Comparative Analysis of the Uptake and Expression of Plasmid Vectors in Human Ciliary and Retinal Pigment Epithelial Cells In Vitro

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Abstract   The retinal pigment epithelium is uniquely suited to gene therapy that uses lipid-mediated DNA transfer due to its high phagocytic activity in situ. We compared the relative efficacy of phagocytosis on the uptake of labeled plasmid vectors by retinal pigment epithelial and ciliary epithelial cells in vitro. Relative levels of endocytosis were then compared with the efficiency of marker transgene expression in these cells. Human retinal pigment epithelial and ciliary epithelial cells from a single donor were isolated and expanded in vitro. Polyplex-mediated transfections were performed using a rhodamine-labeled expression vector for green fluorescent protein. Rhodamine-labeled endosomes were examined by fluorescence microscopy at different time points. Rhodamine labeling and green fluorescent protein expression were analyzed by flow cytometry 48 h after transfection. These gene transfer studies showed that expression of transgenes does occur in both human retinal pigment epithelial and ciliary epithelial cells in vitro. Endocytosis of labeled plasmid vectors occurs at a significantly higher number and density in retinal pigment epithelial cells than in ciliary epithelial cells ($P < 0.04$). However, the efficiency of marker transgene expression is similar in the two cell types. These studies demonstrate that the higher intrinsic phagocytic activity does not enhance the efficacy of transgene expression in retinal pigment epithelial cells in vitro. Both human retinal pigment epithelial and ciliary epithelial cells are competent recipients for lipid-mediated gene transfer, and transgene expression occurs at similar levels in both cell types. J. Cell. Biochem. 83: 671–677, 2001.   © 2001 Wiley-Liss, Inc.

Key words: gene therapy; retina; eye pigment epithelium; ciliary body; transfection; glaucoma; fluorescent probes; gene expression; endocytosis; plasmids

The retinal pigment epithelium (RPE) plays several important roles in maintaining the health of the overlying neurosensory retina. One of these roles is to digest the outer-segment membranes that are shed daily from the retinal photoreceptors. The high intrinsic phagocytic function of the RPE cells in situ can theoretically be used to direct gene therapy to the RPE using lipid-mediated DNA transfer. Liposomes have been employed to transfect marker and therapeutic genes to a variety of ocular and non-ocular tissues, both in vitro and in vivo [Felgner et al., 1987; Behr et al., 1989; Stewart et al., 1992; Nabel et al., 1993; Bebok et al., 1996; Hangai et al., 1996; Kukowska-Latallo et al., 1996; Masuda et al., 1996; Chaum et al., 1999]. These reagents condense DNA and deliver genes to the cell via endocytosis [Wrobel and Collins, 1995; Friend et al., 1996]. Previous work in our laboratory has shown that lipid reagents are an efficient mechanism for transfecting plasmid vectors to target RPE with minimal cytotoxicity [Chaum et al., 1999]. Theoretically, the high phagocytic activity of RPE cells may increase the efficiency of gene transfer and delivery relative to other epithelial cell types in the eye. Enhanced uptake of foreign genes may lead to increased levels of transgene expression in the RPE, however, this has not been demonstrated experimentally.

The ciliary epithelium of the eye (CE) comprises an inner, non-pigmented layer (CNPE) and an outer, pigmented layer (CPE) oriented

with apical surfaces in apposition. At the ora serrata, the CPE is contiguous with the RPE, whereas the CNPE transitions to become the neurosensory retina. The ciliary epithelium is responsible for the formation of the aqueous humor in the eye. The CNPE is primarily responsible for aqueous production through a combination of hydrostatic and oncotic pressure and energy-dependent ion transport mechanisms [Berggren, 1964; Bill, 1973]. Regulation of aqueous humor dynamics and the resulting intraocular pressure (IOP) depends upon the relative contributions of aqueous production and the cholinergic, adrenergic, and mechanical factors regulating outflow facility.

Experimental approaches to the treatment of glaucoma using gene therapy have focused on the delivery of marker genes to the trabecular meshwork [Borras et al., 1998] and potential neuroprotective genes to retinal ganglion cells [DiPolo et al., 1998; Garcia Valenzuela and Sharma, 1998]. An unexplored approach is the delivery of genes that have the potential to modulate aqueous production directly to the CE in vivo. These studies compare the relative efficacy of marker transgene uptake into human RPE and ciliary epithelial cells in vitro by polyplex-mediated gene transfer. Plasmid uptake into cytoplasmic endosomes was compared with GFP expression in the two cell types. Enhanced plasmid uptake was seen in RPE cells compared with ciliary epithelial cells but was not correlated with increased levels of marker gene expression.

METHODS

Cell Culture

Human RPE and ciliary epithelial cells were isolated from cadaver eyes provided by the Mid-South Eye Bank. Globes were incised at the pars plana, and the anterior segment was removed. The vitreous was extracted manually, and the retina was dissected free. The eyecup was washed three times with Dulbecco's minimal essential medium (DMEM, Gibco BRL), and 0.25% trypsin/EDTA was applied for four 15-min digestion cycles. Cells were loosened by aspiration and transferred to DMEM supplemented with fetal calf serum (FCS). Isolated RPE cells were spun at 2,000 rpm for 5 min, resuspended in DMEM with 16% FCS, and plated in 12-well tissue culture plates. The lens was sharply dissected from the anterior seg-

ment of the eye and removed. The iris was sharply incised at the root and was excised en bloc circumferentially. The anterior eyecup was trimmed to remove the pars plana, and the corneal endothelial cells were removed by mechanical abrasion with a Q-tip. The anterior eyecup containing the ciliary body was then washed three times with DMEM, and 0.25% trypsin/EDTA was applied for four 15-min digestion cycles. Ciliary epithelial cells were isolated and cultured as a mixed pigmented and non-pigmented monolayer as described above for RPE cells.

Cultured cells were maintained in DMEM with 16% FCS supplemented with L-glutamine, penicillin, and streptomycin in humidified air with 5% CO_2 at 37°C. The RPE cells grew in a cuboidal monolayer, characteristic of their morphology in the eye in situ. The CE showed both pigmented and non-pigmented phenotypes and were epithelial in morphology (Fig. 1). Both cell types were transfected during the first or second subculture following explantation.

Plasmid DNA

The *pGeneGrip* plasmid (Gene Therapy Systems) is a rhodamine-labeled vector that encodes a promoted green fluorescent protein (GFP) gene. The plasmid nucleic acid is labeled with rhodamine, which renders cell endosomes red when they are visualized using a 510–560 nm excitation filter and a 575 nm barrier filter under the fluorescent microscope and in the flow cytometer. The GFP transgene was maximally expressed in cells 48 h following transfection and visualized in the cell cytoplasm using a 480 ± 30 nm excitation filter and a 535 ± 40 nm barrier filter (fluorescein isothiocyanate [FITC] wavelength used in flow cytometry).

Transfections

Cells were seeded in 6-well tissue culture plates at 1.3×10^5 cells per well and grown until they reached 50% confluence. Transfections were performed using 2 µg of plasmid DNA per well complexed with the polyamidoamine dendrimer *Superfect* (Qiagen) at a ratio of 1:5 (µg:µg) using the manufacturer protocol as previously described [Chaum et al., 1999]. Briefly, 4 µg of plasmid DNA (0.5 µg/µl) was diluted in 192 µl of DMEM without antibiotics or serum in replicate studies (n = 6). Twenty micrograms of *Superfect* was added and the mixture was incubated at room temperature for
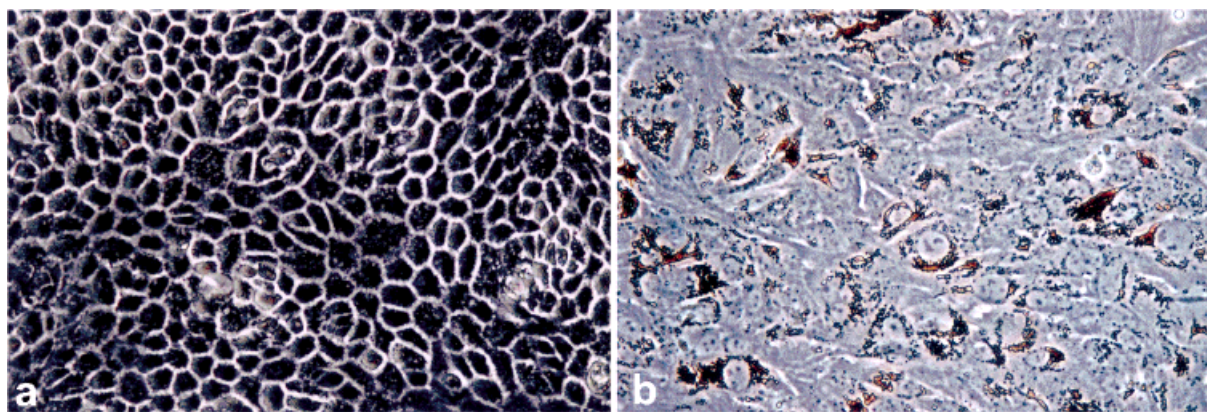
Fig. 1. **a**: Phase contrast micrograph of a primary culture of RPE cells used in transfections. **b**: Phase contrast micrograph of a primary culture of mixed pigmented and non-pigmented ciliary epithelial cells used in transfections.

10 min. The DNA:*Superfect* solution was mixed with 1200 µl of DMEM with 16% FCS. Half of the DNA:*Superfect*:media mixture was placed in a well containing washed RPE cells; the other half on washed CE cells. The cells were incubated for 3 h in an atmosphere of humidified air with 5% CO_2 at 37°C. After transfection, the cells were washed and refed with DMEM with 16% FCS. One well of each 6-well plate served as a negative control and was incubated with plasmid DNA in the absence of the *Superfect* transfection reagent.

At 6, 24, and 48 h after transfection, the cells were examined under the fluorescent photographic microscope. Rhodamine fluorescence was assessed at 6 and 24 h after transfection and representative fields were photographed at both low and high power. Twenty-five cells from both RPE and CE transfections were analyzed at high power with the confocal microscope, twenty-four hours after transfection. Z-series images of each cell were taken using the rhodamine filter and the set of images was overlaid in a single image which was converted to a black-on-white negative so that rhodamine-positive endosomes could be counted. After 48 h, the cell monolayers were trypsinized, neutralized with media, and resuspended in 1 ml of phosphate buffered saline for immediate flow cytometry studies to assess rhodamine and GFP fluorescence.

Flow Cytometry

Cell suspensions were analyzed for both rhodamine and GFP fluorescence in the Beckton Dickinson (Franklin Lakes, NJ) flow cytometer. Control cells, transfected using labeled vector without the lipofection agent, were used to set the gated thresholds of each flow study. Gates were set to confine the control cells to the negative fluorescence groups and served to establish limits for false-positive detection of the autofluorescent pigment granules in these cells. Transfected cells showing rhodamine-positive and/or GFP-positive fluorescence above the gated limits for each experimental control sample were counted as positive and used in the statistical analyses.

RESULTS

Microscopy

Both RPE cells and CE cells were competent to take up and express a labeled plasmid vector by polyplex-mediated gene transfer in vitro. All cells demonstrated rhodamine fluorescence that was relatively uniform across the cell monolayer. The amount of rhodamine label present appeared to be greater in RPE cells than in CE cells, and this impression was validated in the quantitative analyses presented. Conversely, there did not appear to be a detectable difference between RPE cells and CE cells in the number of cells expressing GFP. Flow cytometry analysis 48 h after transfection also confirmed this impression.

Transfected cells showed rhodamine-positive fluorescent endosomes as early as 2 h after transfection and demonstrated marked rhodamine fluorescence in virtually all cells in the cell monolayer within 6 h. A representative low power micrograph of transfected RPE cells demonstrates uniform uptake of labeled plasmid throughout the cell monolayers at 6 h after

transfection (Fig. 2a). Higher power micrographs demonstrate some variability in the number of positive endosomes from cell to cell, but each cell has many labeled endosomes present throughout the cytoplasm (Fig. 2b). The number of labeled endosomes in RPE cells was consistently higher at 6, 24, and 48 h after transfection compared to that seen in CE cells.

CE cell monolayers also demonstrated uniform rhodamine fluorescence 6 h after transfection in vitro. The overall level of fluorescence was weaker in the CE cells than that seen in RPE cells transfected in parallel and under identical conditions because there were fewer rhodamine-positive endosomes per cell (data not shown). Confocal microscopy demonstrated a significant difference between the number of rhodamine-positive endosomes in RPE cells compared to that seen in CE cells 24 h after transfection. Twenty-five cells were analyzed and the number of endosomes per cell was quantified as described in the methods section. The mean number of labeled endosomes per cell for transfected RPE cells was 64.04 (SD $\pm$ 20.81, range 33–117). The mean number of labeled endosomes per cell for transfected CE cells was 25.80 (SD $\pm$ 9.38, range 14–47; $P < 0.038$, paired samples t-test).

Flow Cytometry

Flow cytometric analyses showed a statistically significant difference between the level of total rhodamine fluorescence in RPE and CE cells 48 h after transfection ($P < 0.04$). This difference in the fluorescence reflects a difference in the uptake of labeled plasmid vector in vitro. Mean rhodamine fluorescence above the gated threshold was seen in approximately 10% of transfected RPE cells (mean 9.9 ± 7.3, range 2.5–21.0), but only 0.21% of CE cells (mean 0.21 ± 0.12, range 0.12–0.41; $P < 0.04$, paired samples t-test) under identical transfection conditions. A sample flow cytometry run for RPE and CE cells transfected in parallel is shown in Figure 3.

Despite significantly different levels of plasmid uptake, the mean level of marker gene expression at 48 h, when GFP marker gene expression is maximal, was similar between RPE cells (3.29 ± 1.7, range 1.32–4.75) and CE cells (3.60 ± 0.38, range 3.15–4.03; $P < 0.71$, paired samples t-test). The detection of strong green fluorescence in transfected cells was easily detected by flow cytometry at low voltage in both cultures and demonstrated a similar frequency of transgene expression.

DISCUSSION

There was a marked disparity between the uniformity of rhodamine labeling seen 6 and 24 h after transfection in RPE and CE cell monolayers by microscopy and the mean level of rhodamine fluorescence detected by flow cytometric methods at 48 h. The reasons for the disparity are 2-fold. First, the number of endosomes and the relative level of fluorescence are significantly reduced after 48 h because of lysosomal digestion of the labeled plasmid. Breakdown of the endosomes by fusion with lysosomes leads to loss of rhodamine labeling
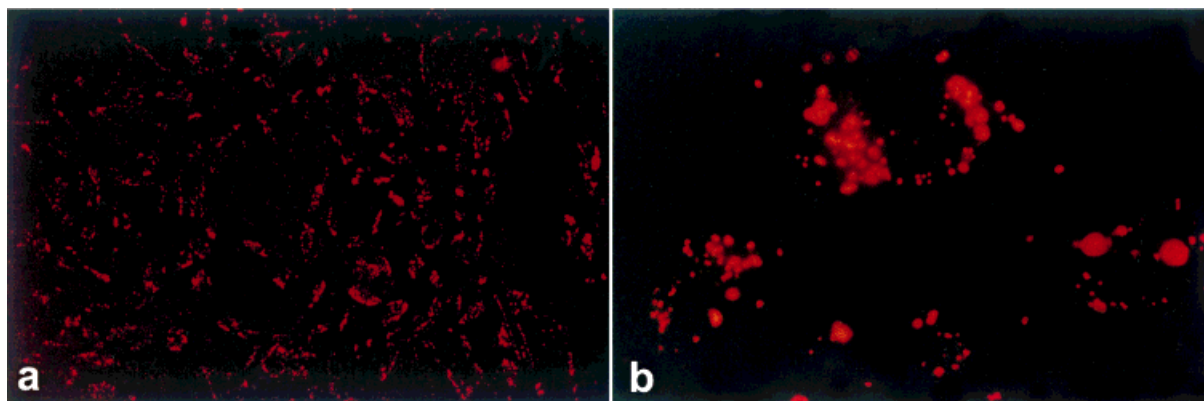
Fig. 2. **a**: Fluorescent micrograph of rhodamine-labeled endosomes in RPE cells 6 h after polyplex-mediated transfection with the *pGeneGrip* vector (100×). Uniform labeling of cells is seen demonstrating generalized uptake of the vector. **b**: Micrograph of rhodamine-labeled endosomes in the RPE cells at higher power (320×). Some variability in uptake is seen, but all cells show many rhodamine-positive endosomes.
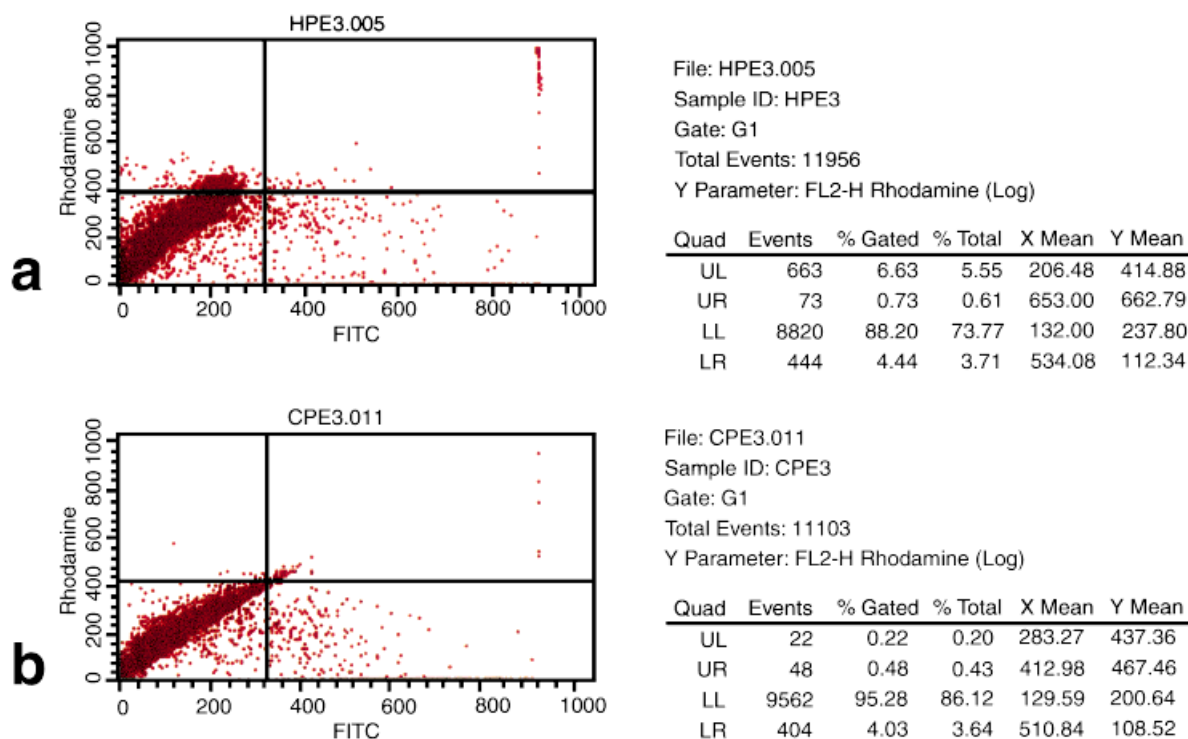
Fig. 3. Flow cytometry study of rhodamine and GFP (FITC) fluorescence, (**a**) in RPE cells and (**b**) in CE cells 48 h after transfection in parallel with the *pGeneGrip* vector under identical conditions. Unlabeled cells are in the lower left (LL) quadrant, rhodamine-labeled cells are in the upper left (UL) quadrant, GFP-labeled cells are in the lower right (LR) quadrant and double-labeled cells are seen in the upper right (UR) quadrant. Gated thresholds were set using sham-transfected control cells in the each experiment.

from the cells and reduced detection by both microscopy and flow cytometry. Microscopic analysis demonstrates low levels of persistent rhodamine fluorescence in transfected cells, but most of these cells cannot be detected by flow cytometry at the gated thresholds set using the control cells.

Second, the lower luminance levels of rhodamine fluorescence (compared to GFP fluorescence) required higher voltages to detect when gating parameters were set. Higher voltages increased the sensitivity of detection but gave rise to more false positives from auto-fluorescence in the control samples. Therefore, voltages were reduced to minimize the false-positive rate in controls. Gating the samples at a higher threshold undercounted cells with weak fluorescence and skewed the sensitivity of flow cytometry towards the most highly labeled cells. Thus, the RPE cells exhibited the greatest fluorescence by flow cytometry because of the larger number of labeled endosomes remaining after 48 h. This higher residual fluorescence reflects the greater number of rhodamine-positive endosomes incorporated

into the RPE cells at the time of transfection and confirms the quantitative results seen by microscopy after 24 h.

There was a statistically significant difference between RPE and CE cells in the uptake of rhodamine-labeled plasmid in vitro as measured by both qualitative and quantitative analysis at 24 h and by flow cytometry at 48 h after transfection. RPE cells showed significantly greater uptake of labeled plasmid by endocytosis than CE cells ($P < 0.04$) due to the higher intrinsic phagocytosis of the RPE cell phenotype in early passage in vitro. However, greater plasmid delivery was not translated into a higher transgene expression frequency in these cells.

Despite significantly greater numbers of plasmid-containing endosomes in RPE cells, transcription efficiency was similar in the two cell types. These studies confirm for the RPE and CE cells the results of gene transfer experiments in non-ocular tissues that showed that the rate-limiting step in polyplex-mediated transgene expression is not the internalization of vector by endocytosis [Zabner et al., 1995].

If delivery of the vector to the nucleus were concentration dependent, then our data suggests that there should be increased transgene expression in RPE cells relative to CE cells. The lack of a correlation between plasmid uptake and transgene expression implies that the rate-limiting step occurs at the level of access to the nucleus or in the control of transcriptional activity once the plasmid reaches the nucleus. Enhanced phagocytic activity and plasmid delivery in RPE cells does not appear to be correlated with enhanced transgene expression in vitro.

Several methods of transfection both in vitro and in vivo have previously been employed to transfect genes into ocular tissues including the retina and RPE. Adenoviruses [Bennett et al., 1994; Abraham et al., 1995; Li and Davidson, 1995; Cayouette and Gravel, 1996] adeno-associated viruses [Jomary et al., 1997], and retroviruses [Sakamoto et al., 1995; Kimura et al., 1996] have been used as transfection agents. The efficiency of viral-mediated gene transfer in the eye is balanced by the relative disadvantage of a lack of tissue specificity using this gene transfer method. Viral transfections deliver genes randomly to various tissue types within the eye following intraocular injection [Mashhour et al., 1994]. Injecting DNA:lipid polyplexes between the retina and RPE in the subretinal space can sequester the DNA and target the RPE, minimizing undesired delivery of transgenes to other ocular tissues. The active transport pump and high intrinsic phagocytic activity of the RPE cells in situ rapidly internalizes the subretinal fluid and DNA into the RPE cells. While this method is less efficient than viral transfer, it has attractive features for intraocular gene therapy applications: it does not require active cell division; it is non-toxic; and it is non-immunogenic [Hug and Sleight, 1991; Stewart et al., 1992; Nabel et al., 1993; Chaum et al., 1999].

The high phagocytic activity of RPE cells does not appear to enhance foreign gene expression in RPE cells in vitro. The RPE cell is, however, a target cell for the application of gene therapy and is competent to express foreign genes. These studies also show for the first time that ciliary epithelial cells are competent to be transfected and express foreign genes by polyplex-mediated gene transfer. Thus, the ciliary epithelium is a potential target tissue for the application of gene therapy to treat glaucoma. Potential approaches might include direct modulation of aqueous production by targeting ion-exchange pumping mechanisms, G proteins or other secondary messengers of signal transduction.

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