrificed at the indicated times, the %ID/g values of tumor and normal organs were determined for each radiopharmacetical, and the T:O ratios (%ID/g tumor divided by the %ID/g organ) were calculated. %ID/g values for the 741F8 sFv' are found in Table 1. The SEM of each listed value was less than 25%. The specificity index was derived by determining the T:O ratios of 74I8 sFv' to 26-10 sFv'.

<table>
<thead>
<tr>
<th>Organ</th>
<th>125I-741F8 sFv'</th>
<th>125I-26-10 sFv'</th>
<th>Specificity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.4</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.2</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.2</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Muscle</td>
<td>3.0</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Heart</td>
<td>1.4</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.7</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Intestine</td>
<td>2.6</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Bone</td>
<td>0.7</td>
<td>0.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Tumorblood ratios were determined by dividing the %ID/g of tumor by the %ID/ml of blood.

Fig. 3. Tumor retention of 125I-741F8 sFv' and 125I-26-10 sFv'. Groups of 4 to 5 C.B17/ICr-scid mice bearing 100 to 200 mg of SK-OV-3 tumor were sacrificed 1, 4, or 24 h following i.v. injections of 125I-labeled 741F8 sFv' (D), Gly4Cys-linked (sFv')2 (M), or 26-10 sFv' (•). The %ID/g in tumor was calculated as described in the text. Columns, mean; bars, SE.

Discussion

In this paper we describe the in vivo properties of sFv-based molecules with the specificity of the anti-c-erbB-2 monoclonal antibody, 741F8. Monovalent 741F8 sFv' and 26-10 sFv' exhibited rapid, biphasic clearance from blood, while a slightly slower clearance was observed with the divalent 741F8 (sFv')2 bridged by Gly4Cys2Gly4. Following i.v. injection, the 741F8 sFv' monomer was selectively retained in SK-OV-3 tumor, with excellent tumor:normal organ ratios.

The specificity of this tumor targeting with 741F8 sFv' was demonstrated by the lack of retention of the anti-digoxin 26-10 sFv', as evaluated by biodistribution studies, gamma camera imaging, and cromocautoradiography studies. Divalent 741F8 (sFv')2 species with extended spacers exhibited similar tumor specificities to those observed with the monomers, with greater tumor retention than was observed with 26-10 [sFv' or (sFv')2], 741F8 sFv' monomers, or 741F8 Fab. This first report of in vivo targeting of tumors by divalent forms of sFv molecules suggests that improved retention of specific divalent (sFv')2 by tumors may have important consequences for diagnostic or therapeutic strategies.

741F8 sFv' species exhibit specific binding to c-erbB-2 by multiple methodologies. Binding of cloned c-erbB-2 ECD to 741F8 (sFv')2 immobilized on a chip in a BIAcore system showed an association constant of approximately 0.5 x 10^10 M^-1 when the slower of the biphasic dissociation rates was utilized. The 741F8 sFv' constructs also bound to SK-OV-3 cells overexpressing c-erbB-2 in live cell binding and competition assays and interfered with binding of labeled 741F8 IgG as determined by flow cytometry studies (data not shown).

Velocity sedimentation and equilibrium sedimentation studies performed with divalent 741F8 (sFv')2 and c-erbB-2 ECD demonstrated that the 741F8 (sFv')2 binds antigen bivalently. Furthermore, given that the molecular weights and sedimentation coefficients of Fab and (sFv')2 are nearly identical, these hydrodynamic data indicate that the free 741F8 (sFv')2 must have a compact shape similar to that of a Fab. Instead of constant domains being connected to V domains in a Fab, there is a disulfide bond between the C-terminal cysteines of each sFv' in an (sFv')2 homodimer. The sedimentation coefficient of 741F8 (sFv')2 is consistent with bottom-to-bottom association of the two sFv' units of the homodimer. Moreover, such a geometry is compat-

Table 3 Biodistribution of divalent (sFv')2 at 24 h

One hundred μg of each listed 125I-labeled sFv' were injected i.v. into C.B17/ICr-scid mice bearing 100- to 300-mg s.c. SK-OV-3 cell human ovarian carcinoma xenografts. Groups of 3 to 6 mice were sacrificed 24 h following injection, and the %ID/g and T:O ratios were calculated as described in the text. The SEM of each value was less than 30%, except where indicated.

<table>
<thead>
<tr>
<th>Organ</th>
<th>741F8 sFv'</th>
<th>741F8 (sFv')2</th>
<th>26-10 (sFv')2</th>
<th>741F8 Gly4Cys-(sFv')2</th>
<th>741F8 MCA-(sFv')2</th>
</tr>
</thead>
<tbody>
<tr>
<td>%ID/g T:O</td>
<td>%ID/g T:O</td>
<td>%ID/g T:O</td>
<td>%ID/g T:O</td>
<td>%ID/g T:O</td>
<td>%ID/g T:O</td>
</tr>
<tr>
<td>Tumor</td>
<td>1.00</td>
<td>1.00*</td>
<td>1.00*</td>
<td>1.00*</td>
<td>1.00*</td>
</tr>
<tr>
<td>Liver</td>
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<td>0.10</td>
<td>13.4*</td>
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</tr>
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<td>16.4*</td>
<td>0.64</td>
<td>16.4*</td>
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<td>49.8*</td>
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<td>0.02</td>
<td>51.2*</td>
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<tr>
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<td>16.5</td>
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<tr>
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<tr>
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<td>58.1*</td>
<td>0.03</td>
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<td>0.03</td>
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<tr>
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<td>11.7*</td>
<td>0.09</td>
<td>11.7*</td>
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</tr>
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</table>

* SEM <40% of depicted value.
TUMOR TARGETING BY ANTI-c-erbB-2 sFv' AND (sFv')2

Fig. 4. Imaging of subcutaneous SK-OV-3 tumors in C.B17/Scid mice by 131I-741F8 sFv' and 131I-26-10 sFv'. Gamma camera images obtained in a mouse treated with 131I-741F8 sFv' and (A) in a mouse treated with 131I-26-10 sFv' (B). The body shape of the mouse is outlined for reference. The mice were imaged at 2 h and 24 h. Camera access time was lengthened to permit acquisition of images at 24 h, when there were lower %ID/g values in tumor and normal organs. Note that with equivalent imaging of the thyroid gland in both mice at 24 h, there are marked differences in tumor imaging.

The specific in vivo localization of 741F8 sFv' and (sFv')2 to tumors expressing c-erbB-2 provides quantitative evidence of specificity for sFv retention which has not been previously reported. Yokota et al. (8) have used microscopic autoradiography and grain counting to show that the retention of CC49 sFv in TAG-72 antigen-positive LS-174T human colon carcinoma xenografts in nude mice is specific when compared with 4-4-20, an sFv with irrelevant specificity. The advantage of that approach rests in its ability to examine the degree of diffusion of the sFv molecules from capillaries and to estimate the degree and uniformity of sFv tumor penetration. The specificity studies presented in the present paper are of importance because specificity is reliably quantitated for multiple organs, and meaningful specificity indices can be derived. The specificity index of monovalent 741F8 sFv', measured by %ID/g, was 13.2:1 for tumor and 0.8 to 2.1 for all tested normal organs (data not shown). This leads to specificity indices for T:O ratios (Table 2) which range from 7.0 (kidneys) to 16.7 (intestines). Comparing divalent 741F8 (sFv')2 with the 26-10 (sFv')2, similar patterns emerge, with specificity indices for sFv retention in tumor of 16.9 [Gly4Cys-linked (sFv')2] and 8.5 [MCA-linked (sFv')2], respectively. Specificity indices for tumor:organ ratios with the MCA-linked 741F8 (sFv')2 ranged from 2.9 (blood) to 13.2 (kidneys), while those for the Gly4Cys-linked 741F8 (sFv')2 ranged from
Fig. 6. Sagittal section autoradiography of mice bearing s.c. c-erbB-2(+) SK-OV-3 tumors in the thigh. A, section of whole mouse demonstrating locations of major organs. B to D, autoradiographs of mice which received i.v. injections labeled sFv'. In B, 125I-26-10 sFv', specific for digoxin, is not observed in the tumor, and activity in the stomach is likely due to dehalogenated iodine or ingestion of contaminated food. In C, 125I-741F8 sFv' monomer and, in D, divalent Gly\(^5\)Cys-linked 125I-741F8 (sFv')\(_2\) are both specifically localized in tumor, with comparatively little activity in normal organs. All mice received a radioactive dose of ~25 to 35 \(\mu\)Ci, \(n = 2\) animals per treatment group. Mice given injections in tail veins with radiopharmaceuticals were sacrificed at 24 h after injection by anesthetizing with chloral hydrate and flash freezing in an acetone-dry ice bath. The frozen mice were embedded in carboxymethylcellulose, and sagittal sections were taken using a PMV-2250 cryocryotome at -17°C. Sections of 50-\(\mu\)m thickness were taken every 250 \(\mu\)m. The sections were then desiccated at -17°C for 3 to 4 days, and autoradiograms were prepared by exposing the sections to LoDose mammography film (DuPont MRF33 BLUE) for various time intervals. The film was developed using standard procedures.

3.4 (spleen) to 6.0 (kidneys). The minor decline in specificity seen with the divalent forms is accounted for by their slightly longer retention in blood and normal organs. These data clearly demonstrate that, following their i.v. administration, both monovalent and divalent forms of 741F8 sFv are specifically retained by SK-OV-3 tumors. This antigen-specific effect precludes any possibility that, after the 1-h time point, passive diffusion and pooling in the tumor interstitium contribute significantly to the localization of these molecules.

The quantitative delivery of monovalent 125I-741F8 sFv' to tumor is low, with only 1%ID/g retained in tumor 24 h after i.v. administration. These values are typical of those reported for other sFv molecules and likely result from their rapid clearance from circulation (6). Thus, optimal tumor selectivity may result in lower retention of sFv by targets. Modified schedules of sFv administration may allow longer blood retention to provide a gradient for sFv penetration into tumor. Although some tumor selectivity may be sacrificed, such losses may be warranted by the more uniform tumor penetration exhibited by sFv proteins over the corresponding Fab, F(ab')\(_2\), or IgG (8). The potential therapeutic value of prolonged sFv infusions over intermittent bolus injections is suggested by the improved outcome observed in a recently reported model of immunotoxin therapy using a fusion protein composed of an anti-c-erbB-2 sFv and Pseudomonas exotoxin (27).

To date, the optimal antibody-based construct for targeted diagnostic and therapeutic strategies has not been identified. IgG molecules have the advantages of divalent binding, but they penetrate tumor...
poorly due to their large size and interact with host effector elements via their Fc domain (1). F(ab')2 fragments of IgG molecules are probably preferable to IgG, but their large size precludes first-pass elimination via the kidneys and limits diffusion through tumors (28). Fab fragments have the advantages of small size, but they suffer from both excess retention in kidneys and their monovalent binding characteristics (29). As presented here, and in other reports (6), SFv monomers exhibit excellent tumor specificity and penetration characteristics, but at the cost of quantitative tumor retention. Pack and Pluckthun (30) have described the production of (SFv')2 molecules using amphophilic helices, but have not described the in vivo characteristics of these proteins. A variety of other SFv-derived molecular species now can be prepared and tested for clinical applications (31).

To determine the relative influences of rapid blood clearance and the monovalent binding of SFv monomers to their targets, we prepared (SFv')2 molecules which exhibit divalent binding characteristics and equivalent binding affinity to parental IgG. Such Mₙ, 53,000 proteins remain below the threshold for renal clearance and indeed are cleared from the blood nearly as rapidly as SFv monomers, apparently without in vivo disruption of the (SFv')2 interchain disulfide bond. The studies presented in this paper demonstrate the superior tumor retention of appropriately constructed 741F8 (SFv')2 over 741F8 SFv'. Since the blood retention of the (SFv')2 is slightly more prolonged than that of the monomer, it was necessary to demonstrate that the increased tumor localizations of the (SFv')2 species were due to their divalent nature rather than to the blood gradient. The localization of the divalent BMH-linked 741F8 (SFv')2 was significantly greater than that of a monovalent Fab fragment of approximately the same size, suggesting that the increased avidity of the (SFv')2 is a factor in its improved tumor retention.

It remains to be demonstrated that the quantitatively limited, but highly specific delivery to tumor of 741F8 SFv' and (SFv')2 will prove useful in targeted diagnostic or therapeutic strategies in patients with c-erbB-2-expressing malignancies. Caution is required when extra tumor retention.

TUMOR TARGETING BY ANTI-c-erbB-2 SFv' AND (SFv')2

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Highly Specific *in Vivo* Tumor Targeting by Monovalent and Divalent Forms of 741F8 Anti-c-erbB-2 Single-Chain Fv

Gregory P. Adams, John E. McCartney, Mei-Sheng Tai, et al.


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