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Bioreducible polyethylenimine-delivered siRNA targeting human telomerase reverse transcriptase inhibits HepG2 cell growth in vitro and in vivo

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ABSTRACT

One new siRNA sequence was found efficient for human telomerase reverse transcriptase (hTERT) gene silencing in vitro in five types of human cancer cells. Then, a biodegradable polyethylenimine containing multiple disulfide bonds (SS-PEI) was successfully applied as a potent non-viral carrier for intracellular delivery of the hTERT siRNA in vitro and in vivo. The SS-PEI could strongly bind siRNA to form nano-sized and positively-charged complexes, but which were readily destabilized to sufficiently release siRNA in a reducing environment. Transfection experiments showed that the complexes of SS-PEI/hTERT siRNA were able to transfect HepG2 cells in vitro, inducing reduced levels of hTERT mRNA and hTERT protein, decreased telomerase activity, cell growth inhibition and significant cell apoptosis. Besides, treatment with the complexes of SS-PEI/hTERT siRNA could inhibit HepG2 tumor growth in a xenograft mouse model. Importantly, the SS-PEI revealed relatively low cytotoxicity in vitro and at an appropriate dose had no adverse effect on liver and kidney functions in vivo. The results of this study indicate that SS-PEI/siRNA-induced hTERT gene silencing provides a promising method for human cancer gene therapy.

1. Introduction

Telomerase is a ribonucleoprotein complex capable of maintaining stable telomere length in cancer cells [1,2]. Telomerase activity is normally highly expressed in almost all cancer cells but not in most normal somatic cells. This feature thus makes telomerase an attractive molecule target for human cancer therapy [3]. An essential component of human telomerase is the human telomerase reverse transcriptase (hTERT). It was indicated that the hTERT was detectable mainly in human cancer cells, but repressed in human somatic cells [4]. Moreover, towards various human cancer cells, such as liver and breast cancer cells, there existed a high level of correlation between hTERT mRNA expression and telomerase activity [5–7]. Further studies suggested that the hTERT appeared to be the rate-limiting determinant of telomerase activity [8,9]. Accordingly, there are increasing researches in inhibiting telomerase activity by targeting hTERT for achieving anti-cancer effects [10–12].

RNA interference (RNAi) induced by small interfering RNA (siRNA) duplexes offers a convenient and efficient approach for down-regulating the expression of hTERT mRNA [13]. The underlying mechanism involves the incorporation of a 21-nucleotide siRNA duplex into a multiprotein RNA-inducing silencing complex, which subsequently specifically identifies and silences target mRNA [14]. However, until now only limited amounts of siRNAs have been available for hTERT gene silencing in human cancer cells. For example, Zaffaroni et al. designed a group of hTERT siRNAs and indicated that two siRNAs were potent to silence hTERT gene in human prostate cancer cell lines [15]. In a recent work, Dong et al. showed that one new hTERT siRNA was efficient to knockdown hTERT gene in human breast cancer cell lines [16]. In addition, in these studies the silencing of hTERT gene in the cancer cells led to appreciably reduced telomerase activity and cell growth inhibition in vitro and in vivo. These research results suggest that siRNA-induced hTERT gene silencing may be an efficient approach for human cancer therapy.

Although RNAi is very promising for human cancer therapy, an essential prerequisite for successful application of RNAi is the development of low-toxic and efficient siRNA delivery systems [17–20]. Cationic polymers as siRNA delivery systems have received much

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attention in the past decade because, compared to recombinant viral carriers, they gain advantages of non-/low immune response in vivo, easy modification, and large-scale manufacturing [21–24]. Among various cationic polymers, polyethylenimine (PEI) is the most used siRNA carrier since it has a few biophysical properties that are favorable for siRNA delivery, including the ability to condense siRNAs into nanoscaled PEI/siRNA complexes and a high buffer capacity to induce facilitated endosomal escape of the complexes [25]. However, further clinical use of PEI is seriously limited by its high cytotoxicity, partially due to the lack of degradability [26]. In order to reduce the cytotoxicity of PEI, a reversible disulfide bond was incorporated into PEI, yielding disulfide-containing (bioreducible) PEI analogs. It has been proven that the disulfide bond is relatively stable extracellularly, but can be degraded inside the cells due to the presence of a high concentration of the glutathione [27,28]. As such, bioreducible PEIs are intracellularly degradable, exhibiting much lower cytotoxicity than their non-degradable counterparts. For example, Kim et al. prepared a group of bioreducible PEIs via Michael-type addition between the *N, N*′-cystaminebisacrylamide and various oligoamines [29] and they showed that these bioreducible PEIs mediated efficient *Fas* siRNA transfection in vitro and meanwhile displayed much lower cytotoxicity than non-degradable 25-kDa PEI [30]. By the same synthesis method, Hahn et al. synthesized a bioreducible PEI polymer with a 2-kDa PEI oligomer and they further conjugated the hyaluronic acid to this PEI for targeted delivery of TGF-β siRNA in vitro and in vivo [31]. However, to the best of our knowledge, no study has been reported on bioreducible polymers for the delivery of siRNA targeting hTERT gene.

In this study, we aim to investigate the potential of bioreducible PEI (SS-PEI) as a carrier system for intracellular delivery of siRNA targeting hTERT gene in vitro and in vivo. We initially designed a group of new hTERT siRNAs and found that one siRNA was efficient for hTERT gene silencing in human cancer cell lines in vitro. The SS-PEI polymer, prepared by chemical coupling of 3′-dithiobispropanoic acid and 800 Da-PEI, was then employed as a carrier to transfer hTERT siRNA into human hepatocellular carcinoma HepG2 cells in vitro and in vivo. Biophysical properties of SS-PEI/siRNA complexes, such as particle size, surface charge and triggered siRNA release, were characterized by dynamic light scattering and gel retardation analysis. The hTERT gene silencing induced by the transfection of SS-PEI/hTERT siRNA in vitro was evaluated by qRT-PCR and Western blotting. Besides this, anti-cancer effect was assessed by hematoxyin and eosin staining and Ki-67 staining, respectively, after intratumoral injection of the complexes into HepG2 tumors in nude mice. Organ distribution of the SS-PEI and its effects on liver and kidney functions in vivo were also examined.

2. Materials and method

2.1. Materials

All chemicals, low molecular weight polyethylenimine (LWPEI, 800 Da), high molecular weight branched polyethylenimine (HWPEI, 25 kDa), 3′-dithiobispropanoic acid (DTPA), 2-morpholinoethanesulfonic acid (MES), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), dithiothreitol (DTT) and N-Hydroxysuccinimide (NHS) were ordered from Aldrich-Sigma.

2.2. hTERT siRNA design and synthesis

hTERT siRNA sequences were designed by using the software tool in the website (http://www.ambion.com/techlib/misc/siRNA_finder). Four sets of double-stranded siRNA sequences (encoded as siRNA1–4) and a non-silencing control siRNA sequence (encoded as siRNA-NC) were listed in Chart 1. These siRNA sequences were submitted to BLAST search to make sure that only the selected hTERT mRNA gene was targeted specifically by these siRNAs. These siRNAs were synthesized by the GenePharma Company, Shanghai, PR. China.

hTERT-siRNA sequences and a non-silencing control siRNA.

Code	Sense sequence
siRNA1	5′-CAUGCGUCGCAAACUCUUUdTdT-3′
siRNA2	5′-AUGCGGCCCCUGUUUCUGGdTdT-3′
siRNA3	5′-GAGCCAGUCUCACCUUCAAdTdT-3′
siRNA4	5′-CGGUGUACGCCGAGACCAAdTdT-3′
siRNA-NC	5′-GGCCUCAGCUGCGCGACGCdTdT-3′

2.3. Synthesis and characterizations of bioreducible polyethylenimine (SS-PEI)

SS-PEI was synthesized by chemical coupling of the 3′-dithiobispropanoic acid (DTPA) with the LWPEI (800 Da) at an equal molar ratio via an EDC/NHS activation reaction. As a typical example, DTPA (2 mmol, 0.42 g), LWPEI (2 mmol, 1.60 g), EDC (5 mmol, 0.96 g) and NHS (5 mmol, 0.57 g) were added into a reaction flask and stirred in MES buffer (pH 6.5, 5 mL). The reaction was performed at room temperature under nitrogen atmosphere. The reaction mixture was allowed to proceed for 2 days, giving a viscous solution. Next, the resulting solution was purified by exhaustive dialysis (3500 g/mol cut-off) with 50 mM NaCl solution (3 × 5 L) and then deionized water for 3 days. SS-PEI polymer was finally collected as a solid powder after freeze-drying (yield: 25%). The ^{1}H NMR spectra (D_2O) and Fourier transform infrared (FTIR) spectra of the SS-PEI polymer were recorded on a Varian Inova spectrometer (300 MHz) and a Spectrum 1000 FT-IR spectrometer, respectively. The molecular weight and polydispersity of SS-PEI were determined by GPC relative to PEO standards as described previously [32].

2.4. Particle size and zeta-potential measurements

SS-PEI/siRNA complexes were prepared by adding a HEPES buffer solution (20 mM, pH 7.4) of SS-PEI (100 μL, varying concentration) to a DEPC solution of siRNA (50 μL, 20 μg/mL), followed by vortexing for 5 s and incubating at room temperature for 30 min. The particle size and surface charge of formed complexes were measured at 25 °C with a Nanosizer NS90 (Malvern Instruments Ltd., Malvern, UK).

2.5. Agarose gel retardation

SS-PEI/siRNA complexes were prepared by adding a HEPES buffer solution (20 mM, pH 7.4) of SS-PEI (10 μL, varying concentrations) to a DEPC solution of siRNA (10 μL, 0.2 μg/mL), followed by vortexing for 5 s and the dispersions were incubated for 30 min at room temperature. Then, 10 μL of HEPES buffer containing DTT or HEPES buffer alone (as a control) was applied to give final DTT concentrations in the range from 20 to 300 mM. The dispersions were incubated for another 30 min. After addition of 2 μL of 6× loading buffer (Fermentas), 10 μL of this mixture was applied onto a 0.7% agarose gel containing ethidium bromide. After development of the gel, siRNA was visualized with a UV lamp using a Tanon Gel system (Tanon Gel Image System, Thermo Scientific).

2.6. Cell culture and hTERT siRNA transfection

Human cancer cells, i.e. HEC, Hela, HepG2, MCF-7 and SW480 (purchased from ATCC, USA), were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin and streptomycin (GIBCO) at 37 °C in a humidified 5% CO_2-containing atmosphere. The cells ($5–7 \times 10^4$ cells/well) were plated in 24-well plates and allowed to grow at least 24 h until 60–70% cell confluence.