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Recombinant single-chain variable fragment antibodies against extracellular epitopes of human multidrug resistance protein MRP3 for targeting malignant gliomas

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Abstract

MRP3, a multidrug resistance protein identified by serial analysis of gene expression as a glioblastoma multiforme (GBM)-associated molecule, is highly expressed in GBM, but not in normal brain cells. Thus MRP3 is a candidate for GBM immunotargeting, but to date, no monoclonal antibody has been isolated that can target an extracellular MRP3 epitope. By phage display, we have isolated three recombinant, fully human, single-chain Fv (scFv) antibodies, M25, M58, and M89, which specifically react with the extracellular N-terminus of human MRP3. In ELISA, these scFvs reacted only with the peptide used for screening and not with other MRP3-derived peptides. Flow cytometric analysis revealed that these scFv fragments bind specifically to viable human GBM cells displaying different MRP3 expression levels, but not to MRP3-null cells. Furthermore, these scFv antibodies failed to react with tumor cells overexpressing other MRP proteins, including MRP1, MRP2, MRP4, and MRP5. M25 and M58 also bound to viable neurospheres. Iodogen-labeled scFvs demonstrated a yield of 56-76%. The immunoreactive fractions of the radiolabeled M25, M58, and M89 scFvs were 32%, 52%, and 69%, respectively. M25 exhibited 20% internalization into D2159MG neurospheres, M58, 33% into D54MG cells, and M89, 26% into D247MG. Immunohistochemical evaluation of human gliomas to determine the localization of MRP3 antigen using scFvs M25 and M58 showed a dense cytoplasmic and membranous staining pattern. These Fv-based recombinant antibodies, which possess superior tumor penetration capabilities and selectively target tumor cells that express MRP3, may potentially be used in immunotherapy and diagnosis for brain tumors and other cancers.

Keywords

GBM; MRP3; scFv antibody; phage display

Introduction

Human multidrug resistance protein 3 (MRP3), also known as cMOAT2 or ABCC3, is an organic anion and multidrug extruding transporter. It confers multidrug resistance on human

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cancer cells by decreasing the intracellular concentration of drugs, which results in cancer treatment failure.[1] Along with several other bacterial and eukaryotic transporters, MRP3 is a member of the C-branch of the ATP binding cassette (ABC transporter).[2] The human MRP gene family has multiple members (MRP1-MRP9),[3,4] among which MRP3 has a molecular weight of 190-200 kDa and is closest in structure to MRP1, with 58% amino acid identity.[2] Functional analysis has revealed that MRP3 is involved in the cellular extrusion of organic anions[5] and can transport monovalent bile acids (as taurocholate and glycocholate).[6] Glucuronate conjugates are preferred substrates for MRP3, whereas nonconjugated organic anions (such as methotrexate), bile acid sulfates (such as taurolithocholate sulfate), and glutathione conjugates are poor substrates for MRP3.[5,6] In normal human tissues, MRP3 is expressed mainly in the liver, adrenal gland, placenta, testis, intestine, colon, and gallbladder, and at a relatively lower level in pancreas, kidney, lung, and tonsils.[7] Hepatic expression of MRP3 has been linked to the concentration of serum bilirubin or its glucuronides, which suggests that these endogenous compounds may be involved in the induction of MRP3.[8] Expression of MRP3 has also been reported in some human cell lines, including Caco-2 and HepG2,[9] and overexpression of MRP3 has been observed in some tumor cell lines that have acquired multidrug resistance.[10,11] MRP3 has also been reported to play a role in progression of adult acute myeloid leukemia.[12]

It has recently been shown that hepatic progenitor cells have high expression levels of functional MRP1 and MRP3, which may have a role in removing either exogenous or endogenous toxins and metabolites from progenitor cells.[13] Bronger et al. have also detected the expression of other MRP family members such as MRP4 and MRP5 in the blood-brain barrier and in glioma cells at the protein level.[14] Localization of MRP1, MRP4, and MRP5 protein in rapidly frozen perilesional samples of several regions of adult human brain was reported by Nies et al.[15] Expression of ABC transporters was also found to be responsible for the highly enriched side population phenotype in a wide variety of stem cells,[16] and other initial studies also showed that they may be active in hematopoietic stem cells as functional regulators.[17] Calatozzolo et al. showed that *MRP3* is hyperexpressed in astrocytomas as the primary resistance to chemotherapy with drugs like cis-platinum (CDDP) and carmustine (BCNU)[18] and that MRP3 can modulate drug sensitivity to certain anticancer agents such as cisplatin, vincristine, and etoposide in human gliomas.[19] Recent research has shown that MRP3 was much less expressed in cancer stem cells. However, after differentiation, the expression of MRP3 notably rose, which suggests that just after differentiation the cells acquired chemotherapeutic resistance via MRP3.[20]

Glioblastoma multiforme (GBM) is the most aggressive tumor type among the astrocytic tumors. Despite recent advances and discoveries in cancer treatment, the prognosis for patients with malignant glioma is very poor. Overexpressed MRP3 protein has been implicated as one of the cell surface targets for GBM by serial analysis of gene expression (SAGE).[21] MRP3 RNA transcripts are highly expressed in GBM tissues as compared with normal brain tissues.[21]

Overexpression of MRP3 in GBM and relative lack of expression in normal brain, along with its localization to the cell membrane, make MRP3 an excellent potential target for immune-based targeted therapy. Monoclonal antibodies detecting internal MRP3 epitopes have been developed, but none have been reported that detect external MRP3 epitopes. However, Fv fragments, the smallest entities that consistently maintain the binding specificity of intact antibody, have gained much interest over the past decade and are being used in a wide range of therapeutic applications, particularly in the targeted therapy against malignant cells. The Fv targeting moiety of the antibody is composed of the heavy- and light-chain variable regions (VH and VL), which are held together by a peptide linker, thereby forming a single-chain Fv molecule (scFv). Furthermore, because of recent advances

in phage display technology, we can now produce scFvs with higher affinity, which circumvents the requirement for an immunization procedure.[22,23] ScFv antibodies have several advantages over conventional antibodies because they are very small. Their smaller size allows them to penetrate into tumor more efficiently and homogenously[24-27] and facilitates faster systemic and normal tissue clearance.[28,29] ScFv fragments are also finding use as tumor imaging agents and for radioimmunotherapy. ScFv-molecules are being developed against a wide spectrum of cancer-specific targets and are being tested in various clinical trials, some of which already present promising anticancer activity.[30,31]

To date, no monoclonal antibody has been isolated that can target an extracellular MRP3 epitope. Here we report, by use of a phage display approach, the first successful isolation of three fully human recombinant scFv antibodies, M25, M58, and M89, which specifically react with the extracellular N-terminus of the human MRP3. The scFvs reacted only with the peptide used for the phage display screening and did not cross-react with other MRP3-derived peptides. We demonstrate herein that the scFv antibody can bind to viable tumor cells with MRP3 expression but not MRP3-null cells. We show that novel recombinant antibody fragments targeting MRP3 may potentially be used for the development of cancer targeting and imaging agents for brain tumors and other cancers expressing MRP3.

Materials and Methods

Cell lines

Human GBM and ependymoma-derived cell lines D247MG, D2159MG, D612EP, and D633EP were established and maintained by our laboratory,[32,33] and 6B-47MG was kindly provided by Dr. David James (University of California, San Francisco). Glioma cell lines were cultured either in zinc option (ZO) medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; ZO-10% FCS) or in knockout DMEM/F-12 low-osmolality neural stem cell medium (NSCM) (Gibco) with supplements. Human ovarian carcinoma 2008 cells and their stable 2008/MRP1-3 and 2008/MRP5 transfectants with different MRP cDNA (MRP1, MRP2, MRP3, and MRP5), as well as the human embryonic kidney MRP4 transfectant cells HEK293/4.63, were kindly provided by Professor P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). All cells were incubated at 37°C in 5% CO_2 and 99% humidity.

Isolation of cells from xenografts

Human glioma and ependymoma xenografts from athymic mice were dissociated with Liberase (Roche, Indianapolis, IN) at a 100-μg/ml concentration. The dissociated cells were filtered through a disposable sieve, and reaction was inhibited with ovomucoid solution. After washing several times with media, the cells were counted and plated in either ZO-10% FCS or in NSCM to isolate neurospheres. The cells were used for FACS and other analyses.

Screening of human naïve scFv phage display library

A human naïve single-chain Fv phage library (Mehta I) containing 15×10^9 diversity was used for the selection of anti-MRP3 scFvs.[34,35] The library was subjected to three successive rounds of biopanning with biotinylated peptides (Anaspec, San Jose, CA) derived from MRP3 protein (PepI, PepII, PepIII, and PepIV, as described and shown in Fig. 1), alternatively with streptavidin-coated beads (Dynal, Invitrogen, Carlsbad, CA) and avidin resin (Promega, Madison, WI). To eliminate phages that bind to streptavidin beads, avidin resin was used in the next round of panning followed by streptavidin beads in the third and final round. Successive reductions in the concentration of the biotinylated peptides were used, 500 nM for the first round, 100 nM for the second, and 20 nM for the third round (Table I). Streptavidin beads were mixed, and the mixture was incubated in a precoated (5%

milk–phosphate-buffered saline [PBS]) tube with continuous rotation. A magnetic force was applied to separate the phages bound to the biotinylated complex. Bound phages were eluted by acidic (200 μl of 0.1 M HCl-glycine, pH 2.0) and alkaline (200 μl of 100 mM triethylamine) treatment. Neutralization was done by adding 400 μl of 1 M Tris HCl. Eluted mixtures were pooled and used to infect the amber-codon-suppressing *E. coli* TG1 cells (A_{600}, 0.5) for 30 min at 37°C, which were later superinfected with VCSM13 interference-resistant helper phage (Stratagene, La Jolla, CA) as described by Amersdorfer and Marks.[36] More phages were grown and subjected to further rounds of selection for the enrichment of putatively positive clones for the respective peptides used for screening. The ratio of phage output to phage input was used to determine the enrichment value (Table I).

Polyclonal and monoclonal phage antibody ELISA

The phage population produced after each round of selection was screened by ELISA for binding to MRP3 peptides and was used to confirm the round-by-round enrichment of antigen-specific phages. Phages from single, infected bacterial colonies were then screened by ELISA to identify the monoclonal phage. For polyclonal phage ELISA, aliquots of phages after each round of selection were added to 96-well plates coated with 10 μg/ml of MRP3-derived peptides conjugated with bovine serum albumin (BSA) in 100 μl of 200 mM $NaHCO_3$, pH 9.6. Plates were incubated overnight for coating with a specific antigen at 4°C. After washing with 0.05% Tween/PBS three times, the plates were blocked with 2% milk-PBS for 2 h at room temperature. Wells were then briefly rinsed with PBS and incubated with phage supernatant (~10^{10} plaque-forming units per well) in 2% milk-PBS for 1 h at room temperature. Plates were further incubated with horseradish peroxidase (HRP)-conjugated mouse anti-M13 antibody (1:5000, Amersham, Piscataway, NJ). Detection was performed by using a Sigmafast OPD *o*-phenylenediamine dihydrochloride tablet set (Sigma, St. Louis, MO). Phages from a single, infected bacterial colony, one from the second round and one from the third round of populations, were then screened by monoclonal phage ELISA. *E. coli* TG1 colonies were picked randomly and expanded, supernatant was added to 96-well plates coated with various MRP3-derived peptides, and ELISA was performed as described above.

DNA sequencing

Phage clones from the second and third rounds of panning were selected, and their DNA was isolated and sequenced by using an ABI377 automatic sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences for positive clones were determined by primers “pelB forward” and “cmyc reverse,” which hybridize the flanking sequence of the insert. Nucleotide sequences of the primers are as follows.

“pelBForward” (located right before the heavy chain):

CATAATGAAATACCTATTGCCTA

“cmycReverse” (located right after the light chain):

CTTATTAGCGTTTGCCATT

Several positive clones were picked on the basis of their ELISA titer, and nucleotide sequences for each of the MRP3-derived peptides were used for screening the phage library.

Production of soluble scFv

Phages expressing M25 and M58 scFvs were selected for the PepI region of MRP3, and phages expressing M89, M23, and M17 scFvs were selected for the PepII, PepIII, and PepIV regions of the MRP3 protein, respectively. ScFv genes were cloned into expression vector pET 22 $b^{(+)}$ (Novagen, Madison, WI) to enable the expression of active and soluble scFv

antibody. Briefly, the scFv-encoding fragment was excised from the phagemid by digesting it with NcoI and Not I restriction endonucleases. It was then ligated to the NcoI- and Not I-digested pET vector, which carries a hexahistidine tag for purification and detection. The scFv antibodies were expressed in *E. coli* BL21 (DE3) gold cells (Stratagene) and purified from the soluble cytoplasmic fraction by metal-ion affinity chromatography (Talon, Clontech, Palo Alto, CA). The M25, M58, and M89 scFvs were chosen for further analysis and characterization on the basis of their production yield, binding affinity, and binding profile with cells expressing MRP3.

Affinity measurements

Kinetic measurements of purified recombinant scFv were determined by surface plasmon resonance using a BIAcore 3000 biosensor (BIAcore, Piscataway, NJ). The streptavidin (SA) chips were immobilized with an individual biotinylated peptide derived from MRP3 protein (e.g., PepI, PepII, PepIII, or PepIV; approximately 1004.2 RU [residual units] of peptide was immobilized) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) balanced salt solution (HBSS) buffer, composed of 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.05% BIAcore surfactant P20. The scFv antibodies were diluted in HBSS buffer and passed over the SA chip at concentrations of 25, 200, and 1000 nM. The flow rate was kept at 30 μl/min, and the injection time was 3 min. Binding constants were calculated from the nonlinear fitting curve by using the BIAevaluation software (BIAcore).

ELISA with purified scFv antibodies

Binding specificity and cross-reactivity of the purified scFvs was determined in ELISA assays for MRP3-derived peptides PepI, PepII, PepIII, and PepIV. A 96-well plate was coated with 10 μg/ml of a specific MRP3-derived peptide conjugated with BSA as antigen vs. BSA and other appropriate control antigens. The rest of the protocol was the same as described above except that the purified scFv antibodies were added in serial dilution to the wells, and the secondary antibody used was HRP-conjugated anti-His antibody (1:5000, Invitrogen).

Flow cytometry

For flow cytometric analysis of M25, M58, and M89 scFv antibodies to test the cell surface MRP3 molecule binding, either live neurospheres (D2159MG) or dissociated cells from xenografts (D2159MG, 6B-47MG, D633EP, and D612EP) were harvested (2×10^5/test), washed, and blocked with 10% FCS. Adherent D54MG, D247MG, and ovarian carcinoma cell lines (2×10^5/test) were harvested with 0.02% EDTA/PBS and fixed by resuspending them in 2-4 ml of 10% formaldehyde. After washing, the cells were blocked with 10% normal goat serum (NGS), with and without saponin, for 20 min. Saponin was added to facilitate the permeabilization of scFvs for examining the total surface receptors. Cells from all the cell lines and xenografts were incubated with corresponding scFv for an hour, on ice for live cells and at room temperature for fixed cells, and were washed twice and incubated with anti-histidine tag (C-terminus)–FITC–conjugated antibody (1:5000, Invitrogen) for an hour, on ice for live cells and at room temperature for fixed cells. Finally, live cells were suspended in PBS and were analyzed. Fixed cells were suspended in 0.5% paraformaldehyde-BSA and freshly analyzed by using a Becton Dickinson FACSort flow cytometer with CellQuest software (BD Biosciences, San Jose, CA).

Immunoreactive fraction determination

Immunoreactive fractions of the radioiodinated M25, M58, and M89 scFvs were determined according to the method described by Lindmo et al.[37] Purified recombinant scFvs (M25, M58, and M89) were labeled with ^{125}I by the Iodogen method, as described by Reist et al.,

[38,39] to a specificity of 1 to 3 μCi/μg and purified by gel filtration on a 10-cm PD-10 Sephadex G-25 (Pharmacia, Pfizer, New York, NY) column.[40]

Fractions of immunoreactive scFvs were calculated by linear exploitation of infinite antigen excess. For these procedures, 1 ml of streptavidin-coated beads (MPG Streptavidin, Pure Biotech, LLC, Middlesex, NJ) were coated with 100 μg of biotinylated PepI or PepII and BSA to measure nonspecific binding. Beads were then rinsed to remove unbound peptides and resuspended in the original volume of Lindmo buffer (115 mM phosphate buffer of pH 7.5, 0.05% BSA, and 0.05% Brij detergent [Sigma]). Triplicate aliquots of beads in increasing volumes (25, 50, and 100 μl) were incubated for 45 min at room temperature with 50 μl of Lindmo buffer containing 5 ng of each of the labeled scFv antibodies. Final volume was adjusted with Lindmo buffer. Beads were washed and separated by using a magnetic separator, and pellets and supernatants were counted in a gamma counter. Specific binding was calculated by subtracting nonspecific binding to BSA-coated beads from the binding of M25, M58, or M89 scFv separately. Plotting the total activity divided by the specifically bound activity versus the reciprocal of the antigen concentration yielded a linear plot, the intercept of which represents the inverse of the immunoreactive fraction.

Internalization assay

Internalization assays were performed with D2159MG, D54MG, and D247MG cell lines, plated at a density of 5×10^5 cells per well in NSCM or ZO medium and incubated at 4°C for 30 min. The medium was removed from each well and replaced with fresh cold medium containing 1 μg/ml of radiolabeled scFv antibody. After 1 h of incubation at 4°C, the medium was removed, and wells were washed twice with 1 ml of cold medium to remove unbound activity. Fresh medium was added to the wells, and plates were placed in an incubator at 37°C for different time periods (0, 1, 2, 4, 8, 20, and 24 h). After each time point, supernatant was removed, and cells were washed twice with medium (pH 2.0) for 10 min to remove surface-bound activity. Finally, the cells were solubilized in 0.5 ml of 0.5 N NaOH (internalized fraction). Each supernatant sample was precipitated by 12% trichloroacetic acid to calculate acid-precipitable counts. All the fractions of the supernatant, acid washes, and the cell pellets were counted in a gamma counter and were expressed as the percentage of activity initially bound to the cells after 1 h of incubation at 4°C.

Immunohistochemistry

Tissue microarrays (TMAs) made of tissues from GBM patients were used for immunohistochemical studies. TMAs were constructed by obtaining cylindrical tissue specimens from regular paraffin blocks and then assembling them into a single block. Immunostaining for MRP3 was performed as previously described.[41] Briefly, TMAs were deparaffinized in xylene, rehydrated in a graded alcohol series, and washed with PBS. Antigen retrieval was performed by placing the slides in 50 mM citrate buffer (pH 6.0) and microwaving it, first for 2 min at full power or until the buffer started to boil and then continuing to microwave for 8 min at 30% power, followed by cooling for 15 min and 2-3 PBS washes of 5 min each. Quenching of endogenous peroxidase was done by incubating the sections in 1% H_2O_2 in methanol for 10 min. Sections were washed in PBS and blocked for 1 h at room temperature in 20% NGS. Sections were then incubated with purified M25 and M58 scFvs separately in 1% BSA-PBS overnight at 4°C. Primary antibody was washed away with PBS, and TMAs were incubated with Penta-His secondary antibody (Qiagen, Valencia, CA) in PBS (1:25) for 1 h at room temperature. Tertiary antibodies were applied by using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Detection of bound tertiary antibodies was performed with diaminobenzidine for 1-5 min. Sections were then counterstained with hematoxylin and mounted. The TMAs were first scanned at 600X magnification using an Aperio Scanscope

XT slidescanner. Automated image analysis was performed using Aperio Image scope (v9.1.19.1567) software and quantified with the Positive Pixel Count algorithm (v2).

Results

Isolation of MRP3-specific single-chain Fv antibodies

A naïve human scFv phage library, Mehta I, with 15×10^9 complexity,[34] was screened for single-chain antibody fragments specific for MRP3 protein. The homology between MRP3 protein and the other members of the human MRP protein family ranges from 33% with MRP5 to 58% with MRP1. Hydrophobicity analysis of the MRP3 amino acid sequence indicates an organization of putative transmembrane domains resembling that suggested for MRP1. The precise membrane topology of MRP3 is complex and remains unclear. MRP3 is most closely related to MRP1, with 58% amino acid identity, and may have similar predicted topology of transmembrane segments. These proteins probably span the membrane 17-18 times, with the NH_2 terminus being extracellular.[42] After analyzing the topology and the antigenicity plot of the MRP3 amino acid sequence, we selected four peptides from extracellular domains that may elicit an immune response. As shown in Fig. 1, two of the selected peptides, PepI and PepII, are from amino acids 1-20 and 21-32 of the amino terminus of the protein. The other two peptides, PepIII and PepIV, encompass amino acids 999-1013 and 1053-1069, respectively, and map to extracellular loop 6, one of the extracellular loops of the MSD3 domain of human MRP3 (Fig. 1).

In order to isolate scFv against the four selected extracellular epitopes, we screened the Mehta I human naïve scFv phage display library, which has a complexity of 15 billion independent clones. It was subjected to three rounds of panning with decreasing concentrations of biotinylated peptides, followed by elution of bound phages and re-amplification of phages in *E. coli* strain TG1. A progressive and marked enrichment for phages was observed after each round of panning, with a total of 68-fold and 390-fold enrichment between the first round and third round of selection in the case of MRP3-derived PepI and PepII, respectively (Table I). There was a total of 126-fold and 72-fold enrichment with PepIII and PepIV, respectively, in the third round of panning (data not shown).

A marked enrichment of specific phage clones was observed after the third round of panning, as exhibited also by polyclonal phage ELISA. Of 192 clones in each peptide screening for monoclonal phage ELISA, 54, 28, 7, and 38 phage clones, respectively, exhibited binding to immobilized MRP3-derived PepI, PepII, PepIII, and PepIV. DNA sequencing was performed with phage DNA of the specific clones, and the amino acid sequence was deduced. Putative clones were chosen for further analysis on the basis of their DNA sequence and their specificity as exhibited by monoclonal phage ELISA. Most of the clones against each peptide had an identical nucleotide sequence, as they converged after three rounds of enrichment. Among all positive clones, according to ELISA titer, M25 and M58 scFvs were selected as best for the PepI region of MRP3 protein, and M89, M23, and M17 were selected as best for the PepII, PepIII, and PepIV regions of the MRP3 protein, respectively. On the basis of the binding analysis of scFv with cells expressing MRP3, we decided not to further pursue the evaluation of M23 and M17.

Characterization of anti-MRP3 scFvs

Expression and purification of M25, M58, and M89 scFv proteins—For expressing large quantities of pure recombinant scFv antibody in bacterial cells, scFv coding sequences were isolated from the pFarber phage display vector by NcoI and NotI digestion and cloned into a pET $22b^{(+)}$ vector in the same orientation. The scFvs expressed in bacterial cells were C-terminally tagged with histidine tag. All scFvs were produced in *E. coli* BL21

(DE3) gold by secretion and were purified from whole cell extracts by metal affinity chromatography using a C-terminal hexahistidine tag. Purified scFvs were analyzed on 4-12% SDS PAGE (Fig. 2A), which revealed a major single band of ~27 kDa.

Binding kinetics, affinity measurements, and specificity—Surface plasmon resonance technology implemented on a BIAcore instrument (BIAcore, Pharmacia) was used to characterize the binding kinetics and affinity of purified scFv antibody with immobilized biotinylated specific peptide. The association and dissociation rates from the sensogram were k_{assoc} of 5.4×10^4 (mol/L-s)$^{-1}$ and a k_{diss} of 1.9×10^{-3} s^{-1} for M25 scFv, k_{assoc} of 7.1×10^4 (mol/L-s)$^{-1}$ and a k_{assoc} of 3.4×10^{-3} s^{-1} for M58 scFv, and k_{assoc} of 5.1×10^4 (mol/L-s)$^{-1}$ and a k_{diss} of 5×10^{-3} s^{-1} for M89. K_A at binding equilibrium, calculated as $K_A = k_{assoc} / k_{diss}$, was 2.8×10^7 (mol/L)$^{-1}$ for M25 scFv, 2.1×10^7 (mol/L)$^{-1}$ for M58 scFv, and 1×10^7 (mol/L)$^{-1}$ for M89 scFv (Fig. 2B).

To determine whether the recombinant scFvs could bind specifically to their corresponding MRP3-derived peptides, we examined soluble, purified scFv antibodies by ELISA assays. All scFvs reacted in a dose-dependent manner with their corresponding MRP3-derived peptides conjugated to BSA. They reacted only with the specific MRP3 peptide that was used to screen the library and did not cross-react with other MRP3 peptides (Fig. 3) or BSA.

Binding of M25, M58, and M89 scFv antibodies to MRP3-expressing tumor cells—To confirm that the purified, soluble M25, M58, and M89 scFvs bound to native MRP3 molecules expressed on the surface of tumor cells, we conducted indirect FACS of M25, M58, and M89 with a number of cell lines, such as D2159MG, 6B-47MG, D633EP, and D612EP. Live tumor cells segregated from human glioma or ependymoma xenografts (D2159MG, 6B-47MG, D633EP, and D612EP) were grown in ZO medium (Fig. 4A), and live neurospheres (D2159MG) were grown in NSCM (Fig 4B). D2159MG neurospheres growing in NSCM showed much better binding than D2159MG cells growing in ZO medium or D2159MG cells derived directly from xenografts (Fig. 4B). Indirect FACS with fixed D247MG cells was also done under both permeabilized and nonpermeabilized conditions to look for total and cell surface binding (Fig. 4C). All of the cells from the xenografts and neurospheres exhibited a right shift in the peak, indicating binding of M25 and M58 to the surface of live cells, as shown in Fig. 4; however, M89 did not exhibit binding to viable cells (data not shown). The shift associated with the binding of M25 was greater than the shift for M58 in all cell lines or neurospheres used and showed that the binding to cell surface is consistent with the affinity measured by the BIAcore biosensor (Fig. 4 and Fig. 2B). D247MG showed a shift in the peak both in permeabilized and in nonpermeabilized cells, indicating the binding of M58 and M89 scFv antibodies to surface MRP3 molecules under the fixed-cell condition. Irrelevant control scFv was unreactive with all of the cell lines (Fig. 4C).

FACS analysis of anti-MRP3 scFv antibodies to viable stable transfectants expressing MRP1-5

In order to rule out the possibility of cross-reaction with other members of MRP families, we analyzed the purified M25 scFv by FACS using ovarian carcinoma cell line 2008 and its transfectants expressing MRP1, MRP2, MRP3, or MRP5. We also analyzed the same purified M25 scFv by FACS using human embryonic kidney cell line HEK293 and its transfectants expressing MRP4 (HEK293/4.63). The analysis revealed that the binding of the M25 scFv antibody was very specific to the MRP3-expressing cells, with no cross-reactivity to the cells expressing other MRPs, as shown in Fig. 5. M58 and M89 also showed no cross-reactivity with other members of MRP-family proteins expressed on the 2008 cell line (data not shown).

Radioiodination and immunoreactivity of purified scFv antibody

Purified M25, M58, and M89 scFv antibodies were radiolabeled with ^{125}I by the Iodogen method and purified by gel filtration. Radiolabeled scFvs were analyzed by gel filtration HPLC using a TSK2000SW column (Tosoh Bioscience, Montgomeryville, PA) and by surface plasmon resonance (BIAcore). Radiolabeling yields were in the range of 56-76% for the M25, M58, and M89 scFv antibodies, and the BIAcore analysis showed almost identical kinetics and affinities as were measured for the corresponding nonradiolabeled scFvs. The immunoreactivity of ^{125}I-labeled M25, M58, and M89 scFvs was measured by using magnetic beads coated with biotinylated PepI (for M25 and M58) or PepII (for M89). Immunoreactive fractions of the labeled M25 and M58 scFv antibodies were 32% and 52%, respectively, and for M89 the corresponding fraction was 69% (data not shown).

Internalization and processing of anti-MRP3 scFvs

Internalization of anti-MRP3 M89 scFv fragments was conducted according to the method described by Reist et al. (1995)[38] using D247MG and D54MG tumor cells to determine whether scFv bound to MRP3 internalizes at physiological temperature. D247MG and D54MG cells were incubated with purified M89 scFv for 1 h at 4°C to allow scFv to bind to the cell surface. The cultures were then adjusted to 37°C, and cell-associated radioactivity was measured at 0, 1, 2, 4, 8, 20, and 24 h. Percentages of radioactive counts for cell culture supernatant, cell surface, and intracellular compartments for different time points were plotted for both cell lines, D247MG and D54MG (Fig. 6A and Fig. 6B, respectively). Cell-surface-associated counts for M89 scFv showed a rapid drop, accompanied by an increase in counts in the cell culture supernatant (Fig. 6). During early time points, from 1 to 4 h, approximately 70%-80% of the associated counts were released to the cell culture supernatant. The radioactivity of scFv associated with the cell culture supernatant increased and accounted for 80% of the radioactive counts for D247MG cells and 90% for D54MG cells of the total cpm recovered after 24 h at 37°C (Fig. 6). The profile showed that the internalization of M89 scFv into D247MG cells gradually increased to a 26% maximum percentage of intracellular counts at the 2-h time point as compared with 25% for M89 scFv in D54MG cells at the 1-h time point. For both D54MG and D247MG, 20% of the M89 scFv counts were detected intracellularly throughout the time course up to the 20-h time point (Fig. 6). In addition, M25 exhibited 20% internalization into D2159MG neurospheres, and M58 showed 33% internalization into D54MG cells (data not shown).

Immunohistochemical analysis

To confirm the specificity of anti-MRP3 scFvs M25 and M58, we examined the expression of MRP3 in paraffin-embedded sections of GBM patient tissue immunohistochemically using purified M25 and M58 scFv antibodies. Immunohistochemistry of M25 revealed a membranous/cytoplasmic binding pattern in 23 (45%) of 51 malignant gliomas. The labeling index varied from 5% to 80% of tumor cells among the positive biopsy cases analyzed (Fig. 7). Immunohistochemistry of M58 also revealed a cytoplasmic and membranous staining pattern in 37 (79%) of 47 malignant gliomas tested. The labeling index among positive cases with M58 scFv-stained sections varied from 5% to 40%. M58 revealed more robust reactivity with GBM cells than was seen with M25.

Discussion

Glioblastoma multiforme is the most prevalent and most aggressive type of brain tumor. In spite of advances in surgery, radiotherapy, and chemotherapy, most patients die within a year of diagnosis. GBM cells express different levels of certain cell surface proteins than the normal surrounding brain cells. Many antigens, such as glycoprotein transmembrane nmb (gpnmb), epidermal growth factor receptor (EGFRwt) and its glioma-associated deletion

variant (EGFRvIII), and the interleukin 13 receptor α2 isoform (IL-13Rα2), have been identified whose expression is clearly up-regulated in brain tumor cells. MRP3 has been identified as one of the GBM-associated molecules,[21] being expressed at high levels in GBM but not in normal brain cells.[31] Therefore, MRP3 is a potentially useful tumor-associated antigen and prognostic predictor for immunotherapeutic approaches for malignant gliomas.

Using recent advances in gene technology, we have developed single fragment chain human antibodies to target GBM tumors.[31,43] We isolated scFv antibodies specific for extracellular MRP3 epitopes by screening a human naïve phage display library with 15×10^9 complexity.[35] These scFv antibodies meet the prime criterion for being a successful therapeutic or diagnostic agent for GBM therapy—high affinity for the target antigen, specificity toward antigen, binding to the extracellular epitope, and efficient internalization into cells.

As localization of the target antigen is an important factor in the selection of a potential target for GBM immunotherapy, we selected four peptides from the extracellular NH_2 terminus and one from the extracellular loop of MRP3 protein, on the basis of an antigenicity plot and a hydrophobicity analysis. Four biotinylated peptides (PepI, PepII, PepIII, and PepIV) were used to screen the phage display library, and we assessed the specificity and sensitivity of all purified scFv antibodies by ELISA assays. There was no cross-reactivity observed among the scFvs isolated from different peptides derived from MRP3 protein. For example, the M25 scFv reacted only with immobilized PepI antigen, and no cross-reactivity was observed with PepII, PepIII, or PepIV. Positive phage clones were sequenced after selection against each of the peptides derived from MRP3. After three rounds of enrichment, we observed that most of the clones against each peptide (PepI, PepII, PepIII, or PepIV) had merged to an identical nucleotide sequence. The M25 and M58 scFvs were selected as optimal binders for the PepI region of MRP3 protein, and M89 for the PepII region.

BIAcore analysis of purified recombinant scFvs on a microsensor chip showed binding affinity in the range of 1×10^7. This level of binding affinity for first-generation, single-chain variable fragments is sufficient for tumor targeting when the antigen is expressed in notably high levels.[44] But for moderately or less expressed antigens, antibody fragments with higher affinities are preferred.[45]

We used FACS to further investigate the binding of purified M25, M58, and M89 scFvs to native MRP3 molecules expressed on the surface of GBM and ependymoma tumor cells, neurospheres, and cells segregated from xenografts. All three scFvs showed a different binding pattern with live cells. Of the three scFvs selected, M25 was able to exhibit greater specific binding with all the cell lines and xenografts used by FACS and was able to show binding specifically with ovarian carcinoma cell line 2008 transfectants expressing MRP3, but there was no binding activity with transfectants expressing MRP1, MRP2, or MRP5. M25 and M58 showed a greater shift with D2159MG neurospheres growing in neural stem cell medium than with D2159MG growing in ZO with FCS, which suggests that MRP3 may be expressed more in cells growing in neural stem cell medium than in those growing in differentiation medium (ZO with FCS). M58 and M89 did not show any cross-reactivity with ovarian carcinoma 2008 transfectant cell lines, including MRP1, MRP2, and MRP5. We also analyzed M58 and M89 under fixed cell conditions in permeabilized and nonpermeabilized cells and found that both scFvs bound specifically to only MRP3-expressing D247MG cells. Additionally, the data supports the immunohistochemistry evaluation, where M25 (45%) and M58 (79%) showed staining with MRP3-expressing glioma biopsy tissues. However, we did not find any staining with M89 by

immunohistochemistry. Normal brain samples did not show any binding with any of the scFvs tested.

Immunoreactive fractions for M25, M58, and M89 scFvs were measured. M89 scFv produced a significantly higher immunoreactive fraction than M25 and M58. We also determined the amount of scFv that was internalized into the cells. This measure is also an important criterion for choosing a scFv antibody as a therapeutic agent, as internalization is required for binding and cytotoxicity of immunotoxins.[46,47] An *in vitro* internalization assay for the purified M89 scFv demonstrated significantly higher amounts of ^{125}I-labeled M89 scFv internalized by D54MG and D247MG cells during the first two hours of the assay, and a good percentage of it remained internalized throughout the assay. D54MG exhibited up to 25% internalization of M89 scFv within the first hour, and then it dropped slowly to 18% between the 2-h and the 20-h time points. For D247MG cells, counts for the internalized M89 scFv dropped to about 16% after 20 h. The pattern of internalization was very similar to that of a previously reported anti-EGFRvIII monoclonal antibody, L8A4, by HC2 20 d2 cells expressing EGFRvIII.[40,43]

In comparison with other anti-MRP3 antibodies, M25, M58, and M89 are completely human single-chain antibodies against cell surface epitopes. To our knowledge, these are the first scFv antibodies isolated against extracellular epitope of MRP3 from a human naïve phage display library. These scFvs, displaying good affinity and specificity, can be used as radiolabeled single-chain fragments for immunotargeting of malignant brain tumors and potentially other MRP3-positive tumors.

In conclusion, we have successfully isolated M25, M58, and M89 anti-MRP3 scFv antibodies that bind selectively to external MRP3 epitopes that are expressed on brain tumor cells. We found no cross-reactivity with normal brain cells and found good binding affinity for first-generation scFv isolates. Because of their good affinity and specificity, these scFvs can be used as a vehicle for radioisotopes or other cytotoxic drugs to target MRP3-expressing cancer cells. Moreover, the affinity of selected antibodies can be improved by affinity maturation in clones developed through construction of mutant antibody libraries. Significantly, these scFvs, with good immunoreactivity and internalization into MRP3-expressing malignant cells, are excellent candidates for immunotoxin development. Thus these MRP3-specific scFvs are potentially valuable reagents for brain tumor diagnosis and targeted therapy.

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Abbreviations

ABC	ATP-binding cassette
BSA	bovine serum albumin
FITC	fluorescein isothiocyanate
GBM	glioblastoma multiforme
HBSS	HEPES balanced salt solution

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGG	high-grade glioma
HRP	horseradish peroxidase
K_A	affinity at binding equilibrium
MRP	multidrug resistance protein
SAGE	serial analysis of gene expression
scFv	single-chain, variable fragment antibody
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMA	tissue microarray

References

1. Deeley RG, Cole SP. Function, evolution and structure of multidrug resistance protein (MRP). Semin Cancer Biol. 1997; 8:193–204. [PubMed: 9441948]
2. Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A. 1999; 96:6914–9. [PubMed: 10359813]
3. Bortfeld M, Rius M, Konig J, Herold-Mende C, Nies AT, Keppler D. Human multidrug resistance protein 8 (MRP8/ABCC11), an apical efflux pump for steroid sulfates, is an axonal protein of the CNS and peripheral nervous system. Neuroscience. 2006; 137:1247–57. [PubMed: 16359813]
4. Kruh GD, Guo Y, Hopper-Borge E, Belinsky MG, Chen ZS. ABCC10, ABCC11, and ABCC12. Pflugers Arch. 2007; 453:675–84. [PubMed: 16868766]
5. Hirohashi T, Suzuki H, Sugiyama Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). J Biol Chem. 1999; 274:15181–5. [PubMed: 10329726]
6. Hirohashi T, Suzuki H, Takikawa H, Sugiyama Y. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). J Biol Chem. 2000; 275:2905–10. [PubMed: 10644759]
7. Scheffer GL, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, Elferink RP, van der Valk P, Borst P, Scheper RJ. Tissue distribution and induction of human multidrug resistant protein 3. Lab Invest. 2002; 82:193–201. [PubMed: 11850532]
8. Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, Akizawa T, Yoshioka M, Sugiyama Y. Characterization of inducible nature of MRP3 in rat liver. Am J Physiol Gastrointest Liver Physiol. 2000; 278:G438–46. [PubMed: 10712264]
9. Kiuchi Y, Suzuki H, Hirohashi T, Tyson CA, Sugiyama Y. cDNA cloning and inducible expression of human multidrug resistance associated protein 3 (MRP3). FEBS Lett. 1998; 433:149–52. [PubMed: 9738950]
10. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res. 1997; 57:3537–47. [PubMed: 9270026]
11. Uchiumi T, Hinoshita E, Haga S, Nakamura T, Tanaka T, Toh S, Furukawa M, Kawabe T, Wada M, Kagotani K, Okumura K, Kohno K, et al. Isolation of a novel human canalicular multispecific organic anion transporter, cMOAT2/MRP3, and its expression in cisplatin-resistant cancer cells with decreased ATP-dependent drug transport. Biochem Biophys Res Commun. 1998; 252:103–10. [PubMed: 9813153]
12. Benderra Z, Faussat AM, Sayada L, Perrot JY, Tang R, Chaoui D, Morjani H, Marzac C, Marie JP, Legrand O. MRP3, BCRP, and P-glycoprotein activities are prognostic factors in adult acute myeloid leukemia. Clin Cancer Res. 2005; 11:7764–72. [PubMed: 16278398]

13. Ros JE, Roskams TA, Geuken M, Havinga R, Splinter PL, Petersen BE, LaRusso NF, van der Kolk DM, Kuipers F, Faber KN, Muller M, Jansen PL. ATP binding cassette transporter gene expression in rat liver progenitor cells. Gut. 2003; 52:1060–7. [PubMed: 12801967]

14. Bronger H, Konig J, Kopplow K, Steiner HH, Ahmadi R, Herold-Mende C, Keppler D, Nies AT. ABCC drug efflux pumps and organic anion uptake transporters in human gliomas and the blood-tumor barrier. Cancer Res. 2005; 65:11419–28. [PubMed: 16357150]

15. Nies AT, Jedlitschky G, Konig J, Herold-Mende C, Steiner HH, Schmitt HP, Keppler D. Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. Neuroscience. 2004; 129:349–60. [PubMed: 15501592]

16. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med. 2001; 7:1028–34. [PubMed: 11533706]

17. Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells. 2002; 20:11–20. [PubMed: 11796918]

18. Calatozzolo C, Gelati M, Ciusani E, Sciacca FL, Pollo B, Cajola L, Marras C, Silvani A, Vitellaro-Zuccarello L, Croci D, Boiardi A, Salmaggi A. Expression of drug resistance proteins Pgp, MRP1, MRP3, MRP5 and GST-pi in human glioma. J Neurooncol. 2005; 74:113–21. [PubMed: 16193381]

19. Haga S, Hinoshita E, Ikezaki K, Fukui M, Scheffer GL, Uchiumi T, Kuwano M. Involvement of the multidrug resistance protein 3 in drug sensitivity and its expression in human glioma. Jpn J Cancer Res. 2001; 92:211–9. [PubMed: 11223551]

20. Jin F, Zhao L, Zhao HY, Guo SG, Feng J, Jiang XB, Zhang SL, Wei YJ, Fu R, Zhao JS. Comparison between cells and cancer stem-like cells isolated from glioblastoma and astrocytoma on expression of anti-apoptotic and multidrug resistance-associated protein genes. Neuroscience. 2008

21. Loging WT, Lal A, Siu IM, Loney TL, Wikstrand CJ, Marra MA, Prange C, Bigner DD, Strausberg RL, Riggins GJ. Identifying potential tumor markers and antigens by database mining and rapid expression screening. Genome Res. 2000; 10:1393–402. [PubMed: 10984457]

22. Clackson T, Hoogenboom HR, Griffiths AD, Winter G. Making antibody fragments using phage display libraries. Nature. 1991; 352:624–8. [PubMed: 1907718]

23. Lerner RA, Kang AS, Bain JD, Burton DR, Barbas CF 3rd. Antibodies without immunization. Science. 1992; 258:1313–4. [PubMed: 1455226]

24. Jain RK. Delivery of molecular medicine to solid tumors. Science. 1996; 271:1079–80. [PubMed: 8599082]

25. Adams GP, Shaller CC, Chappell LL, Wu C, Horak EM, Simmons HH, Litwin S, Marks JD, Weiner LM, Brechbiel MW. Delivery of the alpha-emitting radioisotope bismuth-213 to solid tumors via single-chain Fv and diabody molecules. Nucl Med Biol. 2000; 27:339–46. [PubMed: 10938467]

26. Adams GP, Schier R, McCall AM, Simmons HH, Horak EM, Alpaugh RK, Marks JD, Weiner LM. High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. Cancer Res. 2001; 61:4750–5. [PubMed: 11406547]

27. Adams GP, Weiner LM. Radioimmunotherapy of solid tumors: from fairytale to reality. Cancer Biother Radiopharm. 2001; 16:9–11. [PubMed: 11279802]

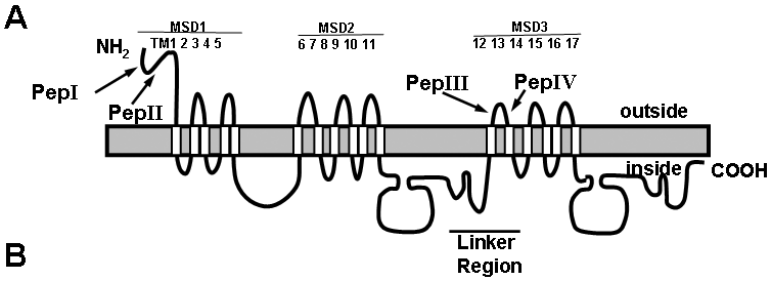
28. Batra SK, Jain M, Wittel UA, Chauhan SC, Colcher D. Pharmacokinetics and biodistribution of genetically engineered antibodies. Curr Opin Biotechnol. 2002; 13:603–8. [PubMed: 12482521]

29. Colcher D, Bird R, Roselli M, Hardman KD, Johnson S, Pope S, Dodd SW, Pantoliano MW, Milenic DE, Schlom J. In vivo tumor targeting of a recombinant single-chain antigen-binding protein. J Natl Cancer Inst. 1990; 82:1191–7. [PubMed: 2362290]

30. Archer GE, Sampson JH, Lorimer IA, McLendon RE, Kuan CT, Friedman AH, Friedman HS, Pastan IH, Bigner DD. Regional treatment of epidermal growth factor receptor vIII-expressing neoplastic meningitis with a single-chain immunotoxin, MR-1. Clin Cancer Res. 1999; 5:2646–52. [PubMed: 10499644]

31. Kuan CT, Reist CJ, Foulon CF, Lorimer IA, Archer G, Pegram CN, Pastan I, Zalutsky MR, Bigner DD. 125I-labeled anti-epidermal growth factor receptor-vIII single-chain Fv exhibits specific and high-level targeting of glioma xenografts. Clin Cancer Res. 1999; 5:1539–49. [PubMed: 10389943]
32. Bigner DD. Biology of gliomas: potential clinical implications of glioma cellular heterogeneity. Neurosurgery. 1981; 9:320–6. [PubMed: 6272152]
33. Bigner DD, Bigner SH, Ponten J, Westermark B, Mahaley MS, Ruoslahti E, Herschman H, Eng LF, Wikstrand CJ. Heterogeneity of Genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. J Neuropathol Exp Neurol. 1981; 40:201–29. [PubMed: 6260907]
34. Mirzabekov T, Kontos H, Farzan M, Marasco W, Sodroski J. Paramagnetic proteoliposomes containing a pure, native, and oriented seven-transmembrane segment protein, CCR5. Nat Biotechnol. 2000; 18:649–54. [PubMed: 10835604]
35. Bai J, Sui J, Zhu RY, Tallarico AS, Gennari F, Zhang D, Marasco WA. Inhibition of Tat-mediated transactivation and HIV-1 replication by human anti-hCyclinT1 intrabodies. J Biol Chem. 2003; 278:1433–42. [PubMed: 12401780]
36. Amersdorfer P, Marks JD. Phage libraries for generation of anti-botulinum scFv antibodies. Methods Mol Biol. 2000; 145:219–40. [PubMed: 10820725]
37. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA Jr. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J Immunol Methods. 1984; 72:77–89. [PubMed: 6086763]
38. Reist CJ, Archer GE, Kurpad SN, Wikstrand CJ, Vaidyanathan G, Willingham MC, Moscatello DK, Wong AJ, Bigner DD, Zalutsky MR. Tumor-specific anti-epidermal growth factor receptor variant III monoclonal antibodies: use of the tyramine-cellobiose radioiodination method enhances cellular retention and uptake in tumor xenografts. Cancer Res. 1995; 55:4375–82. [PubMed: 7671250]
39. Reist CJ, Archer GE, Wikstrand CJ, Bigner DD, Zalutsky MR. Improved targeting of an anti-epidermal growth factor receptor variant III monoclonal antibody in tumor xenografts after labeling using N-succinimidyl 5-iodo-3-pyridinecarboxylate. Cancer Res. 1997; 57:1510–5. [PubMed: 9108453]
40. Reist CJ, Garg PK, Alston KL, Bigner DD, Zalutsky MR. Radioiodination of internalizing monoclonal antibodies using N-succinimidyl 5-iodo-3-pyridinecarboxylate. Cancer Res. 1996; 56:4970–7. [PubMed: 8895752]
41. Simmons ML, Lamborn KR, Takahashi M, Chen P, Israel MA, Berger MS, Godfrey T, Nigro J, Prados M, Chang S, Barker FG 2nd, Aldape K. Analysis of complex relationships between age, p53, epidermal growth factor receptor, and survival in glioblastoma patients. Cancer Res. 2001; 61:1122–8. [PubMed: 11221842]
42. Hipfner DR, Almquist KC, Leslie EM, Gerlach JH, Grant CE, Deeley RG, Cole SP. Membrane topology of the multidrug resistance protein (MRP). A study of glycosylation-site mutants reveals an extracytosolic NH2 terminus. J Biol Chem. 1997; 272:23623–30. [PubMed: 9295302]
43. Kuan CT, Wikstrand CJ, Archer G, Beers R, Pastan I, Zalutsky MR, Bigner DD. Increased binding affinity enhances targeting of glioma xenografts by EGFRvIII-specific scFv. Int J Cancer. 2000; 88:962–9. [PubMed: 11093822]
44. Chowdhury PS, Pastan I. Analysis of cloned Fvs from a phage display library indicates that DNA immunization can mimic antibody response generated by cell immunizations. J Immunol Methods. 1999; 231:83–91. [PubMed: 10648929]
45. Brinkmann U, Pastan I. Immunotoxins against cancer. Biochim Biophys Acta. 1994; 1198:27–45. [PubMed: 8199194]
46. Stirpe F. Ribosome-inactivating proteins. Toxicon. 2004; 44:371–83. [PubMed: 15302521]
47. Daghighian F, Barendswaard E, Welt S, Humm J, Scott A, Willingham MC, McGuffie E, Old LJ, Larson SM. Enhancement of radiation dose to the nucleus by vesicular internalization of iodine-125-labeled A33 monoclonal antibody. J Nucl Med. 1996; 37:1052–7. [PubMed: 8683300]

A



B

MRP3-derived Peptides	Amino Acid Sequence	Amino Acid Position	Transporter Domain
PepI	MDALCGSGELGSKFWDSNLS	1-20	N-Terminus
PepII	VHTENPDLTPCF	21-32	N-Terminus
PepIII	DAMADSRQNNTSLRL	999-1013	Extracellular Loop 6
PepIV	KIRSPQSFFDTTPSGRI	1053-1069	Extracellular Loop 6

FIGURE 1.

Proposed membrane topology of MRP3 and location of peptides used for preparation of scFv antibodies. *(a)* Arrows indicate locations of selected peptides in the extracellular N terminus and the extracellular loop of membrane spanning domain 3 (MSD3) of the MRP3 protein. *(b)* The amino acid sequence and position for MRP3-derived peptides used for panning of the phage display library to screen for anti-MRP3 scFv antibodies.

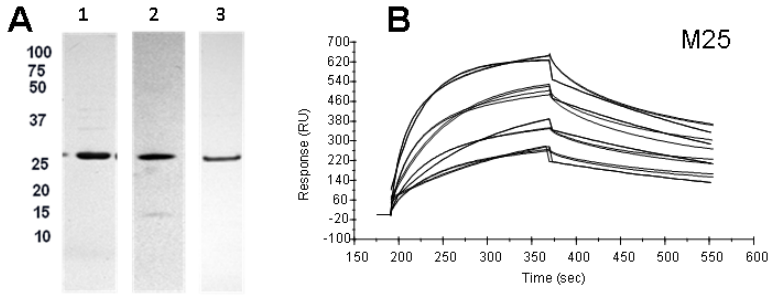


FIGURE 2.

SDS-PAGE and BIAcore analysis of purified M25, M58, and M89 scFv antibody fragments after metal affinity chromatography. *(a)* Purified scFv proteins were analyzed by 4-12% SDS-PAGE. Lanes 1, 2, and 3 correspond to M25 (1 μg), M58 (1 μg), and M89 (500 ng), respectively. Molecular weight standards were in kDa. *(b)* Binding of M25, M58, and M89 scFvs was evaluated with a BIAcore instrument by immobilizing MRP3-derived, biotinylated PepI (for M25 and M58) and PepII (for M89) peptides. The sensogram in panel B shows the binding pattern of M25 scFv. The sensograms for M58 and M89 (not shown) showed the same pattern. The scFv antibodies were diluted in HBSS buffer and passed over the streptavidin-avidin chip at concentrations of 25, 200, and 1000 nM. The association and dissociation rates from the sensogram were k_{assoc} of 5.4×10^4 $(\text{mol/L-s})^{-1}$ and a k_{diss} of 1.9×10^{-3} s^{-1} for M25 scFv, k_{assoc} of 7.1×10^4 $(\text{mol/L-s})^{-1}$ and a k_{diss} of 3.4×10^{-3} s^{-1} for M58 scFv, and k_{assoc} of 5.1×10^4 $(\text{mol/L-s})^{-1}$ and a k_{diss} of 5×10^{-3} s^{-1} for M89. K_A at binding equilibrium, calculated as $K_A = k_{assoc} / k_{diss}$, was 2.8×10^7 $(\text{mol/L})^{-1}$ for M25 scFv, 2.1×10^7 $(\text{mol/L})^{-1}$ for M58 scFv, and 1×10^7 $(\text{mol/L})^{-1}$ for M89 scFv.

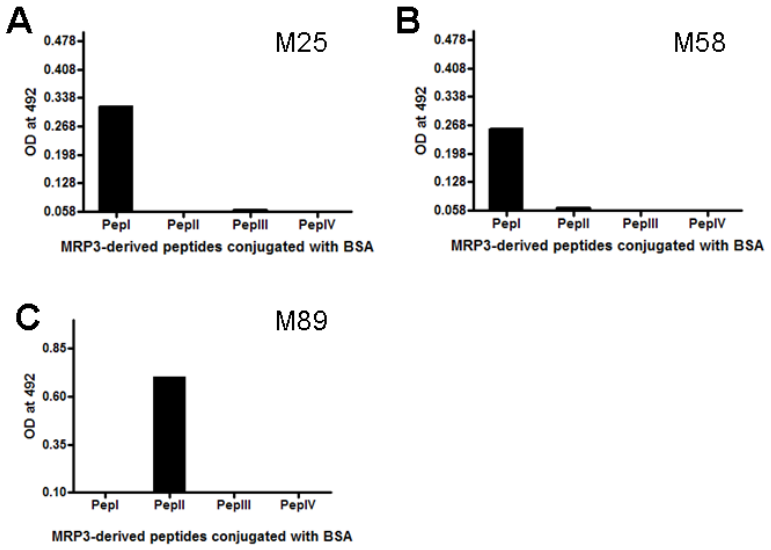


FIGURE 3.

Binding specificity of anti-MRP3 scFvs. *(a)* M25, *(b)* M58, and *(c)* M89 scFvs were analyzed by ELISA with soluble, purified recombinant scFvs. A 96-well ELISA plate was coated with 10 μg/ml of specific MRP3-derived peptides (PepI, PepII, PepIII, and PepIV) conjugated with BSA as antigen. The purified scFv antibodies were added in serial dilution to the wells, and the secondary antibody used was HRP-conjugated anti-His-tag antibody. The M25 *(a)* and M58 *(b)* scFvs reacted only with PepI antigen and not with other MRP3-derived peptides (PepII, PepIII, or PepIV), whereas the M89 scFv *(c)* reacted only with PepII antigen and not with other MRP3-derived peptides (PepI, PepIII, or PepIV).

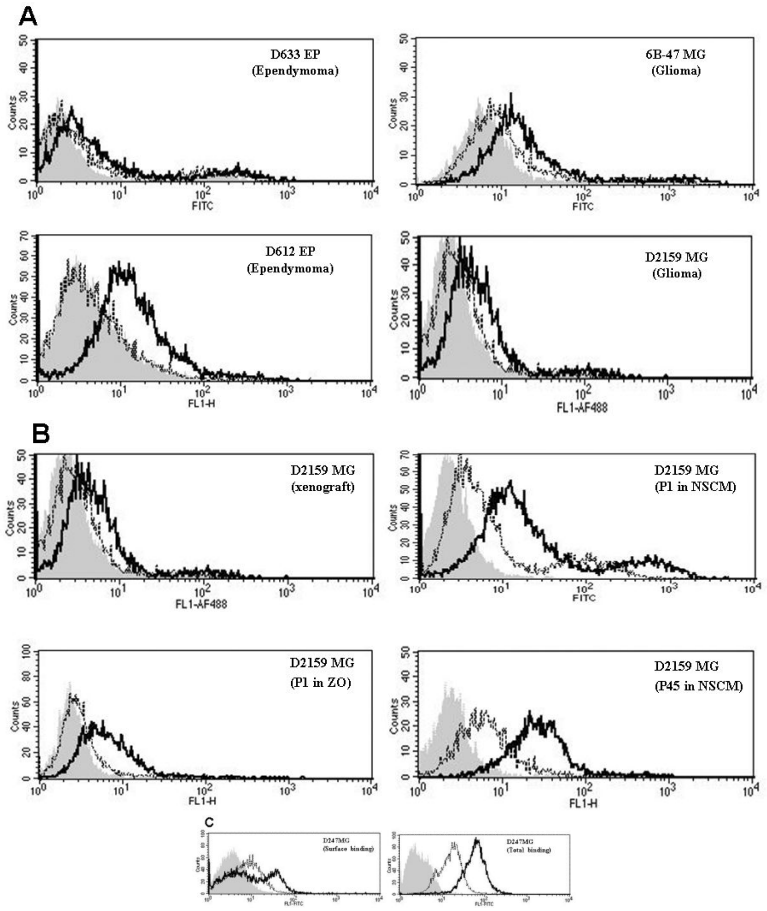


FIGURE 4.

Detection of MRP3 on the surface of malignant glioma (MG) and ependymoma (EP) cells. *(a)* Indirect flow cytometric analysis of MRP3 expression in freshly isolated cells from D633 EP, D612 EP, 6B-47 MG, and D2159 MG xenografts using purified M25 (solid black line) and M58 (dotted black line) scFv antibodies. Filled grey peaks are irrelevant scFv control. *(b)* Live D2159 MG neurospheres growing in NSCM at passage 1 and 45 exhibited better binding with M25 (solid black line) and M58 (dotted black line) than adherent D2159 MG cells growing in ZO medium (ZO; lower left panel) or D2159 MG cells from xenografts (upper left panel). Filled grey peaks are irrelevant scFv control. *(c)* MRP3 expression in permeabilized (total binding) and nonpermeabilized (surface binding) fixed D247 MG cells was done by using purified M89 (solid black line) and M58 (dotted black line) scFv antibodies. Filled grey peaks are irrelevant scFv control.

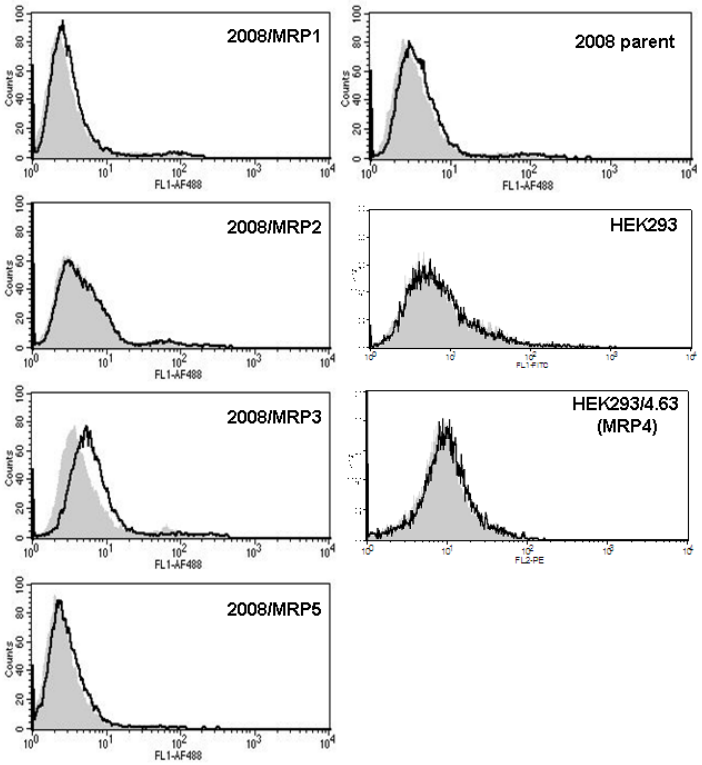


FIGURE 5.

FACS analysis of MRP1-5 overexpressing cells with a purified M25 scFv antibody. Ovarian carcinoma cell line 2008 and its MRP transfectants, 2008/MRP1, 2008/MRP2, 2008/MRP3, and 2008/MRP5, as well as human embryonic kidney cell line HEK293 and its MRP4 transfectant HEK293/4.63, were used to confirm the specific binding of the M25 scFv antibody. Exponentially growing live cells were incubated with purified M25 scFv at 10 μg/ml followed by incubation with HRP-labeled anti-His tag secondary antibody. Filled grey peaks are irrelevant scFv control.

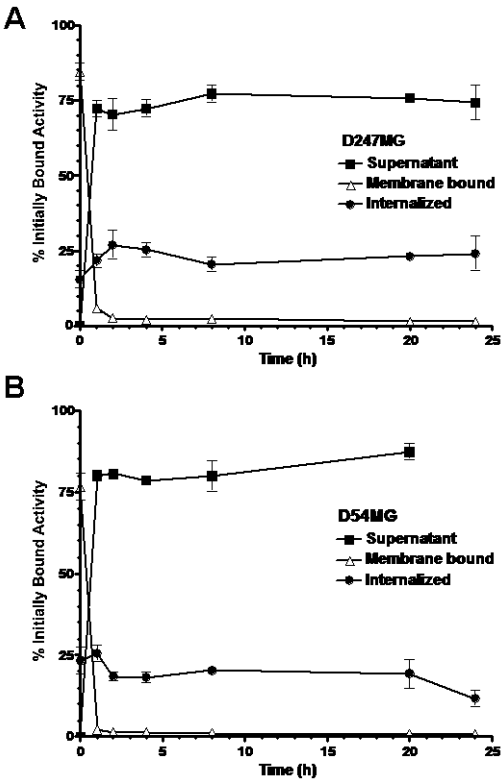


FIGURE 6.

In vitro internalization of ^{125}I-labeled M89 scFv on **(A)** D247MG and **(B)** D54MG malignant glioma cells. The percentage of radioactivity initially bound to cells (membrane bound [▲] or internalized [●]) and in the cell culture supernatant [■] is shown. A marked increase in radioactive counts was observed in the cell culture supernatant, with a slight decrease in counts associated with cell surface. The profile showed that the internalization of M89 scFv into D247MG cells gradually increased to a maximum percentage (26%) of intracellular counts at the 2-h time point compared with 25% in D54MG cells at the 1-h time point; 20% of the M89scFv counts were detected intracellularly throughout the time course up to the 20-h time point for both D54MG and D247MG. Points, average of triplicates; bar, SD.

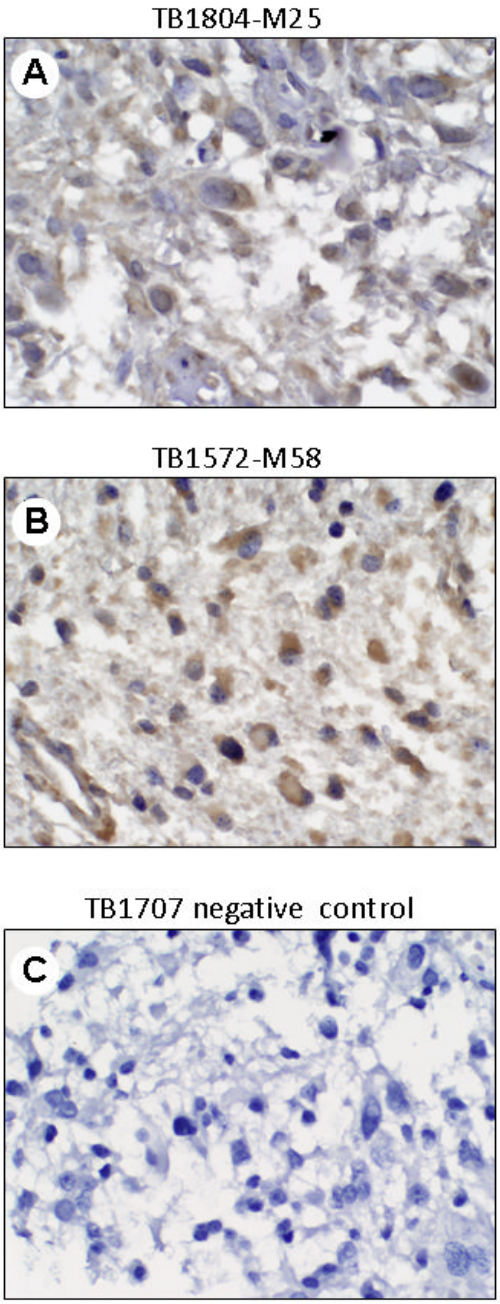


FIGURE 7.

Immunohistochemical demonstration of M25 and M58. Tissue microarrays (TMAs), made of paraffin-embedded sections of tissues from GBM patients, were used for immunohistochemical analysis using purified M25 and M58 scFvs. TMAs were deparaffinized, and antigen was retrieved by boiling the citrate buffer. After washing and blocking with NGS, sections were incubated with purified M25 and M58 scFvs separately in 1% BSA/PBS overnight at 4°C. Anti-penta-His tag was used as secondary antibody, followed by incubation with tertiary antibody from Vectastain Elite ABC kit (Vector Laboratories). Sections were counterstained with hematoxylin. *(a)* Purified M25 scFv showed binding to malignant astrocytic cells in a membranous cytoplasmic pattern, by binding to approximately 50% of the tumor cells (M25, ×40). *(b)* IHC of M58 also revealed

a cytoplasmic/ membranous staining pattern in malignant glioma biopsies. In IHC testing, more tumors (79%) demonstrated more frequent cellular staining with M58 than with M25. *(c)* IHC with an irrelevant scFv antibody at matching concentrations was entirely negative. Magnification, 600 X.

Table I

SCREENING OF HUMAN SCFV NAÏVE PHAGE DISPLAY LIBRARY ON MRP3-DERIVED PEPTIDES*

Panning condition					PepI screening		PepII screening	
Round	**Input**	**Type of Resin**	**Peptide Conc. (nM)**	**Binding Time (h)**	**Output (cfu)**	**Enrichment**	**Output (cfu)**	**Enrichment**
1	1×10^{12}	Streptavidin (200 μl)	500	3	6×10^{5}	1	1×10^{5}	1
2	1×10^{12}	Avidin (40 μl)	100	1.5	5.9×10^{6}	9.8	4.4×10^{6}	44
3	1×10^{12}	Streptavidin (100 μl)	20	1	4.08×10^{7}	68	3.9×10^{7}	390

*Phage input and output was determined by titration, determining phage cfu (colony-forming unit) of infected *E. coli* TG1 cells before and after each round of selection.