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Neural Retina Limits the Nonviral Gene Transfer to Retinal Pigment Epithelium in an In Vitro Bovine Eye Model

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ABSTRACT

We investigated the permeation of liposomal and polymeric gene delivery systems through neural retina into retinal pigment epithelium (RPE) and determined the roles of various factors in permeation and subsequent uptake of the delivery systems by RPE. Anterior parts and vitreous of fresh bovine eyes were removed. Retina was left intact or peeled away. Complexes of ethidium monoazide (EMA)-labeled plasmid DNA and cationic carriers (polyethyleneimine, poly-L-lysine, DOTAP liposomes) were pipetted on the retina or RPE. Two hours later the neural retina was removed, if present, and the RPE cells were detached. Contaminants were removed by sucrose centrifugation, and the RPE cells were analyzed for DNA uptake by flow cytometry. Cellular uptake of FITC-dextrans (molecular weight [mw] 20 000, 500 000 and 2 000 000), FITC-poly-L-lysine (mw 20 000), FITC-labeled oligonucleotide (15-mer), and naked EMA-labeled plasmid DNA was determined after pipetting the solutions on the RPE or neural retina. Location of the fluorescent materials in the retina was visualized with fluorescence microscopy. Neural retina decreased the cellular uptake of DNA complexes by an order of magnitude, the uptake of FITC-dextrans slightly, whereas delivery of polycationic FITC-poly-L-lysine to RPE was almost completely inhibited. Neural retina decreased the cellular uptake of FITC-oligonucleotides, while the uptake of uncomplexed plasmid was always negligible. Conclusions from FACS and fluorescence microscopy were similar: delivery of polymeric and liposomal DNA complexes into RPE are limited by the neural retina. This is due to the size and positive charge of the complexes.

KEYWORDS: gene delivery, intravitreal, retina, liposome, polymer.

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INTRODUCTION

The blood-retinal barrier is known to decrease the penetration of molecules from systemic blood-circulation to the retina and from the vitreous to the blood stream. Blood-retinal barrier is composed of the endothelia of retinal capillaries and retinal pigment epithelium (RPE). This barrier restricts the permeation of large and hydrophilic molecules. In addition, there is some evidence that neural retina is also a diffusional barrier for macromolecules,[1-3] but the barrier properties of neural retina in terms of permeant size and charge are not known. The neural retina is a multilayer consisting of various cell types. Among these layers, the internal and external limiting membranes and the interphotoreceptor matrix, rich in glycosaminoglycans (GAGs),[4] might have barrier properties.

Retinal gene transfer may open new possibilities in the treatment of many severe ocular diseases, such as age-related macular degeneration, proliferative vitreoretinopathy, retinal and choroidal neovascularization, diabetic retinopathy, glaucoma, and retinitis pigmentosa. For example, gene therapies based on neurotrophins and growth factors are possible treatments against retinal degenerations,[5-7] and angiostatic factors have potential in the treatment of neovascular conditions associated with various diseases.[8,9] Viral vectors have been used for successful transfection of neural retina and RPE in vitro and in vivo.[10-20] However, there are safety concerns that may restrict the use of some viral vectors in ocular gene therapy.

Nonviral systems are based on cationic lipids or cationic polymers. They are considered to be safe and biocompatible, but their transfection efficacy in vivo is inadequate and lower than that of many viral vectors. Liposomal and polymeric vectors form complexes with DNA by electrostatic forces and their positive surface charge facilitates their attachment to the cell surface and subsequent endocytosis. In vitro, dividing RPE cells can be transfected by polymeric and liposomal vectors.[21,22]

RPE is an interesting target for gene delivery. For example, transfected RPE could serve as a platform for secretion of neurotrophic or angiostatic factors to the neural retina or to the retinal and choroideal vessels, respectively. RPE cells are

phagocytosing and, therefore, they could take up DNA complexes efficiently if the complexes reach the cell surface. Intravitreal injection would be a clinically feasible and realistic mode of gene delivery to the RPE. However, it was previously shown that the vitreous blocks the permeation of polymeric and liposomal DNA complexes.[23] Vitrectomy might eliminate the problems of the poor vitreal permeation of the gene delivery complexes, although the complete removal of the posterior cortical gel is technically difficult.

The efficacy of intravitreal in vivo transfections of the rat retina with current polymeric and liposomal gene transfer systems is very low (L.P., unpublished data, 2000). However, it is possible to modify the nonviral DNA delivery systems in various ways. For such developments it is important first to know the barriers caused by the ocular environment.

Therefore, we used a novel bovine eye model to investigate the role of the neural retina as a barrier in the RPE delivery of polymeric (poly-L-lysine [PLL] and polyethyleneimine [PEI]) and liposomal (1,2-dioleyl-3-trimethylammonium-propane [DOTAP]) DNA complexes. In addition, permeation of selected macromolecules (poly-L-lysine, dextrans, oligonucleotide, plasmid DNA) was studied in similar conditions.

Materials and Methods

DNA Complexes with Liposomes and Polymers

PEI with a mean molecular weight of 25 kd (Aldrich, St Louis, MO) was used as 10 mM aqueous stock solution.[24] PLL with mean molecular weight of 200 000 was from Sigma (St Louis, MO); it was diluted with water (3 mg/mL). DOTAP was purchased from Avanti Polar Lipids (Pelham, AL). Cationic DOTAP liposomes were prepared by evaporating a chloroform solution of lipids, then resuspending the lipid in water at a concentration of 3.2 mM and sonicating under argon until a translucent lipid solution was obtained.

The cDNA fragment (GFP S65T mutant) that codes for green fluorescent protein was excised from pTR5-DC/GFP (tetracycline regulatable dicistronic/green fluorescent protein) plasmid (a gift from Dr D.D. Mosser, Montreal, Canada)[25] as a BamH1 fragment that was inserted into the BamH1 site of a cytomegalovirus (CMV)-driven pCR3 plasmid (InVitrogen, Carlsbad, CA) to yield the plasmid (pGFP) that was used in the complexes. The pGFP was labeled with ethidium monoazide (EMA; Molecular Probes, Eugene, OR), which forms covalent bonds with DNA bases during photoactivation. The labeling procedure has been described previously.[26,27] The reporter gene plasmid that encodes beta galactosidase under the control of cytomegalovirus promoter was a gift from Dr F.C. Szoka Jr (University of California San Francisco, San Francisco, CA).[28]

Plasmid DNA (pGFP) and the cationic polymers or cationic liposomes were both diluted first to 5% glucose solution. Solutions of DNA and carrier were mixed before transfection to yield complexes at charge ratios of 4:1 (positive charges of carrier over negative charges of DNA; DNA 50 μg/mL). DNA-carrier complexes were prepared at room temperature, and before the experiments the solutions were allowed to remain for at least 20 minutes.

The sizes of the complexes were determined with a NICOMP 380 submicron particle sizer that is based on light scattering (NICOMP Particle Sizing Systems Inc, Santa Barbara, CA). For the measurements, PEI, PLL, and DOTAP were complexed with plasmid DNA (pCMVß) as described above. The complexes were made in 5% glucose and the concentration of DNA was 50 μg/mL. The size distributions were assessed on the basis of NICOMP number-weighted analysis and they showed Gaussian distribution. The mean diameters ± the SD of PEI, PLL, and DOTAP complexes were 114 ± 17 nm, 105 ± 34 nm, and 112 ± 44 nm, respectively.

Labeled Macromolecules

The fluorescein isothiocyanate (FITC)-dextran with mean molecular weight of 20 000 (Sigma) and FITC-dextrans with mean molecular weights of 500 000 and 2 000 000 (Molecular Probes) were used. FITC-dextrans were dissolved in 5% glucose at 3.0 mg/mL.

FITC-labeled PLL and unlabeled PLL of mean molecular weight 20 000 were purchased from Sigma. The FITC-labeled PLL was used at a concentration of 0.1 mg/mL, and then 0.65 mg/mL of unlabeled PLL was added. The PLLs were diluted in 5% glucose.

Phosphorothioate oligonucleotide (sequence 5' TGG CGT CTT CCA TTT 3', molecular weight of 4524.82) with an FITC label attached in the 5'-end of the oligonucleotide and EMA-labeled plasmid (the labeling procedure was described earlier) were diluted in 5% glucose at a concentration of 50 μg/mL. These materials were used for assessing the retinal permeation and cellular uptake of uncomplexed DNA.

Cellular Uptake Experiments with Bovine Eye Model

The bovine eyes were obtained from a local slaughterhouse. Fresh bovine eyes were kept at +9°C. They were cleaned of extraocular material and dipped in 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY) in 0.9% NaCl. The eyes were opened circumferentially ~8 mm behind the limbus and the anterior tissues and the vitreous were separated gently from the neural retina. The neural retina was either left in its place or gently peeled and collected at the optic disc and cut with scissors near the optic nerve head. The optic nerve head was swept with a piece of paper tissue to avoid blood cell contamination from cut vessels. The eyecups were set steady in the 6-well plates. Volume of 500 μL of DNA-carrier complexes, FITC-dextrans, FITC-oligonucleotides,

EMA-labeled plasmid, or 5% glucose were pipetted on the RPE or neural retina in the eye cup. Incubation of 2 hours at +37°C was started in less than 3 hours after the death of the animal. After incubation, the sample solution was removed and the exposed surface of RPE or retina was gently rinsed with 1 mL of phosphate-buffered saline (PBS). If the retina was still present, it was gently removed, the optic nerve head was swept with a piece of paper tissue, and then underlying RPE was rinsed with 1 mL of PBS. The eye cup was kept steady in the well during the separation of neural retina and rinsing.

For the isolation of the RPE cells we used a previously described method[29] with some modifications. Volume of 500 µL of trypsin-EDTA (Gibco) was pipetted on the surface of the RPE. After incubation of 1 to 2 minutes at room temperature, the cells were gently brushed loose from Bruch's membrane with a small brush (Kolibri 1, 888, Synthetic Golden Sable, Germany). Only the cells that were under the surface of the trypsin-EDTA solution were brushed. The loosened cells were collected and fixed immediately with 1% paraformaldehyde and centrifuged at 6000 rpm for 10 minutes (Biofuge Fresco, Heraeus Instruments, Kendro Laboratory Products GmbH, Hanau, Germany). For removal of the contaminants, the cells were suspended in 2 mL of 0.32 M sucrose/50 mM phosphate buffer, pH 7.2, and centrifuged at 2000 rpm for 10 minutes. The supernatant was removed carefully by pipetting and the centrifugation in buffered sucrose was repeated twice. Microscopic evaluation after sucrose centrifugations showed that some samples contained only RPE cells, but occasionally some outer segment fragments, erythrocytes, and pigment granules were still present. After the last sucrose centrifugation, the pellet was washed with 1% paraformaldehyde, centrifuged at 6000 rpm for 10 minutes, and diluted to 400 to 500 µL of 1% paraformaldehyde for flow cytometric analysis.

Flow Cytometry Analysis

Cellular uptake and transgene expression were measured with fluorescence activated cell sorter (FACS-scan flow cytometry, Becton Dickinson, San Jose, CA), equipped with an argon ion laser (488 nm) as the excitation source. Fluorescence of FITC was collected at 525 nm (FL 1) and fluorescence of EMA was collected at 670 nm (FL 3). For each sample, at maximum 10 000 events were collected. The cells were visualized on an FSC (forward angle light scatter) vs SSC (90 degrees light scatter) display. The RPE cells that were living before fixation were selected for analysis by gating; the dead RPE cells and the possible erythrocytes, photoreceptor outer segments, and pigment granules were excluded on the basis of their size and other scattering properties. If less than 1000 living RPE cells could be analyzed from a sample, the result was discarded.

EMA-DNA was used as a marker for intracellular delivery of DNA as described previously.[27] The number of positive events was analyzed from the FL 1 vs FL 3 dot plot. The gate of positive events for each carrier was adjusted so that the negative control was subtracted as background. The controls were unlabeled pGFP for naked plasmid DNA and unlabeled pCMVß (cytomegalovirus beta-plasmid)/carrier complexes in 5% glucose for EMA-DNA complexes. The percentage of the positive cells was calculated as the number of positive events in FL 3 divided by the total number of events in the gate of living RPE cells.

The number of FITC-positive cells after application of FITC-dextran, FITC-oligonucleotide, and FITC-PLL solutions was analyzed from the FL 1 vs FL 3 dot plot. The experiments were performed as described but 5% glucose was used as a control.

The gates for EMA- and FITC-positive cells are visualized in Figure 1.

HPLC Analysis

FITC-oligonucleotide (phosphorothioate, sequence 5' TGG CGT CTT CCA TTT 3') was purchased from A.I.Virtanen Institute (Kuopio, Finland). FITC-oligonucleotide solution at concentration of 50 µg/mL was delivered to RPE or retina as described earlier. Samples of 50 µL were taken from the solution after 15 and 30 minutes or 60 and 120 minutes after the start of the incubation. The sample was centrifuged and the supernatant was stored at –20°C.

The high performance liquid chromatography (HPLC) system was a Beckman System Gold High Performance Liquid Chromatography equipped with a System Gold 168 Detector, 126 Solvent Mixing Module, 507e Autosampler, (Beckman Instruments, Fullerton, CA) and a Rheodyne Model 7725 injector (Cotati, CA) fitted with a 100-µL sample loop. The software was 32 Karat from Beckman Instruments.

The analytical HPLC column was DNA Pac PA-100 column 4 × 250 (Dionex, Sunnyvale, CA). Buffer A was 20% acetonitrile in 0.1 M acetate buffer (pH 8.0) and buffer B was 20% acetonitrile and 0.4 M $NaClO_4$ in 0.1 M acetate buffer (pH 8.0). The gradient consisted of buffer A with increasing amounts of buffer B according to the following scheme: increase from 10% to 35% over 5 minutes and increase to 55% over 25 minutes. The flow rate was 1 mL/min. Column equilibration took 15 minutes. Oligonucleotides eluting from the column were detected by ultraviolet absorption at a wavelength of 260 nm. Suitably diluted standards and samples were loaded to the system through an autosampler.

Histological Analysis

The eyes and solutions were prepared for experiments as described earlier. Volume of 200 µL of PEI, PLL, and DOTAP-

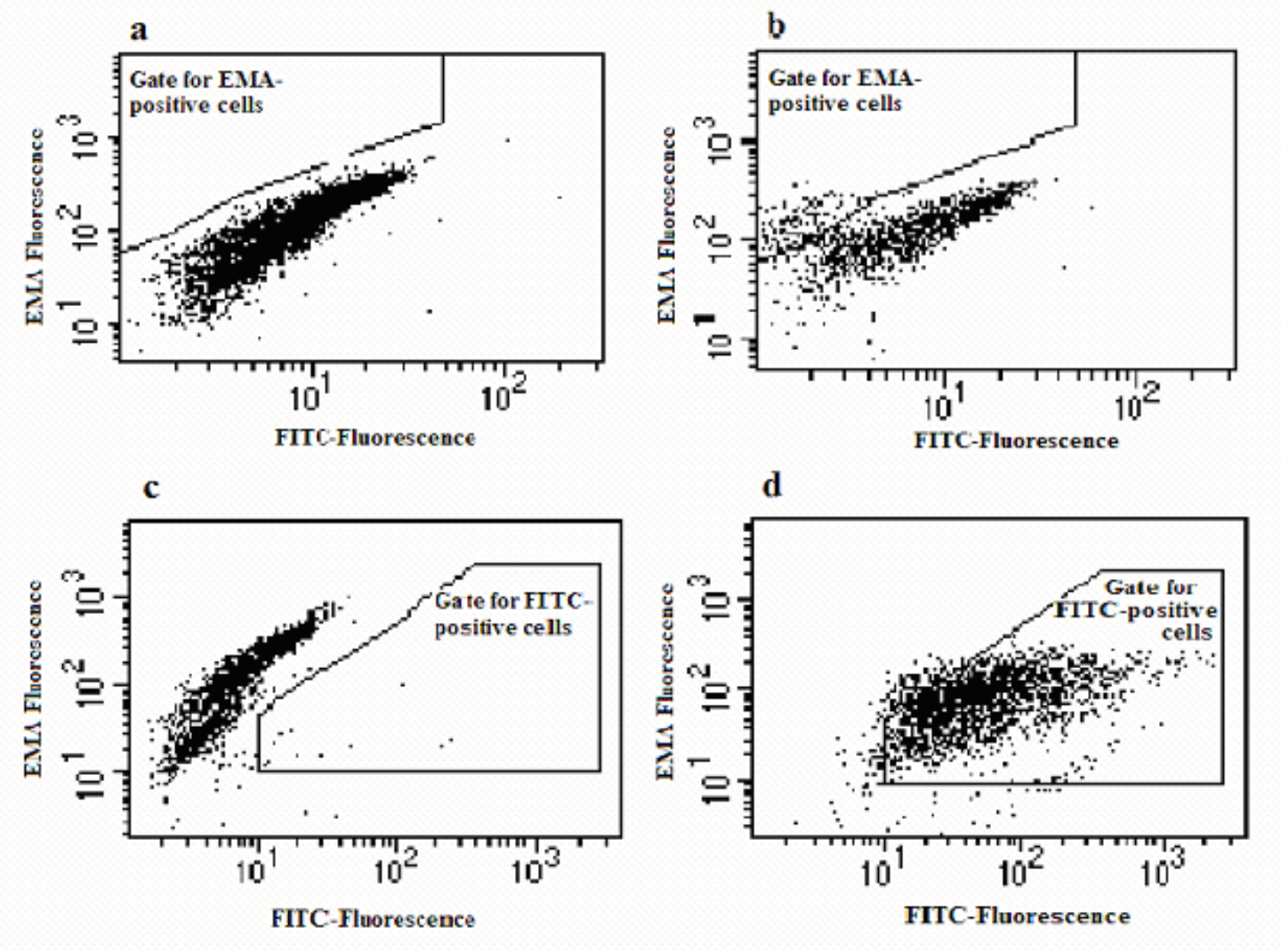

Figure 1. Gating of the EMA-positive and FITC-positive cells. The cells that were treated with complexes with unlabeled DNA, unlabeled DNA/carrier complex or 5% glucose were used for gating the EMA-positive and FITC-positive events. Flow cytometer analysis after treating RPE cells with unlabeled DNA/PEI complexes (a), EMA-labeled DNA/PEI complexes (b), 5% glucose, (c) and FITC-dextran of molecular weight 20 000 (d).

DNA complexes, FITC-labeled oligonucleotides, FITC-dextran of molecular weight 20 000, FITC-PLL of molecular weight 20 000, and 5% glucose was pipetted on neural retinas. Due to the weak fluorescence of the EMA-label, rhodamine-labeled beta-galactosidase encoding plasmid (p-Gene-Grip, Rhodamine/ß-galactosidase Vector, San Diego, CA) was used for the complexation in histological experiments.

After incubation for 2 hours at +37°C, the eye cups were rinsed with PBS, fixed with 4% paraformaldehyde for 30 minutes at room temperature, and rinsed again. Pieces of 8 mm in diameter containing all posterior ocular layers were cut from the area that was exposed to the incubating solution. The samples were frozen in Tissue-Tek O.C.T. Compound (Sakura, Torrance, CA) in isopentane that was kept cold with pieces of dry ice in ethanol. Cryostat cuts of 10 to 14 µm were embedded in glycerol and were evaluated with fluorescence microscope (Nikon Eclipse)/UltraVIEW Confocal Imaging System (PerkinElmer Life Sciences, Cambridge, United Kingdom, connected to Nikon Eclipse TE 300 inverted microscope, Nikon Corporation, Tokyo, Japan). A light microscopic picture was taken of samples from the same area with the fluorescence picture for the localization of the fluorescent probes.

Statistical Analysis of FACS data

The Mann-Whitney *U*-test was used for statistical analysis.

RESULTS

Cellular Uptake of DNA Complexes

According to the FACS analysis, 7% of the cells took up PEI complexes when the complexes were pipetted directly on the RPE cells. When the complexes were delivered on the neural

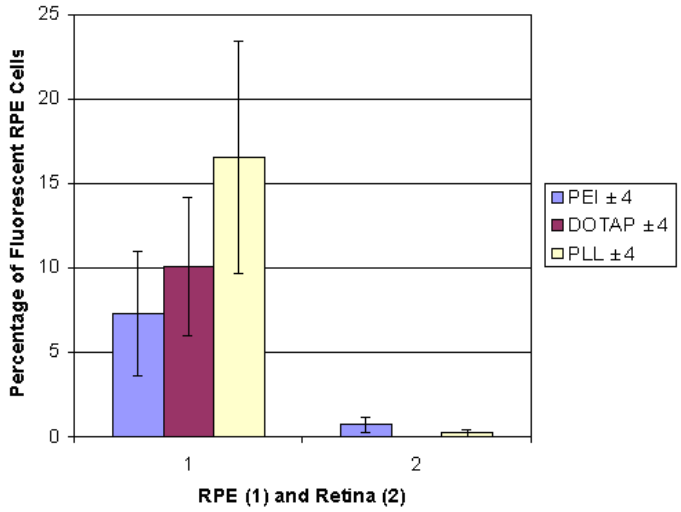

Figure 2. Uptake of carrier/DNA complexes by RPE cells after 2 hours of incubation. All 3 carriers (PEI, DOTAP, and PLL) were examined at charge ratio ±4. The complexes were delivered on RPE (1) and on retina (2). Means ± SEM (n = 5-6) are shown.

retina, the cellular uptake of PEI complexes was decreased to 1% ($P < .05$) (Figure 2). Without the neural retina, the fraction of positive cells, ie, the uptake of DOTAP complexes in the RPE, was 10% and the uptake of PLL complexes was 17%. Neural retina decreased the cellular uptake of DOTAP and PLL complexes practically to zero ($P < .005$ and $P < .05$, respectively) (Figure 2). The presence of the neural retina did not affect the mean fluorescence intensity of the positive cells ($P > .05$). Therefore, the total fluorescence of positive cells decreased significantly ($P < .05$) in all cases. Uptake of plasmid DNA by RPE cells was negligible (less than 1% of the cells were positive).

RPE Uptake of Labeled Macromolecules

FITC-dextrans were used as probe molecules to evaluate the effect of molecular size on permeation through the neural retina. The cellular uptake levels (in terms of percentage of fluorescent cells) of FITC-dextrans with mean molecular weights of 20 000, 500 000, and 2 000 000 to RPE cells were 96%, 36%, and 34%, respectively, when the neural retina was not present (Figure 3A). When the FITC-dextrans were pipetted on the neural retina, the uptake levels in RPE were 87%, 26%, and 10% of positive cells (Figure 3A). The uptake decreased significantly by neural retina in the case of FITC-dextran 20 000 and 2 000 000 ($P < .05$). Due to the variation in the cellular uptake of FITC-dextran 500 000, no statistically significant decrease in uptake was seen in the presence of neural retina. Note that the relative effect of neural retina on FITC-dextran uptake (9% for 20 kd, 29% for 500 kd, and 69% for 2000 kd) was much less than in the case of DNA complexes (89%-100%).

FITC-PLL (mean molecular weight 20 000) was taken up by

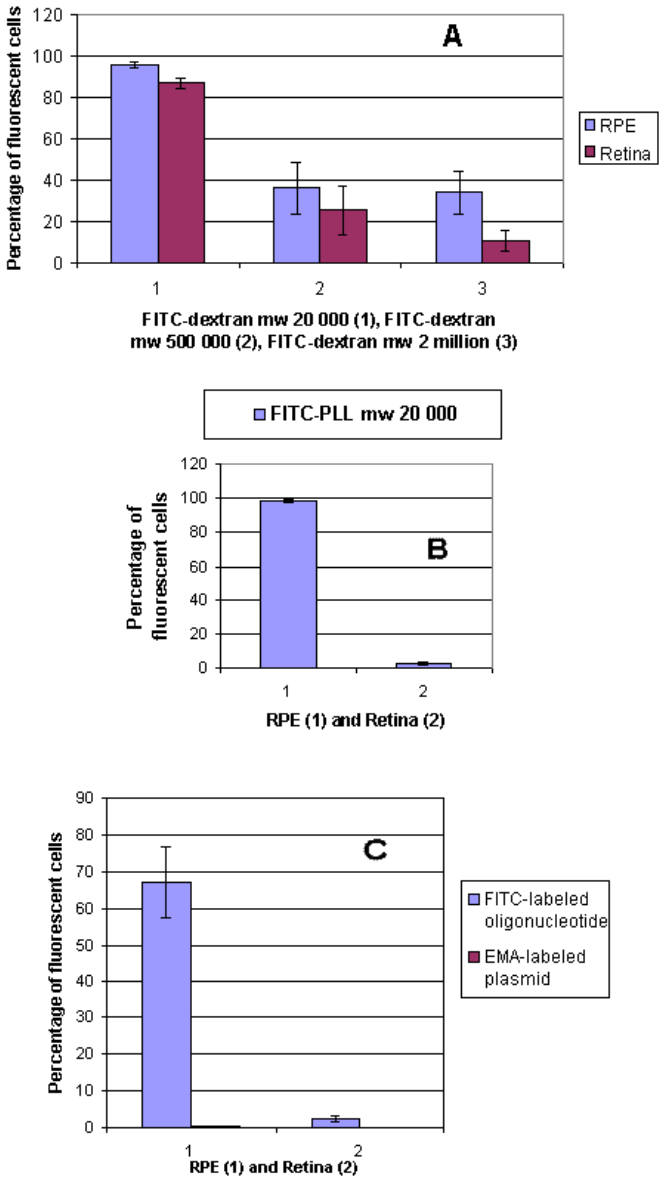

Figure 3. (A) Cellular uptake of FITC-dextrans of molecular weights 20 000, 500 000, and 2 000 000. The dextrans were delivered on the RPE (1) or retina (2) (n = 6-7). (B) Cellular uptake of FITC-PLL of mean molecular weight 20 000. Molecules were delivered on the RPE (1) and retina (2) (n = 6-8). (C) Cellular uptake of FITC-oligonucleotide and EMA-labeled plasmid DNA when they were delivered on RPE (1) or retina (2) (n = 5-6). Means ± SEM are illustrated.

98% of RPE cells without neural retina, but only by 3% of RPE cells when the neural retina was present (Figure 3B). Also, the mean fluorescence intensity and total fluorescence of positive cells decreased significantly ($P < .005$).

FITC-oligonucleotide (mw 4524) was taken up by 67% of RPE cells but the neural retina decreased the uptake to 2% of the cell population ($P < .005$) (Figure 3C). Similarly, also the mean fluorescence intensity and total fluorescence of the

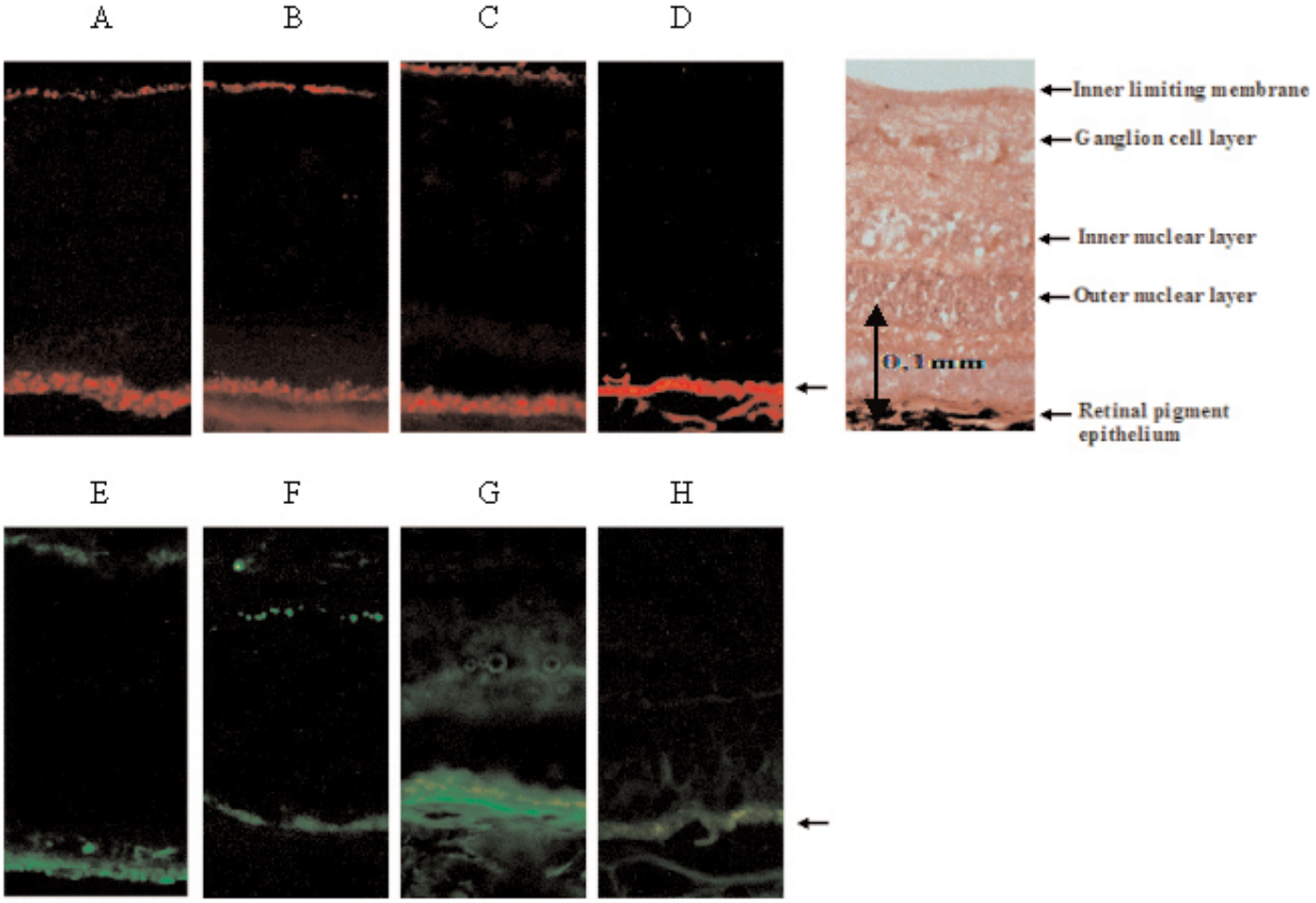

Figure 4. Fluorescence microscopy of retinas after delivering complexes of rhodamine-labeled β-galactosidase DNA with PEI, DOTAP, and PLL (A, B, and C, respectively), FITC-PLL (E), FITC-oligonucleotides (F), and FITC-dextran of molecular weight 20 000 (G) on the neural retina. Retinas incubated with 5% glucose served as controls (D and H). The RPE showed autofluorescence also in control samples (arrows). In all cases of DNA-complexes (A, B, C) and in the case of FITC-PLL and FITC-oligonucleotides (E, F) increased fluorescence is seen in the superficial layers of the retina and in the case of FITC-dextran in the level of outer nuclear layer and RPE. A light photomicrograph of bovine retina shows the layers of retina. The magnification of optics of the fluorescence microscopy was ×20.

positive cells decreased significantly ($P < .05$) in the presence of the neural retina. In the presence of neural retina the concentration of FITC-oligonucleotide in donor solution decreased in 15 minutes by 44% ± 11%, but no further decrease took place in 2 hours. When the FITC-oligonucleotide was pipetted on the RPE cells, the levels in solution decreased by 24% ± 14% during incubation of 15 minutes. These data suggest that FITC-oligonucleotide penetrates into the neural retina, but it does not gain access to the underlying RPE. The uptake of EMA-labeled plasmid into RPE cells was less than 1% and the presence of the neural retina did not change the uptake significantly (Figure 3C).

Fluorescence Microscopy

The fluorescence of rhodamine-labeled DNA-carrier complexes and FITC-PLL were seen only superficially at the level of inner limiting membrane of retina (Figure 4A, B, C, and E). The fluorescent bands were seen in the retinas after incubation with FITC-oligonucletides at the level of inner limiting membrane and ganglion cells and at the level of inner part of the inner nuclear layer (Figure 4F). In the case of FITC-dextran, the RPE showed a bright fluorescence and diffuse fluorescence was seen at the level of photoreceptors (Figure 4G).

DISCUSSION

Although RPE is a promising target for ocular gene therapy, there are only limited data on transfection of RPE cells with nonviral vectors. Dividing, nondifferentiated primary[21,22] and secondary RPE cells (D407)[30,31] can be transfected successfully with many liposomal and polymeric vectors but the efficacy of in vivo transfection of rat retina and RPE cells is very low after intravitreal administration (L.P., unpublished data, 2000). Understanding of the limiting barriers should

give background information for the design of more effective nonviral vectors for retinal gene therapy. However, there is very sparse information on the mechanisms and limiting barriers of nonviral RPE transfection.

After intravitreal injection, the administered molecules must permeate through vitreous and neural retina to reach the RPE. Previously we showed that vitreous limits seriously the permeation of nonviral liposomal and polymeric gene complexes.[23] It also seems that hyaluronan is a major limiting factor in the vitreous. The permeation of the FITC-dextrans was decreased slightly by vitreous, while the transfer of the positively charged PLL into RPE cell molecules was decreased by the vitreous substantially[23] Thus, it appears that the size and especially the positive charge of molecules and DNA complexes limit their mobility in the vitreous. Vitrectomy could minimize the vitreal barrier, but it is difficult to remove the posterior cortical part of vitreous completely. In addition, in vitrectomized eye and in the normal eye the DNA complexes should cross the neural retina in order to reach the RPE.

The neural retina is a soft and fragile multilayer and it is technically demanding to detach it intact from the eye. With our experimental setup it was possible to study the retinal barrier while avoiding mechanical traumas. Since the retina remained in its natural environment, all retinal layers are present in their natural environment, including the interphotoreceptor matrix. Nevertheless, the experiments are done postmortem and, thus, we cannot exclude possible metabolic differences in the model tissues compared with the in vivo situation.

FACS does not distinguish the fluorescence inside the RPE cells from fluorescence attached to the cell surface. It is unlikely that the neutral or negatively charged molecules like dextrans, plasmid DNA, or oligonucleotides would attach firmly to the cell surface, but in the case of positively charged molecules this might happen. However, trypsination of the RPE cells during the detaching procedure cuts the proteoglycans on the cell surface and should separate the attached complexes or molecules from the cell surface. Furthermore, in the permeation experiments, the molecules and complexes must permeate the neural retina before they can reach the surface of the RPE cell. Therefore, this should not affect our conclusions about the role of neural retina as a barrier.

Interestingly, intact RPE takes up DNA complexes (6%-15%), but not naked DNA, if the complexes are administered directly on RPE (Figure 2). This suggests that the DNA complexes can be delivered effectively into both nondividing and differentiated RPE if they can reach the cells in active form. However, in addition to the vitreal barrier, the neural retina also severely limits the access of the complexes to the RPE. Retina decreased the permeation of liposomal and polymeric gene complexes into RPE cells very clearly (Figure 2). One potential barrier is the interphotoreceptor matrix (IPRM) that is located between the outer limiting membrane of the retina and the apical border of the RPE. IPRM takes care of many activities that are essential for retinal function, such as trafficking of metabolites and cell-cell-interactions involved in the outer segment shedding and RPE phagocytosis.[32] IPRM contains glycosaminoglycans (GAG), mainly chondroitin sulfate and hyaluronan[4] that are known to react with DNA cation complexes.[33] Another possible barrier is the inner limiting membrane (ILM), the basement membrane of Müller cells that is contiguous with the vitreous cortex. It contains laminin, collagen, and several proteoglycans[34]; hyaluronan, heparan sulfate, and chondroitin sulfate/dermatan sulfate have been found in animal ILMs.[35,36] ILM may be a mechanical barrier for nonviral complexes, but also interactions between the complexes and GAGs may take place in the ILM. According to fluorescence microscopy, the passage of the complexes through the neural retina seems to be restricted by ILM, the most anterior barrier of neural retina (Figure 4). Both IPRM and ILM contain high levels of GAGs that are known to have interactions with liposomal and polymeric gene complexes.[33] Since polymeric and liposomal complexes of DNA have a cationic surface, they bind to the negatively charged GAGs that may immobilize the complex, relax it, or even release the DNA prematurely.[33]

Our data indicate that the cationic charge is a more important limiting factor than the molecular size in the neural retina barrier. For example, FITC-dextran of mean molecular weight 20 000 permeated the retina well (87% of RPE cells positive), while the permeation of positively charged FITC-PLL of the same mean size was practically blocked (3% of RPE cells positive). Much larger FITC-dextran (mw 2 000 000) permeates better than FITC-PLL (mw 20 000) in the neural retina. In addition, unlike in the case of FITC-PLL, the mean fluorescence intensities of FITC-dextran data in the presence of retina did not show a clear trend. Therefore, no molecular weight dependence was seen in the total fluorescence values. Interestingly, the molecular weight of FITC-dextrans affects the cellular uptake in RPE (without retina), whereas the mean fluorescence intensities were not significantly affected by molecular weight. Therefore, it seems that we cannot rule out the possibility that at high molecular weights (eg, 2 000 000) some cell surface components like proteoglycans might impair the cell uptake. This is suggested by the decreased uptake of FITC-dextrans when molecular weight is increased (Figure 3A). Probably the polycations bind to the negative biomacromolecules of neural retina and therefore are poorly transfered through retina.

The cellular uptake of naked EMA-labeled plasmid DNA to RPE cells was weak (less than 1% positive cells) as expected, but the FITC-labeled oligonucleotide was effectively taken up (67% positive cells). Interestingly, the neural retina decreased substantially the cellular uptake of the FITC-oligonucleotide into RPE. In an additional experiment the change in oligonu-

cleotide concentration in the donor solution was monitored by HPLC. FITC-oligonucleotide concentration decreased rapidly (in 15 minutes) in the donor solution contacting the retina. As oligonucleotide disappears from the solution, but it does not reach RPE, it must be retained and bound in the neural retina. Location of FITC-oligonucleotide binding at the level of the ganglion cells and inner nuclear layer was seen by fluorescence microscopy. Our findings of accumulation of FITC-oligonucleotide to the retina are in line with kinetic studies of Fomivirsen sodium (ISIS 2922) that is an intravitreal antisense oligonucleotide for the local treatment of CMV retinitis.[37-40] Uptake of oligonucleotides by neural cells is not probable,[41] but in general phosphorothioate oligonucleotides are highly bound to proteins.[42] Therefore, the transfer of oligonucleotide to the RPE may be slowed down by the protein binding and gradual release from the protein depot.

Our results suggest that the neural retina forms a substantial barrier for positively charged molecules including polymeric and liposomal gene carrier complexes. Vitrectomy does not solve the penetration problems of the intravitreally administered DNA complexes, because both vitreous and neural retina can individually act as a limiting barrier. Active complexes that overcome this barrier and reach RPE can be taken up effectively into the RPE cells. Barriers of vitreous and neural retina must be taken into account in the development strategies of nonviral intravitreal gene transfer systems targeted to RPE. Successful transfection of RPE would bring new possibilities for the treatment of several severe retinal diseases.

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