Systemic Tumor-targeted Gene Delivery by Anti-Transferrin Receptor scFv-Immunoliposomes[1]

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Abstract

An ideal therapeutic for cancer would be one that selectively targets to tumor cells, is nontoxic to normal cells, and that could be systemically delivered, thereby reaching metastases as well as primary tumor. Immunoliposomes directed by monoclonal antibody or its fragments are promising vehicles for tumor-targeted drug delivery. However, there is currently very limited data on gene delivery using these vehicles. We have recently described a cationic immunoliposome system directed by a lipid-tagged, single-chain antibody Fv fragment (scFv) against the human transferrin receptor (TfR) that shows promising efficacy for systemic *p53* tumor suppressor gene therapy in a human breast cancer metastasis model. However, the extremely low yield of this lipid-tagged scFv limited further downstream development and studies. Here we report a different expression strategy for the anti-TfR scFv, which produces high levels of protein without any tags, and a different approach for complexing the targeting scFv to the liposomes. This approach entails covalently conjugating the scFv to the liposome via a cysteine at the 3′-end of the protein and a maleimide group on the liposome. Our results show that this conjugation does not impair the immunological activity or targeting ability of the scFv. The scFv-cys targets the cationic liposome-DNA complex (lipoplex) to tumor cells and enhances the transfection efficiencies both *in vitro* and *in vivo* in a variety of human tumor models. This scFv-immunoliposome can deliver the complexed gene systemically to tumors *in vivo,* where it is efficiently expressed. In comparison with the whole antibody or transferrin molecule itself, the scFv has a much smaller size for better penetration into solid tumors. It is also a recombinant protein rather than a blood product; thus, large scale production and strict quality control are feasible. This new approach provides a promising system for tumor-targeted gene delivery that may have potential for systemic gene therapy of various human cancers.

Introduction

Direct targeting of cancer cells with gene therapy has the potential to treat cancer on the basis of its molecular characteristics. Although cancer treatments involving gene therapy have substantial promise, many practical obstacles need to be overcome before gene therapy can fulfil its goals in the clinic (1). Perhaps foremost among the issues associated with macromolecular treatments is the efficient delivery of the molecular therapeutics to the site(s) in the body where they are needed (2, 3). The ideal delivery vehicle would be one that could be systemically (as opposed to locally) administered and that would selectively target tumor cells wherever they occur in the body (4). Progress has been made toward developing nonviral, pharmaceutical formulations of genes for *in vivo* human therapy, particularly liposome-mediated gene transfer systems (5, 6). The targeting of cancer cells by liposomes can be achieved by modifying the liposomes, through addition of molecules such as antibodies and antibody fragments, so that they selectively deliver their payload to tumor cells (7). The use of antibody molecules to target defined cell types was actually proposed >100 years ago (8). It took the successful development of hybridoma technology by Köhler and Milstein (9), with the resulting ability to produce Mabs,[3] to make this approach a reality. Monoclonal antibodies are becoming accepted tools for the detection and treatment of cancer. Progress in biotechnology has allowed the derivation of specific recognition domains from Mabs (10). The recombination of the variable regions of heavy and light chains and their integration into a single polypeptide provide the possibility of using single-chain antibody variable region fragments (designated scFv) for targeting purposes (11, 12). A lipid-tagged version of a scFv has been successfully incorporated into liposomes (13–15) and used to target B-lymphocytes *in vitro* (16). Using this lipid-tagged scFv technology, we have established a cationic immunolipoplex system directed by a scFv fragment against the human TfR (17). The lipid-tagged anti-TfR scFv targets the cationic liposome-DNA complex (lipoplex) to tumor cells and enhances the transfection efficiency both *in vitro* and *in*

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[3] The abbreviations used are: Mab, monoclonal antibody; scFv, single-chain antibody variable region fragment; CMV, cytomegalovirus; DDAB, dimethyldioctadecyl-ammonium bromide; DOPE, dioleolyphosphatidyl-ethanolamine; MPB-DOPE, 4-(*p*-maleimidophenyl)butyrate-DOPE; DOTAP, dioleoyltrimethylammonium propane; LB, Luria broth; β-gal, β-galactosidase; GFP, green fluorescence protein; IPTG, isopropyl β-D-thiogalactopyranoside; lipoplex, cationic liposome-DNA complex; TfR, transferrin receptor; wt, wild type.

vivo. In a nude mouse breast cancer xenograft metastasis model, systemic *p53* tumor suppressor gene therapy mediated by the scFv-targeting immunolipoplexes, in combination with conventional chemotherapy, significantly prolonged life span and resulted in long-term survival of the animals (17, 18).

However, in our hands, the level of expression of the lipid-tagged scFv was very low, with a yield <1 mg/l of culture. This low yield limited further downstream development and studies of this immunoliposome. In addition, for clinical use, the Food and Drug Administration prefers a product devoid of any extraneous protein sequences, including the lipoprotein tag present in our recently described scFv (17). To circumvent these problems, we report here a different expression strategy for the anti-TfR scFv, which produces high levels of the antibody fragment protein (26–33 mg/L) without any tags. We have also developed a different approach for complexing the targeting scFv to the liposomes. This approach entails covalently conjugating the scFv to the liposome via a cysteine at the 3′-end of the protein and a maleimide group on the liposome. Our results show that this conjugation does not impair its immunological activity or targeting ability. The resulting scFv-cys targets the cationic liposome-DNA complex (lipoplex) to tumor cells and enhances the transfection efficiencies both *in vitro* and *in vivo* in a variety of human tumor models. In comparison with the whole antibody or transferrin molecule itself, the scFv has a much smaller size for better penetration into solid tumors and is a recombinant protein rather than a blood product; thus, large-scale production and strict quality control is feasible. This new approach provides a promising system for tumor-targeted systemic gene delivery that may have potential for systemic gene therapy of various human cancers.

Materials and Methods

Materials. The cationic lipids, DOTAP, DDAB, the fusogenic neutral helper lipid DOPE, and MPB-DOPE, were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The p53 expression plasmid, pCMVp53, contains the human wild-type *p53* gene under the control of the CMV promoter (19). The control plasmid, pVec, is the empty vector without the p53 cDNA insert.

Cell Lines. Human head and neck cell line JSQ-3, a kind gift from Dr. Ralph Weichselbaum (University of Chicago, Chicago, IL), was derived from a tumor of the nasal vestibule that failed radiotherapy and is maintained in MEM with Earle's salts, supplemented as described previously (20). Human prostate cell line DU145 was maintained in MEM with Earle's salts with 10% fetal bovine serum plus 50 μg/ml each of penicillin, streptomycin, and neomycin and 2 mM L-glutamine. Human breast cancer cell line MDA-MB-435 was maintained in improved MEM supplemented with 10% FBS plus 50 μg/ml each of penicillin, streptomycin, and neomycin and 2 mM L-glutamine. All other cell lines were obtained from the Lombardi Cancer Center Tissue Culture Core Facility or American Type Culture Collection, Inc. and maintained according to their protocols.

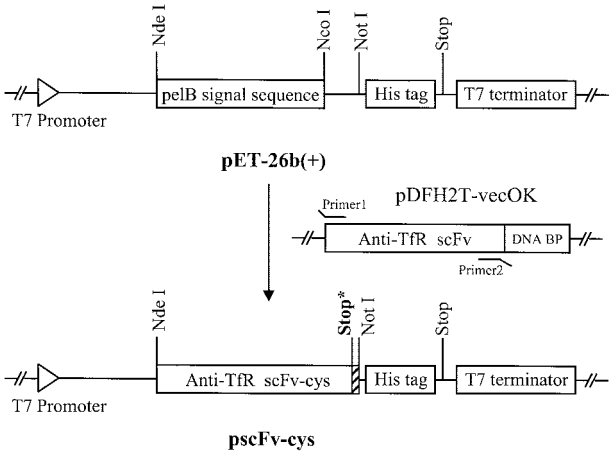

Fig. 1. Construct map of pscFv-cys expression vector for the antihuman TfR scFv-cys. The PCR-amplified scFv-cys cDNA incorporates an extra DNA stop codon (*Stop**) adjacent to the cysteine sequence and before the *Not*I site; therefore, its protein product will not contain the His tag. The scFv-cys cDNA was cloned into pET26(+) using *Nde*I and *Not*I sites to obtain the expression vector, *pscFv-cys,* the protein product of which will not contain the signal sequence and His tag.

Construction of the Expression Vector for Anti-TfR scFv Containing the 3′-Cysteine Residue. The anti-TfR scFv (VH-linker-VK) was obtained from plasmid pDFH2T-VecOK (17, 21) with PCR amplification using a 5′-primer (5′-GGCATATGGAGGTGCAGCTGGTGGAGT-3′) containing a *Nde*I site and a 3′-primer (5′-GGCGCGGCCGCTCAG-CATTTTATCTCCAGCTT-3′) containing the nucleotide sequence for the cysteine residue as well as a *Not*I restriction site. This primer also incorporates an extra DNA stop codon adjacent to the cysteine sequence and before the *Not*I site (Fig. 1). The PCR product was cloned into the *Nde*I and *Not*I sites of the commercial expression vector pET26b(+) (Novagen) to obtain the expression vector for the anti-TfR scFv, pscFv-cys (Fig. 1), the protein product of which will not contain the signal sequence and His tag.

Expression and Purification of scFv-cys. The scFv-cys was expressed in the pscFv-cys-transformed expression host BL21 (λDE3) after induction with 1 mM IPTG (Sigma Chemical Co.). The scFv-cys containing the COOH-terminal cysteine was purified from the inclusion bodies as reported (22) with modification. Briefly, a single colony was inoculated into 200 μl of LB, plated onto an LB-agar plate (with 30 μg/ml kanamycin) and incubated overnight at 37°C. The bacteria was rinsed from the plate with LB, inoculated into 2-l flasks each containing 500 ml of LB with 30 μg/ml kanamycin, and incubated at 37°C and 250 rpm to A_{600} 0.5–0.7. To induce expression of the scFv protein, IPTG was added to the culture at this time to a final concentration of 1 mM, and the incubation continued for an additional 2–8 h. The bacterial cultures were then collected by centrifugation and lysed in 100 ml/l culture of cold 50 mM Tris-HCl/20 mM EDTA (pH 7.4). Fresh lysozyme was added to a final concentration of 200 μg/ml and incubated at 30°C for 30 min. Triton X-100 was then added to a final concentration of 2.5% and incubated at room temperature for 30 min with rotation. The solution was

sonicated with cooling on ice three times for 30 s each, with a 30-s pause, using a probe sonicator (Sonicator Ultrasonic Liquid Processor; Misonix, Farmingdale, NY). The inclusion bodies were isolated by centrifugation at 13,000 × *g* for 15 min. The resulting pellet was washed three times in cold 50 mM Tris-HCl/20 mM EDTA (pH 7.4), containing 1% Triton X-100. The purity and quantity of the inclusion bodies were determined by SDS-PAGE before solubilization.

The isolated inclusion bodies were solubilized in 6 M guanidine-HCl buffer (100 mM Tris-HCl, 6 M guanidine-HCl, 2 mM EDTA, and 200 mM NaCl, pH 8.0) and centrifuged at 12,300 × *g* for 15 min at 4°C to remove insoluble debris. The solubilized protein was purified by gel filtration on a Sephacryl S-200 column (16 × 70 cm) equilibrated and eluted with 6 M guanidine-HCl buffer. 2-Mercaptoethanol was added to the pooled protein fractions to a final concentration of 40 mM, and the mixture was incubated with rotation for 2 h at room temperature and then placed at 4°C for ~20 h. The presence of such a high concentration of guanidine-HCl and the reducing agent results in a totally unfolded protein. Refolding of the scFv-cys protein was accomplished by dialysis at 4°C against decreasing concentrations of guanidine-HCl in the absence of 2-mercaptoethanol. Dialysis was performed for 24 h each against the following concentrations of guanidine-HCl in 100 mM Tris-HCl (pH 8.0) and 200 mM NaCl: 6, 3, 2, 1, and 0.5 M. The last dialysis was against three changes of just 100 mM Tris-HCl (pH 8.0) and 200 mM NaCl. The fourth dialysis solution (of 1 M guanidine-HCl) also contained 2 mM glutathione (oxidized form) and 500 mM L-arginine. These reagents allow the partially refolded protein to form the proper disulfide bonds to produce the correct protein conformation. The final dialyzed protein solution was clarified by centrifugation at 13,000 × *g* to remove any aggregates and analyzed by SDS-PAGE.

Conjugation of scFv-cys to Cationic Liposomes. The purified scFv-cys was first reduced by DTT to obtain monomer scFv-SH. One M DTT was added to the scFv in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) to a final concentration of 1–50 mM. After rotation at room temperature for 5–10 min, the protein was desalted on a 10-DG column (Bio-Rad). The free -SH group was measured by 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent; Refs. 23, 24) and calculated as -SH/protein molar ratio, or number of free -SH per scFv molecule. Cationic liposomes containing 5% molar MPB-DOPE of total lipids in liposome formulations of LipA (DOTAP:DOPE, 1:1 molar ratio) or LipB (DDAB:DOPE, 1:1 molar ratio) were prepared by the ethanol injection method as described (25) with minor modification. Briefly, lipids in ethanol were injected quickly into 55°C water in a test tube while vortexing. The test tube was vortexed for an additional 20 min while cooling to room temperature. For conjugation, the reduced scFv-SH was added to the MPB-liposome at a protein:lipids ratio of 1:10–1:40 (weight ratio). The solution was mixed by gentle rotation for 30 min at room temperature to produce scFv-Lip. Because we have found that the maleimide group is not stable in aqueous solution with a pH >7.0, the liposomes were prepared in water with a pH between 5.0 and 6.5. To evaluate the efficiency of conjugation and the amount of unconjugated scFv present in the scFv-liposome solution, a 4–20% gradient natural (non-SDS) PAGE followed by Western analysis was used. A polyclonal rabbit antibody against the scFv-cys protein was used as the first antibody (produced by AnimalPharm, Healdsburg, CA) and a horseradish peroxidase-labeled mouse antirabbit monoclonal antibody (Sigma Chemical Co.) as the second antibody.

Immunoreactivity of TfRscFv-Immunoliposomes. Indirect cellular ELISA was used to determine the immunoreactivity of the scFv-cys before and after conjugation to liposomes. The ELISA was performed basically as described previously (17), except that the polyclonal rabbit antibody against the scFv-cys protein was used as the first antibody.

Preparation of scFv-Liposome-DNA Complexes (scFv-immunolipoplexes). Plasmid DNA was diluted in water and added to the scFv-Lip at a DNA:lipid ratio of 1:8–1:14 (μg/nmol), as described previously (17). The solution was mixed well for 10 min by inversion several times to produce the scFv-Lip-DNA complex. Four to 20% gradient natural (non-SDS) PAGE followed by Western analysis was also used to assess the percentage of free scFv present in the scFv-liposome-DNA complex solution.

***In Vitro* Gene Transfection.** The *in vitro* gene transfection efficiency of the cys-TfRscFv-liposome complex in tumor cell lines was assessed using the plasmid pLuc, which contains the firefly luciferase gene under control of the CMV promoter, as the reporter gene. The *in vitro* transfection was performed in 24-well plates as described previously (17). Twenty-four h after transfection, the cells were washed and lysed to measure the luciferase activity and protein concentration. The results are expressed as relative light units/μg of protein in the lysate. Another reporter gene, the *Escherichia coli* LacZ gene (*β-gal*) under the control of the CMV promoter in the plasmid pCMVb, was also used in some *in vitro* gene transfection experiments as described previously (19, 20). *β-gal* gene expression levels were measured by enzymatic assay, and the results were given as β-gal milliunits/mg protein in cell lysate (26). The Student's *t* test was used for comparison of *in vitro* data.

***In Vivo* Tumor-targeted Systemic Gene Delivery.** DU145 human prostate cancer cells (1.1×10^7) suspended in MatriGel collagen basement membrane (Collaborative Biomedical Products) were s.c. injected into 4–6-week-old female athymic nude mice, and tumors were allowed to develop. Animals bearing tumors of between 50 and 200 mm^3 were used in the study. The scFv-immunoliposomes carrying the *p53* gene, as well as unliganded LipA-p53, were i.v. injected into the tail vein of the animals at a dose of 25 μg DNA/0.4 ml/animal. As a ligand control, a scFv that does not bind to the tumor cells, CTLscFv, was also conjugated to liposomes, and the resulting CTLscFv-LipA-p53 complex was i.v. tail vein injected. Approximately 60 h after injection, the animals were sacrificed, and the tumors, as well as the liver, were excised. Protein was isolated from the tissues, and 100 μg of each sample (as determined by micro BCA protein concentration assay) were run on a 10% SDS-polyacrylamide gel for Western blot analysis using an anti-p53 monoclonal antibody as described previously (19, 26).

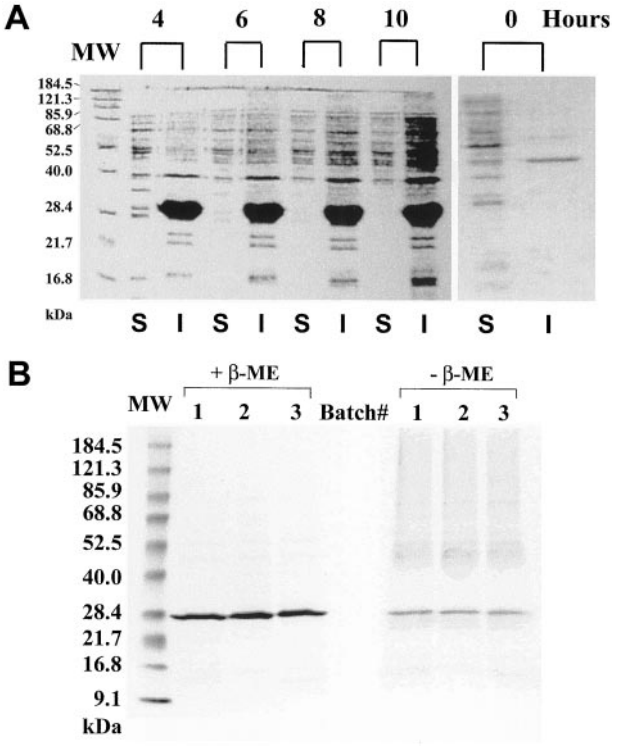

Fig. 2. Expression and purification of scFv-cys protein. *A,* scFv-cys protein levels after IPTG induction. Coomassie blue-stained SDS-PAGE showing the levels of the expressed scFv-cys present in the inclusion bodies (*I*) after induction is shown. *S,* soluble fraction; *B,* purified and refolded scFv-cys protein. Coomassie blue-stained SDS-PAGE showing three batches of scFv-cys protein purified and refolded from inclusion bodies, with and without reducing agent β-mercaptoethanol is shown. Five μg of protein were loaded per lane. The scFv-cys shows a single band at the correct molecular weight (M_r 26,000–28,000). *β-ME,* β-mercaptoethanol; *MW,* protein molecular weight standard.

To further evaluate the *in vivo*-targeted gene delivery to various tumor models by the anti-TfR scFv-immunoliposomes, the plasmid containing another reporter gene coding for GFP, pGFP, was complexed in scFv-immunoliposome, Tf-liposome, or nontargeted liposome (no targeting ligand). In this study, two tumor cell lines, JSQ-3 and DU145, were used to induce xenografts in nude mouse. The complexes were injected via the tail vein (40 μg of DNA/0.6 ml × three injections within 24 h) into nude mice bearing the xenografts. The three i.v. injections were used within 24 h to intensify the reporter protein signal. Twenty-four h after the last injection, the tumors and major organs were excised for Western analysis of GFP expression using a mouse anti-GFP monoclonal antibody, B34 (BabCo, Richmond, CA), in a similar fashion as described previously for *p53* gene expression (17, 19, 26). All animal experiments were performed in accordance with Georgetown University institutional guidelines for the care and use of animals.

Results

Purification of Anti-TfR scFv-cys. The scFv-cys was expressed in the pscFv-cys-transformed expression host BL21 (λDE3) after induction with 1 mM IPTG. The optimal induction time of 4 h was determined to yield the maximum level of protein expression **(**Fig. 2*A***)**. The majority of the TfRscFv protein (~90%) was found not to be soluble but to be contained within the inclusion bodies. Therefore, the scFv-cys containing the COOH-terminal cysteine was purified from the inclusion bodies as described in "Materials and Methods." As shown in Fig. 2*B,* the SDS-PAGE showed a single band of the refolded and purified scFv-cys in the presence of reducing agent 2-mercaptoethanol, with an approximate molecular weight of M_r 26,000–28,000. Without the reducing agent, the scFv-cys has approximately 20–25% of monomer and the rest dimer and oligomers (Fig. 2*B*). In a typical preparation cycle, 40–45 mg of inclusion bodies from 1 liter of culture yielded 26–30 mg of purified scFv-cys. In four separate batches of scFv-cys preparation, the yield of scFv-cys was 26–33 mg/liter of culture (28.2 ± 3.6 mg/l; $n = 4$), accounting for ~30% of total cellular protein.

Table 1 Reduction of anti-TfR scFv-cys

DTT concentration (mM)	-SH:scFv molar ratio
0	0.15
1	0.45
10	1.94
20	2.26
50	3.03

Preparation of scFv-targeted Cationic Immunoliposomes and Immunolipoplexes. To conjugate the anti-TfR scFv to cationic liposomes, the scFv sequence was engineered to include a cysteine at the COOH-terminal. This COOH-terminal cysteine provided a free sulfhydryl group to facilitate the conjugation of the scFv to MPB-liposomes. Because the refolded and purified scFv-cys has approximately 20–25% monomer as shown above, the scFv-cys was reduced first with DTT to obtain the monomer, scFv-SH. The results indicate that between 1 and 10 mM DTT is appropriate for the scFv-cys reduction, which gives rise to approximately one free sulfhydryl per scFv molecule (Table 1). The scFv-SH was conjugated to the maleimide group of MPB-liposome to produce the scFv-liposome. The nucleic acids were then added to the scFv-liposome to form the scFv-liposome-DNA complex (scFv-immunolipoplex). A 4–20% gradient natural (non-SDS) PAGE, followed by Western analysis, indicated that no significant free scFv-cys was detected in either preparations of scFv-liposomes or scFv-lipoplexes (Fig. 3*A*), at a scFv:lipid weight ratio range of 1:15 to 1:45 (Fig. 3*B*), demonstrating that the sulfhydryl-maleimide covalent conjugation between scFv-cys and MPB-liposome is quite efficient. Once conjugated to liposomes, the scFv protein will not be able to enter the PAGE gel. Therefore, only the unconjugated free scFv will be detected. It is difficult, under nondenaturing (non-SDS) conditions, to accurately determine the position of the free scFv-cys monomer. However, we believe that the band indicated by the *arrow* in Fig. 3 represents any unconjugated scFv-cys protein.

Immunoreactivity of scFv-Immunoliposomes. Cellular ELISA was used to determine the immunoreactivity of the scFv-cys and also to determine whether the immunoreactivity of the scFv-cys would be maintained once conjugated to

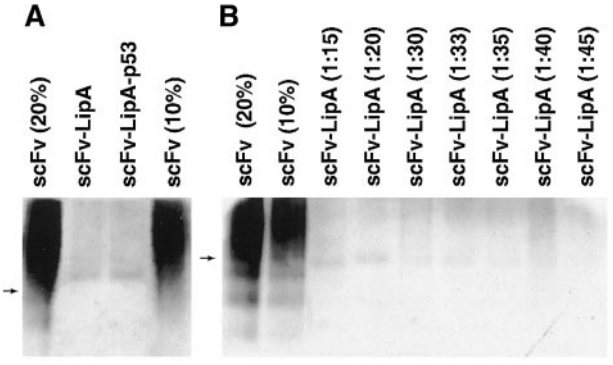

Fig. 3. Efficient conjugation of scFv-cys to MPB-LipA. A 4%–20% gradient natural (non-SDS) PAGE, followed by Western analysis, was used as described in "Materials and Methods" to evaluate the efficiency of conjugation and the amount of unconjugated scFv-cys present in the scFv-liposome solution. No significant free scFv-cys was present in either preparations of scFv-liposomes or scFv-lipoplexes (*A*) at a scFv:lipid weight ratio range of 1:15–1:45 (*B*), demonstrating that the sulfhydryl-maleimide covalent conjugation between scFv-cys and MPB-liposome is quite efficient. *Arrow,* the likely position of free scFv-cys.

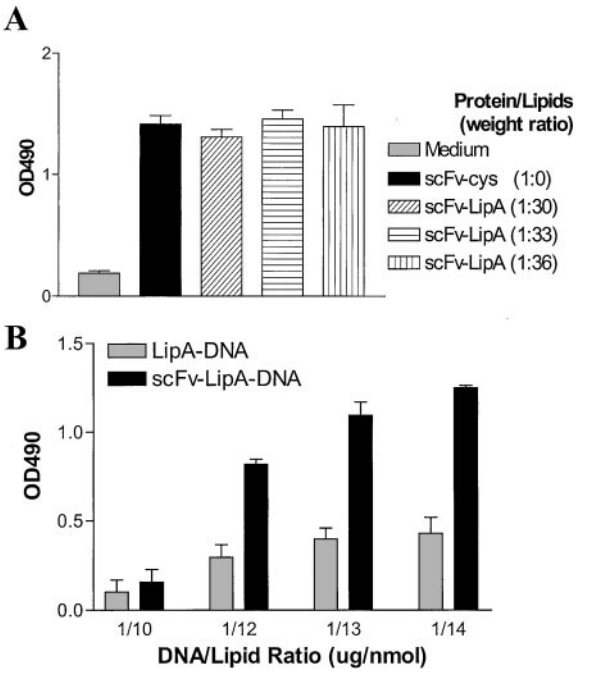

Fig. 4. Immunoreactivity of scFv-immunoliposomes. Cellular ELISA was used as described in "Materials and Methods" to determine the immunoreactivity of the scFv-cys and also to determine whether the immunoreactivity of the scFv-cys would be maintained after conjugated to cationic liposome (*A*) as well as when complexed with DNA (*B*). Its immunoreactivity was well maintained after covalent conjugation to MPB-liposomes (scFv-LipA; *A*) and after their complexing with DNA (scFv-LipA-DNA) using a scFv:lipids weight ratio of 1:30 (*B*). *Bars,* SE.

cationic liposome as well as complexed with DNA. The purified anti-TfR scFv-cys retained its immunoreactivity comparable with that of the parental anti-TfR monoclonal antibody HB21 (at a concentration of 0.6 μg/ml, $A_{490} = 0.43 \pm 0.07$ with scFv-cys and 0.31 ± 0.04 with HB21; $n = 3$). Its immunoreactivity was well maintained after covalent conjugation to MPB-liposomes (scFv-LipA; Fig. 4*A*) and after their complexing with DNA (scFv-LipA-DNA; Fig. 4*B*). As shown in Fig. 4*A,* the scFv-liposomes with protein:lipids weight ratios from 1:30 to 1:36 showed similar binding activities. As for the scFv-LipA-DNA complex (at a scFv:lipids weight ratio of 1:30), the DNA:Lipid ratio of 1:12 to 1:14 μg/nmol yielded good binding to the tumor cells (Fig. 4*B, black columns*). Fig. 4*B* also indicated that the binding of the scFv-targeted lipoplexes (scFv-LipA-DNA, *black columns*) to the tumor cells was significantly higher than that of the liposome lipoplexes without the scFv-cys (Fig. 4*B, gray columns*), demonstrating that this binding is in fact mediated through the attachment of the scFv-cys to the transferrin receptor on the tumor cells.

scFv-Immunoliposome-mediated Gene Transfection of Tumor Cells *in Vitro*. We determined the *in vitro* transfection efficiency of the scFv-cys-liposome complex in human cancer cell lines, MDA-MB-435 and DU145, using the firefly luciferase gene as a reporter gene. As shown in Fig. 5, the scFv-cys-immunoliposomes (*black columns*) have very high transfection activity *in vitro*, 4–10-fold more efficient than the untargeted liposomes (*gray columns*). Moreover, they appear to be even more efficient (2-fold) than the liposome targeted by transferrin itself (Fig. 5, ▤). Thus, they seem to retain their immunoreactivity and can bind to their target receptor. The liposome conjugated with a negative control scFv-cys (CTLscFv; Fig. 5, ▥) that does not bind to the tumor cells showed no increase in transfection activity as compared with the untargeted liposome (Fig. 5, *A* and *B, gray columns*), demonstrating this improved transfection activity of the scFv-immunoliposomes is mediated by the anti-TfR scFv. In our *in vitro* optimization study, the percentage of MPB-DOPE present in the immunoliposomes also seems to be a critical factor, with 5% molar MPB-DOPE apparently being the optimal concentration. Those containing a higher percentage of MPB-DOPE evidenced a lower transfection activity and reduced stability. On the basis of these findings, 5% molar MPB-DOPE was used in the remainder of these studies.

The transfection efficiencies of cationic liposomes targeted by scFv-cys in various human cancer cell lines as well as a normal human fibroblast cell line, H500, were also analyzed using β-*gal* as the reporter gene. As shown in Fig. 6*A,* the anti-TfR scFv-immunoliposomes showed high transfection activity in tumor cells in a DNA dose-dependent manner, with very limited activity in the normal cells. This tumor cell preference in gene transfer may result from the optimized liposome formulation and high level of TfR as well as high efficiency of TfR recycling on the tumor cells. The anti-TfR scFv-targeted immunoliposomes have higher transfection activity (around 2–6-fold) than that of the untargeted liposomes in various human cancer cell lines, including lung cancer (H358), liver cancer (Hep3B), colon cancer (HT29), and prostate cancer (DU145; Fig. 6*B*), as well as head and neck cancer (JSQ-3; Fig. 6*A*) and breast cancer (Fig. 5). Moreover, as was observed with the most of the cancer cell lines, the scFv-cys seemed to be a better targeting molecule than the transferrin ligand (Figs. 5 and 6). Thus, this increased transfection efficiency with the scFv-liposomes is

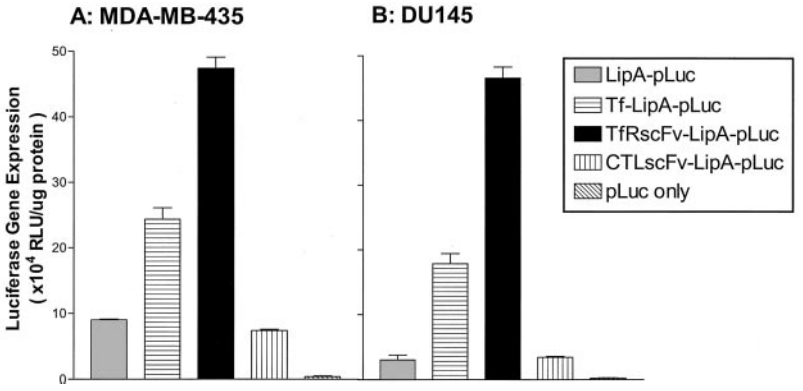

Fig. 5. scFv-immunoliposome-mediated *in vitro* gene transfection of tumor cells. Transfection efficiency was assessed using the firefly luciferase gene as a reporter gene, and the result is given as relative light units/μg protein, as described previously (17). The scFv significantly increased the transfection efficiency of cationic liposomes. This complex was 4–10-fold more efficient as compared with nontargeted LipA and 2-fold more efficient than the liposome targeted by transferrin itself. The negative control antibody CTLscFv, which does not bind to tumor cells, showed no increase in transfection activity as compared with the untargeted liposome, demonstrating that this improved transfection activity of the scFv-immunoliposomes is mediated by the anti-TfR scFv. *A,* MDA-MB-435 human breast cancer cells; *B,* DU145 human prostate cancer cells. *Bars,* SE.

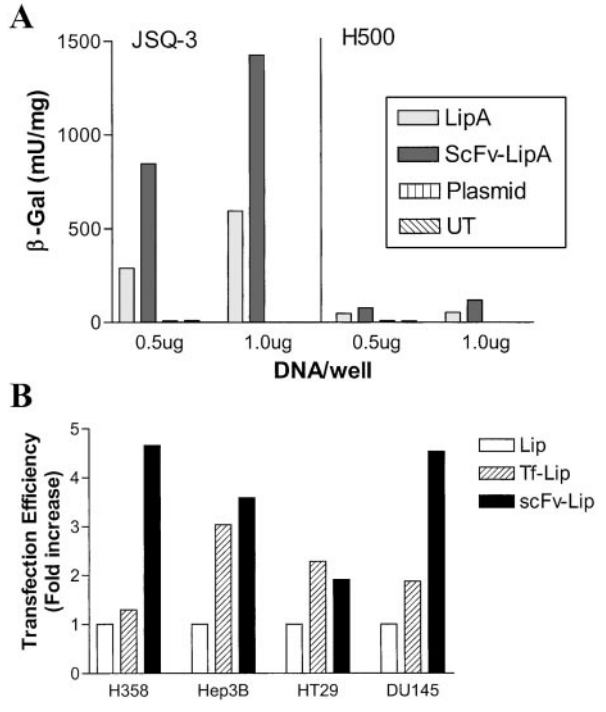

Fig. 6. The transfection efficiencies of scFv-immunoliposomes in various human cancer cell lines as well as a normal human fibroblast cell line, H500. Transfection efficiency was assessed using *β-gal* as the reporter gene. The *β-gal* enzymatic assay was used, and results are given as milliunits of *β-gal*/mg protein (19, 20). *A,* the scFv-immunoliposomes showed high transfection activity in JSQ-3 tumor cells but only very limited activity in the normal human fibroblast cell line H500. *B,* the anti-TfR scFv-targeted immunoliposomes showed higher transfection activity (approximately 2–6-fold) than that of the untargeted liposomes in various human cancer cell lines. This increased transfection efficiency with the scFv-liposomes is apparently a generalized phenomenon applicable to a broad spectrum of tumor cell types with low activity in normal cells.

apparently a generalized phenomenon applicable to a broad spectrum of tumor cell types.

Tumor-targeted Systemic Gene Delivery by scFv-Immunoliposomes *in Vivo*. To examine the ability of the scFv-immunoliposomes to deliver *p53* gene specifically to tumor tissue *in vivo,* scFv-LipA-p53, scFv-LipB-p53 or the untargeted LipB-p53, and the scFv-LipA-pVec (empty vector) were injected i.v. into nude mice bearing DU145 s.c. xenograft tumors. The two liposome formulations, LipA (DOTAP:DOPE, 1:1 molar ratio) and LipB (DDAB:DOPE, 1:1 molar ratio), were used here for *in vivo* optimization. Sixty h after the single i.v. injection, the tumors and liver were excised, and protein was isolated for Western analysis. The experiment was repeated three times with representative results presented in Fig. 7. As shown in Fig. 7*A,* the DU145 tumor from the animal i.v. injected with scFv-LipA-p53 or the scFv-LipB-p53 displayed a high level of expression of exogenous wtp53, as indicated by the intense p53 signal in an additional lower band, consistent with our previous reports (17, 19). Although it appears that the LipA composition was somewhat better than the LipB, both liposome compositions worked, demonstrating the general nature of this method. Only the endogenous mouse p53 protein was evident in the liver of these animals. In contrast, only the endogenous mouse p53 protein was evident in the tumors excised from the mice injected with the scFv-LipA-pVec or the naked p53 plasmid DNA (Fig. 7*A*). A small increase in p53 expression also was observed in the DU145 tumor with the untargeted LipB-p53. Thus, the scFv-immunoliposomes delivered and expressed the *wtp53* gene preferentially in the tumors.

To evaluate the effect of scFv:lipid ratios on the *in vivo* transfection efficiencies of the immunoliposomes, scFv-LipA-p53 (at different scFv protein:lipids weight ratios), CTLscFv-LipA-p53 or the untargeted LipA-p53, and the Tf-LipA-p53 were injected i.v. into nude mice bearing DU145 s.c. xenograft tumors. As observed in Fig. 7*A,* the DU145 tumor from the animal i.v. injected with the scFv-immunoliposome (scFv-LipA-p53) displayed a high level of expression of exogenous p53 (Fig. 7*B*). The scFv:lipid weight ratio at 1:33 showed better *in vivo* gene expression than 1:30, found to be the best ratio for *in vitro* gene transfection. In contrast, only the endogenous mouse p53 protein was evident in the liver of these animals. A small increase in p53 expression also was observed in the DU145 tumor with the unliganded LipA-p53. Therefore, the scFv:lipids weight ratio of 1:33 seems to be the better formulation for *in vivo* tumor-targeted gene delivery. These results demonstrate that the exogenous *p53* gene can be systemically delivered to, and efficiently expressed in, the tumor *in vivo* by the scFv-immunoliposomes.

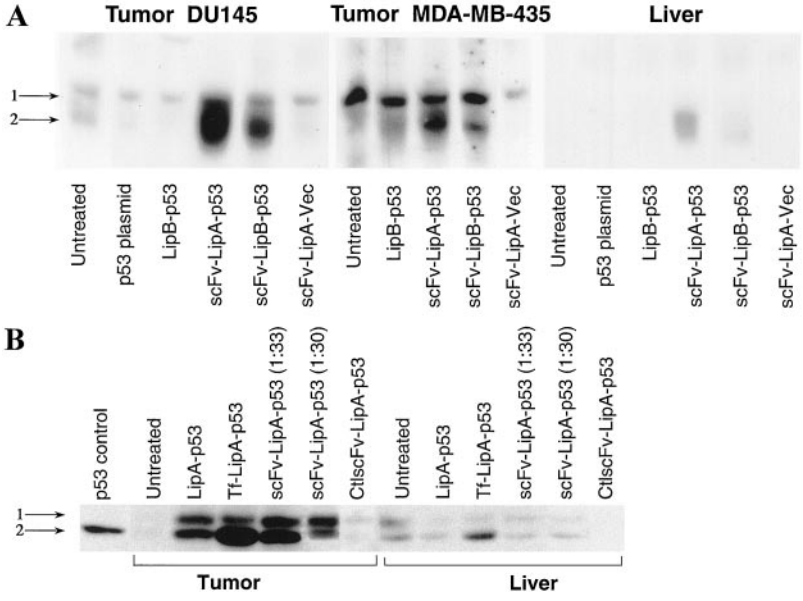

Fig. 7. Exogenous wtp53 expression in DU145 xenograft tumors after i.v. injection of scFv-LipA-p53. *A,* athymic nude mice carrying DU145 and MDA-MB-435 xenograft tumors were i.v. injected with scFv-LipA-p53, scFv-LipB-p53, Tf-LipA-p53, scFv-LipA-pVec, and untargeted LipB-p53 at a DNA dose of 25 μg/mouse. Sixty h later, the animals were euthanized, the tumor and liver were excised, and protein was isolated for Western analysis. The tumors from the animal i.v. injected with the scFv-LipA-p53 or the scFv-LipB-p53 displayed a high level of expression of exogenous wtp53, as indicated by the intense p53 signal in the additional lower band. *B, in vivo* optimization of scFv-LipA. scFv-LipA-p53 (at different scFv protein to lipids weight ratios), CTLscFv-LipA-p53 or the untargeted LipA-p53, and the Tf-LipA-p53 were injected i.v. into nude mice bearing DU145 s.c. xenograft tumors. The scFv:lipid weight ratio at 1:33 showed better *in vivo* gene expression than 1:30. The later was the best ratio for *in vitro* gene transfection. The scFv: lipids weight ratio of 1:33 seems to be the better *in vivo* formulation for tumor-targeted gene delivery. *Arrow 1,* endogenous p53; *arrow 2,* exogenous wtp53. This experiment was repeated three times with representative results shown here.

To evaluate and compare the capability of the anti-TfR scFv-immunoliposomes for *in vivo* targeted gene delivery to various human tumors, the nude mouse model bearing two human tumor s.c. xenografts (JSQ-3 and DU145) in each animal was used. The plasmid containing another reporter gene coding for GFP was complexed in the scFv-immunoliposome, the Tf-liposome, or the unliganded liposome (*i.e.,* no targeting ligand). The complexes were injected via the tail vein, 40 μg DNA/injection. To intensify the reporter protein signal, three i.v. injections were given within 24 h. Twenty-four h after the last i.v. injection, the tumors and major organs were excised for Western analysis of GFP expression. As shown in Fig. 8, the tumors in the scFv-LipA-pGFP injected mouse showed significant expression of the *GFP* gene whereas only minimal expression was observed in the tumors in the animals injected with untargeted LipA-pGFP. In both the DU145 and JSQ-3 tumor xenografts, the anti-TfR scFv-immunoliposome resulted in much better reporter gene expression than the Tf-liposome (Fig. 8), consistent with the *in vitro* gene transfection results. This experiment was repeated two more times with the representative results shown in Fig. 8. The advantage of using this animal model is that we can compare the *in vivo* gene expression efficiencies of the tumor-targeted gene delivery system in more than one tumor type simultaneously in the same animal. Certain *in vivo* reporter gene expression profiling can be obtained through this experiment that may provide a comparative data for *in vivo* formula optimization of our gene delivery system. The scFv-immunoliposome-mediated systemic gene delivery was tumor specific in that the major organs, such as liver, showed no obvious gene expression (Fig. 8), consistent with our previous report with the lipid-tagged scFv-immunoliposomes (17).

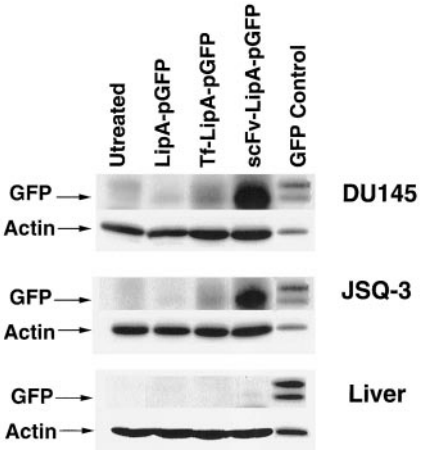

Fig. 8. *In vivo* systemic gene delivery and expression by the optimized scFv-LipA using a reporter gene coding for GFP. The complexes scFv-LipA-pGFP, Tf-LipA-pGFP, and untargeted LipA-pGFP were injected via the tail vein into nude mice bearing two human tumor s.c. xenografts (DU145 and JSQ-3). Three i.v. injections, at a DNA dose of 40 μg/injection, were given within 24 h. Twenty-four h after the last i.v. injection, the tumors and liver were excised for Western analysis of GFP expression. The tumors in the scFv-LipA-pGFP-injected mouse showed significant expression of the *GFP* gene, whereas only minimal expression was observed in the tumors in the animals injected with untargeted LipA-pGFP. The reporter gene expression was significantly higher in the tumor from the scFv-LipA-pGFP-injected mice compared with those receiving the Tf-LipA-pGFP. This experiment was repeated two more times with the representative results shown here.

Discussion

A variety of gene delivery systems have been tried including viruses and liposomes. The infectivity that makes viruses attractive as delivery vectors also poses their greatest drawback. Residual viral elements can be immunogenic, cytopathic, or recombinogenic. Recent events have highlighted the potential complications associated with viral vectors (27). The generation of novel viruses with new targets for infection also raises the theoretical possibility that, once introduced into patients, these viruses could be transformed via genetic alteration into new human pathogens. Consequently, a sig-

nificant amount of attention has been directed at nonviral vectors for the delivery of molecular therapeutics (5, 6). The most extensively used method of nonviral gene delivery is the lipoplex, a complex between cationic liposomes and DNA. The liposome approach offers a number of advantages over viral methodologies for gene delivery. Most significantly, they lack immunogenicity. Moreover, because liposomes are not infectious agents capable of self-replication, they pose no risk of evolving into new classes of infectious human pathogens. Cationic liposomes are composed of positively charged lipid bilayers that can be complexed to negatively charged, naked DNA by simple mixing (28). The resulting complex, formed by a combination of electrostatic attraction and hydrophobic interactions, has a net positive charge that facilitates transfection of the cells (4, 5).

The transfection efficiency of cationic liposomes can be dramatically increased when they bear a ligand recognized by a cell surface receptor (6). Receptor-mediated endocytosis represents a highly efficient internalization pathway present in eukaryotic cells (29). The presence of a ligand on a liposome facilitates the entry of DNA into cells through initial binding of ligand by its receptor on the cell surface, followed by internalization of the bound complex. Transferrin receptors levels are elevated in various types of cancer cells and correlate with the aggressive or proliferative ability of tumor cells (30–32). Therefore, TfR is a potential target for drug delivery in the therapy of malignant cell growth (33, 34). In our laboratory, we have developed transferrin-complexed cationic liposomes with tumor cell transfection efficiencies in head and neck cancer cells of 60–70%, as compared with only 5–20% by cationic liposomes without ligand (19, 20).

In addition to use of ligands that are recognized by receptors on tumor cells, specific antibodies can also be attached to the liposome surface, enabling them to be directed to specific tumor cell surface antigens (35–37). These immunoliposomes can deliver therapeutic drugs to a specific target cell population (38). Park *et al.* (39) found that anti-HER-2 monoclonal antibody Fab fragments conjugated to liposomes could bind specifically to breast cancer cell line SK-BR-3 that overexpresses HER-2. The immunoliposomes were found to be internalized efficiently by receptor-mediated endocytosis via the coated pit pathway and also possibly by membrane fusion. Moreover, the anchoring of anti-HER-2 Fab fragments enhanced their inhibitory effects (39, 40). More recently, a number of studies have been published that have used antibodies against tumor-specific antigens coupled to liposomes to target tumor cells for delivery of prodrugs and drugs *in vitro* or *in vivo* (36, 37, 41–45). These studies demonstrated the usefulness of immunoliposomes for tumor-targeting drug delivery. The recombination of the variable regions of heavy and light chains and their integration into a single polypeptide provides the possibility of using this single-chain antibody fragment for targeting purposes. A scFv is formed by connecting the component VH and VL variable domains from the heavy and light chains, respectively, with an appropriately designed linker peptide (11, 12). The linker bridges the COOH terminus of the first variable region and NH_2 terminus of the second, ordered as either VH-linker-VL or VL-linker-VH. The binding site of a scFv can replicate both the affinity and specificity of its parent antibody combining site (11). Currently, there is very limited data published using scFv-directed immunoliposomes for targeted gene delivery. We have recently prepared an immunoliposome targeted by a lipid-tagged scFv based on the anti-TfR Mab, 5E9, which contains the complete antibody binding site for the epitope of the transferrin receptor recognized by this Mab, as a single polypeptide chain of M_r ~30,000 (17). Our work indicated that the combination of cationic liposome-gene transfer and scFv-immunoliposome techniques is a promising system for tumor-targeted gene therapy (17, 18). The anti-TfR scFv has a number of advantages over the whole antibody or transferrin molecule itself (17–20): (*a*) scFv has a much smaller size than Tf, producing a smaller immunolipoplex giving better penetration into solid tumors; (*b*) unlike Tf, the scFv is a recombinant protein, not a blood product; and (*c*) large-scale production and strict quality control of the recombinant scFv, as well as scFv-immunolipoplex, are feasible.

Because of the low yield of the lipid-tagged scFv as described above, we constructed a new expression vector for production of the single-chain protein. The scFv produced from this vector did not contain the lipid tag. In the absence of this tag, another method was devised to attach the purified scFv protein to the cationic liposomes. A cysteine moiety was introduced into the 3′-end of the scFv protein. Reduction of this cysteine results in a free sulfhydryl group that is capable of being covalently conjugated to MPB-liposomes via a thioether bond, thus targeting the liposome to cells expressing the transferrin receptor. After purification, SDS-PAGE showed a single band of the refolded and purified scFv-cys protein with the molecular weight of M_r ~26,000. Our results show that this conjugation does not destroy its immunological activity or targeting ability. Our data demonstrated that the anti-TfR scFv-cys improved the transfection efficiency of cationic liposomes to the various tumor cell lines *in vitro* and *in vivo*, including the human cancers of prostate, breast, and head and neck.

Numerous studies have demonstrated that the *in vitro* optimized liposome formulation may not always be the best for *in vivo* gene delivery (5, 6) because the microenvironment that lipoplexes encounter *in vivo* is very different from that experienced *in vitro* (46). To determine whether there were differences between optimal liposome formulations for *in vitro* and *in vivo* delivery, the ability of i.v.-administered scFv-immunoliposomes, prepared using two different cationic liposome formulations, LipA and LipB, to target and deliver wtp53 to prostate xenograft tumors was determined in our laboratory. Although both compositions were able to deliver the p53 to the tumor, liposome composition LipA was somewhat more efficient, resulting in a higher level of expression (Fig. 7*A*). Interestingly, in this instance, the lipid formulation optimized for *in vitro* use (LipA) was also superior *in vivo*. scFv:total lipid ratios were also compared for *in vivo* targeting ability. Varying the ratio of single-chain protein to lipid *in vivo*, we found that the optimal *in vivo* ratio (Fig. 7*B*) was somewhat lower than that found to be optimal in the *in vitro* setting. The *in vitro* optimal DNA:lipid ratio of the scFv-immunolipoplex was found to be 1 μg/12 nmol (17). How-

ever, *in vivo* optimization data showed that a slightly higher DNA:lipid ratio (1 μg/13–14 nmol) gave better reporter gene expression in the tumor after systemic administration in a nude mouse tumor model. This observation supports the notion that *in vivo* optimization is critical, and the *in vivo* optimal formulation cannot be predicted merely by *in vitro* results (5, 6). Our findings also emphasize the fact that for immunoliposome therapy to be effective, other factors besides antibody selection and targeting ability, such as liposome composition, antibody:lipid, and DNA:lipid ratios, are critical and must be taken into account when designing the therapeutic complex.

In conclusion, the anti-TfR scFv-cys antibody fragment improved the transfection efficiency of cationic liposomes to the various tumor cell lines both *in vitro* and *in vivo*. The scFv-immunoliposomes can deliver the complexed gene systemically to tumor *in vivo* and are efficiently expressed. This new approach provides a promising system for tumor-targeted gene delivery that may have potential for use in the systemic gene therapy of various human cancers.

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