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Control of solid tumor growth in mice using EGF receptor targeted RNA replicase-based plasmid DNA

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Abstract

Previously, it was shown that treatment of tumor-bearing mice with an RNA replicase-based plasmid that produces double-stranded RNA when transfected into tumor cells significantly inhibited the tumor growth. In the present study, the feasibility of further improving the anti-tumor activity of the RNA replicase-based plasmid by targeting it into tumors cells was evaluated. An epidermal growth factor (EGF)-conjugated, PEGylated cationic liposome was developed to deliver the RNA replicase-based plasmid, pSIN- β , into EGFR-over-expressing human breast cancer cells (MDA-MB-468) *in vitro* and *in vivo*. Delivery of the pSIN- β using the EGF receptor-targeted liposome more effectively controlled the growth of MDA-MB-468 tumors (and human epidermoid carcinoma A431 tumors) in mice than using un-targeted liposome. The pSIN- β carried by the EGFR-targeted liposome caused the complete regression of MDA-MB-468 tumors in mice, likely due to the enhancement of its pro-apoptotic, anti-proliferative, and anti-angiogenic activities. Tumor-targeted RNA replicase-based plasmid holds a strong potential in tumor therapy.

Keywords

Apoptosis; plasmid uptake; liposomes; dsRNA therapy; breast tumor; epidermoid carcinoma

Cancer therapy has improved dramatically over the past few years, but chemotherapy remains commonly used in clinics. Traditional chemotherapeutic agents generally have a single tumor-killing mechanism, making it easier to develop resistance and requiring multiple chemotherapeutic agents for combination therapy. An ideal chemotherapy agent would be highly selective for cancer cells and utilize multiple killing mechanisms to ensure tumor cell death.

It is known that double-stranded (dsRNA) molecules have multiple direct and indirect pro-apoptotic, anti-proliferative, and anti-angiogenic activities [1–3]. Moreover, dsRNA is a known agonist of Toll-like receptor 3 (TLR 3), and the interaction between dsRNA and TLR3 triggers both innate and adaptive immunities [4–6]. Double-stranded RNA is also a potent inducer of type I interferons (IFN- α/β), which are pro-apoptotic and anti-angiogenic

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as well [3, 7]. For decades, synthetic dsRNA, such as polyriboinosinic-polyribocytidylic acid (poly (I:C)), had been evaluated in preclinical and clinical trials for cancer therapy [1, 2, 7–12], but the severe adverse effects from high doses of systemic dsRNA limited its clinical application [2, 9, 10, 13, 14]. Therefore, novel strategies are sought to more effectively take advantage of the multiple anti-tumor mechanisms of dsRNA, while minimizing its adverse effects.

Data from recent studies showed that intracellular dsRNA is more effective than extracellular dsRNA in promoting cells to undergo apoptosis and in orchestrating adaptive immune responses [5, 15, 16]. Previously, we proposed and demonstrated the feasibility of using an RNA replicase-based plasmid to generate dsRNA intracellularly in the transfected cells to control tumor growth in culture and in mice [17]. The Sindbis viral RNA replicase-based plasmid, pSIN- β , contains cytomegalovirus promoter-driven non-structural protein genes (nsp1–4) that encode the Sindbis viral RNA replicase complex. The β -galactosidase gene in the pSIN- β is driven by a viral subgenomic promoter [17, 18]. Previous data showed that transfection of cells with a similar alphavirus RNA replicase-based plasmid appeared to mimic cell infection by an alphavirus, because the RNA replicase mediated the production of dsRNA intracellularly [4, 19]. In a previous study, we showed that treatment of tumor-bearing mice with the pSIN- β plasmid complexed with cationic liposomes peritumorally significantly inhibited the growth of model mouse tumors pre-established in mice and, in many cases, caused complete tumor regression [17], suggesting the use of RNA-replicase based plasmid as a novel tumor molecular therapy.

In the present study, in order to further improve the specific killing of tumor cells by the dsRNA produced by the pSIN- β plasmid, we tested the feasibility of targeting the pSIN- β plasmid into tumors cells that over-express EGFR. EGFR is over-expressed in many cancer cells including glioblastoma, breast cancer, colorectal, head and neck cancer [20]. For example, Klign *et al.* found that, depending on the method of detection, EGFR was over-expressed in 14–90% of human breast cancer cells with a mean percentage of EGFR positivity of 45% (5,232 patents) [21]. EGFR consists of an extracellular ligand-binding domain, a single transmembrane spanning region, and an intracellular region containing a kinase domain [20]. EGFR over-expression is involved in the control of tumor cell proliferation, metastasis, and angiogenesis [20]. Various EGFR targeting agents have been developed, including those targeting the extracellular domain (e.g., anti-EGFR MAb 225) and tyrosine kinase inhibitors (e.g., ZD1839 (Iressa)) [22, 23]. Clinical data of such EGFR targeting agents have been promising, but they are highly dependent on intact EGFR [20]. We intended to utilize the over-expressed EGFR as the target to more specifically deliver the pSIN- β plasmid into tumor cells by complexing it with cationic liposomes surface-conjugated with EGF, a known ligand to EGFR. Previously, it was shown that EGF conjugated onto liposomes facilitated the internalization of the liposomes by EGFR-over-expressing tumor cells [24].

Materials and methods

Plasmids and cells

Plasmid pSIN- β was kindly provided by Dr. Richard Weiss (University of Salzburg, Salzburg, Austria). The pEGFP C1 plasmid was obtained from Addgene, Inc. (Cambridge, MA). Plasmids were amplified in *E. coli* DH5 α under selective growth conditions and purified using a QIAGEN midiprep kit (Valencia, CA) according to the manufacturer's instruction. Large scale plasmid purification was performed by GenScript (Piscataway, NJ). Human breast adenocarcinoma cells (MDA-MB-468, # HTB-132, MDA-MB-231, # HTB-26, MCF-7, # HTB-22) and human epidermoid carcinoma cells (A431, # CRL-1555) were from the American Type Culture Collection (ATCC) and cultured in DMEM medium

(Invitrogen, Carlsbad, CA). EL4/PSA cells, kindly provided by Dr. Pavel Pisa in the Karolinska Hospital Institute (Stockholm), were cultured in DMEM medium as well (Invitrogen). All media was supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μ g/ml of streptomycin (all from Invitrogen). It was shown previously that EGFR expression was not detectable in EL-4 cells [25]. The density of EGFR on MDA-MB-468, MDA-MB-231, and MCF-7 cells was reported to be 1×10^6 , $1-2 \times 10^5$, and 1×10^4 per cell, respectively [26–28].

Construction of pSIN-EGFP plasmid

To construct pSIN-EGFP plasmid, the enhanced green fluorescent protein (EGFP) gene from the pEGFP C1 plasmid was PCR-amplified with primers EGFP F 5'-ACAAGTTCTAGAATGGTGAGCAAGGGCGAG-3' and EGFP R 5'-CCTAGAGCATGCTTACTTGTACAGCTCGTC-3'. The PCR product was digested with *Xba*I and *Sph*I, and the EGFP gene was used to replace the β -galactosidase gene downstream of the subgenomic promoter in the pSIN- β . Positive clones were screened for insert using restriction digestion and further confirmed by DNA sequencing (ABI 3730XL DNA analyzer, Applied Biosystems, Foster City, CA).

Preparation of EGF-conjugated, PEGylated liposomes (EGF-PEG-liposomes)

Cationic liposomes were prepared using cholesterol (Sigma-Aldrich, St. Louis, MO), egg phosphatidylcholine (Avanti Polar Lipids, Inc, Alabaster, AL), and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti) at a molar ratio of (4.6:10.8:12.9). Lipids were mixed in a 20-ml glass scintillation vial followed by solvent evaporation and the formation of a thin film by placing the vial under a constant stream of nitrogen gas. HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (20 mM, 150 mM NaCl, pH 7.4) was added to the film and incubated at room temperature with intermittent vigorous vortexing for 6–24 h. The liposome suspension was forced through polycarbonate filters (1.0, 0.4, and 0.1 μ m, sequentially) using a mini-extruder (Avanti) [29]. The final concentration of DOTAP in the liposomes (DOTAP liposomes) was 10 mg/ml.

Recombinant murine EGF was from Peprtech Inc. (Rocky Hill, New Jersey). Prior to the conjugation, EGF was thiolated with 2-iminothiolane (Traut's reagent, Sigma-Aldrich). Protein was diluted in PBS (0.01 M with 2.5 mM EDTA, pH 8.0), followed by the addition of Traut's reagent (20 X molar excess). The mixture was incubated for 1 h at room temperature. The protein was purified/desalted using a PD10 column (GE Biosciences, Piscataway, NJ). Thiolated EGF (0.5 mg) was mixed with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol) 2000] (DSPE-PEG(2000)-maleimide micelles) (Avanti) at a molar ratio of 8.3:568 in PBS (0.1 M, pH 7.4). The mixture was stirred under nitrogen gas for 24 h at room temperature, mixed with an equal volume of DOTAP liposomes, and incubated at 60 °C for 1 h. Un-conjugated proteins were removed by gel permeation chromatography (GPC, Sepharose® 4B, 5 $\times$ 135 mm). The EGF protein concentration in the final EGF-conjugated, PEGylated liposomal preparation (EGF-PEG-liposomes) was determined using a CBQCA Protein Quantitation Kit (Invitrogen). DOTAP concentration in the liposomes was determined following a previously reported method [30]. Briefly, in the presence of chloroform, methyl orange (Fisher Scientific, Pittsburgh, PA) reacts with DOTAP, a cationic lipid, to form chloroform-soluble, yellow colored complexes. The intensity of the yellow color in chloroform is proportional to the concentration of methyl orange/cationic lipid complexes measured spectrophotometrically at 415 nm [30]. EGF-free PEGylated liposomes (PEG-liposomes) were prepared similarly by mixing the equivalent amount of DSPE-PEG(2000)-maleimide micelles with preformed DOTAP liposomes.

The particle size and zeta potential of the cationic liposomes, PEG-liposomes, and EGF-PEG-liposomes were measured using a Malvern Zetasizer® Nano ZS (Westborough, MA).

Preparation of plasmid-liposome complexes (lipoplexes) and their sensitivity to DNase I

The plasmid-liposome lipoplexes were prepared by mixing equal volumes of plasmid DNA (25 µg) in solution and various amounts of liposomes in suspension [17]. The mixture was incubated at room temperature for at least 15 min before further use. To evaluate the extent to which the plasmid DNA was protected from DNase digestion by complexing with the liposomes, the lipoplexes were incubated with 2 U/µg of DNase I (Fermentas, Glenn Burnie, MD) in a total volume of 400 µl in Tris-HCl buffer (pH 7.5, 10 mM) containing MgCl₂ (2.5 mM). Samples were placed at 37 °C for 1 h, and the reaction was stopped by 10 min of incubation at 60 °C in the presence of EDTA (5 mM). The DNA and liposomes were disassociated from each other by incubation (2 h) at room temperature in the presence of NaCl (2 M). DNA was extracted using phenol/chloroform and analyzed by electrophoresis using 1% agarose gel stained with ethidium bromide [31]. Band intensities were quantified using the GeneSnap software from Syngene G-box (Syngene, Frederick, MD).

Plasmid DNA uptake assay

Cells (1×10^5) were seeded in 24-well plates ($n = 6-12$) and incubated at 37 °C, 5% CO₂ for 24 h or until 60% confluency. The pSIN-β plasmid was labeled using a *LabelIT*® fluorescein nucleic acid labeling kit (Mirus, Madison, WI) according to the manufacturer's instruction. Freshly labeled pSIN-β (0.75 µg) was complexed with the EGF-PEG-liposomes or the PEG-liposomes (DOTAP, 12.9 µg) and incubated for at least 15 min at room temperature. The resultant lipoplexes were added to each well and incubated for 1 h at 37 °C, 5% CO₂. Cells were washed with PBS and lysed using Triton X-100 (0.5% in 20 mM Tris, 100 mM NaCl, and 1 mM EDTA) following by incubation at -80 °C for 1 h. The fluorescence intensity was measured at 492/518 nm in a black bottom plate using a BioTek Synergy® Multi-Mode Microplate Reader (Winooski, VT). To understand whether the uptake of the lipoplexes was mediated by the EGF-EGFR interaction, cells were pre-incubated with free EGF (0.1 mg/ml) at 37 °C, 5% CO₂ for 1 h before the addition of the lipoplexes.

Plasmid DNA uptake detected by fluorescence microscopy

MDA-MB-468 or MCF-7 cells (2×10^6) were seeded on poly-D-lysine-coated glass coverslips and incubated in 6-well plates at 37 °C, 5% CO₂ for 24 h. Cells were further incubated in the presence of fluorescein-labeled pSIN-β/EGF-PEG-liposome lipoplexes or PEG-liposome lipoplexes (DNA:DOTAP, 3.75 µg:64.7 µg) in reduced growth medium for 1 h at 37 °C. After the incubation, cells were washed twice with PBS and fixed in 3% paraformaldehyde for 20 min at room temperature. Cells were washed with PBS three times, and coverslips were mounted on slides using a mounting medium (vectashield H-1200 with 4',6-diamidino-2-phenylindole (DAPI)) from Vector laboratories (Burlingame, CA). Cells were viewed using an Olympus BX60 Microscope (Olympus America, Inc., Center Valley, PA).

In vitro cell transfection and apoptosis assay

MDA-MB-468 cells (1×10^7) were seeded and incubated at 37 °C, 5% CO₂ for 24 h or until 60% confluency followed by transfection using pEGFP C1 or pSIN-EGFP (40 µg) complexed with Lipofectamine® (Invitrogen). After 24 h incubation at 37 °C, 5% CO₂, cells were detached using 0.05% trypsin/EDTA and re-suspended in PBS with 2% FBS. GFP positive cells were sorted using a FACSAria II Cell Sorter (BD Biosciences, San Jose, CA), re-suspended in fresh medium, and seeded into a 96-well plate (5,000 cells per well). As a

control, un-transfected cells were also passed through the cell sorter. Cells were stained 0 and 72 h later using a Guava Nexin kit, which contained annexin V and 7-amino actinomycin D (7-AAD), according to the manufacturer's instruction and analyzed using a Guava EasyCyte 8HT Flow Cytometry System (Millipore, Hayward, CA). GFP positive cells were gated and analyzed for annexin V and 7-AAD staining. Analysis was performed using the FlowJo Flow Cytometry Analysis Software (Ashland, OR).

Animal studies

All animal studies were carried out following National Institutes of Health guidelines for animal care and use. Animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Female athymic nu/nu mice (6–8 weeks) were from Charles River laboratories, Inc. (Wilmington, MA). Mice were subcutaneously injected in the right flank with MDA-MB-468 or A431 cells (1×10^7) admixed with BD Matrigel™. When tumors reached an average diameter of 5 mm for the MDA-MB-468 cells and 6.5–7 mm for the A431 cells, the pSIN-β/EGF-PEG-liposome lipoplexes or the pSIN-β/PEG-liposome lipoplexes (DNA:DOTAP, 25:431 μg) were injected subcutaneously peritumorally (s.c., p.t.) for 14 consecutive days [17]. Tumor size was measured using a digital caliper, and tumor diameter was calculated using the following equation: tumor diameter = (length + width)/2.

Hematoxylin and eosin (H&E) staining

Tumors were fixed in formalin and embedded in paraffin prior to sectioning. Sections of 7 μm were cut and stained with H&E.

Immunohistochemical staining for EGFR, CD31, and Ki67 and TUNEL assay

MDA-MB-468 tumors were harvested from mice that had been treated for 10 d with the pSIN-β/EGF-PEG-liposome or pSIN-β/PEG-liposome lipoplexes. Tumors were fixed in formalin, embedded in paraffin, and sectioned. After deparaffinization, section was subjected to antigen retrieval by microwaving in sodium citrate (10 mM, pH 6.0) for 20 min, washed in PBS with 0.2% Triton-X 100, and incubated with PBS with 0.1% Tween 20, 5% horse serum albumin (HSA) for 1 h. Slides were incubated for 1 h at 37 °C with anti-EGFR-Alexa fluor 488 (Millipore, diluted 1/1,000) in 5% HSA in PBS. After incubation, slides were washed with PBS. Terminal deoxynucleotidyltransferase dUTP nick end-labeling (TUNEL) analysis was performed with an *in situ* cell death detection kit from Roche Diagnostics (Indianapolis, IN). Briefly, the TUNEL reaction mix (50 μl) was placed on the sections and incubated for 60 min at 37 °C. Sections were washed with PBS three times, mounted using the vectashield H-1200 mounting medium, and analyzed using an Olympus BX60 Microscope. Moreover, tumor sections were also stained with antibodies against Ki67 and CD31 as markers of cell proliferation and angiogenesis, respectively, in the Histology Core at the University of Texas M.D. Anderson Center Science Park Research Division (Smithville, TX). Slides were examined under a bright-field microscope. The number of blood vessels per 14.6 mm² (n = 13–37 per treatment) and the percent of Ki67 positive cells in an area of 0.04 mm² (n = 9 per treatment) were determined.

Statistical analysis

Statistical analyses were completed using ANOVA followed by the Fisher's protected least significant difference procedure. Mouse survival curves were compared using the Kaplan–Meier survival analysis (GraphPad Prism®, La Jolla, CA). A p-value of 0.05 (two-tail) was considered statistically significant.

Results

Preparation and characterization of pSIN- β /EGF-PEG-liposome lipoplexes

EGF-conjugated, PEGylated cationic liposomes (EGF-PEG-liposomes) were prepared by mixing cationic DOTAP liposomes with EGF-conjugated DSPE-PEG(2000) micelles followed by 1 h of incubation at 60 °C. After the removal of the un-conjugated EGF, the concentrations of the EGF and DOTAP in the EGF-PEG-liposomes were determined to be $1.94 \pm 0.08 \mu\text{g/ml}$ and $6.9 \pm 2.0 \text{ mg/ml}$, respectively. The EGF-free, PEGylated cationic liposomes (PEG-liposomes) were prepared similarly except that an equivalent amount of DSPE-PEG(2000) micelles were not conjugated with EGF before they were mixed with the preformed cationic DOTAP liposomes. The diameter and zeta potential of the EGF-PEG-liposomes were $143 \pm 1 \text{ nm}$ and $2.2 \pm 0.1 \text{ mV}$. The PEG-liposomes were $134 \pm 2 \text{ nm}$, with a zeta potential of $13.3 \pm 0.7 \text{ mV}$. The liposomes were then complexed with the pSIN- β plasmid to form lipoplexes.

The following experiments were completed to identify the optimal ratio of pSIN- β to liposomes in the lipoplexes. Various amounts of EGF-PEG-liposomes were complexed with a fixed amount of pSIN- β to form different pSIN- β /EGF-PEG-liposome lipoplexes. The particle diameters and the zeta potentials of the resultant lipoplexes are shown in Fig. 1A. At the ratio of 8.63:1 (DOTAP vs. pSIN- β , w/w), the lipoplexes appeared unstable and aggregated (Fig. 1A), whereas lipoplexes prepared at other ratios had a smaller size of around 200 nm (Fig. 1A). The zeta potential of the lipoplexes peaked at the DOTAP lipid to DNA ratio of 8.63:1 as well (Fig. 1A).

Shown in Fig. 1B is the fraction of undigested (intact) pSIN- β plasmid when complexed with increasing amount of EGF-PEG-liposomes, and then digested with DNase I. As expected, increasing the ratio of the liposomes to plasmid DNA protected more DNA from DNase I digestion. At the ratio of 17.25:1 (DOTAP vs. pSIN- β), about 70% of the pSIN- β was protected from the DNase I digestion (Fig. 1B). At the ratios of 34.5:1 and 86.25:1, almost 100% of the pSIN- β was protected from DNase I digestion.

In order to identify the liposomes (or DOTAP lipid) to DNA ratio that was optimal for transfecting the pSIN- β into tumor cells, the uptake of the pSIN- β in various lipoplexes by the MDA-MB-468 cells was evaluated, and the ratio of 17.25:1 was found optimal based on the highest level of cellular uptake of the fluorescein-labeled pSIN- β at that ratio (Fig. 1C). The 17.25:1 ratio was the optimal ratio for the PEG-liposomes as well (Fig. 1C). Therefore, the lipoplexes prepared at the DOTAP to pSIN- β ratio of 17.25:1 (w/w) were used for further studies. Shown in Fig. 1D are the diameters and zeta potentials of the PEG-liposomes and the EGF-PEG-liposomes, before and after complexing with the pSIN- β plasmid at the DOTAP to DNA ratio of 17.25:1 (w/w). For comparison, the diameter and zeta potential of the un-PEGylated DOTAP liposomes are also included (Fig. 1D).

In vitro uptake of the pSIN- β in lipoplexes by cells expressing different levels of EGFR

The uptake of fluorescein-labeled pSIN- β by cells expressing different levels of EGFR was evaluated after the cells were incubated for 1 h with lipoplexes prepared with the EGF-PEG-liposomes or the PEG-liposomes. The extent to which the pSIN- β was taken up by the EL-4 cells was not dependent on whether the pSIN- β was in the lipoplexes prepared with EGF-PEG-liposomes or with the EGF-free PEG-liposomes, as indicated by the ratio of 1 in Fig. 2A. It was previously reported that EGFR expression was not detected in EL-4 cells [25]. However, in the cells that express various levels of EGFR, the ratio of the uptake of the pSIN- β in the EGF-PEG-liposome lipoplexes over that in the PEG-liposome lipoplexes was correlated to the EGFR density on the cells. The ratio was the largest in the MDA-MB-468 cells, followed by the MDA-MB-231 cells, and then by the MCF-7 cells (Fig. 2A). It is

known, and our RT-PCR data also confirmed, that the relative expression of the EGFR in the cell lines was MDA-MB-468 > MDA-MB 231 > MCF-7 (data not shown).

To further investigate whether the uptake of the pSIN- β /EGF-PEG-liposome lipoplexes was mediated by the binding of the EGF on the lipoplexes to the EGFR on cell surface, cells were incubated with free EGF for 1 h prior to the addition of the lipoplexes. The uptake of the pSIN- β in the EGF-PEG-liposome lipoplexes was significantly inhibited in the MDA-MB-468 cells (Fig. 2B), but not in the MCF-7 cells (Fig. 2C). Moreover, the uptake of the pSIN- β in the PEG-liposome lipoplexes by both MDA-MB-468 cells and MCF-7 cells was not significantly affected by pre-incubation of the cells with free EGF (Fig. 3C).

Finally, the fluorescent microscopic images in Figure 3 showed that after 1 h of incubation, the uptake of the fluorescein-labeled pSIN- β in the EGF-PEG-liposome lipoplexes by the MDA-MB-468 cells was more extensive than by the MCF-7 cells. However, the uptake of pSIN- β was significantly less in both cell lines when it was complexed with the EGF-free PEG-liposomes (Fig. 3).

The pSIN- β plasmid was more effective at controlling the growth of MDA-MB-468 tumors in mice when complexed with the EGF-PEG-liposomes than with the EGF-free, PEG-liposomes

The anti-tumor activities of the pSIN- β complexed with the EGF-PEG-liposomes or the EGF-free, PEG-liposomes against MDA-MB-468 tumors were evaluated *in vivo*. As shown in Fig. 4, the pSIN- β /EGF-PEG-liposome lipoplexes were more effective than the pSIN- β /PEG-liposome lipoplexes at controlling the tumor growth. Starting on day 15, tumors in mice that were treated with the pSIN- β /EGF-PEG-liposome lipoplexes became significantly smaller than in mice that were treated with the pSIN- β /PEG-liposome lipoplexes (Fig. 4A). On day 76, tumors in all mice that were treated with the pSIN- β /EGF-PEG-liposome lipoplexes regressed completely. No tumor mass was visible or palpable thereafter. Tumors in mice that were treated with the pSIN- β /PEG-liposome lipoplexes partially regressed. The median survival time for mice treated with sterile PBS was 57 d, 161 d for mice that were treated with the pSIN- β /PEG-liposome lipoplexes (Fig. 4B).

Greater pro-apoptotic, anti-proliferative, and anti-angiogenic activities were detected in tumors in mice that were treated with pSIN- β complexed with the EGF-PEG-liposomes than with the PEG-liposomes

MDA-MB-468 tumors in mice were treated for 10 d with pSIN- β /EGF-PEG-liposome lipoplexes or PEG-liposome lipoplexes; tumors were harvested 24 h after the last treatment for histological assays. Extensive apoptosis (i.e., TUNEL positive staining) was detected in tumors in mice that were treated with the pSIN- β /EGF-PEG-liposome lipoplexes, and the apoptotic cells were co-localized with EGFR positive cells (Fig. 5). In contrast, significantly fewer apoptotic cells were detected in tumors in mice that were treated with the pSIN- β /PEG-liposome lipoplexes or with sterile PBS (Fig. 5). Data in Fig. 6 showed that the pSIN- β also induced apoptosis when transfected into MDA-MB-468 cells in culture. The pSIN-EGFP, instead of pSIN- β , was used because the EGFP gene in the pSIN-EGFP allowed the sorting and gating of the transfected cells based on the expression of the EGFP gene. Cells were transfected with pEGFP C1 or pSIN-EGFP plasmids. Twenty-four hours later, GFP positive cells were sorted and cultured for 72 hours. Cells were stained with annexin V and 7-AAD to determine the extent of apoptosis at 0 and 72 h. At 0 h, the percent of viable cells, cells in the early apoptotic stage, or in the late apoptotic stage were not different regardless of the plasmid used for transfection. However after 72 h, all GFP positive cells transfected with the pSIN-EGFP plasmid were in the late apoptotic stage, whereas the percent of viable

GFP positive cells that were transfected with the pEGFP C1 plasmid increased by almost three-fold (Fig. 6B).

Typical H&E images of MDA-MB-468 tumors after treatment with PBS, pSIN- β complexed with PEG-liposomes, or pSIN- β complexed with EGF-PEG-liposomes were shown in Fig. 7A (H&E). A moderate regression of tumors was observed in mice that were treated with pSIN- β complexed with the PEG-liposomes, while a significant regression of tumors was observed in mice that were treated with pSIN- β complexed with the EGF-PEG-liposomes (Fig. 7A, H&E). Tumors in mice that were treated with PBS exhibited a high level of Ki67 positive staining, a marker of cell proliferation (Fig. 7A); and a lower percent of Ki67 positive cells was detected in tumors in mice that were treated with pSIN- β complexed with the PEG-liposomes. However, the percent of Ki67 positive cells in tumors in mice that were treated with pSIN- β complexed with the EGF-PEG-liposomes was significantly lower ($> 50\%$) than that in mice that were treated with pSIN- β complexed with the PEG-liposomes ($p < 0.001$) (Figs. 7A, B). CD31 is a marker of endothelial cells. Anti-CD31 staining revealed extensive vascularization in tumors in mice that were treated with PBS (Fig. 7A). The extent of CD31 positive staining was decreased in tumors in mice that were treated with the pSIN- β /PEG-liposome lipoplexes, relative to that in mice that received PBS ($p = 0.003$) (Fig. 7C). Finally, a significantly lower extent of CD31 positive staining was detected in tumors in mice that were treated with pSIN- β complexed with the EGF-PEG-liposomes than with the PEG-liposomes ($p < 0.001$) (Figs. 7A, C).

The pSIN- β plasmid was more effective against the A431 tumors in mice when complexed with the EGF-PEG-liposomes than with the EGF-free PEG-liposomes

It was reported that A431 cells over-express EGFR as well ($1-3 \times 10^6$ per cell) [32]. When left untreated, A431 tumors grew aggressively in nude mice, with a median mouse survival time of 17 days (Fig. 8). A431 tumor-bearing mice that were treated with the pSIN- β /PEG-liposome lipoplexes had a median survival time of 15 days (Fig. 8). However, the median survival time for A431 tumor-bearing mice that were treated with the pSIN- β /EGF-PEG-liposome lipoplexes was 28 days, significantly longer than the median survival time of mice that were treated with the pSIN- β /PEG-liposome lipoplexes ($p = 0.017$) (Fig. 8).

Discussion

Previously, it was shown that pSIN- β , a plasmid DNA that encodes the Sindbis viral RNA replicase complex (non-structure proteins 1–4) had anti-tumor activity, likely due to the pro-apoptotic dsRNA generated by the RNA replicase complex expressed in tumor cells transfected with the plasmid [17]. The ability of the dsRNA and the CpG motifs on the plasmid to activate innate immunity likely also contributed to the observed anti-tumor activity [17, 33, 34]. In the present study, the hypothesis that actively targeting the RNA replicase-based plasmid into tumor cells will further improve the anti-tumor activity of the plasmid was tested. It was shown that targeting the pSIN- β plasmid into tumors that over-express the EGFR significantly improved its anti-tumor activity, likely because the targeted pSIN- β induced more tumor cell to undergo apoptosis and exhibited enhanced anti-proliferative and anti-angiogenic activities.

It is well documented that dsRNA has anti-tumor activities. However, data in clinical trials showed that the anti-tumor activity from systemically dosed synthetic dsRNA was weak, inconsistent, and associated with severe adverse effects [2, 9, 10, 14]. Recently, interest in further exploring the anti-tumor activity of dsRNA is revived, largely due to the findings that when delivered intracellularly, dsRNA induced more extensive tumor cell death, and can more effectively activate innate immunity and orchestrate the induction of adaptive immune responses [5, 15, 16, 35]. Instead of utilizing synthetic dsRNA, we decided to

deliver a plasmid DNA that encodes the Sindbis viral RNA replicase genes, and thus enable the transfected cells to produce dsRNA. It is expected that dsRNA tumor therapy using the RNA replicase-based plasmid DNA has the following advantages: (i) Plasmid DNA is relatively more stable than dsRNA; (ii) Plasmid DNA purified from bacteria has an inherent anti-tumor activity, due to the immuno-stimulatory activity of the unmethylated CpG motifs on the plasmid [33, 34]; and (iii) only cells that are transfected with the RNA replicase-based plasmid will produce dsRNA, and thus being exposed to the dsRNA. The fact that only cells transfected with the RNA replicase-based plasmid will produce dsRNA is important, because the dsRNA is generated endogenously and is expected to be cytotoxic mainly to the cells with the intracellular dsRNA. In other words, delivering RNA replicase-based plasmid DNA into tumor cells can potentially take advantage of the potent anti-tumor activity of intracellular dsRNA, while minimizing the adverse effects caused by the direct injection of synthetic dsRNA. The present study was devised to actively target the RNA replicase-based plasmid into tumor cells to more specifically allow the tumor cells to take up the plasmid, generate intracellular dsRNA, and then undergo apoptosis by committing “suicide”.

EGFR is expressed universally, but over-expressed (10–1000-fold) in many tumor cells [36, 37]. It has been exploited as a target to develop a number of novel tumor therapeutics (e.g., Gefitinib and Cetuximab) [22, 23]. EGFR was also used as a target to facilitate the internalization of liposomes surface-conjugated with an EGFR ligand (such as the EGF) by tumor cells that over-express EGFR [24, 38]. In the present study, EGF was chemically conjugated onto the cationic liposomal carrier of the pSIN- β plasmid to target the pSIN- β into EGFR-over-expressing human tumor cells *in vitro* and *in vivo*. Murine EGF was used because the *in vivo* studies in the present study were carried out in mice, and data from previous studies showed that murine EGF has similar biological activities as the human EGF [39]. Human breast adenocarcinoma cells MDA-MB-468, MDA-MB-231, and MCF-7 were used because they express different levels of EGFR (1×10^6 , 2×10^5 , and 1×10^4 per cell, respectively) [26, 27]. *In vitro* data showed that the EGF-conjugated cationic liposomes more effectively delivered the pSIN- β plasmid into EGFR-expressing tumor cells than the EGF-free liposomes, and the extent to which the tumor cells took up the pSIN- β carried by the EGF-conjugated liposomes was correlated to the density of the EGFR on the cell surface. The facilitated uptake of the pSIN- β plasmid in the EGF-conjugated liposomes by the EGFR-over-expressing tumor cells was mediated by the EGF-EGFR interaction, because pre-incubation of the tumor cells with free EGF significantly inhibited the uptake of the plasmid (Fig. 2), whereas pre-incubation with free EGF did not significantly affect the uptake of the pSIN- β plasmid complexed with the EGF-free PEG-liposomes (Figs. 2B, C). It was noticed that the uptakes of the pSIN- β /PEG-liposome lipoplexes by different cells were not identical. For example, a comparison of data in Fig. 2B and Fig. 2C revealed that the uptake of the pSIN- β /PEG-liposome lipoplexes by the MCF-7 cells was higher than the uptake of the same pSIN- β /PEG-liposome lipoplexes by the MDA-MB-468 cells and the MDA-MB-231 cells (data not shown). The pSIN- β /PEG-liposome lipoplexes are physically nanoparticles; the extent to which a specific cell line can take up them is likely determined mainly by the cell's endocytosis activity.

In athymic mice with pre-established human MDA-MB-468 tumors, treatment with the pSIN- β carried by the EGF-conjugated liposomes more effectively controlled the tumor growth than with the same pSIN- β carried by EGF-free liposomes (Fig. 4). In fact, the EGFR-targeted pSIN- β completely eliminated the MDA-MB-468 tumors in all mice (Fig. 4). When the A431 epidermoid carcinoma was used, mice that were treated with the pSIN- β carried by the EGFR-targeted liposomes survived significantly longer than mice that were treated with pSIN- β carried by the un-targeted liposomes (Fig. 8). The pSIN- β was unable to cause the regression of the A431 tumors in the present study, likely because a large number

of tumor cells were implanted, and the tumors grew aggressively, more than 1 mm a day. Moreover, the pSIN- β treatment was not started until the tumors reached 6–7 mm in diameter.

In vivo, the pSIN- β carried by the EGFR-targeting liposomes induced significantly more tumor cells to undergo apoptosis than the pSIN- β carried by the non-targeted liposomes (Fig. 5), which may explain why the EGFR-targeted pSIN- β generated a more potent anti-tumor activity. The enhanced anti-proliferative and anti-angiogenic activities from the pSIN- β complexed with the EGF-PEG-liposomes as shown in Figure 7 may have also contributed to the more potent anti-tumor activity from the pSIN- β /EGF-PEG-liposome lipoplexes. Because immuno-compromised athymic mice were used for the *in vivo* studies, it is unlikely that adaptive immune response had played a significant role in the anti-tumor activity from the pSIN- β plasmid, although data from a previous study showed that in immuno-competent mice, T cell-mediated adaptive immune response contributed to the anti-tumor activity of the pSIN- β plasmid [17]. However, it is expected that the activation of innate immunity by the pSIN- β plasmid, the dsRNA produced by the plasmid, and the type I interferons induced by the dsRNA may have contributed, to a certain extent, to the anti-tumor activity observed.

In the present study, EGFR was used as the target for the delivery of the RNA replicase-based plasmid. EGFR is over-expressed in a variety of tumor cells, but many other targets such as folate receptor or integrins are also over-expressed in many tumors cells and may be potentially exploited to target the RNA replicase-based plasmid into specific tumor cells of interest. Finally, the RNA replicase-based plasmid was given by peritumoral injection, which is not ideal for the administration to many tumors, but tumors such as glioblastoma, head and neck tumors, non-metastasized melanoma and lung cancer are localized and may be ideal tumors for molecular therapy using the RNA replicase-based plasmid by intratumoral or peritumoral injection. Indeed, clinical studies of intratumoral injection for gene therapy have already been performed [40–42]. Tumors in the livers may also be treated by localized injection [43, 44]. The feasibility of treating orthotopic and spontaneous tumors in mice with the RNA replicase-based plasmid will be evaluated.

Conclusions

Treatment with an RNA replicase-based plasmid that can generate dsRNA in tumor cells transfected with the plasmid represents a promising cancer molecular therapy. Targeting the RNA replicase-based plasmid more specifically into tumor cells further improved the anti-tumor activity of the plasmid, likely by enhancing its pro-apoptotic, anti-proliferative, and anti-angiogenic activities.

Future Perspective

Efforts utilizing synthetic dsRNA to control tumor growth in animal models and in clinical trials were met with only limited success in the past decades, despite the multiple potential tumor killing mechanisms of dsRNA. The present study represents a departure from the conventional paradigm in tumor dsRNA therapy. Instead of dosing with synthetic dsRNA directly, an RNA replicase-based plasmid was actively targeted into tumor cells, enabling the cells to produce their own dsRNA and then commit 'suicide'. In the next 5–10 years, we expect more studies further validating and improving the efficacy of this plasmid DNA-based tumor dsRNA therapy, which will provide the underpinnings for future translational studies of this approach.

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** of considerable interest

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Executive Summary

- Previously, the RNA replicase-based plasmid, pSIN- β , was shown to inhibit solid tumor growth in mice, and the dsRNA produced by cells transfected with the plasmid was shown to have contributed significantly to the observed anti-tumor activity.
- In the present study, it was shown that targeting the RNA replicase-based plasmid into EGFR-over-expressing tumor cells significantly improved the anti-tumor activity of the plasmid.
- Cationic liposomes surface conjugated with EGF were the carrier of the RNA replicase-based plasmid.
- The enhancement of the anti-tumor activity of the RNA replicase-based plasmid by targeting it into EGFR-over-expressing tumors was confirmed in two human tumor models xenografted in nude mice.
- Targeting the RNA replicase-based plasmid into EGFR-over-expressing tumors enhanced the pro-apoptotic, anti-proliferative, and anti-angiogenic activities of the plasmid.

Fig. 1. Physicochemical parameters of the pSIN-β/EGF-PEG-liposome lipoplexes

(A). Mean diameter and zeta potential of pSIN-β/EGF-PEG-liposome lipoplexes at various plasmid to liposome ratios. Equal volumes of DNA (25 μg) and EGF-PEG-liposomes were mixed and allowed to incubate at room temperature for at least 15 min.

(B). Complexation of the pSIN-β with liposomes protected it from DNase I. pSIN-β/EGF-PEG-liposome lipoplexes prepared at various plasmid to liposome ratios were incubated with DNase I. DNA was extracted and analyzed using 1 % agarose gel stained with ethidium bromide. Weight ratios of DOTAP lipid to pSIN-β for the DNA bands in the inset are 1.73 (lane 1), 3.45 (lane 2), 8.63 (lane 3), 17.25 (lane 3), 34.5 (lane 5), and 86.25 (lane 6). The experiment was repeated twice with similar results.

(C). The uptake of pSIN- β by MDA-MB-468 cells in culture. Cells (1×10^5 /well) were incubated with fluorescein-labeled pSIN- β complexed with EGF-PEG-liposomes or PEG-liposomes at various ratios for 1 h at 37 °C, washed, and lysed before measuring the fluorescence intensity (492/518 nm).

(D). Mean diameter and zeta potential of PEG-liposomes or EGF-PEG-liposomes before and after complexation with pSIN- β at the ratio of 17.25:1 (w/w, pSIN- β , 25 μ g). Except in B, data shown are from at least 3 independent determinations. S.E.M. was not included in C for clarity. (LP, liposomes; DNA, pSIN- β ; LP-PEG, PEG-liposomes; LP-PEG-EGF, EGF-PEG-liposomes).

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Fig. 2. *In vitro* uptake of the pSIN- β in EGF-PEG-liposome lipoplexes or PEG-liposome lipoplexes by tumor cells expressing different levels of EGFR

(A). Cells (1×10^5 /well) were incubated with fluorescein-labeled pSIN- β ($0.75 \mu\text{g}$) complexed with EGF-PEG-liposomes (LP-EGF) or PEG-liposomes (LP) ($12.9 \mu\text{g}$) for 1 h at 37°C , washed with PBS, and lysed with Triton X-100 to measure fluorescence intensity. Data shown are the fluorescence intensity in cells incubated with pSIN/LP-EGF divided by that in cells incubated with pSIN/LP.

(B, C). Pre-incubation of cells (1×10^5 /well) with free EGF inhibited the uptake of the pSIN- β /EGF-PEG-liposome lipoplexes by the MDA-MB-468 cells (B), but not by the

MCF-7 cells (C). Cells were pre-incubated with EGF (0.1 mg/ml) at 37 °C for 1 h before the addition of the lipoplexes. Data shown are mean $\pm$ S.E.M. (n = 6–12).

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Fig. 3. Fluorescent images of cells incubated with fluorescein-labeled pSIN- β -liposome lipoplexes MDA-MB-468 or MCF-7 cells (2×10^6) were incubated with fluorescein-labeled pSIN- β complexed with EGF-PEG-liposomes (LP-PEG-EGF) or PEG-liposomes (LP-PEG) for 1 h at 37 °C. After incubation, cells were washed twice with PBS, fixed in 3 % paraformaldehyde, washed again, and mounted using vectashield medium. Cell nucleus was stained with DAPI (blue) (bar = 50 μ m).

Fig. 4. pSIN- β was more effective at controlling the growth of MDA-MB-468 tumors in mice when complexed with the EGF-PEG-liposomes

MDA-MB-468 (1×10^7) cells were established in athymic mice ($n = 5-8$) and treated when tumors reached an average diameter of 5 mm with the lipoplexes for 14 consecutive days.

(A). Tumor growth curves. Starting from day 15, the values of the pSIN/LP-PEG-EGF and the pSIN/LP-PEG were different from each other ($p < 0.05$).

(B). Mouse survival curves ($p = 0.03$, pSIN/LP-PEG-EGF vs. pSIN/LP-PEG).

Fig. 5. pSIN- β induced more apoptosis in MDA-MB-468 tumors in mice when complexed with the EGF-PEG-liposomes than with the PEG-liposomes

MDA-MB-468 tumors in nude mice ($n = 4$) were treated with pSIN- β complexed with EGF-PEG-liposomes (LP-PEG-EGF) or PEG-liposomes (LP-PEG) for 10 consecutive days.

Tumors were fixed in formalin, embedded in paraffin, sectioned, and stained against EGFR with anti-EGFR alexa fluor 488 (green) and apoptosis using an *in situ* cell death detection kit based on TUNEL analysis (red). Cell nucleus was stained with DAPI (blue), (bar = 50 μ m).

Fig. 6. pSIN-EGFP induced MDA-MB-468 cells to undergo apoptosis in culture

Cells (1×10^7) were transfected with pSIN-EGFP (40 μ g) or pEGFP C1 (40 μ g) and sorted for GFP positive cells 24 h later. The sorted cells were staining with Annexin V-PE and 7-AAD 0 and 72 h after sorting.

(A). Flow cytometry graphs of cells after annexin V and 7-AAD staining. Analysis was performed on GFP positive cells only. Upper right quadrant represents cells in late apoptotic stage; lower right, cells in early apoptotic stage; lower left, viable cells. Cells in the PBS group were passed through the cell sorter, but not GFP-gated.

(B). A comparison of the % of GFP positive cells that were in the late apoptotic stage, early apoptotic stage, or were viable 0 or 72 h after cell sorting. Data shown are mean $\pm$ S.D. from three replicates.

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Fig 7.

(A). Images of MDA-MB-468 tumor tissues after H&E staining or staining with anti-Ki67 or anti-CD31. MDA-MB-468 tumors in nude mice were treated with pSIN-β complexed with EGF-PEG-liposomes (LP-PEG-EGF) or PEG-liposomes (LP-PEG) for 10 consecutive days before they were harvested, fixed in formalin, embedded in paraffin, sectioned, and stained.

(B). The % of Ki67 positive cells in tumors in mice that received different treatments. % of Ki67 positive cells was calculated based on the total number of brown cells divided by the total number of cells. An area of 0.04 mm² was analyzed (n = 9).

(C). The average number of blood vessels per 14.6 mm² (n = 13–37 per treatment).

Fig. 8. A431 tumor-bearing mice survived longer when treated with the pSIN- β /EGF-PEG-liposome lipoplexes than with the pSIN- β /PEG-liposome lipoplexes

Nude mice ($n = 6-7$) were implanted with A431 cells (1×10^7). When tumors reached an average diameter of 6.5–7 mm, treatment with pSIN- β /EGF-PEG-liposome lipoplexes or pSIN- β /PEG-liposome lipoplexes was initiated and continued for 14 consecutive days (pSIN/LP-PEG-EGF vs. pSIN/LP-PEG, $p = 0.017$).