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Induction of Gene into the Rabbit Eye by Iontophoresis: Preliminary Report

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Purpose: To assess the transfer of 6-carboxyfluorescein (6-FAM)-labeled phosphorothioate oligonucleotides(S-ODNs) into the ocular tissues, their stability, and possibility of injury to the ocular tissues.

Methods: The S-ODNs(2 mL/eye)were transduced noninvasively into albino rabbit eyes.The iontophoresis group consisted of 6 rabbits (12 eyes); the control group consisted of 2 rabbits (4 eyes) given eye drops containing S-ODNs. Aqueous humor and vitreous humor were collected after iontophoresis, subjected to electrophoresis with a fluorescence DNA sequencer and analyzed by the Gene Scan program. Frozen sections, 10-μm thick, were prepared for observation under a fluorescence microscope. A plasmid 4.7 kbp in size that expresses green fluorescent protein (GFP) was induced into the 18 eyes of 9 rabbits by the same procedure.

Results: In the iontophoresis group, S-ODNs were detected in the anterior chamber 5 minutes after electrophoresis began and in the vitreous after 10 minutes. These S-ODNs maintained the same length as at the initial synthesis. The S-ODNs could also be detected in the posterior retina 20 minutes after electrophoresis. No evidence of degeneration or inflammation due to the above procedure was found in the ocular tissues. Fluorescence showing GFP gene expression was found in the cornea, the anterior chamber angle, and the ciliary subepithelial tissues.

Conclusions: These findings show that iontophoresis is an effective method to induce genes into the rabbit eye. **Jpn J Ophthalmol 2001;45:31–39** © 2001 Japanese Ophthalmological Society

Key Words: Gene transduction, green fluorescent protein, iontophoresis, phosphorothioate oligonucleotide.

Introduction

In antisense therapy, the expression of target genes is regulated by the administration of antisense oligodeoxynucleotides. Their high rate of transduction into tissue and their stability are important factors in the therapy. Various methods, including different virus vectors, have been developed to transduce genes into cells.[1–5] However, at present, no single method can transduce genes efficiently in a manner that is safe to the target tissue. Furthermore, in order to improve the stability of oligonucleotides in tissue, phosphoric acids are modified by a sulfurization agent to form phosphorothioate oligonucleotides (S-ODNs). This facilitates the incorporation of oligonucleotides into cells and makes it more difficult for the oligonucleotides to be hydrolyzed by biological enzymes.

In the present study, 6-carboxyfluorescein (6-FAM)-labeled S-ODNs were administered to rabbit eyes noninvasively using iontophoresis to study S-ODN migration to such ocular tissue as the retina,

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to monitor their stability in the eye, and to assess the likelihood of their causing ocular tissue injury. In addition, plasmids were transduced in the same manner in order to incorporate alien genes into the eye. The present paper deals with the results of these experiments.

Materials and Methods

Preparation and Detection of 6-FAM-Labeled S-ODNs

A 23-base antisense oligonucleotide of human aldose reductase mRNA (TACCGCTCGGTAGATTCTGAGTT) was synthesized using an automatic synthesizer (DNA/RNA Synthesizer 392; Applied Biosystems, Foster, CA, USA). This sequence was chosen because we are currently using it to suppress the gene expression of human aldose reductase in transgenic mice. The oxidation of automatic synthesis was changed to sulfurization using tetraethylthiram-disulfide to modify every base. In addition, a labeling agent, 6-FAM, was used during the final cycle to label the 5′-end of each oligonucleotide. The synthesized S-ODNs were then purified by reverse phase high-performance liquid chromatography to eliminate free 6-FAM and to purify the longest oligonucleotide. The purified 6-FAM-labeled S-ODNs were then analyzed with a fluorescence DNA sequencer to check their purity based on the pattern of migration. No free oligonucleotides nor any extremely short oligonucleotides were found. The 6-FAM-labeled S-ODNs that were extracted from the vitreous humor and aqueous humor were subjected to electrophoresis, analyzed with a fluorescence DNA sequencer (377; Perkin-Elmer, Cetus, Norwalk, CT, USA), and evaluated by Gene Scan program (Figure 1). The main detection of the wavelength was 532 nm. Unlike other modified oligonucleotides, stereoisomers are easily formed with S-ODNs, and as the result three peaks were seen.

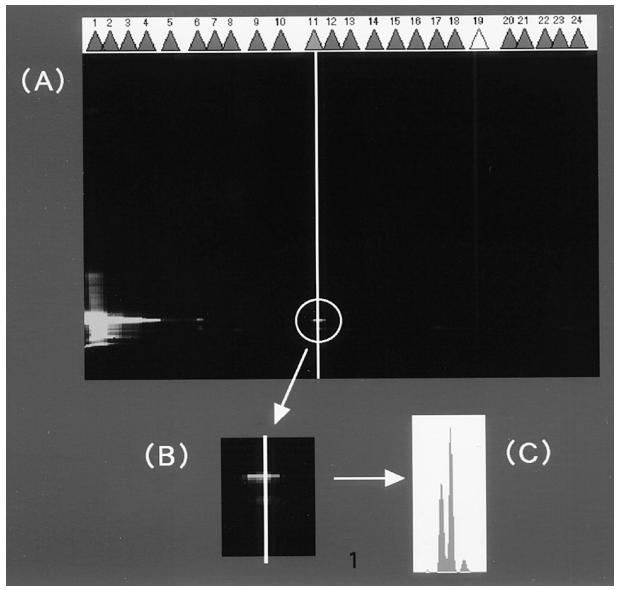

Figure 1. Analyzing images of original 6-carboxyfluorescein (6-FAM)-labeled phosphorothioate oligonucleotides (S-ODNs). Electrophoresis image (**A**) and enlarged image (**B**) of 6-FAM-labeled S-ODNs were obtained on scanning lane 11 of fluorescence DNA sequencer. No free oligonucleotides nor any extremely short oligonucleotides were found. Evaluation image (**C**) obtained by Gene Scan shows waveform related to number of bases of 6-FAM-labeled S-ODNs. Unlike other modified oligonucleotides, stereoisomers are easily formed with S-ODNs; therefore, three peaks were seen.

Induction of 6-FAM-Labeled S-ODNs into Rabbit Eyes by Iontophoresis and Their Detection

An Ito Ion Therapy Apparatus (Handaya, Tokyo) was used to perform iontophoresis. As nucleic acids are negatively charged, we believed that they would migrate along the electrical current into the eye. Female New Zealand white rabbits, weighing from 2.0 to 3.0 kg, were used. The animals were maintained and used in accordance with the ARVO statement on the treatment of animals in ophthalmic and vision research. Six rabbits (12 eyes) were used in the iontophoresis group and 2 rabbits (4 eyes) served as controls. The 6-FAM-labeled S-ODNs were dissolved in an artificial aqueous humor (BSS Plus; Santen, Osaka), and its concentration was adjusted to 10 pmol/μL. Next, rabbits were given an injection of a mixture of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (10 mg/kg) to produce general anesthesia, and a glass eyecup, equipped with a negative electrode, was placed on the cornea. After pouring 2 mL of the 6-FAM-labeled S-ODNs solution into the eyecup, a positive electrode was connected to the back of each rabbit. The electrical current was then increased gradually, so as to minimize the effect of iontophoresis on the circulation system, until it reached 1.5 mA after 2 minutes (Figure 2). The source of electricity was 100 V of alternating current. After reaching 1.5 mA, iontophoresis was performed for either 5, 10, or 20 minutes. Then rabbits were immediately administered a lethal dose of pentobarbital sodium (50 mg/kg) intravenously. The eyeballs were removed, and thoroughly washed with physiological saline to wash out excess 6-FAM-labeled S-ODN solution. The aqueous humor and vitreous humor were collected immediately. A 3-μL sample of these solutions was analyzed with a

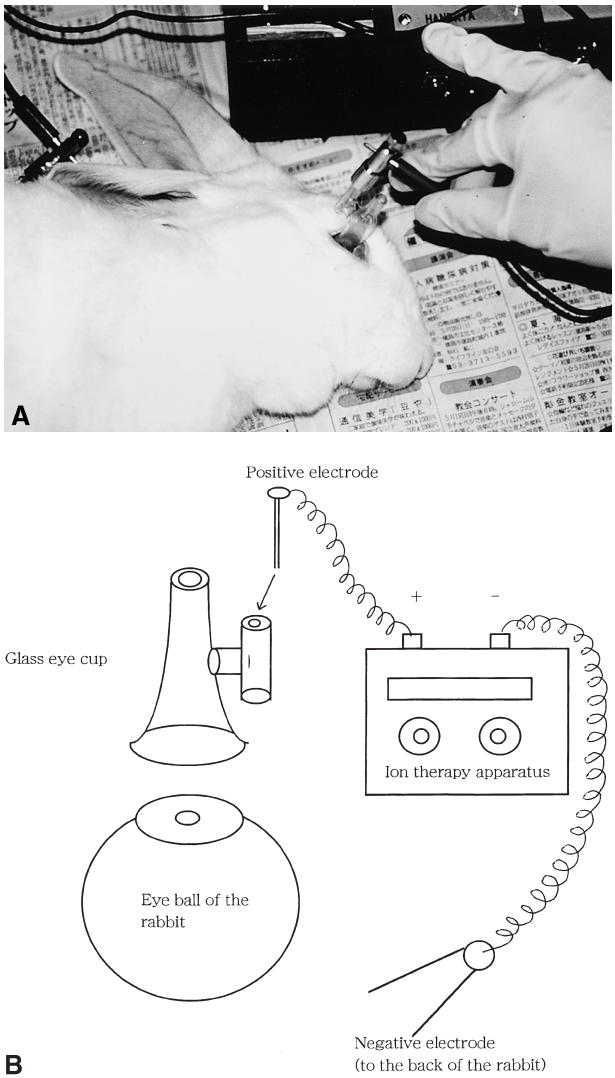

Figure 2. (**A**) Transduction method of gene by iontophoresis. Ito Ion Therapy Apparatus was used to perform iontophoresis. Glass eyecup, equipped with negative electrode, was placed on cornea. After pouring 2 mL of 6-carboxyfluorescein-labeled phosphorothioate oligonucleotides solution into eyecup, positive electrode was connected to back of rabbit. Electrical current was then increased gradually until it reached 1.5 mA after 2 minutes. Source of electricity was 100 V, alternating current. (**B**) Schematic view of iontophoresis.

fluorescence DNA sequencer and evaluated by the Gene Scan program. For each experiment at three different times of iontophoresis, the 4 eyes of 2 rabbits were analyzed to confirm reproducibility.

To confirm the transduction of S-ODNs into the ocular tissues, frozen sections at 10-μm thickness were prepared and examined under a fluorescence microscope (Olympus, Tokyo). For control, the same concentration of S-ODN solution was administered as eye drops 3 times every 20 minutes to the 4 eyes of 2 rabbits. These rabbits were euthanized with the same procedure used in the iontophoresis group 20 minutes after final instillation of the eye drops. Immediately thereafter, the aqueous humor and vitreous humor were collected, and frozen sections were prepared in the same manner as in the iontophoresis group.

Ocular Tissue Injury

Nonfluorescent S-ODNs at a concentration of 10 pmol/μL were transduced into both eyes of 1 white rabbit using iontophoresis for 20 minutes. The right eye was removed after 4 days and the left eye after 7 days, and the animal was euthanized with a lethal dose of pentobarbital sodium administered intravenously. Following the removal of these eyeballs, frozen sections at 10-μm thickness were prepared and stained with hematoxylin and eosin.

Induction of Plasmids into Ocular Tissue Using Iontophoresis

Plasmids 4.7 kbp in size that express green fluorescent protein (GFP) by a cytomegalovirus promoter (pEGFP-N1; Clontech, Palo Alto, CA, USA) were used. The protein produced by this plasmid becomes fluorescent when exposed to ultraviolet rays. Unlike

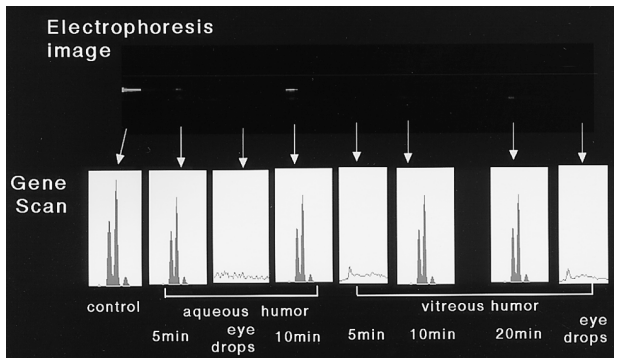

Figure 3. Detection of phosphorothioate oligonucleotides (S-ODNs) in aqueous and vitreous humor after iontophoresis. Following transduction of 6-carboxyfluorescein (6-FAM)-labeled S-ODNs into rabbit eyes using iontophoresis, S-ODNs were detected in aqueous humor and vitreous humor at 5 and 10 minutes, respectively. In addition, images obtained with DNA sequencer showed that molecular weight of recovered 6-FAM-labeled S-ODNs was same as that of original; their waveforms evaluated by Gene Scan program were also identical. In contrast, no 6-FAM-labeled S-ODNs were detected in eyes receiving S-ODN eye drops 3 times every 20 minutes, with no accompanying iontophoresis.

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other bioluminescent markers, this protein does not require other proteins, substrates or cofactors.

Three solutions containing either 0.5, 5, or 50 μg/mL of this plasmid were prepared, and 2 mL of each was then put into an eyecup placed on the cornea. Iontophoresis was then performed for 10, 20, or 30 minutes. Both eyes of each rabbit were used, for a total of 18 eyes of 9 rabbits. Four days after iontophoresis, rabbits were euthanized with a lethal dose of pentobarbital sodium administered intravenously, and the eyes were then removed and washed with physiological saline. Frozen sections at 10-μm thickness were prepared and examined under a fluorescence microscope. Frozen sections were also stained with hematoxylin and eosin to detect ocular tissue injuries. For comparison, the 50 μg/mL plasmid solution was administered as eye drops every 20 minutes for an hour to the 4 eyes of 2 rabbits.

These rabbits were euthanized 4 days later in the same manner as the iontophoresis group. Then the eyes were removed, and frozen sections at 10 μm were prepared and examined under a fluorescence microscope.

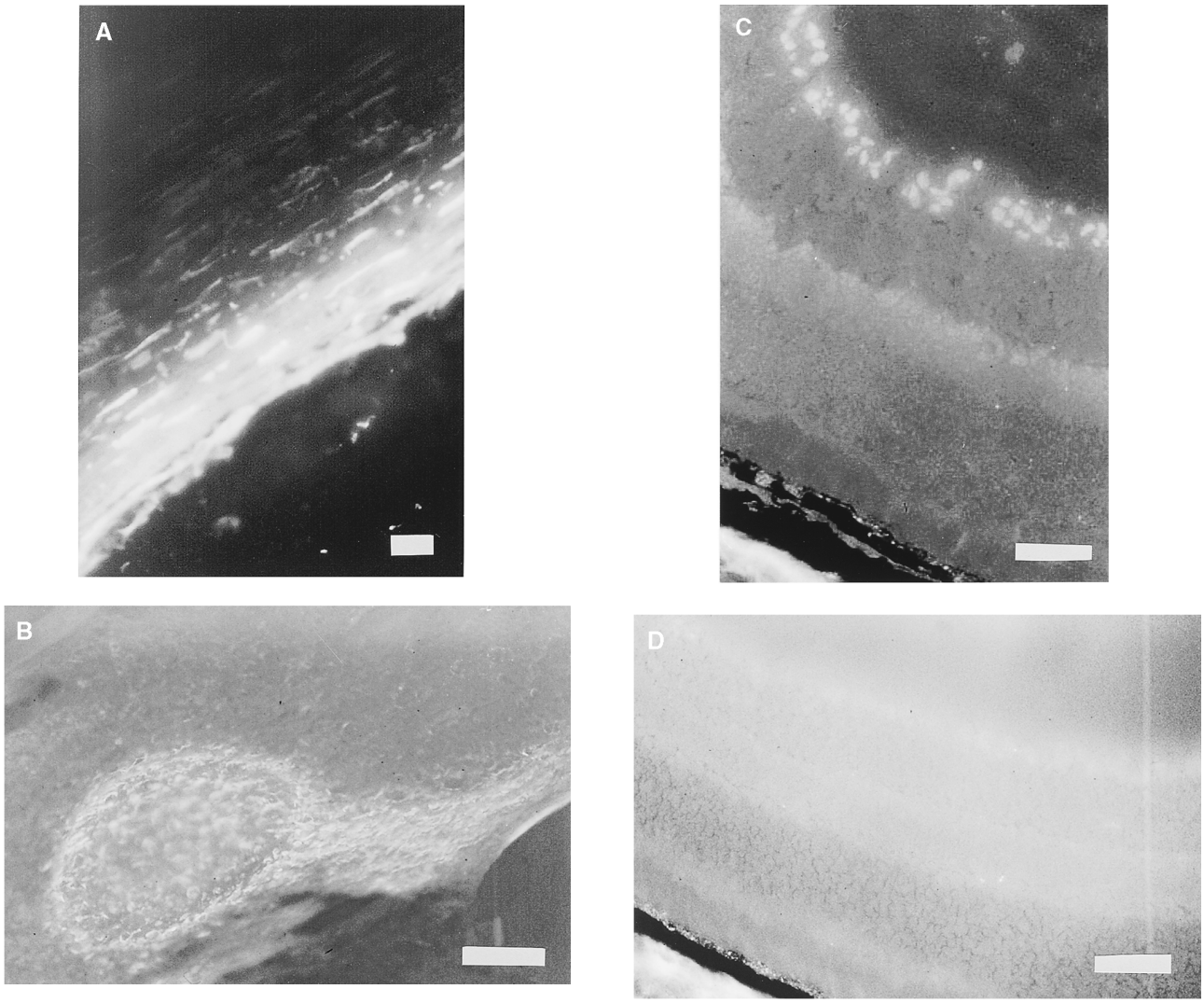

Figure 4. Frozen sections of ocular tissues after transducing 6-carboxyfluorescein-labeled phosphorothioate oligonucleotides by iontophoresis. Intense fluorescence was confirmed in each layer of cornea (**A**), and in tissue surrounding Schlemm's canal (**B**) 5 minutes following iontophoresis. Relatively intense fluorescence was confirmed in ganglion cell layer of peripheral retina (**C**), but no fluorescence in posterior retina (**D**) 10 minutes following iontophoresis. Relatively intense fluorescence was confirmed in ganglion cell layer to outer nuclear layer of posterior retina (**E**) 20 minutes following iontophoresis. Bars = 50 μm.

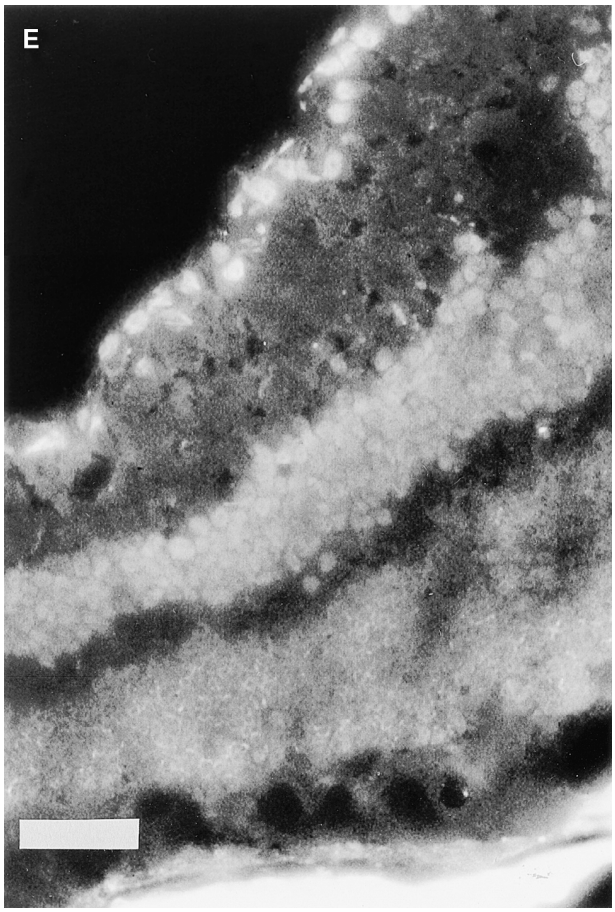

Figure 4. Continued.

Results

Five minutes following transduction of 6-FAM-labeled S-ODNs into rabbit eyes using iontophoresis, the S-ODNs were detected in the aqueous humor. They were not detected in the vitreous humor at 5 minutes after iontophoresis, but could be seen at 10 minutes (Figure 3). Images obtained with the DNA sequencer showed that the molecular weight of recovered 6-FAM-labeled S-ODNs was the same as that of the original, and their waveforms, evaluated by Gene Scan, were also the same. In contrast, no 6-FAM-labeled S-ODNs were detected in the eyes when the solution was administered as eye drops three times every 20 minutes for an hour without iontophoresis.

Figure 4 shows the findings in the frozen sections of the cornea and the angle following 5 minutes of iontophoresis. Intense fluorescence was confirmed in each layer of the cornea. In the angle, intense fluorescence was seen in the tissue surrounding Schlemm's canal. Ten minutes after iontophoresis, 6-FAM-labeled S-ODNs had reached the peripheral area of the retina, and at 20 minutes they were detected in the deep layer of the posterior retina. In contrast, no fluorescence was detected in any ocular tissue following the administration of the 6-FAM-labeled S-ODN solution as eye drops.

Even following the transduction of the 6-FAM-labeled S-ODN solution using iontophoresis, examination by slit-lamp microscope revealed that the transparency of all corneas had been maintained and no abnormal findings were observed. The results of hematoxylin and eosin staining showed no signs of degeneration or inflammation in the ocular tissues at either 4 or 7 days following iontophoresis. No systemic changes were seen in these animmals after iontophoresis.

Frozen sections were prepared from eyes to which plasmids had been transduced using iontophoresis. When the eyes were subjected to iontophoresis for more than 20 minutes using either the 5 or 50 mg/mL plasmid solution, fluorescence indicating the expression of the GFP gene was seen in the cornea, angle, and ciliary subepithelium. There was a tendency for the fluorescence to be stronger after longer intervals of iontophoresis, but no clear differences were observed. Although a relatively high degree of fluorescence was seen in the scleral tissue, it was hard to distinguish the fluorescence originating from the GFP from the relatively strong autofluorescence found in the sclera. No fluorescence was seen in the retinal tissue of any of the rabbits (Figure 5). When the plasmid solution was administered as eyedrops, no fluorescence was detected in any ocular tissue.

Discussion

Antisense therapy is a type of gene therapy that regulates the expression of target genes with the administration of DNA fragments having complementary sequences (anti-sense oligonucleotides). In this therapy, high rates of transduction of alien genes, including oligonucleotides, into cells and the stability of transduced genes are important factors in determining efficiency. In order to transduce such alien genes as oligonucleotides and plasmids into cells, various techniques using a variety of different virus vectors and liposomes have been developed. Retrovirus vectors have long been studied and modified. These vectors have already been determined as safe for human use. Dunaief et al[1] injected retinal pigment epithelia infected with retrovirus under the retina of infant mice and reported that a target gene was transduced in the photoreceptor cells. In addition, Kido et al[2] obtained the same result using an opsin promoter.

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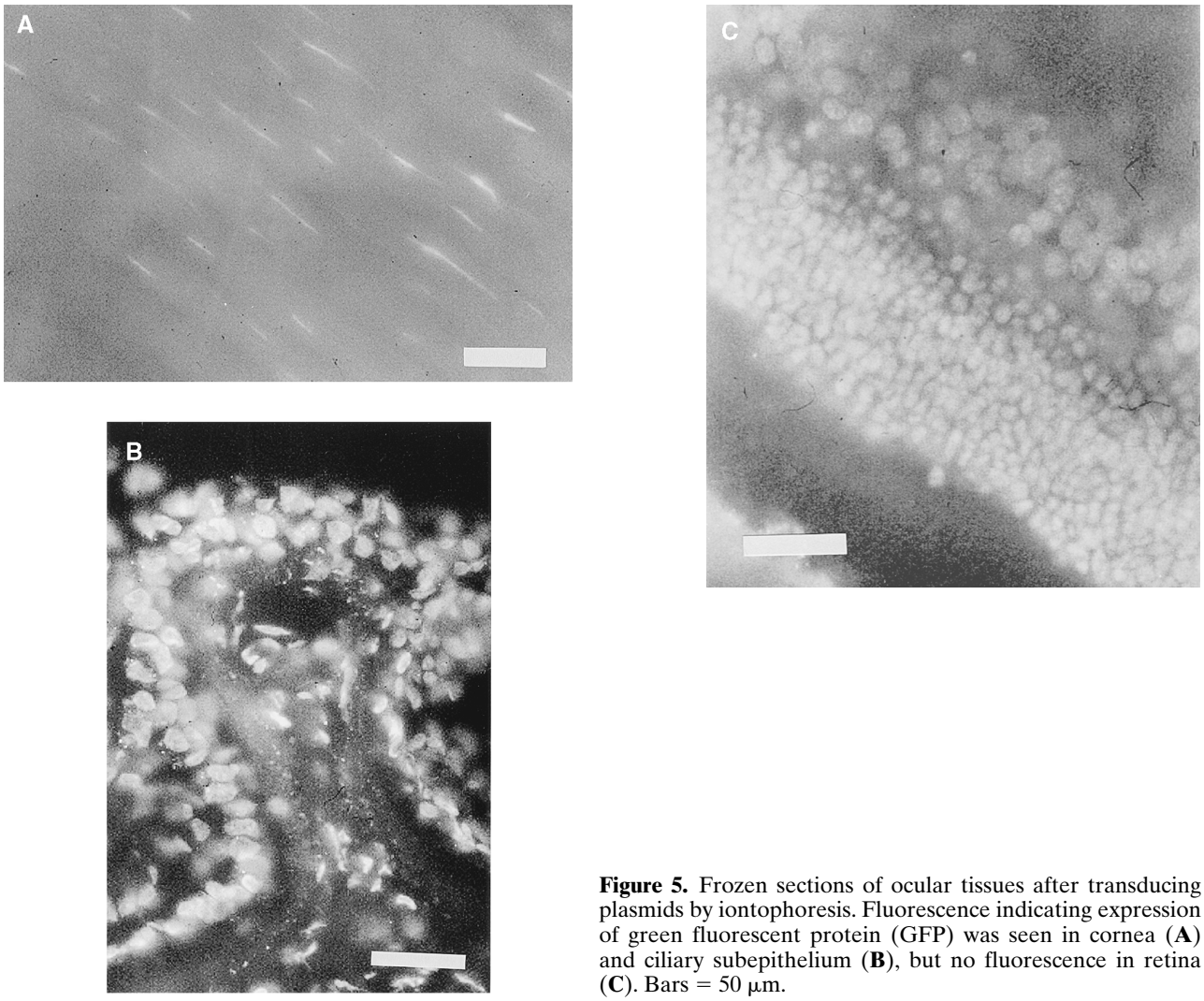

Figure 5. Frozen sections of ocular tissues after transducing plasmids by iontophoresis. Fluorescence indicating expression of green fluorescent protein (GFP) was seen in cornea (**A**) and ciliary subepithelium (**B**), but no fluorescence in retina (**C**). Bars = 50 μm.

It appears that once retrovirus vectors are incorporated, the resulting gene expression is maintained for a long time. The rate of transduction is not necessarily high, and the transduction occurs only in dividing cells. In contrast, adenovirus vectors can efficiently transduce genes into cells in the interphase. Abraham et al[3] inserted hemeoxygenase-1 gene into an adenovirus vector and then injected it in the vitreous. The gene expression was confirmed in the corneal endothelium, iris, lens, and retina. However, adenovirus vectors are transient manifestation vectors, and neutralizing antibodies that target the virus itself can reduce the rate of transduction. Adeno-associated virus (AAV) vectors do not have these disadvantages, and as a result have been closely studied recently. These vectors are not pathogenic, and can be incorporated by nondividing cells at a specific site on a chromosome. Flannery et al[4] inserted the opsin promoter into an adeno-associated virus vector and then injected it into subretina. The reported rate of transduction in the neighboring photoreceptor cells was nearly 100%. However, genetically recombinant AAV vectors may not remain in the host chromosomes for long periods of time. Furthermore, their production systems are somewhat complicated and, as a result, high-titer virus solutions are difficult to produce. Currently, HIV vectors, herpes simplex virus (HSV) vectors, and papilloma virus vectors are being developed. The characteristics of these virus vectors are determined by the natural characteristics of the base virus. Thus, their development is dependent on the target disease and organ. In addition to these virus vectors, positively charged liposomes are used for transduction. However, the rate of transduction is often extremely poor

and the degree of cytotoxicity is high. Despite this limitation, a technique that fuses liposome and viral coats of hemagglutinating virus of Japan (HVJ; Sendai virus) was introduced recently. The resulting membrane-fused liposome was found to transduce genes more efficiently and to be less cytotoxic. Hangai et al[5] filled this membrane-fused liposome with S-ODNs and injected it into the vitreous of mice, and the S-ODNs were transduced into retinal tissue.

Thus, there are advantages and disadvantages associated with each of the current transduction techniques. Daily progress is being made in this field of study, however, and we believe that eventually safer and more reliable ophthalmological gene therapies will be developed as a result.

It is possible to introduce various charged drugs into the eye with the use of iontophoresis. No special vectors are needed, and given the low cost, the therapy can be repeated many times. In addition, as the target compounds are actively transported, they can be incorporated into any cells.

In the field of ophthalmology, iontophoresis was used in the 1980s to introduce antibiotics into the eye, and in particular to the vitreous body.[6–8] Aside from antibiotics, Grossman and Lee[9] performed iontophoresis using ketoconazole, and Lam et al[10] did so using dexamethasone. When compared to subconjunctival or retrobulbar injections, the migration of these agents into the vitreous and retina was found to be extremely favorable, and the therapeutic concentrations of the drugs were maintained for a relatively long period of time.

However, iontophoresis has only recently been used to transduce nucleic acids into the body. Most studies have so far used oligonucleotides. To the best of our knowledge, however, none of these studies were done in ophthalmology, but came from other fields of medical research. Robinson et al[11] reported that anti-sense oligonucleotides were transduced in porcine aorta using a charged balloon catheter. Geest et al[12] found that transduced nucleotides were decomposed by biological enzymes.

As both nucleic acids and cells are negatively charged, it is difficult to transduce genes into cells. However, by using a positive electrode as a counter electrode, genes can be transferred following an electrical current.

Therefore, in the present study, 6-FAM-labeled S-ODNs and plasmid 4.7 kb in size that expressed GFP were transduced noninvasively into the eyes with iontophoresis, and their movements in the eyes and gene expression were examined by fluorescence microscopy and a fluorescence DNA sequencer.

The oligonucleotide used in the present study was synthesized with an automatic synthesizer. In order to transduce oligonucleotides into the body, S-ODNs are used to counteract the greater susceptibility of natural oligonucleotides to hydrolysis by biological enzymes. When compared to natural oligonucleotides, S-ODNs are more likely to be incorporated by cells, and less likely to be hydrolyzed by biological enzymes. Additionally, S-ODNs bind more strongly to target RNA with a high degree of affinity toward target areas. When an RNA/S-ODN hybrid is formed, RNaseH can break the target RNA strands. Given these advantages, S-ODN has been drawing a lot of attention from scientists.

The results of the present study showed that once iontophoresis-induced transduction was begun, S-ODNs reached the anterior chamber through the cornea within 5 minutes, and the posterior retina within 15 minutes. Based on images obtained with the DNA sequencer, the molecular weight of S-ODNs used for transduction was the same as that of S-ODNs collected from the anterior chamber or vitreous. Additionally, as their waveforms were the same, S-ODNs must not have been decomposed when they passed through such ocular tissue as the cornea, suggesting that they could act as an antisense therapeutic agent in the eye. Furthermore, S-ODNs could not be detected in the control eye when given as eyedrops, suggesting that the migration of S-ODNs to the eye was accelerated by iontophoresis.

In transcorneal iontophoresis, no abnormal findings were observed in the cornea where the electric current passed through, and no inflammatory or degenerative findings were detected in other ocular tissues. However, Yoshizumi et al[13] reported 1–3-mm burn injuries of the retina and choroid following the transscleral method. Additionally, Lam et al[14] confirmed the following findings after more than 5 minutes of iontophoresis: necrosis of the retinal pigment epithelium, loss of the outer segment, thinning of the inner and outer nuclear layers of the retina, and formation of a glial membrane. Burn injuries could have been minimized by placing a transducing agent solution in an eyecup; a procedure that is normally done to prevent the electrode from touching the eye. Furthermore, the effect of iontophoresis on the posterior retinal tissue could have been reduced by placing a counter electrode on the back of the rabbit to ensure a sufficient distance between the two electrodes. Further studies investigating this in more detail are needed.

The molecular weights of plasmids are markedly higher than those of oligonucleotides. In the present

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study, the transduction of plasmids into the retina could not be definitively confirmed. Nonetheless, fluorescence indicative of GFP expression was confirmed in the cornea, angle, and ciliary subepithelium. These findings suggest that plasmids passed through the cornea within 20 minutes, and that they accumulated in the angle along the flow of the aqueous humor. Even though fluorescence was undetectable in the retinal tissue, it was detectable in the ciliary subepithelium. Plasmids traveling through the uveoscleral flow from the angle to the ciliary subepithelium may have caused this effect. Although intense fluorescence was detected in the scleral tissue, it was difficult to distinguish between fluorescence originating from the GFP and autofluorescence found in the sclera. However, given the above, the GFP gene could have easily been expressed in the scleral tissue.

Plasmids were not transduced in the retina probably because they decomposed before reaching the retina. Plasmids were not modified to resist biological enzymes, unlike the oligonucleotides, which were modified by phosphorothioate-processing to resist hydrolysis.

Our findings demonstrate that oligonucleotides and other relatively large molecules such as plasmids may be transduced into ocular tissues. Oligonucleotides are mainly used in antisense therapy to suppress the expression of genes that cause disease. Robinson et al[15] directly injected antisense oligonucleotides for vascular endothelium growth factor (VEGF) mRNA into the vitreous of mice with experimentally induced proliferative retinopathy, and found that the neogenesis of retinal vessels could be suppressed to some degree. The effect of antisense therapy involving oligonucleotides is temporary, and as such, future therapy should be targeted to the neovascularization diseases or diseases of short duration such as infections or acute uveitis in which a temporary suppression of the causative gene is sufficient. In order to maintain the effect of antisense therapy, oligonucleotides must be administered continuously.

Since iontophoresis is easier and cheaper than other forms of therapy, this type of repeated continual administration is quite feasible. Therefore, the present technique may be effective against chronic diseases such as diabetic retinopathy. Future studies to investigate the inhibitory effect of iontophoresis on gene expression, the duration of its effect, and the degree of ocular tissue injuries caused by long-term administration are needed.

Nevertheless, if plasmids can be transduced, the expression of various genes in cells becomes possible. Aside from the antisense technique, degenerative diseases of the cornea and retina may be treated by plasmid transduction with the introduction of missing genes. Iontophoresis is also effective in this type of long-term administration. In the present study, the plasmids that were transduced in retinal tissue, decomposed in the eye and as a result they could not be detected. However, as phosphorothioate-processing cannot be used with plasmids, vectors must be modified to improve the stability of plasmids in the eye. Additionally, as large molecular weight compounds must be subjected to iontophoresis for long periods of time, the efficacy of iontophoresis may be improved by adjusting the site of electrode attachment or by adjusting the apparatus used to protect ocular tissues. Ascertaining the possible expression of genes in specific tissue by modifying promoters is an interesting topic for future research.

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