Gene Delivery and Transfection in Human Pancreatic Cancer Cells using Epidermal Growth Factor Receptor-Targeted Gelatin Nanoparticles

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ABSTRACT

Type B gelatin-based engineered nanovectors system (GENS) was developed for systemic gene delivery in the treatment of pancreatic cancer. The surface of thiolated gelatin nanoparticles was modified with epidermal growth factor receptor (EGFR) specific peptide that targets on the EGFR receptor of Panc-1 cells and this system could release the payload under reducing environment, such as high intracellular glutathione concentrations. Plasmid DNA expressing enhanced green fluorescent protein (EGFP) or wild type p53 (wt-p53) were physically encapsulated in the nanoparticles formed by the solvent displacement process. With efficient and stable encapsulation, EGFR-targeted nanoparticles showed rapid internalization and payload release in Panc-1 cells within 30 minutes. Both quantitative and qualitative analysis of transgene expression showed that the EGFR-targeted nanoparticles had significantly greater efficiency and better therapeutic effect than all the other systems.

Keywords: gene therapy, gelatin nanoparticles, pancreatic cancer

1. INTRODUCTION

More than 32,000 patients are diagnosed with pancreatic cancer in the United States per year and the disease is associated with very high mortality [1]. Urgent need exist to develop novel clinically-translatable therapeutic strategies that can improve on the dismal survival statistics of pancreatic cancer patients. Although gene therapy in cancer has tremendous promise, the major challenge has been in the development of safe and effective delivery system. The main objective of this study is to develop a safe and effective non-viral gene delivery system for therapeutic genes.

Gelatin is one of the most versatile natural biopolymer, widely used in food and pharmaceutical products. Previous studies have shown that type B gelatin could physical encapsulate DNA, which preserves the supercoiled structure of the plasmid and improves the transfection efficiency upon intracellular delivery. By thiolation of gelatin, the sulfhydril groups could be introduced into the polymer and would form disulfide bond within nanoparticles, which stabilize the whole complex and once disulfide bond is broken due to the presence of glutathione in cytosol, payload would be released [2]. Poly(ethylene glycol) (PEG)-modified GENS, when administered into the systemic circulation, provide long-circulation times and are preferentially targeted to the tumor mass due to the hyper-permeability of the vasculature by the *enhanced permeability and retention* effect [3]. Studies have shown over-expression of EGFR receptor on Panc-1 human pancreatic adenocarcinoma cells. In order to actively target on this pancreatic cancer cell line, epidermal growth EGFR specific peptide was conjugated on the particle surface through a PEG spacer.

Most anti-tumor gene therapies target on tumor suppressor genes or oncogenes [4]. The p53 pathway functions as a critical signaling pathway in cell growth, which regulates apoptosis, cell cycle arrest, metabolism and other processes [5]. In pancreatic cancers, most cells have mutations in p53 protein, causing the loss of apoptotic activity. With the introduction of wt-p53, the apoptosis could be repaired and this would trigger cell death in cancer cells [6].

Based on the above rationale, we have designed EGFR targeting peptide-modified thiolated gelatin nanoparticles for wt-p53 gene delivery and evaluated delivery efficiency and transfection in Panc-1 cells.

2. EXPERIMENTAL METHODS

Type B gelatin (pI 4.5 to 5.5) was thiolated by incubation with 2-iminothiolane, a cyclic thioimidate compound which reacts with primary amine group of gelatin. Ethanol was used as desolvation solvent and added to the gelatin/plasmid solution. Enhanced green fluorescent protein (EGFP) or wt-p53 expressing DNA was loaded at 0.5% (w/w). With continuous stirring condition, thiolated gelatin nanoparticles were prepared. Glyoxal (40%v/v) was added at desired time interval to crosslink nanoparticles. Particles were collected by ultracentrifugation at 16,000 rpm for 30 min. The pellet was resuspended into phosphate buffer and surface of the nanoparticles was modified with heterobifunctional poly(ethylene glycol) (mPEG-NHS or MAL-PEG-NHS). Another round of unltracentrifugation was operated to separate the nanparticles and then EGFR-targeting peptide (C-*GGG*-YHWYGYTPQNVI) were conjugated on to surface of nanoparticles through covalent bond between C-*GGG* spacer and maleimide.

Thiolated gelatin nanopartilces in suspension were characterized for mean particle size, size distribution and zeta potential by Zetasizer Nano® (Malvern). Lyophilized

nanoparticles were mounted on aluminum sample mount, sputter coated with gold-palladium and observed with Hitachi 4900 field emission scanning electron microscope (SEM) at 3kV. To determine the surface modifications of PEG and peptide, freeze-dried nanoparticles were analyzed by electron spectroscopy for chemical analysis (ESCA), performed at the National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO), University of Washington (Seattle, WA).

By digesting the particles with protease solution for 30 minutes, plasmid was released and measured for loading efficiency with PicoGreen® dsDNA assay. The stability of plasmid was confirmed with gel electrophoresis after treatment with protease or DNAase.

The expression level of epidermal growth factor receptors (EGFR) in different pancreatic cancer cell lines were examined in western blot.

CellTiter 96® AQueous one solution cell proliferation assay (MTS) was used to analyze the effect of nanoparticles on cell proliferation. 0.5-6mg/ml different nanoparticles were used to treated cells for 6 hours. Poly(ethyleneimine) (PEI) was used as a positive control.

Rhodamine B isothiocyanate (RBITC)-labeled nanoparticles, encapsulated with PicoGreen®-labeled plasmid, were used for intracellular delivery and trafficking studies in Panc-1 cells. Additionally, EGFP plasmid encapsulated nanoparticles were administered in Panc-1 cells and the transgene expression was qualitatively and quantitatively analyzed with fluorescence microscopy and ELISA, respectively.

Wt-p53 expressing plasmid DNA was administered into Panc-1 cells using the control and EGFR-targeted nanoparticles. RT-PCR was used to analysis mRNA level of downstream transcription factors of p53 after transfection. Chromatin Condensation/ Membrane Permeability/ Dead Cell Apoptosis Kit and iCys® Research Imaging Cytometer was used to analyze apoptosis after treatment. Apo-ONE® Homogeneous Caspase-3/7 Assay kit was used to examine the pro-apoptotic activity after transfection of wt-p53 plasmid DNA.

3. RESULTS

3.1 Synthesis and Chatacterization of EGFR Targeted Nanoparticles

The nanoparticles prepared by desolvation were characterized for particle size and zeta potential. The average size and surface charge of the particles prepared from thiolated gelatins with different degrees of thiolation are listed in Table 1. The mean particle diameters of different nanoparticles were between 150-250 nm. Thiolated nanoparticles have smaller size compared to gelatin nanoparticles, might due to the disulfide bridge formation inside particle. With different surface modification, size of nanoparticles has increased. The zeta potential of different formulations were around -20 mV. With SEM analysis, the sizes, surface morphology and spherical shape of nanoparticles were observed and corresponding to Zetasizer result. DNA loading efficiency of gelatin, thiolated gelatin nanoparticles was higher than 95% (Table 1). With surface modification of PEG and peptide, loading efficiency has decreased to 65%.

Formulation	Nanoparticle Diameter (nm)	Zeta Potential (mV)	Plasmid DNA Loading Efficiency (%)
Gel NP	151.4 ± 23.5	-17.1 ± 5.23	95.6 ± 2.2
SH-Gel NP	132.6 ± 17.9	-24.6 ± 5.16	97.0 ± 3.8
SH-Gel-PEG	179.0 ± 30.9	-22.3 ± 9.50	85.8 ± 9.3
SH-Gel PEG Peptide	230.8 ± 41.5	-18.1 ± 4.02	64.8 ± 8.0

Table 1. Particle size, surface charge, and plasmid DNA encapsulation efficiency of control and EGFR-targeted gelatin and thiolated gelatin nanoparticles

High-resolution C_{1S} scans of electron spectroscopy for chemical analysis (ESCA) was used to analyze surface component of thiolated gelatin (SHGel NP), PEG-modified thiolated gelatin (SH-Gel PEG) and EGFR targeting peptide-modified thiolated gelatin nanoparticles (SH-Gel PEG Peptide). The results (Table 2) show peak intensities of the C-H (hydrocarbon), C-O (ether), and C=O (carbonyl) groups at 285.0, 286.3, and 288.1 eV, respectively. The ether C-O signal has increased after PEG modification and decreased after peptide conjugation. While nitrogen composition has decreased after PEG modification and increased after peptide modification, which confirmed the presence of EGFR-targeting peptide on the nanoparticles. ESCA analysis has further confirmed PEG and peptide surface modification.

Formulation	C 1s (%)	O 1s (%)	N 1s (%)
SH-Gel NP	59.3±0.8	22.9±0.5	12.9±0.1
SH-Gel-PEG	58.2±0.6	28.0±1.2	9.5±0.7
SH-Gel PEG Peptide	56.7±0.8	25.9±0.7	12.3±0.6
Formulation	C-C (%)	C-O, N (%)	C=O (%)
SH-Gel NP	51.5	26.6	21.9
SH-Gel-PEG	17.1	63.1	19.8
SH-Gel PEG Peptide	33.1	42.8	24.1

Table 2. C_{1S} high-resolution scans of electron spectroscopy for chemical analysis (ESCA)

3.2 Stability of Encapsulated Plasmid DNA

Agarose gel electrophoresis was used to examine the stability of encapsulated plasmid DNA due to process condition as well as after exposure to protease and DNAse.

Based on the results, both control and EGFR targeted thiolated gelatin nanoparticles can efficiently encapsulate plasmid DNA and preserve intact supercoiled plasmid structure, protect them during intracellular transport.

3.3 Baseline EGFR Expression in Pancreatic Cancer Cells

Two human pancreatic adenocarcinoma cell lines (Panc-1 and Capan-1) were analyzed by western blot for EGFR expression. Human ovarian adenocarcinoma (SKOV3) and murine fibroblast ((NIH-3T3) cells were chosen as positive and negative controls, respectively. Beta-actin was analyzed as protein loading control. Panc-1 cells has shown higher EGFR expression compared to Capan-1 and was used for the following *in vitro* studies.

3.4 Cytotoxicity of Control and Surface-Modified Thiolated Gelatin Nanoparticles

In order to evaluate the cellular interaction of nanoparticles, cytotoxicity assays were carried out after treatment with nanoparticles. 0.5-6mg/ml control and surface modified thiolated gelatin nanoparticles were used to treated Panc-1 cells for 6 hours, Poly(ethyleneimine) (PEI), a cationic cytotoxic polymer was used as a positive control. Based on the results in Figure 1, both the control and the surface-modified nanoparticles were relatively safe and biocompatible in Panc-1 cells even at high concentrations, with comparison to PEI. The following studies were carried out with 1mg/ml nanoparticles.

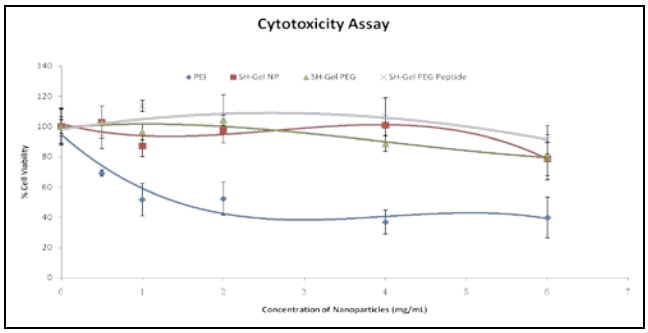

Figure 1. Percent cell viability as a function of nanoparticle formulation concentrations in Panc-1 cells as evaluatedby tetrazolium dye (MTS) assay

3.5 Receptor Mediated Cell Uptake in Panc-1 Cells

To confirm surface accessibility of EGFR-targeting peptide and receptor-mediated endocytotic uptake of nanoparticles, a system was designed by labeling each component with different fluoresences for visualization of nanoparticles uptake and trafficking in cells. Rhodamine B isothiocyanate (RBITC) was used to conjugated on thiolated gelatin by reaction with amine group. After dialysis and lyophilization, RBITC labeled thiolated gelatin could be used for nanoparticles preparation. Before desolvation, PicoGreen® was mixed with plasmids for 1 minute and labeled plasmids were added to thiolated gelatin solution. With this labeling system, plasmid DNA, nanoparticles and cell nucleus could be identified. Laser scanning confocal fluorescence microscopy was used to take images at different time points, from 15 minutes to 6 hours. By comparing the images of different formulations, peptide conjugated gelatin nanoparticles showed the fast uptake and plasmid release within 30 minutes.

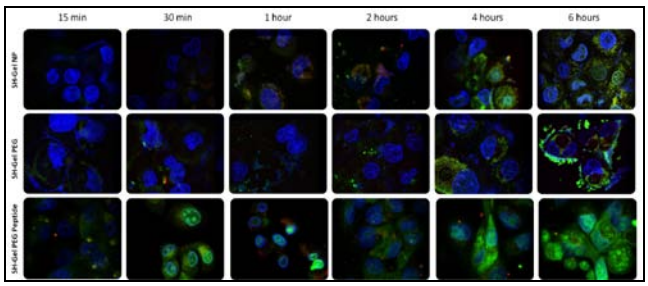

Figure 2. Confocal fluorescence microscopy analysis of DNA-encapsulated nanoparticle uptake and trafficking in Panc-1 cells. (red=rhodamine-labeled nanoparticles, green=PicoGreen-labeled plasmid DNA, and blue=DAPI-labeled nucleus)

3.6 Qualitative and Quantitative *In Vitro* Transfection of Enhanced Green Fluorescent Protein

Fluoresence microscopic analysis and ELISA were used to measure quanlitative and quantitative GFP tranfection efficiency in Panc-1 cells upon administrtion of unmodified, PEG-modified and EGFR peptide-modified thiolated gelatin nanoparticles. Plasmid delivered by EGFR-targeted nanoparticles resulted to have highest levels of GFP expression after 48 hours relative to other controls, including Lipofectin®-complexed DNA.

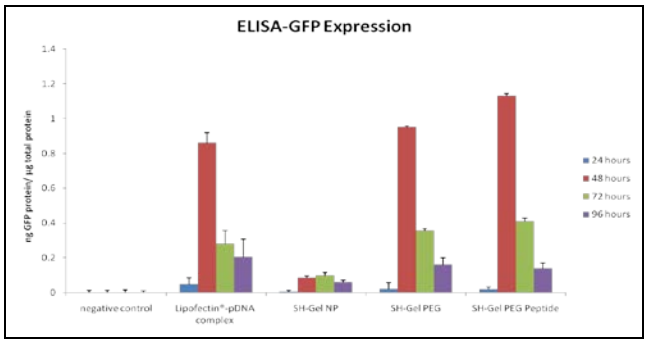

Figure 3. EGFP expression by an ELISA as a function of time post-administration of plasmid DNA in control and EGFR-targeted nanoparticles

3.7 *In Vitro* Tranfection with Wild-Type p53 Plasmid in Panc-1 Cells

Wild type p53 plasmids were extracted from *E. coli* and encapsulated into nanoparticles to study the apoptotic therapeutic effect. Panc-1 cells were treated with particles for 6 hours and transfected for additional 24, 48, 72, and 96 hours.

Since p53 could induce apoptosis in cells and in order to accomplish this function, many downstream transcription factors are involved and directly regulated by expression of wt-p53. Among them, Bax, caspase-3, caspase-9, DR5, PUMA and Apaf-1 are up-regulated by expression of p53 and while Bcl-2, survivin are down-regulated. In order to examine the levels of these transcription factors, RNA was extracted from Panc-1 cells after 48 hours post-transfection and used for RT-PCR. The products were evaluated with gel electrophoresis and bands were analyzed with ImageJ. Based on the results showed in Figure 4, survivin decreased significantly with the treatment of EGFR targeted thiolated gelatin nanoparticles compared to other treatments, no obvious change was seen in Bcl-2 and the rest transcription factors increased with targeted nanoparticles treatment.

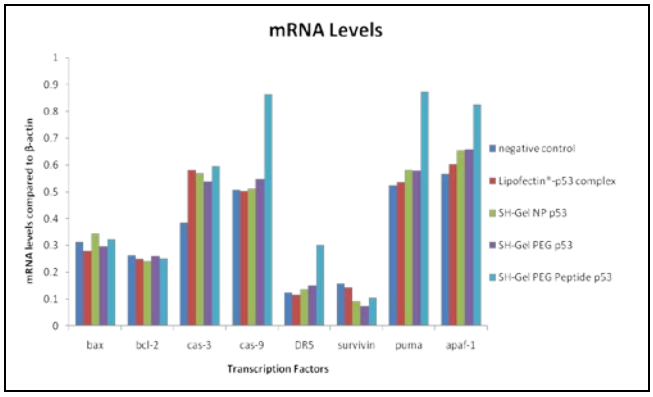

Figure 4. The mRNA levels of downstream factors of wt-p53 expression were compared by RT-PCR after 48 hours post-transfection.

After wt- p53 transfection, Chromatin Condensation/ Membrane Permeability/ Dead Cell Apoptosis kit was used to differentiate apoptotic cells, necrotic cells and live cells with different dyes. iCys® Research Imaging Cytometer from CompuCyte (Westwood, MA) was used to analyze and compare apoptosis levels after treatment. Based on the fluorescence microscopic images, the intensities of all colors was recorded and plotted versus counts. The percentages for different populations were calculated. Compared to the negative control, apoptotic cells fold changes were calculated out and listed in Figure 5. EGFR targeted thiolated gelatin nanopaticles have showed the highest apoptotic cell population after post-transfection. Analysis of caspase 3/7 activity also showed that EGFR-targeted nanoparticles had rapid internalization and highest level of apoptotic activity in Panc-1 cells.

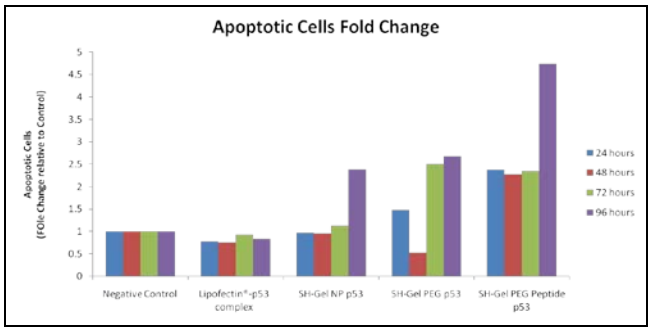

Figure 5. Cytometric analysis of pro-apoptotic activity in control wt-p53 transfected Panc-1 cells using iCys® Imaging Cytometer

4. CONCLUSIONS

These preliminary results suggest that EGFR-targeted gelatin nanoparticles have efficient DNA loading and rapid interlization, payload release in cancer cells, compared to other unmodified systems. Targeting gelatin system can serve as a safe and efficient DNA delivery system for gene therapy in the treatment of pancreatic cancer.

5. ACKNOWLEDGEMENTS

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