Transferrin-Conjugated Liposome/IL-12 pDNA Complexes for Cancer Gene Therapy in Mice

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Abstract: Transferrin (T_f) has been used as a targeting ligand for delivering liposome/interleukin-12 (IL-12) pDNA complexes to cancer cells mostly due to the greater number of transferrin receptors (T_fR) found on tumor cells than on normal cells. T_f was conjugated to liposomes via the reaction of MPB-PE with thiol groups of T_f introduced by a heterobifunctional cross-linking agent, N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Four days after C-26 inoculation when the tumor volume reached ~100 mm^3, tumor-bearing Balb/c mice were injected intravenously with T_f-liposome/IL-12 pDNA complexes twice a week for 3 weeks. Significant suppression of tumor growth was achieved in the group treated with the T_f-liposome/IL-12 pDNA complexes, with a dose of 10 μg of IL-12 pDNA showing the highest suppression effect among the tested doses. Similar results were obtained when the therapy was initiated one day after tumor inoculation, although in this case 30 μg IL-12 pDNA/T_f-liposome complexes showed a significant suppression of tumor growth between 19 and 23 days after tumor inoculation. This result indicates that the transferrin receptor-targeted liposomal system is an efficient delivery agent of therapeutic genes, such as IL-12, in mice and that its potential clinical use warrants further research investigation.

Keywords: targeting, gene delivery, T_f-liposome, IL-12, C-26.

Introduction

Gene therapy has become important as a novel therapeutic candidate for human diseases,[1-3] but not a single gene delivery system is ideal for the use in clinic yet. The use of cationic liposomes as a non-viral delivery vector for gene delivery is becoming increasingly prevalent in the field of gene therapy. Even with relatively low transfection efficiency, precipitation problem and cytotoxicity in some cases, non-viral gene delivery systems have been reported easy and safe for both *in vitro* and human clinical use.[4-6]

Transfection efficiency of cationic liposomes could be increased when complexed with targeting ligands, such as transferrin (T_f), employing a receptor-mediated endocytosis mechanism.[7] Level of transferrin receptor (T_fR) is found to be elevated in various types of cancer cells,[8] and it is correlated with the aggressive or proliferative ability of tumor cells.[9] Therefore T_fR is considered to be useful as a prognos-tic tumor marker and as a potential target for chemotherapeutic drug delivery in the control of malignant cell growth.[10]

Interleukin-12 (IL-12) is a polypeptide produced predominantly by B-lymphocytes and macrophages, and it has pleiotropic effects on T-cells and natural killer (NK) cells, including stimulation of lymphokine production and promotion of cytolytic activity.[11,12] Anti-tumor effects of IL-12 have been observed both *in vitro* and *in vivo* in different murine tumor models.[13-19] Based on its biological activities, IL-12 has been examined for its capacity to induce an antitumor effect, and therapeutic activity has been observed in various murine tumor models.[20-24] The effects observed in these models include substantial growth inhibition and prolongation of survival.

We have previously reported the physico-chemical properties and *in vitro* transfection efficiency of T_f-liposome/IL-12 pDNA complexes in cell culture system.[25] Herein, we employed T_f-liposome/IL-12 pDNA complex system for cancer-targeted gene therapy in mice and investigated the

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therapeutic efficacy of T_f as a targeting ligand for C-26 bearing mice with this regard.

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). Transphosphatidylated egg phosphatidylethanolamine (TPE) was bought from Avanti Polar Lipids (Pelham, AL). Murine colon carcinoma cell line, C-26, was purchased from Korean Cell Line Bank (Seoul, Korea). Plasmid (p2CMVmIL-12) containing the coding sequences for the p35 and p40 subunits of murine IL-12 vector was kindly provided by Prof. Sung-Wan Kim at the University of Utah (Salt Lake City, UT). Bacterial strain DH5 was purchased from Qiagen (Valencia, CA). All reagents for cell culture were purchased from Gibco BRL (Grand Island, NY). All other materials were reagent grade or better.

Preparation of DDAB-Liposomes. Synthesis of maleimidophenyl butyl-phosphatidyl ethanolamine (MPB-PE) was performed using the method of Martin *et al.*.[26] Liposomes were prepared by the reverse-phase evaporation method.[27] Briefly, DDAB : Chol : MPB-PE at a molar ratio of 20 : 20 : 1 was dissolved in 1 mL of chloroform. After evaporation of solvent under argon at room temperature, the formed 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 evaporator. Liposomes were downsized by extrusion through 0.2 μm polycarbonate membranes 20~30 times using a Liposofast extrusion device (Avestin, Toronto, Canada). The whole extruder was autoclaved prior to use and all reagents and glassware used for liposome preparation were also sterilized.

Preparation of Transferrin-Conjugated Liposomes (T_f-Liposomes). Transferrin was modified to have reactive thiol groups by the method of Carlsson *et al.*.[28] Briefly, 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 derivative transferrin (PDP-T_f) was separated from reactants by gel chromatography on a Sephadex G-75 column. Concentration of separated PDP-T_f was determined spectrophotometrically at 280 nm. Then 30 μL of 1 M DTT was added to 1 mL of PDP-T_f and stirred for 30 min at room temperature. Separation of sulfhydryl-transferrin (SH-T_f) from other reactants was done by gel chromatography on a Sephadex G-75 column. The SH-T_f solution was sterilized by filtering

through a 0.2 μm pore size Nalgene membrane and conjugation was initiated by mixing equal amounts of MPB-PE-liposomes with SH-T_f overnight at room temperature. T_f-conjugated liposomes were separated from unconjugated liposomes by metrizamide flotation method with a slight modification.[29]

C-26 Tumor Model in Balb/c Mice. Six-week-old female Balb/c mice were purchased from Daehan-Biolink Co., Ltd. (Eum-Sung, Chung-Buk, Korea) and used as hosts for the C-26 murine colon carcinoma. Typically, 1×10^6 cells, which were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), were inoculated subcutaneously on each flank of a donor mouse, and four to five days after inoculation when tumor size has reached about 100 mm^3, a tumor inoculum was prepared by protease-collagenase tumor isolation method.[30] Mice were then injected subcutaneously with 1×10^6 cells per mouse and they were randomized into 7 groups, each group having 5~7 mice.

Administration of T_f-liposome/IL-12 pDNA Complexes. Treatment was initiated either four days or one day after tumor inoculation. T_f-liposome/IL-12 pDNA complexes were prepared in PBS (pH 7.4) and the complexes were injected intravenously into the tumor-bearing Balb/c mice at a dose of 3, 10, and 30 μg IL-12 pDNA per mouse. PBS and Lipofectin™/IL-12 pDNA complexes were used as the negative and positive control, respectively. Injection was performed two times a week for three weeks (6 injections in total). Tumor size (volume) was measured using a Vernier's caliper across its two perpendicular diameters, and its volume was calculated using the formula;

$$V = (SD)^2(LD)/2$$

, where SD and LD represent the smallest and the largest diameter of each tumor, respectively.

Results and Discussion

Characterization of T_f-Liposome Conjugate. Schematic diagram of transferrin-conjugated liposome is shown in Figure 1 and more detailed results from characterization and *in vitro* transfection of T_f-liposome/IL-12 pDNA complexes were reported elsewhere.[25]

4-Day Therapy Experiment. Four days after tumor inoc-
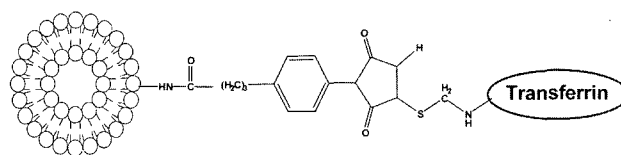
Figure 1. Schematic diagram of a transferrin-conjugated liposome.
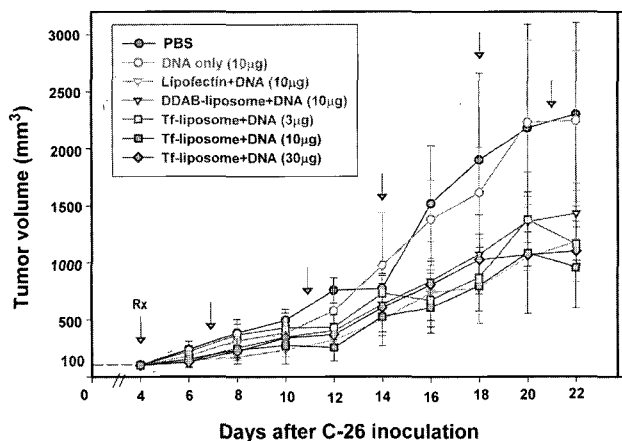
Figure 2. The 4-day therapy of C-26 tumor-bearing mice. Mice were injected intravenously, two times a week for three weeks, with various formulations of IL-12 pDNA with T_f-liposome with Lipofectin^{TM} as a control. Data is shown as mean tumor volume ± S.D.(n= 5~7).

ulation into Balb/c mice when the tumor volume reached about 100 mm^3, intravenous (i.v.) administration of various T_f-liposome/IL-12 pDNA formulations was initiated. As shown in Figure 2, free IL-12 pDNA (not as a complex form) showed no therapeutic effect up to 10 μg. However, the same amount of IL-12 pDNA complexed with T_f-liposome showed significant suppression of tumor growth and the effect was dose-dependent up to 10 μg. However, tumor suppression effect from 30 μg IL-12 pDNA complexed with T_f-liposome was slightly lower than that from 10 μg IL-12 pDNA complexed with T_f-liposome. This could be related with the organ toxicity in liver and/or spleen due to the administration of higher amount of pDNA as the mice treated with the complex of 30 μg IL-12 pDNA with T_f-liposome showed about 2-fold enlarged livers and spleens than other groups (data not shown). Even though the tumor growth was suppressed to a certain extent in all treated groups, none showed a complete regression of tumor, presumably due to the relatively large tumor size (about 100 mm^3) at the time of treatment.

Numbers of survived mice 23 days after treatment with 3 or 10 μg T_f-liposome/IL-12 pDNA complexes were much higher than those with 30 μg T_f-liposome/IL-12 pDNA complexes as shown in Table I (6 or 5 out of 6 mice vs. 2 out of 7 mice). In other words, over 90% of the mice survived when 3 or 10 μg T_f-liposome/IL-12 pDNA complexes were administered, whereas only 28% survived when 30 μg T_f-liposome/IL-12 pDNA complexes were administered. This is also speculated to be due to the organ toxicity from higher amount of pDNA administered

1-Day Therapy Experiment. With the expectation of better anti-cancer effect, it was also proposed to initiate the therapy one day, not four days, after tumor inoculation. How-

Table I. Survival of Mice from 4-Days or 1-Day Therapy Experiment

Formulations	# of Survived Mice (23 days after tumor inoculation)	
	4-day therapy	1-day therapy
PBS (control)	2/5	1/5
DNA only (10 μg)	4/5	3/5
Lipofectin^{TM}+DNA (10 μg)	1/6	4/5
liposome + DNA (10 μg)	4/6	4/5
T_f-liposome+DNA (3 μg)	6/6	5/5
T_f-liposome+DNA (10 μg)	5/6	4/6
T_f-liposome+DNA (30 μg)	2/7	3/5
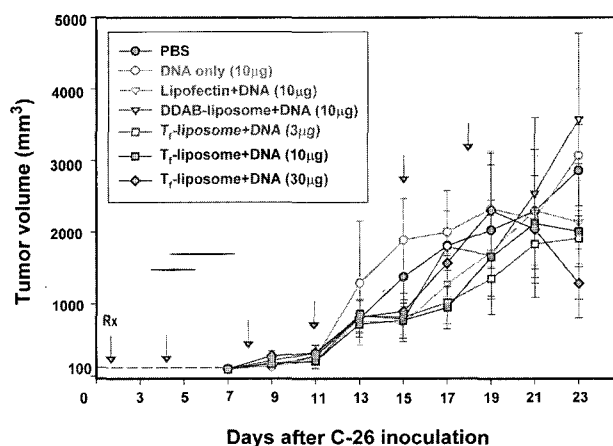
Figure 3. The 1-day therapy of C-26 tumor-bearing mice. Mice were injected intravenously, two times a week for three weeks, with various formulations of IL-12 pDNA with T_f-liposome with Lipofectin^{TM} as a control. Data is shown as mean tumor volumes ± S.D.(n= 5~7).

ever, the results from the 1-day therapy experiment were not much better than those from 4-day therapy experiment. As shown in Figure 3, the most suppression of tumor growth was obtained with 3 or 10 μg T_f-liposome/IL-12 pDNA complexes by 19 days after tumor inoculation and this result is similar to that from previously described 4-day therapy experiment. On the other hand, 30 μg T_f-liposome/IL-12 pDNA complexes resulted in significant suppression of tumor growth at later stages between 19 days and 23 days after tumor inoculation. Possible explanation for this is that one day is too short for tumor-bearing mice to establish enough tumor burden and the resulting transferrin receptors on its surface for efficient endocytosis of therapeutic materials.

Numbers of survived mice 23 days after tumor inoculation is shown in Table I and the result was similar to that from 4-day therapy experiment. As shown in Table I, treatment with

3 or 10 μg T_f-liposome/IL-12 pDNA complexes showed highest survival rate over 80%, whereas 60% survival was seen with 30 μg T_f-liposome/IL-12 pDNA complexes. Nonetheless, the overall survival rate of 1-day therapy experiment was higher than that of 4-day experiment, confirming the importance of early initiation of tumor therapy.

Conclusions

Development of safe and efficient non-viral gene delivery systems applicable to human use is one of the major huddles for the successful outcome of human gene therapy, especially when considering the unknown risks of viral gene delivery systems. We report here the use of transferrin as an efficient targeting ligand for gene delivery using cationic liposome as a carrier. As tumor cells replicate more rapidly than the normal ones, they need to uptake more iron ions in the form of transferrin through transferrin receptors. For this reason, transferrin has been used in targeted drug delivery systems for a long time and proved to be safe and efficient in this regard. We have successfully conjugated transferrin to maleimidophenyl butyl-phosphatidyl ethanolamine (MPB-PE) using heterobifunctional cross-linking agent (SPDP) via thio-ether linkage and liposomes were prepared using T_f-conjugated MPB-PE and cationic DDAB lipid. Among the physico-chemical properties of T_f-liposome and/or its DNA complex include the size, shape, zeta-potential and gel retardation properties as reported elsewhere.[25] Significant suppression of tumor growth was achieved by treatment with 10 μg T_f-liposome/IL-12 pDNA complexes among tested both in 4-day therapy and 1-day therapy experiment. The survival rate was also highest at 10 μg T_f-liposome/IL-12 pDNA complexes, though 1-day therapy experiment exerted better results than 4-day therapy one.

In conclusion, T_f-conjugated DDAB liposome could be useful in targeted gene delivery *in vivo* and further studies will open the opportunity for this system to be in humans in the future.

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