Increased Affinity Leads to Improved Selective Tumor Delivery of Single-Chain Fv Antibodies

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ABSTRACT

M, 25,000 single-chain Fv (scFv) molecules are rapidly eliminated from the circulation of immunodeficient mice, yielding highly specific retention of small quantities of scFv in human tumor xenografts. We postulated that the specific retention of scFv in tumor could be enhanced by engineering significant increases in the affinity of the scFv for its target antigens. Affinity mutants of the human anti-HER2/neu (c-erbB-2) scFv C6.5 were generated by site-directed mutagenesis, which target the same antigenic epitope with a 320-fold range in affinity (3.2 x 10^-7 to 1.0 x 10^-9 M). In vitro, the Kd of each scFv correlated closely with the duration of its retention on the surface of human ovarian carcinoma SK-OV-3 cells overexpressing HER2/neu. In biodistribution studies performed in scid mice bearing established SK-OV-3 tumors, the degree and specificity of tumor localization increased significantly with increasing affinity. At 24 h after injection, tumor retention of the highest affinity scFv was 7-fold greater than that of a mutant with 320-fold lower affinity for HER2/neu. Because the rapid renal clearance of scFv may blunt the impact of improved affinity on tumor targeting, the distributions were also assayed in the absence of renal clearance (e.g., in mice rendered surgically anephric). In this model, the peak tumor retentions of the two higher affinity scFv approximated that reported previously for IgG targeting the same SK-OV-3 tumors in scid mice with intact kidneys. In contrast, the mutant with the lowest affinity for HER2/neu failed to accumulate in tumor, indicating the presence of an affinity threshold that must be exceeded for active in vivo tumor uptake. These results indicate that affinity can significantly impact the in vivo tumor-specific retention of scFv molecules.

INTRODUCTION

The intrinsic properties of immunoglobulin molecules and their fragments regulate the in vivo biodistribution properties of these molecules in tumor-bearing hosts. Intact IgG molecules are large (M, 150,000) glycoproteins that exhibit a slow systemic clearance, leading to poor tumor targeting specificity (1). Furthermore, IgG intratumoral diffusion is limited by size to a penetration rate of 1 mm every 2 days, potentially resulting in heterogeneous deposition in tumor (2). Smaller antibody-derived molecules include enzymatically produced scFv, with improved affinity on tumor targeting, the distributions were also assayed in the absence of renal clearance (e.g., in mice rendered surgically anephric). In this model, the peak tumor retentions of the two higher affinity scFv approximated that reported previously for IgG targeting the same SK-OV-3 tumors in scid mice with intact kidneys. In contrast, the mutant with the lowest affinity for HER2/neu failed to accumulate in tumor, indicating the presence of an affinity threshold that must be exceeded for active in vivo tumor uptake. These results indicate that affinity can significantly impact the in vivo tumor-specific retention of scFv molecules.

MATERIALS AND METHODS

Production of C6.5 scFv and C6.5-based scFv Affinity Mutants. C6.5 is a HER2/neu binding human scFv isolated from a nonimmune phage antibody library (12, 14). C6O98A was identified during alanine scanning of the C6.5 Vh CDR3 and differs from C6.5 by one amino acid, having a glycine to alanine substitution at Vh residue 98 (13). C6ML3-9 was isolated from a phage antibody library of C6.5 mutants and differs from C6.5 by three amino acid substitutions in the Vh CDR3 (13). For the work described here, these C6.5-based scFv affinity mutants were used to examine the effect of affinity and koff on in vitro and in vivo tumor retention of scFv.

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3 The abbreviations used are: scFv, single-chain Fv; CT, chloramine-T; T:O, tumor:organ; scid, severe combined immunodeficient; %IDg, percent injected dose per gram; MoAb, monoclonal antibody; PE, phycoerythr.
Epitope Retention Assay. The ability of an unlabeled C6.5 affinity mutant to displace biotinylated C6.5 was determined using flow cytometry. One microgram of biotinylated C6.5 was added along with unlabeled scFv (C6.5, C6ML3-9, C6G98A, or 26-10) at a competitor:C6.5-biotin molar ratio of 1:1 or 10:1 to 1 × 10^6 SK-OV-3 human ovarian carcinoma cells (#HTB 77; American Type Culture Collection, Rockville, MD), which overexpress HER2/neu. Reactions were performed in a total volume of 100 μl of FACS buffer (PBS with 0.1% sodium azide, pH 7.2). After a 15-min incubation at 37°C, the cells were washed twice with 2 ml of FACS buffer and fixed by the addition of 100 μl of 1% paraformaldehyde. Fifty μl of a 1:800 dilution of streptavidin-phycocerythrin (TAGO, Inc., Burlingame, CA) were then added to each tube; the samples were incubated for 15 min at 4°C and then washed twice with FACS buffer. The degree of fluorescence was determined by analysis on a FACSCan flow cytometer (Becton Dickinson, San Jose, CA) as described (19).

Cell Surface Retention Assay. To assess the impact of affinity on the retention of the scFv affinity mutants with cell-bound HER2/neu, an in vitro cell surface retention assay was performed. In this assay, 0.1% sodium azide was included to prevent artificially prolonged retention of the scFv-biotin resulting from internalization. Twelve μg of biotinylated scFv was incubated with 1.2 × 10^7 SK-OV-3 cells in a total volume of 0.5 ml of FACS buffer for 30 min at room temperature. The cells were centrifuged at 500 × g for 5 min at 4°C, washed with 10 ml of ice-cold FACS buffer two times, and then resuspended gently in 12 ml of FACS buffer at 37°C. The cell suspensions then were incubated at 37°C with gentle shaking in a water bath. To decrease the rebinding of dissociated biotinylated scFv to the cells, at 15, 30, 45, 60, 90, and 120 min after commencing the incubation, the suspensions were pelleted at 500 × g, the supernatants were aspirated, and the cells were gently resuspended in fresh FACS buffer (37°C). Immediately after each round of pelleting and resuspension, 0.5-ml aliquots containing 5 × 10^6 cells were removed to triplicate (i.e., at 0, 15, 30, 45, 60, 90, and 120 min), placed on ice for 5 min and centrifuged at 500 × g for 5 min at 4°C. After removing the supernatants from the aliquots, the cells were gently resuspended in 50 μl of ice-cold FACS buffer containing 50 μl of a 1:800 dilution of streptavidin-PE, incubated on ice for 30 min, and washed twice with FACS buffer at 4°C. The cells were fixed with 1% paraformaldehyde, and the degree of fluorescence was determined by analysis on a FACSCan flow cytometer as described above.

Radioiodination. All scFv were labeled with 125I using the CT method as described previously (19). Briefly, 1.0–2.0 μg of protein were combined with 125I (NEZ033H; DuPont NEN, Wilmington, DE) at an iodine:protein ratio of 1:10. Ten μg of CT (Sigma Chemical Co., St. Louis, MO) were added per 100 μg of protein, and the resulting mixture was incubated for 3 min at room temperature. Unincorporated radiiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method (20). The final specific activity of the 125I-scFv molecules were about 1.0 μCi/μg. The quality of the radiopharmaceuticals was evaluated by high-performance liquid chromatography, SDS-PAGE, and a live cell-binding assay. The high-performance liquid chromatography analysis was performed using a Superox 75 column (Pharmacia). Eluted fractions were collected and counted in a gamma well counter (Beckman) (5). The elution profiles consistently demonstrated that greater than 99% of the radioactivity was associated with the protein peak. The radiodiobinated scFv molecules were evaluated by SDS-PAGE. Reduced and nonreduced SDS-PAGE were run using 12% gels (10 × 12 cm; 120.75HMC-10P; Jule, Inc., New Haven, CT; Refs. 5 and 21). PAGE, Reduced and nonreduced SDS-PAGE were run using 12% gels (10 × 12 cm; 120.75HMC-10P; Jule, Inc., New Haven, CT; Refs. 5 and 21). The results of the live cell-binding assays indicated that the radiodiobinated scFv were still reactive with cell surface HER2/neu. The activity associated with the cell pellet was 61.2, 59.0, 5.3, and 2.7%, respectively, for C6ML3-9, C6.5, C6G98A, and 26-10. The poor retention of the C6G98A was consistent with its rapid dissociation rate from the HER2/neu.

Biodistribution Studies. Four to 6-week-old inbred C.B17/irr-scid mice or outbred Icr:Ha(ICH)-scid mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. SK-OV-3 cells (2.5 × 10^6) in log phase were implanted s.c. on the abdomens of the mice. After 6–8 weeks, the tumors had achieved sizes of ~100 mg, and Lugol’s solution was placed in the drinking water to block thyroid accumulation of radiodine. Two days later, biodistribution studies were initiated.

Initial studies were performed with escalating doses to determine the quantity of each scFv required to saturate the tumor antigen pool. Doses of 5, 25, 125, or 625 μg of radiodiobinated C6.5, C6ML3-9, or 26-10 scFv (negative control) were selected based upon our previous observation that a 125-μg dose of the C6.5 scFv is capable of saturating the tumor antigen pool.4 The scFv molecules were administered by tail vein injection to cohorts of three tumor-bearing outbred scid mice. The mice were sacrificed at 4 h after injection, and the tumors and organs were removed, weighed, and counted in a gamma counter to determine the percentage of the injected dose localized per gram of tissue (%ID/g) for each labeled scFv (5, 22). The degree of tumor-specific retention was determined by subtracting the value of the 26-10 (irrelevant) tumor:blood ratios from that observed with each dose group for the anti-HER2/neu scFv molecules. Saturation was defined as the dose above which specific retention did not increase.

To study the impact of affinity on tumor retention, 125I-scFv (C6.5, C6ML3-9, C6G98A, or 26-10) was administered to cohorts of six inbred scid mice by tail vein injection at the dosage determined above to just saturate the tumor antigen with C6ML3-9 (25 μg). Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model 2007; Canberra, Meridian, CT). Blood samples and whole-body counts of the mice were determined immediately after injection and just prior to euthanization. Groups of six mice were sacrificed at 4 and 24 h after injection; tumor, organ, and blood retentions were determined as described above. The mean and SE for each group of data were calculated, and T:O ratios were determined. Significance levels were determined using the Wilcoxon rank sum test.

Additional studies were performed on surgically neoprectomized mice to determine the impact of affinity on biodistribution in the absence of renal clearance. Male scid mice bearing established s.c. SK-OV-3 tumors were lightly anesthetized with metofane (Mallinckrodt Veterinary, Mundelein, IL), and contralateral retroperitoneal incisions were made. The kidneys were identified, ligated with 4-0 silk, and excised from the mice with special care taken to prevent damage to the adrenal blood supply. The incisions were then closed with surgical staples. Biodistribution studies were initiated 1 h later. Twenty μg of 125I-scFv (C6.5, C6ML3-9, or C6G98A) were administered to cohorts of three neoprectomized, inbred scid mice by tail vein injection. Total injected doses were determined as described above. Blood samples and whole-body counts of the mice were collected immediately after injection and just prior to euthanization. Mice were sacrificed at 4 and 24 h after injection, and tumor, organ, and blood retentions were determined as described above. The mean and SE for each group of data were calculated, and T:O ratios were determined. Significance levels were determined using the Wilcoxon rank sum test.

RESULTS

Amino Acid Sequences and Affinity Constants of C6.5-derived scFv Molecules. The amino acid sequences of C6G98A and C6ML3-9 differ from each other and from the C6.5 scFv from which they were derived by only a few amino acids in the V_H and V_L CDR3 domains (Table 1). The binding kinetics (k_a and k_μ) of C6G98A, C6.5, and C6ML3-9 for HER2/neu ECD were determined using surface plasmon resonance, and the results were used to calculate the K_d (Table 1). The K_d of the parental molecule (C6.5) was 1.6 × 10^{-8} M, which was in close agreement to the K_d of 2.0 × 10^{-8} M determined previously by Scatchard analysis for radiolabeled C6.5 binding to HER2/neu expressing SK-OV-3 cells (12). Mutant K_d ranged from

4 Unpublished data.
Table 1: Affinity and binding kinetics of C6.5-based scFv

<table>
<thead>
<tr>
<th>scFv clone</th>
<th>$K_a$ (M)</th>
<th>$k_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$V_H$ CDR3</th>
<th>$V_L$ CDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6.5</td>
<td>$1.6 \times 10^{-8}$</td>
<td>$4.0 \times 10^{-5}$</td>
<td>$6.3 \times 10^{-3}$</td>
<td>HDVGYCSSNCAKWEYFOH</td>
<td>AAADDLSLGWV</td>
</tr>
<tr>
<td>C6ML3-9</td>
<td>$1.0 \times 10^{-7}$</td>
<td>$7.8 \times 10^{-5}$</td>
<td>$7.8 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6G98A</td>
<td>$3.2 \times 10^{-7}$</td>
<td>$4.1 \times 10^{-5}$</td>
<td>$1.4 \times 10^{-1}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20-fold less than C6.5 (C6G98A, $K_a = 3.2 \times 10^{-7}$ M) to 16-fold greater than C6.5 (C6ML3-9, $K_a = 1.0 \times 10^{-9}$ M), resulting in a 320-fold range of affinities and a 170-fold range of $k_{off}$.

**Determination of Epitope Conservation.** To ensure that the fingerprint specificity of C6.5 mutants was maintained, a competition assay was performed. In this assay, biotinylated C6.5 was mixed with each unlaunched mutant scFv in either a 1:1 or 1:10 molar ratio. Following an incubation with SK-OV-3 cells overexpressing HER2/neu, the amount of C6.5-biotin bound to the surface of the cells was determined by flow cytometry. Each C6.5 mutant successfully competed with C6.5-biotin for the target antigen, whereas the 26-10 scFv negative control had no effect (Table 2). As expected, the highest affinity C6.5 mutant (C6ML3-9) displayed significant binding competition even at the lowest molar ratio. C6G98A, the scFv with the lowest affinity, required the highest molar ratio to compete with biotinylated C6.5.

**In Vitro Cell Surface Retention.** The in vitro cell surface retention of biotinylated C6.5 and C6ML3-9 were determined, both to verify the observed differences in $k_{off}$ and to confirm that binding to the antigen in the BIAcore instrument had biological significance. The lowest affinity clone, C6G98A, was not assayed in vitro because it exhibited a dissociation from the cell surface that was too rapid to quantify by flow cytometry. SK-OV-3 cells were washed with biotinylated scFv and washed to remove unbound scFv, and the cells were diluted in buffer at 37°C. The cells were washed at intervals to remove dissociated scFv-biotin, and aliquots were removed, fixed with paraformaldehyde to prevent dissociation of the bound scFv-biotin, and incubated with streptavidin-PE. Samples were analyzed by flow cytometry to determine the percent of biotinylated scFv bound to the cell surface. Representative results from one of two separate experiments are presented in Fig. 1. The half-life ($t_{1/2}$) of the scFv on the cell surface was approximately 2.5 min for C6.5 and 11 min for C6ML3-9. The 26-10 control scFv did not bind to the cells. These values agreed closely with the $t_{1/2}$ calculated from the $k_{off}$ as determined by surface plasmon resonance (1.6 min for C6.5 and 13 min for C6ML3-9), assuming a simple exponential decay ($e^{-kt}$, where $k = k_{off}$).

**Biodistribution Studies.** Preliminary biodistribution studies were performed to determine the dose of scFv required for tumor antigen saturation. Escalating doses of radioiodinated anti-HER2/neu C6.5, C6ML3-9, or the irrelevant 26-10 scFv were administered to tumor-bearing scid mice. The mice were sacrificed at 4 h after about one elimination phase half-life ($t_{1/2}$), and tumor:blood ratios were determined. The dose of each anti-HER2/neu scFv required to saturate the tumor antigen pool was determined after subtracting the activity attributable to blood and extravascular spaces as defined by the irrelevant 26-10 scFv. Saturation of the HER2/neu on the SK-OV-3 tumors within the first 4 h after injection occurred at doses of 25 μg (C6ML3-9) and 125 μg (C6.5).

Twenty-four-h biodistribution studies then were performed in scid mice bearing s.c. SK-OV-3 tumors to examine the role of affinity on the specificity and degree of tumor retention. These assays used $^{125I}$-labeled forms of C6G98A, C6.5, C6ML3-9, and a negative control scFv (26-10) at the dose of 25 μg, which was determined above to achieve early tumor saturation with C6ML3-9. The HER2/neu-specific scFv were selected to provide the following stepwise increase in affinity: C6G98A (3.2 × 10$^{-7}$ M), C6.5 (1.6 × 10$^{-8}$ M), and C6ML3-9 (1.0 × 10$^{-9}$ M). The biodistribution studies revealed a correlation between the affinity and the %ID/g of the radioiodinated scFv retained in tumor (Table 3). The greatest degree of tumor retention was observed with $^{125I}$-C6ML3-9 (1.42 ± 0.23 %ID/g). At 24 h, significantly less tumor retention was achieved with $^{125I}$-C6.5 (0.80 ± 0.07 %ID/g) and C6G98A (0.19 ± 0.04 %ID/g). The tumor retention of the lowest affinity clone $^{125I}$-C6G98A was significantly less than that of C6.5 ($P = 0.0001$) and was identical to that of the negative control $^{125I}$-26-10. The T:O ratios also reflected the greater retention of higher-affinity species in tumor (Table 3). For example, tumor:blood ratios of 17.2, 13.3, 3.5, and 2.6 and tumor:liver ratios of 26.2, 19.8, 4.0, and 3.1 were observed for C6ML3-9, C6.5, C6G98A, and 26-10, respectively, at 24 h.

We postulated that the rapid systemic elimination of the small scFv

![In vitro cell surface retention assay.](image)
molecules would limit their perfusion of the tumors, thus hindering the assessment of the role of affinity on tumor targeting. Accordingly, biodistributions were performed with $^{125}$I-labeled C6.5, C6G98A, and C6ML3-9 in surgically nephrectomized mice to determine the impact of affinity in the absence of renal elimination. Although blood and normal organ retentions were similar for all three scFv molecules, the rate of accumulation in tumor was closely correlated with affinity for HER2/neu (Table 4). Tumor retention of C6ML3-9 scFv peaked at 17.3 %ID/g by 4 h after administration and remained constant through the end of the study, whereas C6.5 scFv accumulated in the tumors during the course of the study, peaking between the 4- and 24-h time points. The lowest affinity scFv, C6G98A, failed to accumulate in tumor over time and remained at below 10 %ID/g throughout the assay. However, in a separate biodistribution study performed in nephrectomized scid mice, the 24-h tumor retention of C6G98A was found to be 10-fold greater than that observed with the 26-10 negative control scFv (data not shown). These results suggest that the continued presence of C6G98A is capable of overcoming its low affinity, leading to the active maintenance of measurable tumor concentrations.

**DISCUSSION**

This is the first reported examination of the effects of affinity on tumor targeting by antibody based molecules that bind to the same epitope and antigen. The C6.5 scFv and its affinity mutants differed from each other by only one to three amino acid residues, yet differed in affinity for the same epitope of HER2/neu by 320-fold. The potential impact of affinity on the successful targeting and retention of MoAb in tumors has led to two disparate theories. Fujimori et al. (23) has postulated that high affinity MoAb will not be affected by the selective targeting of scFv to solid tumors.

### Table 3

Twenty-four-h biodistribution of $^{125}$I-labeled C6.5 and its affinity mutants in tumor-bearing scid mice

Twenty-five $\mu$g of iodinated scFv were administered to SK-OV-3 tumor-bearing scid mice by i.v. tail vein injection. Cohorts of six mice were sacrificed at 24 h after injection, and the %ID retained per gram of tissue or mL of blood was determined and the T:O ratios calculated. Values are presented for major organs of interest. Unless otherwise indicated, SEs were $\leq$36% of the indicated value.

<table>
<thead>
<tr>
<th>scFv</th>
<th>26-10</th>
<th>C6G98A</th>
<th>C6.5</th>
<th>C6ML3-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (Organs)</td>
<td>%ID/g</td>
<td>T:O</td>
<td>%ID/g</td>
<td>T:O</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.18</td>
<td>0.19</td>
<td>0.80</td>
<td>1.42</td>
</tr>
<tr>
<td>Blood</td>
<td>0.07</td>
<td>0.06</td>
<td>0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>Liver</td>
<td>0.06</td>
<td>0.10</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>Spleen</td>
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<td>0.06</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>Lung</td>
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<td>0.05</td>
<td>0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>Kidney</td>
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<td>0.22</td>
<td>0.11</td>
<td>0.15</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.18</td>
<td>0.18</td>
<td>0.15</td>
<td>0.35</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.02</td>
<td>0.93</td>
<td>0.05</td>
<td>0.03</td>
</tr>
</tbody>
</table>

### Table 4

Biodistribution of C6.5-based scFv molecules in anephric tumor-bearing scid mice

Twenty $\mu$g of $^{125}$I-labeled C6G98A ($K_d = 3.2 \times 10^{-7}$ M), C6.5 ($K_d = 1.6 \times 10^{-8}$ M) or C6ML3-9 ($K_d = 1.0 \times 10^{-9}$ M) were administered by i.v. tail vein injection to anephric scid mice bearing s.c. SK-OV-3 tumors (n = 3 mice/time point). Tissue and blood retention of the radiolabeled scFv molecules were determined as described in the text. Results are expressed as the %ID/g tissue (or mL blood). T:O ratios are presented in parentheses. SEs were less than 25% of the associated value, unless noted.

<table>
<thead>
<tr>
<th>scFv</th>
<th>26-10</th>
<th>C6G98A</th>
<th>C6.5</th>
<th>C6ML3-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (Organ)</td>
<td>%ID/g</td>
<td>T:O</td>
<td>%ID/g</td>
<td>T:O</td>
</tr>
<tr>
<td>Tumor</td>
<td>9.3</td>
<td>8.3</td>
<td>12.7</td>
<td>17.3</td>
</tr>
<tr>
<td>Blood</td>
<td>11.8</td>
<td>7.0</td>
<td>12.4</td>
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</tr>
<tr>
<td>Liver</td>
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<td>4.3</td>
<td>3.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Spleen</td>
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<td>5.1</td>
<td>4.8</td>
<td>4.4</td>
</tr>
<tr>
<td>Lung</td>
<td>10.2</td>
<td>5.7</td>
<td>10.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Stomach</td>
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<td>21.7</td>
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</tr>
<tr>
<td>Muscle</td>
<td>2.9</td>
<td>2.9</td>
<td>2.3</td>
<td>2.1</td>
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</table>

$^{a}$ SE = 53% of the associated T:O ratio value.
antigenic patches, whereas $^{125}$I-labeled control MoAb was uniformly distributed throughout the tumor (24). In contrast with the theory of Fujimori et al. (23), evidence for increased affinity prolonging the tumor retention of radiolabeled antibody comes from studies using B72.3, a MoAb with a specificity for the pancarcinoma antigen TAG-72. The tumor targeting of B72.3 was compared to a series of higher affinity second generation MoAbs, each reactive with a different epitope of TAG-72 (25). In this study, the second generation MoAb CC49 ($K_a = 6 \times 10^{-11}$) localized human tumor xenografts in athymic nude mice better than did the lower affinity molecule B72.3 ($K_a = 2 \times 10^{-9}$). However, in subsequent clinical trials, the second generation MoAb CC49 failed to display improved tumor retention over that observed for B72.3 (26). As the authors noted, however, it was unlikely that affinity was the only variable affecting the tumor localization properties. Other factors, such as accessibility and quantity of the targeted epitopes, may have influenced the results. Furthermore, different distribution patterns would likely be achieved with smaller species such as the scFv molecules used in the present study. Rigorous examination of this issue will require the study of antibody-based molecules of various sizes with a range of affinities for an identical epitope.

Despite the large (320-fold) increase in affinity, the actual amounts retained at 24 h for the highest affinity scFv (1.4% ID/g tumor) remain much less than those observed for IgG molecules in similar model systems. The improved quantitative targeting of IgG molecules in these animals has been thought to be related to the prolonged clearance of IgG in mice; typically, the $t_{1/2} \beta$ of IgG in mice is on the order of 107 h (27), whereas the $t_{1/2} \beta$ of most scFv is approximately 3.5 h (28). The rapid renal clearance of scFv may blunt the impact of improved affinity on tumor targeting by decreasing opportunities for these molecules to diffuse to tumor sites. Accordingly, we sought to examine the impact of affinity in a model system where clearance had been negated. This was accomplished by determining the biodistribution of labeled scFv in mice following the surgical excision of their kidneys.

The studies in anephric mice resulted in two observations of significance:

(a) The peak tumor retentions for the two higher affinity scFv approximated those observed with IgG targeting the HER2/new antigen on the same SK-OV-3 tumors in scid mice with functional kidneys (21% ID/g for scFv, 20% for IgG; Ref. 29). The specificity of tumor retention was surprisingly good, despite the absence of renal clearance, and improved over time. Thus, if renal clearance can be negated, scFv molecules can target tumors with as much quantitative deposition as IgG molecules, and with greater specificity. Because their large size decreases their diffusion coefficients, IgG molecules are ill-suited to overcome the many morphological and physiological features that limit the accessibility of antibodies to tumors. For example, IgG will diffuse 100 μm in 1 h, 1 mm in about 2 days, and 1 cm in 7 months (2). Decreasing the size of antibody-based molecules increases their interstitial diffusion rates and tumor distribution. Because scFv are smaller and diffuse through tumors more efficiently, the targeting of tumor cells in malignant masses is likely to be more homogeneous than with IgG (8). These results are in agreement with Buchegger’s observation of the impact of size on the maintenance of sufficient antibody fragment in circulation to achieve significant tumor targeting (30). Working with a similar nephrectomized mouse model, Buchegger et al. (30) reported significantly greater tumor retention of a radiolabeled Fab fragment as compared with the parent IgG.

(b) The second observation of significance from the studies in anephric mice is that higher affinity scFv do exhibit improved quantitative and selective tumor retention. The importance of affinity is underscored by the minimal tumor retention achieved with the lowest affinity variant, C6G98A. Although the C6G98A did exhibit 10-fold greater tumor retention than the negative control 26-10 scFv, its low affinity was insufficient to mediate an accumulation in tumor over time. Thus, C6G98A never approached the peak tumor retentions achieved with C6ML3-9 and C6.5, clearly, an affinity threshold that must be crossed to achieve selective retention in the in vivo setting. In this model system, the in vivo threshold exceeds the in vitro threshold for reasons that are not clearly understood. When a molecule with 320-fold higher affinity was examined in the same model, its tumor retention was significantly higher, and tumor/blood ratios improved to 3.5 to 1, despite the absence of renal clearance of the molecule. Because these studies were performed using molecules radioiodinated by the CT method, substantial dehalogenation occurred, as evidenced by the large accumulation of radiodine in the stomach at 24 h. Accordingly, estimates of tumor retention may be artificially low, not reflecting the presence of scFv that have lost their label (data not shown). This issue is being addressed by labeling modified scFv molecules using methodologies to create covalent associations between the radiodine and the proteins (31); we have found that such strategies greatly improve the quantity and specificity of tumor targeting. The studies reported here used protein doses of scFv molecules that saturated available tumor antigen so that continued accumulation of the highest affinity scFv, C6ML3-9, may have been limited by this factor. However, peak tumor retention was achieved more rapidly, e.g., after only 4 h, than with the lower affinity scFv, C6.5. Furthermore, this level of tumor retention persisted as the blood levels of C6ML3-9 dropped, yielding improved tumor:normal organ ratios at 24 h as compared with C6.5.

Advances in protein engineering techniques and the use of phage display make it possible to create small, human antibody-based targeting molecules, such as scFv, and engineer affinity to values not achievable by rodent immunization (11). For example, we have further increased the affinity of C6.5-based scFv another 100-fold to a $K_a = 1.3 \times 10^{-11}$ M (13). The in vivo tumor targeting properties of this and related molecules require study. The use of extremely high-affinity scFv molecules may further improve in vivo targeting, particularly when the $k_{on}$ rates of the molecules exceed the $t_{1/2} \beta$ of the molecules in circulation. Given the results presented here, however, it is likely that rapid clearance will still impose a ceiling on the amount of administered protein that can accumulate at tumor sites. Strategies to retain small size and reduce renal clearance require examination. Alternatively, the scFv molecules can be used as building blocks to create small, bivalent, scFv-based molecules, such as diabodies. These molecules can bind bivalently to cell surface antigens, resulting in a large increase in functional affinity, but are still below the renal threshold and thus rapidly cleared from the circulation. For example, constructing a diabody from the V-genes of C6.5 improves affinity 40-fold ($K_a = 4.0 \times 10^{-10}$ M) and yields a 6-fold increase in specific tumor retention at 24 h compared to the C6.5 scFv (6.5% ID/g versus 1.0% ID/g for C6.5 scFv). Improved quantitative delivery may also partially result from a longer $t_{1/2} \beta$ for the diabody (6.4 h versus 3.5 h for the scFv molecules). Construction of diabodies from the V-genes of higher affinity C6.5-based scFv molecules may lead to further improvements in specific targeting. These and other potential modifications to the structures of scFv will enhance the ability of these molecules to function as delivery vehicles that can achieve the needed balance of quantitative and selective tumor retention to be effective in cancer therapy strategies.

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References


Increased Affinity Leads to Improved Selective Tumor Delivery of Single-Chain Fv Antibodies


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