Cationic Nanoparticles Effectively Delivered VEGF SiRNA in A549 Cells

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ABSTRACT

Vascular endothelial growth factor (VEGF) plays a crucial role in the production of new blood vessels (angiogenesis) needed for cell growth and proliferation, and tumor expansion. Therefore, inhibition of angiogenesis via targeting the VEGF signalling pathway offers a target for cancer therapy. Small interfering RNAs (SiRNAs) are specific inhibitors of mRNA, block the translation step, and inhibit the expression of protein in the tumor cells. The challenge with SiRNA as therapeutic agents is its inherent instability in biological fluids. We propose to overcome this by encapsulating the SiRNA in nanoparticles, which has the potential to provide improved effectiveness and safety for cancer treatment. In this study, we validated this approach of inhibition of VEGF levels using SiRNA in lung cancer cells. Cationic nanoparticles of SiRNA were prepared and characterized. No significant injury to the cells was observed after cells were treated with SiRNA nanoparticles. Cationic Liposomes of VEGF SiRNA showed the most effecacy in inhibiting expression of VEGF in A549 lung cancer cells.

Keywords: SiRNA, Cationic nanoparitlces, Vasculare epitheliar growth factor, Cancer therapy

1 INRODUCTGION

VEGF has been identified as a potential target for lung cancer treatment. Studies have shown that small cell lung cancer (SCLC) cells express functional VEGF receptors, suggesting that attenuating VEGF signal-pathway by reducing levels of VEGF might effectively treat SCLC by retarding growth and migration of the cancer cells (Tanno et al., 2004). Bevacizumab, an anti-VEGF antibody, was the first drug to show a survival benefit in patients with lung cancers when it was added to traditional chemotherapy. However, there is a risk for toxicities associated with bevacizumab therapy that is common for all chemotherapeutic agents (Ferrara et al., 2004).

Among novel anticancer agents, small interfering RNAs (SiRNAs) have shown potential because of their high specificity, high efficiency, and low toxicity. SiRNA cleaves mRNA to stop synthesis of cellular proteins such as VEGF. Compared to antibodies, SiRNA is more easily directed to intracellular factors, including transcription factors. Furthermore, gene inhibition is exquisitely selective, even down to the level of single nucleotide polymorphisms. However, SiRNAs for therapeutic application face a series of hurdles. Several processes including renal filtration and excretion, uptake by phagocytes, aggregation with serum proteins, and enzymatic degradation rapidly reduce SiRNA concentrations after *in vivo* delivery (Whitehead et al., 2009).

The pharmacokinetic profiles of drugs can be altered by encapsulating them in small, specialized nanocarriers. Traditionally, lipid based particulate nanocarriers are called liposomes or liposomal nanoparticles. Liposomal products have been successfully used to increase drug stability (circulation times). For example, doxorubicin given in liposomes has a half-life of 55 hours, five times longer compared to its traditional intravenous formulation. Studies on SiRNA encapsulated in liposomes for cancer therapy have recently been conducted (De Fougerolles et al., 2007). Liposomes have several of advantages for delivery of SiRNA: First, particles with sizes of 5 - 200 nm have high transport across cell membranes into the cytoplasm; Second, the encapsulated drug will be released gradually, providing longer circulating half-life; Third, this range of particle sizes has a high distribution in specific organs such as liver, lung, kidney, and tumor (Torchilin, 2005). Importantly, nanoparticles also appear to accumulate in tumors due to enhanced permeability and retention (Wu, et al., 1993).

To overcome limitations of traditional lung cancer therapy, we propose to develop a novel reagent that is stable, specific, and effective to inhibit the overexpression of VEGF, thereby limiting cancer cell

proliferation. This will be achieved by engineering a multifunctional inhibitor composed of a nanocarrier that protects SiRNA from degradation, and improves SiRNA pharmacokinetics and distribution into cancer cells as well as a therapeutic moiety that is targeting an inhibitor (SiRNA) on the specific survival factor of lung cancer. The first steps in this studies were to develop the nanoparticle carrier system and to test whether targeted delivery of SiRNA would effectively and specifically inhibit VEGFs in lung cancer cells.

2 EXPERIMENTS

2.1 Preparation and Characterization of VEGF SiRNA Nanoparticles

An SiRNA (sense: 5′-GGAGUACCCUGAUGAGAU CdTdT-3′, antisense: 5′-GAUCUCAUCAGGGUA C UCCdTdT-3′) targeting isoform 189 of human VEGF or a complex of SiRNA and polyethyleneimine (PEI) was incorporated into cationic liposome nanocarriers. The structure of three types of nanocarriers are illustrated in **Figure 1**. SiRNA and PEI (P:N = 1:20) complex were prepared by mixing an aliquot of PEI and SiRNA solutions. The resulting solution was then incubated for 30 min at room temperature for complexation to occur. Liposomes were prepared by the hydration method described previously (Bai and Ashan, 2010). Briefly, lipid mixtures were dissolved in an organic solvent, and a dry thin lipid film was prepared in a round-bottom flask using a Buchi R-114 Rotavapor (Buchi Laboratories AG, Postfach, Switzerland). Subsequently, the flask was kept under vacuum for 2 hours to ensure complete removal of residual solvent. The dry lipid film was hydrated with an RNase-free buffered solution of SiRNA at 40±2°C. The dispersion thus obtained was vortexed and subjected to ultrasound for 2 minutes. The final formulation was extruded through 0.1 μm polycarbonate membrane filters (Costar Nucleopore, Cambridge, Massachusetts). Each formulation included 500 mg of cholesterol, 250 mg of phosphocholine, and 100 mg of, 1,2-dioleoyl-3-trimethylammonium-propane, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(poly-ethyleneglycol)-2000] and 1 μM of VEGF SiRNA. The morphology of the particles was examined using a Hitachi S-3400-II transition electronic microscopy TEM (Hitachi High Technologies America Inc., Schaumburg, IL) equipped with an image analyzer. A drop (~50 μl) of the formulations was placed to grid until the grid surface nearly dried. Then another drop of negative phosphotungstic acid (PTA) was added onto the grid. The grid was air-dried, and examined under TEM.

2.2 Cytotocixity of SiRNA Nanoparticles

Experiments were addressed the nonspecific cell toxicity of SiRNA liposomal nanoparticles measured as cell viability using 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described (Bai et al., 2007; Keller et al., 2005). The 16HBE14O$^-$ human bronchial epithelial cells were seeded in flat-bottom, 96-well micro-titer tissue culture plates. After cells grew confluence, they were incubated with 20 μl of different formulations for 4 hours, respectively. The test samples contained 30 pmol/L SiRNA or nanoparticles containing equivalent amounts of SiRNA. Sodium dodecyl sulfate (SDS) was used at 0.1% as a positive cytotoxic control. After 4 hours, MTT (5 mg/ml) solution was added to each well and the cells were incubated at 37°C for 4 hours. Next, the solution in each well was removed and acidified isopropyl alcohol was added. Finally, the plates were incubated at 37 °C for 1 hour and absorbance was measured on an xMark™ microplate absorbance spectrophotometer (Life Science Research, Hercules, CA) at 570 nm. Each assay was performed on eight samples and cell viability was expressed as the percentage of MTT released by cells exposed to SiRNA formulations or SDS compared to cells incubated with saline alone (negative control).

2.3 Efficacy Studies of SiRNA Nanoparticles

The efficacy of SiRNA on the inhibition of VEGF expression were tested by decreased levels of VEGF in lung cancer cells A549 (Bai et al., 2008; Li et al., 2008). A549 Cells were seeded on a 6-well plate. After 24 hours of cell growth, the buffer solution and optimized 30 pmol of SiRNA formulations were added to the cells. The conditioned medium was collected after 24 and 48 hours, and the medium was spun at 800 g for 3 minutes at 4 degree Celsius to remove cell debris. The supernatant was frozen at -20 degree Celsius and subjected to analysis to determine the amount of VEGF secreted from the cells by enzyme-linked immunosorbent assay (Quantikine® human VEGF immuno-detection kit, R&D Systems, Minneapolis, MN), following the manufacturer's instruction.

3 RESULTS AND DISCUSSON

The experiments were performed to test the efficacy and cytotoxicity of negatively charged VEGF SiRNAs. The SiRNA liposome nanoparticles were prepared by hydration method as described previously (Bai and Ahsan, 2009A, Bai et all, 2009B, and Bai and Ahsan, 2010). SiRNA and SiRNA-PEI complex liposomes were visualized by transition electron microscopy (TEM) **(Figure 2)**. The size of liposomes was 100-150 nm, which was acceptable for SiRNA delivery. The liposomes were relatively small in size and showed a uniform surface. Liposomal particles were evenly distributed and no agglomerates were observed in the photographs. Interestingly, incorporation of PEI into the nanoparticles has not significantly affected surface morphology, porosity, and particle–particle interaction. This could be explained by the small amount of PEI (2 nmol/ml) in the formulations.

To evaluate the cytotoxicity of the liposomes to 16 HBE14o$^-$ cells, the viability of the cells was assessed with the MTT assay. MTT, a tetrazolium salt, is cleaved by mitochondrial dehydrogenase in living cells to form a measurable, dark blue product called formazan. Damaged or dead cells have reduced dehydrogenase activity and diminished formazan production. Results of the MTT test showed high levels of viability of 16 HBE14o$^-$ cells after incubation with saline (**Figure 3**); in contrast, only 45% of 16 HBE14o$^-$ cells were viable following treatment with 0.1% SDS ($p < 0.05$, data not shown). Viability of cells treated with VEGF SiRNA nanoparticles was not significantly different from saline treated cells **(Figure 3)**. Overall, no significant cytotoxicity was produced by all the liposomes of SiRNA. With an SiRNA concentration of 30 pmol/mL, the liposomal VEGF SiRNA was the most effective at inhibiting the expression of VEGF with about 50% and 40% decreases at 24 and 48 hours, respectively **(Figure 4)**. Appreciable decreases of VEGF level in medium were observed when cells were treated by all the SiRNA formulations. There is no significant difference among treatment groups ($p > 0.05$). The VEGF with about 50% decrease is significantly different from other SiRNA nanoparticles and buffer control group. Also, SiRNA-PEI liposomes showed about 30% decrease of VEGF secretion. Data in the Figure 4 indicated that the liposomal formulation of SiRNAs was the efficacious in inhibiting VEGF levels in the A549 Cells.

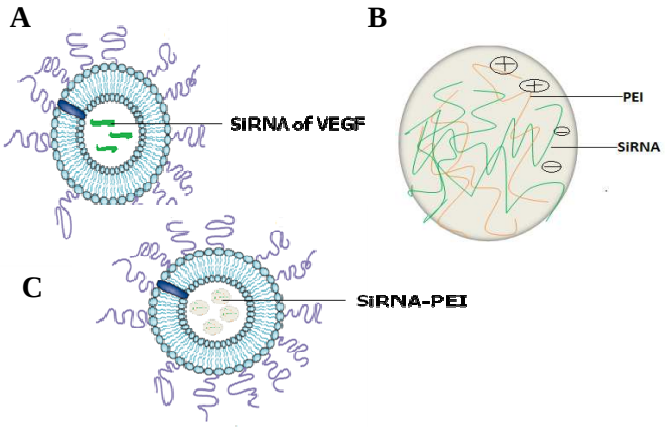


Figure 1. Schematic structures of VEGF liposome (A), SiRNA-PEI complex (B), and SiRNA-PEI liposome (C) (Modified from Torchilin, 2005).

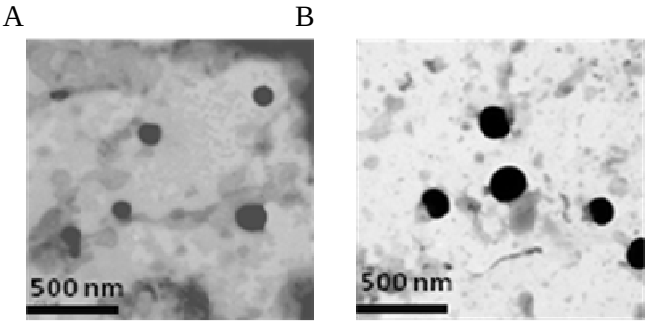


Figure 2. Transition electron micrographs of (A) SiRNA liposomes and (B) SiRNA-PEI liposomes.

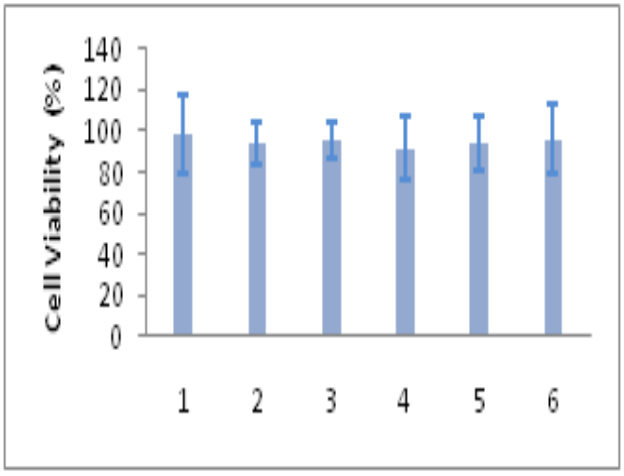
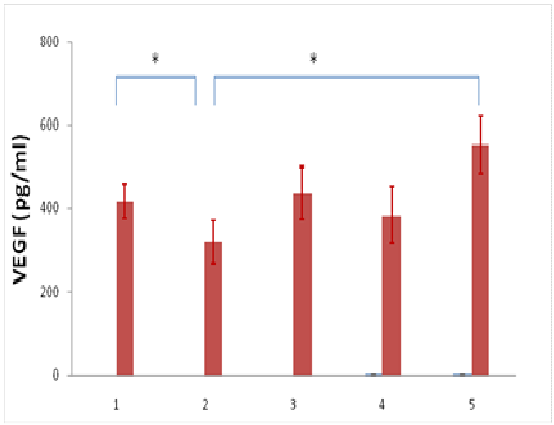


Figure 3. Viability of lung epithelial cells after treatments of (1) SiRNA, (2) SiRNA liposomes, (3) SiRNA-PEI, (4) SiRNA-PEI liposomes, (5) PEI, and (6) blank liposome compared to medium-treated cells ($p > 0.05$).

A



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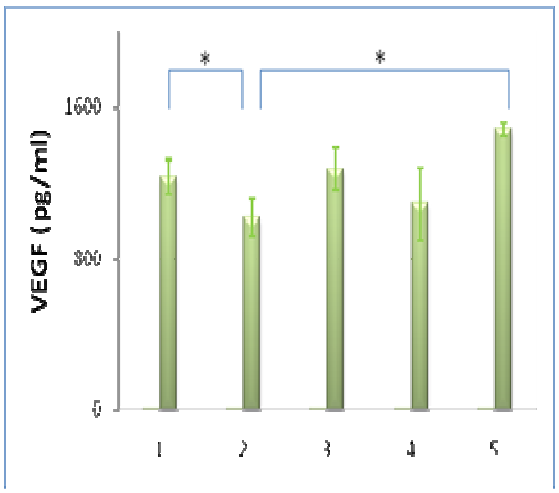

Figure 4. Amount of VEGF in culture media of A549 cell after treatments of 1: SiRNA; 2: SiRNA liposomes; 3: SiRNA-PEI complex; 4: SiRNA-PEI liposomes; 5: buffer control for 24 hours (A) and 48 hours (B) (* indicated the significant difference among groups, $p < 0.05$).

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