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Polyplex-Mediated Gene Transfer into Human Retinal Pigment Epithelial Cells In Vitro

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Abstract The human retinal pigment epithelium (RPE) is a potential target tissue for directed transfer of candidate genes to treat age-related macular degeneration (AMD). The RPE is uniquely suited to gene therapy protocols that use liposome-mediated DNA transfer because of its high intrinsic phagocytic function in vivo. In these studies, we examined the efficacy of human RPE cell uptake and expression of the green fluorescent protein (GFP) and neomycin resistance marker genes by polyplex-mediated gene transfer in vitro. The effects of varying DNA and polyplex concentration and ratios on GFP transgene expression were examined. A narrow range of experimental conditions were found to maximize transgene expression; most important were the DNA concentration and the DNA:polyplex ratio. The transfection efficiency for human RPE cells was reproducibly 20% in vitro by this method and reached a maximum level of expression after 48 h. There was a rapid decline in gene expression over 2 weeks following polyplex-mediated gene transfer, but stable integration does occur at low frequencies with and without selection. J. Cell. Biochem. 76:153–160, 1999.

Key words: gene transfer; retinal pigment epithelium; liposome-mediated gene transfer; dendrimer; green fluorescent protein; cytotoxicity

Diseases of the retinal pigment epithelium (RPE) are responsible for the important blinding disorder age-related macular degeneration (AMD) and certain forms of retinitis pigmentosa [Tasman, 1991]. The identification of mutations in genes involved in the phototransduction cascade in patients with retinitis pigmentosa [Dryja et al., 1990; Farrar et al., 1991; Lem et al., 1992; Maw et al., 1997] suggests that gene therapy may be a potential approach to the treatment of these retinal diseases. The genetics of AMD is still poorly understood; however, there is evidence that certain growth factors enhance RPE cell cycle kinetics and phenotype in vitro [Schweigerer et al., 1987; Waldbillig et al., 1991; Takagi et al., 1994; Gupta et al., 1997] and may delay or prevent retinal degeneration in animal models in vivo [Faktorovich et al., 1990; Cayoutte and Gravel, 1997; McLaren and Inana, 1997]. These studies suggest that the RPE is a potential target tissue for the transfer of candidate growth factor genes to treat AMD.

The RPE serves several important functions in maintaining the health of the overlying neurosensory retina. In particular, (1) it plays an important role in the production of trophic hormones, which have both paracrine and autocrine function [Waldbillig et al., 1991; Takagi et al., 1994], and (2) it digests outer-segment membranes, which are shed daily from the retinal photoreceptors. This phagocytic function is critical, as defects in uptake and digestion, as seen in the RCS rat model, result in retinal degeneration [LaVail, 1981]. Age-related phagocytic dysfunction of the RPE is thought to result in the subretinal accumulation of lipofuscin deposits called drusen, the hallmark of patients with AMD [Young, 1987]. Enhancement of autocrine and phagocytic functions in aging RPE cells are potential therapeutic approaches to the treatment of AMD using gene therapy.

The high intrinsic phagocytic function of RPE cells can theoretically be used to direct gene therapy to the RPE using liposome-mediated

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DNA transfer. Liposomes have been employed to transfect marker and therapeutic genes to a variety of ocular and nonocular tissues, both in vitro and in vivo [Behr et al., 1989; Stewart et al., 1992; Nabel et al., 1993; Caplen et al., 1995; Abdallah et al., 1996; Bebok et al., 1996; Hangai et al., 1996; Kukowska-Latallo et al., 1996; Masuda et al., 1996; Vitiello et al., 1996; Chaum et al., 1998].

Commercially available liposome reagents are composed of cationic lipid or polymer molecules that bind to and condense DNA in a purely physicochemical manner, forming, respectively, lipoplex or polyplex molecules [Felgner et al., 1987, 1997; Yang and Huang, 1997]. These lipid-DNA complexes are capable of delivering genes to the cell via endocytosis [Wrobel and Collins, 1995; Friend et al., 1996; Xu and Szoka, 1996]. Lipoplex and polyplex-mediated gene transfer allows for efficient transfection of large plasmid DNA sequences. The method is associated with low toxicity, does not require active cell division, and is nonimmunogenic [Hug and Sleight, 1991; Stewart et al., 1992; Nabel et al., 1993], all attractive features for application to intraocular gene therapy.

We have examined the efficacy of polyplex-mediated gene transfer into the RPE using the starburst polyamidoamine dendrimer *Superfect* (Qiagen) and have identified transfection conditions which enhance gene transfer efficiency into human retinal pigment epithelial cells in vitro.

MATERIALS AND METHODS

Cell Culture

Human RPE cells were isolated from cadaver eyes provided by the Lion's Eye Bank (Maryland) and grown in tissue culture. 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 eye cup was washed three times in minimal essential medium (MEM)(Gibco-BRL), and 0.25% trypsin was applied for four 15-min digestion cycles. Cells were loosened by gentle aspiration and transferred to MEM supplemented with 10% fetal calf serum (FCS). Isolated cells were spun at 3,000 rpm for 5 min, resuspended in MEM/10% FCS, and plated in 12-well tissue culture plates.

Cells were maintained in MEM/10% FCS supplemented with L-glutamine, penicillin, and streptomycin in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. The cells maintained many of the characteristics present in the human eye including cuboidal monolayer growth and production of pigment granules for several passages in vitro. Melanin pigment synthesis diminished with subsequent passaging in vitro. The cells used were in the third to eighth passage in culture and showed no significant changes in growth characteristics when used during these passages. Cytokeratin staining of cells in culture (Sigma Chemical Co.) confirmed their identification as a homogeneous population of retinal pigment epithelial cells (data not shown).

Plasmid DNA

The plasmid encoding the marker genes employed in these experiments (pEGFP-N1; Clontech) contains the green fluorescent protein (GFP) gene from *Aequorea victoria* under the control of a cytomegalovirus (CMV) promoter. The pEGFP-N1 plasmid also contains a selectable neomycin resistance gene (neo^r) under the control of an SV40 promoter. The plasmid was amplified in *Escherichia coli* and purified on maxiprep nucleic acid columns (Qiagen).

Lipoplex and Polyplex Reagents

We tested several commercially available cationic liposome-based gene transfer reagents including *Lipofectin, Lipofectamine, Cellfectin, DMRIE* (Gibco-BRL) and *Superfect* (Qiagen). Gene transfer studies with the starburst polyamidoamine dendrimer *Superfect* consistently yielded the highest gene transfer efficiency of 18–20% and are reported in this article. There was negligible cell toxicity based on morphologic assessment and in situ cell death assays.

A series of gene transfer experiments were carried out to determine the effect of numerous variables on gene transfer efficiency in culture. These variables included (1) duration of exposure to DNA, (2) ratio of DNA to *Superfect* at fixed reagent concentration, (3) ratio of DNA to *Superfect* at fixed DNA concentration, and (4) varying concentration of DNA and *Superfect* at fixed DNA:*Superfect* ratio.

Transfections

Cells were seeded in 6-well tissue culture plates at 1.3×10^5 cells per well and grown for 48–72 h in MEM/10% FCS at which time they reached ~70% confluency. Cells were washed

once with phosphate-buffered saline (PBS) before transfection. Plasmid DNA was diluted in serum-free MEM before the addition of the *Superfect* reagent. Varying amounts of DNA and lipid were used according to the criteria of individual experiments.

Cells were routinely exposed to the DNA:*Superfect* mixture for 2 h. Longer duration exposure to the DNA:*Superfect* mixture did not result in significantly increased transfection frequencies. After transfection, cells were washed once with PBS, and the media (MEM/10% FCS) was replaced. Cells were observed at 48 h after transfection by fluorescent microscopy. The number of fluorescent cells was counted in 19 fields per well. The average number of positive cells per field was calculated and the total number of positive cells per well was calculated based on the known area of the well and the number of fields per well. Transfection efficiency was expressed as the average percentage of cells that exhibited fluorescence in each well.

To determine conditions that resulted in optimal transfection efficiency, the ratio of DNA to *Superfect* was varied (Table I). The amount and concentration of DNA and *Superfect* were also varied while maintaining a fixed ratio of DNA:*Superfect*.

Duration of Transgene Expression

GFP-positive cells were counted 48 h after transfection and twice weekly thereafter. The total percentage of fluorescent cells were determined at each time interval under nonselective culture conditions. Parallel transfections were performed and the RPE cells were cultured in G418 (500 µg/ml) supplemented media to select for clones capable of sustained expression of the neo^r gene in vitro. Neomycin-resistant clones were isolated and propagated in G418 (125 µg/ml)-supplemented media. The neomycin resistant clones also demonstrated GPF transgene expression in vitro.

TABLE I. Summary of Transfection Conditions Used to Evaluate the Effect of Variations in DNA and *Superfect* Dose, Concentration, and Ratios on GPF Marker Gene Expression in Human RPE Cells In Vitro

	DNA:*Superfect*	DNA (µg)	DNA (µg/ml)	*Superfect* (µg)	*Superfect* (µg/ml)
Figure 1	1:1	30.0	42.25	30	42.25
	1:5	6.0	8.45	30	42.25
	1:15	2.0	2.82	30	42.25
	1:30	1.0	1.41	30	42.25
	1:50	0.6	0.85	30	42.25
Figure 2	1:5	2.0	2.82	10	14.09
	1:15	2.0	2.82	30	42.25
	1:30	2.0	2.82	60	84.51
	1:50	2.0	2.82	100	140.85

RESULTS

Effect of DNA:*Superfect* Ratio on Transfection Efficiency

We examined the expression of the GFP transgene under the control of a CMV promoter as a function of the ratio of DNA to *Superfect*. The DNA amount and concentration or *Superfect* amount and concentration were varied in a series of studies. Transfection efficiency was also determined while varying the concentration and amount of both DNA and *Superfect* while maintaining the optimal DNA:*Superfect* ratio determined in the initial studies.

The results presented in Figure 1 demonstrate the gene transfer efficiency of the DNA:*Superfect* complex using a fixed amount and concentration of *Superfect*, while varying the amount and concentration of DNA. Transgene expression was observed over a wide range of DNA:*Superfect* ratios with maximal gene transfer efficiency occurring at a ratio of 1:5.

The studies presented in Figure 2 examined the gene transfer efficiency of the polyplex us-

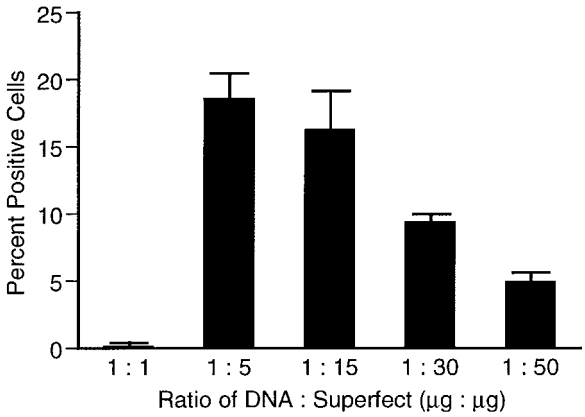

Fig. 1. Effect of varying DNA:*Superfect* ratio on green fluorescent protein (GFP) marker gene transfer efficiency in human retinal pigment epithelial cells in vitro. DNA amount and concentration were varied, while *Superfect* amount and concentration remained fixed (Table I). Average percentage of GFP positive cells per tissue culture well from gene transfer experiments (with standard deviations).

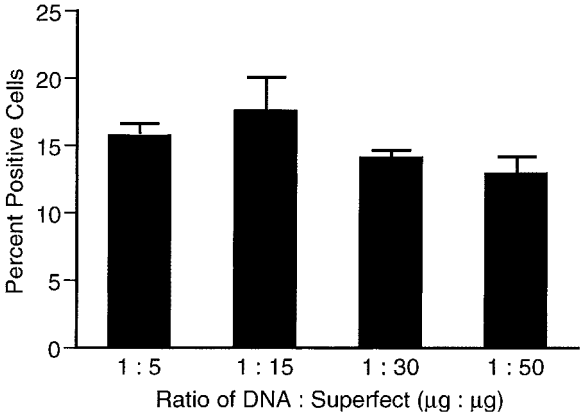

Fig. 2. Effect of varying DNA:*Superfect* ratio on green fluorescent protein (GFP) marker gene transfer efficiency in human retinal pigment epithelial (RPE) cells in vitro. *Superfect* amount and concentration were varied, while DNA amount and concentration remained fixed (Table I). Average percentage of GFP-positive cells per tissue culture well from gene transfer experiments (with standard deviations).

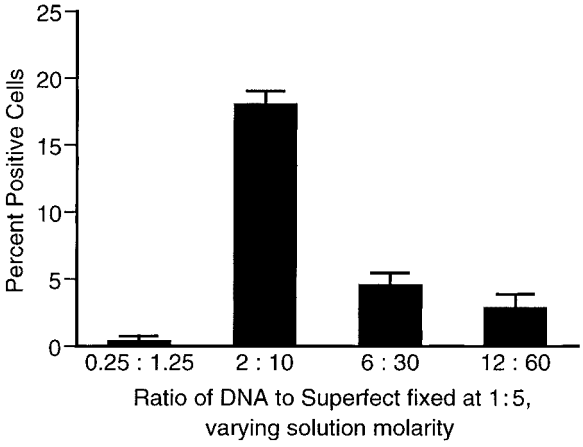

Fig. 3. Effect on gene transfer efficiency of varying the amount and concentration of both DNA and *Superfect* while maintaining fixed DNA:*Superfect* ratio. Average percentage of green fluorescent protein (GFP)-positive cells per tissue culture well from gene transfer experiments (with standard deviations).

ing a fixed amount and concentration of DNA while varying the amount and concentration of the *Superfect* reagent. These studies show that efficient gene transfer in human RPE cells can be seen to occur across a range of DNA:*Superfect* ratios when a minimal concentration of DNA (2 µg) is present. A minimal amount of DNA appears to be critical to achieving optimal transgene expression in vitro; however, increasing the amount of DNA did not improve the efficiency of transgene uptake and expression. In addition, the *Superfect* reagent does not appear to be toxic to the RPE cells, even in excess, under these experimental conditions.

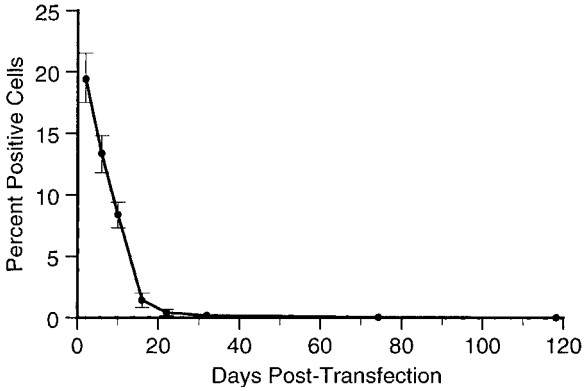

Fig. 4. Green fluorescent protein (GFP) expression decay curve in human retinal pigment epithelial (RPE) cells after polyplex-mediated gene transfer in vitro under nonselection conditions. Average percentage of GFP-positive cells per tissue culture well from multiple gene transfer experiments (with standard deviations).

Cytotoxicity was assessed after transfection in vitro. No appreciable cytotoxicity was identified in any of these experiments as determined by morphologic evaluation of the cell cultures (Fig. 5). The absence of demonstrable toxicity was confirmed by apoptosis assays, using the TUNEL reaction (Sigma Chemical Co.)(data not shown).

Effect of Varying Amount and Concentration of DNA and *Superfect* at a Fixed Ratio

The studies presented in Figure 3 show the gene transfer efficiency of the DNA:*Superfect* complex using a fixed DNA:*Superfect* ratio (1:5), while varying the amount and concentration of both the DNA and the *Superfect* reagent. These studies confirmed that maximal gene transfer efficiency occurs at a DNA:*Superfect* ratio of 1:5. Optimal gene transfer efficiency occurred when 2 µg of DNA and 10 µg of *Superfect* were employed. Transfection with <2 µg of DNA yielded poor expression frequencies even when performed at the optimal 1:5 ratio. These data confirmed the requirement of a minimal amount of DNA necessary to optimize the transfection efficiency.

Cytotoxicity was not observed when optimal conditions were employed in transfections. However, significant cytotoxicity was seen under conditions in which there is a higher concentration of both DNA and *Superfect* while maintaining the optimal 1:5 ratio. Gene transfer efficiency decreased to less than 5% at higher

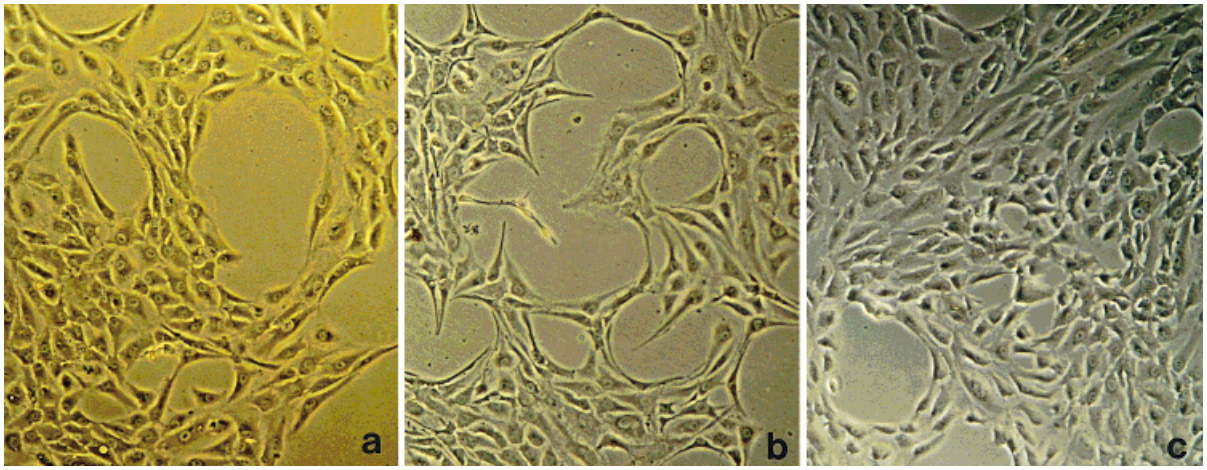

Fig. 5. **a:** Photomicrograph of cultured retinal pigment epithelial cells before transfection. **b:** 12 h. **c:** 18 h after *Superfect*-mediated gene transfer in vitro. There is no morphologic evidence of cytotoxicity induced by the transfection protocol. The morphology also appears unchanged at 24 h and 48 h post-transfection.

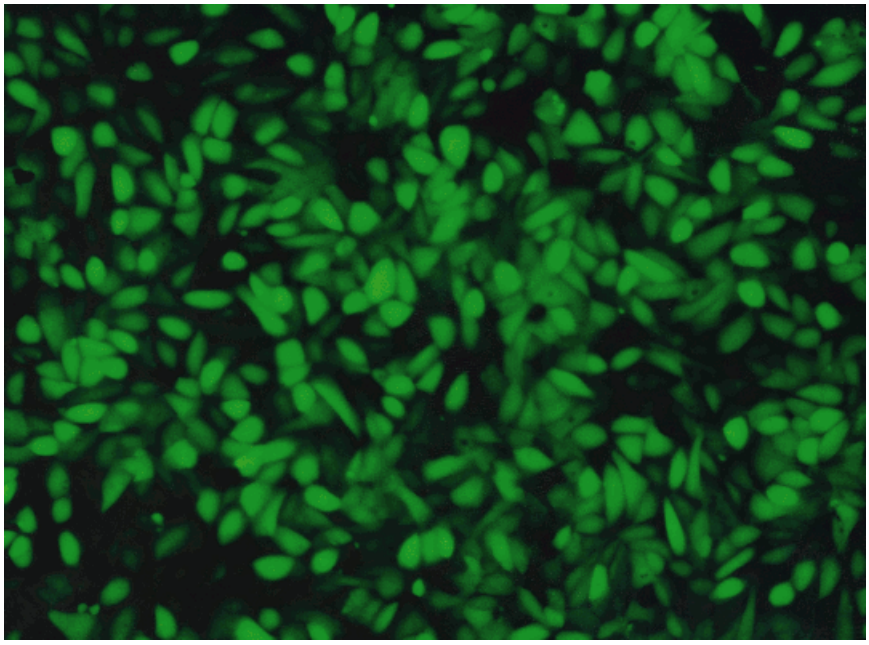

Fig. 6. Photomicrograph of a human retinal pigment epithelial cell clone expanded in media containing G418 after polyplex-mediated gene transfer in vitro. All cells are green fluorescent protein (GFP)-positive, indicating constitutive expression of both the GFP and the neomycin resistance transgenes after transfection.

concentrations despite the optimal 1:5 DNA: *Superfect* ratio. This decrease in expression was seen to be due primarily to in vitro cell death.

Duration of Transgene Expression

The curve exhibited in Figure 4 shows the percentage of cells expressing the GFP transgene over time after *Superfect* transfection. GFP expression is maximal at 48 h after gene transfer and is reproducibly ~20% at optimal DNA: *Superfect* ratios and concentrations. There is a rapid decline in GFP expression after transfection. Transgene expression decreases 90% by 16 days after transfection and continues to decline at a slower rate thereafter. At 118 days after transfection, GFP transgene expression in RPE cells under nonselection conditions was 0.03%.

Although the marker gene expression frequency is low after 4 months in vitro, we believe that these cells reflect stable integration of the marker gene into the RPE cell DNA and constitutive transgene expression. These cells have been maintained under conditions in which there is no selective advantage for the cell to express the GFP gene in order to survive. The

ongoing expression under nonselective conditions in vitro suggests that the GFP gene is stable within the RPE cells and is likely integrated into the host cell DNA. The frequency of stable integration of DNA transfected using this gene transfer method in other cell lines ranges from 0.25 to 16 $\times$ 10^{-4} [Kukowska-Latallo et al., 1996]. Our long-term expression frequency of 3 $\times$ 10^{-4} compares favorably with published results.

We selected for stable incorporation of the plasmid by culturing transfected RPE cells in tissue culture medium containing G418 at 48 h after transfection. We have expanded RPE cell clones that are capable of surviving and proliferating in media containing G418 (Fig. 6). These G418-resistant clones, we believe, reflect stable integration and expression of the neo^r and GFP transgenes in the host RPE cell line in vitro.

DISCUSSION

Several methods of transfection have been employed to transfect genes into the retina, RPE, and vitreous cavity, both in vitro and in vivo. These methods include adenoviruses [Bennett et al., 1994, 1996; Li et al., 1994; Abraham et al., 1995; Li and Davidson, 1995; Cayouette and Gravel, 1996; Sullivan et al., 1996] adeno-associated virus [Jomary et al., 1997] and retroviruses [Sakamoto et al., 1995; Kimura et al., 1996]. Whereas most viral transfection systems have the advantage of high-frequency integration into the host cell genome, they have certain disadvantages in the application of gene therapy for intraocular disease, depending on the virus used for gene transfer. These disadvantages include transient gene expression, a requirement for cell division, inability to control the tissue target, viral immunogenicity, and the potential for recombination with host cell viruses, depending on the viral vector used.

We have established the conditions necessary for the efficient polyplex-mediated transfer of genes into human RPE cells in vitro. There appear to be minimal toxic effects of polyplex-mediated gene transfer on human RPE cells in culture. These studies establish conditions that minimize cytotoxic effects, while maximizing gene transfer efficiency in human RPE cells in vitro, using the polyplex dendrimer *Superfect*.

Maximal gene transfer efficiency in human RPE cells in vitro occurs at a DNA to *Superfect* ratio of 1:5. Complex formation between DNA and dendrimers occurs due to the electrical charge interactions between the molecules. The charge ratio of the DNA to *Superfect* dendrimer molecule is estimated to range from 1:5 to 1:50 depending on the plasmid [Kukowska-Latallo et al., 1996]. Our data reflect this relationship. Transfection efficiency is poor with a DNA: *Superfect* ratio of 1:1, as most of the DNA anionic charge remains exposed, hindering theoretical binding to the hydrophobic cell membrane and endocytosis. Progressively higher ratios of dendrimer to DNA showed an optimal transfection efficiency between 1:5 and 1:15 in human RPE cells. At higher ratios, gene transfer efficiency declines. This may reflect competition for membrane binding and internalization between polyplexes and unbound dendrimer molecules. With increased competition for access to cell membranes and endocytic vesicles, less DNA may be internalized and transfer efficiency may decrease. The decreased gene transfer efficiency at higher ratios does not appear to be attributable to increased cytotoxicity based on our results.

These studies show that gene transfer in human RPE cells occurs across a range of DNA: *Superfect* ratios when the DNA concentration remains fixed and is sufficient to permit transfer of a minimal amount of DNA molecules, approximately 2 μg/1.3 $\times$ 10^5 cells. There appears to be a minimal quantity of DNA molecules required to achieve optimal transgene expression in vitro. Increasing the amount of DNA present with the transfer reagent did not yield an increase in transgene expression in our studies. Similar results with other cells lines suggest that the mechanism of DNA uptake in vitro is saturable [Caplen et al., 1995]. The reagent does not appear to be toxic to the RPE cells, even in significant excess. Nor does excess *Superfect* appear to inhibit gene transfer efficiency if there is sufficient DNA present. There may be saturable competition for membrane vesicles if there is enough DNA present, to permit adequate polyplex formation, even in the presence of excess *Superfect* reagent.

Toxicity assays were performed in parallel with transfection experiments, once optimal conditions were established. These studies showed that the optimal conditions used for polyplex gene transfer of DNA to human RPE cells in culture had negligible toxicity. Only when high concentrations of both DNA and *Superfect* were used was cytotoxicity seen. Gene

transfer efficiency may be reduced at higher DNA:*Superfect* concentrations, but the decrease in expression appeared to be due primarily to cell death. Similar results have been seen with liposome-mediated gene transfer in other cell lines [Caplen et al., 1995].

We have demonstrated that human RPE cells are capable of taking up marker genes and expressing them in vitro. Uptake has been shown to be reliable and reproducible, however, polyplex-mediated gene expression is variable and in most cells transient. We have selected and expanded neo^r RPE cell clones which are capable of surviving and proliferating in media containing G418, demonstrating sustained selectable transgene expression in vitro.

The ability to transfect these cells and effect long-term expression of specific promoted genes has important implications for the development of gene transfer technology for the treatment of retinal disease. Integration of transfected genes is critical to the success of gene therapy as a treatment for disease because only integrated transgenes will be expressed on a sustained basis. These studies have shown that polyplex-mediated gene transfer in vitro, using synthetic dendrimer molecules appears to be capable of introducing stable integration of both selectable and nonselectable marker genes by transfection into RPE host cell DNA. This methodology has the potential to be useful in the clinical application of gene transfer directed to the RPE in vivo.

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