[CANCER RESEARCH 61, 4750–4755, June 15, 2001]

High Affinity Restricts the Localization and Tumor Penetration of Single-Chain Fv Antibody Molecules[1]

Gregory P. Adams,[2] Robert Schier,[3] Adrian M. McCall,[4] Heidi H. Simmons, Eva M. Horak, R. Katherine Alpaugh, James D. Marks,[5] and Louis M. Weiner[5]

Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 [G. P. A., A. M. M., H. H. S., E. M. H., R. K. A., L. M. W.], and Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, California 94110 [R. S., J. D. M.]

ABSTRACT

Antitumor monoclonal antibodies must bind to tumor antigens with high affinity to achieve durable tumor retention. This has spurred efforts to generate high affinity antibodies for use in cancer therapy. However, it has been hypothesized that very high affinity interactions between antibodies and tumor antigens may impair efficient tumor penetration of the monoclonal antibodies and thus diminish effective *in vivo* targeting (K. Fujimori *et al.,* J. Nucl. Med., *31:* 1191–1198, 1990). Here we show that intrinsic affinity properties regulate the quantitative delivery of antitumor single-chain Fv (scFv) molecules to solid tumors and the penetration of scFv from the vasculature into tumor masses. In biodistribution studies examining a series of radioiodinated scFv mutants with affinities ranging from 10^{-7}-10^{-11} M, quantitative tumor retention did not significantly increase with enhancements in affinity beyond 10^{-9} M. Similar distribution patterns were observed when the scFv were evaluated in the absence of renal clearance in anephric mice, indicating that the rapid renal clearance of the scFv was not responsible for these observations. IHC and IF evaluations of tumor sections after the i.v. administration of scFv affinity mutants revealed that the lowest affinity molecule exhibited diffuse tumor staining whereas the highest affinity scFv was primarily retained in the perivascular regions of the tumor. These results indicate that antibody-based molecules with extremely high affinity have impaired tumor penetration properties that must be considered in the design of antibody-based cancer therapies.

INTRODUCTION

Tumor antigen density and interstitial tumor pressure are known to contribute to antibody-based tumor targeting. The requirement for a large differential in antigen expression between normal tissue and the tumor has long been accepted. Detailed studies by Jain *et al.* (1) have described how high interstitial pressures in solid tumors limit the penetration and distribution of MAb[6] and other macromolecules. Whereas a rigorous examination of the impact of binding affinity has not been performed, a threshold affinity clearly exists below which antibodies will not durably bind to tumor antigen (2). Because slow k_{off} rates mediate the prolonged *in vitro* retention of MAb on tumor cells, it has been assumed that the best tumor targeting will be achieved with the highest affinity antibodies. However, Weinstein has postulated the existence of a "binding site barrier" effect in which MAbs with very high affinity (*e.g.,* prolonged k_{off}) for tumor antigens stably bind to the first encountered tumor antigen. This would lead to patchy and incomplete tumor perfusion and could be associated with suboptimal therapeutic effects when therapeutic efficacy is dependent upon uniform delivery to tumor cells (3, 4). Accordingly, antibodies with moderate affinity may be advantageous as they could effectively penetrate into tumors and exhibit moderately durable retention on tumor cells. Identification of the optimal affinities for tumor targeting should improve antibody-based therapeutics.

We have reported previously on the production of C6.5, a human scFv that is specific for the HER-2/*neu* (c-erbB-2) proto-oncogene product overexpressed in a variety of carcinomas (5). Using site-directed mutagenesis of the variable light chain and variable heavy chain CDR3 regions, we have generated a series of affinity mutants from the original C6.5 scFv. These bind to the same epitope of HER-2/*neu* with affinities ranging from 10^{-6}-1.5×10^{-11} M (6). Using lower affinity variants, we have reported that a threshold affinity of between 10^{-7} and 10^{-8} M is required to observe detectable tumor retention in mice with intact kidneys at 24 h after injection (2). In the current study, we examine the impact of very high affinity on *in vivo* tumor targeting.

MATERIALS AND METHODS

scFv Affinity Mutants. The anti-HER-2/*neu* scFv molecules (C6.5 and its affinity mutants) were produced and expressed from TG1 *Escherichia coli* growing in shake flasks as described previously (5, 6). Fully functional scFv were purified from periplasmic preparations by affinity chromatography directed against the His_6 Tag, followed by high performance liquid chromatography size exclusion chromatography using a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden; yield ~1 mg/liter). K_d values for the scFv molecules were calculated from association and k_{off} rate constants measured on immobilized HER-2/*neu* extracellular domain using surface plasmon resonance on a BIAcore instrument (Pharmacia) as described previously (5, 6). 26–10 scFv, specific for digoxin, was generously provided by Dr. James Huston (Creative BioMolecules, Hopkinton, MA) for use as an irrelevant control.

Rabbit polyclonal antiserum specific for the C6.5 family of scFv molecules was generated by immunizing two New Zealand White rabbits (Taconic Labs, Germantown, NY) with C6.5 scFv in Complete Freund's Adjuvant (initial injections) or Incomplete Freund's Adjuvant (subsequent injections). Bleeds were obtained from the auricular artery. The reactivity of the antiserum to C6.5 and its affinity mutants were determined by direct comparison to preimmunization test bleed samples on the BIAcore instrument (data not shown). Polyclonal antibodies that were reactive with myc were removed by adsorption against c-myc peptide (PP06; CalBiochem, San Diego, CA) using an AminoLink Plus Immobilization kit (#44894; Pierce, Rockford, IL).

Radioiodination. All scFv were labeled with iodine-125 using the chloramine-T method as described previously (7). Briefly, 1.0–2.0 mg of protein were combined with iodine-125 (#NEZ033H; DuPont NEN, Wilmington, DE) at an iodine:protein ratio of 1:10. A total of 10 μg of chloramine T (Sigma Chemical Co., St. Louis, MO) was added per 100 μg of protein, and the resulting mixture was incubated for 3 min at room temperature. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method (7). The final specific activity of each ^{125}I-scFv was ~1.0 mCi/mg. The quality of the radioiodinated scFv molecules

Received 8/16/00; accepted 4/18/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by National Cancer Institute Grant CA65559, an appropriation from the Commonwealth of Pennsylvania, the Bernard A. and Rebecca S. Bernard Foundation, and the Frank Strick Foundation.

[2] To whom requests for reprints should be addressed, at Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. E-mail: gp_adams@fccc.edu.

[3] Present address: Xerion Pharmaceuticals GmbH, Fraunhoferstrasse 9, D-82152 Martinsried, Germany.

[4] Present address: Faculty of Education, University of Melbourne, Victoria, Australia, 3010.

[5] The Marks and Weiner laboratories contributed equally to the work.

[6] The abbreviations used are: MAb, monoclonal antibody; scFv, single-chain Fv; IHC, immunohistochemistry; IF, immunofluorescence; k_{off}, disassociation rate; pI, isoelectric point.

Downloaded from http://cancerres.aacrjournals.org/cancerres/article-pdf/61/12/4750/2486333/4750.pdf by guest on 24 April 2024

was evaluated by SDS-PAGE and in a live cell binding assay. Nonreduced SDS-PAGE were run using 12% gels (10 cm × 12 cm; #12D.75HMC-10P; Jule, Inc., New Haven, CT; Refs. 7 and 8). Migration of the scFv was detected by autoradiography at −70°C using Kodak X-ray film with Kodak X-Omatic regular intensifying screens. Greater than 98% of the nonreduced ^{125}I-scFv preparations migrated on SDS-PAGE as ~26 kDa, indicating their monomeric nature. The immunoreactivity of the radiopharmaceuticals was determined in a live cell binding assay using HER-2/*neu* expressing SK-OV-3 cells (7). A total of 10 ng of labeled scFv in 100-μl PBS was added in triplicate to 5×10^6 SK-OV-3 cells in 15-ml polypropylene centrifuge tubes. The cells were incubated for 30 min at room temperature, washed with 2.0 ml of PBS, and centrifuged for 5 min at 500 × *g*. Supernatants were separated from the cell pellets, both were transferred to 12 × 75 counting tubes, and the percentage of activity associated with the cell pellet was determined. The results of the live cell binding assays indicated that the radioiodinated scFv were still reactive with cell surface HER-2/*neu*. The activities associated with the cell pellets were 89.7, 72.4, 61.2, 59.0, 5.3, and 2.7%, respectively, for the 1.5×10^{-11} M, 1.2×10^{-10} M, 1.0×10^{-9} M, 1.6×10^{-8} M, 3.2×10^{-7} M, and negative control 26–10 scFv. The retention of the C6G98A was consistent with its rapid k_{off} rate from the HER-2/*neu* antigen.

Biodistribution Studies. Biodistribution studies were performed as described previously (9). Briefly, 2.5×10^6 SK-OV-3 cells in log phase were implanted s.c. on the abdomens of 4–6-week-old inbred C.B17/Icr- *scid* mice (acquired from the Fox Chase Cancer Center Laboratory Animal Facility). After 8 weeks, the tumors were ~100 mg in size, and Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. A total of 2 days later, 20 μg of one of the ^{125}I-scFv were administered to cohorts of five to six mice by tail vein injection. Injected doses were determined by counting the mice on a Series 30 multichannel analyzer/probe system (probe model #2007; Canaberra, Meridian, CT). Groups of six mice were sacrificed at 24 h after injection; tumor, organ, and blood retentions were determined as described (9). The SE for each group of data was calculated.

To determine the impact of affinity on biodistribution in the absence of renal clearance, studies were also performed using surgically nephrectomized mice as described previously (9). Briefly, 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. Both kidneys were identified, ligated, and excised from the mice. The incisions were then closed with surgical staples, and biodistribution studies were initiated 1 h later. A total of 1 μg of each tested ^{125}I-scFv molecule was administered to cohorts of three nephrectomized, 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 before euthanization. Mice were sacrificed at 4 and 24 h after injection, and tumor, organ, and blood retentions were determined as described above. Significance was determined using an unpaired *t* test.

IHC Detection. A total of 100 μg of unlabeled anti-HER-2/*neu* 10^{-7} M scFv or 10^{-11} M scFv were administered by i.v. injection into groups of three surgically nephrectomized *scid* mice bearing established SK-OV-3 tumors. The mice were euthanized 24 h later; the tumors were excised and fixed in formalin. Sections were cut and mounted on slides for IHC and IF studies. The tissue sections were deparaffinized and rehydrated using standard methodology. Primary antibodies were applied to the sections; antimouse CD-31 (platelet/endothelial cell adhesion molecule 1; PharMingen #01951D) were applied to detect endothelial cells (tumor blood vessels); and polyclonal rabbit anti-C6.5 scFv were applied to detect the scFv affinity mutants. After the application of the primary antibodies, an adenomatous polyposis coli detection system was used using 3,3′-diaminobenzidine as the substrate for anti-C6.5 scFv staining and Ni^{++}-3,3′-diaminobenzidine as the substrate for anti-CD31 MAb staining.

IF. Tumor sections mounted on slides were deparaffinized and rehydrated. Autofluorescence was reduced by washing the samples three times for 10 min in sodium borohydride (1 mg/ml). The sections were then blocked with 3% BSA for 30 min to reduce nonspecific binding. Excess BSA was removed by washing three times for 10 min with 1% BSA/PBS containing 0.1% Tween 20 (BSA-PBST). Sections were treated sequentially with the primary antibodies, rabbit anti-C6.5 scFv antiserum and rat antimouse CD-31 MAb, and each was allowed to incubate for 1.5 h at 37°C. After each incubation, samples were washed four times with BSA-PBST. Secondary antibodies were added sequentially to prevent cross-reactions. Goat antirabbit Rhodamine Red X (Jackson Immunoresearch) was applied and incubated for 45 min at 37°C in the dark. Rabbit antirat FITC (Sigma Chemical Co.) was then applied to the same sections and allowed to incubate for 45 min at 37°C in the dark. The sections were then washed four times with BSA-PBST and twice with dH_2O. Prolong (Molecular Probes) self-hardening antifade solution was applied to the sections that were then sealed with coverslips. Sections were imaged using the 10 × and 40 × Plan Fluor objectives on a Nikon TE800 upright microscope coupled to a Quantix cooled CCD camera (Photometrics). FITC and Rhodamine Red X were detected with EX460-500 BA510-560 and EX510-560 BA590 filter sets, respectively. Images were acquired as separate channels for each fluorochrome using ISEE software (Inovision Corp.), and the channels were merged to form the final images.

RESULTS

Biodistribution Studies. We initially investigated the biodistribution of radioiodinated scFv in tumor-bearing *scid* mice using molecules representing ~10-fold jumps in affinity for HER-2/*neu* (3.2×10^{-7} M, 1.6×10^{-8} M, 1.0×10^{-9} M, 1.2×10^{-10} M, and 1.5×10^{-11} M). A total of 20 μg of ^{125}I-labeled scFv were administered by i.v. injection into *scid* mice bearing established s.c. SK-OV-3 human ovarian carcinoma tumor xenografts. The mice were euthanized 24 h later, and the quantity of radiolabeled scFv retained in tumor, normal tissues, and blood was determined. As observed previously, affinities $>10^{-7}$ M were required for the quantity of radiolabeled scFv retained in the tumor to be significantly greater than that observed with the negative control 26–10 antidigoxin scFv (Fig. 1; Ref. 2). Specific tumor retention was 0.2% ID/g for the 1.6×10^{-7} M scFv and 1.4% ID/g for the 1×10^{-9} M scFv, respectively. As the affinity for HER-2/*neu* increased from 1×10^{-9} M to 1.2×10^{-10} M, a plateau was observed in the quantity of scFv localizing in the tumor that persisted through the highest affinity scFv examined (Fig. 1). Of note, whereas the quantity of radiolabeled scFv retained in tumor plateaued with increasing affinity, the quantity remaining in blood and to a lesser extent in the normal organs increased in the high affinity range (Fig. 1). To determine whether decreased availability because of rapid systemic clearance of the scFv was responsible for the plateau in tumor retention, the same panel of scFv molecules was evaluated in surgically nephrectomized *scid* mice. As the small size of the scFv molecules leads to first pass elimination via the kidneys, scFv blood

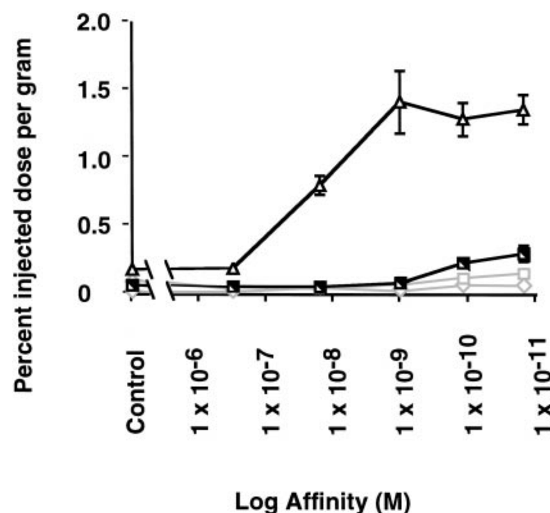

Fig. 1. Twenty-four h biodistribution of ^{125}I-labeled anti-HER-2/*neu* scFv affinity mutants in SK-0V-3 tumor-bearing *scid* mice. Cohorts of six mice were given 20 μg of one of the affinity mutants and were then euthanized 24 h later. The percentage of the injected dose of each affinity mutant localized per gram of tumor (—△—), blood (—◩—), liver (—□—), muscle (—◇—), and spleen (—○—) is presented. *Bars*, SE.

Downloaded from http://cancerres.aacrjournals.org/cancerres/article-pdf/61/12/4750/2486333/4750.pdf by guest on 24 April 2024

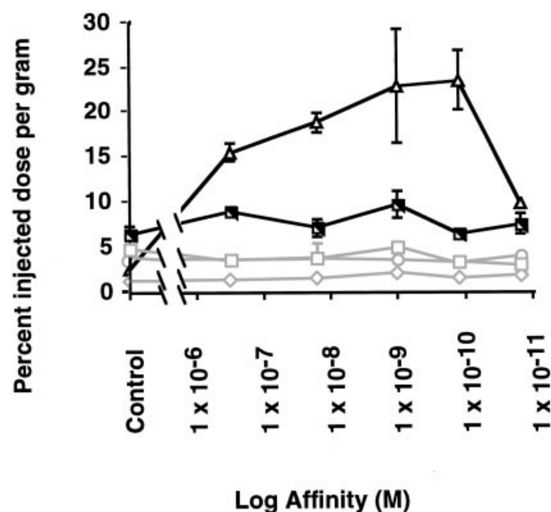

Fig. 2. Twenty-four h biodistribution of ^{125}I-labeled anti-HER-2/*neu* scFv affinity mutants in anephric SK-0V-3 tumor-bearing *scid* mice. Cohorts of three mice were given 1 μg of one of the affinity mutants and were then euthanized 24 h later. The percentage of the injected dose of each affinity mutant localized per gram of tumor (—△—), blood (—◪—), liver (—□—), muscle (—◇—), and spleen (—○—) is presented. *Bars*, SE.

concentrations were significantly higher (average values ranged from 6.5% ID/ml to 10% ID/ml at 24 h) in the anephric mice (Fig. 2). In the absence of systemic clearance, significantly higher concentrations of all tested scFv molecules were retained in tumor (Fig. 2). However, a plateau was still observed in the quantities retained, and a significant drop ($P = 0.015$) in tumor retention was observed as affinity was increased from 1.2×10^{-10} M to 1.5×10^{-11} M. These results suggest that rapid clearance alone did not blunt the effects of affinity on tumor retention and that tumor localization of the highest affinity scFv clone was impaired. Furthermore, as all of the affinity mutants exhibited similar retentions in well-perfused organs (*e.g.,* liver and spleen) and in poorly perfused organs (*e.g.,* muscle), it appears that the only factor impacting on tumor localization was binding affinity (Fig. 2).

Recent reports have indicated that the charge balance (pI) of antibody-based molecules can impact on their clearance from circulation and their quantitative retention in tumors (10). Accordingly, we sought to determine whether differences in the pI of the scFv molecules could explain the biodistribution results. The scFv molecules with affinities ranging from 10^{-7}-10^{-9} M had pI values that were >9, whereas the two scFv with affinities $>10^{-9}$ M had pI values of ~7 (data not shown). These pI values indicate that the low and moderate affinity scFv would have an overall positive charge at physiological pH, and the highest affinity scFv would have a neutral charge. To determine whether these differences in pI had an impact on the tumor targeting properties of the molecules, the *myc* tag was removed from the COOH terminus of the 10^{-11} M affinity scFv. As the tag contained three negatively charged aspartic acid residues, the pI of the modified scFv was raised to >9, resulting in an overall charge at physiological pH that was similar to those of the lower affinity scFv. The pI 7 and pI >9 forms of the 10^{-11} M scFv were radioiodinated, and their 24-h biodistributions were evaluated in *scid* mice bearing s.c. SK-OV-3 tumors. In these studies, the tumor retention of the pI modified and native forms of the scFv were not significantly different (data not shown). Thus, differences in pI do not explain the tumor-retention properties of the C6.5 scFv series of affinity mutants.

Tumor Penetration Studies. IHC and IF studies were performed to determine the impact of affinity on the tumor penetration of antitumor scFv molecules in the anephric mouse model. A total of 100 μg of low affinity (3.2×10^{-7} M) or high affinity (1.5×10^{-11} M) anti-HER-2/*neu* scFv were administered by i.v. injection into groups of three *scid* mice bearing s.c. SK-OV-3 tumors. Tumors were excised 24 h later and fixed in formalin, and sections were evaluated by IHC and IF. The distribution of the anti-HER-2/*neu* scFv molecules was determined using rabbit polyclonal antibodies specific for the scFv molecules. The locations of tumor blood vessels were identified with a commercial rat antimouse CD-31 (platelet/endothelial cell adhesion molecule 1) MAb that specifically binds to endothelial cells. Whereas the low affinity scFv was distributed diffusely throughout vascularized regions of the tumors, the high affinity scFv was primarily detected within several cell diameters of the blood vessels (Fig. 3). These observations indicate that penetration from blood vessels into a solid tumor is impaired by high affinity for the target antigen.

DISCUSSION

This is the first thorough examination of the effects of affinity on *in vivo* tumor targeting. Determination of the desirable physical properties that are required for selective tumor targeting and maximal tumor penetration would facilitate the engineering of antibody-based constructs for the immunotherapy of solid tumors. The results of the current study provide conclusive evidence of the existence of the binding site barrier effect first postulated by Weinstein (11). Until now, a series of antibodies with a wide range of affinities for the same epitope of a solid tumor antigen was unavailable. Accordingly, evidence to support the binding site barrier theory was limited. The most compelling results were obtained by studying MAbs targeting different epitopes of a tumor antigen in *in vitro* multicell tumor spheroids (12) and in *in vivo* analysis of the tumor penetration of an isotype-matched pair of an antitumor MAb and an irrelevant control MAb (4). However, because of the use of different target epitopes and antibody isotypes in the former studies, and the lack of a low affinity MAb in the latter studies, the interpretation of these results is difficult.

Here, we have used a series of affinity mutants of the C6.5 scFv to demonstrate that high affinity limits the tumor localization and intratumoral diffusion of small antibody-based molecules. In biodistribution studies, quantitative tumor retention of the radiolabeled scFv molecules plateaued at affinities $>1 \times 10^{-9}$ M. Furthermore, because of increased blood levels, affinities $>10^{-9}$ M were actually associated with lower tumor:blood ratios. This effect persisted even after the elimination of variables that could alter efficient tumor targeting, such as scFv pI and systemic clearance. Finally, as the anti-HER-2/*neu* scFv used in the current studies targeted the same epitope and differed from each other by no more than eight amino acids, the only major difference between them was affinity for HER-2/*neu*.

IHC and IF studies were performed to determine whether the ability of the scFv molecules to diffuse into tumors was associated with the degree of localization in the tumor. Using these techniques, we observed that a low affinity (3.2×10^{-7} M) anti-HER-2/*neu* scFv exhibited broad diffusion from the vasculature into the tumor, whereas the highest affinity (1.5×10^{-11} M) scFv generally failed to traverse more than 2–3 cell diameters. The moderately high affinity (1×10^{-9} M) scFv revealed an intermediate staining pattern (data not shown). These studies indicate that the affinity for HER-2/*neu* dictates the degree of scFv penetration from blood vessels into tumors.

Solid tumors are characterized by high interstitial pressures. This phenomenon, described in detail by Jain *et al.* (13), severely limits the diffusion of macromolecules from the blood vessels into the tumor. For example, a Fab fragment that is twice the size of the scFv molecules used in this study would take 16 h to move a distance of 1 mm into a solid tumor. Although affinity is independent of interstitial pressure, the ability of scFv molecules to diffuse into the tumor is not. We believe that the binding site barrier effect results from a combination of high affinity and high interstitial pressure. In the studies

Downloaded from http://cancerres.aacrjournals.org/cancerres/article-pdf/61/12/4750/2486333/4750.pdf by guest on 24 April 2024

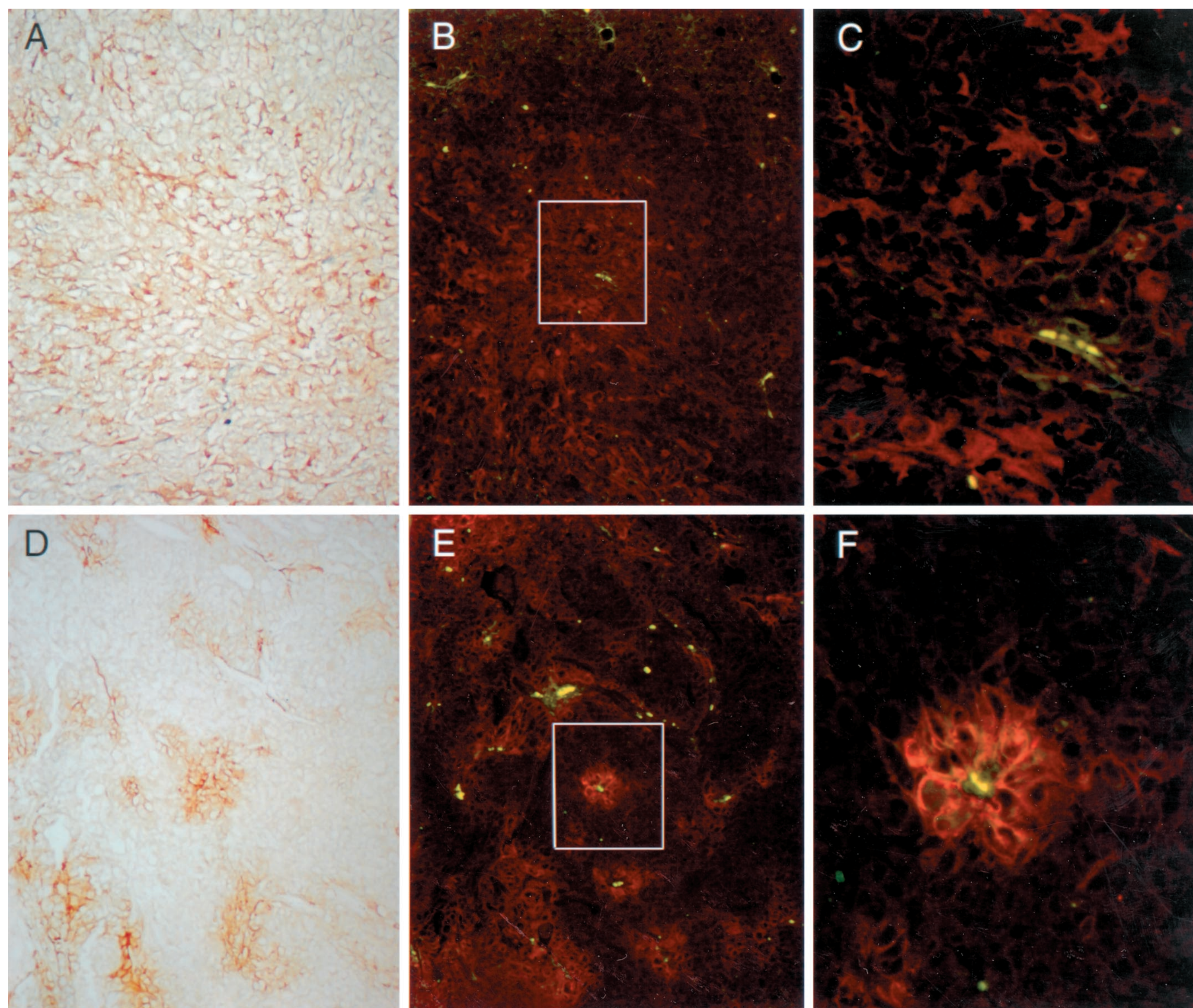

Fig. 3. IHC and IF examination of the *in vivo* distribution of anti-HER-2/*neu* scFv molecules relative to the location of tumor vasculature in anephric SK-OV-3 tumor-bearing *scid* mice. *A,* low affinity (3.2×10^{-7} M) anti-HER-2/*neu* scFv (*brown*) displays a diffuse staining pattern relative to tumor blood vessels (*black/blue*) by IHC. In *B,* IF examination of nearby section of same tumor (3.2×10^{-7} M anti-HER-2/*neu* scFv = *red* and tumor blood vessels = *green*) again indicates diffusion from the blood vessels. *C,* higher magnification of boxed area in *B*. In *D,* IHC examination of the localization of the high affinity (1.5×10^{-11} M) anti-HER-2/*neu* scFv (*brown*) relative to tumor blood vessels (*black/blue*) reveals a focal staining pattern with short diffusion from the vascular bed. In *E,* IF examination of nearby section of same tumor (1.5×10^{-11} M anti-HER-2/*neu* scFv = *red* and tumor blood vessels = *green*) again indicates a lack of diffusion from the tumor blood vessels. *F,* higher magnification of boxed area in *E*. Original magnifications: ×10 (*A, B, D,* and *E*) and ×40 (*C* and *F*).

described above, it is conceivable that the rapid off rate (1.3×10^{-1} s^{-1} or <10 s) of the low affinity scFv allowed it to diffuse into the tumor through a series of associations and dissociations from HER-2/*neu* antigen on the surface of tumor cells. In contrast, when a high affinity scFv molecule diffused into the tumor, the slow off rate (1×10^{-5} s^{-1} or ~28 h for our highest affinity molecule) mediated a long association that precluded the docking of other scFv to the same antigen. This would impede high affinity scFv from traversing tumors using sequential binding to and dissociation from unoccupied antigens (Fig. 4). Alternatively, low affinity scFv may diffuse through tumors without impediments posed by tumor antigen reservoirs. Accordingly, diffuse penetration is ultimately achieved but at the expense of limited specific targeting. As the affinity for a tumor-related antigen rises, this penetration is progressively impeded, but specific targeting improves. The data presented here suggests that exceptionally high affinity retards scFv penetration, although perivascular tumor cells are very efficiently targeted. These results thus suggest that scFv affinity properties impose constraints on the movement of antibody molecules through tumors that must be considered in addition to size, valence, and hydrostatic tumor pressures.

It is unlikely that shed HER-2/*neu* extracellular domain antigen in the tumor microenvironment was responsible for these observations. If this were the case, preadministration of excess unlabeled scFv would saturate the shed antigen pool and facilitate increased tumor localization. However, a 5-fold larger dose of the unlabeled highest affinity (1.5×10^{-11} M) scFv decreased the tumor localization of subsequently administered radioiodinated scFv (data not shown). Additionally, the significantly lower tumor localization of the highest affinity scFv in anephric mice suggests that the binding site barrier cannot be rapidly broached by simply increasing the dose of scFv to saturate accessible antigen. It is possible that the binding site barrier effect can be overcome over long periods of time through continuous

Downloaded from http://cancerres.aacrjournals.org/cancerres/article-pdf/61/12/4750/2486333/4750.pdf by guest on 24 April 2024

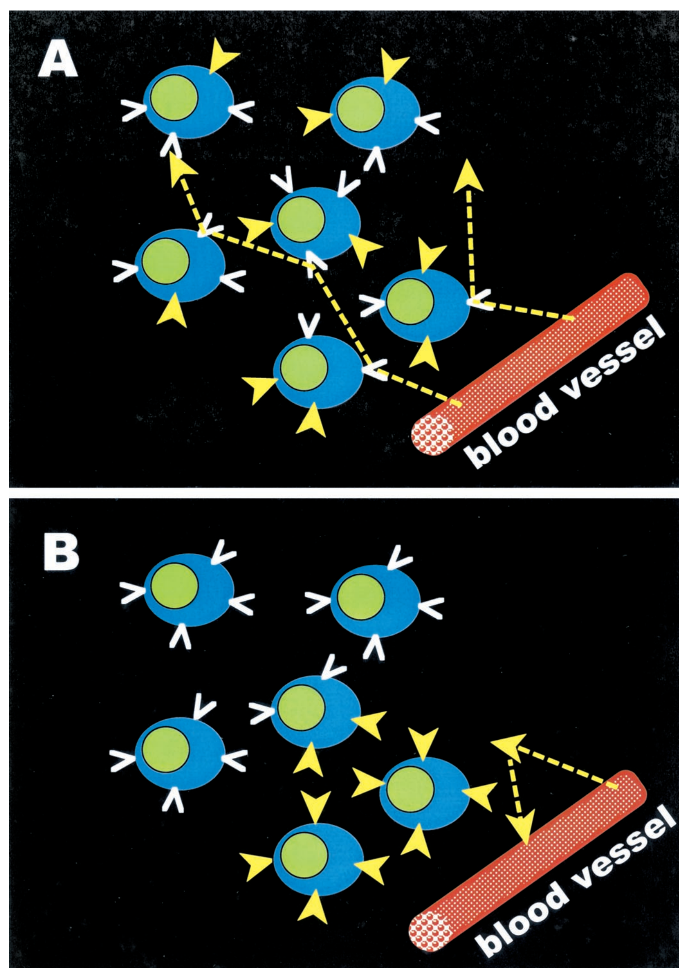

Fig. 4. A model displaying the impact of affinity on the diffusion of scFv molecules into a solid tumor during the first 24 h after administration. In *A,* in a setting of high interstitial pressure, low dissociation affinity scFv molecules (▲) can rapidly diffuse into the tumor using sequential binding to and dissociation from unoccupied target antigen (*V*). The path traveled by the scFv is indicated (----). In *B,* under the same conditions, high affinity scFv will durably occupy the target antigen pool that can be reached in a single diffusion step. scFv that subsequently enter the tumor from the blood vessel will be unable to reach free antigen, and the high interstitial pressure will force them back out of the tumor.

exposure to high affinity molecules, mediating the eventual diffusion of the scFv molecules throughout the tumor. However, as rising blood urea nitrogen levels are lethal to surgically nephrectomized mice within 48 h of the procedure, it was not possible to test this hypothesis in the current model. The contribution of affinity to the tumor retention and penetration of intact MAb molecules remain to be experimentally determined. It is possible that the slower clearance dictated by the larger size may predominate over affinity. Although studies examining the tumor targeting of two MAbs specific for different epitopes of the antigen tumor-associated glycoprotein 72 revealed a localization advantage for the higher affinity molecule (14), variables other than affinity (*e.g.,* differences in accessibility of the target epitope) could have been responsible. Thus, it is likely that a rigorous examination of the type performed here, using matched reagents with differing affinities, will be required to identify conclusively the effects of affinity for tumor targeting using intact MAb.

These results suggest that high affinity scFv molecules may be inappropriate vehicles when rapid and uniform tumor penetration is needed. For example, antibodies can be used to deliver highly cytotoxic moieties such as radionuclides, toxins, or chemotherapeutic agents (15–17). In such circumstances, it is desirable for tumor targeting to be rapid and highly efficient to minimize nontargeted toxicity to normal organs lacking the antigen. scFv are desirable vehicles for such applications because of their small size and rapid systemic clearance. The results presented here indicate that exceedingly high affinity scFv display impaired tumor penetration properties that may decrease the efficacy of scFv-targeted therapies. These considerations may not be as important when unconjugated antibodies are used to perturb cellular signaling or to promote antibody-dependent cellular cytotoxicity (18). Several IgG-based antibodies with such properties have demonstrated efficacy or promise in clinical trials (18–20). In this setting, the antibody is less toxic to normal tissues and can safely circulate at high concentrations in the blood for prolonged periods, allowing the binding site barrier to be slowly overcome. Consequently, high affinity, intact immunoglobulins that more efficiently inhibit targeted ligand-receptor interactions may be preferable to lower affinity variants that are less efficient. Accordingly, the appropriate affinity for antibody-based therapeutics will likely depend upon the antibody structure and the intended therapeutic application.

ACKNOWLEDGMENTS

We thank Jonathan Boyd of the Fox Chase Cancer Center confocal microscope facility, Catherine Renner and Bonnie Mason of the Fox Chase Cancer Center Research Pathology Facility, Anthony Lerro of the Fox Chase Cancer Center Laboratory Animal Facility, and Ellen Wolf and Shada Rabone for their excellent technical assistance.

REFERENCES

1. Jain, R. K. Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. Cancer Res., *50* (Suppl.)*:* 814s–819s, 1990.
2. Adams, G. P., Schier, R., Marshall, K., Wolf, E. J., McCall, A. M., Marks, J. D., and Weiner, L. M. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Cancer Res., *58:* 485–490, 1998.
3. Fujimori, K., Covell, D. G., Fletcher, J. E., and Weinstein, J. N. A modeling analysis of monoclonal antibody percolation through tumors: a binding site barrier. J. Nucl. Med., *31:* 1191–1198, 1990.
4. Juweid, M., Neumann, R., Paik, C., Perez-Bacete, M. J., Sato, J., van Osdol, W., and Weinstein, J. N. Micropharmacology of monoclonal antibodies in solid tumors: direct experimental evidence for binding site barrier. Cancer Res., *52:* 5144–5153, 1992.
5. Schier, R., Marks, J. D., Wolf, E., Apell, G., Wong, C., McCartney, J., Bookmna, M., Huston, J., Weiner, L., and Adams, G. *In vitro* and *in vivo* characterization of a human anti-c-erB2 single chain Fv isolated from a filamentous phage antibody library. Immunotechnology, *1:* 73–81, 1995.
6. Schier, R., McCall, A., Adams, G. P., Marshall, K. W., Merritt, H., Yim, M., Crawford, R. S., Weiner, L. M., Marks, C., and Marks, J. D. Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. J. Mol. Biol., *263:* 551–567, 1996.
7. Adams, G. P., McCartney, J. E., Tai, M-S., Oppermann, H., Huston, J. S., Stafford, W. F., Bookman, M. A., Fand, I., Houston, L. L., and Weiner, L. M. Highly specific *in vivo* tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv. Cancer Res., *53:* 4026–4034, 1993.
8. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
9. Adams, G. P., Schier, R., McCall, A. M., Crawford, R. S., Wolf, E. J., Weiner, L. M., and Marks, J. D. Prolonged *in vivo* tumor retention of a human diabody targeting the extracellular domain of human HER2/*neu*. Br. J. Cancer, *77:* 1405–1412, 1998.
10. Pavlinkova, G., Beresford, G., Booth, B. J. M., Batra, S. K., and Colcher, D. Charge-modified single chain antibody constructs of monoclonal antibody CC49: generation, characterization, pharmacokinetics, and biodistribution analysis. Nucl. Med. Biol., *26:* 27–34, 1999.
11. Fujimori, K., Covell, D. G., Fletcher, J. E., and Weinstein, J. N. Modeling analysis of the global and microscopic distribution of immunoglobulin G, F(ab′)2, and Fab in tumors. Cancer Res., *49:* 5656–5663, 1989.
12. Langmuir, V. K., Mendonca, H. L., and Woo, D. V. Comparisons between two monoclonal antibodies that bind to the same antigen but have differing affinities: uptake kinetics and ^{125}I-antibody therapy efficacy in multicell spheroids. Cancer Res., *52:* 4728–4734, 1992.
13. Jain, R. K., and Baxter, L. T. Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: significance of elevated interstitial pressure. Cancer Res., *48:* 7022–7032, 1988.

Downloaded from http://cancerres.aacrjournals.org/cancerres/article-pdf/61/12/4750/2486333/4750.pdf by guest on 24 April 2024

14. Colcher, D., Minelli, F. M., Roselli, M., Muraro, R., Simpson-Milenic, D., and Schlom, J. Radioimmmunolocalization of human carcinoma xenografts with B72.3 second generation monoclonal antibodies. Cancer Res., *48:* 4597–4603, 1988.
15. Press, O. W., Eary, J. F., Appelbaum, F. R., Martin, P. J., Badger, C. C., Nelp, W. B., Glenn, S., Butchko, G., Fisher, D., Porter, B., Matthews, D. C., Fischer, L. D., and Bernstein, I. D. Radiolabelled-antibody therapy of B-cell lymphoma with autologous bone marrow support. N. Engl. J. Med., *329:* 1219–1224, 1993.
16. Vitetta, E. S., Fulton, R. J., May, R. D., Till, M., and Uhr, J. W. Redesigning nature's poisons to create antitumor reagents. Science (Wash. DC), *238:* 1098–1104, 1987.
17. Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S., Casazza, A. M., Firestone, R. A., Hellstrom, I., and Hellstrom, K. E. Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science (Wash. DC), *261:* 212–215, 1993.
18. Fan, Z., and Mendelsohn, J. Therapeutic application of antigrowth factor receptor, antibodies. Curr. Opin. Oncol., *10:* 67–73, 1998.
19. Pegram, M., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Tripathy, D., Baly, D., Baughman, S. A., Twaddell, T. Glaspy, J. A., and Slamon, D. J. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J. Clin. Oncol., *16:* 2659–2671, 1998.
20. Maloney, D. G. Preclinical and Phase I and II trials of rituximab. Semin. Oncol., *26:* 74–78, 1999.

Downloaded from http://cancerres.aacrjournals.org/cancerres/article-pdf/61/12/4750/2486333/4750.pdf by guest on 24 April 2024