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Reduction of Oncogene Expression in Cancer Cells Using siRNA Delivery Systems

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ABSTRACT – Recently, siRNA has been emerging as new therapeutic agents for various diseases such as cancers and infectious diseases. However, the evaluation for delivery systems for siRNA has not been fully done. In this study, we designed and delivered siRNA of oncogenic E6 and E7 proteins to several cell lines and tested the delivery efficiencies of various cationic nonviral delivery vectors. Of cationic delivery systems tested in this study, lipid-based Lipofectamine revealed higher delivery efficiency of siRNA to cervical cancer cell line, SiHa, compared to other delivery systems. Notably, the polyethylenimine, which showed the comparable delivery efficiencies in plasmid DNA, did not show significant delivery of siRNA in cervical cancer cells. These results indicate that the mechanisms involved in siRNA delivery might be different from those in plasmid DNA delivery, and that cationic lipid-based delivery vehicles deliver siRNA with higher efficiency to intracellular target sites.

Key words – siRNA, Delivery systems, Polyethylenimine, Lipofectamine, Cervical cancer cells

Recently, there has been emerging interest for small interfering RNA (siRNA). RNA interference is a new modality in gene therapy which can elicit down-regulation of gene expression and has enormous potential in the treatment of various diseases such as cancers and infectious diseases.[1,2] In RNA interference, double stranded RNA induces the degradation of sequence specific mRNA. The siRNA molecules have been reported to trigger the RNA interference systems and inhibit mRNA expression *in vivo*.[3]

However, although the evaluation for delivery systems for siRNA has not been fully done, the therapeutic application of siRNAs is largely dependent on the development of a delivery vehicle that can efficiently deliver the siRNAs to target cells. Cationic liposomes represent one of the few examples that can reach these requirements.[4] These agents are composed of positively charged lipid bilayers and can be complexed to negatively charged siRNA duplexes. In cases of plasmid DNA, numerous studies have reported the delivery efficiencies of cationic nonviral delivery systems. Previous studies have reported that cationic polymers such as linear polyethylenimines (PEI, m.w. 25 KD) may have higher *in vitro* and *in vivo* gene delivery efficiency as compared to branched PEI of similar molecular weight (m.w. 25 KD), or cationic lipid-based Lipofectamine.[5] Among PEI, lower molecular weight PEI was shown to be more effective in hematopoietic progenitor cells and human CD34+ cells, than higher molecular weight PEI.[6] For siRNA delivery, despite its clinical significance and impacts,[7] there has been lack of understanding for the effects of molecular weights, N/P ratios, and chemical natures of nonviral vectors on the cellular delivery.

In this study, we designed and delivered siRNA of cervical oncogenic E6 and E7 proteins[8] to several cell lines and tested the delivery efficiencies of various cationic nonviral delivery derivatives. Here, we report that the mechanisms involved in siRNA delivery might be different from those in plasmid DNA delivery, and that cationic lipid-based delivery vehicles deliver siRNA with higher efficiency to intracellular target sites in various cell types.

Materials and Methods

Design of siRNA

E6 and E7 siRNA target sequences used in this study were designed by running the programs provided in the following sites; http://jura.wi.mit.edu/bioc/siRNAext/[9] and http://ambion.com/techlib/misc/siRNA_finder.html. The sequence was submitted to a BLAST search against the human genome sequence to ensure that no gene of the human genome was targeted. For E6 and E7 siRNA synthesis, the primers 5'-aacaaaccgttgtgtgatttg-3' and 5'-aagtgtgactctacgcttcgg-3' were used as

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Table I–*The Target Sequences of siRNA Used for HPV16 E6 and E7*

HPV16 E6
5'-aac aaa ccg ttg tgt gat ttg-3'
Anti-sense: 5'-aac aaa ccg ttg tgt gat ttg *cct gtc tc-3'
Sense: 5'-aac aaa tca cac aac ggt ttg *cct gtc tc-3'
HPV16 E7
5'-aag tgt gac tct acg ctt cgg-3'
Anti-sense: 5'-aag tgt gac tct acg ctt cgg *cct gtc tc-3'
Sense: 5'-aac cga agc gta gag tca cac *cct gtc tc-3'

*Leader sequence: T7 promoter primer sequence

described in Table I.

Synthesis of siRNA

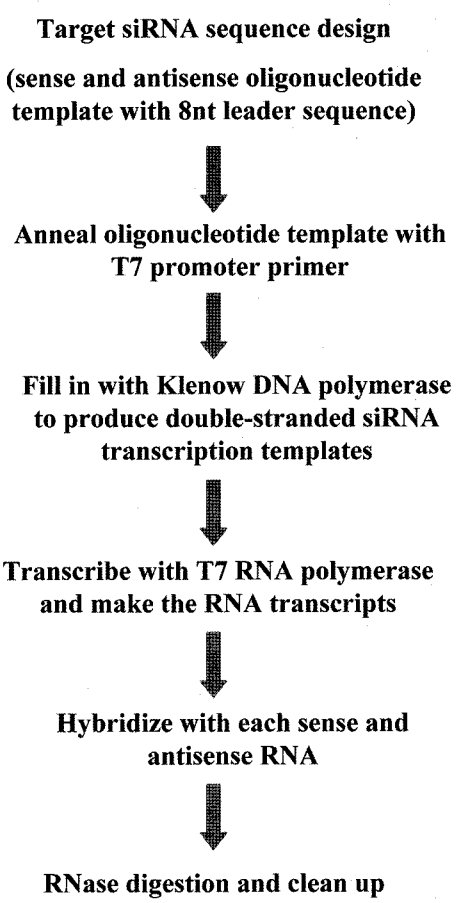
To synthesize the E6 and E7 siRNA, we used the Silencer™ siRNA construction kit (Ambion, TX, USA). In brief, we first prepared two template oligonucleotides with 21 nucleotides encoding the siRNA and 8 nucleotides complementary to the T7 promoter primer. The 2 template oligonucleotides were hybridized to a T7 promoter primer provided with the construction kit. The 3' ends of the hybridized DNA oligonucleotides were extended by the Klenow DNA polymerase to create double-stranded siRNA transcription templates. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and the resulting RNA transcripts were hybridized to create dsRNA. The leader sequences and DNA template were removed by digesting the dsRNA with a ribonuclease and deoxyribonuclease, and the resulting siRNA was purified by glass fiber filter binding (Scheme 1).

Cell cultures

SiHa and CaSki cell lines, derived from human cervical carcinomas, were known to contain integrated human papillomavirus (HPV) type 16 genome, about one to two copies (SiHa) and 600 copies (CaSki),[8] respectively. An HPV-18 genome-positive cell line HeLa, and a mouse melanoma cell line B16-F10 were used in this study. CaSki cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. Other cell lines were cultured in DMEM medium with 10% fetal bovine serum. As antibiotics for the culture medium, penicillin (100 units/mL) and streptomycin (100 μg/mL) were used and the cells were cultured at 37°C and 5% CO_2 in air.

Cellular delivery of siRNA or fluorescent dsRNA

For delivery of RNA molecules, the cells were trypsinized and subdivided into 12-well plates without antibiotics, with 70% confluence per well. After 24 h the cells were treated with



Scheme 1–Scheme of siRNA production process.

E6 and E7 siRNA and fluorescein-labeled double-stranded RNA (BLOCK-iT™ Fluorescent Oligo, Invitrogen, CA, USA) in naked form or complexed with LipofectAmine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. In the case of PEI-mediated delivery, siRNA or fluoresceint dsRNA were complexed to PEI (m.w. 2 and 25 KD, branched and linear form, Aldrich, WI, USA) at the N/P ratio of 10/1. PEI was added to RNA molecules by adding appropriate amounts while vortexing and the resulting mixtures were incubated for 10 min at room temperature to allow the complexation process.[6] The siRNA and fluorescence dsRNA concentration was 50 pmole per each cell culture well. The final volume of culture medium was 1 mL per well. Cells were harvested for real-time PCR and FACS, and observed under fluorescence microscope at 24 h after treatment.

FACS measurement of fluorescent dsRNA and real-time PCR

The delivery efficiency of various vectors was measured by FACS and real-time PCR. The conditions for running real-time RT-PCR are described in Table II. The cells treated with flu-

Table II–*Human Specific Primers and Sizes of Real-Time RT-PCR*

Gene	Sequences (5'-3')	Amplicon size (bp)
hGAPDH	F: gag cca aaa ggg tca tca tct c	200
	R: cca cag tct tct ggg tgg cag	
E6	F: atg cac caa aag aga act gca atg	200
	R: gca tat gga ttc cca tct c	
E7	F: atg cat gga gat ac acct ac	300
	R: tta tgg ttt ctg gga aca gat g	

orescent dsRNA were collected and washed twice with ice-cold PBS. Cells were then stained with propidium iodine (Sigma) (5 μg/mL) and analyzed by using a three-color FACSCalibur flow cytometer equipped with CELLQUEST PRO software (Becton Dickinson, NJ, USA). The total RNAs were extracted from the siRNA-treated cells and cDNAs were synthesized as a PCR template for real-time PCR (Corbett Research, Mortlake, Australia). Real-time RT-PCR was performed using a SYBR green dye (Qiagen, Hilden, Germany). The forward and reverse primers for each variant are described in Table II. DNA polymerase was first activated at 95°C for 15 min, denatured at 95°C for 15 s, and annealed/extended at 58 for 18 s, for 45 cycles according to the manufacturer's protocol. All PCR reactions were performed in triplicate. Relative quantitation of gene expression was carried out using the delta-delta Ct method (Rotor-Gene 6.0.19, Corbett Research, Mortlake, Australia). Each value of the experimental sample was divided by the normalized control sample value (human glyceraldehyde-3-phosphate dehydrogenase; hGAPDH) to generate the relative expression levels of E6 or E7 in SiHa cells. Statistical analysis of data was performed using one-way analysis of variance. Duncan's multiple range test was used as a post hoc test.

Results and Discussions

Design and synthesis of siRNA for E6/E7 oncogenes

In this study, we selected siRNA for E6 and E7 of human papillomavirus (HPV) genes. The significance of E6 and E7 genes in the oncogenesis of cervical cancers is widely known. The siRNA target sequences were searched using the search engines provided by a commercial source. The target sequence was designed to be specific for E6 and E7, respectively.

The siRNA was synthesized *in vitro* using T7 RNA polymerase systems. For *in vivo* delivery of siRNA, plasmid-based siRNA is also being used. However, *in vitro* the RNAi effects of siRNA were known to be higher following treatment of the cells using *in vitro* synthesized siRNA as compared to plasmid-based siRNA. Therefore, in this study, we used the *in vitro* synthesized E6- and E7-specific siRNA. The sequences of siRNA for E6 and E7 are described in Table I.

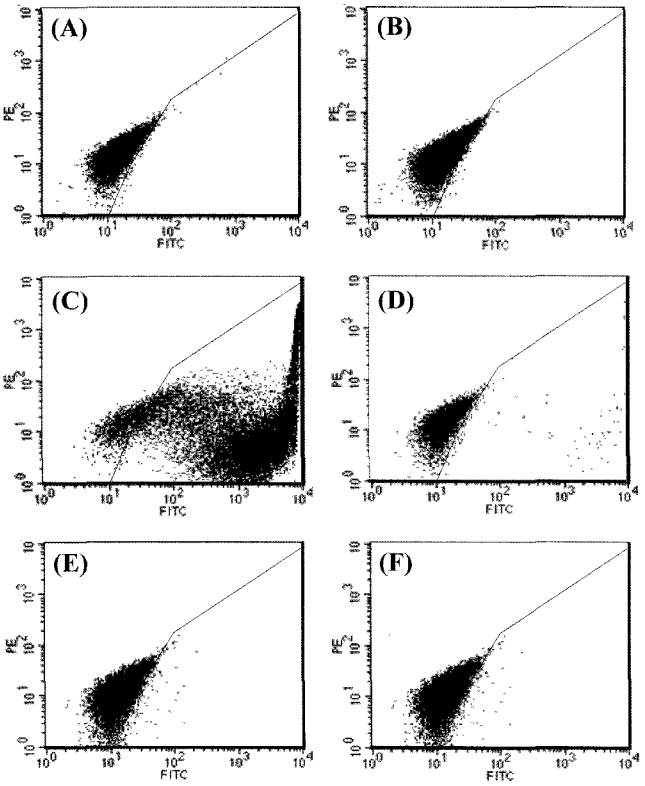


Figure 1–FACS analysis for the cellular uptake efficiency of fluorescein-labeled dsRNA in SiHa cells. Untreated control cells (A), cells treated with fluorescent dsRNA without any transfection reagents (B), and the cells treated with fluorescent dsRNA complexed with Lipofectamine (C), PEI 2KD (D), 25 KD (E), and linear 22 KD (F) were analyzed by FACS, respectively.

FACS analysis of siRNA delivery

To test the efficiencies of siRNA delivery into the cells, we treated HPV16-positive cancer cell line, SiHa, with fluorescent dsRNA. The cellular delivery was tested using FACS instrument. Compared to untreated cells (Figure 1A), naked siRNA-treated cells (Figure 1B) did not show any significant increases in positive fractions of cells with fluorescence signal. Similarly, the cells treated with siRNA/PEI complexes did not significantly differ from the untreated cells (Figure 1D-F). However, siRNA/Lipofectamine-treated cells showed the significantly higher fractions of the cells positive with FITC fluorescence markers (Figure 1C). The percentage of the cells positive with fluorescence marker of siRNA was 92% for Lipofectamine, followed by branched PEI 2 KD (1.55%), branched 25 KD (1.08%), and linear 22 KD (0.35%).

Effect of siRNA on the oncogene expression in cervical cancer cells

We next studied the changes in the levels of mRNA expression of siRNA following delivery with various cationic vectors. The expression of E6 and E7 was tested in SiHa cells using real-time RT-PCR. The expression of E6 oncogenes was significantly inhibited following delivery of siRNA using Lipofectamine. Consistent with the observation using FACS analysis, various PEI vectors did not show significant reduction of mRNA levels of E6 (Figure 2A). In case of E7, the expression of the oncogene was reduced about 80% following delivery of E7-specific siRNA using Lipofectamine. The treatment of the cells with E7-specific siRNA/PEI complexes did not exert significant reduction in the mRNA levels of E7 (Figure 2B).

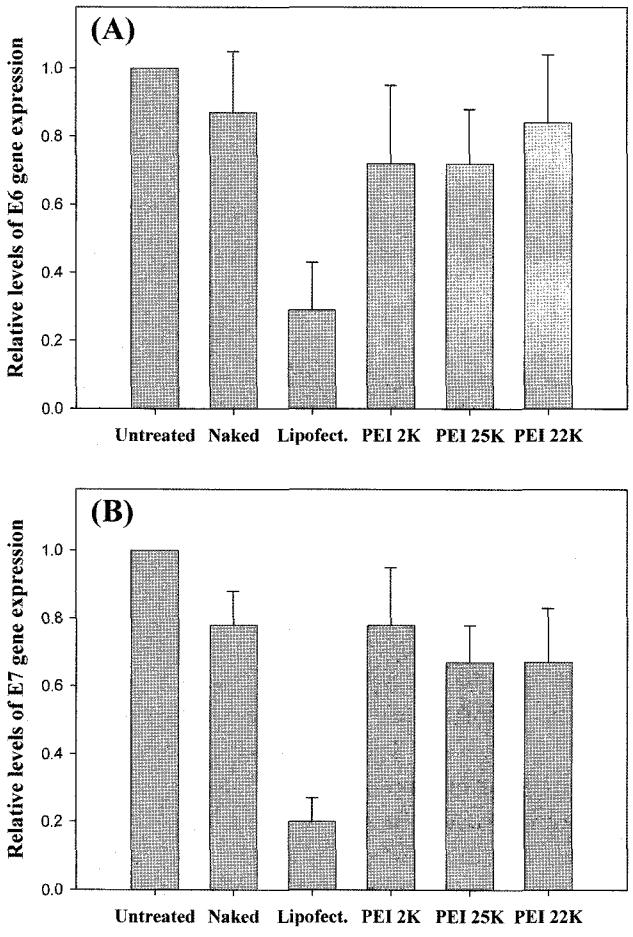


Figure 2–E6 and E7 mRNA expression levels in cervical cancer cells. Reverse transcription was performed on total RNA isolated from SiHa cells treated with E6 (A) and E7 (B) siRNA delivered using various vectors. The cDNAs were used for real-time PCR using primers specific for E6, E7, and the housekeeping gene GAPDH. Values of mRNA amounts were normalized to GAPDH expression and presented relative to E6 and E7 expression. The data are the mean ± S.E. from three independent experiments.

Notably, although the system for *in vivo* application of siRNA through PEI complexation was reported that siRNA targeting the HER-2 receptor resulted in a marked reduction of tumor growth,[10] we did not observe any distinctive effect of PEI depending on its molecular weight, and branched or linear types. Previous studies reported that the delivery efficiencies of plasmid DNA were highly affected by the molecular weight, N/P ratios, and the types of PEI (branched or linear forms).[11, 12] We previously showed that the DNA delivery efficiencies of PEI to hematopoietic progenitor cells could be enhanced by using lower molecular weight PEI (2 KD), and at the higher N/P ratios.[6] Currently, it is unlikely that we can expect the modulation of PEI-mediated siRNA delivery efficiencies using these physicochemical factors as the efficiency of PEI-mediated delivery is substantially lower than Lipofectamine-mediated siRNA delivery. Although we need to elucidate the mechanisms by which the efficiency of PEI-mediated siRNA delivery is unexpectedly low, we can not exclude the possibility that the low efficiency might be contributed in part by the small size of siRNRA. Such a relatively small size of siRNA may result in the altered charge force and conformational change upon complexation with PEI. There exists the possibility that the cellular traffficking pathway of PEI and Lipofectamine may result in the differences in siRNA delivery efficiencies.

Cellular delivery of FITC-dsRNA in cervical cancer and other cell lines

The higher siRNA delivery efficiency of Lipofectamine was studied in other cells. In addition to SiHa cells, we tested Caski, HeLa derived from HPV18-positive cell, and a mouse melanoma cell line, B16F10. To visualize the siRNA delivery efficiencies, we employed the fluorescence-labeled dsRNA. Following delivery with linear PEI 22 KD-complexed siRNA, very dim signal was observed in every cell type tested in this study. In contrast, siRNA/Lipofectamine-treated cells showed fluorescence in high fractions of the observed cells. The higher siRNA delivery efficiency of Lipofectamine was not limited to SiHa cells. The fluorescene of siRNA was observed in high fractions of the cells including Caski, HeLa, and B16F10 cells, used in this study (Figure 3A-D).

It has been currently reported that the development of sepsis in mice following the modified liposome, a lipopolysaccharide, injection, was significantly inhibited by pre-treatment of the animals with anti-TNF-α siRNA.[13] Furthermore, liposome complexation of siRNA as pharmaceutical drugs might offer a powerful tool for future therapeutical application. Therefore, our observation that cationic lipid-based siRNA showed the

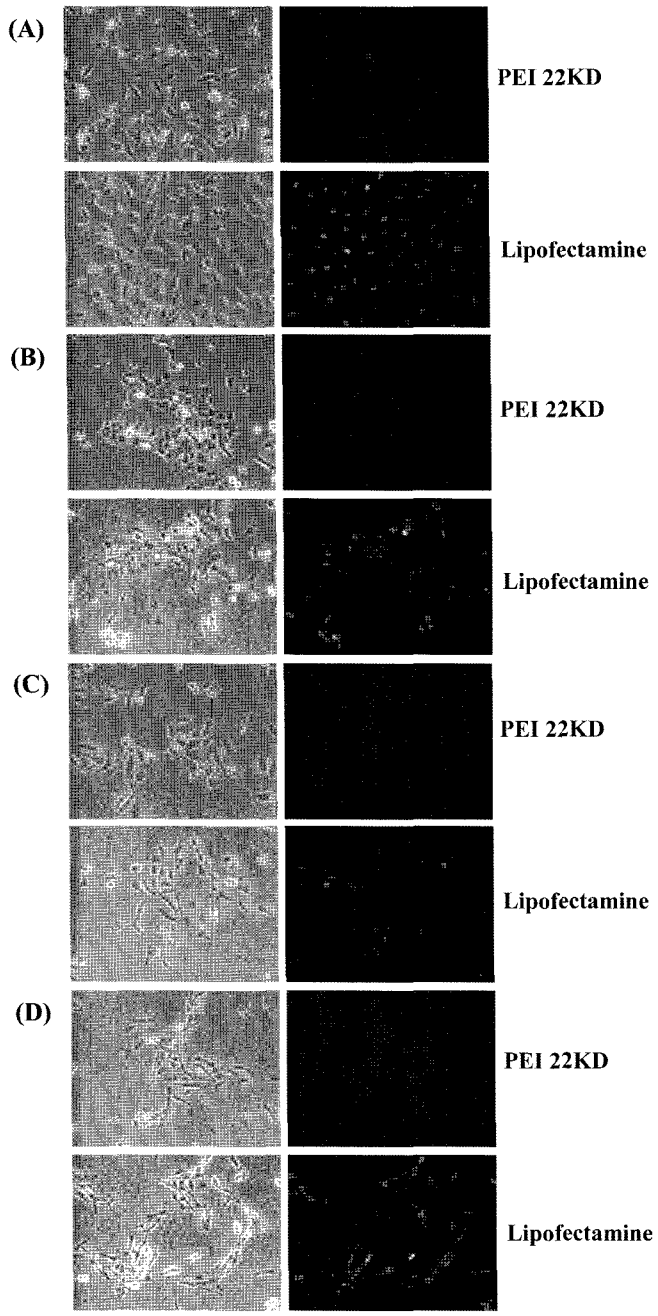


Figure 3–Delivery of fluorescein-labeled dsRNA to various cell lines. Fluorescein-labled dsRNA complexed to Lipofectamine or PEI 22KD was added to SiHa (A), CaSki (B), HeLa (C), or B16-F10 (D) cells. At 24 hour after treatment, cells were washed and observed under the fluorescence microscope. Left panel shows phase-contrast images and right panel shows the fluorescence images.

higher delivery efficiency in wide ranges of cell types might be promising for diverse *in vivo* application of siRNA. However, on the other hand, the nonspecific high delivery of siRNA to variety of cell types may cause undesirable side effects. To prevent the side effects of siRNA due to the nonspecific delivery, it will be necessary to develop the targeting machinery in delivery vehicles and to design cell type-specific siRNA targeting sequences in the near future.

Conclusion

Our results suggest that the mechanisms involved in siRNA delivery might be different from those in plasmid DNA delivery. Moreover, in various cell lines, cationic lipid-based delivery vehicles delivered siRNA with higher efficiency to intracellular target sites than did PEI of different molecular weights and branched/linear forms.

Acknowledgements

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