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Dual gene targeted multimeric siRNA for combinatorial gene silencing

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ABSTRACT

Simultaneous silencing of multiple up-regulated genes is an attractive and viable strategy to treat many incurable diseases including cancer. Herein we report that multimerized siRNA conjugate composed of two different siRNA sequences in the same backbone shows more efficient inhibition of the two corresponding target genes at one time than physically mixed multimerized siRNA conjugates. Two model siRNAs against VEGF and GFP gene were chemically crosslinked via cleavable and noncleavable linkages for the preparation of dual gene targeted multimeric siRNA conjugates (DGT multi-siRNA). Cleavable DGT multi-siRNA with reducible disulfide linkages exhibited significantly higher gene silencing efficiencies at mRNA and protein expression levels than noncleavable DGT multi-siRNA, the physical mixture of naked siRNA, and that of single gene targeted multimeric siRNA (SGT multi-siRNA) with eliciting negligible immune response. DGT multi-siRNAs against two therapeutic siRNAs, anti-survivin and anti-bcl-2 targeted siRNA, also showed greatly enhanced apoptotic effect. This approach for concurrent suppression of combinatorial therapeutic target genes using cleavable multimeric siRNA structure can be potentially used for improved therapeutic efficacy.

1. Introduction

Small interfering RNAs (siRNAs) that show excellent target-specific gene silencing capacity with a small dose have been widely utilized as a powerful tool for diverse biomedical and therapeutic applications [1–3]. However, several challenges such as delivery efficiencies, degradation in serum, immune response, and safety issues caused by cationic carriers are still unsolved for clinical applications [4–10]. To address these issues, identification of more potent siRNA sequence, chemical modification of siRNA for preventing enzymatic degradation and reducing innate immune response, and more importantly, development of safe and effective cationic delivery carriers are critical. Recently, diverse siRNA structures have been vigorously investigated to reduce the effective dose of siRNA by chemical and genetic modifications [11–14]. Chemically modified siRNAs could endow enhanced therapeutic efficacy by increasing stability against serum containing enzymes and prolonging blood-circulation time. In particular, siRNA has been conjugated with various lipophilic molecules, hydrophilic polymers such as polyethylene glycol (PEG), targeting ligands, and cell penetrating peptides to increase the target-specific intracellular delivery efficiency *in vitro* and *in vivo* [15–19]. In our recent studies, we showed that multimeric siRNA structure (multi-siRNA) enabled to exhibit significantly enhanced gene silencing efficiencies, compared to naked siRNA, by complexing with less charged and less cytotoxic cationic carriers to form more stable and compact nano-sized polyelectrolyte complexes due to increased charge density and chain flexibility [20,21].

A combinatorial RNAi (co-RNAi) technology, using multiple shRNA (small hairpin RNA)s and siRNAs that can simultaneously silence multiple target genes, was introduced as a new strategy to enhance therapeutic effects of siRNAs for treating cancer [22,23]. Silencing only a single gene involved in a disease-state cell might show a limited therapeutic effect, since other disease-related genes involved in the signal transduction pathway network are likely to function complementarily and/or to be up-regulated. In this sense, an siRNA delivery system incorporating multiple siRNA sequences to target multiple disease-related genes can be an attractive and powerful approach to effectively and simultaneously shut-down the critical pathways involved in a disease-state cell of interest. Several multiple siRNA delivery systems, such as a genetically engineered viral vector embedding multiple target sequences and a cocktail formulation of multiple siRNA complexes have been reported [23–29]. It was recently observed that siRNA-induced silencing of a particular gene in the resistant virus within cells showed limited success due to the facile genetic mutation, but simultaneous gene silencing of multiple mutated genes could make

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the virus intolerable to survive. Thus it is highly desirable to develop multi-gene targeted siRNA delivery systems that can silence multiple disease-related genes in each cell at the same time, thereby achieving synergistic therapeutic effects [23,30,31].

In this study, a new class of multiple siRNA delivery system based on multimeric siRNA structure was developed to effectively inhibit two disease-related target genes at the same time. Two kinds of model siRNAs, green fluorescent protein (GFP) and vascular endothelial growth factor (VEGF), were chemically crosslinked via a reducible disulfide bond to prepare dual gene targeted multimeric siRNA conjugates (DGT multi-siRNA). The two GFP and VEGF siRNA monomers end-functionalized with thiol groups at both ends were randomly multimerized to produce oligomeric siRNA species. Previously, we have shown that multimeric siRNA homo-conjugates prepared by either GFP or VEGF siRNA, when complexed with selected cationic polymers, enabled to form more stable and compact nano-sized complexes than naked ones, resulting in far enhanced gene silencing efficiencies [20]. It was hypothesized that the GFP and VEGF multimeric siRNA hetero-conjugates are likely to have different physical properties such as chain stiffness and charge distribution from either the GFP or the VEGF multimeric homo-conjugate (single gene targeted multi-siRNA, SGT-multi-siRNA), resulting in different nano-structured complexes. Linear polyethylenimine (Mw. 25,000, LPEI 25k) was employed to produce nanocomplexes with naked and multi-siRNA, and the resultant complexes were transfected to cells. Dual gene silencing efficiencies of DGT multi-siRNA complexes were evaluated as compared to those of the physical mixture of GFP and VEGF SGT multi-siRNA complexes. The LPEI complexes prepared with the two monomeric siRNAs were used as a control. Intracellular uptake of the two GFP and VEGF siRNAs delivered by DGT and SGT multi-siRNA complexes was quantified. Two apoptosis-related siRNAs, anti-survivin and anti-bcl-2 siRNA, were also multimerized to evaluate their dual gene silencing effects and cellular apoptotic levels. It was anticipated that the intracellular delivery of two or more siRNAs crosslinked in the form of multi-siRNA conjugates would have far superior multiple gene silencing effects to that prepared by physical mixing of the two SGT-multi-siRNA conjugates.

2. Materials and methods

2.1. Materials

siRNAs modified with a thiol group at their 3′ end of sense and anti-sense strand against human vascular endothelial growth factor (VEGF), green fluorescent protein (GFP), survivin, bcl-2, and primers for semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) were purchased from Bioneer Co. (Daejeon, South Korea). The sequences of each siRNA and primer are shown in Table 1. Dithiobis-maleimidoethane (DMTE) and 1,8-bis-maleimidodiethyleneglycol ($BM(PEG)_2$) were provided from Pierce (Rockford, IL). GFP over-expressing MDA-MB-435, PC-3, and A549 cell lines were kindly donated by Samyang Co. (Seoul, South Korea), and HeLa and MCF-7 cell lines were obtained from Korean Cell Line Bank (Seoul, South Korea). Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood kindly donated from the Republic of Korea National Red Cross Daejeon Chapter using lymphocyte separation medium-LSM (Fisher scientific, Pittsburgh, PA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), RPMI medium 1640 (RPMI-1640), LysoTracker® Red DND-99, and Quant-iT™ RiboGreen® RNA Reagent were obtained from Invitrogen (Carlsbad, CA), and N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N- trimethylammonium methylsulfate (DOTAP) was provided from Roche Diagnostics (Indianapolis, IN). LPEI 25k was purchased from Polysciences, Inc. (Warrington, Pennsylvania). TRI Reagent®, reverse transcriptase, and *Taq* DNA polymerase were obtained from Ambion, Inc. (Texas, USA), Promega (Madison, USA), and Takara (Tokyo, Japan), respectively. Quantikine human VEGF immune-detection kit and human IFN-α ELISA kit were purchased from R&D systems (Minneapolis, MN) and PBL Biomedical (Piscataway, NJ), respectively. ApoScan™ Annexin V FITC apoptosis detection Kit was obtained from BioBud (Seoul, South Korea) and cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). *Taq* Man® MicroRNA Reverse Transcription kit, Custom *Taq* Man® Small RNA assay, *Taq* Man® Universal Master MixII were purchased from Applied Biosystems (Foster city, USA).

Table 1
Sequences of siRNAs and primers for RT-PCR.

Name		Sequences
VEGF siRNA		5′-GGAGUACCCUGAUGAGAUCdTdT-3′ (sense)
		3′-dTdTCCUCAUGGGACUACUCUAG-5′ (anti-sense)
GFP siRNA		5′-GCAAGCUGACCCUGAAGUUdTdT-3′ (sense)
		3′-dTdTCGUUCGACUGGGACUUCAA-5′ (anti-sense)
Survivin siRNA		5′-AAGGAGAUCAACAUUUUCAdTdT-3′ (sense)
		3′-dTdTUUCCUCUAGUUGUAAAAGU-5′ (anti-sense)
Bcl-2 siRNA		5′-CCGGGAGAUAGUGAUGAAGdTdT-3′ (sense)
		3′-dTdTGGCCCUCUAUCACUACUUC-5′ (anti-sense)
VEGF primers	Forward	5′-AGGAGGGCAGAATCATCACG-3′
	Reverse	5′-GATCCGCATAATCTGCATGGT-3′
GFP primers	Forward	5′-TGGTGAGCAAGGGCGAGGAG-3′
	Reverse	5′-GGGGGTGTTCTGCTGGTAGT-3′
Survivin primers	Forward	5′-GCACCACTTCCAGGGTTTAT−3′
	Reverse	5′-CTCTGGTGCCACTTTCAAGA-3′
Bcl-2 primers	Forward	5′-GTCCAAGAATGCAAAGCACA-3′
	Reverse	5′-ACATCTCCCGCATCCCACT-3′
β-actin primers	Forward	5′-GTGGGGCGCCCCAGGCACCAGGGC-3′
	Reverse	5′-CTCCTTAATGTCACGCACGATTTC-3′

2.2. Synthesis of dual gene targeted multi-siRNA conjugates (DGT multi-siRNA)

To produce DGT multi-siRNA conjugates, VEGF siRNA (VEGF siRNA) and GFP siRNA (GFP siRNA) were randomly multimerized using the synthetic scheme described in our previous study with a slight modification [20]. Briefly, 50 nmoles of VEGF siRNA and 50 nmoles of GFP siRNA with thiol groups at both 3′ ends were mixed together after deprotection of end thiol groups by dithiotreitol (DTT) treatment. The two end-thiolated GFP and VEGF double strand siRNAs were crosslinked in 50 μL of PBS solution (pH 7.4) with 100 nmoles of DTME or $BM(PEG)_2$ in DMSO. DTME and $BM(PEG)_2$ were used as crosslinkers to produced cleavable and noncleavable multi-siRNA, respectively (Fig. 1). To synthesize SGT multi-siRNA conjugates, either GFP or VEGF double strand siRNAs modified with thiol group at both 3′ ends were used for multimerization. After overnight reaction, residual crosslinkers were removed by dialysis using MWCO 10,000 membrane for 1 day. Final products were analyzed by 12% polyacrylamide gel electrophoresis (180 V, 40 min) and ethidium bromide (EtBr) staining. To confirm the presence of reducible disulfide linkages in the cleavable DGT multi-siRNA, 10 mM of DTT was treated to both of cleavable and noncleavable DGT multi-siRNA for 15 min and the reduced products were analyzed by 12% polyacrylamide gel electrophoresis. Quantitative values of each band intensity were calculated with the electrophoresis result by a densitometry using an Image J software (National Institute of Health, USA; http://rsb.info.nih.gov/ij/).

2.3. Comparison of biological activities and immune activation between cleavable and noncleavable DGT multi-siRNA

To compare biological activities of target gene inhibition for cleavable and noncleavable DGT multi-siRNA, GFP over-expressing MDA-MB-435 cells (human breast carcinoma cells) were seeded in a 12-well plate with 10% serum containing DMEM medium at a density of 1.5×10^5 cells/well and transfected with various concentrations of DGT multi-siRNA complexes prepared with LPEI 25k at an N/P ratio 20 in serum free medium for 5 h. After further incubation in serum containing fresh medium for 48 h, the cells were lysed using 1% Triton X-100 solution and centrifuged to remove cell debris. GFP containing supernatants were analyzed with spectrofluorometer (SLM-AMINCO 8100, SLM Instruments Inc., Urbana, IL) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. To determine IFN-α levels activated by siRNA, human PBMCs were isolated from whole blood with a standard density centrifugation technique using lymphocyte separation medium. The cells were resuspended in a 96-well plate with 10% serum containing RPMI-1640 medium at a density of 2.5×10^5 cells/well. Cleavable/noncleavable DGT multi-siRNA (154 nM) complexes prepared with LPEI 25k or DOTAP at an N/P ratio 20 or a weight ratio 7.5 were then added to the cells. After 1 day, the culture medium was collected and the amount of released IFN-α was quantified by an enzyme-linked immunoadsorbent assay (ELISA) kit according to the manufacturer's protocol.

2.4. Co-inhibition of target gene expression using VEGF and GFP DGT multi-siRNA

To verify target gene inhibition, three kinds of GFP over-expressing cancer cells, MDA-MB-435, PC-3 (human prostate cancer cell), and A549 (human lung adenocarcinoma epithelial cell), were maintained in 10% FBS containing DMEM medium (MDA-MB-435 cells) or RPMI-1640 medium (PC-3 and A549 cells) supplemented with 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2 humidified atmosphere. For VEGF and GFP target protein inhibition assay,