Targeted drug delivery to mesothelioma cells using functionally selected internalizing human single-chain antibodies

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Abstract

Mesothelioma is a malignancy of the mesothelium and current treatments are generally ineffective. One promising area of anticancer drug development is to explore tumor susceptibility to targeted therapy. To achieve efficient, targeted intracellular delivery of therapeutic agents to mesothelioma cells, we selected a naive human single-chain (scFv) phage antibody display library directly on the surface of live mesothelioma cells to identify internalizing antibodies that target mesothelioma-associated cell surface antigens. We have identified a panel of internalizing scFvs that bind to mesothelioma cell lines derived from both epithelioid (M28) and sarcomatous (VAMT-1) types of this disease. Most importantly, these antibodies stain mesothelioma cells *in situ* and therefore define a panel of clinically represented tumor antigens. We have further exploited the internalizing function of these scFvs to achieve targeted intracellular drug delivery to mesothelioma cells. We showed that scFv-targeted immunoliposomes were efficiently and specifically taken up by both epithelioid and sarcomatous mesothelioma cells, but not control cells, and immunoliposomes encapsulating the small-molecule drug topotecan caused targeted killing of both types of mesothelioma cells *in vitro*. [Mol Cancer Ther 2008;7(3):569–78]

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Introduction

Mesothelioma is a deadly disease caused by malignant transformation of the mesothelium. Although the exact cause of this disease is unknown, there is a strong association with exposure to asbestos (1, 2). Despite this knowledge, exposure to asbestos continues due to disturbance of naturally occurring asbestos and asbestos used in existing buildings. For example, the destruction of the World Trade Center led to the discharge of massive amounts of asbestos and other hazardous materials (3), exposing thousands of people at Ground Zero and nearby areas (4). The prognosis for patients with malignant mesothelioma is universally poor and treatment is generally ineffective (5). One chemotherapeutic agent, pemetrexed (Alimta), has been approved by the Food and Drug Administration for use with cisplatin to treat patients with pleural mesothelioma. Although the pemetrexed/cisplatin combination can prolong life and improve the lung function of mesothelioma patients, it does not cure the disease (6). New treatment strategies are needed for both localized and metastatic forms of mesothelioma.

One promising area of anticancer drug development is to explore tumor susceptibility to targeted therapy. Cancer cells differ from normal cells in a variety of ways, one of which is the molecular composition of the cell surface. The altered surface chemistry allows tumor cells to respond efficiently to external signals for growth and survival and to interact directly with a variety of host tissue elements to migrate, enter the circulation, extravasate, and become colonized at a distant site. Besides serving as markers for malignant cells, tumor cell surface molecules are valuable targets for therapy due to their relatively easy accessibility to targeting molecules administered to the bloodstream or extracellular space. The modular nature of tumor recognition and killing functions provides an experimental platform for constructing targeted therapeutics. In principle, a variety of antineoplastic agents can be attached to affinity agents that recognize tumor-associated cell surface molecules to achieve targeted killing (7, 8).

Unfortunately, very few mesothelioma cell surface markers are known (9). For example, mesothelin is a cell surface antigen of unknown function that has been reported to be expressed in epithelioid mesothelioma cells (10). Serologic tests and treatment studies based on mesothelin are under active development (11). Mesothelin, however, is also expressed on normal mesothelial cells and is not expressed on sarcomatous mesothelioma (12), a particularly recalcitrant subtype of mesothelioma. Other cell surface molecules such as CD44, the 67-kDa laminin receptor, and a subset of voltage-dependent anion channels have also been reported to be expressed at an elevated level in

mesothelioma cells (13). Those molecules, however, are also found in many other types of tumor as well as in normal tissues. Thus, much work is needed to identify additional cell surface markers with more restricted patterns of tissue distribution and more specific associations with mesothelioma, of both epithelioid and sarcomatous subtypes.

Differential gene expression-based approaches have been widely used for discovery and identification of tumor markers, but they face important limitations, especially in analyzing tumor-associated post-translational modifications (14). In contrast, regardless of the exact chemical makeup, the tumor cell surface epitope space can be mapped by complementary monoclonal antibodies. Monoclonal antibodies are able to discern subtle differences in antigen structure and conformation and recognize antigenic determinants of diverse chemical composition with high affinity and specificity (15, 16). As such, the tumor cell surface epitope space can be efficiently mapped by high-affinity interactions with monoclonal antibodies (17). Isolating these epitopes enables the antibodies to achieve specific binding to neoplastic cells, an ability that could be used in applications such as induction of antibody-dependent cell cytotoxicity or inhibition of signaling pathways involved in tumor cell migration, growth, and survival. In addition, antibodies targeting internalizing tumor epitopes could be exploited to achieve specific intracellular delivery of chemotherapeutic drugs (7, 18, 19).

We report here the identification of a panel of internalizing monoclonal antibodies that target mesothelioma cell surface antigens. We have used a naive phage antibody display library to probe the cell surface epitope space of live mesothelioma cells and identified human single-chain Fv fragments (scFv) that target mesothelioma-associated internalizing cell surface epitopes present on both epithelial and sarcomatous cell lines. We have further showed that several of these scFvs are capable of mediating targeted intracellular drug delivery to mesothelioma cells, suggesting that they may be useful for developing targeted therapies against mesothelioma.

Materials and Methods

Materials

Reagents for scFv purification and characterization: nitrilotriacetic acid-nickel agarose beads (Qiagen) and EZ-Link Sulfo-NHS-LC-Biotin (Pierce). Reagents for fluorescence-activated cell sorting (FACS): streptavidin-phycoerythrin (Invitrogen/BioSource) and biotin-labeled polyclonal anti-Fd antibody (Sigma-Aldrich). Reagents for immunohistochemistry: streptavidin-horseradish peroxidase (Sigma-Aldrich), 3,3′-diaminobenzidine (Sigma-Aldrich), and hematoxylin (Vector Laboratories). Reagents for immunoliposomes and cytotoxicity study: 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid (Invitrogen/Molecular Probes); 1-2-distearoyl-3-*sn*-glycerophosphocholine and methoxy polyethylene glycol-distearoyl phosphatidylethanolamine (Avanti Polar Lipids); and cholesterol (EMD/Calbiochem) and β-(*N*-maleimido)propionyl polyethylene glycol-1,2-distearoyl-3-*sn*-phosphoethanolamine (Nektar Therapeutics) and Cell Counting Kit-8 (Dojindo). Topotecan was a kind gift of the Taiwan Liposome Company.

Cell Lines and Primary Cells

All cell lines were obtained from the American Type Culture Collection unless otherwise indicated. The benign prostatic hyperplasia line (BPH-1) was obtained from Dr. Jerry Cunha (University of California-San Francisco; ref. 20). This line is easy to grow *in vitro* and is therefore often used as a control in our high-throughput phage antibody screening experiments (17, 21). The M28 and VAMT-1 cell lines were obtained from Dr. Brenda Gerwin (National Cancer Institute; ref. 22). The nonmalignant primary mesothelial cells were generated from benign ascites from patients under an approval (as below; ref. 23). The hTERT-transduced LP9 cell line (LP9/hTERT) was obtained from Brigham and Women's Hospital (24) and cultured in DMEM/F-12 supplemented with 10% bovine calf serum, 10 ng/mL EGF, 100 IU/mL penicillin, and 100 μg/mL streptomycin. All other cell lines were maintained in RPMI 1640 supplemented with 10% bovine calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Human Tissues

Informed consent was obtained from each subject or subject's guardian. The protocol for tissue acquisitions was approved by the institutional review board and in accordance with an assurance filed with and approved by the Department of Health and Human Services. Surgically removed mesothelioma tissues were fast frozen with liquid nitrogen and processed for immunohistochemistry studies.

Phage Antibody Selection and Characterization

A naive phage antibody display library containing 5×10^8 members was used in this study (25). The library was created by subcloning human scFv gene repertoires from a naive phagemid (26) into a phage vector for multivalent display (25, 27). The library was preincubated with control cells (BPH-1 and LP9/hTERT) at 4°C for 4 h to reduce binders to common cell surface antigens as described (17). The depleted library was further incubated with 10^6 M28 cells at 37°C for 1 h in medium containing 10% FCS, washed thrice with PBS, once with 100 mmol/L glycine/150 mmol/L NaCl (pH 2.8), lysed with 100 mmol/L triethylamine, neutralized with 1 mol/L Tris-HCl (pH 7.0), and used to infect log-phase TG1 and to produce polyclonal phage antibodies (17). Polyclonal phage antibodies from the first round of selection were further selected on VAMT-1 cells (round 2) using procedures described above and used to produce polyclonal phage antibodies that were selected again on live M28 cells (round 3). Output of this round 3 selection was screened by FACS on M28 and VAMT-1 cells, respectively, to identify binders to both cell lines (17). ScFvs were sequenced to determine the number of unique clones as described (17).

To further study binding specificity, a panel of tumor cell lines and control cells (described in Results) were incubated with 21 monoclonal phage antibodies. Bound phage

antibodies were detected with biotin-labeled anti-M13 and streptavidin-phycoerythrin and analyzed by FACS (17). Hierarchical analysis of mean fluorescence intensity values was done using GeneCluster 3.0 (28), and the results were visualized in Java Treeview (29).

Production of scFvs

To produce soluble scFvs, genes encoding scFvs were spliced into an expression vector imparting a c-myc and a hexahistidine tag at the COOH terminus (17). To produce soluble $(scFv)_2$, a second vector was used to impart a cysteine and a hexahistidine tag at the COOH terminus (17). Following IPTG induction, antibody fragments were purified from bacterial periplasmic space on nitrilotriacetic acid-nickel beads (17). For FACS and immunohistochemistry studies, scFvs were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer's instructions.

K_d Measurement

Mesothelioma cell lines (M28 and VAMT-1) were grown to 90% confluency in RPMI 1640 supplemented with 10% FCS. Cells were harvested by brief digestion with trypsin (0.2%) in 2 mmol/L EDTA/PBS. Biotinylated scFvs were incubated with 10^5 cells for 4 h at 4°C in PBS/0.25% bovine serum albumin. Bound scFvs were detected by streptavidin-phycoerythrin and analyzed by FACS as described previously (30, 31). Data was curve fitted and K_d values were calculated using GraphPad Prism (GraphPad Software).

Immunohistochemistry Study

Frozen sections of mesothelioma and control tissues were stained with biotinylated scFvs (250 nmol/L) at room temperature for 1 h. A scFv (N3M2) that does not bind to mesothelioma cell lines by FACS was used as a control for all experiments. Bound scFvs were detected by streptavidin-horseradish peroxidase using 3,3′-diaminobenzidine substrate as described (17). The stained tissues were counter-stained with hematoxylin, dried in 70%, 95% and 100% ethanol, mounted and analyzed by a board-certified pathologist (S.L.N.).

Liposome and Immunoliposome Preparation

Fluorescently labeled unilamellar liposomes were prepared according to the repeated freeze-thawing method of Szoka et al. (32). Liposomes were composed of the diacylphospholipid, 1-2-distearoyl-3-*sn*-glycerophosphocholine, cholesterol, methoxy polyethylene glycol-distearoyl phosphatidylethanolamine, and the lipophilic fluorescent marker, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid, combined in a 200:133:1:1 molar ratio. Liposomal topotecan of an identical lipid composition was prepared using a modified gradient loading and stabilization procedure, with sucrose octasulfate employed as an intraliposomal trapping agent (33). One modification from the published method (33) was that the drug entrapping solution was diethylammonium sucrose octasulfate (0.65 mol/L SO_4, pH 5.5). Topotecan (molecular weight, 421.45 Da) was added at a ratio of 350 g (0.830 mol) drug/mol phospholipids and the pH was adjusted to 6.5 with 1 N HCl before initiating loading at

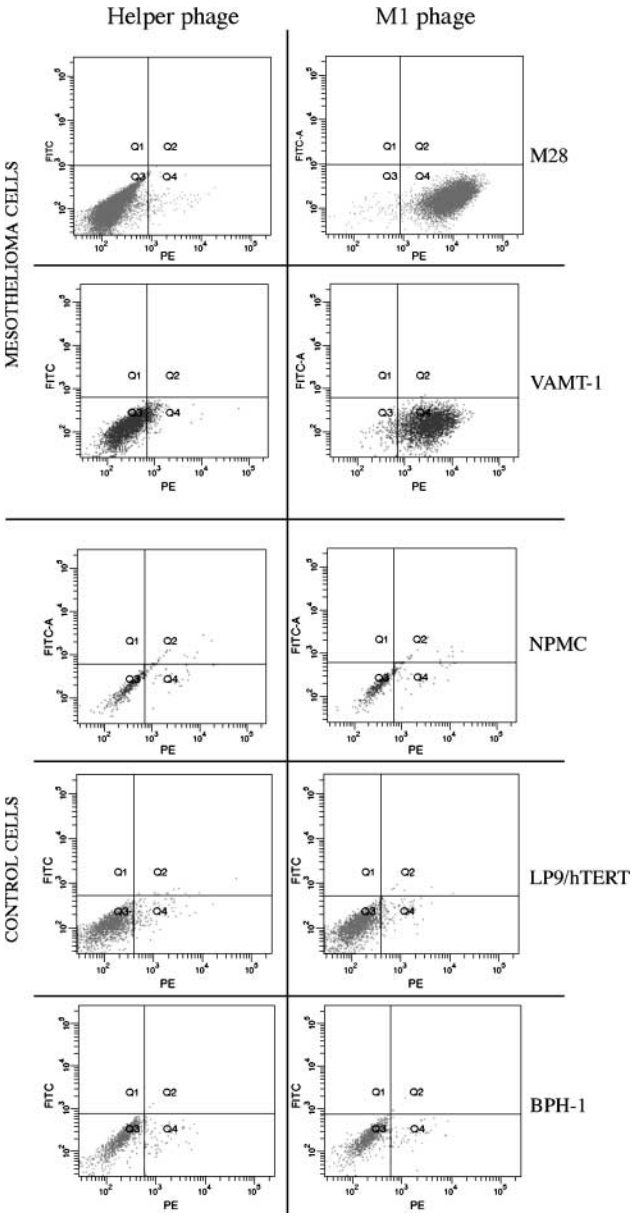

Figure 1. FACS analysis of phage antibody binding to mesothelioma cells. Binding patterns of the M1 scFv. M1 scFv binds to both mesothelioma cell lines but not the control nontumorigenic cells. Helper phage was used as the negative control in the binding study.

60°C for 30 min. The resulting liposomal topotecan was subsequently placed on ice for 15 min and purified on a Sephadex G-75 column to remove unencapsulated drug.

To construct immunoliposomes, $(scFv)_2$ were reduced with 20 mmol/L mercaptoethylamine, purified using a Sephadex G-25 gel filtration column, and eluted with HEPES-buffered saline [5 mmol/L HEPES, 145 mmol/L NaCl, 3.4 mmol/L EDTA (pH 7.0)]. To create an active surface for conjugation, micellar solutions of β-(*N*-maleimido)propionyl polyethylene glycol-1,2-distearoyl-3-*sn*-phosphoethanolamine were inserted into liposomes by

incubation at 60°C for 30 min at the ratio of 0.5 mol % of the liposomal phospholipids (34). Reduced scFv were incubated with the activated liposomes overnight at room temperature at 30 μg/μmol phospholipids, corresponding to ~60 scFv per liposome (35). An excess of 2-mercaptoethanol (2 mmol/L final concentration) was added to derivatize all unreacted maleimide groups, and scFv-conjugated immunoliposomes were purified on a Sepharose CL-4B gel filtration column. To quantify encapsulated topotecan, the liposome samples (5-20 μL) were dissolved in 1 mL acidic methanol [90% methanol (v/v) and 10% 0.1 mol/L H_3PO_4 (v/v)] and the absorbance was read at 375 nm. Samples were analyzed in triplicate.

Internalization Study

For fluorescence microscopy experiments, cells were grown to 80% confluency in 24-well plates and coincubated with nontargeted or targeted liposomes labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid (15 μmol/L phospholipids) for 4 h at 37°C. The cells were washed with PBS and examined with a Nikon Eclipse TE300 fluorescence microscope. For FACS analysis, cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid–labeled liposomes or immunoliposomes at 37°C for 2 h, removed from the dish by trypsin digestion (we did not observe cell membrane damage caused by trypsin treatment using the trypan blue exclusion assay), exposed to glycine buffer (pH 2.8; 150 mmol/L NaCl) at room temperature for 5 min to remove surface-bound liposomes, and analyzed by FACS (LSRII; BD Biosciences). Mean fluorescence intensity values were used to calculate the percentage of internalized liposomes (resistant to glycine treatment) over total cell-associated liposomes (before glycine treatment).

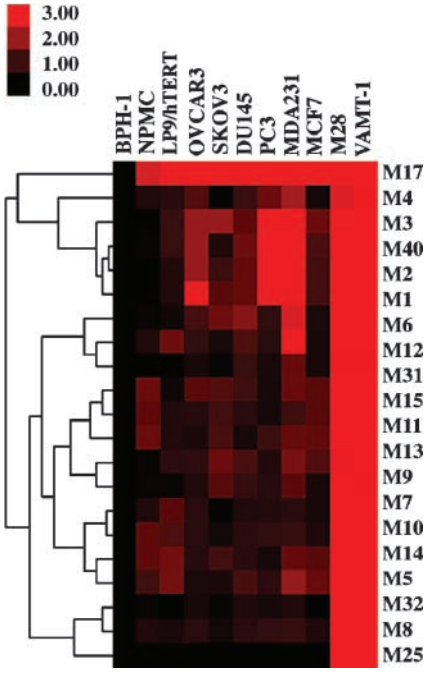

Figure 2. Cluster analysis of cell surface binding patterns. Binding of the 21 scFvs against a panel of tumor and control cell lines was studied by FACS, and logarithmic mean fluorescence intensity values were analyzed by hierarchical analysis.

In vitro Cytotoxicity Study

Cells were plated at 6,000 per well in 96-well plates and incubated with liposomal drugs or free drug at varying concentrations (0-10 μg/mL) for 2 h at 37°C. After removal of the drug, the cells were washed once with RPMI 1640 supplemented with 10% FCS and incubated for an additional 70 h at 37°C. The cell viability was assayed using Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions. The data are expressed as the percent of viable cells compared with that of untreated control cells.

Results

Selection of scFvs Targeting Mesothelioma

We used a nonimmune, multivalent phage display library that contains >500 million different scFvs (25, 26) as a source of a random-shaped affinity repertoire to define the antigenic profile characteristic of the mesothelioma cell surface. The phage display library was preabsorbed against a panel of normal cell lines to remove binders to common cell surface molecules (17). Two mesothelioma cell lines were used as targets for selection: M28, which is derived from tumors of the epithelioid type, and VAMT-1, which is derived from tumors of the sarcomatoid type (22, 36). The preabsorbed naive phage antibody library was incubated with live M28 and VAMT-1 cells. To recover internalized phage antibodies preferentially, surface-bound phage that failed to internalize were removed by a low pH glycine solution (17, 37). Internalized phages were recovered by lysing the cells and were amplified in *Escherichia coli*. Because we are interested in developing therapeutics against all subtypes of mesothelioma, we alternated M28 and VAMT-1 as targets for selection to identify antibodies targeting both tumor subtypes. Outputs of the second and third rounds of selections were screened on the mesothelioma cells to identify binding antibodies. Ninety-five unique scFvs that recognized both M28 and VAMT-1 cells (Fig. 1) were identified. Twenty one of these scFvs were chosen for further study.

Tumor Recognition and Specificity

To further study tumor reactivity and specificity of these phage antibodies, we did comparative FACS analysis using a panel of tumor and control human cell lines. In addition to mesothelioma lines (M28 and VAMT-1), the tumor cell lines used were two prostate cancer lines (PC3 and DU-145), two ovarian cancer lines (OVCAR3 and SKOV3), and two breast cancer lines (MDA231 and MCF7). The control cells used were BPH-1 cells, which serves as a general control for cell surface expression of markers involved in growth in culture, nonmalignant primary mesothelial cells, and LP9/hTERT, an immortalized mesothelial cell line derived from normal human mesothelium (24). The FACS data were compiled and the binding patterns were studied by cluster analysis (Fig. 2). All 21 phage antibodies bound

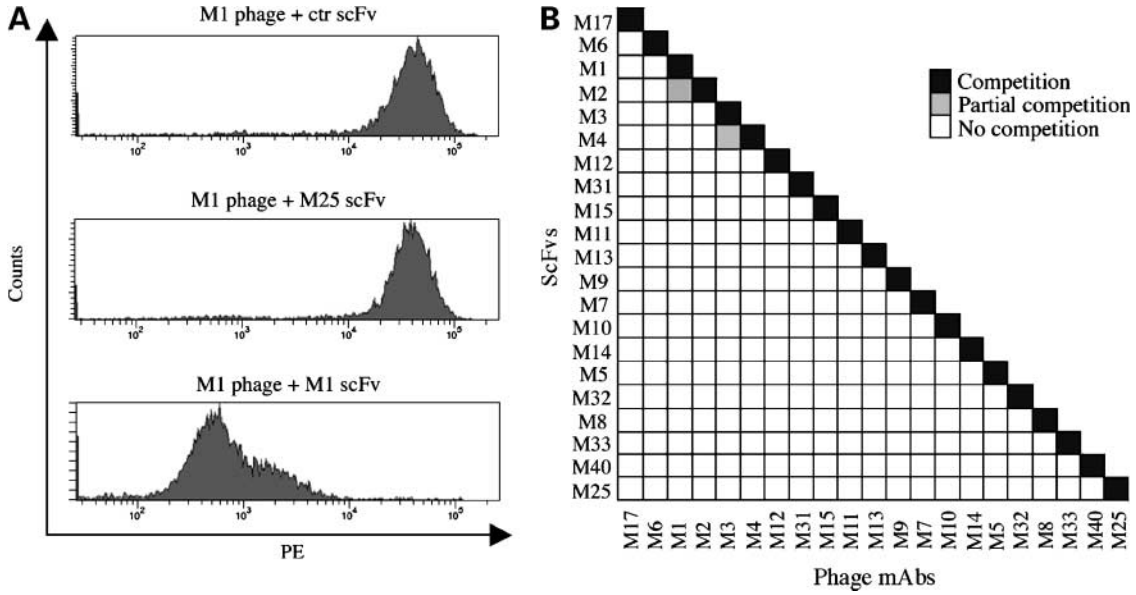

Figure 3. Epitope analysis by competition between scFvs and phage antibodies. **A,** specific competition of phage antibody binding by corresponding soluble scFv. M1 scFv but not M25 scFv competed with the M1 phage binding to M28 cells, indicating that M1 and M25 scFvs bind to different cell surface epitopes. **B,** patterns of competition between scFvs and their corresponding phage antibodies. Partial competitions were observed between M1 and M2 and M3 and M4, indicating overlapping epitopes.

strongly to both mesothelioma cell lines studied, whereas none bound to the control BPH-1 cells. Fifteen of 21 phage antibodies did not bind to either BPH-1 or nonmalignant primary mesothelial cells, and 7 of 21 did not bind to any of the three control cell lines, including LP9/hTERT (Fig. 2). One antibody, M25, binds exclusively to mesothelioma cells but not any of the control cells or other tumor cells (Fig. 2). Thus, this antibody may recognize a mesothelioma-specific cell surface antigen. Cluster analysis of phage antibody binding patterns suggests that this panel of scFvs bind to diverse cell surface receptors, with varying degrees of tumor association and mesothelioma specificity.

Affinity Measurement and Epitope Profiling

For biological and therapeutic applications, it is often required to convert phage antibody into soluble antibody fragments such as the scFvs. Soluble scFvs can be used to determine binding affinity to target cells and to conjugate to effector molecules or nanoparticles to achieve therapeutic effects. We converted all 21 phage antibodies into (His_6)-tagged scFvs by splicing the scFv genes into a bacterial expression vector (17). We produced and purified monomeric scFvs and used them for affinity and epitope studies.

We used FACS analysis to determine binding affinities for seven of the scFvs on live mesothelioma cells. Soluble monomeric M1 and M25 scFvs bind to M28 cells with affinities of 30 nmol/L (Supplementary Fig. S1)[6] and 50 nmol/L (data not shown), respectively. For the seven scFvs studied, the measured binding affinities on M28 cells ranged from 20 to 240 nmol/L.

To determine if these scFvs bind to distinct epitopes, we did competition experiments using 300 nmol/L soluble scFvs to compete with phage binding. As shown in Fig. 3*A*, soluble scFvs were able to compete off binding by the corresponding parental phage, indicating that the soluble scFvs have the same binding specificity as that of the phage antibody and that it is feasible to use the competition experiment to determine nonoverlapping epitopes. The full results of the competition experiments are shown in Fig. 3*B*. With the exception of 4 phage antibodies (two pairs of near neighbors by cluster analysis), the remaining 17 antibodies bind to distinct epitopes. Two pairs of scFvs bind to overlapping but not identical epitopes (Fig. 3*B*) as evidenced by partial competition. We conclude that the 21 scFvs recognize at least 19 unique epitopes, 17 of which are unique and 2 partially overlapping.

Binding to Mesothelioma Cells *In situ*

To determine if scFvs selected on mesothelioma cell lines recognize tumor cells *in situ* in clinical specimens, we did immunohistochemistry studies using biotin-labeled scFvs. We studied all three subtypes of mesothelioma, that is, epithelioid, sarcomatoid, and mixed subtype. All scFvs bind to the three subtypes of mesothelioma tissue (Supplementary Table S1).[6] This is consistent with our selection scheme that was designed to identify scFvs targeting both M28 and VAMT-1 cell lines. There was an intense staining of mesothelioma cells (Fig. 4*A-D*), with minimal staining of the control normal mesothelium (Fig. 4*E*). These experiments show that scFvs selected on tumor cell lines bind to mesothelioma antigens present in actual cases, which may be attractive targets for therapeutic intervention.

[6] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

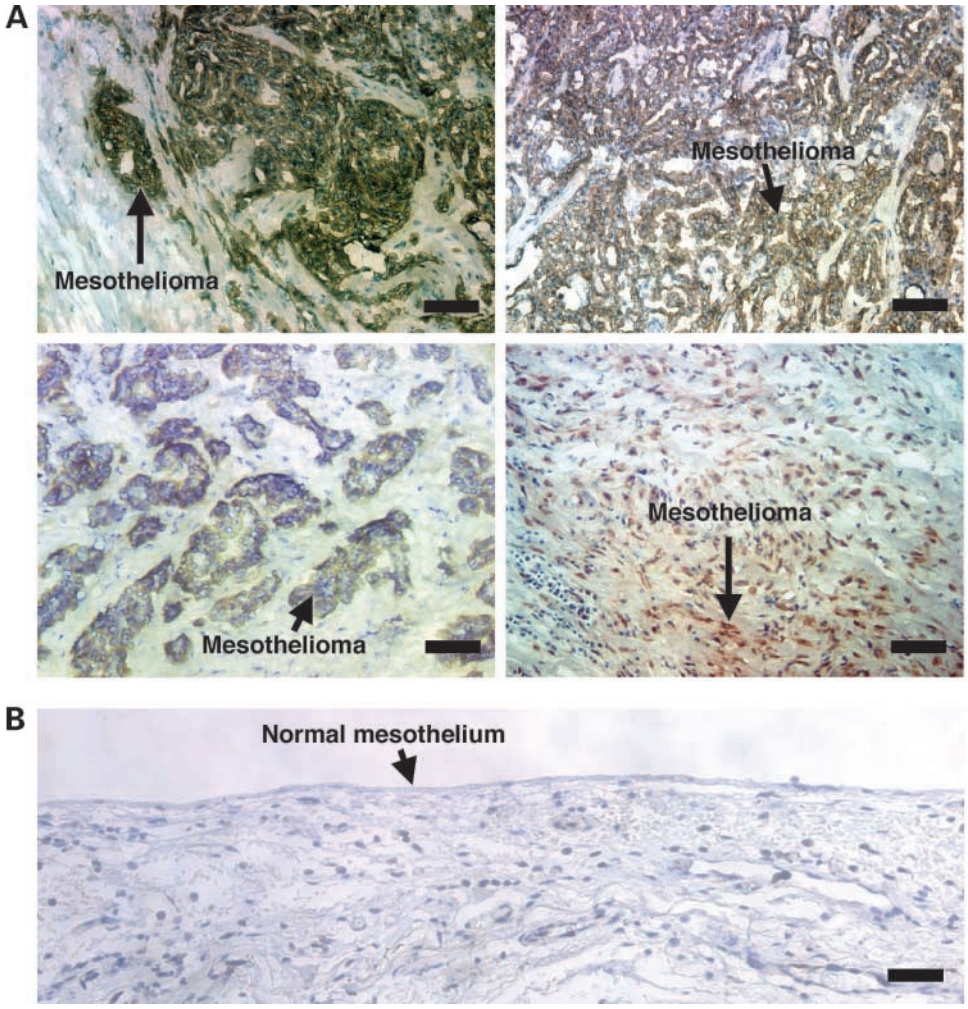

Figure 4. Immunohistochemistry studies. **A,** biotin-labeled soluble scFvs were used to stain mesothelioma tissues of epithelioid (cases 1-3) and sarcomatoid types (case 4). Cases 1, 3, and 4 were stained with the M1 scFv; case 2 was stained with the M25 scFv. Arrows, representative mesothelioma cells. **B,** normal pleural mesothelium (*arrow*) stained with the M1 scFv. Neither M1 nor M25 scFv (data not shown) stain normal pleural mesothelium (data not shown). Bar, 50 μm.

Mesothelioma Cell-Selective Intracellular Payload Delivery

Our phage antibodies were selected for their internalizing functions. One of the therapeutic applications of internalizing antibodies is targeted delivery of payloads to tumor cells. To study targeted delivery of nanoparticles, we constructed M1 and M25 scFv-targeted immunoliposomes labeled with a fluorescent lipid molecule, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid, and monitored internalization by both epithelioid and sarcomatous mesothelioma cell lines. Intracellular uptake was determined by fluorescence microscopy, as evidenced by the punctuate perinuclear staining pattern (Fig. 5*A*), and FACS (Fig. 5*A-C*) following surface stripping of noninternalized liposomes. The M1 scFv-targeted immunoliposomes were efficiently delivered intracellularly to both subtypes of mesothelioma cells, whereas nontargeted liposomes were not (Fig. 5*B*). The fraction internalized was about 40% at 2 h and 60% to 70% at 8 h (Fig. 5*C*). The delivery was mesothelioma cell specific; there was no uptake of immunoliposomes by control BPH-1 cells that were not recognized by the M1 scFv (Fig. 5*B*). Similar results were obtained with the M25 scFv-targeted immunoliposomes (data not shown). These experiments show that internalizing scFvs are indeed capable of mediating targeted payload delivery to both epithelioid and sarcomatous mesothelioma cells and, as such, may be suited for the development of targeted therapeutics.

Targeted Killing of Mesothelioma Cells by Immunoliposomal Topotecan

To evaluate the therapeutic potential of internalizing scFvs further, we constructed M1 and M25 scFv-targeted immunoliposomes encapsulating the anticancer drug topotecan and studied their cytotoxic effects on mesothelioma and control cells. Compared with drug-loaded, nontargeted

liposomes, the M1 scFv-targeted immunoliposomes showed significantly increased cytotoxicity toward mesothelioma cells (Fig. 6*A*). The scFv-targeted and the nontargeted drug-loaded liposomes showed no significant cytotoxicity toward control BPH-1 cells (Fig. 6*B*). Tested on M28 cells, the half-maximal effective concentration (EC_{50}) estimated for the M1 and M25 scFv-targeted immunoliposomal topotecan was 0.625 μg drug/mL (1.483 μmol/L) and 0.750 μg drug/mL (1.780 μmol/L), respectively, whereas the EC_{50} of the nontargeted liposomal topotecan was 2.50 μg/mL (5.93 μmol/L; Supplementary Table S2). Similar results were obtained with VAMT-1 cells (Supplementary Table S2).[6] Thus, scFv-mediated targeted delivery of liposome-encapsulated topotecan to mesothelioma cells improves both the potency and the specificity of the cytotoxic activity. This result shows the potential value of a targeting mechanism in payload delivery to tumor cells and in improving the specificity of conventional chemotherapeutics.

Discussion

Mesothelioma is an intractable tumor with no curative treatment to date. In a first step toward developing targeted therapeutics against mesothelioma, we sought to identify internalizing antibodies that target mesothelioma-associated cell surface antigens. Taking a functional approach, we have used a nonimmune phage antibody library as an unbiased random-shaped affinity repertoire to select for tumor-targeting scFvs on live mesothelioma cells. The selection methodology was optimized to enrich for scFvs that efficiently target internalizing epitopes (17, 37, 38), providing a means of efficient intracellular payload delivery to mesothelioma cells. We identified 95 unique

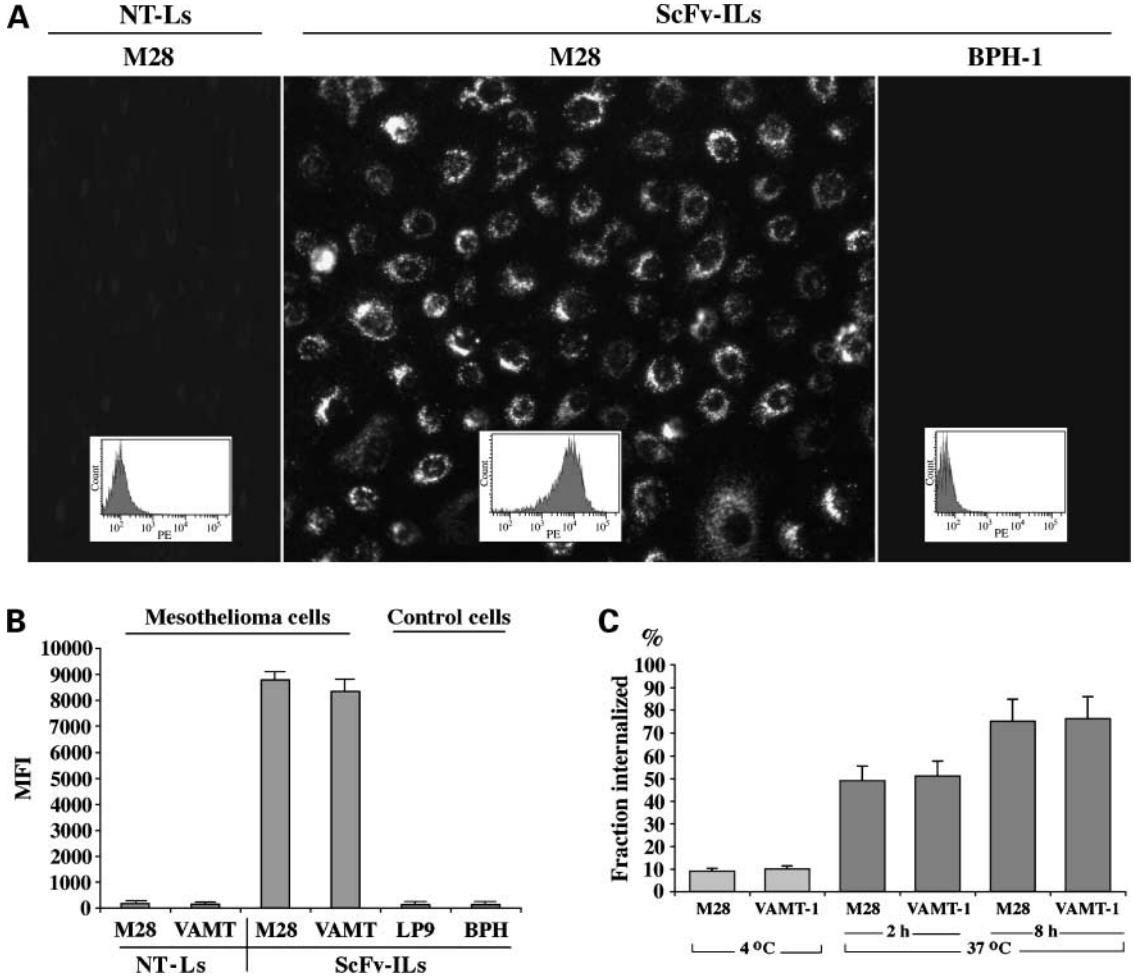

Figure 5. Internalization and targeted payload delivery. **A,** uptake of M1 scFv-targeted immunoliposomes and nontargeted liposomes by M28 cells was studied by fluorescence microscopy and FACS (*insets*) after 4-h incubation at 37°C. **B,** quantification of FACS-based uptake analysis of immunoliposomes and NT-Ls on a panel of mesothelioma and control cells. The experiment was done in duplicates. Bars, SE. **C,** analysis of fraction internalized. After incubation at 37°C for the indicated periods, the fraction internalized was calculated from immunoliposomes associated with target cells after a glycine wash, which removed about 90% of noninternalized, surface-bound immunoliposomes (based on data obtained at 4°C). The experiment was done in duplicates. Bars, SE.

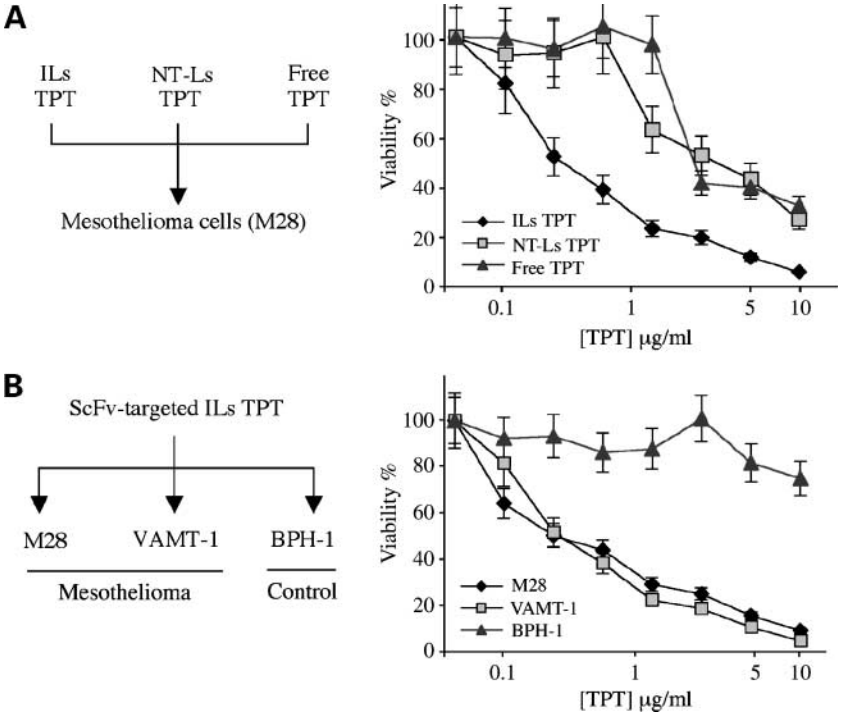

Figure 6. Cytotoxicity of scFv-targeted immunoliposomes encapsulating the small-molecule drug topotecan (*TPT*). **A,** scFv-mediated efficient intracellular delivery of liposomal drugs. Left, experimental scheme; right, viability curve, showing the benefit of a targeting mechanism, provided by the M1 scFv, in the targeted killing of mesothelioma cells (M28). NT-Ls TPT, nontargeted liposomal topotecan. **B,** targeted drug delivery leads to tumor-specific cytotoxicity. Left, experimental scheme; right, viability curve. Both mesothelioma cell lines but not the control BPH-1 cells were killed by the immunoliposome topotecan, showing the specificity of targeted cell killing.

mesothelioma-targeting scFvs, 21 of which were further characterized by FACS profiling on tumor cell lines, immunohistochemistry on mesothelioma tissue samples, and *in vitro* internalization/payload delivery assays. All 21 scFvs bind to both epithelioid and sarcomatoid type mesothelioma cell lines. In addition, all 21 scFvs stain mesothelioma cells *in situ* and therefore recognize clinically represented tumor antigens expressed on all mesothelioma subtypes. Two of the scFvs, M1 and M25, were shown to be capable of targeted intracellular payload delivery into mesothelioma cells. Cluster analysis and competition experiments indicate that the 21 scFvs bind to 17 unique epitopes and two pairs of overlapping epitopes. These properties make this panel of scFvs attractive candidates for therapeutic development.

A novel feature of this panel of scFvs is that they recognize all subtypes of mesothelioma. Many previously identified markers, such as mesothelin, recognize only the epithelioid mesothelioma, but not the sarcomatoid subtype, a particularly recalcitrant form of this disease (39). Because we selected mesothelioma-targeting antibodies from an antibody library, selection conditions could be manipulated to enrich for scFvs with desired properties. By alternating the selection target between epithelioid and sarcomatoid cell lines, we were able to select for scFvs targeting both subtypes, therefore broadening therapeutic applicability.

Our study also shows that although mesothelioma is notorious for resistance to conventional chemotherapy (40, 41), it may nonetheless be susceptible to targeted therapy. Immunoliposomes encapsulating the small-molecule drug topotecan and targeted by the M1 or M25 scFvs showed efficient and selective killing of mesothelioma but not control cells. Topotecan, an inhibitor of the nuclear enzyme topoisomerase I, exists in two forms. At acidic pH, topotecan is mainly in the active ring-closed lactone form (42). At neutral (physiologic) or alkaline pH, the drug is converted to a ring-open carboxylate form, which has poor membrane permeability, and thus poor cellular uptake and cytotoxicity (43, 44). Therefore, the use of a liposome carrier for topotecan is particularly relevant to its therapeutic effects (45). Immunoliposomes can be constructed to have a long circulating half-life and to be nonimmunogenic (46). As such, immunoliposomes represent one form of targeted therapy that can be used to exploit the internalizing function of this panel of scFvs.

For therapeutic development, it is important to identify antibodies binding to clinically represented tumor antigens. In this study, we selected the phage antibody library on mesothelioma cell lines and further studied their binding patterns to mesothelioma tissues to identify scFvs that target tumor cells *in situ*. A very high percentage of our scFvs selected on mesothelioma cell lines were found to bind to mesothelioma tissues. This is rather surprising as our previous studies on other tumors such as prostate cancer have indicated that selection on tumor cell lines often generates antibodies that do not bind to tumor cells *in situ*, and novel selection methods such as laser capture microdissection are required for identification of antibodies binding to tumor cells *in situ*. There are several possible explanations for this discrepancy. (*a*) The mesothelioma cell lines used in this study were obtained relatively recently (22), whereas the prostate cancer lines have been

cultured for nearly 30 years (47). As such, the mesothelioma cell lines may have fewer culture artifacts and resemble more closely mesothelioma cells *in situ* (9). (*b*) We focused our study on scFvs that bind to both epithelioid and sarcomatoid cell lines, further reducing the chance of selecting for scFvs binding to artifacts caused by culture conditions. Regardless of the exact cause, we have taken advantage of the cell surface antigen similarity between mesothelioma cell lines and mesothelioma cells in tissues and identified a panel of scFvs that targets clinically relevant tumor markers.

We are identifying mesothelioma antigens bound by our panel of internalizing scFvs using a combination of approaches, including screening a novel yeast cDNA surface display library that we have developed (48, 49), and analyzing immunoprecipitation products by mass spectrometry (50). Some of the selected scFvs, such as M1, target several other tumor cell lines such as breast and prostate cancer lines in addition to mesothelioma lines, suggesting that they bind to antigens commonly expressed by tumor cells. Mesothelioma thus shares cell surface markers with other tumors; therefore, treatments developed for other neoplastic diseases may also be effective against mesothelioma. Other scFvs, such as M25, target mesothelioma cells with very high specificity. Thus, the identification of these mesothelioma-specific antigens may reveal etiologies and pathogenic pathways unique to this disease and facilitate the development of early diagnostics and therapeutic strategies against mesothelioma.

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