Tumor-Targeting Nanocomplex Delivery of Novel Tumor Suppressor RB94 Chemosensitizes Bladder Carcinoma Cells \textit{In vitro} and \textit{In vivo}

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Abstract

\textbf{Purpose:} RB94, a truncated form of RB110, has enhanced tumor suppressor potency and activity against all tumor types tested to date including bladder carcinoma. However, efficient, systemic delivery of the gene encoding RB94 specifically to tumors, is an obstacle to clinical application as an anticancer therapeutic. We have developed a systemically given, nanosized liposome DNA delivery system that specifically targets primary and metastatic disease. The ability of RB94, delivered via this nanocomplex, to sensitize bladder carcinoma to chemotherapy \textit{in vitro} and \textit{in vivo} was assessed.

\textbf{Experimental Design:} The nanocomplex is an RB94 plasmid encapsulated by a cationic liposome, the surface of which is decorated with a tumor-targeting moiety, either transferrin (Tf/Lip/RB94) or an antitransferrin receptor single-chain antibody fragment (TfRScFv/Lip/RB94). The ability of the complex to sensitize human bladder carcinoma HTB-9 cells to chemotherapeutics was assessed \textit{in vitro} by XTT assay. \textit{In vivo} tumor specificity and efficacy were tested in mice carrying HTB-9 tumors by PCR and tumor growth inhibition, respectively.

\textbf{Results:} Transfection with Tf/Lip/RB94 significantly sensitized HTB-9 cells to chemotherapeutic agents \textit{in vitro}. Tumor specificity of the complex was shown in an orthotopic bladder tumor model by immunohistochemistry and PCR. Moreover, in mice bearing subcutaneous HTB-9 tumors, the combination of systemically given Tf/Lip/RB94 or TfRScFv/Lip/RB94 plus gemcitabine resulted in significant ($P < 0.0005$) tumor growth inhibition/regression and induction of apoptosis.

\textbf{Conclusions:} Use of our tumor-targeting nanocomplex to specifically deliver the potent tumor suppressor RB94 efficiently to tumors has potential as a more effective treatment modality for genitourinary and other cancers.

There is increasing emphasis on the development and use of nonviral delivery methods for cancer gene therapy, including cationic liposomes. Features of cationic liposomes that make them versatile and attractive for DNA delivery include lack of immunogenicity or biohazardous activity (reviewed in refs. 1–3). Moreover, cationic liposomes have been proved to be safe and efficient for \textit{in vivo} gene delivery (reviewed in refs. 4, 5). More than 102 clinical trials using cationic liposomes for gene delivery, 78 in the United States alone, have been approved.\textsuperscript{3,4} At least six liposome-based products are on the market (3).

The transfection efficiency of cationic liposomes can be dramatically increased when they bear a ligand recognized by a cell surface receptor, such as transferrin, which facilitates entry of DNA into cells through internalization of the complex via receptor-mediated endocytosis, a highly efficient internalization pathway (6, 7). TfR levels are elevated in various types of cancer, recycle during internalization in rapidly dividing cells (8–10), and correlate with the aggressive or proliferative ability of tumor cells, making TfR a potential target for anticancer drug delivery. This is also the case with bladder cancers (11–13). Moreover, higher recurrence rate, histologic grade, and pathologic grade were also found to be associated with elevated level of TfR in bladder tumors (13–15).

Studies using transferrin-cationic liposome complexes as tumor-targeting systemic delivery vehicles for wt p53 gene therapy of head and neck, prostate, and breast cancers have been successfully undertaken \textit{in vitro} and \textit{in vivo} (16–18).
of this complex resulted in a 70% to 80% in vitro transfection efficiency in JSQ-3 cells (derived from a radiation resistant squamous cell carcinoma of the head and neck) and was at least 2-fold to 3-fold more efficient than transfection with the same liposome lacking transferrin (16, 17).

Using the β-galactosidase reporter gene, we showed that expression of this systemically delivered ligand-liposome complex has a high degree of tumor selectivity. Strong β-gal staining was present in both the primary xenograft tumors and metastases, with little staining evident in normal tissues or organs, including liver, lung, bone marrow, or gut (16–18). Whereas neither p53 gene therapy nor radiation alone was sufficient to eliminate tumors long-term, replacement of the normal p53 gene via this systemically delivered complex rendered head and neck xenograft tumors significantly more sensitive to radiation and chemotherapy in vivo, resulting not only in growth inhibition, but in long-term (18 months) tumor elimination (16). These data show a pronounced synergistic effect of the combination therapy and provide proof of principle for the utility of this ligand-facilitated cationic liposome delivery system in cancer gene therapy. Combining gene therapy with more conventional cancer treatment may represent a significant improvement over traditional therapies alone.

In addition to the use of ligands, specific antibodies can also be attached to the liposome surface, enabling them to deliver therapeutic drugs to a specific target cell population (3, 19–21). Immunoliposomes are being used for a variety of therapeutic uses, including delivery of antisense molecules as anti-HIV agents (22), chemotherapeutics (23), and plasmid DNA (24). Thus, the combination of cationic liposome-gene transfer and immunoliposome techniques seems to be a promising system for targeted gene therapy.

Whereas the majority of antibody-targeted molecules in the clinic and in clinical trials contain intact monoclonal antibody (mAb), including chimeric and humanized forms (3), progress in biotechnology has permitted the construction of specific recognition domains derived from mAb that have better pharmacokinetic profiles, while simultaneously reducing the immunogenicity associated with whole antibodies. These include Fab’ and scFv fragments (25). The recombination of the variable regions of heavy and light chains and their integration into a single polypeptide provides the possibility of using single-chain antibody derivatives (designated scFv) for targeting purposes. The binding site of an scFv can replicate both the affinity and specificity of its parent antibody (23).

A scFv based on the anti-TfR mAb 5E9 (26, 27) contains the complete antibody binding site for the epitope of the TfR recognized by this mAb as a single polypeptide chain of ~28 kDa (TfRsFv). This TfRsFv is formed by connecting the component VH and VL variable domains from the heavy and light chains, respectively, with an appropriately designed linker peptide. The linker bridges the COOH terminus of the first variable region and NH2 terminus of the second, ordered as either VH-linker-VL or VL-linker-VH. We have modified our transferrin ligand targeting lipoplex, replacing transferrin with this TfRsFv fragment. Previous studies using the TfRsFv targeting lipoplex showed that this nanosized immunoliposome was able to deliver the wtp53 gene specifically and efficiently to tumor cells in vitro and in vivo (28, 29), resulting in increased survival (28). We have also used this complex to deliver small interfering RNA (30, 31) and imaging agents (32).

The tumor suppressor protein RB94 is produced by translation of the wild-type RB gene from the second in-frame AUG codon and lacks the NH2 terminal 112 amino acids present in RB110 (33). RB94 has markedly increased tumor suppressor potency compared with RB110 and is active against all tumor types examined to date, despite their specific genetic defects, including both RB(+) and RB(-) tumors (33–35). Moreover, no resistance to RB94 has been found in any cancer cells or cancer cell types examined to date, based on the fact that a cancer cell has never been able to be isolated >3 weeks after transfection with and expression of RB94. In addition, no
cytotoxicity to normal human cells has been associated with RB94 (33–35). Therefore, its therapeutic index should be high.

In this paper, we have focused on the delivery of the RB94 gene by our nanoliposome complexes (both transferrin and TfRscFv targeted) and have examined the ability of these complexes to sensitize human bladder carcinoma cells to conventional chemotherapeutic agents in vitro and in vivo. We have undertaken our studies in bladder cancer because a phase I clinical trial is planned using our targeted systemic delivery approach with the RB94 gene primarily in patients with metastatic RB-negative bladder cancer.

Materials and Methods

Cell culture. RB-negative human bladder carcinoma cell line HTB-9 and normal human umbilical vein endothelial cell line CRL1730 (HUV-EC-L) were obtained from American Type Culture Collection. HTB-9 was cultured in RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 50 μg/mL each of penicillin, streptomycin, and neomycin, plus 10% heat-inactivated fetal bovine serum. CRL1730 was cultured in HAM’s F12K medium (Invitrogen) with 0.1 mg/mL Heparin, 0.03 to 0.05 mg/mL endothelial cell growth supplement, and cultured in HAM’s F12K medium (Invitrogen) with 0.1 mg/mL Heparin, 0.03 to 0.05 mg/mL endothelial cell growth supplement, plus L-glutamine, penicillin, streptomycin, and neomycin and fetal bovine serum as above.

pSCMV-RB94 clones. A 3.1-kb BamHI-HindIII restriction digest fragment of the original RB94 clone (PEW13; ref. 33) was cloned into a high-expression vector where the RB94 gene is under the control of a modified cytomegalovirus promoter to yield pSCMV-RB94 (Fig. 1A) and propagated in bacterial host TOP 10 F’ (Invitrogen). Plasmid DNA was prepared using the Qiagen endofree kit with a resultant 260:280 ratio of >1.9, >80% supercoiled molecules and endotoxin levels of 3 to 4 Eu/mg DNA.

Complex preparations and in vitro transfections. The transferrin-liposome-DNA complexes were prepared, and transfection was done as previously described (16). The complexes using TRscFv as the targeting moiety were prepared, and transfection was done as previously described (36). Nontumor-specific CD2 scFv was a kind gift from Dr. John deKruif (Crucell Holland BV). The sizes of the complexes (number values) were measured by dynamic light scattering using a Malvern Zetasizer 3000HS.

In vitro Western blot and immunochemical analysis. In vitro Western blot analysis, the cells were seeded in six-well plates at 2 × 105 cells per well. After 24 h, they were transfected as previously described (16, 36). The detached cells were collected by centrifugation of the culture medium at 200 × g and 4°C for 7 min. The adherent cells were mechanically detached by scraping, suspended in PBS, and pelleted as above. The pellets were combined, washed once with PBS, and incubated at 4°C in 50 to 100 μL of cold lysis [radioimmunoprecipitation assay (RIPA)] buffer for 20 min. The resulting lysate was passed several times through a 21-gauge needle, and protein concentration was measured (Pierce Protein concentration assay reagent). The proteins were denatured, separated by discontinuous 10% polyacrylamide/SDS gel electrophoresis, and the protein concentration was measured (Pierce Micro bicinchoninic acid protein assay reagent). The proteins were denatured, separated by discontinuous 10% polyacrylamide/SDS gel electrophoresis, and transferred to nitrocellulose membrane. RB94 protein was identified using 1:10,000 dilution of RB mAb (QED Bioscience, Inc.), followed by a peroxidase-conjugated antitumor IgG antibody (Santa Cruz), enhanced chemiluminescence (Amersham) detection, and exposure to film.

For the immunohistochemical studies, the HTB9 cells were cultured at 2 × 105 cells per well in six-well plates containing a coverslip. At 24 h posttransfection, cells on the coverslip were fixed and stained for RB94, as previously described (34).

Western blot of in vivo samples. For Western blot analysis of in vivo samples, the mice were i.v. injected with the Tf/Lip/RB94 or the TRscFv/Lip/RB94 complexes, and the appropriate controls at the amount of plasmid DNA indicated in Results for each experiment. At the indicated time after the last injection, tumor, liver, and lungs were excised from the mice, flash frozen in liquid N2, pulverized using a Bessman tissue pulverizer (Fisher Scientific), and homogenized in RIPA buffer. The protein was isolated, and RB94 expression was analyzed, as described above for cell culture. In some cases, paraffin sections from the same tissues were examined for RB94-positive cells, as previously described (34).

2.3-Bis[2-methoxy-4-nitro-5-sulophenyl]-2H-tetrazolium-5-carboxamide inner salt (XTT) assay. For the XTT in vitro drug sensitivity assay, either HTB-9 bladder cancer or normal CRL1730 endothelial cells were seeded in 96-well plates at 5 × 103 cells per well. After 24 h, the cells were transfected with the specific complex or control (at 0.05 μg RB94 plasmid DNA), as previously described (16, 36). Twenty-four hours posttransfection, the medium was replaced with complete medium without drug or with varying concentrations of cisplatin (CDDP, 0.1-10 μmol/L) or gemcitabine (0.01-100 μmol/L), followed by incubation at 37°C for an additional 72 h, at which time untreated cells reached ~100% confluency. Cell viability was determined by an XTT assay following the manufacturer’s protocol (Roche Applied Sciences). The IC50 value, the drug concentration resulting in 50% cell kill, was interpolated from the graph of the log of drug concentration versus the fraction of surviving cells.

PCR for detection of exogenous RB94 DNA in mouse tissues. DNA was isolated from mouse tissues using the high pure PCR template kit (Roche Applied Science) as per manufacturer’s instructions. To specifically amplify exogenous RB94 DNA, the forward primer (5’-ATG GTG ATG CGG TTT TG-3’) is a sequence in the cytomegalovirus promoter in the vector backbone, whereas the reverse primer (5’-ACA TGG GAG GTG AGA GTA TA-3’) is a sequence in the RB94 gene insert, yielding an ~850 bp fragment. DNA PCR was done using Bioline DNA polymerase (Bioline Co.) and a 25-cycle amplification using the following conditions: 94°C 1 min for one cycle; 94°C 30 s, 53°C 1 min, 72°C 1 min for 25 cycles; 72°C 10 min for extension. The PCR products were run on an Agarose gel, stained with ethidium bromide, and photographed.

Detection of cleaved caspase-3 17-kDa subunit as an indicator of apoptosis. Blood was obtained from mice in the presence of sodium heparin (0.015 USP units/μL blood collected) and centrifuged twice at 0.1 × g at 4°C for 10 min to separate the plasma from the blood cells. The 17-kDa fragment of cleaved caspase-3 was isolated from the plasma via chromatography. Equal volumes were analyzed by Western blot analysis using the cleaved caspase-3 (Asp175) antibody (Cell Signaling) and the enhanced chemiluminescence Western blot kit (Amer sham). This polyclonal antibody is specific for the large fragment (17 kDa) of activated caspase-3.

Induction of bladder tumors in mice. Orthotopic HTB-9 bladder tumors were induced in female nude mice, as previously described (37, 38)

In vivo chemosensitization of HTB-9 xenograft tumors. Female athymic nude (nCR nu/nu) mice (4-6 weeks old) were s.c. inoculated on the lower back above the tail with 5 × 106 HTB-9 cells suspended in Matrigel collagen basement membrane (BD Biosciences). Approximately 10 to 13 d post-injection, the tumors averaged 50 to 100 mm3.

Mice bearing subcutaneous xenograft tumors of ~100 mm3 were divided into groups (4-10 mice per group). The targeted liposome complex (either Tf/Lip/DNA or TRscFv/Lip/DNA) carrying the pSCMV-RB94 plasmid DNA was i.v. injected thrice a week via the tail vein. pSCMV-RB94 plasmid DNA at 10 to 20 μg was given per injection per mouse. Gemcitabine administration was initiated 6 to 20 h after the first i.v. injection and was given i.v. twice weekly at a dose of 60 mg/kg/mouse per injection. The in vivo response was evaluated based upon the changes in tumor volume over time. Tumor sizes were measured weekly by a third party in a blinded manner at the Georgetown University Medical Center animal facility. Tumor volume (L × W × H) in mm3 is
compositions A (DOTAP/DOPE) and D (DOTAP/cholesterol) to the human bladder carcinoma cell line HTB-9. We found that liposome D would yield the highest transfection efficiency in the human series of different liposomes to determine which compositions system(16,17) and the luciferase gene as a reporter, we tested a few clones for further study. The orientation and sequence authenticity of the inserts and enzyme digestion and partial DNA sequence analysis to verify several of the resulting pSCMV-RB94 clones by restriction into the pSCMV high-expression vector (Fig. 1A), we analyzed Bam cell membranes. The positively charged, as is desired for optimal interactions with the complexes. Although, as expected, addition of the ligand reduces the positivity of the complex measured ~166 nm with a ζ potential of 32. The Tf/Lip/RB94 complex was ~108 nm with a ζ potential of 9.4, whereas TfRscFv/Lip/RB94 was ~207 nm with a ζ potential of 6.6, demonstrating that these complexes are in the nanosize range. The polydispersity or width of the complex size distribution is within the recommended range for all of the complexes. As expected, addition of the ligand reduces the positivity of the ζ potential, the complexes remain positively charged, as is desired for optimal interactions with cell membranes.

**Results**

**Size determination.** The size of the ligand-targeted liposome RB94 complex was analyzed by dynamic light scattering on a Malvern Zetasizer 3000HS. The unliganded liposome/RB94 complex measured ~166 nm with a ζ potential of 32. The Tf/Lip/RB94 complex was ~108 nm with a ζ potential of 9.4, whereas TfRscFv/Lip/RB94 was ~207 nm with a ζ potential of 6.6, demonstrating that these complexes are in the nanosize range. The polydispersity or width of the complex size distribution is within the recommended range for all of the complexes. Although, as expected, addition of the ligand reduces the positivity of the ζ potential, the complexes remain positively charged, as is desired for optimal interactions with cell membranes.

**RB94 expression in HTB-9 cells.** After subcloning a 3.1-kb BamHI-HindIII fragment of the original RB94 clone (PEW13) into the pSCMV high-expression vector (Fig. 1A), we analyzed several of the resulting pSCMV-RB94 clones by restriction enzyme digestion and partial DNA sequence analysis to verify the orientation and sequence authenticity of the inserts and chose several clones for further study.

Using the established transferrin-targeted liposomal delivery system (16, 17) and the luciferase gene as a reporter, we tested a series of different liposomes to determine which compositions would yield the highest transfection efficiency in the human bladder carcinoma cell line HTB-9. We found that liposome compositions A (DOTAP/DOPE) and D (DOTAP/cholesterol) were the most efficient (data not shown). HTB-9 cells were subsequently transfected with the various RB94 plasmids complexed with Tf/LipD. Twenty-four hours posttransfection, RB94 expression was assessed by Western analysis using an anti-RB2 mAb (QED Bioscience, Inc.). Purified RB94 protein is included on the gel as a positive control and for verification of RB94 positioning. In all cases, a significantly higher level of RB94 protein expression was observed with the pSCMV-RB94 clones (X452-X456) compared with that of the original construct (X457; Fig. 1B). An RB94 band is present in X457 upon longer exposure (data not shown). Glyceraldehyde-3-phosphate dehydrogenase levels show equal protein loading. Moreover, RB94 expression was not observed in normal endothelial cell line CRL1730 after transfection with Tf/LipD/RB94, indicating the tumor cell specificity of this complex (not shown).

We chose pSCMV-RB94 clone X455 for use in the remainder of the studies. High-RB94 expression level achieved using this clone when compared with the original pCMV-RB94 construct is shown at the single-cell level by immunohistochemical analysis in Fig. 1C. Clear nuclear staining is evident in the HTB-9 cell transfection with Tf/Lip complex carrying the original pCMV-RB94 construct (Fig. 1C, top). However, after transfection under identical conditions using Tf/Lip encapsulating the construct with the high-expression promoter, strong staining is detected not only in the nucleus but also in cytoplasm (Fig. 1C, bottom). The broad arrows indicate the RB94-expressing cells with strong nuclear and cytoplasmic staining.

**RB94-mediated sensitization of bladder tumor cells to chemotherapy.** We assessed the ability of RB94 delivered via the transferrin-targeted liposome complex to sensitise RB94-negative HTB-9 cells to conventional chemotherapeutic agents gemcitabine and CDDP by means of the XTT cytotoxicity assay. IC50 values, the drug concentration yielding 50% growth inhibition, were calculated and represent the degree of
chemosensitization. In these experiments LipD was used. Cells that were transfected only with the chemotherapeutic agent and were not transfected with the Tf/Lip complex are designated as UT. A dramatic increase in sensitization of HTB-9 cells to both drugs with the Tf/LipD/RB94 complex was observed (Fig. 2A and B).

The HTB-9 cells transfected with the Tf/Lip/RB94 complex at 0.05 μg RB94 plasmid DNA have an IC50 for gemcitabine of 0.009 μmol/L, compared with 2.8 μmol/L for cells transfected with the complex carrying empty vector (Tf/Lip/Vector), a 31.1-fold increase in sensitivity of the bladder cancer cells to the chemotherapeutic agent (Fig. 2A). This increased sensitivity was not present in cells treated with the complex minus DNA (Tf/Lip only). These results show that the observed sensitization is RB94 related and is not due to nonspecific cytotoxicity from the delivery system.

A similar high degree of chemosensitization of HTB-9 cells to CDDP occurred after treatment with Tf/LipD/RB94 at a DNA dose of 0.1 μg. In this instance, even without CDDP, the effect of Tf/LipD/RB94 on the tumor cells is so potent that only 40% of the cells survive; thus an IC50 value could not be calculated (Fig. 2B). However, if we assume that the IC50 for Tf/LipD/RB94 is <0.01 (the lowest dose of CDDP used), then transfection of HTB-9 cells with this complex results in a >55-fold increase in cell killing by CDDP compared with that observed with cells treated with the complex carrying empty vector.

To show the tumor cell specificity of Tf/LipD/RB94 induced chemosensitization, a similar assay using normal human endothelial cell line CRL1730 and gemcitabine was done. In this experiment, the DNA dose was 0.1 μg, twice that used above with HTB-9 cells. In addition, the range of gemcitabine concentrations (0-10,000 μmol/L) was 1,000-fold higher with these normal cells than that used with the tumor cell line (0-10 μmol/L). No significant sensitization over that seen with gemcitabine alone (UT) was observed after transfection with Tf/LipD/RB94 or the control complexes (Fig. 2C). Thus, use of the Tf/Lip complex to deliver RB94 did not result in nonspecific cytotoxicity in normal cells. These findings in normal cells also indicate the safety and potential for reduced side effects with our tumor cell-specific delivery system.

**Tumor-specific targeting and in vivo RB94 expression.** To begin to assess the potential of this ligand-liposome-RB94 complex as a clinical agent, we tested the ability of the systemically given complex to deliver RB94 specifically and efficiently to tumor tissue in an animal model. Tf/Lip/RB94 (with liposome formulation D) was i.v. given via the tail vein to nude mice bearing s.c. HTB-9 xenograft tumors. The animals received two i.v. injections within 24 hours at 60 μg RB94 plasmid DNA/injection. As a control, we also injected mice with LipD-RB94 minus the targeting transferrin ligand (UL). Approximately 16 hours after the second injection, we sacrificed the mice, harvested tumor, liver, and lung, and isolated protein for Western analysis. Protein (80 μg) was loaded per lane. There was strong expression of RB94 in the tumor from the animal that received Tf/LipD/RB94 complex, whereas there was no clear signal evident in the tumor from the mouse injected with the unliganded complex (Fig. 3A). More importantly, there was no RB94 expression in the liver and only minimal expression in the lungs of the animal treated with Tf/LipD/RB94. These findings show the tumor specificity of this complex. Actin expression, as an internal control for protein loading, was comparable in all samples. It has previously been shown using Ponceau Red staining that although equal amounts of protein are present on the membrane, actin levels are lower in the liver compared with other organs (34).

The properties of the antitransferrin receptor single-chain antibody fragment (TfRscFv) make it attractive as a potential targeting moiety for tumor-specific delivery. We have shown that the tumor specificity and transfection efficiency of the nanocomplex with TfRscFv are at least as good, if not better, than those observed when transferrin is used (28, 29). Therefore, we assessed the ability of the i.v. given liposome-RB94 complex, targeted by the TfRscFv molecule (TfRscFv/Lip/RB94), to deliver RB94 specifically to the tumor. In this study, we compared the targeting ability and transfection efficiency of the complex using either liposome formulation A or formulation D. The complex without TfRscFv (unliganded) was also i.v. injected as a control. The mice received three i.v. tail vein injections over 24 hours at 40 μg RB94 plasmid DNA/injection. Forty-eight hours after the last injection, tumor and liver were excised and protein was isolated for Western blot analysis. As seen with the transferrin-targeted complex, there was a high level of RB94 expression evident in the tumor and not in the liver of the animals receiving the i.v. TfRscFv/Lip/RB94 complex (Fig. 3B). A high incidence of
tumor-specific RB94 transfer and expression was also seen by immunohistochemical analysis of tissue from the same experiment. Numerous RB94 staining cells were observed in the tumor from the mouse that received the targeted LipA/RB94 complex in Fig. 3B. Liver from the same mouse whose tumor is shown in A. C, tumor from a mouse that received the untargeted LipA/RB94 complex in Fig. 3B. D, liver from the same mouse whose tumor is shown in C. E, PCR analysis of tumor bearing bladder and normal tissues from two individual mice bearing RB94-negative HTB-9 tumors which had been injected thrice over 24 h with the TFRscFv/LipA/RB94 complex at 24 μg DNA per mouse per injection. Lanes 1 to 4 are from mouse 1, whereas lane 6 is from a separate tumor-bearing mouse. Lane 1, liver; lane 2, large intestine; lane 3, kidney; lanes 4 and 6, bladder with tumor; lane 5, water blank; M, size markers (500 and 1000 bp hyperladder V and IV, respectively; Bioline Co.).

In a separate experiment, tumor specificity was also shown by DNA PCR using samples from tumor-containing bladders, as well as from normal tissues, of mice i.v. injected thrice over 24 hours with the TFRscFv/LipA/RB94 complex at 24 μg DNA per mouse per injection. DNA was isolated from the tissues, and PCR was done as described in Materials and Methods. As shown in Fig. 4E, a strong RB94-specific signal was evident in the bladder tumors from two individual mice (lanes 4 and 6). In contrast, only a very weak RB94 DNA band is detectable in the normal liver (lane 1), large intestine (lane 2), or kidney (lane 3). Thus, complexes with both LipA and LipD and with both Tf and TFRscFv can lead to tumor-specific transfection and expression of RB94 after i.v. administration in tumors located both subcutaneously or within the bladder.

Cleaved caspase-3 in plasma as a measure of RB94-induced apoptosis. We have recently shown in vitro that TUNEL positivity is an early marker of RB94-induced cancer cell death, whereas caspase-3 cleavage is a later event.5 Thus, delivery and expression of RB94 to tumor cells in vivo should result in induction of apoptosis. Detection of the 17-kDa fragment of cleaved caspase-3 in the plasma of tumor-bearing mice treated with RB94 would be indicative of ongoing apoptosis. Athymic nude mice bearing
subcutaneous HTB-9 xenograft tumors were i.v. injected thrice within 24 hours with the liganded liposome D complex carrying the RB94 gene (40 μg RB94 plasmid DNA per mouse per injection), with either Tf or TfRscFv as the targeting molecule. As controls, other mice were i.v. injected with the complex without targeting ligand (unliganded), or with a nontumor-specific single-chain molecule (CD2) as the ligand. Sixteen hours after the last injection, the animals were sacrificed, blood was taken, and plasma was isolated, as described in Materials and Methods. Western analysis of the expression of the 17-kDa fragment of cleaved caspase-3 is shown in Fig. 5. The 17-kDa cleaved caspase-3 protein was evident in the plasma from all three of the mice that received the TfRscFv/Lip/RB94 complex.

In vivo response of HTB-9 xenograft tumors to the combination of ligand-liposome-RB94 and gemcitabine. The response of subcutaneous HTB-9 xenograft tumors to the combination of targeted-liposome-RB94 and gemcitabine was assessed in a nude mouse tumor model. This study also compared the efficacy of the complex made with LipA and LipD, as well as the effect of the combination treatment with that of single-agent therapy (gemcitabine or Tf/LipA/RB94 alone). Mice (four to eight per group) each bearing xenograft tumors of ~100 mm³ were treated with the combination of i.v. given Tf/LipA/RB94 (eight mice) or Tf/LipD/RB94 plus gemcitabine (eight mice). Additional groups of animals received no treatment (eight mice) or treatment with gemcitabine only (four mice) or Tf/LipA/RB94 only (four mice). Each group received a total of 20 i.v. injections of the complex (10 μg DNA per injection) and 14 i.p. injections of gemcitabine (60 mg/kg per injection). Differences in tumor volume (mean ± SE) in the various treatment groups over time were used to assess the in vivo response. A low DNA dose of 10 μg was used here to be able to assess the presence of synergy between the immunoliposome and gemcitabine. Treatment ended on day 49. At this low-DNA dose, <1 month after the end of treatment (day 78), the animals in both single-agent groups were sacrificed due to excessive tumor burden (thus, statistical analysis could not be done; Fig. 6A). In contrast, 3 months posttreatment (day 130), the tumors in the groups treated with the combination of either Tf/LipA/RB94 or Tf/LipD/RB94 plus gemcitabine were showing regression. These results not only show an improved tumor response but also that the combination of the two therapeutic agents is more effective than either agent alone.

Fig. 5. Detection of cleaved caspase-3 in vivo after treatment with TfRscFv/LipD/RB94. Western blot analysis of the level of the 17-kDa cleaved caspase-3 protein, a marker for apoptosis, in plasma from HTB-9 tumor-bearing mice 16 h after treatment with complete tumor-targeting nanocomplex (Tf/Lip/RB94 and TfRscFv/Lip/RB94), complex minus the targeting moiety (unliganded), or the complex bearing a nontumor-specific ligand (CD2). The mice received three i.v. tail vein injections over 24 h at 40 μg DNA per mouse per injection. Protein was obtained, and Western blot analysis was done as described in Materials and Methods. The second and third lanes represent plasma from two individual mice that received the unliganded complex, and the fifth and sixth lanes represent plasma from two individual mice that received the TfRscFv/Lip/RB94 complex.

Fig. 6. Tumor response of the HTB-9 xenograft tumor model to ligand-targeted liposome delivery of RB94. HTB-9 tumors were induced in female athymic nude mice as described in Materials and Methods. A. Mice bearing tumors of ~100 mm³ were i.v. tail vein injected with Tf/LipA or Tf/LipD complexed RB94 plasmid DNA (10 μg DNA per mouse per injection) alone or in combination with gemcitabine (Gem). The last injection was on day 49. Points, mean of four to eight tumors per group; bars, SE. B. Mice bearing tumors of 50 to 100 mm³ were i.v. tail vein injected with scL complexed RB94 plasmid DNA or empty vector (20 μg DNA per mouse per injection) in combination with gemcitabine. Unliganded Lip/RB94 complex was also given in combination with the chemotherapeutic agent. The last injection was on day 28. Points, mean of 4 to 10 tumors per group; bars, SE.
response to combination therapy but also show that both LipA and LipD work equally well in vivo.

A second in vivo study was done, in which TIRscFv was used as the targeting moiety (Fig. 6B). Liposome formulation D was used in this experiment. In addition to the group of mice that received the combination of TIRscFv/LipD/RB94 plus gemcitabine (10 mice), groups of mice remained untreated (four mice) or were treated with unliganded LipD/RB94 plus gemcitabine (four mice) or with the combination of the TIRscFv/LipD complex carrying empty vector plus gemcitabine (five mice). This latter group was included to verify that the tumor response observed was due to the presence of RB94 and not to a liposomal effect. The mice received 13 i.v. injections of the complex (20 μg of RB94 plasmid DNA per mouse per injection) and nine i.p. administrations of gemcitabine (60 mg/kg per injection). Treatment ended on day 28. As above, differences in tumor volume (mean ± SE) in the various treatment groups over time were used to assess the in vivo response. As was observed with TIRscFv/Lip/RB94, the tumors in the group receiving the combination of TIRscFv/LipD/RB94 plus gemcitabine showed no increase in tumor size and even evidenced tumor regression on day 150, ~4 months after the end of treatment. In contrast, the untreated group was humanely euthanized by day 50 due to tumor burden. The group that received the unliganded complex plus gemcitabine or the complex carrying empty vector plus gemcitabine experienced a significant increase in tumor size. The differences in tumor volumes between the TIRscFv/LipD/RB94 plus gemcitabine and both control groups, Lip/RB94 plus gemcitabine and TIRscFv/LipD/vector plus gemcitabine, were highly statistically significant by Student’s t test at $P < 0.0005$ and $P < 0.0076$, respectively. The decrease in tumor size shown at 90 days for the two control groups was the result of removal of fluid that had accumulated in the tumors.

Discussion

Although many mAb-based agents are in use as anticancer therapeutics, they possess some drawbacks and limitations. Adverse toxic reactions can occur due to interactions between Fc receptors on normal tissues and mAb. Moreover, with regards to use as therapeutic agents in the treatment of cancer, the large size of the intact mAb (~155 kDa) limits their ability to diffuse from the capillaries into the solid tumor (39) and thus their potential efficacy. Thus, new approaches using antibody fragments, such as scFv molecules, are being developed.

For a number of reasons, the antitransferrin receptor single-chain antibody fragment (TIRscFv) has advantages in human use over the full mAb or even the transferrin molecule itself in targeting liposomes to cancer cells with elevated TfR levels. (a) The size of the scFv (~28 kDa) is much smaller than that of the transferrin molecule (~80 kDa) or the parental mAb (~155 kDa). The scFv-liposome-DNA complex may thus exhibit better penetration into small capillaries characteristic of solid tumors. The nanosize of this complex lends support to this theory. (b) The scFv also has practical advantages related to its production as a recombinant protein because large-scale production of the TIRscFv will be required for the phase I RB94 trial. (c) The scFv is a recombinant molecule (not a blood product like transferrin) and therefore presents no issues related to potential contamination with blood borne pathogens. (d) Without the Fc region of the mAb, the problem of nonantigen-specific binding through Fc receptors is eliminated.

We have previously shown that this single-chain antibody fragment can target an i.v. given cationic liposome/payload complex preferentially to tumors (28–32) resulting, e.g., in increased survival in various mouse models of human cancer (28, 31, 36). Moreover, our TIRscFv/LipD/DNA nanocomplex carrying the wtp53 gene has been approved to enter phase I clinical trials for gene therapy of cancer.

The efficacy of our approach can be attributed in part to the nanosize of the complex carrying payloads as diverse as plasmid DNA (28, 29, 36, 40), small interfering RNA (30, 31), and even imaging agents (32). The encapsulation of the payload within the ligand-decorated liposome helps maintain this small size. As it has been shown that the payload is indeed encapsulated within the targeting molecule decorated liposome for other molecules, such as imaging agents (32), small interfering RNA (31), and other plasmid DNAs (40), it is likely that this is also the case with the RB94 constructs.

Here, we further show the potential of this platform nanotechnology by systemically delivering the RB94 tumor suppressor gene preferentially and efficiently to tumors in a mouse model of human bladder cancer. The specificity was shown not only by Western analysis but also by immunohistochemistry and DNA PCR. The very faint bands seen in the normal tissues with PCR are likely a result of the complex still in circulation in the blood stream and/or the presence of macrophages in the tissues, particularly liver and kidney, as we have previously shown (16, 18). Nonsterically stabilized liposomes are taken up by the macrophages of the reticuloendothelial system (41).

It is now well accepted that single-agent gene therapy is far less effective than when used in combination with conventional therapeutic modalities. Therefore, the emphasis of the in vivo studies presented here was on the testing of the combination of the ligand-targeted liposome RB94 complexes with chemotherapeutic agent gemcitabine rather than on single-agent treatment. These results not only showed the ability of the combination of nanoliposome complex-delivered RB94 and a conventional chemotherapeutic agent to inhibit bladder tumor growth but also the importance of the targeting molecule and that this antitumor effect is RB94 specific. Thus, they also indicate the potential of this approach as an anticancer treatment in the clinic.

We believe that RB94 may be an ideal gene to consider for this type of systemically given gene therapy because it has been effective in all tumor types studied to date. No human cancer cells have yet been identified that have survived transfection with an RB94 construct (33–35). Therefore, no resistance to this therapeutic has yet been observed. In addition, RB94 has not been found to be cytotoxic to various normal cell types, including fibroblasts, endothelial cells as shown here, or urothelial cells. Furthermore, as shown with the CRL1730 cells, the use of the tumor-targeting delivery system did not affect this lack of cytotoxicity in normal cells.

Our initial planned phase I study will involve the systemic treatment of RB-negative tumors, such as bladder and prostate tumors with TIRscFv/Lip/RB94 as a single agent to show safety and proof of principle for tumor-specific targeting. It is our plan, however, to add gemcitabine in future studies because we believe the combination will provide an enhanced therapeutic modality for the treatment of genitourinary and other cancers.
Cancer Therapy: Preclinical

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Tumor-Targeting Nanocomplex Delivery of Novel Tumor Suppressor RB94 Chemosensitizes Bladder Carcinoma Cells

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