

Systemic p53 Gene Therapy of Cancer with Immunolipoplexes Targeted by Anti-Transferrin Receptor scFv

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Abstract

Background: A long-standing goal in genetic therapy for cancer is a systemic gene delivery system that selectively targets tumor cells, including metastases. Here we describe a novel cationic immunolipoplex system that shows high *in vivo* gene transfer efficiency and anti-tumor efficacy when used for systemic p53 gene therapy of cancer.

Materials and Methods: A cationic immunolipoplex incorporating a biosynthetically lipid-tagged, anti-transferrin receptor single-chain antibody (TfRscFv), was designed to target tumor cells both *in vitro* and *in vivo*. A human breast cancer metastasis model was employed to evaluate the *in vivo* efficacy of systemically administered, TfRscFv-immunolipoplex-mediated, p53 gene therapy in combination with docetaxel.

Results: The TfRscFv-targeting cationic immunolipoplex had a size of 60–100 nm, showed enhanced tumor cell binding, and improved targeted gene delivery and transfection efficiencies, both *in vitro* and *in vivo*. The p53 tumor suppressor gene was not only systemically delivered by the immunolipoplex to human tumor xenografts in nude mice but also functionally expressed. In the nude mouse breast cancer metastasis model, the combination of the

p53 gene delivered by the systemic administration of the TfRscFv-immunolipoplex and docetaxel resulted in significantly improved efficacy with prolonged survival.

Conclusions: This is the first report using scFv-targeting immunolipoplexes for systemic gene therapy. The TfRscFv has a number of advantages over the transferrin (Tf) molecule itself: (1) scFv has a much smaller size than Tf producing a smaller immunolipoplex giving better penetration into solid tumors; (2) unlike Tf, the scFv is a recombinant protein, not a blood product; (3) large scale production and strict quality control of the recombinant scFv, as well as scFv-immunolipoplex, are feasible. The sensitization of tumors to chemotherapy by this tumor-targeted and efficient p53 gene delivery method could lower the effective dose of the drug, correspondingly lessening the severe side effects, while decreasing the possibility of recurrence. Moreover, this approach is applicable to both primary and recurrent tumors, and more significantly, metastatic disease. The TfRscFv-targeting of cationic immunolipoplexes is a promising method of tumor targeted gene delivery that can be used for systemic gene therapy of cancer with the potential to critically impact the clinical management of cancer.

Introduction

As we enter the new millennium, effective treatment of human cancers remains a challenge. With the recent advancements in human genomics and cancer genetics, molecular therapy, especially gene therapy, is becoming a promising strategy for the treatment of cancer (1–3). A long-standing goal in gene therapy for cancer is a stable, low toxic, systemic gene delivery system that selectively targets tumor cells, including metastases (4). Current gene therapy approaches employ either viral or non-viral vector systems (5). Progress has been made toward developing non-viral, pharmaceutical formulations of genes for *in vivo* human therapy, particularly cationic liposome-mediated gene transfer systems (6–8). One

disadvantage of cationic liposomes is that they lack tumor specificity and have relatively low transfection efficiencies as compared to viral vectors. However, this can be dramatically improved when the liposomes bear a ligand recognizing a cell surface receptor (9). Transferrin receptors (TfR) levels are elevated in various types of cancer cells (10,11) and correlate with the aggressive or proliferative ability of tumor cells (12). Therefore, TfR levels are considered useful as a prognostic tumor marker, and TfR is a potential target for drug delivery in the therapy of malignant cells (13,14). In our laboratory, we have developed transferrin-targeting cationic liposomes with tumor cell transfection efficiencies of 60%–70% in squamous cell carcinoma of head and neck, as compared to only 5–20% by cationic liposomes without the targeting ligand (15). This system was shown to have the capability of systematically delivering the p53 tumor suppressor gene to pre-established xenograft tumors and sensitizing them to radiotherapy, resulting in long term tumor regression in animals receiving the combination radiotherapy (16).

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In further developing a targeted gene delivery system aimed at the TfR, we have established a cationic immunolipoplex system directed by a single-chain antibody Fv fragment against the human transferrin receptor (TfRscFv). The TfRscFv targets the cationic liposome-DNA complex (lipoplex) to tumor cells and enhances the transfection efficiencies both *in vitro* and *in vivo*. In a nude mouse breast cancer metastasis model systemic p53 tumor suppressor gene therapy mediated by the TfRscFv-targeting immunolipoplexes, in combination with conventional chemotherapy, significantly prolonged the life-span and resulted in long term survival of the animals.

Materials and Methods

The p53 expression plasmid, pCMVp53, contains the human wild-type (wt) p53 gene under the control of the cytomegalovirus (CMV) promoter (16). The control plasmid, pVec, is the empty vector without the p53 insert. The plasmid pBP100 used to detect the functional expression of wtp53 was kindly provided by Dr. Arnold Levine, Rockefeller University, NY. The cationic lipids, dioleoyltrimethylammonium propane (DOTAP), dimethyldioctadecylammonium bromide (DDAB) and the fusogenic neutral lipid dioleoylphosphatidylethanolamine (DOPE), or cholesterol (Chol), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Human head and neck cancer cell line JSQ-3, derived from a tumor of the nasal vestibule that failed radiotherapy, was maintained in Minimum Essential Medium with Earle's salts (EMEM), supplemented as previously described (15). This cell line, a kind gift from Dr. Raplh Weichselbaum, University of Chicago, was maintained in Improved Minimum Essential Medium (IMEM) with 10% FBS plus 50 $\mu\text{g}/\text{ml}$ each of penicillin, streptomycin and neomycin and 2 mM L-glutamine. Human prostate cancer cell line DU145 was maintained in EMEM with 10% FBS plus 50 $\mu\text{g}/\text{ml}$ each of penicillin, streptomycin and neomycin and 2 mM L-glutamine. Both cell lines were purchased from ATCC. All three cell lines have mutant p53.

Construction of the Expression Vector for Anti-TfR scFv

To construct the expression vector we used the vector pLP1 kindly provided by Dr. John de Kruif (Utrecht University, The Netherlands). This vector contains an amino acid linker sequence between the *E. coli* lipoprotein signal peptide (LPP) and the scFv cloning site (17,18), and also contains both *c-myc* and *His₆* tag sequences that can be used for purification and detection of the expressed scFv (Fig. 1). The plasmid pDFH2T-vecOK containing the anti-TfR scFv from the 5E9 antibody, which recognizes the human transferrin receptor (CD71)(19), was obtained from Dr. David Fitzgerald, NCI, NIH, Bethesda, MD. Since there are no convenient restriction enzyme sites for cloning of the

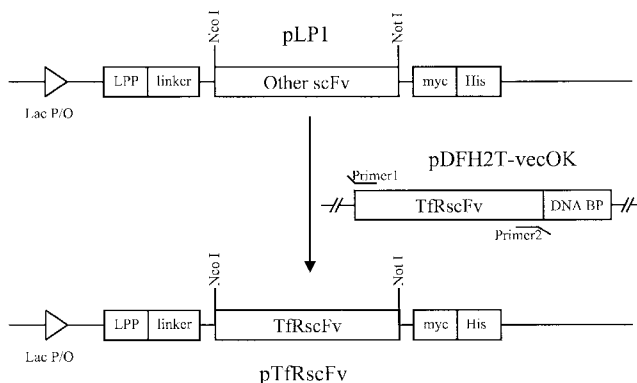


Fig. 1. Construction of pTfRscFv, the expression vector for the lipid-tagged anti-human transferrin receptor scFv (TfRscFv). The vector pLP1 contains an amino acid linker sequence between the *E. coli* lipoprotein signal peptide and the scFv cloning site (LPP and linker), and also contains both *c-Myc* and *His₆* tag sequences (*myc* and *His*). The PCR-amplified TfRscFv cDNA from the vector pDFH2T-vecOK was cloned into pLP1 using *Nco*I and *Not*I sites to obtain the expression vector, pTfRscFv.

TfRscFv sequence, PCR was carried out to amplify the scFv cDNA from pDFH2T-vecOK. This PCR used a 5' primer (Primer1, 5'GGCCCATGGAGGTGCAGCTGTGG 3') containing a *Nco*I site and a 3' primer (Primer 2, 5' CCGGAATTCGCGGCCGCTTTTATCTCCAGCTTGTC 3') containing a *Not*I site, as shown in Fig. 1. The TfRscFv cDNA was then cloned into pLP1 using *Nco*I and *Not*I sites to obtain the expression vector for the lipid-tagged TfRscFv, pTfRscFv (Fig. 1).

Expression and Purification of TfRscFv

The TfRscFv was expressed in the pTfRscFv-transformed expression host SF110F' after overnight induction with 1mM IPTG. The TfRscFv protein was isolated from the bacterial membrane using Triton X-100 as described by de Kruif et al (18). The TfRscFv was purified from the detergent-solubilized membranes by metal-chelating chromatography with a HiTrap column (Pharmacia), which binds the His₆ tag on the expressed TfRscFv. The TfRscFv was stored in 20 mM HEPES, 150 mM NaCl, pH 7.4, containing 1% n-octyl β -D-glucoside (OG) (Sigma), at a concentration of 1 mg/ml.

Preparation of the TfRscFv-immunolipoplexes by Lipid-film Solubilization Method

5 μmol lipids (DOTAP/DOPE, 1:1 molar ratio, for LipA) in chloroform were evaporated under reduced pressure to obtain a dry lipid film in a glass round-bottom flask. To the lipid film was added 500 μg TfRscFv in 0.5 ml 20 mM HEPES, 150 mM NaCl, pH 7.4, containing 1% OG, incubated 10–20 minutes at room temperature and then vortexed to solubilize the lipid membrane. 2 ml sterile water was added to dilute the TfRscFv-lipid mixture. The solution was briefly sonicated to clarity in a bath-type sonicator at

20°C. The resultant TfRscFv-liposome is an almost clear solution with a limited amount of residual OG detergent. The immunoliposomes made by this method are designated TfRscFv-LipA1.

Preparation of TfRscFv-immunoliposomes by the Direct Anchoring Method

The preformed cationic liposomes were prepared basically as previously described (15,16). Briefly, 10 ml pure water was added to 20 μ mol lipids prepared as dry lipid film in a glass round-bottom flask and sonicated in a bath-type sonicator for 10–30 min at room temperature (for liposomes containing DOPE) or at 65°C (for liposomes containing cholesterol (Chol)). The cationic liposomes prepared were almost clear solutions. Their lipid compositions and molar ratios were as follows: (1) *LipA*: DOTAP:DOPE, 1:1; (2) *LipB*: DDAB:DOPE, 1:1; (3) *LipC*: DOTAP:Chol, 1:1. For attaching TfRscFv to the preformed liposomes, various amount of TfRscFv in 20 mM HEPES, 150 mM NaCl, pH 7.4, containing 1% OG, was added to the liposomes while vortexing, at volume ratios from 1:3 to 1:10. The mixture was vortexed for approximately 5–10 minutes more to obtain a clear TfRscFv-immunoliposome solution, designated TfRscFv-LipA2.

Immunoreactivity of TfRscFv-immunoliposomes Determined by ELISA and Immunofluorescence

Indirect cellular enzyme-linked immunosorbent assay (ELISA) was employed to determine the immunoreactivity of the TfRscFv before and after attachment to liposomes. Confluent JSQ-3 cells in 96-well plates were fixed with 0.5% Glutaraldehyde in PBS for 10 min at room temperature. The plate was blocked with 5% fetal bovine serum (FBS) in PBS at 37°C for 30 min. The TfRscFv, TfRscFv-immunoliposomes or unliganded liposomes were added to each well in duplicate and incubated at 4°C overnight. After three PBS-washes, the anti-*c-myc* monoclonal antibody, 9E10 (10–30 μ g/ml in PBS with 3% FBS) was added to each well and incubated at 37°C for 60 min. After three PBS-washes, HRP-labeled goat-anti-mouse IgG (Sigma) diluted 1:1000 in PBS with 3% FBS, was added to each well and incubated for 30 min at 37°C. The plate was washed three times with PBS and 100 μ l of the substrate, 0.4 mg/ml OPD in citrate phosphate buffer (Sigma), was added to each well. The color-development was stopped by adding 100 μ l 2 M sulfuric acid to each well. The plate was read by an ELISA plate reader (Molecular Devices Corp.) at 490 nm.

For immunofluorescent staining studies, TfRscFv-LipA1 or TfRscFv-LipA2, free TfRscFv, non-targeted LipA, or FITC-labeled human transferrin (Tf-FITC) were incubated with the tumor cells (JSQ-3 or DU145) for 60 min on ice. After three washes with ice-cold PBS/3% FBS, the second antibody, anti-*c-Myc* monoclonal antibody 9E10 (Pharmacia) which binds

the *c-Myc* sequence in the c-terminus of TfRscFv, was added at 30 μ g/ml and incubated on ice for 45–60 min. After three washes with ice-cold PBS/3% FBS, the cells were stained with FITC-labeled sheep-anti-mouse IgG (Sigma), 1:60 dilution in PBS/5% FBS, for 30 min on ice. The cells were then washed and fixed with 0.4% paraformaldehyde at 4°C overnight. The stained cells were analyzed on a FAC-Star flow cytometer (Becton & Dickinson, Franklin Lakes, NJ).

In Vitro Gene Transfection

Liposome-mediated gene transfection of tumor cells was performed as described previously (15,16,20). In these studies the reporter construct used contained the *E. coli* Lac Z gene under the control of the CMV promoter (pCMVb), the same promoter used in pCMVp53 (16). TfRscFv-liposomes made at a concentration of 1.8 mM lipids and varying amounts of protein (16 to 22 μ g/ μ mol lipids) were mixed with plasmid DNA at a ratio of 1 μ g DNA/8–14nmol lipids, in a manner similar to our other ligand-liposomes (20). Co-transfection of pCMVp53 with an expression plasmid (pBP100) which contains the luciferase reporter gene under the control of a p53 responsive promoter, was performed essentially as previously reported with some modifications (21). Briefly, Tf-LipA complexed to 0.3 μ g/well of pBP100 (Tf-LipA-BP100) was added to JSQ-3 cells seeded in a 24 well plate as previously described (15). Subsequently, 0.3 μ g DNA/well of TfRscFv-LipA2-p53, TfRscFv-LipA2-pVec or TfRscFv-LipA2 minus DNA (equivalent lipids) were added in triplicate. The co-transfection was performed identically to that for other reporter genes and the luciferase activity was assayed 48 hours later (15,16,20).

In Vivo Studies

For *in vivo* p53 gene delivery studies, nude mouse xenografts were induced by the subcutaneous injection of 2.5×10^6 JSQ-3 cells on the lower back above the tail of 4–6 week old female athymic nude (NCr nu/nu) mice. The TfRscFv-LipA2 was prepared by the direct anchoring method at a final concentration of 1.8 mM lipids and 20 μ g/ml protein. The TfRscFv-LipA2-pCMVp53 immunolipoplex was prepared by adding pCMVp53 DNA to TfRscFv-LipA2 at a ratio of 1 μ g DNA/14nmol lipids. 25 μ g pCMVp53 complexed with either TfRscFv-LipA2 or non-targeted liposomes (total volume 0.3 ml) were intravenously injected into tumor-bearing nude mice via tail vein. Two days after injection, the tumors as well as major organs, were excised and the p53 protein expression was assessed by Western analysis as previously described (16).

For *in vivo* efficacy studies, a nude mouse tumor metastasis model was employed (22). 435/LCC6 cells (8×10^6) were intravenously injected into 4–6 wk old female athymic nude mice. Ten days later, the

mice were randomly divided into 5 mice/group and systemic treatment with TfRscFv-LipA2-pCMVp53, with or without docetaxel (TaxotereTM, Rhone-Poulenc Roprer Pharmaceuticals, Inc., Colleagueville, PA), was begun. Intravenous injections of the complex, at a DNA dose of 20 μ g/0.3ml per i.v., were administered twice a week, for a total of 10 injections. The immunolipoplexes were freshly prepared before each injection by mixing pCMVp53 DNA with TfRscFv-LipA2 at a ratio of 1 μ g DNA/14nmol lipids. As controls, groups of mice were also treated with docetaxel alone, with the untargeted LipA-pCMVp53 plus drug, or with the human transferrin-targeting lipoplex Tf-LipA-pCMVp53, with or without drug. The Tf-LipA-pCMVp53 was prepared as previously described (16). For combination treatment groups, docetaxel stock of 10mg/ml was added directly to the liposome-DNA solutions before injection to reach a dose of 7.5 mg/kg body weight. A separate study indicated that adding stock docetaxel directly to immunolipoplex solution did not change the integrity and size of the complex (data not shown). All solutions for i.v. injection were labeled with Solution Numbers and administered by the technicians from the Georgetown University Animal Facility without knowledge of their individual contents. The animal deaths were recorded daily. All animal experiments were performed in accordance with Georgetown University institutional guidelines for the care and use of animals.

Characterization of TfRscFv-immunoliposomes and Immunolipoplexes

The sizes and zeta potentials of the TfRscFv-Lip and TfRscFv-LipA-pCMVp53 were analyzed by photon correlation spectroscopy (PCS) using the Malvern Zetasizer 3000HS (Malvern Instruments, Inc., UK). The measurement was performed according to the manufacturer's instruction with the following settings: temperature 25°C; scattering angle 90; analysis mode CONTIN. Each measurement was repeated 2–3 times for size and 5–8 times for zeta potential. The results are presented as the mean \pm SE of at least three preparations.

Statistical Analysis

The student's *t*-test was used for comparison of *in vitro* data. For *in vivo* nude mice efficacy studies, the Kaplan-Meier Survival Analysis was performed using GraphPad Prism 3.02, GraphPad Software, San Diego, CA. The Mantel-Haenszel Logrank Test from the same software package was employed for comparison of individual survival curves.

Results

Expression of Anti-TfR scFv

To facilitate subsequent preparation of immunoliposomes, the biosynthetically lipid-tagged scFv system described by Laukkanen et al. (17) and de

Kruif et al. (18) was employed. Fig. 1. shows the construction of the expression vector for the lipid-tagged scFv, designated TfRscFv. In this expression vector construct, a sequence for the *E. coli* lipoprotein signal peptide and the *E. coli* lipoprotein N-terminal 9 amino acids (LPP) were inserted as described by Laukkanen et al. (23,24). The insertion of these sequences will lead to fatty acid acylation of the expressed signal peptide on the scFv in the *E. coli* host and its insertion into the bacterial membrane (17). Purification of the lipid modified scFv from the bacterial membrane results in an active molecule that can attach to liposomes via its lipid tag (18,23,24). The purified TfRscFv was analyzed by SDS-PAGE and Western Blot using anti-c-Myc antibody 9E10, with the TfRscFv showing a band of the size of about 30 kDa (data not shown).

Preparation of TfRscFv-targeted Cationic Immunoliposomes and Immunolipoplexes

The lipid-tagged TfRscFv-immunoliposomes are prepared basically by two methods: a lipid-film solubilization method and a direct anchoring method. The lipid-film solubilization method is modified from the detergent dialysis method, which was originally described by Laukkanen et al. (24) and de Kruif et al. (18) for neutral or anionic liposomes. This method has been found to be suitable for attaching lipid-tagged TfRscFv to cationic liposomes as well. The second method for attaching lipid-tagged antibody fragments is the direct anchoring method that is particularly useful for attaching the TfRscFv to only the outer surface of preformed liposomes. The remaining OG and the unincorporated TfRscFv can be removed by column chromatography with Sepharose CL-4B or Sephacryl S-500. However, separation experiments, i.e., ultrafiltration with Centricon-100 (Amicon), or Sepharose CL-4B (Pharmacia) chromatography, demonstrated that virtually all of the lipid-tagged TfRscFv molecules added have been attached or anchored to the cationic liposomes (data not shown). Therefore, it is unnecessary to have a further purification step to remove the unattached TfRscFv.

The TfRscFv-immunolipoplexes were prepared by mixing equal volumes of TfRscFv-immunoliposomes with plasmid DNA at various DNA/lipid ratios, either in serum-free medium for *in vitro* studies, or in water for *in vivo* experiments, as described in detail in Methods.

Immunoreactivity of TfRscFv-immunoliposomes

To analyze the immunoreactivity of TfRscFv before and after incorporation into cationic liposomes, indirect cellular ELISA was employed using human TfR-positive head and neck cell line JSQ-3 as the target cells. ELISA results are expressed as optical density at 490 nm (OD490), mean \pm SD, *n* = 2. The immunoliposomes TfRscFv-LipA1 and TfRscFv-LipA2

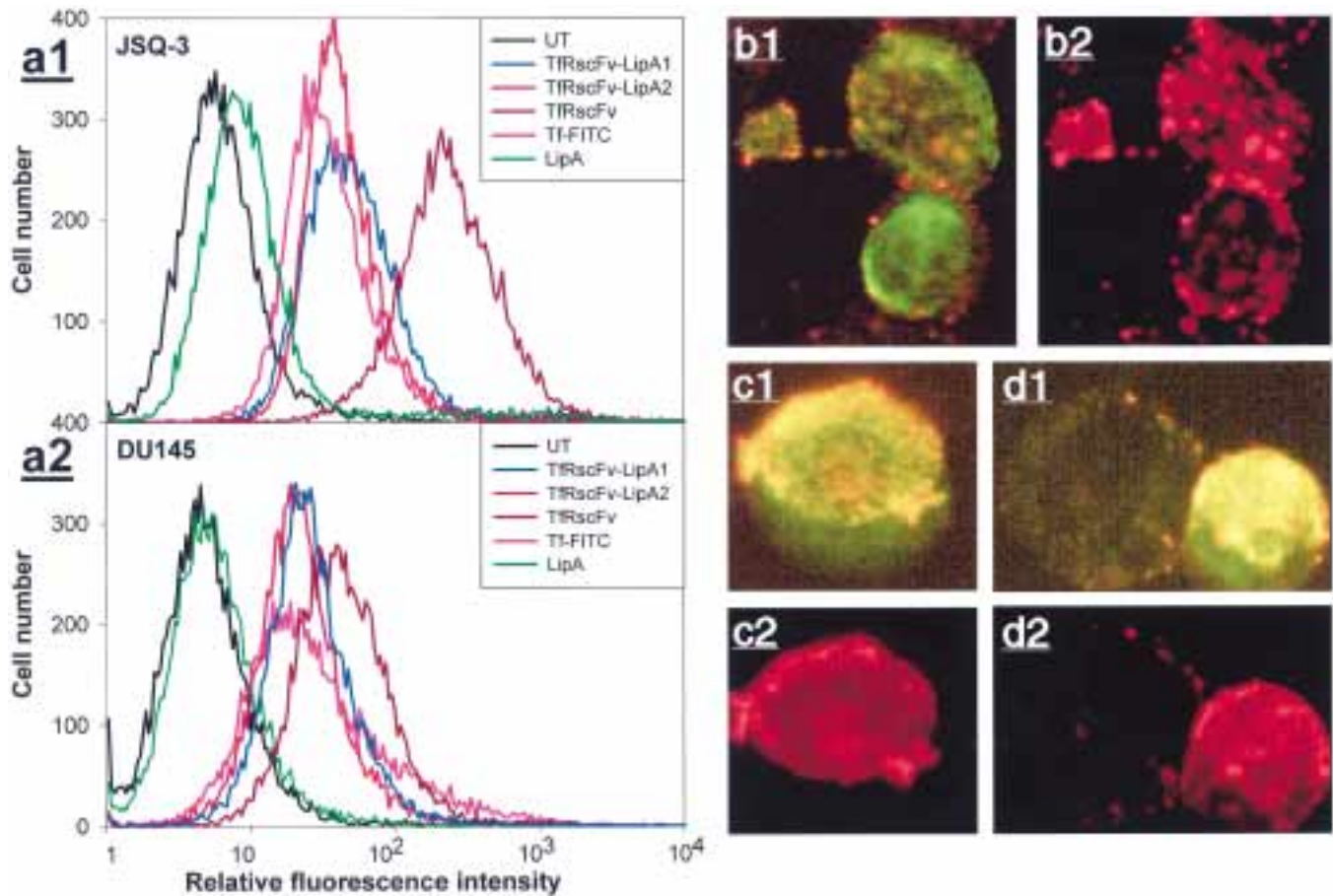


Fig. 2. Binding of TfRscFv-liposomes to tumor cells. The human head and neck cancer JSQ-3 cells (a1) and prostate cancer DU145 cells (a2) were incubated with TfRscFv-LipA1, TfRscFv-LipA2, free TfRscFv, non-targeted liposomes (LipA) or FITC-labeled human transferrin (Tf-FITC). After immunofluorescent staining with anti-c-Myc antibody followed with FITC-labeled sheep-anti-mouse IgG antibody, the cells were analyzed by flow cytometry. UT: untreated control cells. TfRscFv-LipA1 and TfRscFv-LipA2 binds to tumor cells comparable to that observed with human transferrin. The stained cells were also observed under a fluorescence microscope. (b1), (c1) and (d1) are TfRscFv-LipA2-treated JSQ-3 cells observed with FITC filter, the green fluorescence indicates the presence of TfRscFv, (b2), (c2) and (d2) are the corresponding fields observed with rhodamine filter, the red fluorescence indicates the presence of liposomes.

yielded OD490 values of 1.134 ± 0.038 and 1.386 ± 0.004 , respectively, similar to that of the free TfRscFv (1.024 ± 0.037). Unliganded liposome LipA had an OD490 value of only 0.142 ± 0.036 . These results demonstrate that the TfRscFv retained its immunoreactivity after incorporation into liposomes. Pre-incubation with free cationic liposomes did not significantly reduce the binding of the immunoliposomes to the tumor cells, demonstrating that the binding is through the targeting ligand TfRscFv. The slightly higher binding activity of TfRscFv-LipA2 than TfRscFv-LipA1 ($p = 0.0113$) may be due to the fact that the direct anchoring method ensures virtually all TfRscFv added is incorporated into the outer surface of the liposomes, whereas the TfRscFv-LipA1 made by the lipid-film solubilization method may have at best about 50% of TfRscFv on the surface.

Immunofluorescent staining followed by FACS analysis and fluorescent microscopy was also employed to assess the specific binding of TfRscFv-

liposomes to the target cells. As shown in Fig. 2 (a1), incubation of JSQ-3 cells with TfRscFv-LipA1 or TfRscFv-LipA2 resulted in a fluorescence shift identical to that observed with Tf-FITC, indicating the tumor cell binding of the immunoliposome. TfRscFv-LipA1 showed the same binding activity to tumor cells as TfRscFv-LipA2. Similar results were also observed with prostate tumor cell line DU145 (Fig. 2 a2). Indirect immunofluorescent staining with TfRscFv-liposomes (where liposomes had been labeled with rhodamine-DOPE) confirmed the binding of TfRscFv-targeting liposomes to JSQ-3 cells (Fig. 2 b-d). The TfRscFv-LipA2-treated JSQ-3 cells showed the concurrence of the green fluorescence (indicating the presence of TfRscFv, Fig. 2 b1, c1, d1) and red fluorescence (indicating the presence of liposomes, shown in Fig. 2 b2, c2, d2). The results demonstrate that TfRscFv does indeed direct the binding of liposomes to the tumor cells. In addition, TfRscFv-LipA2 showed substantial binding to

tumor cells, demonstrating that incorporation into the liposome complex did not inactivate the immunological activity of TfRscFv, and that TfRscFv can direct liposomes to bind to target tumor cells.

Optimization of TfRscFv-Immunoliposome-Mediated Gene Transfection of Tumor Cells *In Vitro*

To assess the transfection efficiency of TfRscFv-liposomes, *in vitro* transfection experiments were performed with plasmid pCMVb that uses the *E. coli* LacZ gene as a reporter (20). The level of LacZ gene expression in transfected JSQ-3 cells (correlating with transfection efficiency) was assessed by β -galactosidase (β -Gal) enzymatic assay (15,16). As shown in Fig. 3, the attachment of TfRscFv significantly increased the transfection efficiency of cationic liposomes (2- to 2.4-fold over that observed with the non-targeted liposomes (TfRscFv-LipA1 vs. LipA, $p = 0.005$; TfRscFv-LipA2 vs. LipA, $p = 0.0119$; two-tailed *t*-test). This increase in gene transfection is comparable to that of human transferrin. Here too we observed higher efficiency with TfRscFv-LipA2 than with TfRscFv-LipA1, correlating with their immunoreactivities in the ELISA results described above. The difference observed here might also be due to more TfRscFv being present on the surface of TfRscFv-LipA2 immunoliposomes. Thus, subsequent experiments focused on TfRscFv-LipA2 made by the direct anchoring method.

To examine the effects of DNA/lipid ratio and lipid composition on the transfection efficiencies of the immunolipoplexes, JSQ-3 cells were transfected with TfRscFv-targeting cationic lipoplexes

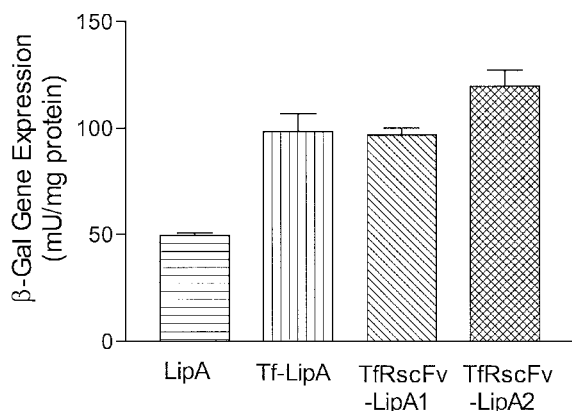


Fig. 3. TfRscFv facilitated liposome-mediated gene transfection of JSQ-3 tumor cells *in vitro*. Transfection efficiency was assessed by the β -galactosidase enzymatic assay and is given as mU β -galactosidase/mg protein (15,16). The attachment of TfRscFv significantly increased the transfection efficiency of cationic liposomes, (2- to 2.4-fold) as compared to non-targeted LipA. The gene transfection efficiency of TfRscFv-LipA1 (prepared by the lipid-film solubilization method) or TfRscFv-LipA2 (prepared by the direct anchoring method) is comparable to that of the human transferrin targeting lipoplex (Tf-LipA). TfRscFv-LipA2 showed slightly higher transfection efficiency than TfRscFv-LipA1, correlating with their immunoreactivities in the ELISA results in Fig. 2.

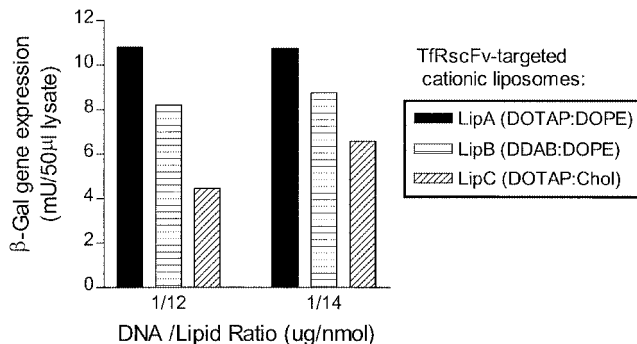


Fig. 4. Effects of DNA/lipid ratio and lipid composition on the transfection efficiencies of TfRscFv-immunolipoplexes. JSQ-3 cells were transfected with TfRscFv-targeting cationic lipoplexes prepared with different lipid compositions and at various DNA/lipid ratios, using the LacZ gene as the reporter (16). TfRscFv-LipA showed superior transfection activity over TfRscFv-targeting LipB or LipC. DNA/lipid ratios at 1/12 to 1/14 μ g/nmol gave the best and similar efficiencies *in vitro*.

prepared with different lipid compositions and at various DNA/lipid ratios (16,20). As shown in Fig. 4, TfRscFv-targeted LipA (DOTAP:DOPE, molar ratio 1:1) showed superior transfection activity over other liposome compositions, i.e., LipB (DDAB:DOPE) or LipC (DOTAP:Chol) (molar ratio 1:1). The liposome composition LipA was therefore chosen for further optimization. With this composition, DNA/lipid ratios at 1/12 to 1/14 μ g/nmol gave the best result and had similar efficiencies *in vitro* (Fig. 4).

To optimize TfRscFv-LipA2 mediated gene transfection, different amounts of TfRscFv were also incorporated into cationic liposomes by the direct anchoring, with final protein/lipids ratios of 16–22 μ g/ μ mol. Both human breast cancer cell line MDA-MB-435 and head and neck cell line JSQ-3 were transfected with TfRscFv-LipA2-pCMVb. Fig. 5 shows that TfRscFv-targeting liposomes gave 2- to 4-fold higher reporter gene expression than the non-targeted liposomes in both cell lines. The immunoliposomes with 22 μ g protein/ μ mol lipids showed higher transfection efficiency, but in later *in vivo* experiments, they tended to precipitate and were not stable when complexed with DNA. Therefore, the 18–20 μ g/ μ mol of TfRscFv/lipids were chosen as optimal ratios for further study.

To further confirm this optimized formulation for TfRscFv-immunolipoplexes, we employed a second reporter gene, the firefly luciferase gene in the plasmid pLuc, in the *in vitro* transfection assay. The transfection assay was carried out as previously described (16). Both MDA-MB-435 and prostate cancer cell line DU145 were transfected with TfRscFv-LipA2-pLuc. As shown in Fig. 6, the presence of the TfRscFv significantly increased the transfection efficiency of the cationic liposomes. Based on the recombinant luciferase standard (Sigma), the

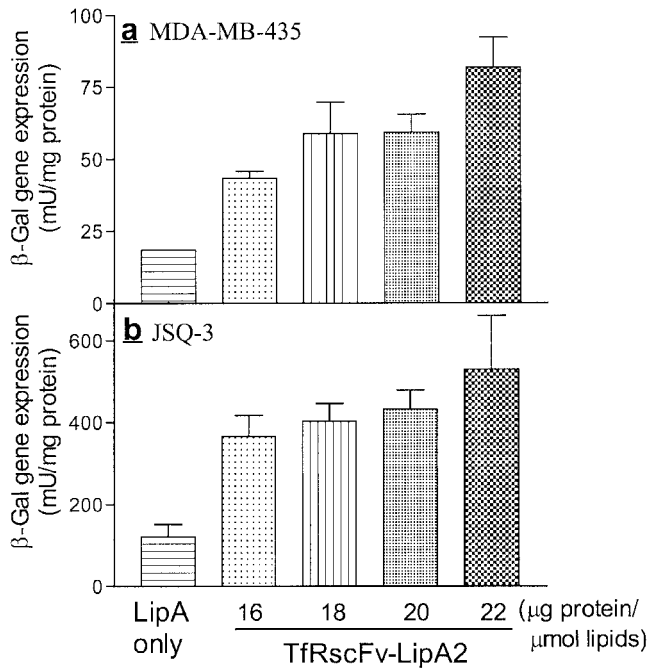


Fig. 5. Optimization of protein/lipid ratios for TfRscFv-LipA2. Different amounts of TfRscFv were incorporated to cationic liposomes by the direct anchoring method, with final protein/lipids ratios of 16–22 $\mu\text{g}/\mu\text{mol}$. Human breast cancer cells MDA-MB-435 (a) and head and neck cancer cells JSQ-3 (b) were transfected as described in Methods.

resulting luciferase gene expression of 80,000 RLU/ μg protein in MDA-MB-435 cells and 40,000 RLU/ μg protein in DU145 cells, corresponded to 10% and 5% of total cellular protein, respectively. For both cell lines, the TfRscFv-immunoliposomes were 10-fold more efficient than the unliganded LipA and 2- to 3-fold more efficient than Tf-targeting liposomes. Control scFv (CTLscFv)-LipA2 showed the same activity as LipA, demonstrating that this increased efficiency is via the specific targeting ligand, TfRscFv.

Functional Exogenous P53 Expression In Tumor Cells Transfected By TfRscFv-LipA2-pCMVp53 Immunoliposomes In Vitro

The functional expression of exogenous wtp53 in JSQ-3 tumor cells transfected with the TfRscFv-targeting immunoliposomes was assessed by co-transfection of pCMVp53 with plasmid pBP100, which contains the luciferase reporter gene under the control of a p53 responsive promoter (21). Consequently, the higher the level of exogenous p53 expression, the higher the level of luciferase activity. Co-transfection experiments were performed by transfecting JSQ-3 cells with pBP100 complexed with Tf-LipA (Tf-LipA-pBP100) together with the same amount of pCMVp53 complexed with TfRscFv-LipA2 (TfRscFv-LipA2-p53). To serve as controls, either TfRscFv-LipA2 without DNA (TfRscFv-LipA2

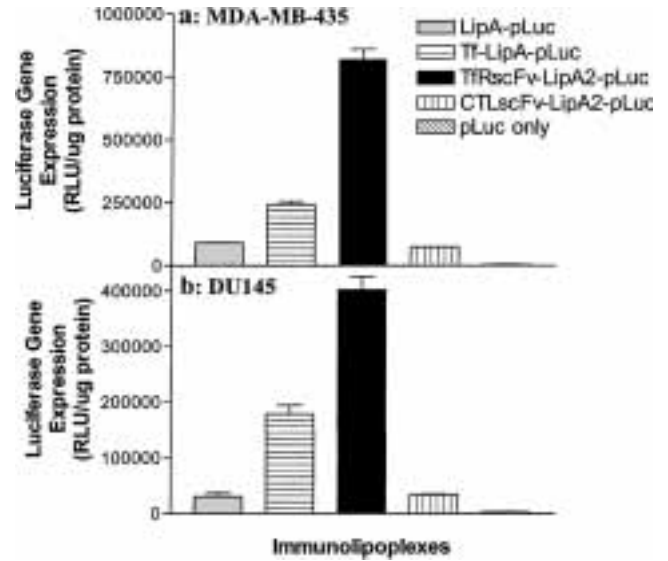


Fig. 6. TfRscFv-immunolipoplex-mediated gene transfection *in vitro*, using another reporter gene, the firefly luciferase gene. (a) Human breast cancer cell line, MDA-MB-435. (b) Human prostate cancer cell line, DU145. Transfection efficiency was assessed by the Luciferase assay and is given as relative light units (RLU)/ μg protein, as previously described (16). The TfRscFv significantly increased the transfection efficiency of cationic liposomes. TfRscFv-LipA2-pLuc was 10-fold more efficient than non-targeted LipA-pLuc and 2- to 3-fold more efficient than Tf-LipA-pLuc. Control scFv (CTLscFv)-LipA-pLuc showed the same activity as LipA-pLuc.

only) or complexed with the same amount of control plasmid pCMVpVec (TfRscFv-LipA2-pVec) were co-transfected with Tf-LipA-pBP100. Using TfRscFv as the targeting ligand for cationic liposomes increased the transfection efficiency and enhanced the expression of functional wtp53 protein, indicated by the increased p53-responsive luciferase activity, as compared to non-targeting lipoplex LipA-p53 ($p = 0.0263$). As shown in Fig. 7, TfRscFv-LipA2-p53 co-transfection gave 54-fold more p53-responsive luciferase activity than TfRscFv-LipA2-pVec ($p = 0.0001$) and 11-fold more than TfRscFv-LipA2 only ($p = 0.0002$). Once again, the level of p53 expression in the cells transfected with the TfRscFv-LipA2-p53 immunolipoplex was at least comparable to that with transferrin-targeting lipoplex ($p = 0.0526$) (Fig. 6). These results (Fig. 7) demonstrated that, via this targeted gene delivery system, exogenous wtp53 protein was expressed efficiently and functionally.

Characterization of the TfRscFv-targeting Immunoliposomes

For characterization of the immunolipoplex, the sizes and zeta potential were measured by photon correlation spectroscopy (PCS), also known as dynamic laser light scattering (DLS). The immunolipoplex was prepared according to the optimized formulation for *in vivo* injection. The cationic liposome alone (LipA) had a size of 29.3 ± 7.2 nm in

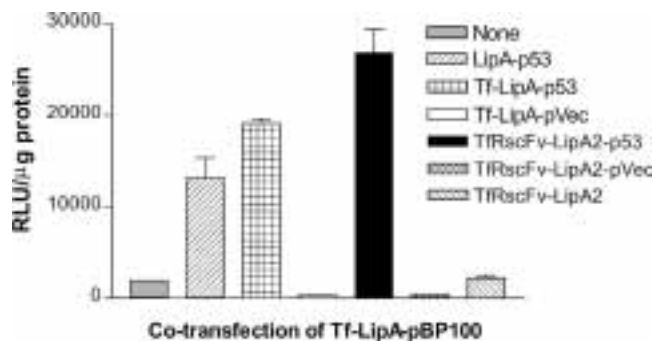


Fig. 7. Functional exogenous p53 expression in JSQ-3 cells transfected by TfRscFv-LipA2-p53 immunolipoplex *in vitro*. JSQ-3 cells were co-transfected in triplicate by Tf-LipA-complexed plasmid pBP100, which contains the luciferase reporter gene under the control of a p53 responsive promoter (21), together with p53 plasmid complexed with TfRscFv-LipA2. TfRscFv-LipA2-p53 co-transfection gave 54-fold more p53-responsive luciferase activity than TfRscFv-LipA2-pVec ($p = 0.0001$) and 11-fold more than TfRscFv-LipA2 only ($p = 0.0002$), demonstrating that exogenous wtp53 protein was expressed functionally by this gene delivery system.

diameter (mean \pm S.D., $n = 3$). The incorporation of TfRscFv into the cationic liposome by the direct anchoring method, TfRscFv-LipA2, resulted in a size of 25.2 ± 0.6 nm in diameter. Therefore, the incorporation of TfRscFv into the liposome did not cause significant change in liposomal size ($p > 0.05$). The size of the TfRscFv-LipA2 corresponds well to the 20–35 nm size of small unilamellar vesicles (SUV) (25). When complexed with plasmid DNA at an optimal DNA/lipid ratio of $1 \mu\text{g}/14$ nmol, the immunolipoplex has a size of 92.9 ± 8.9 nm in diameter and a net positive charge of 52.7 ± 1.8 mV ($n = 3$). More importantly, the immunolipoplex in 5% dextrose is quite stable, with a size of 81.8 ± 2.0 nm 24 hr after preparation when stored at 4°C . This difference in size is not significant when compared with the freshly prepared immunolipoplex ($p > 0.05$).

Systemic p53 Gene Delivery by TfRscFv-Immunolipoplex in a Mouse Xenograft Tumor Model

To examine the ability of TfRscFv-targeting liposome to deliver the p53 gene systemically to tumor tissue *in vivo*, TfRscFv-LipA2-p53 or untargeted LipA-p53 was injected via tail vein into nude mice bearing JSQ-3 subcutaneous xenograft tumors. JSQ-3 is a squamous cell carcinoma of head and neck cell line with mutant p53 (4,20). Two days after injection, as shown by Western analysis in Fig. 8, the tumor from the mouse injected with the TfRscFv-LipA2-p53 immunolipoplex displayed a very intense additional lower band. This is indicative of a high level expression of the exogenous wtp53 protein, represented by this slightly faster mobility p53 band. It should be noted that an intense endogenous p53 band was also observed. This

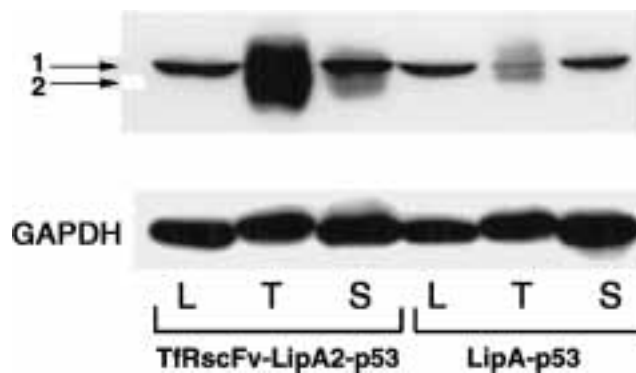


Fig. 8. Exogenous wtp53 expression in JSQ-3 xenograft tumors after i.v. injection of TfRscFv-LipA2-p53. Athymic nude mice carrying JSQ-3 xenograft tumors were i.v. injected with either TfRscFv-LipA2-p53 or the unliganded LipA-p53. 48 hrs later the animals were euthanized, the tumor, liver and skin excised, and protein isolated for Western analysis. L = liver; T = tumor; S = skin; Arrow 1 = Endogenous p53; Arrow 2 = Exogenous wtp53. This experiment was repeated three times with representative results shown here. A high level of expression of the exogenous wtp53 protein is evident in the tumor from the mouse injected with the TfRscFv-LipA2-p53 immunolipoplex, while only limited expression of the exogenous p53 is observed in skin and only endogenous mouse p53 was evident in liver of the same mouse. In contrast, a much lower level of exogenous p53 expression was observed in the tumor from the mouse injected i.v. with the unliganded lipoplex, LipA-p53.

is consistent with earlier reports that exogenous wtp53 induced endogenous p53 gene expression, possibly through the wtp53 transcriptional autoregulation (26,52). However, only limited expression of the exogenous p53 is evident in skin and only endogenous mouse p53 was evident in liver of the same TfRscFv-LipA2-p53 immunolipoplex injected mouse. In contrast, a much lower level of exogenous p53 expression was observed in the tumor from the mouse i.v. injected with the unliganded LipA-p53 lipoplex (Fig. 8). The TfRscFv-immunolipoplex mediated systemic gene delivery was tumor-specific in that the liver showed no obvious exogenous p53 gene expression (Fig. 8), nor did other organs (data not shown). This experiment was repeated three times with representative results shown in Fig. 8. These results demonstrate that the exogenous p53 gene can be systemically delivered to, and efficiently expressed in, the tumor *in vivo* by the TfRscFv-targeting immunolipoplex.

Increased Survival in a Nude Mouse Metastasis Model of Human Breast Cancer After Treatment with TfRscFv-LipA2-p53 in Combination with Docetaxel

To examine the *in vivo* efficacy of the systemic p53 gene therapy with the TfRscFv-LipA2-p53 immunolipoplex, a nude mouse tumor metastasis model was employed. This tumor model uses a human breast cancer cell line, 435/LCC6, derived from mouse ascites of tumor cell line MDA-MB-435 (22). In this well-defined model, the mice begin to develop

symptoms and die within a reproducible time period of about 30 days. Fig. 9(a) shows the animal survival curves of the nude mice after various treatments and Fig. 9(b) shows the result of a repeat experiment focusing on comparison of the two targeting ligands, TfRscFv and Tf. Docetaxel alone offered no benefit in terms of survival (median survival time 32 days), indicating that the tumor is highly chemoresistant. The systemic administration of TfRscFv-LipA2-p53 via the tail vein in combination with docetaxel significantly extended the life-span of the animals beyond that of untreated control or the docetaxel alone group ($p < 0.0001$, Kaplan-

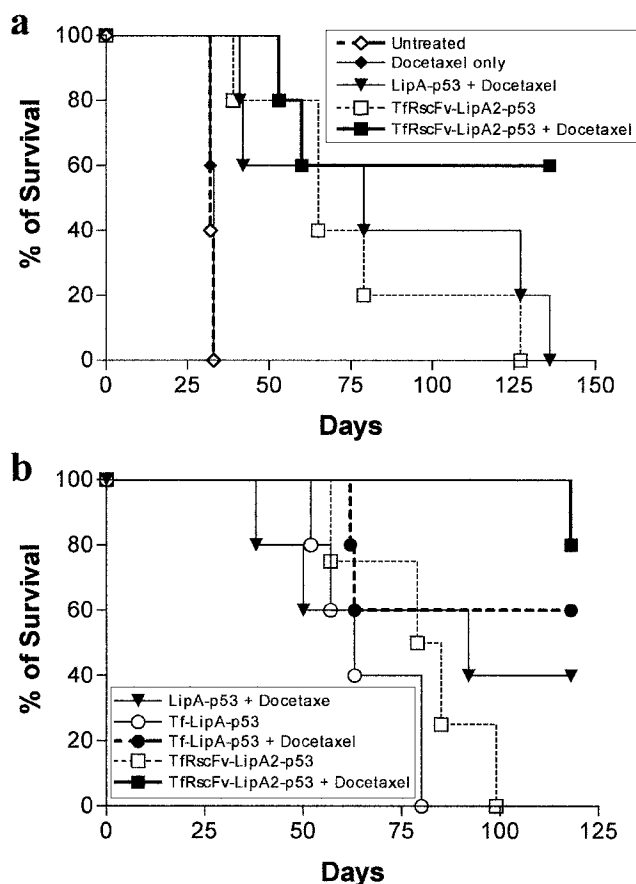


Fig. 9. Increased survival in a nude mouse metastasis model of human breast cancer after treatment with TfRscFv-LipA2-p53 in combination with docetaxel. Beginning 10 days after tumor cell injection, twice weekly doses of TfRscFv-LipA2-p53 (20 μ g p53 DNA/0.3 ml) with or without docetaxel (7.5 mg/Kg) were intravenously administered (5 animals per group). A total of 10 injections were administered. (a) shows the animal survival curves of the nude mice after various treatments and (b) shows the result of a repeat experiment focusing on comparison of the two targeting ligands, TfRscFv and Tf. The systemic administration of TfRscFv-LipA2-p53 via tail vein in combination with docetaxel significantly extended the life-span of the animals. Long term survivors were observed in the animals receiving the combination of TfRscFv-LipA2-p53 and docetaxel, with 60% in the first study (A) and 80% in the second study (B), while no long term survivors were observed in animals receiving either treatment alone.

Meier Survival Analysis and Mantel-Haenszel Logrank Test). Moreover, TfRscFv-LipA2-p53 and docetaxel combination therapy showed significantly improved efficacy over the immunolipoplex without docetaxel ($p < 0.005$). Interestingly, the survival of animals treated with TfRscFv-targeting lipoplex plus docetaxel was not significantly different than that of the Tf-targeting lipoplex plus docetaxel ($p > 0.1$), but both treatments showed much better anti-tumor effect than that of the non-targeting lipoplex plus docetaxel ($p < 0.05$). More importantly, long term survivors were observed in the animals receiving the combination of TfRscFv-targeting p53-lipoplex and docetaxel, with 60% in the first study (Fig. 9a) and 80% in the second study (Fig. 9b), while no long term survivors were observed in animals receiving either treatment alone. The systemic administration of the TfRscFv-immunolipoplex did not seem to be toxic to the animals. The results demonstrate that anti-TfR single-chain antibody can target the lipoplex specifically to tumors *in vivo* as efficiently as transferrin itself. This targeting leads to enhanced efficacy of systemic p53 gene therapy.

Discussion

Receptor-mediated endocytosis represents a highly efficient internalization pathway in eukaryotic cells (9,27,28). The presence of a ligand on a lipoplex facilitates the entry of DNA into cells through binding of the ligand by its receptor on the cell surface followed by internalization of the bound complex. Once internalized, sufficient DNA escapes the endocytic pathway to be expressed in the cell nucleus. In addition to utilizing ligands to recognize corresponding receptors on tumor cells, specific antibodies can also be attached to the liposome surface to direct them to specific tumor surface antigens (29,30). These immunoliposomes can deliver therapeutic drugs to a specific target cell population (31). Park et al. (32) reported that anti-HER-2 monoclonal antibody (MAb) Fab fragments conjugated to liposomes could bind specifically to a 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. Suzuki et al. (33) used anti-transferrin receptor monoclonal antibody conjugated immunoliposomes to deliver doxorubicin more effectively in human leukemia cells *in vitro*. Huwyler et al. (34) used anti-TfR monoclonal antibody immunoliposomes to deliver daunomycin to rat glioma (RT2) cells *in vivo*. These studies demonstrate the utility of immunoliposomes for tumor-targeted drug delivery. Therefore, the combination of cationic liposome-gene transfer and immunoliposome techniques would be a promising system for targeted gene delivery.

In the present study, we employed a biosynthetically lipid-tagged recombinant single chain Fv

antibody fragment based on the anti-TfR monoclonal antibody 5E9, TfRscFv, to target cationic liposomes. The lipid-tagged TfRscFv can be incorporated into cationic liposomes with ease via either the lipid-film solubilization method or the direct anchoring method. The advantage of the direct anchoring method is that the TfRscFv is attached to the preformed liposomes, so that all TfRscFv are present on the surface of the liposomes and thereby available for binding to targets. This has been confirmed by their relatively higher binding activity and transfection efficiencies. The TfRscFv-targeting immunolipoplex showed enhanced tumor cell binding, improved gene delivery and transfection efficiencies both *in vitro* and *in vivo*. The p53 tumor suppressor gene was systemically delivered to the tumor xenografts in nude mice and functionally expressed. The duration of the functional p53 gene expression and the pharmacokinetics still need further studies. Our previous studies with Tf-lipoplexes indicate that p53 transgene expression in human tumor cells and xenografts are transient, peak at 48–72 hrs post transfection injection and diminish in 7–10 days (4,16,28). The TfRscFv-immunolipoplex should have a similar pattern. This transient gene expression gives us the capability to control the transgene expression level and supports the twice a week i.v. administration strategy employed in the *in vivo* efficacy studies.

The TfRscFv has advantages over the Tf molecule itself for a number of reasons. First, the size of the TfRscFv (~30kDa with lipid tag) is much smaller than that of the Tf molecule (~80kDa). Thus, the TfRscFv-liposome-DNA complex may exhibit better penetration into small capillaries characteristic of solid tumors. Second, as a recombinant protein, the smaller scFv also has practical advantages related to its production. Large scale production and strict quality control of the scFv as well as scFv-immunolipoplex will be required for this therapy to be used in human trials. Third, the scFv is a recombinant molecule, not a blood product like human Tf, and therefore presents no regulatory issues related to potential contamination with blood borne pathogens. Additional advantages of using the scFv relate to the fact that Tf interacts with the TfR with high affinity only after the ligand is loaded with iron. Large-scale production of liposomes containing iron-loaded Tf may present practical challenges. Use of scFv enables the TfR on tumor cell to be targeted by a liposomal therapeutic complex that does not contain iron (itself implicated in cancer (35)). Fourth, without the Fc region, the problem of non-antigen-specific binding through Fc receptors is eliminated (36).

The size of a gene therapeutic is a critical parameter for its design, preparation, optimization and quality control. We employed photon correlation spectroscopy (PCS) to measure and monitor the sizes of the TfRscFv-immunoliposomes and immunolipoplexes.

The permeable size of the capillaries in a tumor is usually believed to be about 100 nm in diameter (37,38). Therefore, the TfRscFv-Liposome-DNA immunolipoplexes prepared in this study are theoretically able to pass through the capillary wall to reach the target. Our *in vivo* results showing increased transgene expression in tumor and enhanced efficacy of systemic p53 gene therapy indicate that is very likely to be the case. This size limit is a prerequisite for systemic delivery systems, especially for genes. The PCS technique provides a convenient and robust way to measure the size and surface charge of a complex system. The preliminary analysis of TfRscFv-immunolipoplexes by electron microscopy (EM) with negative staining confirmed the results of PCS, and also provided insights into the structure and molecular arrangement of the complexes (Xu et al., Manuscript in preparation).

Abnormalities in the tumor suppressor gene, p53, have been identified in over 50% of breast cancers and have been linked to resistance to chemotherapy (39,40). A role for p53 in the control of cellular proliferation by the regulation of cell cycle events and induction of programmed cell death (apoptosis) has been established (4). Since it appears that most anti-cancer agents work by inducing apoptosis (41,42), inhibition of, or changes in this pathway may lead to failure of therapeutic regimens (4,28). Loss or malfunction of this p53-mediated apoptotic pathway is one mechanism by which tumors become resistant to chemotherapy (43). Restoration of normal p53 function may therefore sensitize resistant breast cancers to treatment (4,28). We have previously shown that the restoration of p53 function via p53 gene therapy resulted in sensitization of breast tumors to chemotherapeutic agents (4,28,44). This sensitization is due to the restoration of p53-dependent apoptotic pathway in the tumor cells (4). The sensitization of tumors to chemotherapy could result in lowering the required effective dose of chemotherapeutic agents, correspondingly lessening the severe side effects, while improving the efficacy and decreasing the incidences of recurrence. Moreover, this combination approach can be applied to both primary and recurrent tumors, as well as metastases (4).

Docetaxel, a novel member of the taxoid family, has shown greater potency than paclitaxel in the treatment of advanced breast cancer and certain other solid tumors (45,46). Thus, docetaxel is becoming an important part of the current first-line chemotherapy regime for human breast cancer (49–51). Its anti-tumor activity also relates to the induction of cellular apoptosis (45,47,48). Therefore, restoration of functional p53 and the p53 mediated apoptotic pathway could result in increased efficacy of docetaxel. In fact, here we observed that the combination of docetaxel chemotherapy, and systemic administration of the wtp53 gene delivered by the TfRscFv-immunolipoplex did show improved efficacy

with prolonged animal life-span in a human breast cancer metastasis model. Moreover, the systemic administration of the immunolipoplexes did not seem to be toxic to the animals. This study demonstrates the proof-of-principle of using TfRscFv-targeting immunolipoplexes with a defined size for systemic p53 gene therapy to improve the outcome of conventional chemotherapy. Thus, the TfRscFv-targeting of cationic immunolipoplex is a promising strategy to develop a targeted gene delivery system for systemic gene therapy of cancer.

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