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Nanoparticle-mediated target delivery of TRAIL as gene therapy for glioblastoma

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Abstract

Human tumor necrosis factor α -related apoptosis-inducing ligand (TRAIL) is an attractive cancer therapeutic because of its ability to induce apoptosis in tumor cells while having a negligible effect on normal cells. However, the short serum half-life of TRAIL and lack of efficient *in vivo* administration approaches have largely hindered its clinical use. Using nanoparticles (NPs) as carriers in gene therapy is considered as an alternative approach to increase TRAIL delivery to tumors as transfected cells would be induced to secrete TRAIL into the tumor microenvironment. To enable effective delivery of plasmid DNA encoding *TRAIL* into glioblastoma (GBM), we develop a targeted iron oxide NP coated with chitosan-polyethylene glycol-polyethyleneimine copolymer and chlorotoxin (CTX), and evaluate its effect in delivering *TRAIL in vitro* and *in vivo*. NP-TRAIL successfully delivers *TRAIL* into human T98G GBM cells and induces secretion of 40 pg/ml of TRAIL *in vitro*. Transfected cells show 3-fold increased apoptosis as compared to the control DNA bound NPs. Systemic administration of NP-TRAIL-CTX to mice bearing T98G derived flank xenografts results in near zero tumor growth, and induces apoptosis in tumor tissue. Our results suggest that NP-TRAIL-CTX could potentially serve as a targeted anticancer therapeutic for more efficient TRAIL delivery to GBM.

Graphical Abstract

A chitosan-PEG grafted PEI copolymer coated iron oxide nanoparticle (NP) can effectively deliver TRAIL encoding plasmid DNA into glioblastoma (GBM) cells with no notable cytotoxicity demonstrated *in vitro*. *In vivo* systemic administration of chlorotoxin (CTX) activated NP-TRAIL complexes in mice bearing GBM xenografts induces apoptosis of tumor cells and a sustainable reduction of tumor burden.

Keywords

glioblastoma; TRAIL; gene therapy; transfection; apoptosis

1. Introduction

Glioblastoma (GBM) is the most common and deadly primary brain tumor in humans.^[1] Even with aggressive surgical resection combined with radio-chemotherapy, the prognosis for GBM patients remains dismal with a median survival of approximately 14 months after initial diagnosis.^[2, 3] The highly invasive and infiltrative nature of GBM prevents complete resection and the inherent resistance to current radiation and chemotherapeutic agents limits their usefulness as adjuvant therapies.^[4]

Gene therapy is considered to hold unique promise to overcome treatment resistance.^[5–7] Suicidal genes or genes expressing therapeutic proteins and pro-drug activated enzymes such as Bcl-2-related protein, herpes simplex virus type-1 thymidine kinase, bone morphogenetic protein 2, cytosine deaminase, and human tumor necrosis factor α -related apoptosis-inducing ligand (TRAIL), have attracted research interests due to their capacity in inducing apoptosis in cancer cells.^[8–10] TRAIL is a TNF cytokine superfamily member, which forms a homotrimer that crosslinks death receptors (DRs) on the cell surface leading to downstream signaling of apoptosis.^[11, 12] TRAIL is an attractive anticancer agent due to its ability to induce apoptosis in various types of tumors.^[13–15] It is a strong candidate for GBM treatment because the majority of GBM express DRs,^[16] and recombinant TRAIL

displays good tolerability in pre-clinical and early-phase clinical studies.^[17, 18] Importantly, early-phase clinical trials demonstrated that it has no negative effect on non-neoplastic tissue.^[19–22] In addition, TRAIL has shown a therapeutic effect when combined with chemo or radio-therapy in pre-clinical studies.^[15, 23]

Much effort has been devoted on delivering recombinant TRAIL using cell-based vehicles and vectors at nanoscale or microscale, but the translation of TRAIL-based therapies into clinical use for GBM treatment remains limited by the failure to deliver sufficient protein into the tumor to generate a therapeutic effect.^[24–26] TRAIL gene therapy can potentially overcome this limitation by delivering *TRAIL* encoding DNA specifically to tumor cells so that they express and secrete this therapeutic protein into the tumor microenvironment.^[27, 28] This strategy has been utilized in treating several types of cancer using both viral and non-viral based vectors.^[29, 30] However, gene delivery to GBM is still hindered by low *in vivo* gene transfection efficiency with most delivery vehicles, especially with the presence of the blood-brain barrier (BBB) that has to be overcome before any therapeutics can reach tumor sites in the brain.^[31]

With this in mind, we designed a nanoparticle (NP) delivery vehicle for targeted gene transfection in GBM cells. The NP is comprised of an iron oxide core coated with a shell of chitosan- polyethylene glycol (PEG) grafted polyethyleneimine (PEI) copolymer (CP-PEI) and conjugated with chlorotoxin (CTX). Iron oxide is used as the core material because of its biocompatibility, biodegradability, and intrinsic superparamagnetic properties, and can serve as a magnetic resonance imaging (MRI) contrast agent for disease diagnosis and treatment monitoring.^[32, 33] Chitosan is a natural polymer derived from crustacean shells and has ample functional groups allowing for attachment of functional ligands.^[34] The PEG grafted chitosan (herein termed CP) serves as a stabilizer that prevents particle agglomeration and leads to excellent biodistribution and blood half-life.^[35] We previously demonstrated that CP-PEI based NPs can effectively complex and deliver plasmid DNA to GBM cells *in vitro* and *in vivo*.^[36] These NPs were derivatized with CTX to enhance their affinity towards GBM by binding to cell surface lipid rafts containing MMP-2 and Annexin A2,^[37, 38] and increase the *in vivo* transfection efficiency of gene delivery NPs.^[34] Furthermore, CTX can promote BBB penetration through receptor-mediated transcytosis.^[35, 39] In this study, we use the same CP-PEI based NPs to deliver *TRAIL* encoding plasmid DNA to study its efficacy in treating GBM. We first characterized the physicochemical properties of the NPs. We then examined the effect of NP bound *TRAIL* (NP-TRAIL) on cell viability and apoptosis in human T98G GBM cells *in vitro*, and the ability of CTX conjugated NP-TRAIL (NP-TRAIL-CTX) to induce apoptosis and reduce the tumor growth of T98G derived flank xenografts in mice *in vivo*.

2. Results and Discussion

2.1. NP Development and Characterization

The physicochemical properties of NPs, such as size and surface charge, are key factors determining their pharmacokinetics. Therefore, hydrodynamic sizes and zeta potentials of NP (i.e., the base NP with no TRAIL and CTX loaded), NP-TRAIL, and NP-TRAIL-CTX were characterized using dynamic light scattering (DLS). Z-average diameters of NP and

NP-TRAIL were 34.2 ± 5.4 nm and 50.0 ± 1.1 nm, respectively (Figure 1a). Sizes of NP-TRAIL slightly increased to 57.1 ± 1.4 nm after CTX conjugation. Importantly, the size of NP-TRAIL-CTX is within the preferred range (10–100 nm) for *in vivo* navigation and evasion of reticuloendothelial system and renal clearance.^[40, 41]

Zeta potential is another important physicochemical property for DNA delivery applications.^[42] The positive surface charge correlates with the capacities of NPs to electrostatically complex, protect, and transfect plasmid DNA into cells. NP exhibited a zeta potential value of 17.75 ± 0.64 mV (Figure 1b). In agreement with the hydrodynamic size measurement, zeta potential was similar before and after CTX conjugation (16.70 ± 2.78 mV vs. 18.63 ± 1.27 mV), suggesting that CTX attachment does not compromise NP and TRAIL plasmid DNA complexation. TEM images revealed the spherical morphology and small size (10–12 nm) of the NP core (Figure 1c). Further, NP-TRAIL-CTX were well dispersed with no aggregation observed under TEM. This can be attributed to PEG coating on the surface of NPs and electrostatic repulsion between positively charged NPs. This suggests that DNA is fully incorporated within the polymer coating of a single NP rather than in aggregates of multiple NPs.

The ability of NPs to complex plasmid DNA was evaluated using a gel retardation assay where NPs bound DNA would not migrate down the gel. Naked plasmid DNA can freely migrate on agarose gel, whereas both NP formulations indicated complete binding of DNA as no DNA was observed in the gel (Figure 1d). After incubated with heparin, a competing reagent that disrupts the electrostatic interaction between DNA and NP, DNA became visible in both samples. This confirms DNA is bound within the NPs and CTX conjugation does not inhibit the complexing capacity of NPs. Taken together, the characterizations suggest that this NP system can potentially function well as a vehicle for delivery of plasmid DNA.

2.2. In Vitro Delivery of TRAIL

Efficacy of NP mediated delivery of TRAIL was evaluated *in vitro*. TRAIL encoding plasmid DNA was transfected in T98G cells using NP-TRAIL with NP loaded with salmon DNA as a control (NP-control). qRT-PCR revealed TRAIL mRNA only in NP-TRAIL transfected T98G cells as compared to untreated and NP-control treated cells (Figure 2a). Since TRAIL can be released as a soluble ligand, we also used an ELISA assay to evaluate the expression of TRAIL in cell culture supernatants. In agreement with the qRT-PCR measurement, NP-TRAIL treated cells produced 11.68 ± 0.13 pg of TRAIL in 300 μ L cell culture medium 6 days after transfection, while no TRAIL expression was detected in either untreated or NP-control treated groups (Figure 2b). Moreover, ELISA revealed a 24-fold more TRAIL in cell culture medium than that intracellularly (11.68 ± 0.13 vs. 0.49 ± 0.08 pg) indicating the vast majority of expressed TRAIL was secreted.

This is important for gene therapies since secreted therapeutic proteins could affect surrounding untransfected cells, which greatly reduces the transfection efficiency required to achieve a therapeutic effect. Previous studies have demonstrated that delivery of recombinant TRAIL using NPs can cause apoptotic activity of GBM.^[24] In the current study, the concentration of secreted TRAIL is about 40 pg/ml, which is comparable with those reported in other studies where TRAIL was delivered by vehicles such as virus, neural

stem cells, and lipofectamine.^[29, 43, 44] Furthermore, multiple serial treatments required by recombinant TRAIL is not a necessity for our approach, which is preferable in clinical use.

2.3. *In Vitro* Toxicity of NP-TRAIL to Human GBM Cells

Having demonstrated NP-mediated *TRAIL* gene delivery and expression in T98G cells, we investigated the therapeutic effects of NP-TRAIL *in vitro*. Six days post-transfection, cell viability was tested using the Alamar blue (AB) assay (Figure 3a). As expected, NP-control showed little toxicity with approximately 81.9 ± 3.0 % viability. As a comparison, NP-TRAIL led to greatly reduced cell viability (51.4 ± 1.9 %). The AB results were also corroborated by optical imaging of cells shown in Figure 3b. Untreated and NP-control treated T98G cells exhibited similar normal cell morphologies whereas NP-TRAIL treated cells had significantly lower cell numbers and dramatic toxicity can be directly visualized.

2.4. Apoptotic Effect of NP-TRAIL on GBM Cells *In Vitro*

We examined the degree of apoptosis in T98G cells using Annexin V-FITC and PI double-staining. In accordance with the cell viability measurement, NP-TRAIL treated cells showed positive staining for both Annexin V-FITC and PI by fluorescence microscopy (Figure 4a). As a comparison, no fluorescence signal was detected from either the untreated or NP-control treated cells. Additionally, DAPI staining showed significant nuclear fragmentation in NP-TRAIL treated cells whereas untreated and NP-control treated cells showed little evidence of apoptosis. Quantification of apoptosis using flow cytometry revealed a 47.9 ± 3.2 % apoptotic cell population by NP-TRAIL transfection. In contrast, untreated and NP-control treatment showed 13.1 ± 3.4 % and 17.9 ± 2.6 % Annexin V-FITC and PI double positive populations (Figure 4b and c).

The *TRAIL* encoding plasmid DNA used here is expressed as a fusion with EGFP, which was shown to not affect its therapeutic efficacy.^[45] In order to examine if EGFP expression has a negative effect on Annexin V-FITC staining, flow cytometry and microscopy analysis was conducted on NP-TRAIL treated T98G cells with and without Annexin V-FITC labeling. Both assays revealed that green fluorescence intensity of NP-TRAIL transfected cells after 6 days were similar with untreated T98G cells (Figure S1). We believe that EGFP expression had receded into background color 6 days after NP-TRAIL transfection because of plasmid DNA dilution through cell division and impairment as the majority of T98G cells underwent apoptosis. Therefore, the green fluorescence intensity of EGFP was significantly lower than that from Annexin V-FITC labeled NP-TRAIL treated cells (Figure S1), which demonstrates the reliability of the apoptosis analysis results.

The effect of NP-TRAIL on cell cycle progression in GBM cells was also examined. Six days after transfection with NP-TRAIL, T98G cells were labeled with PI and the DNA content was analyzed using flow cytometry. As shown in Figure 5, NP-TRAIL treatment caused an increase in the proportion of cells in the G2-M phase (54.6% vs. 20.8% for untreated and 13.7% for NP-control) and a corresponding decrease in the proportion of cells in the S and G1 phases. This G2-M cell cycle arrest in NP-TRAIL treated cells is in agreement with the apoptosis results and provides further evidence of NP-TRAIL treatment efficacy in GBM cells.

TRAIL is an attractive anticancer agent due to its ability to induce apoptosis in a variety of tumor cell types including GBM.^[13–15, 24] In line with these studies, NP-TRAIL caused cytotoxicity occurred through induction of the apoptosis in GBM cells. In fact, TRAIL can activate both extrinsic and intrinsic mitochondrial apoptotic pathways in T98G that was identified as a p53 mutant cell line.^[11, 46–48] In the current study, cell cycle status was analyzed 6 days after NP-TRAIL treatment rather than 24 hr or 48 hr, the duration that were used for apoptosis characterization in other studies.^[49, 50] At this late stage at 6 days, apoptosis might also rely on the intrinsic mitochondrial apoptotic pathway, accompanied by p53 associated G2-M phase cell cycle arrest in response to DNA damage.^[51–55]

2.5. *In Vivo* Antitumor Effect of NP-TRAIL-CTX

The therapeutic efficacy of NP delivery of *TRAIL* encoding plasmid DNA was determined *in vivo* by treating athymic nude mice harboring T98G flank tumors with NP-TRAIL-CTX or NP-control-CTX. Here, CTX was attached to the NP as it is crucial to incorporate targeting agents on the surface of gene delivery vehicles in order to improve *in vivo* transfection efficiency specifically in the tumor.^[56, 57] Our previous studies show that conjugating CTX to the surface of NPs increases NP distribution throughout the tumor and *in vivo* transfection efficiency.^[34, 58] Mice treated with NP-TRAIL-CTX showed rapid shrinkage in tumor size followed by slow growth whereas mice treated with NP-control-CTX showed a consistent increase in tumor size over time (Figure 6b). This suggests that the NPs were able to deliver TRAIL encoding plasmid DNA into tumor cells for TRAIL expression, secretion, and induction of apoptosis.

To determine the degree of apoptosis caused by NP-TRAIL-CTX treatment, xenograft tumors were collected 9 days after NP injection, and apoptosis analyzed through TUNEL staining. NP-TRAIL-CTX induced more apoptosis as compared to NP-control-CTX as evidenced by the large number of TUNEL-positive tumor cells (Figure 6c). This correlates well with the *in vitro* apoptosis results and shows the ability of the NPs to deliver *TRAIL* gene to tumors for expression and subsequent promotion of apoptosis in transfected and surrounding cells (Figure 6a). The high level of apoptosis in tumor tissues induced by NP-TRAIL-CTX administration suggests that this strategy has clinical potential for GBM treatment.

Targeted gene delivery is crucial for gene therapy and much efforts have been devoted to improve efficient NP targeting and penetration into tumors. The targeting capacity of CTX to human brain tumors by binding to MMP-2 and Annexin A2 is well established by previous studies.^[34, 37, 38, 59] Moreover, Annexin A2 has been identified highly expressed on the surface of neovasculature.^[60–63] This could provide an additional target for TRAIL delivery as endothelial cells that were targeted with NP-TRAIL-CTX could also produce TRAIL and secrete it into the tumor microenvironment. Here, we have also incorporated another level of targeting by using TRAIL, as its therapeutic effect is specific to tumor cells.^[64] Therefore, even if NP-TRAIL-CTX were taken up to a significant degree by any off-target tissue, no negative response to TRAIL expression would be expected.

Another big impediment to the clinical translation of TRAIL therapeutics lies in the heterogenous resistant of tumors to TRAIL, which is due to the expression of decoy

receptors and several internal countervail cell signaling.^[46, 65, 66] Our NP system is ideal in overcoming these obstacles by co-delivery of *TRAIL* and inhibitors of the resistant mechanisms. Furthermore, our treatment can be combined with other gene therapeutics targeting multiple signaling pathways for a synergetic efficacy.

3. Conclusion

In this study we developed a CTX activated iron oxide NP coated with chitosan-PEG-PEI copolymer with appropriate properties for plasmid DNA delivery to tumor. We demonstrated that NPs loaded with the *TRAIL* gene are able to transfect GBM cells to express and secrete sufficient quantities of TRAIL to promote apoptosis *in vitro*. Importantly, we found that NP-mediated TRAIL gene delivery in a GBM flank xenograft mouse model induced significant apoptosis in the tumor and effectively reduced tumor burden. These results suggest NP-TRAIL-CTX as a potential strategy for improving localized delivery of TRAIL to GBM.

4. Experimental Section

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise specified. All tissue culture reagents were purchased from Life Technologies unless otherwise specified.

Plasmid DNA Preparation

The plasmid pEGFP-TRAIL was purchased from Addgene (plasmid # 10953).^[45] It was propagated in DH5 α *E. Coli* and purified using the Plasmid Giga Kit (Qiagen). Salmon DNA purchased from Sigma-Aldrich was used as the control plasmid DNA.

NP synthesis

Chitosan-PEG (CP), chitosan-PEG-PEI copolymers (CP-PEI), and iron oxide nanoparticles with a siloxane PEG monolayer (IOSPM) were synthesized as described previously.^[36, 67, 68] The IOSPM was coated with CP-PEI copolymer through crosslinking N-succinimidyl iodoacetate (SIA) and 2-Iminothiolane (Traut's reagents) (Molecular Biosciences) before purification using a S-200 sephacryl resin (GE Healthcare) equilibrated with 20 mM HEPES buffer (pH 7.4). NPs were complexed with plasmid DNA at a NP:DNA weight ratio of 1:2 (iron mass:DNA mass) prior to CTX attachment using SIA and Traut's reagent.

NP characterization

For transmission electron microscopy (TEM) analysis, 5 μ L of the NP-TRAIL-CTX (60 μ g/mL) were placed on a formvar/carbon coated 300 mesh copper grid (Ted Pella). After 5 min, NP solution was removed and the grid was allowed to dry overnight before imaging using a Tecnai G2 F20 transmission electron microscope (FEI) operating at a voltage of 200 kV. All hydrodynamic size and zeta potential analyses were acquired in HEPES buffer (pH 7.4) using a DTS Zetasizer Nano analyzer (Malvern Instruments).

DNA binding was characterized using gel retardation assay. NP-TRAIL and NP-TRAIL-CTX were prepared by mixing 0.5 µg NP with 1 µg plasmid DNA in 60 µl of 20 mM HEPES buffer (pH 7.4). The complex was treated with heparin (50 µL of 1000 units/ml Heparin/1 µg DNA) and incubated for 30 min at room temperature to disrupt the electrostatic interaction between NP and DNA. Both heparin treated and untreated samples were subjected to electrophoresis on 1 % agarose gel for about 30 min at 120 V. Gels were stained with 0.5 µg/ml ethidium bromide and visualized using a Bio Rad Universal Hood II Gel Doc System.

Cell culture

Human GBM derived T98G cells were purchased from American Type Culture Collection. T98G cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % FBS and 1 % penicillin/streptomycin (0.5 mg/ml) at 37 °C in a humidified atmosphere with 5 % CO₂. The medium was changed every 2–3 days.

In vitro gene transfection

Twenty-four hours after plating T98G cells at a concentration of 25,000 cells/ml (1 ml/well) in 12 well plates, transfection was performed by replacing the cell culture medium with 1 ml of NP-TRAIL complex-containing medium (2 µg plasmid DNA per well). Four hours after transfection, the complex-containing medium was removed and replaced with fresh growth medium.

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from T98G cells using the Qiagen RNeasy kit (Qiagen). cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. Human β-actin served as a reference gene. SYBR Green PCR Master mix (Bio-Rad) was used for template amplification with a primer for each of the transcripts in a Bio-Rad CFX96 real-time PCR detection system. Quantitative amplification was monitored by the level of fluorescence reflecting the cycle number at the detection threshold (crossing point) using a standard curve. Thermocycling for all targets was carried out in a solution of 20 µl containing 0.2 µM primers (Integrated DNA Technologies) and 4 pg of cDNA from the reverse transcription reaction under the following conditions: 95 °C for 2 min, 40 cycles of denaturation (15 sec, 95 °C), annealing (30 sec, 55 °C), and extension (30 sec, 72 °C). The primers are 5'-CGGTTCCGATGCCCTGAGGCTC-3'/5'-CGTCACACTTCATGATGGAATTG-3 and 5'-GTCTCTCTGTGTGGCTGTAAC-3'/5'-CTCTCTGAGGACCTCTT TCTCT-3' for β-actin and TRAIL, respectively.

Enzyme-Linked Immunosorbent Assay (ELISA)

Production of TRAIL protein levels following NP-TRAIL transfection in culture supernatants and cell lysates were measured by ELISA (Quantikine Human TRAIL Immunoassay; R&D Systems). Briefly, T98G cells were transfected with NP-TRIAL as described above. After 6-day incubation, medium was collected. Both supernatants and cell lysates were tested for TRAIL expression by following the manufacturer's specifications. Final color was evaluated at 450 nm (OD₄₅₀) on a microplate reader (Molecular Devices).

Cell viability assay

The Alamar blue (AB) assay was used to evaluate cell viability following the manufacture's protocol (Life Technologies). Briefly, T98G cells were plated and treated with NP-TRAIL as previously described. Six days after treatment, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) three times before adding 10% AB solution in complete growth medium to the wells. The samples were incubated at 37 °C for 2 hr. Then the AB solution was transferred to a 96-well plate, and fluorescence at an excitation wavelength of 556 nm and an emission wavelength of 586 nm was measured on a microplate reader (Molecular Devices).

Apoptotic assay

The extent of apoptosis *in vitro* was determined by Annexin V and propidium iodide (PI) double staining using the Apoptotic, Necrotic & Healthy Cells Quantification Kit (Biotium). T98G cells were seeded on glass coverslip one day prior to treatment. Then, the cells were treated with NP-TRAIL or NP-control as described previously. Six days after NP treatment, cells were collected and washed with DPBS. Annexin V binding buffer (500 µl) mixed with 5 µl Annexin V-fluorescein isothiocyanate (FITC) and PI was added to cells, followed by incubation at room temperature in the dark for 15 min. The stained cells were then stained by DAPI to indicate nucleus, and visualized using a Nikon ECLIPSE TE2000-S microscope.

For flow cytometric analysis, T98G cells were stained with Annexin V-FITC and PI following the same protocol and analyzed with a FACS Canto flow cytometer (BD Biosciences). Data were analyzed with FlowJo (Ashland).

Cell cycle distribution analysis

Six days following NP-TRAIL treatment, cells were harvested, washed and fixed with 70% ethanol at 4 °C overnight, followed by treatment of 0.5 µg/ml RNase and 50 µg/ml PI at 4 °C for 3 hrs. The cells were then analyzed for their DNA content using a BD FACS Canto flow cytometer (BD Biosciences). All data were analyzed with FlowJo (Ashland). Results are presented as percentages of cells in the various phases, G0/1, S, G2/M.

In vivo studies

All animal studies were performed in accordance with the University of Washington Office of Animal Welfare guidelines for the humane use of animals, and all procedures were reviewed and approved by the Institutional Animal Care and Use Committee. For the flank xenograft model, 6-week-old female NOD-SCID mice (Jackson Laboratories) were anesthetized using 1.5% inhaled isoflurane and 6×10^6 T98G cells in 150 µl of a 1:1 mixture of RPMI media and Matrigel (BD Biosciences) were injected subcutaneously into their right flank. Palpable T98G xenografts developed within 6 days and tumors were measured using an external caliper and the volume was calculated using the formula: $4\pi/3 \times (\text{length}/2) \times (\text{width}/2)^2$. Animals with 12 days-old xenografts (3 animals per group) were randomly divided into 3 groups: untreated, NP-control treated, and NP-TRAIL treated. Animals in the control group did not receive any treatment. Each animal in treated groups received one injection of NP-control or NP-TRAIL at 20 µg plasmid DNA. The treatment was terminated

after 38 days; tumor tissues were harvested on day 9 and submitted for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

Statistical Analysis

All of the data were statistically analyzed to express the mean $\pm$ standard deviation (SD) of the mean. Statistical significance was set at $p < 0.05$ and tested with Student's t -test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Physicochemical properties of NP-TRAIL-CTX. (a) Hydrodynamic size of NP, NP-TRAIL and NP-TRAIL-CTX in HEPES buffer (pH 7.4) determined by DLS. (b) Zeta potential of NP, NP-TRAIL, and NP-TRAIL-CTX in HEPES buffer (pH 7.4). (c) Representative TEM image of NP-TRAIL-CTX. Scale bar in the magnified image represents 10 nm. (d) Agarose gel image of naked *TRAIL* encoding plasmid DNA, NP, NP-TRAIL, NP-CTX, and NP-TRAIL-CTX with and without heparin treatment in the gel retardation assay. A 1 kb DNA Ladder was used as marker.

Figure 2.

In vitro evaluation of TRAIL expression following NP-TRAIL treatment. (a) T98G cells were transfected with NP-TRAIL, with untreated cells and NP-control treated cells as controls. TRAIL mRNA expression was quantified by qRT-PCR at 6 days after transfection. (b) At day 6 after NP-TRAIL treatment, the concentration of secreted TRAIL in culture supernatant and intra-cellular TRAIL was analyzed by ELISA.

Figure 3. TRAIL gene therapy induces cellular toxicity *in vitro*. (a) Viability of T98G cells on day 6 post-transfection as determined by AB assay. (b) Bright-field microscopy images of transfected T98G cells on day 6 post transfection. Scale bars correspond to 10 μm .

Figure 4.

TRAIL gene therapy induces apoptosis in T98G cells *in vitro*. (a) Representative fluorescence images of T98G cells co-stained with Annexin V-FITC and PI 6 days following treatment. Scale bars represent 10 μ m. (b) Apoptosis analysis of T98G cells with flow cytometry 6 days after NP-TRAIL treatment. (c) Quantification of Annexin V-FITC and PI positive apoptotic cell populations from panel b. Data are from three independent experiments. * represents $p < 0.01$.

Figure 5. Representative cell cycle phase distribution of T98G cells 6 days after NP-TRAIL transfection. Results were the average of three independent experiments.

Figure 6. NP-mediated TRAIL gene delivery shows therapeutic efficacy *in vivo*. (a) Schematic of mice subjected to one injection of NP-TRAIL-CTX. (b) Volumes of T98G flank xenograft tumors of mice treated with NP-TRAIL-CTX and NP-control-CTX once tumor became palpable. All values are mean $\pm$ SD of determinations made in 3 animals. ** indicates $p < 0.05$. (c) TUNEL staining revealed significant apoptosis in tumors from NP-TRAIL-CTX treated mice. Scale bars represent 200 μ m.