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Efficient Transfer of Intact Oligonucleotides into the Nucleus of Ligament Scar Fibroblasts by HVJ-Cationic Liposomes Is Correlated with Effective Antisense Gene Inhibition[1]

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The efficacy of two different cationic liposomes, Lipofectin and hemagglutinating virus of Japan (HVJ)–cationic liposomes, on nuclear uptake of fluorescence-labeled phosphorothioate oligodeoxyribonucleotide (S-ODN) by ligament scar fibroblasts and suppression of decorin mRNA expression when antisense decorin S-ODN was transferred was investigated. There was no significant difference in nuclear uptake of fluorescent ODN between the two methods. However, only HVJ-cationic liposomes had a significant effect on suppression of decorin mRNA expression levels. To address the discrepancy, the molecular integrity of the transferred ODN in the cells was assessed by analysis of fluorescence resonance energy transfer (FRET) within double-fluorescence-labeled S-ODN. More than 70% of the ODN transfected by HVJ-cationic liposomes remained intact within the nucleus at 20 h after transfection, while the majority of the ODN transferred by Lipofectin was degraded at this point. These results suggest a strong relationship between the nuclear integrity of transfected antisense ODN and its suppression of target mRNA expression.

Key words: antisense oligonucleotides, fluorescence resonance energy transfer, liposome, Sendai virus.

Antisense oligonucleotides (ODNs) are potential therapeutic agents which inhibit gene expression in a sequence-specific manner. Although the precise mechanism of antisense action has not been fully elucidated (*1, 2*), it is widely accepted that RNase H hydrolyses the RNA strand of an RNA-ODN duplex and is probably responsible for the major antisense effects of ODNs (*3*). Further, recent study demonstrated that expression of target mRNA does not begin to decline until after antisense ODN is seen in the nucleus (*4*). These findings together suggest that antisense ODNs function mainly within the nucleus. Therefore, it is reasonable to presume that success in antisense therapy depends upon efficient delivery of ODNs into the cell nucleus. Cationic lipids are widely used for this purpose and have been shown to enhance the antisense effect of ODNs *in vitro* in several types of cells (*5–7*). ODNs associated with cationic liposomes are presumably introduced into cells by endocytosis (*5, 8*). Therefore, a significant amount of the ODN might be degraded in the endocytic pathways before reaching the nucleus. Recently, a highly efficient gene delivery method using hemagglutinating virus of Japan (HVJ, Sendai virus) to enhance the fusion of anionic liposomes to cell membranes has been developed (*9, 10*). The HVJ-mediated gene delivery system using cationic lipids for the liposomes has also been shown to be an effective method of delivering genes to cells (*11*). Using this technique, transfer of antisense ODN specific for the proteoglycan decorin has been shown to effectively decrease decorin mRNA levels in scar fibroblasts (*12*).

The present study was undertaken to investigate the effectiveness of cationic liposomes (Lipofectin®) and HVJ-cationic liposomes in delivering ODNs into the nuclei of rabbit ligament scar fibroblasts *in vitro*. While both methods effectively delivered ODNs into the nuclei of these cells, only HVJ-cationic liposome method suppressed target mRNA expression. We hypothesized that the integrity of

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Abbreviations: HVJ, haemagglutinating virus of Japan; ODN, oligodeoxyribonucleotide; RT-PCR, reverse transcription polymerase chain reaction; FRET, fluorescence resonance energy transfer; 6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; BSS, balanced salt solution; PBS, phosphate-buffered saline; DOTMA, *N*-(1-(2,3-dioleyloxy)propyl)-*N,N,N*-trimethyl-ammonium chloride; DOPE, dioleolylphosphatidylethanolamine; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; bp, base pair(s).

ODN transfected into nuclei by these two methods might differ, and tested this hypothesis by fluorescence resonance energy transfer (FRET) analysis (*13*, *14*). FRET is an interesting example of a fluorescence-related phenomenon. When the fluorescence spectrum of one fluorophore (donor) overlaps with the excitation spectrum of another fluorophore (acceptor), and when the donor and the acceptor are in close physical proximity, the excitation of the donor induces the emission of fluorescence from the acceptor as if the acceptor had been excited directly, while the donor's own fluorescence decreases. Uchiyama *et al.* demonstrated the visualization of FRET under the fluorescence microscope in sea uchin eggs after microinjection of double fluorescence-labeled ODNs, but found that enzymatic digestion of ODNs eliminated the efficiency of FRET in eggs. Based on those results, they proposed potential use of FRET for the detection of only intact ODNs in living cells after transfection (*13*). The results of FRET analysis showed that HVJ-cationic liposomes method were over three times more efficient in delivering intact ODNs within the nuclei of transfected cells than lipofectin. These results suggest a correlation between the integrity of transfected ODN within the nuclei and its effectiveness in suppressing targeted mRNA expression.

MATERIALS AND METHODS

Synthesis of Oligonucleotides and Selection of Sequence Targets—The sequences of phosphorothioate ODN against rabbit decorin were: antisense, 5′-GGA-TGA-GAG-TTG-CCG-TCA-TG-3′; sense, 5′-CAT-GAC-GGC-AAC-TCT-CAT-CC-3′ (−1 to +19 of the rabbit sequence). This antisense ODN specifically inhibits decorin mRNA in primary cultured rabbit medial collateral ligament (MCL) scar fibroblasts and in MCL scar tissue *in vivo* (*12*). The antisense sequence selected for further studies yielded the best suppression of decorin mRNA levels among five sequences tested in preliminary experiments. The other antisense sequences tested were: 5′-GTT-GCC-GTC-ATG-ATT-TAT-TT-3′ (−9 to + 11); 5′-AGG-ATG-AGA-GTT-GCC-3′ (0 to +15); 5′-TGA-GAG-TTG-CCG-TCA-TGA-3′ (−2 to +16); and 5′-GAG-AGT-TGC-CGT-CAT-GAT-3′ (−3 to +15). The first of these sequences showed modest inhibition (~50%) of decorin mRNA levels *in vitro*, while the other three sequences yielded no inhibition of decorin mRNA levels in primary cultures of rabbit MCL scar cells. The sequence of the 6-carboxyfluorescein (FAM)–labeled phosphorothioate ODN was 5′-CTT-CGT-CGG-TAC-CGT-CTT-C-3′ (19 bp). 6-FAM was labeled on the 3′ and 5′ ends of the ODN. These ODNs were synthesized and purified by the University of Calgary Regional DNA Synthesis laboratory. The sequence of the double- labeled phosphorothioate ODN was 5′-CGG-GGC-TGA-TGA-GGC-CGA-AAG-GCC-GAA-ACG-GCT-3′ (33 bp). The chemically synthesized ODN was labeled with 6-carboxytetramethylrhodamine (TAMRA) and 6-FAM on the 3′ and 5′ ends, respectively, and purified as previously reported (*13*, *14*).

Preparation of HVJ-Cationic Liposomes and Cationic Liposomes (Lipofectin)—HVJ-cationic liposomes were prepared as described previously (*11*, *15*). Briefly, a lipid mixture which contained 6 mg of phosphatidylcholine, 3 mg of cholesterol, and 0.75 mg of 3β-[*N*-(*N*,*N*-dimethylaminoethane)carbamoyl]cholesterol was dissolved in chloroform and then dried with a rotary evaporator. The dried lipid mixture was hydrated with 200 μl of balanced salt solution (BSS; 140 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.5) containing ODN (20 nmol). Liposomes were prepared by vortexing and extrusion. The liposomes were fused with HVJ that had been inactivated by ultraviolet irradiation. The HVJ-liposome complexes were purified by ultracentrifugation through a 30% (w/w) sucrose layer at 62,800 ×*g* for 90 min. Cationic liposomes (Lipofectin™/Gibco BRL) composed of *N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethyl-ammonium chloride (DOTMA) and dioleolylphosphatidylethanolamine (DOPE) in a 1:1 ratio (w/w) were prepared according to the manufacturer's instructions. Briefly, the ODN was diluted into 100 μl of prewarmed Opti-MEM (Gibco BRL) in polystyrene plastic culture tubes. Lipofectin was diluted similarly. The prediluted ODN and Lipofectin mixtures were combined in polystyrene plastic culture tubes. After 15 min, 800 μl of prewarmed Opti-MEM was added. This mixture was immediately used for transfection.

Cell Culture—Primary rabbit medial collateral ligament scar fibroblasts were obtained from ligament scar 3 wk post-injury (*16*) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO_2.

Fluorescence (6-FAM) Uptake Study—Before transfection, 2×10^5 cells were cultured on a coverslip in each well of a 6-well plate. After 24 h, the cells were incubated with: (a) naked 6-FAM–labeled ODN; (b) 6-FAM-ODN in Lipofectin (either 10 or 20 μg/ml); (c) 6-FAM-ODN in HVJ-cationic liposomes for 8 h. The concentration of ODN was adjusted to 0.1, 1, or 10 μM in each experiment. After transfection, the cells were washed once with DMEM and twice with phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4), then fixed with 4% formalin in PBS at room temperature for 15 min. The cells were mounted and observed with a fluorescence microscope (Axiophoto, Zeiss, Jena, Germany) after nuclear staining with Hoechst 33258 dye (Sigma, St. Louis, MO), and the ratio of the number of FAM-6-fluorescent nuclei to that of Hoechst dye-stained (total) nuclei was calculated using an image analysis system (Kontron Elektronik, Germany). For the nuclear fluorescence uptake analysis, an average of 4 fields taken at a magnification of 200 was used (*17*). Data were analyzed using ANOVA (one-way) and Student's *t*-test. All reported experiments were performed in triplicate.

Decorin Antisense Study and Semi-Quantitative RT-PCR—Since RNase H hydrolyses the RNA strand of a RNA-DNA duplex and appears to be responsible for the major antisense effects of ODNs (*3*), degradation of the target mRNA was measured by RT-PCR to evaluate the efficacy of transfection. Sense ODN complimentary to the antisense sequence was used as control.

Cells (2×10^5) were cultured in 6-well cell culture plates. After 24 h, the cells were transfected using: (a) naked antisense or sense ODNs for decorin (1 μM); (b) antisense or sense ODNs for decorin (1 μM) in Lipofectin (20 μg/ml); (c) antisense or sense ODNs for decorin (1 μM) in HVJ-cationic liposomes. After 8 h of incubation, ODNs were removed and cells were washed with DMEM, then cultured further in DMEM supplemented with 10% FCS for 40 h. The medium was then removed, 1 ml of TRIzol™ (Gibco/BRL, MD) was added to each well, and total RNA was iso-

lated from the cells by the TRIspin method (*18*). Semi-quantitative RT-PCR was performed on the extracted RNA. Briefly, 1 μg of total RNA was reverse-transcribed using the StrataScript™ RT-PCR kit (Stratagene, CA), and aliquots of cDNA were amplified by PCR using primer sets specific for rabbit decorin and a constitutively expressed housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The rabbit decorin 5′ primer (nucleotides 780 to 800) was 5′-TGT-GGA-CAA-TGG-TTC-TCT-GG-3′; the 3′ primer (nucleotides 1239 to 1250) was 5′-CCA-CAT-TGC-AGT-TAG-GTT-CC-3′. The rabbit GAPDH 5′ primer (nucleotides 211 to 230 of rabbit sequence) was 5′-TCA-CCA-TCT-TCC-AGG-AGC-GA-3′; the 3′ primer (nucleotides 488 to 507) was 5′-CAC-AAT-GCC-GAA-GTG-GTC-GT-3′. The rabbit-specific primers were synthesized and purified by the University of Calgary Regional DNA Synthesis Laboratory. Cloning and sequencing of the amplified cDNA fragments confirmed the identity of the products. Optimal conditions for semi-quantitative RT-PCR have been described previously (*19–22*) and assessments were in the linear range of the assay.

Fluorescence Resonance Energy Transfer (FRET) Study—Cells (1×10^5) were cultured in 6-well plates. After 24 h, 4 μg of double-fluorescence–labeled ODN in Lipofectin or in HVJ-liposomes was added to the cultured cells as described above. After 20 h of transfection, FRET was microscopically visualized using a fluorescence microscope with two special filters (Diaphoto-TMD; Nikon, Tokyo) as previously described (*13*, *14*). Briefly, green images were observed through a band-pass filter at 510–560 nm, and red images were observed through a cutoff filter at wavelengths above 580 nm. For both types of imaging, infrared radiation was eliminated with a heat-absorbing filter. The excitation light, obtained at 470–490 nm by passage through a band-pass filter, was removed from the fluorescence signal by use of a dichroic mirror with a wavelength cut-off of 510 nm. All filters and the mirror were purchased from Olympus (Tokyo). When excited with blue light at 470–490 nm, intact ODN has a specific fluorescence spectrum with peaks at 515 nm (6-FAM; a green image) and over 580 nm (TAMRA; a red image). Fluorescence intensity over 580 nm is detectable because of the FRET phenomenon. Unless the double-labeled ODN is intact, the fluorescence peak over 580 nm (red) almost completely disappears, while the fluorescence peak at 515 nm increases. Thus, green fluorescence within the nuclei is indicative of the accumulation of ODN regardless of the degradation of ODN, while the presence of red fluorescence within the nuclei demonstrates the accumulation of only intact ODN.

Nuclear fluorescence uptake analysis was performed as described above. For statistical analysis, Student's *t*-test was used. All experiments were performed in triplicate.

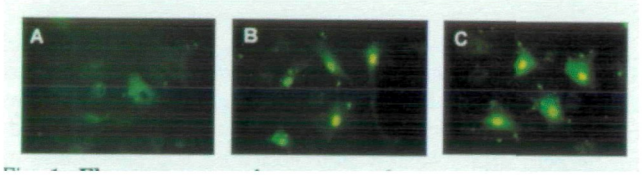

Fig. 1. **Fluorescence microscopy of scar cells transfected with 1 μM naked 6-FAM–labeled ODN (A), 1 μM 6-FAM-ODN in Lipofectin (20 μg/ml) (B), and 1 μM 6-FAM-ODN in HVJ-cationic liposomes (C).** (×250). Increased nuclear uptake of ODN is evident in cells treated with Lipofectin (B) and by HVJ-cationic liposomes (C).

TABLE I. **Quantitative assessment of the nuclear uptake of a 6-FAM–labeled ODN into ligament scar fibroblasts using various lipid and ODN concentrations.**

Delivery method	Conc. of lipid (μg/ml)	Conc. of ODN (μM)	% nuclear uptake of fluorescence
Naked ODN		0.1	0
		1	0
		10	0
Lipofectin	10	0.1	0.3
		1	13.3
		10	18.6
	20	0.1	4.3
		1	70.3
		10	68.4*
HVJ-cationic liposomes		0.1	8.7
		1	74.6
		10	82.6

*Decrease in cell viability noted.

RESULTS

Nuclear Uptake of 6-Carboxyfluorescein (FAM)–Labeled ODN—To evaluate the intracellular distribution of transfected ODN, scar cells were visualized by fluorescent microscopy. Incubation of MCL scar fibroblasts with naked ODNs resulted in weak staining in the cytoplasm of the cells regardless of the concentration of the ODN (Fig. 1A and Table I). ODNs complexed with Lipofectin showed an improved cellular uptake, and nuclear fluorescence was observed in a majority of the cells. Nuclear uptake of fluorescence varied with the concentration of lipid and ODN, and most efficient uptake was observed with 20 μg/ml Lipofectin and 1 μM ODN (approximately 70% of the cells had nuclear fluorescence, Fig. 1B and Table I). At 10 μM ODN, a decrease in cell viability was detected (approximately 20% dead cells). HVJ-cationic liposomes also enhanced cytoplasmic and nuclear uptake of the ODNs in scar cells. At both 1 μM or 10 μM ODN, more than 70% of MCL scar fibroblasts exhibited nuclear fluorescence and cell viability was maintained (Fig. 1C and Table I).

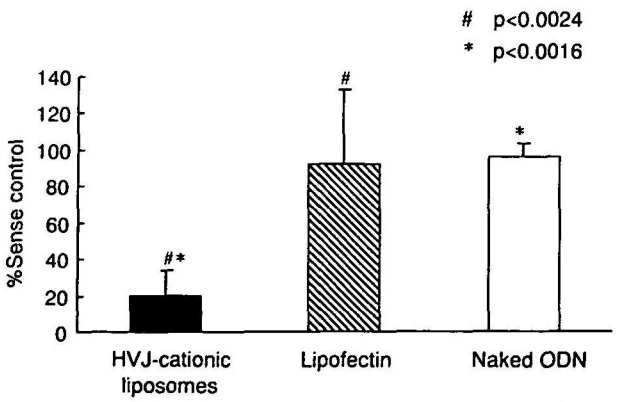

Fig. 2. **Ratio of decorin mRNA expression in antisense ODN (1 μM) treated ligament scar fibroblasts to that in sense ODN (1 μM) treated scar fibroblasts 48 h after treatment with naked antisense ODN, antisense ODN in Lipofectin (20 μg/ml), and in HVJ-cationic liposomes.**

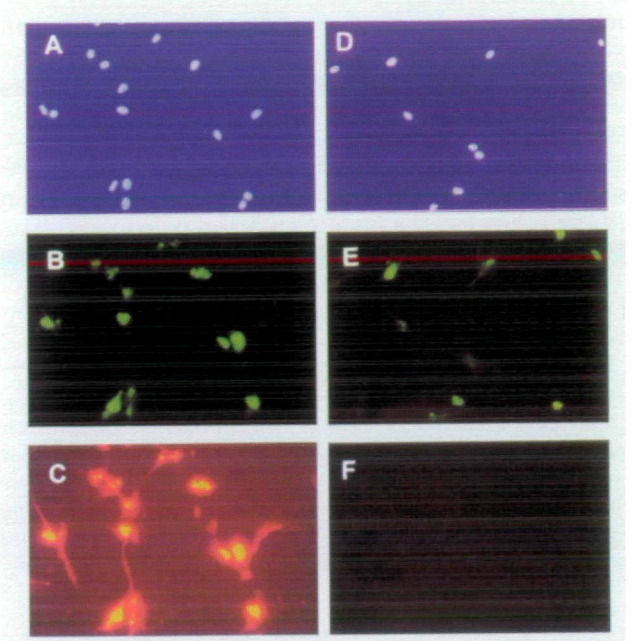

Fig. 3. **Fluorescence microscopy of ligament scar cells in which double-fluorescence-labeled ODN was, transfected by use of HVJ-cationic liposomes (A, B, C) and Lipofectin (D, E, F).** Pictures were taken at 20 h after transfection. (A, D) Hoechst 33258 (nuclear) stain. (B, E) 6-Carboxyfluorescein (6-FAM) detection by MNIB filter (excitation wavelength 470–490 nm, absorption wavelength 515 nm). (C, F) 6-Carboxytetramethylrhodamine (TAMRA) detection by a special filter (excitation wavelength 470–490 nm, absorption wavelength > 580 nm). (×250).

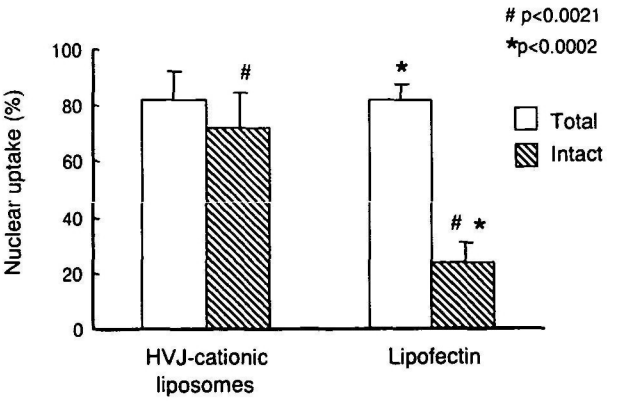

Fig. 4. **Percentage of cells with the fluorescence of total (both intact and degraded) ODN (white) and of intact ODN (gray) within the nuclei when transfected with Lipofectin and by HVJ-cationic liposomes.**

Decorin Antisense Study—From the above results, all subsequent decorin antisense experiments were performed using ODN at a concentration of 1 μM, and for the Lipofectin-mediated delivery, 20 μg lipid/ml of culture medium was used. Forty-eight hours after transfection, when RT-PCR testing was performed on the RNA extracted from MCL scar fibroblasts incubated with naked decorin antisense ODN, no suppression of decorin mRNA levels (97.4 ± 6.79% of sense control) (Fig. 2) could be detected. Likewise, no significant suppression of decorin mRNA expression was observed when the cells were transfected with lipofectin containing the decorin antisense ODN (93.1 ± 40.4% of sense control). In contrast, transfection with HVJ-cationic liposomes containing the decorin antisense ODN led to a significant suppression of decorin mRNA expression levels (20.3 ± 14.0% of sense control; $p < 0.002$) (Fig. 2). In the present antisense study, no significant difference in the expression levels of decorin mRNA was found between sense ODN-treated scar fibroblasts and untreated scar fibroblasts.

FRET Study—Although Lipofectin facilitated the accumulation of ODN within the cell nucleus, antisense decorin ODN delivered into nuclei did not suppress decorin mRNA levels when assayed by RT-PCR. To monitor the integrity of ODN within the cell nucleus, FRET analysis was performed. When transfected using Lipofectin, 82.7 ± 5.03% of MCL scar fibroblasts showed green fluorescence, corresponding to nuclear fluorescence by Hoechst 33258 (Fig. 3, A and B). However, the majority of the cells emitted no red fluorescence (Fig. 3F). Nuclear uptake analysis showed that only 24.0 ± 7.21% of the cells emitted red fluorescence within the nuclei (Fig. 4). Therefore, the Lipofectin-mediated delivery method led to the degradation of a high percentage of the double-labeled ODN in the majority of ODN-bearing cells, either before nuclear localization or within the nucleus. In contrast, most of the cells transfected with HVJ-cationic liposomes emitted bright red fluorescence in addition to the green fluorescence corresponding to nuclear fluorescence (Fig. 3, A, B, and C), suggesting that a high percentage of ODN was intact in the majority of transfected cells. Nuclear fluorescence uptake analysis showed that 82.7 ± 9.45% of the cells emitted green fluorescence and 72.7 ± 12.1% of the cells emitted red fluorescence within the nuclei (Fig. 4).

DISCUSSION

For effective antisense therapy, a vector should be used which efficiently delivers ODNs into cells and allows them to reach their target without degradation. In this regard, the present study clearly demonstrated the usefulness of HVJ-cationic liposomes for the effective delivery of antisense ODN. Although both HVJ-cationic liposomes and lipofectin were effective in accumulating ODN in the nuclei of transfected cells, HVJ-liposomes proved to be the more efficient in suppressing the target mRNA expression with antisense ODN.

Interestingly, such differences in antisense effect between these two vector systems appeared to correlate with the results of FRET analysis. This analysis showed that 88% (72.7% intact ODN divided by 82.7% labeled cells) of HVJ-cationic liposome-treated cells bearing intra-nuclear ODN fluorescence showed FRET after 20 h of culture. In contrast, only 29% (24.0% intact ODN divided by 82.7% labeled cells) of Lipofectin-treated cells bearing intra-nuclear ODN fluorescence did so. The extent of FRET is extremely sensitive to the distance between the two fluorophores (6-FAM and TAMRA), being inversely proportional the sixth power of the distance (*14*). Accordingly, even a subtle dissociation of the two fluorophores by some degradation of ODN can lead to the elimination of FRET. Therefore, the discrepancy in FRET phenomenon between the two different vector systems could be interpreted as reflecting the difference in the integrity of ODN within the nuclei.

Haemagglutinating virus of Japan (HVJ, Sendai virus) is well known to enhance the fusion of liposomes to cell membranes (*10*). Therefore, ODN trapped in HVJ-cationic liposomes could be directly transferred into the cytoplasm without lysosomal trafficking and possible degradation. In contradistinction, with the use of cationic liposomes (including Lipofectin), the major cellular uptake mechanism of ODN is considered to be endocytosis (*5*, *8*), creating the potential for the degradation of ODN in lysosomes. Therefore, the difference in the cellular uptake mechanisms of ODN between these two vector systems might influence the integrity of ODN transferred into the nucleus.

The nucleus has been regarded as a major location of antisense action, with the binding of antisense ODN with its target mRNA and subsequent degradation of the bound mRNA by a nuclear enzyme, RNase H (*1*, *3–5*). Taken together, these results obtained with two different ODN delivery methods suggest that the integrity of ODN within the nuclei of target cells is of critical importance for effective suppression of a specific target molecule in antisense therapy.

In summary, the present study demonstrates that HVJ-cationic liposomes enhance the effective delivery of ODN into cultured cells with preservation of the integrity of the ODN, leading to significant antisense suppression of target mRNA expression. In this study, rabbit ligament scar fibroblasts were used, but similar experiments with other cell types have also indicated that HVJ-cationic liposomes deliver plasmid DNA, ribozyme, and ODN effectively (*11*, *15*). Therefore, this method could be applicable to numerous gene therapies and a variety of cell targets.

REFERENCES

1. Crooke, S.T. and Bennett, C.F. (1996) Progress in antisense oligonucleotide therapeutics. *Annu. Rev. Pharmacol. Toxicol.* **36**, 107–129
2. Gerwirtz, A.M., Stein, C.A., and Glazer, P.M. (1996) Facilitating oligonucleotide delivery: helping antisense deliver on its promise. *Proc. Natl. Acad. Sci. USA* **93**, 3161–3163
3. Wagner, R.W. (1994) Gene inhibition using antisense oligodeoxynucleotides. *Nature* **372**, 333–335
4. Marcusson, E.G., Bhat, B., Manoharan, M., Bennett, C.F., and Dean, N.M. (1998) Phosphorothioate oligodeoxyribonucleotides dissociate from cationic lipids before entering the nucleus. *Nucleic Acids Res.* **15**, 2016–2023
5. Bennett, C.F., Chiang, M.Y., Chan, H., Shoemaker, J.E., and Mirabelli, C.K. (1992) Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* **41**, 1023–1033
6. Colige, A., Sokolov, B.P., Nugent, P., Baserga, R., and Prockop, D.J. (1993) Use of an antisense oligonucleotide to inhibit expression of a mutated human procollagen gene (COL1A1) in transfected mouse 3T3 cells. *Biochemistry* **32**, 7–11
7. Ollikainen, H., Lappalainen, K., Jaaskelainen, I., Syrjanen, S., and Pulkki, K. (1996) Liposomal targeting of bcl-2 antisense oligonucleotides with enhanced stability into human myeloma cell lines. *Leuk. Lymphoma* **24**, 165–174
8. Wattiaux, R., Jadot, M., Laurent, N., Dubois, F., and Wattiaux-DeConnick, S. (1996) Cationic lipids delay the transfer of plasmid DNA to lysosomes. *Biochem. Biophys. Res. Commun.* **227**, 448–454
9. Kaneda, Y., Iwai, K., and Uchida, T. (1989) Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science* **243**, 375–378
10. Kaneda, Y., Uchida, T., Kim, J., Ishiura, M., and Okada, Y. (1987) The improved efficient method for introducing macromolecules into cells using HVJ (Sendai virus) liposomes with gangliosides. The improved efficient method for introducing macromolecules into cells using HVJ (Sendai virus) liposomes with gangliosides. *Exp. Cell Res.* **173**, 56–69
11. Saeki, Y., Matsumoto, N., Nakano, Y., Mori, M., Awai, K., and Kaneda, Y. (1997) Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and in vivo gene transfer. *Hum. Gene Ther.* **8**, 2133–2141
12. Nakamura, N., Timmermann, S.A., Hart, D.A., Kaneda, Y., Shrive, N.G., Shino, K., Ochi, T., and Frank, C.B. (1998) A comparison of in vivo gene delivery methods for antisense therapy in ligament healing. *Gene Ther.* **5**, 1455–1461
13. Uchiyama, H., Hirano, K., Kashiwasake-Jibu, M., and Taira, K. (1996) Detection of undegraded oligonucleotides in vivo by fluorescence resonance energy transfer. Nuclease activities in living sea urchin eggs. *J. Biol. Chem.* **271**, 380–384
14. Ota, N., Hirano, K., Warashina, M., Andrus, A., Mullah, B., Hatanaka, K., and Taira, K. (1998) Determination of interactions between structured nucleic acids by fluorescence resonance energy transfer (FRET): selection of target sites for functional nucleic acids. *Nucleic Acids Res.* **26**, 735–743
15. Kitajima, I., Hanyu, N., Soejima, Y., Hirano, R., Arahira, S., Yamaoka, S., Yamada, R., Maruyama, I., and Kaneda, Y. (1997) Efficient transfer of synthetic ribozymes into cells using hemagglutinating virus of Japan (HVJ)-cationic liposomes. Application for ribozymes that target human t-cell leukemia virus type I tax/rex mRNA. *J. Biol. Chem.* **272**, 27099–27106
16. Ross, S.M., Joshi, R., and Frank, C.B. (1990) Establishment and comparison of fibroblast cell lines from the medial collateral and anterior cruciate ligaments of the rabbit. *In Vitro Cell. Dev. Biol.* **26**, 579–584
17. Toritsuka, Y., Nakamura, N., Lee, S.B., Hashimoto, J., and Yasui, N. (1997) Osteoclastogenesis in iliac bone marrow of patients with rheumatoid arthritis. *J. Rheumatol.* **24**, 1690–1696
18. Reno, C., Marchuk, L., Sciore, P., Frank, C.B., and Hart, D.A. (1997) Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. *Biotechniques* **22**, 1082–1086
19. Marchuk, L., Sciore, P., Reno, C., Frank, C.B., and Hart, D.A. (1998) Postmortem stability of total RNA isolated from rabbit ligament, tendon and cartilage. *Biochim. Biophys. Acta* **1379**, 171–177
20. Hart, D.A., Boykiw, R., Sciore, P., and Reno, C. (1998) Complex alterations in gene expression occur in the knee ligaments of the skeletally mature multiparous rabbit during pregnancy. *Biochim. Biophys. Acta* **1397**, 331–341
21. Hart, D.A., Sciore, P., Boykiw, R., and Reno, C. (1998) Pregnancy induces complex changes in the pattern of mRNA expression in knee ligaments of the adolescent rabbit. *Matrix Biol.* **17**, 21–34
22. Boykiw, R., Sciore, P., Reno, C., Marchuk, L., Frank, C.B., and Hart, D.A. (1998) Altered levels of extracellular matrix molecules mRNA in healing rabbit ligaments. *Matrix Biol.* **17**, 371–378