Macromolecular Research, Vol. 13, No. 3, pp 218-222 (2005)

Biodistribution and Genotoxicity of Transferrin-Conjugated Liposomes/DNA Complexes in Mice

Sang Mi Lee and Jin-Seok Kim*

College of Pharmacy, Sookmyung Women's University, Seoul 140-724, Korea

Yu-Kyoung Oh

Pocheon CHA University, Pocheon, Korea

Yong-Bok Lee

College of Pharmacy, Chonnam National University, Gwangju 500-757, Korea

Hongkee Sah

College of Pharmacy, Catholic University of Daegu, Daegu, Korea

Received January 26, 2005; Revised March 30, 2005

Abstract: Transferrin-conjugated liposomes (T_f-liposomes) were made and formulated with pCMVluc DNA to form a lipoplex. Among the various formulations studied, the T_f-liposome: pCMVluc DNA complex at a ratio of 5:1 (wt/wt) showed the highest transfection efficiency, which was twice that of Lipofectin™ on HeLa cells. The maximum tolerated dose (MTD) of this lipoplex formulation from a single intravenous injection was over 10 mg/kg in healthy ICR mice. The RT-PCR results showed that the highest level of luciferase mRNA was detected in the lungs, followed by the liver, spleen, heart and kidneys, after an intravenous injection into mice. Two weeks after the injection, the levels of luciferase mRNA decreased gradually in the liver, spleen, heart, and kidney, but not in the lungs. The micro-array study showed that the cancer-related genes, including the bcl 6 gene, were highly up-regulated by the treatment with T_f-liposome/ pCMVluc DNA complex on HeLa cells, indicating that there were possible interactions between the host chromosomal DNA and the T_f-liposome within the cells. The results obtained from this study are expected to be useful for designing a safe and efficient gene delivery system using transferrin-conjugated liposomes.

Keywords: gene delivery; T_f-liposome, biodistribution, microarray, MTD.

Introduction

Gene delivery systems based on non-viral origin, such as cationic liposomes or polymers, have attracted much attention for both non-clinical and clinical studies.[1-3] Even with the weak point of low transfection efficiency, non-viral gene delivery systems have been reported easy and safe especially for human clinical use.[4,5] Especially, cationic liposome with a targeting ligand attached shows great potential for use in clinical studies as it can overcome the problem of low transfection efficiency of non-viral gene delivery systems to a certain extent. Transferrin (T_f) has widely been used as a targeting ligand to improve the transfection efficiency of cationic liposomes, employing a receptor-mediated endocytosis mechanism.[6] Levels of transferrin receptor (T_fR) are found to be elevated in various types of cancer cells, including cervical and breast cancers[7], which is correlated with the aggressive or proliferative ability of tumor cells. Therefore, T_fR is considered to be useful as a prognostic tumor marker and also as a potential target for chemotherapeutic drug delivery in the control of malignant cell growth.[8]

We have preciously reported the intracellular trafficking of T_f-liposome/pCMVluc DNA complex within the cells using a double-labeled fluorescence technique.[9] As a continuing study, the work described herein demonstrates the biodistribution of transgene expression after intravenous injection of T_f-liposome/pCMVluc DNA complex in mice and also the micro-array study for the elucidation of possible genotoxity due to the T_f-liposome/ pCMVluc DNA formulation on HeLa cells.

*e-mail: jskim@sdic.sookmyung.ac.kr
1598-5032/06/218-05

Experimental

Materials. Dimethyldioctadecyl-ammonium bromide (DDAB), cholesterol (Chol), dithiothreitol (DTT), 4-(*p*-maleimidophenyl)-butyric acid *N*-hydroxy succinimide ester (SMPB), *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and transferrin (T_f) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Transphosphatidylated egg phosphatidylethanolamine (TPE) was bought from Avanti Polar Lipids (Pelham, AL, USA). Human cervical cancer cell line (HeLa) was purchased from the Korean Cell Line Bank (Seoul, Korea). Plasmid (pCMVluc) containing the coding sequences for luciferase was purchased from Gibco BRL (Grand Island, NY, USA). Bacterial strain DH5α was purchased from Qiagen (Valencia, CA, USA). For the RT-PCR study, random primers were purchased from Promega (Madison, WI, USA). Trizol reagent was from Gibco BRL (Grand Island, NY, USA). The dNTP mixture and RNase H were from Takara (TaKaRa, Japan) and other reagents including 5x first-strand buffer, 0.1 M DTT, RNase OUT, and M-MLVRT were from Invitrogen (Carlsbad, CA, USA). 10x PCR buffer and Super Taq were from SUPER-Bio (Suwon, Korea). Microarray (Tween-Chip™) was purchased from Digital Genomics (Seoul, Korea). Female ICR mice were purchased from Dae-Han Bio Inc. (Chungbuk, Korea). All other materials were of reagent grade or better.

Preparation of T_f-Liposomes. Liposomes were prepared by the reverse-phase evaporation method.[10] For the maleimidophenyl butyl-phosphatidyl ethanolamine (MPB-PE) containing liposome, DDAB:Chol:MPB-PE at a molar ratio of 20:20:1 was dissolved in 1 mL of chloroform. After evaporation of solvent at room temperature, dry lipid film was suspended in 1 mL of freshly hydrated diethyl ether, to which was added 0.7 mL of phosphate buffered saline (PBS, pH 7.4). The mixture was vigorously vortexed for 1 min and ether was then eliminated by a rotary evaporation. Liposomes were downsized by extrusion through 0.2 μm polycarbonate membranes 20 to 30 times using a Liposofast extrusion device (Avestin, Toronto, Canada). For the preparation of transferrin-conjugated liposomes (T_f-liposomes), transferrin was modified to have reactive thiol groups by the method of Carlsson *et al.*[11] And, 5 mg of transferrin was dissolved in 5 mL of 0.1 M PBS (pH 7.4) and the SPDP solution was freshly prepared at 20 mM in methanol. After 30 min reaction (SPDP:T_f=25:1 molar ratio) with stirring, the pyridyl-dithiopropionate derivatized transferrin (PDP-T_f) was separated from reactants by gel chromatography on a Sephadex G-75 column. Then, 30 μL of 1 M DTT was added to 1 mL of PDP-T_f and stirred for 30 min at room temperature. The T_f-conjugated liposomes were separated from unconjugated liposomes by metrizamide flotation method with a slight modification.[12]

***in vitro* Transfection Using HeLa Cells.** HeLa cells were seeded at a density of 1×10^4 cells/mL in 96-well plates 24 h prior to transfection. T_f-liposome/pCMVluc DNA complexes were prepared by mixing pCMVluc DNA and T_f-liposome in PBS at an appropriate charge ratio and incubated for 1 h at room temperature to achieve complex formation. Cells were rinsed with serum-free medium and then covered with 50 μL of MEM before T_f-liposome/pCMVluc DNA complexes were added. T_f-liposome/pCMVluc DNA complexes were added gently to cells in a volume of 50 mL per well. After incubation for 4 h (5% CO_2, 37°C) the medium was replaced with MEM containing 10% FBS, and the cells were further incubated for 48 h. Cells were then washed with PBS, and the lysis buffer was added to each well. The level of gene expression in the lysates was evaluated by measuring the light production by luciferase assay. The protein content of the lysates was measured by the bicinchoninic acid protein assay using bovine serum albumin as the standard.

Determination of MTD for T_f-Liposome/pCMVluc DNA Complex in Mice. The maximum tolerated dose (MTD) for T_f-liposome/DNA complexes after a single i.v. injection was determined using healthy female ICR mice (24-28 g). Experimental mice were randomly divided into 6 groups, each group containing 6 mice. The groups included a PBS-injected control group and 5 groups which received various T_f-liposome/ pCMVluc DNA complexes (0.5, 1, 2.5, 5, and 10 mg pCMVluc DNA/kg body weight) over 30 days period by weighing individual mice once a day.

Biodistribution Study of T_f-Liposome/ pCMVluc DNA Complex by RT-PCR. T_f-liposome/pCMVluc complexes were administered into ICR mice (6 weeks old, female) as a single i.v. dose of 100 μL (70 μg of plasmid DNA) via the tail vein. Mice were sacrificed at the indicated times after the injection and the following tissues were then taken for the RT-PCR analysis; spleen, liver, heart, lung, and kidney. Organ distribution of pDNA was studied using RT-PCR method with β-actin as a control. Briefly, total RNA was extracted using Trizol reagent according to the manufacturer's instructions and cDNA synthesis was performed with 6.5 μg of total RNA using random hexamer primers. The reaction was carried out in a total volume of 25 μL for 15 min at room temperature and then heated to 70°C to denature the enzyme. LUC-sense and antisense primers produced an 491-bp DNA in size.

LUC-sense primer:
5'-AGAACTGCCTGCGTGAGATT-3'
LUC-antisense primer:
5'-GCCCATATCCTTGCCTGATA-3'

As a control, β-actin sense and antisense primers produced an 230-bp DNA in size.

β-actin sense primer:
5'-GATGGTGGGAATGGGTCAGAAG-3'

β-actin antisense primer:
5'-TCATCTTTTCACGGTTGGCCTT-3'

The final concentrations for all PCR components in a 50 μL volume were as follows: 10 mM dNTP mixture, 10 pmole of each primer, 1 μL of extracted genomic DNA, and 2.5 units of Super Taq (SUPER-Bio) in 10x PCR Buffer (SUPER-Bio). RT-PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, S. San Francisco, CA, USA) using the program of 33 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min, respectively.

Microarray Study from Total RNA of HeLa Cells. Total RNA was isolated from the treated HeLa cells using Trizol reagent according to the manufacturer's protocols. Briefly, cells were lysed directly in 96-well micro-plates by adding Trizol reagent, followed by incubation at R.T. and addition of chloroform. The isolated RNA was further cleaned up using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) before the microarray experiment. Appropriate amount of control RNA mixture and 2 μL of oligo(dT) primer was added to 20 μL of total RNA, followed by prehybridization and hybridization. The image was scanned using a ScanArray Lite (Packard BioChip Technology, Ontario, OR, USA).

Results and Discussion

***T_f*-Liposome/pCMVLuc Complex.** Transferrin (T_f) was conjugated to DDAB:Chol:MPB-PE liposomes by thioether linkage (-S-). Size and zeta-potential of *T_f*-liposome/pDNA complexes were determined. Diameter of *T_f*-liposome/pDNA complex determined by dynamic laser-light scattering (DLS) method was 367±12 nm and that of unconjugated DDAB liposome/pDNA complex was 316±17 nm. So, the *T_f*-conjugated liposome/DNA complex was slightly larger than the unconjugated DDAB liposome/pDNA complex. Though liposomes were filtered through the membrane with the 0.2 μm pore size, the size of the liposome/pDNA complexes was found to be slightly bigger than that of the pore size off the membrane. This phenomenon is thought to be caused by aggregation of liposomes during the reaction between *T_f*-SH and MPB-PE liposome and the further following storage. Gel retardation assay was performed using various liposome : DNA ratios. When the ratio was between 5:1~10:1 (w/w), the most compact complex was formed with a slight negative zeta-potential being around -2~0 mV. More detailed physico-chemical properties of *T_f*-liposome/pDNA complexes were reported elsewhere.[9]

MTD of *T_f*-Liposome/pDNA Complex in Mice. In order to examine the maximum tolerated dose (or MTD) of *T_f*-liposome/DNA complexes in mice, healthy female ICR mice (24-28 g) were used for a single intravenous injection via tail-vein over 30 days period by weighing individual mice once a day. The percent (%) weight change on day *n* of individual mice was calculated as follows:

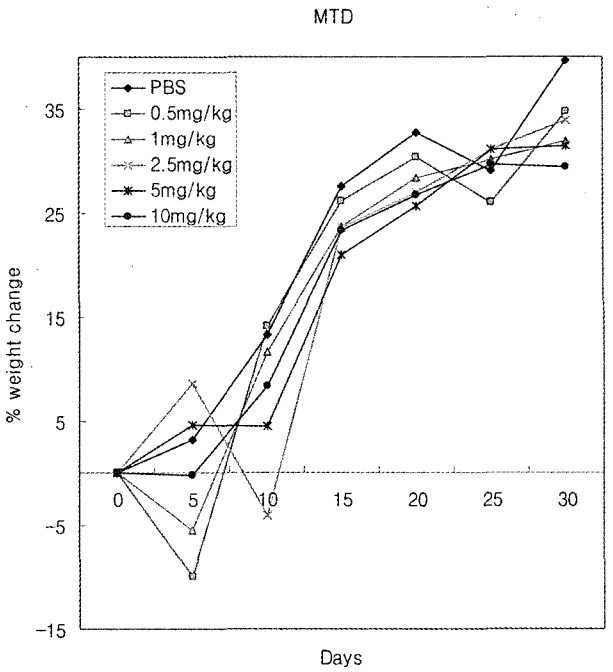

Figure 1. Determination of the maximum tolerated dose (MTD) after a single i.v. injection of *T_f*-liposome/pDNA complexes into ICR mice.

$$\%\ \text{weight change on day } n = \frac{(\text{weight on day } n - \text{weight on day } 0)}{(\text{weight on day } 0)} \times 100$$

As shown in Figure 1, no dose caused a weight loss greater than 15% of original weight at any time. The MTD of *T_f*-liposome/DNA complexes in ICR mice was thought to be higher dose than 10 mg/kg, the maximum dose used, for a single i.v. injection of the formulation.

Biodistribution Results. To study the distribution of pDNA after a single i.v. injection into the mice, RT-PCR analysis was conducted following intravenous administration of *T_f*-liposome/pDNA complexes. Total mRNA was isolated from spleen, liver, lung, heart, and kidney at various time points from 1 day to 4 weeks post-injection. Figure 2 shows a typical semi-quantitative RT-PCR result of luciferase gene from a tissue with β-actin as a standard. One day after injection, exogenously delivered pDNA was barely detected in the spleen, whereas lung showed relatively long-term detection up to 4 weeks. Spleen, liver and heart tissue showed a clear detection up to around 1 week. Semi-quantitative analysis of RT-PCR results was also performed using various amounts of standard mRNA and the results are shown in Figure 3. As shown in Figure 3, luciferase mRNA was detected in all tissues as early as 1 day after injection. The highest mRNA level was detected in the lung, followed by heart, liver and spleen. By 2 weeks post-injection, the level of mRNA decreased in all tissues except in the lung. The

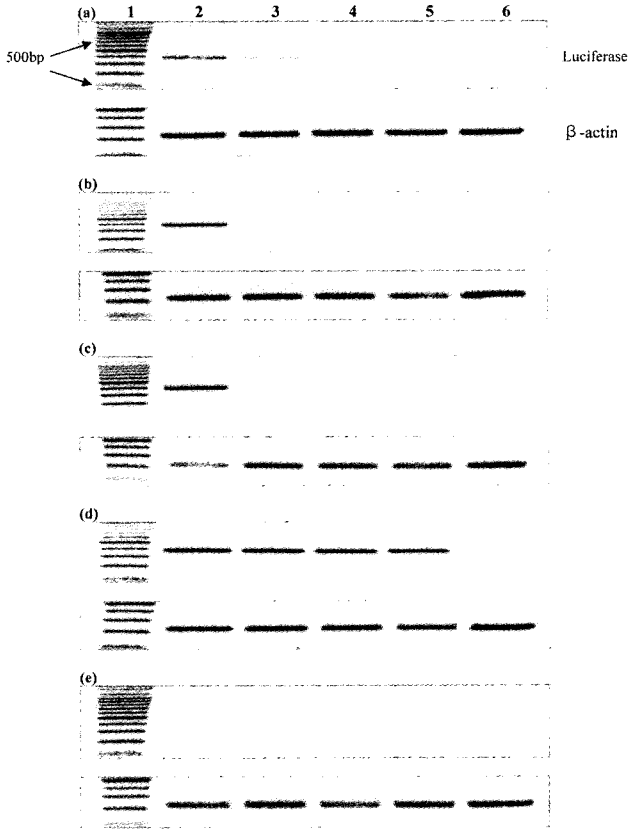

Figure 2. RT-PCR of tissues after a single i.v. injection of T_f-liposome/pDNA complexes into mice. (a) spleen, (b) liver, (c) heart, (d) lung, and (e) kidney. Lanes 1: DNA ladder, 2: 1day, 3: 1 week, 4: 2 weeks, 5: 4 weeks, and 6: control. Positive PCR product size is 388 bp (upper) and β-actin for standard is 230 bp (lower).

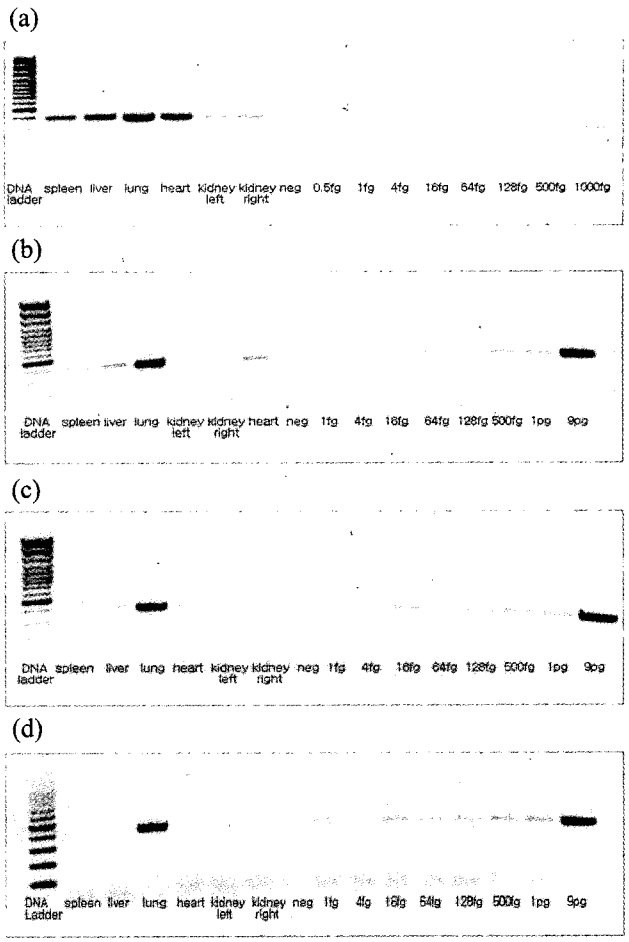

Figure 3. RT-PCR at various time points after a single i.v. injection of T_f-liposome/DNA complexes; (a) 1 day, (b) 1 week, (c) 2 weeks, and (d) 4 weeks post-injection (neg ; negative control, pg; picogram, fg; femtogram).

most rapid decrease in mRNA level occurred in the heart and liver, whereas the least decrease did in the lung. Interestingly enough, picogram levels of mRNA were still detected in the lung 4 weeks after injection.

Microarray Result. To elucidate whether the presence of cationic delivery system (T_f-liposome) might interact with host chromosomal DNA and result in any harmful effect to the host cells, cDNA microarray assay using 384 kinds of cancer- and cell cycle-related genes (DNA Tween-Chip^{TM}, Digital Genomics Inc., Seoul, Korea). As shown in Figures 4 and 5, some oncogenes, including BCL6, CCL5, AKT1, and CXADR, were found to be over expressed with normalized ratio of 7 or above. This is presumably due to the interaction of cationic complex with the anionic genetic component within the host cells.

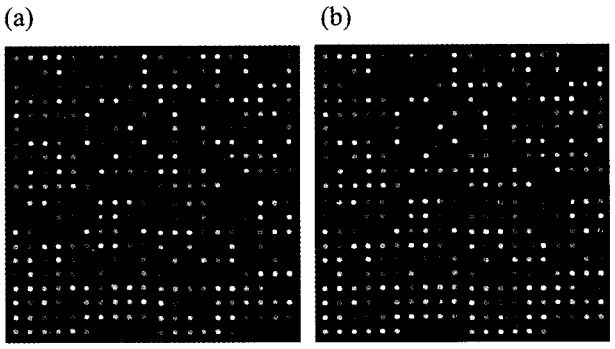

Figure 4. Image map of cDNA chip: (a) upper image and (b) lower image.

Conclusions

Success of human gene therapy using non-viral gene delivery systems lies mainly in the development of safe and efficient delivery system. Even though the DNA delivery efficiency is one of the most important factors governing the overall success, safety issue is also important when it comes to the human use. Elucidation of intracellular traf-

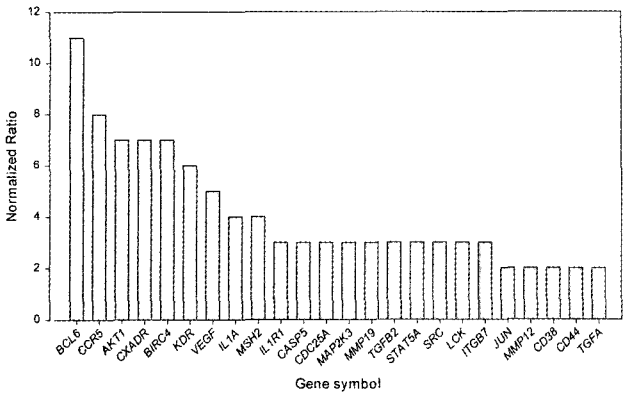

Figure 5. Normalized ratios of selected gene symbols.

ficking and the possible interaction of T_f-liposome/pDNA complexes with the endogenous components remain very important issues not only in improving transfection efficiency but in the possible toxicity (or safety) of non-viral gene delivery system. As a continuing work of previous report, we hereby report the MTD and biodistribution of T_f-liposome/pDNA complexes in ICR mice after a single i.v. injection and the possible interaction of it with the host chromosomal DNA in HeLa cells. T_f-liposome/pDNA complexes seem to be distributed throughout the major organs in mice, even though the lung was found to be the most preferred organ among tested. The i.v. injection of DNA with T_f-liposome showed that the highest level of mRNA existed in the lung at any time points. Between 1-day and 1-week, the mRNA level decreased significantly in most tissues except in the lung, where the mRNA level remained unchanged. Although the high level of mRNA was detected in heart and liver at the early time points, it decreased rapidly after 1 week. Even though liver and spleen are known to express higher level of transferrin receptors on their surface,[13] exogenously delivered DNA conjugated with T_f-liposome might be taken up by Kupffer cells and degraded without significant transgene expression.[14]

Microarray study showed that some oncogenes, such as Bcl6 and Ccr5, were found to be highly over expressed from the treatment of T_f-liposome/pDNA complexes onto the HeLa cells. As the complex exerted a net positive charge, it might be possible for the complex to interact with an anionic component within the cells, which might result in the abnormal over expression of some genes. Further investigation need to be done for more detailed nature of the interaction and the resulting over expression of such oncogenes.

Acknowledgements. This work was supported by a grant (M10414010003-04N1401-00310) from the Ministry of Sciences and Technology (MOST), Korea.

References

(1) G. Y. Wu and C. H. Wu, *J. Biol. Chem.*, **262**, 4429 (1987).
(2) E. J. Oh, J. Y. Shim, and J. S. Kim, *Macromol. Res.*, **11**, 19 (2003).
(3) S. Son, S. Y. Chae, C. Choi, M. Y. Kim, V. G. Ngugen, M. K. Jang, and J. W. Nah, *Macromol. Res.*, **12**, 573 (2004).
(4) X. Gao and L. Huang, *Gene Ther.*, **2**, 710 (1995).
(5) R. I. Mahato, A. Rolland, and E. Tomlinson, *Pharm. Res.*, **14**, 853 (1997).
(6) P. Cheng, *Human Gene Ther.*, **7**, 275 (1996).
(7) E. Wagner, D. Curiel, and M. Cotton, *Adv. Drug Del. Rev.*, **14**, 113 (1994).
(8) K. Thorstensen and I. Romslo, *Scand. J. Clin. Lab. Invest. Suppl.*, **215**, 113 (1993).
(9) S. Lee and J. S. Kim, *Arch. Pharm. Res.*, **28**, 93 (2005).
(10) F. Szoka and D. Papadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194 (1978).
(11) J. Carlsson, H. Devin, and R. Axon, *Biochem. J.*, **173**, 723 (1978).
(12) F. Martin, T. Heath, and R. New, in *Liposomes: A Practical Approach*, R. New, Ed., IRL Press, Oxford, 1990, pp 163-182.
(13) P. Ponka and C.N. Lok, *Int. J. Biochem.Cell Bio.*, **31**, 1111 (1999).
(14) R. Kirchesis, L. Wightman, A. Schreiber, and E. Wagner, *Gene Ther.*, **8**, 28 (2001).