In Vivo Delivery of Phosphorothioate Oligonucleotides Into Murine Retina

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Objectives: To determine the fate of phosphorothioate oligonucleotides (S-ODNs), which are commonly used for antisense strategy, in murine retina in vivo with the use of fluorescein isothiocyanate (FITC)–labeled S-ODNs, and to evaluate our fusogenic liposome system that may facilitate the delivery of S-ODNs.

Methods: The FITC-labeled S-ODNs were encapsulated in liposomes, which were then coated with the envelope of inactivated hemagglutinating virus of Japan (HVJ; Sendai virus) by fusion (HVJ liposomes). Intravitreal injection of naked FITC-labeled S-ODNs or of the HVJ liposomes was done in ICR mice. After fixation, cryo-sections and flat-mounted retinas were prepared and examined by fluorescence microscopy.

Results: Injection of naked FITC-labeled S-ODNs at 3 µmol/L exhibited weak fluorescence in 13% of the cells in the ganglion cell layer. When the concentration was increased to 30 µmol/L, high fluorescence was seen in 59% of cells in the ganglion cell layer at this time. This fluorescence diminished within a day. In contrast, injection of HVJ liposomes containing FITC-labeled S-ODNs at 3 µmol/L resulted in high fluorescence in 44% of the cells in the ganglion cell layer at 1 hour, and this fluorescence lasted for up to 3 days. This treatment also resulted in high fluorescence within retinal vessel walls, and weak fluorescence in photoreceptor cells.

Conclusions: Intravitreally injected S-ODNs were rapidly eliminated from neural retina, and the use of HVJ liposomes could improve the delivery of S-ODNs. This method may be a potentially useful system for the antisense-based therapies for retinal diseases.


Antisense oligonucleotides (ODNs) show great promise as agents to inhibit the expression of specific genes that regulate physiological functions or mediate various diseases. Recent studies have shown that antisense ODNs can exert pharmacological effects on the retina in vivo after their direct intravitreal injection. The usefulness of intravitreal injection of antisense ODNs against cytomegalovirus RNA is currently being studied for the treatment of cytomegalovirus retinitis in patients with the acquired immunodeficiency syndrome. Thus, antisense ODNs appear to have potential as powerful tools for retinal research and even for the treatment of some serious retinal diseases.

Unfortunately, the effectiveness of antisense ODNs is limited by their poor cellular uptake and subsequent degradation by endocytic-lysosomal pathways remain major obstacles. To circumvent these problems, efficient delivery systems for ODNs are essential. Numerous methods have been explored, and some have succeeded in enhancing the efficiency of delivery of ODNs to various cells. Although several experiments have shown that antisense ODNs can inhibit cellular and viral gene expression in the retina by their intravitreal injection, the fate of ODNs in the retina has not yet been determined, and efficient systems for their delivery into the retina have not been described.

We previously described a simple and efficient system to deliver macromolecules such as DNA and RNA into cells with the use of liposomes and viral coats of hemagglutinating virus of Japan (HV); Sendai virus. In this system, the viral envelope proteins (hemagglutinin-neuraminidase and fusion proteins) on liposomes mediate liposome-cell mem-
MATERIALS AND METHODS

SYNTHESIS OF FITC-LABELLED S-ODNs

Scramble S-ODN (5’-CTTCGTCGGTACCGTCTT-3’) was synthesized and purified over a nucleic acid purification column (NAP 10, Pharmacia Fine Chemicals, Piscataway, NJ). This sequence was randomly selected, and its complementatory sequence (5’-AAGACGGTAGACGGAAGG-3’) does not correspond to any known messenger RNA. The FITC was labeled on the 3’ and 5’ ends of the S-ODNs by means of phosphoramidite chemistry.

PREPARATION OF HVJ LIPOSOMES

Bovine brain phosphatidylserine (sodium salt) was purchased (Avanti Polar Lipids, Alabaster, Ala), as were egg yolk phosphatidylcholine and cholesterol (Sigma Chemical Co, St Louis, Mo).

The HVJ liposomes were prepared as described previously, with a slight modification. The HVJ liposomes were prepared as described previously, with a slight modification. Briefly, 10 mg of a dried lipid film consisting of phosphatidylserine, phosphatidylcholine, and cholesterol (1:4:8.2 in weight ratio) was mixed with FITC-labeled S-ODNs, shaken vigorously, and sonicated to form liposomes. The HVJ (Z strain) was purified as described previously. The purified HVJ was inactivated by UV irradiation (110 erg/mm² per second) for 3 minutes immediately before use. The liposome suspension (0.5 mL, containing 10 mg of lipids) was mixed with the inactivated HVJ (30 000 hemagglutinating units) in a total volume of 4 mL of balanced saline solution. The mixture was incubated for 5 minutes at 4°C, and then for 30 minutes with gentle shaking at 37°C. Free HVJ was removed from the HVJ liposomes by sucrose density gradient centrifugation. The HVJ liposome solution was 8-fold concentrated by centrifugation for 30 minutes at 27 000g.

IN VIVO DELIVERY

All animal experiments adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. ICR mice (6-week-old male mice) were anesthetized with an intramuscular injection of ketamine hydrochloride, and their pupils were dilated with 1% atropine sulfate and 0.5% phenylephrine. The intravitreal injections were made via a pars plana approach with a 33-gauge beveled needle under an ophthalmic operating microscope. Eyes that sustained major surgical trauma, such as retinal breaks, retinal detachment, or retinal hemorrhage, were excluded from histological examination. The HVJ liposome solutions (3 µL) containing FITC-labeled S-ODNs (3 µmol/L) or naked FITC-labeled S-ODNs (3 µmol/L or 30 µmol/L) were injected.

RESULTS

DELIVERY OF NAKED S-ODNs

To determine the distribution of intravitreally injected naked S-ODNs in retina, we examined cross sections by fluorescence microscopy. Intravitreal injection of naked FITC-labeled S-ODNs, 3 µmol/L, resulted in weak fluorescence primarily in the GCL when examined 1 hour after injection (Figure 1, A, and Figure 2, A). Little fluorescence was detectable in other areas at this time. Injection of 10-fold naked FITC-labeled S-ODNs (30 µmol/L) increased the number of positive cells in the GCL and also enhanced the intensity of their fluorescence (Figure 1, B, and Figure 2, B). In addition, this treatment resulted in diffuse high fluorescence in the inner plexiform layer, scattered fluorescence in the first row on the vitreal side of the inner nuclear layer, and very weak fluorescence in the retinal vessel walls and the outer plexiform layer 1 hour after injection (Figure 1, B). However, the fluorescence in the inner neural retina...
Figure 1. Cross sections of retina intravitreally injected with naked fluorescein isothiocyanate (FITC)–labeled phosphorothioate oligonucleotides (S-ODNs) or hemagglutinating virus of Japan (HVJ) liposomes containing FITC-labeled S-ODNs. A through C, Eyes injected with naked FITC-labeled S-ODNs. A, 3 µmol/L, 1 hour after injection. B, 30 µmol/L, 1 hour after injection. Note low fluorescence in retinal blood vessels (arrows). C, 30 µmol/L, 1 day after injection. Note high fluorescence in the retinal pigment epithelial cells with nuclei that appeared as dark spots (arrows). D, Control eyes injected with HVJ liposomes containing nonlabeled S-ODNs. E through G, Eyes injected with HVJ liposomes containing 3-µmol/L FITC-labeled S-ODNs. E, 1 hour after injection. Note high fluorescence in retinal blood vessels (arrows). F, 3 days after injection. G, 7 days after injection. Note high fluorescence in the retinal pigment epithelial cells, where nuclei appeared as dark spots (arrows). GCL indicates ganglion cell layer; ONL, outer nuclear layer; bar, 25 µm.
Figure 2. Flat-mounted retina focused on the ganglion cell layer. A through C, Eyes injected with naked fluorescein isothiocyanate (FITC)-labeled phosphorothioate oligonucleotides (S-ODNs). A, 3 µmol/L, 1 hour after injection. B, 30 µmol/L, 1 hour after injection. C, 30 µmol/L, 1 day after injection. This fluorescence on day 1 was detected only in small localized areas. D and E, Eyes injected with hemagglutinating virus of Japan (HVJ) liposomes containing 3-µmol/L FITC-labeled S-ODNs. D, 1 hour after injection. E, 5 days after injection. Note that many cells showed fluorescence seemingly in the cytoplasm, but scattered cells showed fluorescence in the nuclei. F, Retina stained with 5% propidium iodide for 2 minutes. G and H, Low-power fluorescent micrographs showing widespread distribution of fluorescence on day 1 in eyes injected with HVJ liposomes (G) and rapid elimination of fluorescence in eyes injected with 30-µmol/L naked FITC-labeled S-ODNs at the same time (H). Bar indicates 25 µm (A through F); 100 µm (G and H).
cent micrographs of flat-mounted retinas showed that this fluorescence in the GCL was widely distributed throughout the retina on day 1, while little fluorescence was seen in control eyes injected with naked FITC-labeled S-ODNs, 30 µmol/L (Figure 2, G and H). On days 5 through 7, there were many cells with nuclei that appeared as dark spots and that showed fluorescence seemingly in the cytoplasm. At these times, scattered cells (7.8% on day 5; 2.7% on day 7) showed fluorescence in the nuclei (Figure 2, E). Little fluorescence was detectable in control eyes injected with HVJ liposomes containing nonlabeled S-ODNs (Figure 1, D).

The HVJ liposome-mediated transfer of S-ODNs also resulted in high fluorescence in retinal vessel walls. All of the eyes injected with HVJ liposomes containing FITC-labeled S-ODNs exhibited intense fluorescence in retinal vessel walls 1 hour and 1 day after injection (Figure 1, E). Although decreased, this fluorescence was still detectable in 3 of 6 eyes 3 days after injection, but not on day 5 or 7. Autofluorescence of elastic fibers can be converted to a dark red color by counterstaining with eriochrome black T. 26 However, the fluorescence in the vessel walls was unchanged by this treatment (data not shown). In contrast, the eyes injected with naked FITC-labeled S-ODNs showed little fluorescence in the vessel walls (Figure 1, A and B). Furthermore, there appeared to be diffuse weak fluorescence in the outer nuclear layer, which was decreased but still detectable in some cells 7 days after injection (Figure 1, E-G). Fluorescence was not observed in the retinal pigment epithelium 1 hour and 1 day after injection, but was detected at 3 to 7 days (Figure 1, E-G). This fluorescence in the retinal pigment epithelial cells appeared to be localized only in the cytoplasm.

**PATHOGENICITY**

There was no histological evidence of retinal damage or infiltration of inflammatory cells in the eyes intravitreally injected with HVJ liposomes. In these eyes the fundus showed a blurred appearance. However, the vitreous became clear except for some mild haziness around the clumped liposomes on localized retina by the following day, but this had cleared completely by day 5. Throughout the study, all animals appeared healthy and showed no changes in their consumption of food and water. No animals died before scheduled death.

**COMMENT**

Intravitreally injected naked S-ODNs were rapidly eliminated from murine retina. This poor stability could be markedly improved by using our fusogenic liposomes (HVJ liposomes). The instability of ODNs has been partially overcome by using nuclease-resistant analogues, such as phosphorothioate modification. In this study, however, intravitreally injected naked S-ODNs were almost completely eliminated from neural retina within 1 day, even at high concentrations (Figure 1, C, and Figure 2, C). This rapid degradation will limit the use of antisense S-ODNs in the retina. In addition, high concentrations of S-ODNs (30 µmol/L) were needed to deliver them into high percentages of cells in the GCL (Figure 3).

**DElIVERY BY HVJ LIPOSOMES**

Transfer by HVJ liposomes increased significantly the number of the cells in the GCL transfected with S-ODNs. Intravitreal injection of HVJ liposomes containing S-ODNs (3 µmol/L in HVJ liposomes) resulted in high fluorescence in 44% of the cells in the GCL 1 hour after injection (P<.001 vs direct injection of 3-µmol/L naked fluorescein isothiocyanate (FITC)–labeled phosphorothioate oligonucleotides (S-ODNs) (Student t test). HVJ indicates hemagglutinating virus of Japan.
Since high oligonucleotide concentrations are thought to generate cytotoxic effects, injection of as high a concentration of S-ODNs as 30 μmol/L may exert toxic effects on the retina. Moreover, antisense effects must be interpreted with caution, because there is a growing body of evidence as to possible nonspecific effects of ODNs, especially of S-ODNs, on various biological actions.1,3,26-35 It is thought that nonspecific effects might be negligible if the concentrations of ODNs are kept low. The percentage of cells in the GCL transfected with S-ODNs was increased and cellular localization of S-ODNs was prolonged by using HVJ liposomes containing low concentration of S-ODNs. This improved delivery of S-ODNs by HVJ liposomes may, at least in part, solve these problems and widen the usefulness of antisense S-ODNs in the retina.

The use of HVJ liposomes resulted in high fluorescence in retinal vascular endothelium and weak fluorescence in the photoreceptors, whereas injection of naked S-ODNs produced little fluorescence in these cells (Figure 1, A, B, and E). The HVJ attaches to the cell surface by recognizing sialic acids. Sialic acids are abundant in retinal vascular endothelium.30 Therefore, HVJ liposomes may fuse better with retinal vessels. The present result raises the possibility that HVJ liposomes may be a promising drug delivery system to treat retinal blood vessels selectively with minimal effects on systemic vasculature.

Fluorescence in retinal vascular endothelium disappeared earlier than the neuronal cells, though the vascular fluorescence was far more intense (Figure 1, E and F). The reason is uncertain in this study, but one possible explanation is that intracellular kinetics and metabolism of S-ODNs vary with the cell types.

The present study did not demonstrate sufficiently in vivo stability of S-ODNs in the retina. We used S-ODNs labeled with fluorescein. Many researchers also used oligonucleotides labeled with chemical markers such as fluorescein to monitor their intracellular kinetics and stability. Decrease and disappearance of the fluorescence in the nuclei are thought to reflect degradation of oligonucleotides.37 Therefore, the prolonged existence of fluorescence in neural retina in this study would reflect the increased stability of S-ODNs by HVJ liposome-mediated introduction, though the results did not necessarily indicate the real stability.

Antisense S-ODNs have therapeutic potential in various retinal diseases. It has been already reported that intravitreal injection of antisense S-ODNs against cytomegalovirus RNA is effective for the treatment of cytomegalovirus retinitis in patients with the acquired immunodeficiency syndrome.7,8 Various strategies for serious retinal diseases could be devised, depending on the spectrum of the retinal cell types to which HVJ liposomes can target S-ODNs. For example, protection of retinal ganglion cells will be an attractive therapeutic target in retinal ischemic diseases and glaucoma,54,59 because retinal ganglion cell death in these diseases has been suggested to occur by apoptosis, a genetically regulated cell death.40-42 This may be achieved by inhibiting gene products that mediate apoptosis or by inhibiting the expression of N-methyl-D-aspartate receptors.43,44 Antisense S-ODNs against N-methyl-D-aspartate receptors were shown to be protective in a brain model of ischemic injury.45 The retinal large vessels appeared to be the effective target of HVJ liposomes. Retinal angiogenic diseases such as proliferative diabetic retinopathy and sickle cell retinopathy may be managed by antisense S-ODNs, for example, against vascular endothelial growth factor receptors,46 integrins αvβ3 and αvβ5,47 c-myc oncoprotein, or cell cycle–regulatory genes. Further studies should be undertaken to determine effects of antisense S-ODNs against these candidate pathogenic gene products with the use of animal models of retinal diseases.

In summary, this study has shown that intravitreal injection of S-ODNs was not effective in delivering them into neural retina in vivo, and the use of HVJ liposomes could improve the delivery of S-ODNs. This method may not only extend the usefulness of antisense S-ODNs in studies of the function and regulation of specific gene products in the retina but also open new avenues to antisense-based treatment of currently devastating retinal diseases.

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REFERENCES


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Figure 1. Cross sections of retina intravitreally injected with naked fluorescein isothiocyanate (FITC)–labeled phosphorothioate oligonucleotides (S-ODNs) or hemagglutinating virus of Japan (HVJ) liposomes containing FITC-labeled S-ODNs. A through C, Eyes injected with naked FITC-labeled S-ODNs. A, 3 µmol/L, 1 hour after injection. B, 30 µmol/L, 1 hour after injection. Note low fluorescence in retinal blood vessels (arrows). C, 30 µmol/L, 1 day after injection. Note high fluorescence in the retinal pigment epithelial cells with nuclei that appeared as dark spots (arrows). D, Control eyes injected with HVJ liposomes containing nonlabeled S-ODNs. E through H, Eyes injected with HVJ liposomes containing 3-µmol/L FITC-labeled S-ODNs. E, One hour after injection. Note high fluorescence in retinal blood vessels (arrows). F, Three days after injection. G, Seven days after injection. Note high fluorescence in the retinal pigment epithelial cells, where nuclei appeared as dark spots (arrows). GCL indicates ganglion cell layer; ONL, outer nuclear layer; bar, 25 µm.
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