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## **Transferrin- Liposome-Mediated Systemic p53 Gene Therapy in Combination with Radiation Results in Regression of Human Head and Neck Cancer Xenografts**

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**Published on:** 10 Dec 1999 - Human Gene Therapy (Mary Ann Liebert, Inc.)

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# Transferrin–Liposome-Mediated Systemic p53 Gene Therapy in Combination with Radiation Results in Regression of Human Head and Neck Cancer Xenografts

LIANG XU, KATHLEEN F. PIROLLO, WEN-HUA TANG, ANTONINA RAIT, and ESTHER H. CHANG

## ABSTRACT

The use of cationic liposomes as nonviral vehicles for the delivery of therapeutic molecules is becoming increasingly prevalent in the field of gene therapy. We have previously demonstrated that the use of the transferrin ligand (Tf) to target a cationic liposome delivery system resulted in a significant increase in the transfection efficiency of the complex [Xu, L., Pirollo, K.F., and Chang, E.H. (1997). *Hum. Gene Ther.* 8, 467–475]. Delivery of wild-type (wt) p53 to a radiation-resistant squamous cell carcinoma of the head and neck (SCCHN) cell line via this ligand-targeted, liposome complex was also able to revert the radiation resistant phenotype of these cells *in vitro*. Here we optimized the Tf/liposome/DNA ratio of the complex (LipT) for maximum tumor cell targeting, even in the presence of serum. The efficient reestablishment of wtp53 function in these SCCHN tumor cells *in vitro*, via the LipT complex, restored the apoptotic pathway, resulting in a significant increase in radiation-induced apoptosis that was directly proportional to the level of exogenous wtp53 in the tumor cells. More significantly, intravenous administration of LipT–p53 markedly sensitized established SCCHN nude mouse xenograft tumors to radiotherapy. The combination of systemic LipT–p53 gene therapy and radiation resulted in complete tumor regression and inhibition of their recurrence even 6 months after the end of all treatment. These results indicate that this tumor-specific, ligand–liposome delivery system for p53 gene therapy, when used in concert with conventional radiotherapy, can provide a new and more effective means of cancer treatment.

## OVERVIEW SUMMARY

A transferrin–cationic liposome system was optimized for efficient and tumor-specific gene delivery both *in vitro* and *in vivo*. The replacement of wild-type p53 by lipT–p53 restored the wtp53 function and apoptotic pathway. The systemic delivery of the LipT–p53 to established nude mouse xenograft model of human squamous cell carcinoma of the head and neck markedly sensitized the tumor to radiation therapy. The combination of systemic p53 gene therapy and conventional radiotherapy resulted in tumor regression and inhibition of their recurrence long-term. This strategy may provide a more effective treatment modality for human head and neck cancer.

## INTRODUCTION

WILD-TYPE (wt) p53 plays a crucial role in apoptotic pathways leading to cell death (reviewed in Bates and Vousden, 1999). The lack of functional p53 is believed to contribute to the failure of many cancers to respond to conventional therapies (reviewed by Ruley, 1996; Roth, 1998). For example, the presence of mutant (mt) p53 has been shown to correlate with increased radiation resistance in some human tumors and cell lines, possibly because of the loss of the p53-dependent apoptotic pathway (Lee and Bernstein, 1993; O'Connor *et al.*, 1993; McIlwrath *et al.*, 1994; Bristow *et al.*, 1996; Nielson and Maneval, 1998; Meng and El-Deiry, 1999). Therefore, reestablishing wt p53 function in tumor cells may restore the p53-me-

diated apoptotic pathway, resulting in more efficient treatment modalities.

Progress has been made toward developing nonviral, pharmaceutical formulations of genes for *in vivo* human therapy, particularly cationic liposome-mediated gene transfer systems (Felgner *et al.*, 1995; Templeton *et al.*, 1997; Huang and Vironchatapan, 1999). However, liposomal systems in general suffer from low transfection efficiencies when compared with those observed with viral vector delivery systems. The transfection efficiency of cationic liposomes can be dramatically increased when a ligand recognizing a cell surface receptor is included in the complex. The presence of the ligand facilitates the entry of the DNA–liposome complex into the cell through the initial binding of ligand by its receptor on the cell surface, followed by internalization of the bound complex via receptor-mediated endocytosis (Cheng, 1996), a highly efficient internalization pathway present in eukaryotic cells (Christiano and Curiel, 1996; Wagner, 1999). We have previously reported that the addition of the transferrin (Tf) ligand to a cationic liposome complex resulted in an *in vitro* transfection efficiency of 70–80% in squamous cell carcinoma of the head and neck (SCCHN) as compared with only 5–20% transfection by the cationic liposome complex without Tf (L. Xu *et al.*, 1997). Transferrin receptor (TfR) levels are known to be elevated in various types of cancer cells including breast (Inoue *et al.*, 1993) and prostate cancers (Keer *et al.*, 1990), even those prostate cancer cell lines derived from human lymph node and bone metastases (Grayhack *et al.*, 1979; Chackal-Roy *et al.*, 1989; Rossi and Zetter, 1992), as well as squamous cell carcinomas (Thorstensen and Romsio, 1993; Miyamoto *et al.*, 1994), and correlate with the aggressive or proliferative ability of tumor cells (Elliott *et al.*, 1993).

Squamous cell carcinoma of the head and neck is one of the most difficult forms of cancer to treat successfully. Approximately 40,000 new cases are diagnosed in the United States each year, with more than 12,000 individuals succumbing to this devastating disease annually (Landis *et al.*, 1998). Despite advances in the preferred forms of therapy, i.e., surgery and radiation, and now even chemotherapy, no major improvement in efficacy and survival has occurred over the past several decades. Approximately 70% of patients with advanced disease (stage III–IV) will still die from their illness, primarily from recurrent disease, which occurs in more than 60% of patients (Dimery and Hong, 1993). Even 25% of those with early stage disease (stage I–II) are incurable (van Dongen and Snow, 1997). Resistance to ionizing radiation by a subset of the tumor cells is believed to account for a significant portion of these clinical failures. Therefore, a new treatment modality that results in sensitization of these tumors to standard radiation therapy would have a marked effect on the clinical outcome of this disease for many patients.

In our previous report (L. Xu *et al.*, 1997), we demonstrated that wt p53 was efficiently delivered to SCCHN tumor cells *in vitro* by the Tf–liposome complex. The high-level expression of this exogenous wt p53 in the Tf–liposome–DNA-transfected cells resulted in the reversion of the radioresistant phenotype of the SCCHN cells in a DNA dose-dependent manner. In the present study, we further optimized the transferrin–cationic liposome system for systemic p53 gene therapy of SCCHN and demonstrate that the transduced wt p53 restored wt p53 func-

tion in these cells, leading to increased radiation-induced apoptosis in a p53 DNA-dependent manner. These findings indicate that the reversal of the radiation resistance of the SCCHN cells observed *in vitro* in our previous report was due to this restoration of the p53-dependent apoptotic pathway.

Most significantly, we examined the ability of transfected wt p53 to sensitize SCCHN to radiation *in vivo*. Our findings demonstrate that this transferrin–cationic liposome system could preferentially deliver the p53 gene to preestablished SCCHN xenograft tumors and sensitize them to radiotherapy, resulting in long-term tumor regression in animals receiving the combination therapy. This sensitization of SCCHN tumors, through the combination of systemic p53 gene therapy and ionizing irradiation, has the potential for use as a new therapeutic modality for the treatment not only of initial malignancies, but also for the larger challenge of recurrent disease.

## MATERIALS AND METHODS

### Cell culture

Human head and neck cell line JSQ-3, derived from a tumor of the nasal vestibule that failed radiotherapy, was a generous gift from R. Weichselbaum (University of Chicago; Weichselbaum *et al.*, 1993). The cell line was maintained in minimum essential medium with Earle's salts (EMEM) with supplements as previously described (L. Xu *et al.*, 1997).

### Plasmids

The *Escherichia coli lacZ* gene, driven by a simian virus 40 (SV40) promoter in expression plasmid pSVb, and the firefly luciferase gene driven by the cytomegalovirus (CMV) promoter in plasmid pCMVLuc, were employed as reporter genes (both from Promega, Madison, WI). The p53 expression plasmid pCMVp53 contains the 1.7-kb human wt p53 cDNA under the control of the CMV promoter, followed by the SV40 polyadenylation signal. The control plasmid pCMVpRo contains the same p53 cDNA in the reversed orientation, also under the control of the CMV promoter. All the plasmids were expanded in *E. coli* DH5 $\alpha$  and purified with plasmid Mega/Giga kits (Qiagen, Valencia, CA). The purified plasmids were quantified spectrophotometrically with  $A_{260}/A_{280}$  values of approximately 1.95. Agarose gel (0.8%) electrophoresis confirmed that >95% of the plasmid DNA was supercoiled.

### Preparation of transferrin–liposome–DNA complex

Cationic liposomes consisting of 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) were prepared as previously described (L. Xu *et al.*, 1997). The final concentration of liposomes was 2  $\mu$ mol/ml. Holo-transferrin (Tf, iron saturated; from Sigma, St. Louis, MO) was dissolved in pure water at 5 mg/ml. The Tf–liposome–DNA complex for *in vitro* experiments was prepared as described except that the serum-free medium EMEM was used instead of Hanks' balanced salt solution (HBSS) (L. Xu *et al.*, 1997). The prepared Tf–liposome (designated LipT)–DNA complex was used for *in vitro* cell transfection freshly within 1 hr of preparation, although on

the basis of transfection efficiencies it was found to be stable for at least 24 hr. Agarose gel electrophoresis was employed to assess the DNA retardation by complexing with LipT.

#### *Optimization of LipT system by luciferase assay*

The LipT system was optimized for JSQ-3 transfection using the luciferase assay. JSQ-3 cells ( $5 \times 10^4$ /well) were plated in a 24-well tissue culture plate. Twenty-four hours later, the cells were washed once with EMEM without serum, and 0.3 ml of EMEM without serum or antibiotics was added to each well. EMEM (0.2 ml) with the Tf-liposome-pCMVLuc (LipT-Luc) complex containing 1.0  $\mu$ g of plasmid DNA complexed with different amounts of cationic liposome and transferrin was added to the cells, usually in triplicate. After a 5-hr incubation at 37°C and 5% CO<sub>2</sub>, 0.5 ml of EMEM, supplemented with 20% fetal bovine serum and hydrocortisone (1  $\mu$ g/ml), was added to each well. Twenty-four hours later, the cells were washed once with phosphate-buffered saline (PBS), lysed with 1  $\times$  reporter lysis buffer (100  $\mu$ l/well; Promega), and the expressed luciferase activities were measured with the luciferase assay system (Promega) on a luminometer (Xu *et al.*, 1999). A recombinant firefly luciferase (Promega) standard was used at each measurement so as to convert the luminometer readings of relative light units (RLU) to the amount of luciferase equivalent expressed. Protein concentrations of the cell lysates were measured with a DC protein assay kit (Bio-Rad, Hercules, CA). The results were expressed as micrograms of luciferase equivalent per milligram of total protein.

#### *In vitro transfection of JSQ-3 cells by LipT-pSVb*

A quantitative  $\beta$ -galactosidase colorimetric assay was employed as described previously (L. Xu *et al.*, 1997). Purified  $\beta$ -galactosidase (Boehringer Mannheim, Indianapolis, IN) was used as standard. The results were expressed as milliunits (mU) of  $\beta$ -galactosidase equivalent per milligram of total protein. For histochemical studies of Tf-liposome-pSVb transfection, 60% confluent JSQ-3 cells in a 24-well tissue culture plate were transfected for 5 hr with 1.2  $\mu$ g of pSVb with or without LipT as described above. After an additional 2 days in culture, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal), and transfection efficiency was calculated as the percentage of blue-stained cells as previously described (L. Xu *et al.*, 1997, 1999).

#### *Western blot analysis*

To determine the DNA-dose response of p53 gene expression,  $2 \times 10^5$  JSQ-3 cells/well were plated in a six-well tissue culture plate. After 24 hr, cells were washed once with EMEM without serum and antibiotics, transfected with 1 ml of transfection solution containing LipT-p53 or LipT-pRo containing up to 4  $\mu$ g of plasmid DNA per  $2 \times 10^5$  cells, at the ratio of 1  $\mu$ g of DNA per 10 nmol of liposome per 15  $\mu$ g of Tf in EMEM. Five hours after transfection, 1 ml of EMEM supplemented with 20% fetal bovine serum (FBS) and hydrocortisone (1  $\mu$ g/ml) was added and cultured for an additional 48 hr. The transfected cells were collected and lysed in RIPA radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and Western blot analysis was performed as previously de-

scribed (L. Xu *et al.*, 1997, 1999). For the time course of p53 gene expression,  $2 \times 10^5$  JSQ-3 cells were transfected with 2  $\mu$ g of pCMVp53 or pCMVpRo complexed with LipT. The cells were collected for Western blot analysis every 24 hr up to 5 days after transfection and applied. To analyze the radiation effect on p53 gene expression, JSQ-3 cells were transfected with LipT-p53, or LipT-pRo (2  $\mu$ g of DNA per  $2 \times 10^5$  cells per well in a six-well plate) for 2 days, then trypsinized and irradiated in suspension with graded doses (up to 6 Gy) of <sup>137</sup>Cs  $\gamma$  rays in a J.L. Shepard and Associates Mark I irradiator (L. Xu *et al.*, 1997). The irradiated cells were replated and cultured for 2 or 4 days before being harvested for Western blot analysis.

#### *p53 and radiation-induced apoptosis*

To study p53-induced apoptosis, JSQ-3 cells were transfected with LipT-p53 or LipT-pRo (1 to 3  $\mu$ g of DNA per  $2 \times 10^5$  cells) as described above, and both the attached and floating cells were collected every day for 3 days for apoptosis staining. For radiation-induced apoptosis, the cells were transfected for 2 days, and then trypsinized and irradiated as described above. The replated cells were collected 4 days later for apoptosis staining. The collected cells were stained with the annexin V-fluorescein isothiocyanate (FITC) kit (Trevigen, Gaithersburg, MD) according to the manufacturer's protocol. The stained cells were analyzed on a flow cytometer (FACStar; Becton Dickinson, Franklin Lakes, NJ).

#### *In vivo gene transfection in nude mouse JSQ-3 xenograft model by intravenous injection of LipT-pSVb*

The transferrin-liposome, liposome-pSVb, and pSVb DNA complexes were prepared as previously described (L. Xu *et al.*, 1997) at a ratio of 1  $\mu$ g of DNA per 10 nmol of liposome per 12.5  $\mu$ g of transferrin. However, sterile 5% dextrose was used in place of HBSS. The nude mouse tumor model was established by subcutaneous injection of  $2.5 \times 10^6$  JSQ-3 cells in the flank of 4- to 6-week-old female athymic nude mice (NCR nu/nu). pSVb DNA (30  $\mu$ g) complexed with Tf-liposome in a 300- $\mu$ l total volume was injected into each mouse via the tail vein, using a 1-ml syringe and a 30-gauge needle. In the control groups, either liposome-pSVb or pSVb DNA without liposome was injected. Two and 10 days after DNA injection, the tumors as well as mouse organs were excised, cut into 1-mm-thick slices, washed once with PBS, and fixed with 2% formaldehyde-0.2% glutaraldehyde for 4 hr at room temperature. The fixed tumor slices were washed four times with PBS, each for 1 hr, and stained with X-Gal solution plus 0.1% Nonidet P-40 (NP-40) (pH adjusted to 8.5) at 37°C overnight. The stained tumor slices were embedded and sectioned by normal histological procedures and counterstained with nuclear fast red. Four sections per tumor were examined to evaluate the  $\beta$ -galactosidase gene expression, as indicated by the blue-stained cells.

#### *In vivo systemic p53 gene therapy in combination with radiation*

JSQ-3 cells ( $2.5 \times 10^6$ ) were injected subcutaneously into the lower back above the tail of athymic NCR nu/nu nude mice. Seven to 10 days later, the tumors grew to approximately 40–50

mm<sup>3</sup> at the injection site. Freshly prepared LipT-p53 or LipT-pRo containing 8  $\mu$ g of DNA in 300  $\mu$ l of 5% dextrose was intravenously injected per mouse via the tail vein twice a week for a total of five injections. Forty-eight hours after the initial intravenous injection, the animals were secured in a lead restraint that permitted only the tumor area to be exposed to  $\gamma$  irradiation, and the first fractionated dose of 2.5 Gy of <sup>137</sup>Cs ionizing radiation was administered. Thereafter, the animals were given 2.5 Gy every 48 hr to a total dose of 25 Gy. For comparison, a group of untransfected mice, as well as a group of mice injected with LipT-p53, received no radiation. The tumor sizes were measured weekly in a blinded manner by a third party at the Georgetown University Animal Facility. All animal experiments were performed in accordance with Georgetown University institutional guidelines for the care and use of animals.

#### Histological analysis of JSQ-3 xenografts after combination treatment

Subcutaneous JSQ-3 tumors were excised from the animals at various times after the end of the treatment. The tumor tissues were formalin fixed, sectioned, and stained with H&E according to common histological procedure.

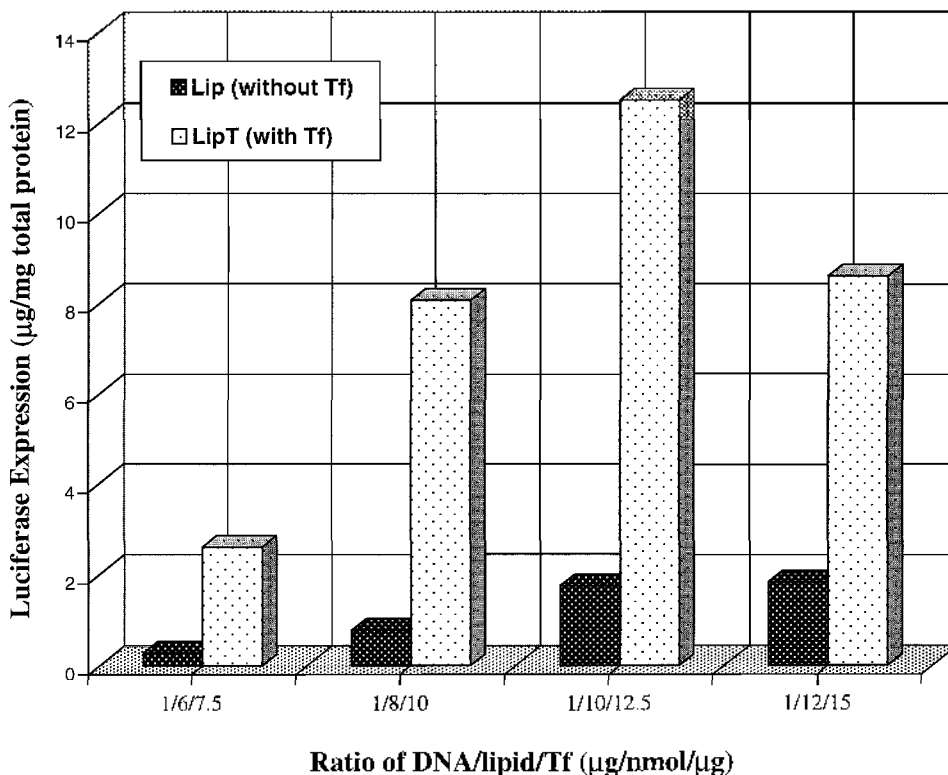
#### Statistical analysis

The Student *t* test and two-way ANOVA was performed with GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

## RESULTS

#### Optimization of transferrin-liposomes for transfection of head and neck cancer cells

LipT, the liposome complex based on cationic lipids DOTAP and DOPE with transferrin, was prepared as previously described (L. Xu *et al.*, 1997). To optimize the transfection efficiency, a plasmid designated as pCMVLuc that contains the firefly luciferase gene driven by the cytomegalovirus (CMV) promoter was employed as reporter gene. JSQ-3 cells were transfected with different DNA-lipid-Tf ratios of LipT-pCMVLuc (LipT-Luc). Figure 1 shows that transferrin significantly enhanced the transfection efficiency of cationic liposomes. Under optimal conditions, i.e., a DNA:lipid:Tf ratio of 1  $\mu$ g:10 nmol:12.5  $\mu$ g, luciferase was expressed at  $12.5 \pm 1.1$   $\mu$ g/mg total protein, representing 1.25% total protein, 7- to 10-



**FIG. 1.** Optimization of transferrin-liposome system by luciferase assay. In a representative experiment,  $5 \times 10^4$  JSQ-3 cells per well in a 24-well plate were transfected with Tf-liposome-pCMVLuc (LipT-Luc), containing 1.0  $\mu$ g of plasmid DNA complexed with various amounts of cationic liposome and transferrin. Twenty-four hours later, the cells were lysed and the expressed luciferase activities were measured with the luciferase assay system, with a recombinant firefly luciferase to convert the luminescence readings of relative light units (RLU) to the amount of luciferase equivalent expressed. The results were expressed as micrograms of luciferase equivalent per milligram of total protein. The DNA:lipid:Tf ratio of 1  $\mu$ g:10 nmol:12.5  $\mu$ g gave the best transgene expression:  $12.5 \pm 1.1$   $\mu$ g/mg total protein, or 1.25% total protein.

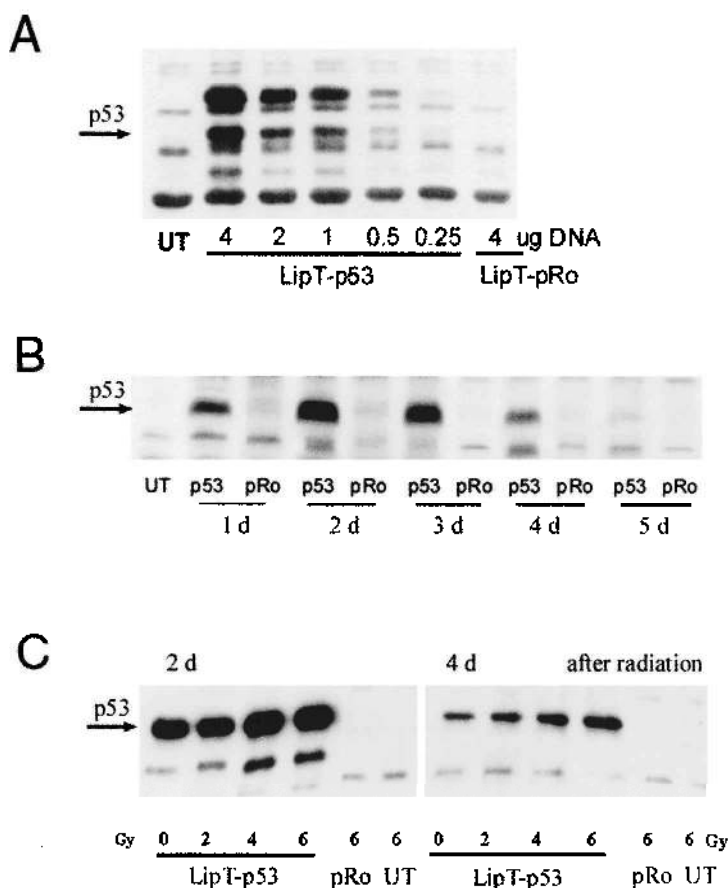
TABLE 1. *IN VITRO* TRANSFECTION EFFICIENCIES FOR JSQ-3 CELLS<sup>a</sup>

Transfected with:	Transfection efficiency (%)	
	Without serum	With serum
pSVb alone	0	0
Lip-pSVb	20	<10
LipT-pSVb	70-80	40-50

<sup>a</sup>JSQ-3 cells (60% confluent), cultured in a 24-well plate, were transfected for 5 hr with 0.5 ml of transfection solution containing 1.2  $\mu$ g of pSVb. After an additional 2 days in culture, the cells were fixed and stained with X-Gal. Transfection efficiency was calculated as the percentage of blue-stained cells. LipT-pSVb, transferrin-liposome-pSVb complex; Lip-pSVb, liposome-pSVb without transferrin.

fold more than liposome alone without transferrin. The results agreed with our previous report (L. Xu *et al.*, 1997). This ratio was used as a basic ratio for *in vivo* optimization below.

In the quantitative  $\beta$ -galactosidase assay, the JSQ-3 cells transfected under the optimal conditions, with 0.5  $\mu$ g of DNA per  $10^5$  cells of LipT-pSVb, expressed  $15.04 \pm 0.60$  mU/mg total protein of  $\beta$ -galactosidase without serum, and  $10.95 \pm 0.15$  mU/mg in the presence of serum. In histochemical studies, transfection with LipT-pSVb resulted in 70-80% of the cells being transfected (see Table 1). The presence of serum during transfection slightly reduced transfection efficiency, but even with serum, 40-50% of the cells stained blue, while cationic liposome without ligand gave only 10-20% transfection efficiency. These results demonstrated that using Tf as a ligand significantly increased the transfection efficiency of cationic liposomes, even in the presence of serum.



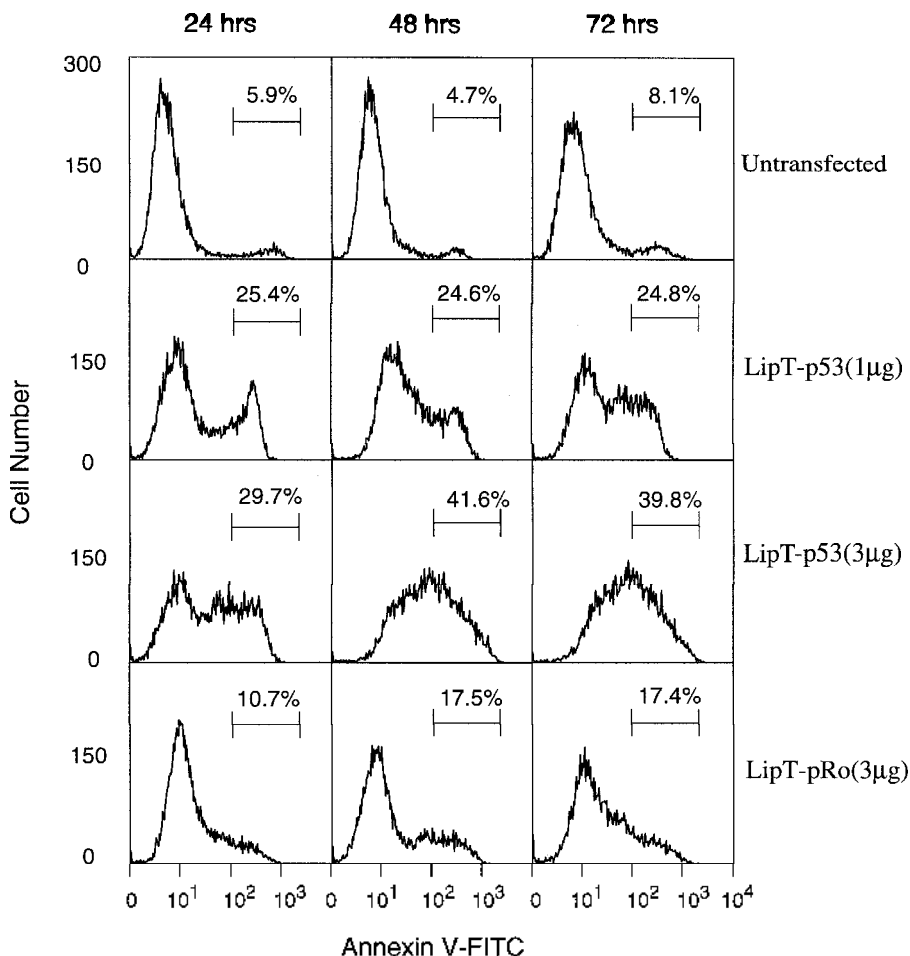
**FIG. 2.** Expression of exogenous wt p53 protein in LipT-p53-transfected JSQ-3 cells *in vitro* and its relationship to radiation. UT, Untransfected JSQ-3 cells; Lip T-p53, cells transfected with transferrin-liposome complexed to p53 plasmid pCMVp53; LipT-pRo, transferrin-liposome complexed to pCMV-pRo, the plasmid containing p53 gene in the reverse orientation. The arrows indicate exogenous wt p53. (A) DNA dose response of LipT-p53 transfection. JSQ-3 cells were transfected in a six-well plate for 5 hr with increasing amounts of LipT-p53 or LipT-pRo as described in Materials and Methods. Forty-eight hours later the cells were collected and lysed for Western blot. A total of 40  $\mu$ g of protein was loaded per lane. (B) Time course of exogenous wt p53 expression in JSQ-3 cells. The cells were transfected with LipT-p53 or LipT-pRo at 2  $\mu$ g of plasmic DNA per  $2 \times 10^5$  cells. At the indicated time, the cells were collected and lysed for Western blot analysis as described above. (C) Effect of  $\gamma$  irradiation on wt p53 gene expression JSQ-3 cells were transfected with LipT-p53 or LipT-pRo (2  $\mu$ g of DNA per  $2 \times 10^5$  cells) for 2 days, then trypsinized and irradiated at graded doses up to 6 Gy of  $^{137}\text{Cs}$   $\gamma$  rays. The irradiated cells were replated and cultured for a further 2 and 4 days before collecting for Western blot analysis. The  $\gamma$  irradiation can enhance and/or stabilize the exogenous wt p53 expression in a radiation dose-dependent manner.

*Expression of exogenous wild-type p53 protein in LipT-p53-transfected JSQ-3 cells*

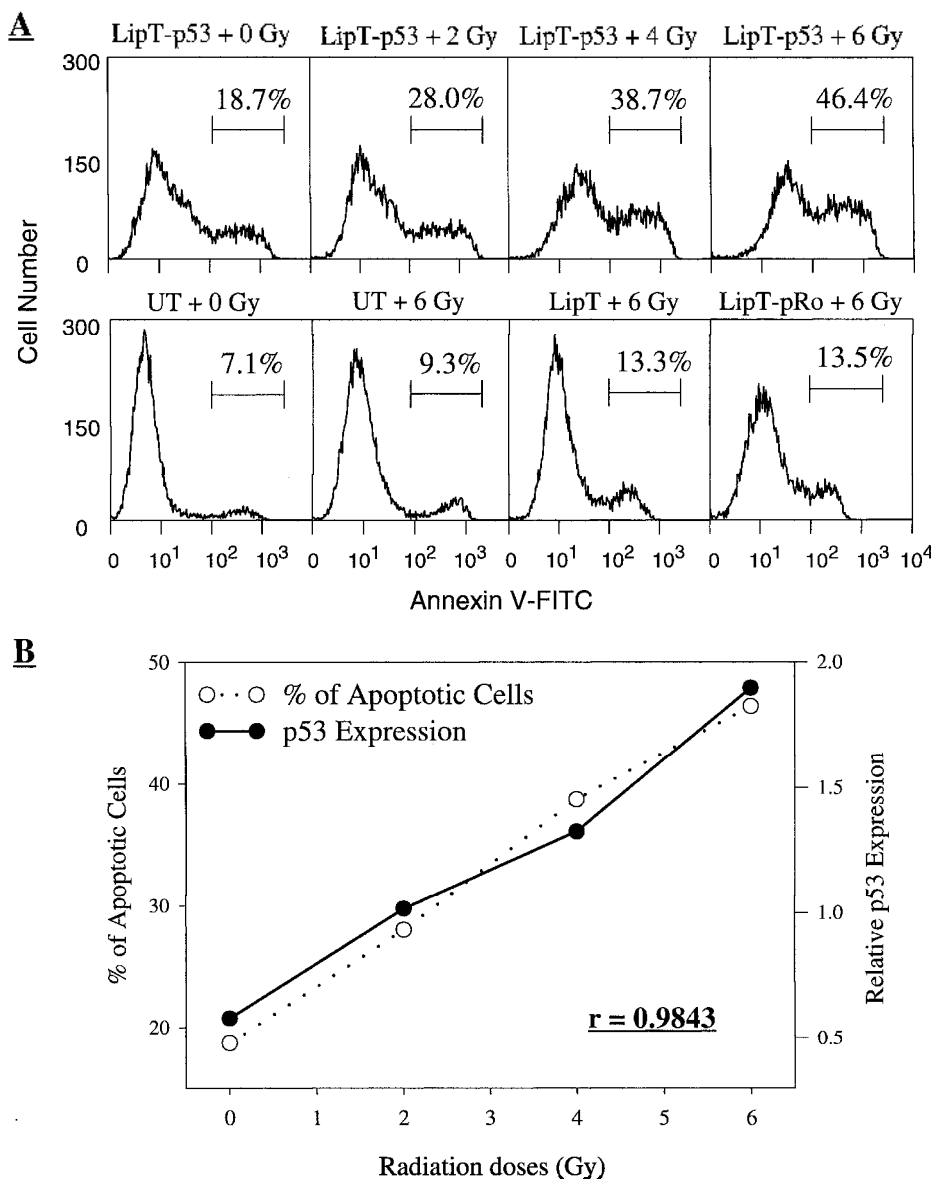
Western blot analysis was employed to demonstrate that the LipT-transduced wt p53 was being expressed in JSQ-3 cells. As shown in Fig. 2A, transfection with increasing doses of p53 expression plasmid pCMVp53 complexed with LipT (LipT-p53) resulted in a DNA dose-dependent expression of wt p53, while no exogenous p53 expression was evident in JSQ-3 cells transfected with LipT-pRo, which carries the wt p53 cDNA in reverse orientation. Wild-type p53 was expressed 24 hr after LipT-p53 transfection and peaked on the second day (Fig. 2B). Only traces of exogenous p53 were detected 5 days after transfection, indicating that LipT-mediated wt p53 expression was transient (Fig. 2B). When the cells were irradiated 48 hr after LipT-p53 transfection, i.e., at the peak of exogenous wt p53 expression, the level of wt p53 protein increased with increasing doses of  $\gamma$  irradiation (Fig. 2C). The level of p53 protein still present even 4 days postirradiation (i.e., 6 days posttransfection) indicates that  $\gamma$  irradiation can enhance and/or stabilize the exogenous wt p53 expression in a radiation dose-dependent manner, analogous to the situation observed in cells with naturally occurring wt p53.

*Apoptosis induced by p53 transfection and  $\gamma$  irradiation*

To examine the effect of wt p53 restoration on the induction of apoptosis, JSQ-3 cells were transfected with LipT-p53 or LipT-pRo, then stained on days 1 to 3 with annexin V-FITC, which binds specifically to phosphatidylserine present on apoptotic cells. As shown in Fig. 3, a clear induction of apoptosis is observed with LipT-mediated wt p53 restoration, in a DNA dose-dependent manner. The percentage of apoptotic cells peaked on the second day of transfection, correlating with the levels of wt p53 expression in the cells as revealed by Western blot (Fig. 2B). To examine the effect of irradiation on the induction of apoptosis, the transfected cells were irradiated with various doses 48 hr after transfection. Two to 4 days later, the cells were stained with annexin V-FITC and analyzed by flow cytometry. As shown in Fig. 4A,  $\gamma$  irradiation induced a significant increase in apoptosis only in LipT-p53-transfected cells, from 18.7% (0 Gy) to 38.7% (4 Gy) and 46.4% (6 Gy) 2 days after irradiation (or 4 days after transfection). No increase was observed with untransfected (UT) cells or those transfected with LipT or LipT-pRo. This increase was also radiation dose dependent and correlated, as indicated in Fig. 4B, with the wt



**FIG. 3.** LipT-p53 transfection-induce d apoptosis. JSQ-3 cells were transfected with LipT-p53 or LipT-pRo, then stained from day 1 to day 3 with annexin V-FITC, which bound specifically to phosphatidylserine present on apoptotic cells. A clear induction of apoptosis is observed in LipT-mediated wt p53 restoration, in a DNA dose-dependent manner.



**FIG. 4.** The effect of irradiation on the induction of apoptosis. **(A)** The transfected cells were irradiated at various doses 48 hr after transfection. Two to 4 days later, the cells were stained with annexin V-FITC and analyzed by flow cytometry.  $\gamma$  Irradiation induced a significant increase in apoptosis only of LipT-p53-transfected cells. **(B)** The increase was radiation dose dependent and correlated with the wt p53 expression levels from Western blot data.

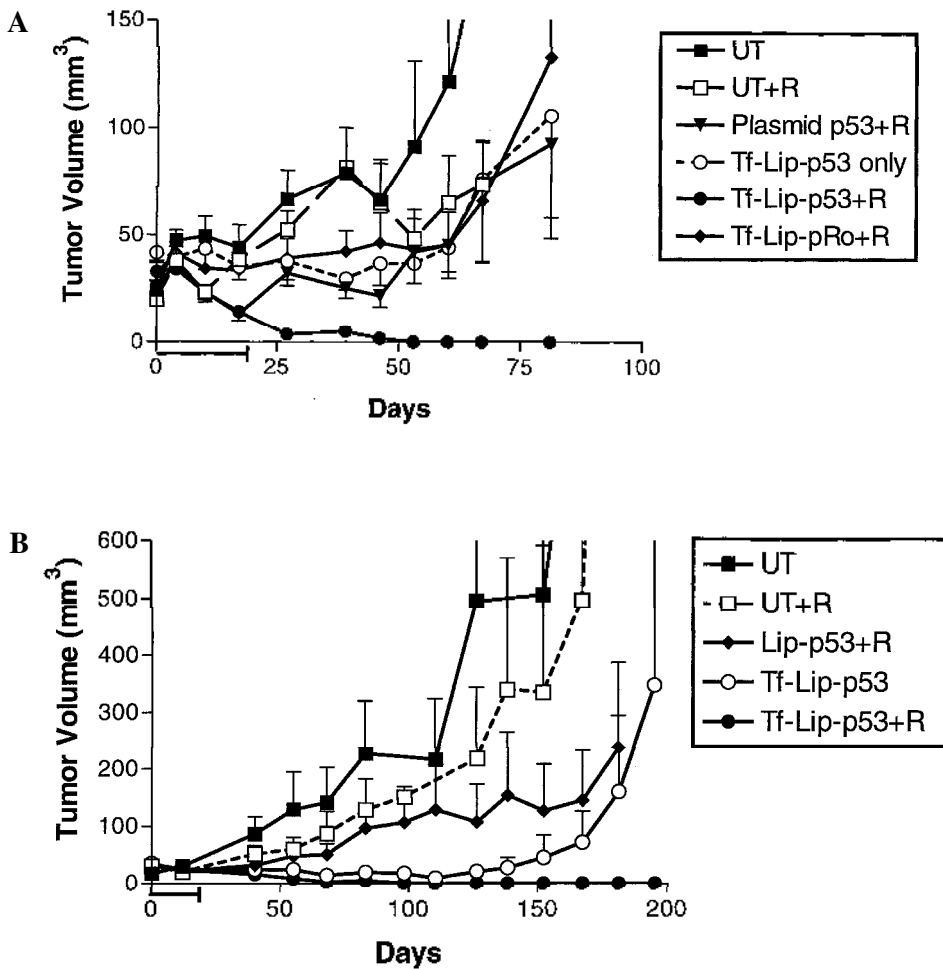
p53 expression levels shown in Fig. 2C ( $r = 0.9843$ ), demonstrating that the radiation enhancement of apoptosis was proportional to the wt p53 level in cells, i.e., the more wt p53 expressed, the more apoptotic cells induced.

#### *In vivo transfection of systemically delivered LipT-pSVb complex*

To examine the ability of the Tf-containing liposomes to selectively target tumor tissue *in vivo*, LipT-pSVb or the pSVb plasmid alone was injected intravenously into nude mice bearing JSQ-3 subcutaneous xenografts. After either 2 or 10 days, the tumors and major organs were examined by histochemical staining with X-Gal. At 2 days, the tumors in mice injected with LipT-pSVb showed reporter gene expression representing an

*in vivo* transfection efficiency of approximately 20–30% with one intravenous injection (Color Plate 1A and B). In contrast, with pSVb plasmid alone, without liposome, less than 1% of the tumor cells stained for reporter gene expression (Color Plate 1D). Ten days after intravenous administration of LipT-pSVb, both the percentage of positive cells and the intensity of blue staining in the tumors were significantly reduced (Color Plate 1C), indicating that the LipT-mediated systemic transfection was transient. Vital organs in mice injected with LipT-pSVb showed staining only of macrophages, such as histiocytes of the lung (Color Plate 1E) or Kupffer cells of the liver (Color Plate 1F), whereas the lung alveolar cells and hepatocytes remained unstained. No staining was evident in the lymphoblastic growth centers in the spleen (data not shown). In summary, the histochemical staining indicates that delivery of the reporter gene by

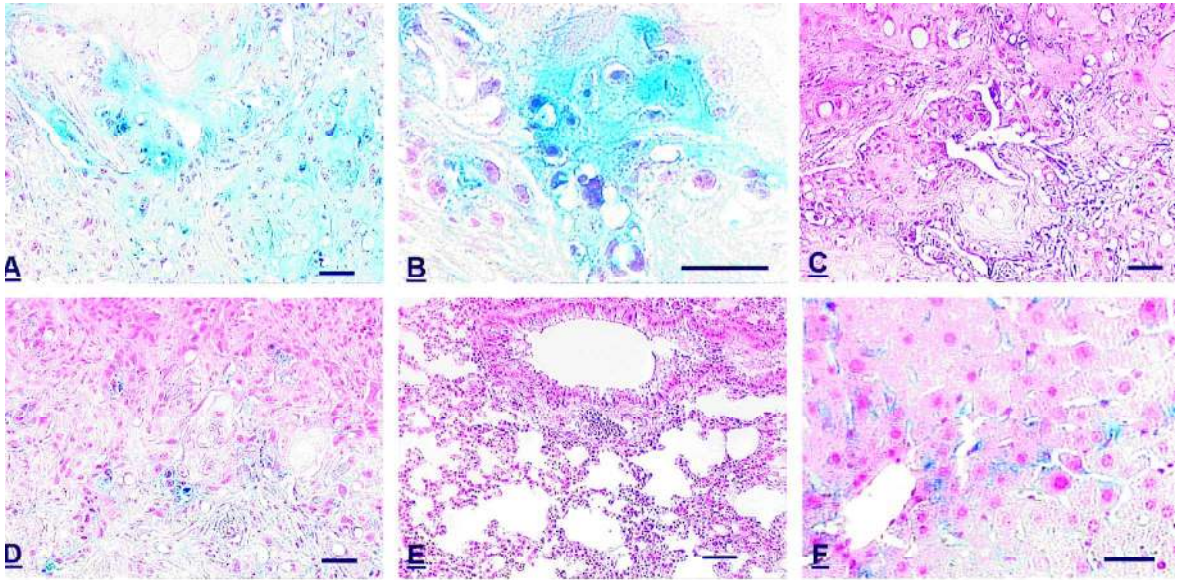




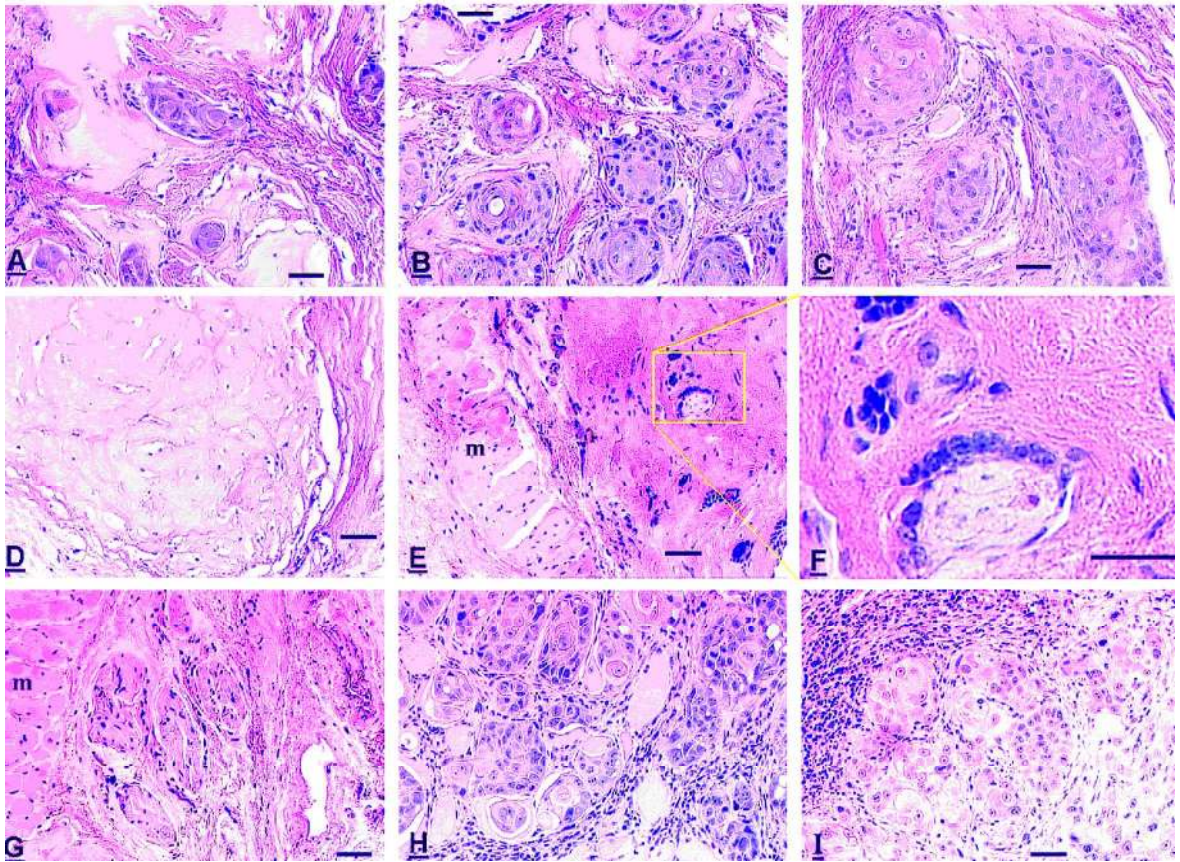
**FIG. 5.** Effect of the combination of LipT-p53 and radiation treatment on JSQ-3 xenografts *in vivo*. Day 0 represents the tumor volume prior to initiation of treatment. The last point represents 81 days (A) or 195 days (B) posttreatment. While there was no significant difference between the three control groups, the difference between the controls and the combination LipT-p53-treated group was highly significant. The standard error for the combination group was negligible. The bar indicates the duration of treatment (a total of five intravenous LipT injections and 25 Gy of radiation).

**Color Plate 1.** *In vivo* LipT-mediated systemic transfection of  $\beta$ -galactosidase reporter gene in nude mouse JSQ-3 xenografts. The  $\beta$ -galactosidase-expressing cells stained blue. (A) JSQ-3 xenograft tumor from a mouse injected intravenously with LipT-pSVb, 48 hr postinjection; (B) second, independent xenograft tumor from a mouse injected intravenously with LipT-pSVb, 48 hr postinjection; (C) JSQ-3 tumor treated intravenously with LipT-pSVb, 10 days after injection; (D) tumor from mouse injected intravenously with pSVb alone, 48 hr postinjection; (E) lung from a mouse carrying a JSQ-3 xenograft tumor and injected intravenously with LipT-pSVb, 48 hr postinjection; (F) liver from a mouse carrying a JSQ-3 xenograft tumor and injected intravenously with LipT-pSVb. Bars: 50  $\mu\text{m}$ .

**Color Plate 2.** Histological analysis of JSQ-3 xenografts after combination treatment. Subcutaneous JSQ-3 tumors were excised from the animals at various times after and the end of treatment with radiation and/or intravenously administered LipT-p53 or LipT-pRo. The mass was formalin fixed, sectioned, and stained with H&E. Shown is tumor or residual mass from animals that received (A) LipT-p53 plus 25 Gy, 1 day posttreatment; (B) LipT-pRo plus 25 Gy, 1 day posttreatment; (C) no intravenous treatment but 25 Gy of radiation, 1 day posttreatment; (D) LipT-p53 plus 25 Gy, 1 month posttreatment; (E and G) LipT-p53 plus 25 Gy (two individual tumors), 6 months posttreatment; (F) enlargement of the area indicated in (E), showing dead tumor cells being engulfed by Langerhans cells; (H) LipT-p53, without radiation treatment, 6 months posttreatment; (I) LipT-pRo plus 25 Gy, 6 months posttreatment. m, Normal muscle. Bars: 50  $\mu\text{m}$ .



Color Plate 1



Color Plate 2

LipT is selective, with the human xenograft being most heavily stained.

#### *Effect of the combination of LipT-p53 and radiation treatment on JSQ-3 xenografts in vivo*

Two independent experiments with SCCHN (JSQ-3) xenograft tumors have been performed with similar results (Fig. 5A and B). Mice bearing subcutaneous JSQ-3 tumors of approximately 25–40 mm<sup>3</sup> were injected, via the tail vein, with LipT-p53 twice weekly (a total of five injections) and only the tumor areas were exposed to fractionated doses (2.5 Gy) of  $\gamma$  radiation, every other day to a total of 25 Gy. As shown in Fig. 5A and B, short-term radiation effects on tumor growth were evident in cells transfected using the control LipT-pRo. There was only minimal tumor growth inhibition in the animals that received LipT-p53 without radiation. In contrast, in both experiments all the tumors that received the combination of LipT-p53 plus radiation exhibited virtually complete regression and showed no signs of recurrence ( $p < 0.001$ ). In the case of the longer maintained experiment (Fig. 5B), the tumor-bearing animals in control groups had died or were humanely euthanized because of excessive tumor burden by 6 months postirradiation. However, the combination treatment group (p53 plus irradiation) still showed no sign of tumor regrowth (Fig. 5B).

Histology of the small residual “tumor nodule” present in some of the animals, which was static at less than 10% of the original tumor volume over the entire course of the experiment, is shown in Color Plate 2. In the mice that had received LipT-p53 plus radiation, it is evident that mostly dead and dying tumor cells, many being engulfed by macrophages, and few live tumor cells remain 1 day after completion of treatment (Color Plate 2A). In contrast, although radiation effects are evident, live nests of tumor cells are clearly visible in the mice that received LipT-pRo plus radiation (Color Plate 2B). For comparison, an untreated tumor receiving radiation alone is shown in Color Plate 2C. Color Plate 2D shows, 1 month posttreatment, residual tissue at the site of the original tumor in an animal that received LipT-p53 plus radiation. No live tumor cells were evident; only scar tissue and a few multinuclear Langerhans cells were present. By 6 months posttreatment, only mature scar tissue and numerous macrophages or Langerhans cells in the process of surrounding and engulfing the remains of the dead tumor cells, are evident in the residual tumor mass from the animal in the combination treatment group (Color Plate 2E and G). An enlargement of this is shown in Color Plate 2F. In contrast, large areas of live cancer cells can be seen in the tumor from an animal receiving LipT-p53 without radiation (Color Plate 2H) or LipT-pRo plus radiation (Color Plate 2I). These observations were confirmed by an experienced pathologist and are consistent with our previous results in studies with intratumorally injected, adenoviral vector carrying wt p53 in conjunction with radiation (Pirolo *et al.*, 1997). These experiments using a mouse cancer model demonstrate that the combination of transferrin-targeted systemic liposomal p53 gene therapy and conventional radiotherapy was markedly more effective than either treatment alone.

## DISCUSSION

The development of viral and nonviral methods for the introduction of genes into cells *in vivo* has made therapeutic interventions involving gene therapy a reality. The introduction of wt p53 has been reported to suppress the growth of various types of malignancies, both *in vitro* and in mouse xenograft models. However, most of the *in vivo* results are obtained with viral p53 gene vectors and local administration (Fujiwara *et al.*, 1994; Clayman *et al.*, 1995; Liu *et al.*, 1995; reviewed by Nielson and Maneval, 1998). To overcome the limitations of the viral vectors, including the immunogenicity and biosafety issues, and to realize the ultimate goal of systemic cancer gene therapy, nonviral gene delivery, especially ligand-directed tumor-targeted systemic gene delivery, is an attractive alternative.

We have previously reported that wt p53 replacement by a nonviral gene delivery system, transferrin-liposome-DNA complex, sensitized SCCHN cells to radiation *in vitro* (L. Xu *et al.*, 1997). This system was further optimized for systemic gene delivery *in vivo*. The results described above demonstrate that the transferrin-cationic liposome system can deliver genes systemically to xenograft tumors *in vivo*, after intravenous administration. Twenty to 30% *in vivo* transfection efficiency was achieved via intravenous injection of the LipT-DNA complex. The LipT-mediated p53 gene transfer apparently restored a functional apoptotic pathway in the SCCHN cells, thereby sensitizing the tumors to radiation therapy. Total tumor regression was observed in a nude mouse xenograft model of human SCCHN by a systemically delivered nonviral p53 vector in combination with conventional radiation therapy. The combination of systemic LipT-p53 gene therapy and radiotherapy for SCCHN was markedly more effective than either treatment alone. This observation is consistent with our report of radiosensitization by folate-liposome-mediated p53 gene therapy (Xu *et al.*, 1999). Although systemic LipT-p53 alone showed suppressive effects on tumor growth, as observed here and also by others (Lesoon-Woo *et al.*, 1995; M. Xu *et al.*, 1997), the tumor would ultimately recur after the treatment ceased. However, when LipT-p53 was combined with conventional radiotherapy, the established tumors regressed for up to 6 months after the end of treatment without recurrence. In the small residual “tumor nodules” that were static at less than 10% of the original tumor volume, only scar tissue and numerous macrophages or multinuclear Langerhans cells, in the process of surrounding and engulfing the remains of the dead tumor cells, were observed. Therefore, host macrophages might be involved in the treatment response (discussed below).

Lack of functional p53 results in the inability of the tumor cell to initiate programmed cell death (apoptosis) in response to DNA damage induced by radiation or other agents. Replacement of wild-type p53 should therefore restore the apoptotic response to irradiation (Liu *et al.*, 1995). We have demonstrated previously that adenovirus mediated restoration of wt p53, in combination with radiation treatment, was able not only to inhibit tumor growth, but also to completely eliminate established radiation-resistant SCCHN xenograft tumors (Pirolo *et al.*, 1997). This adenoviral wt p53 replacement also restored the G<sub>1</sub> checkpoint and the  $\gamma$  radiation-induced apoptotic pathway in the tumor cells, and therefore sensitized the tumors to



radiotherapy (Chang *et al.*, 1997). The results presented here demonstrate that (1) transferrin-liposome-mediated p53 gene delivery can efficiently restore wt p53 function and the apoptotic pathway; (2)  $\gamma$  irradiation can enhance and/or stabilize exogenous wt p53 expression in a radiation dose-dependent manner; (3) radiation induces a significant increase in p53-dependent apoptosis; and (4) this increase is radiation dose dependent and correlates with the exogenous wt p53 level in tumor cells. Further work is now underway to analyze the LipT-p53-mediated changes in p53 downstream effectors or related molecules, e.g., p21 WAF1, Bcl-2, MDM2, p19ARF, etc., as well as cell cycle-related analysis.

The host defense system plays an important role in interactions between therapeutic regimens and target tissue, especially in the case of cancer gene therapy. With transferrin-targeted cationic liposome gene delivery, although only a fraction of tumor cells were transfected, the combination therapy resulted in tumor regression without recurrence 6 months after the treatment stopped. Antiangiogenesis may be an important mechanism involved in the system, as observed by M. Xu *et al.* (1997). Liu *et al.* (1999) reported that systemic p53 gene delivery by cationic liposome inhibited angiogenesis by inducing endogenous thrombospondin-1 expression. Natural killer (NK) cells, which are abundant in the nude mouse, may also play a role in the tumor regression, as suggested by Hall *et al.* (1998). As mentioned above, host macrophages are involved in the tumor response. Systemic LipT-p53 gene therapy plus radiation resulted in tumor cell death, likely through the induction of apoptosis. These apoptotic cells were phagocytosed by macrophages and possibly also by dendritic cells. The macrophages and dendritic cells can then serve as antigen-presenting cells to stimulate the host immune system (Lotze and Jaffe, 1999). In the case of the athymic nude mouse, which lacks T lymphocytes, NK cells may be involved. Peron *et al.* (1999) suggested a dendritic cell-NK cell link, in antitumor therapy. Currently, we are testing LipT-p53 systemic gene therapy in immune-competent syngenic tumor models to explore the immunological and molecular mechanisms.

In conclusion, the transferrin-liposome system is a highly efficient and tumor-specific gene delivery system both *in vitro* and *in vivo*. The replacement of wild-type p53 by LipT-p53 restored the wt p53 functional apoptotic pathway. The systemic delivery of LipT-p53 to established human SCCHN xenografts markedly sensitized the tumors to radiation therapy. The combination of systemic p53 gene therapy and conventional radiotherapy resulted in tumor regression and inhibition of their recurrence long-term. This strategy may provide a more effective treatment modality for human head and neck cancer.

## ACKNOWLEDGMENTS

We thank Ms. Tina Wilson for assistance with the *in vivo* animal studies, Dr. Laiman Xiang for assistance with tissue culture, Drs. Karen Creswell and Wen-Hui Zhu for assistance with flow cytometry analysis, and Dr. Williams Alexander for his critical review of the manuscript. This work was supported in part by NCI Grant R01 CA 45158 (E.C.) and Synergene Therapeutics, Inc.

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Received for publication July 20, 1999; accepted after revision September 20, 1999.