Barriers to productive transfection of trabecular meshwork cells

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Purpose: A critical function of trabecular meshwork cells is to degrade cellular debris, including DNA. We hypothesize that low transfection efficiencies of primary human trabecular meshwork (HTM) cell cultures with plasmid DNA are a function of retained capacity to efficiently degrade exogenous DNA in vitro.

Methods: To determine mechanisms responsible for low transfection efficiencies of cultured HTM cells, steps of DNA entry into cytoplasm and nucleus were characterized. Following synchronization with sequential serum starvation and serum reintroduction, the HTM cell cycle was characterized using 5-bromo-2-deoxyuridine incorporation into replicating DNA. HTM cells were transfected during S-phase with plasmid DNA encoding green fluorescence protein (GFP) or plasmid DNA conjugated with Cy3. In some experiments, cells were treated with a DNase I inhibitor, 100 nM auranitricarboxylic acid. Uptake of plasmid DNA was measured by intracellular fluorescence of Cy3 and productive transfection efficiency was measured by intracellular fluorescence of GFP.

Results: HTM cells enter S-phase between 18 and 20 h after synchronization. Plasmid DNA reached the cytosolic compartment in 95% of transfected cells, regardless of synchronization. Synchronization dramatically increased productive transfection efficiency in HTM cells, from 3.0 to 9.0%. DNase I inhibition increased productive transfection efficiency of HTM cells two fold.

Conclusions: Cultured HTM cells have a lower transfection efficiency than other primary ocular cell cultures, likely due partially to cytoplasmic digestion of DNA. We suggest that the difficulties in transfecting cultured HTM cells may be related to the filter function of the cells in vivo where the cells must degrade exogenous DNA.

The conventional drainage pathway of the human eye consists of the trabecular meshwork (TM) and Schlemm’s canal (SC). The juxtacanalicular (JCT) region of the TM and/or the inner wall of SC provide the primary resistance to aqueous humor outflow and generate intraocular pressure [1,2]. Proper functioning of these two components depends on efficient upstream filtration of aqueous humor by TM cells on lamellar beams [3]. When the inner TM, the filter, is overloaded with exfoliation material, pigment, or DNA (frequently found in the aqueous humor [4-6]), the JCT, TM, and SC inner wall (the resistor) become impaired and intraocular pressure rises [2,7]. Efficient filtration is made possible by the unique architecture of the TM, which mixes aqueous and maximizes the cell surface area coming in contact with debris in the aqueous humor. Upon contact, human (H) TM cells remove debris from the aqueous humor by phagocytosis [8] and neutralize peroxides and oxygen free radicals using intracellular enzymes [9-12].

Characterizing phagocytic behavior and other responsibilities of HTM cells has been largely accomplished in cultured cells [13,14]. One obstacle in using cultured HTM cells as models to study outflow physiology has been low expression of heterologous proteins by HTM cells after transfection with plasmid DNA. When examined in the most basic way, there are four main barriers to efficient expression: (1) plasmid DNA must cross the plasma membrane into the cytosol, (2) DNA must traverse the cytosol without being degraded or mislocalized, (3) DNA must cross the nuclear membrane so that transcription machinery can be utilized and, (4) transcription must occur [15]. Although different promoters can be employed to optimize transcription, the first three steps are more difficult to address and are the focus of this paper. When HTM cells are transfected, efficiency, as evidenced by protein expression, is extremely low. Possible explanations for this observation include decreased capacity to take up exogenous DNA, increased capacity to degrade exogenous DNA in the cytosol, decreased ability to permeate the nuclear membrane, or a combination of all three.

In the present study we explore these possibilities. We hypothesize that difficulties in transfecting HTM cells may be related to the filter function of HTM cells in vivo, and that HTM cells in culture, like their in vivo counterparts, retain a high capacity to efficiently take up and degrade exogenous DNA. To test this hypothesis, we monitor the entry of plasmid DNA into the cytoplasm and nucleus of HTM cells under different conditions. We show that HTM cells readily take up DNA into their cytoplasm, however HTM cells are extremely inefficient at expressing the transfected protein. In order to increase the utility of transfected primary cultures, this study identifies two complementary ways to increase productive transfection efficiency of HTM cells.

METHODS

Cell culture: Primary cultures of two cell types, human trabecular meshwork (HTM) and human retinal pigment epithe-
lium (HRPE), were isolated from human cadaver eyes and used in the present study. Cadaver eyes were obtained from the National Disease Research Interchange (Philadelphia, PA), Vision Share (ApeX, NC), and Donor Network of Arizona (Phoenix, AZ). HTM cells were isolated from TM tissue obtained from human donors without history of glaucoma were used in the present study (ages 3 months, 35 years, 68 years, 67 years, and 71 years). HRPE cells were isolated from posterior eye poles by carefully dissecting away the retina and digesting the eye cup with trypsin as described previously [17]. The two HRPE cell lines used in the present study were from human donors without history of eye disease. HTM cells were cultured in low glucose Dulbecco’s modified Eagles medium (DMEM, Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (FBS, Gemini, Woodland, CA) and penicillin, streptomycin and glutamine (PSG-100 U/ml, 0.1 mg/ml, 0.29 mg/ml, respectively, Invitrogen). HRPE cells were cultured in high glucose DMEM with 10% FBS and PSG. All cells were maintained in humidified air containing 5% CO₂ at 37 °C. Results from all experiments were obtained from at least two cell lines from each cell type.

Cell cycle analysis/BrdU labeling: HTM cells were plated at about 30% confluency (4.2x10⁴ cells/cm²) onto glass coverslips in 6 well culture plates with serum-containing medium. Twenty-four h after plating, cells were serum starved in DMEM containing 0.1% Triton X-100 (three times, 1 ml/well) and incubated for one hour at 25 °C in blocking buffer containing Cy3-conjugated anti-mouse IgGs (1:500 anti-DNase I IgG; Sigma, Dallas, TX). After a 2 h incubation with BrdU, cells were fixed in 4% paraformaldehyde, stained with DAPI, and mounted onto glass slides. Cells were visualized by ultraviolet and immunofluorescence microscopy and counted from digital images taken from random fields for a total of 300 cells per coverslip.

Plasmid preparation and Cy3-labeling of plasmid DNA: Plasmid DNA was prepared by growing up green fluorescent protein (GFP) containing bacteria (pEGFP-C1; Clontech Laboratories, Palo Alto, CA) overnight from glycerol stocks and using traditional alkaline lysis miniprep and phenol/chloroform extraction. Plasmid purity and presence was confirmed by agarose gel electrophoresis and spectrophotometry. The day prior to transfection experiments, plasmid DNA encoding GFP was labeled with Cy3 using a Mirus Label IT Tracker Cy3 kit as per manufacturers instructions (Mirus, Madison, WI). Briefly, Cy3 reagent was incubated with plasmid DNA (at a ratio of 1.5:2) and precipitated using ethanol and 5 M NaCl. To confirm plasmid labeling, absorbance was read at 550 nm (the appropriate wavelength for Cy3) and found to be 0.08 for labeled plasmid compared to 0.0 for unlabeled plasmid when using equivalent quantities of DNA (as determined by absorbance at 260 nm).

Transient transfection of plasmid DNA: HTM and HRPE cells were seeded onto glass coverslips at about 30% confluency (4.2x10⁴ cells/cm²) in serum-containing medium. Cells were split into two groups 24 h after plating: synchronized or unsynchronized. For synchronized cells, serum-containing medium was replaced with DMEM containing 0.2% lactalbumin hydrolysate. After 24 h of serum-starvation, cells were reintroduced to medium containing 10% serum. At various times, ranging from 18-20 h after the reintroduction of serum, cells were transfected for 5 h using Lipofectamine reagent according to manufacturers instructions (Invitrogen, San Diego, CA) using a DNA: Lipofectamine ratio of 1.6:1 and a total transfection volume of 1 ml. Unsynchronized cells remained in serum-containing medium for the duration of the experiment. In all transfections, plasmid DNA encoding GFP was included at a concentration of 4 µg/9.8 cm² well. Control wells were mock transfected using transfection reagent only. In some experiments, cells were treated with the DNase I inhibitor, aurintricarboxylic acid (ATA, 100 nM, Sigma), during the 5 h transfection period. After transfection, the cells were returned to ATA-free medium for 24 h. The cells were fixed in PBS containing 4% paraformaldehyde, stained with DAPI, and mounted onto glass slides. Cells were visualized by ultraviolet and immunofluorescence microscopy and counted from digital images taken from random fields for a total of 300 cells per coverslip.

α-DNase immunocytochemistry: HTM and HRPE cells were plated on glass coverslips to about 60% confluency (8x10⁴ cells/cm²). Cells were fixed in 4% paraformaldehyde, blocked in 10% goat serum in 1X PBS plus 0.1% TX-100, and incubated overnight at 4 °C in 1:500 anti-DNase I IgG (Sigma) in blocking solution. Cells were washed with PBS containing 0.1% Triton X-100 (three times, 1 ml/well) and incubated for 1 h at 25 °C in blocking buffer containing Cy3-conjugated anti-rabbit IgGs (1:2000 dilution; Jackson Immunoresearch, West Grove, PA). Cells were washed (three times, 1 ml/well) and counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml; Sigma). Coverslips were mounted in antifade (90% glycerol, 10% 1 M Tris pH 8.0, 0.2% n-propyl gallate) on glass slides and labeled cells were viewed by indirect immunofluorescence and ultraviolet microscopy. BrdU-labeled cells were counted from digital images taken from random fields for a total of 300 cells per coverslip.

Statistical analyses: Two tail, paired Student’s t-tests assuming equal variance were used to determine whether experimental data were different from controls. An α level of 0.01 was chosen.
RESULTS
Experiments were undertaken to determine obstacles that interfere with transfection efficiency of HTM cells in culture. Strategies were designed based on the hypothesis that HTM cells may have an increased ability to degrade DNA due to their physiology and function in vivo.

HTM cell cycle analysis: Decreasing the time plasmid DNA spends in the cytosol increases the chances of it crossing the nuclear membrane before being degraded. To minimize the time plasmid DNA is present in the cell cytoplasm during transfections, we needed to characterize the HTM cell cycle to determine the precise time of breakdown of the nuclear envelope. This information provides a time window to expose dividing cells to plasmid DNA for transient transfections. Synchronizing cell cycles enables the timing of transfections so that plasmid DNA begins to enter the cytosol during S phase, immediately before the breakdown of the nuclear membrane that accompanies mitosis. To synchronize HTM cell cycles, cells were serum-starved for 24 h and then exposed to serum. During 2 h intervals (12-14, 14-16, 16-18, 18-20, 20-22, 22-
24, and 24-26 h) after reintroduction to serum, cells were treated with BrdU. Control experiments were executed in parallel using unsynchronized cultures that were treated with BrdU at identical times. BrdU, a synthetic nucleotide, incorporates into replicating DNA, thus providing a good indicator of S phase. Figure 1A shows a representative microscopic field of HTM cells labeled with BrdU (arrowheads). Figure 1B summarizes the times tested, indicating that BrdU labeling peaked around 18-20 h in synchronized cells; with more than three times the labeling of unsynchronized cells. Thus, the S phase of HTM cells in culture occurs between 18-20 h after stimulation of proliferation from a quiescent population. We chose to use HRPE cells as our control primary cell type partly because the cell cycle of HRPE cells has been previously described [18]. In HRPE, incorporation of radiolabeled nucleotides (another indicator of S phase) also peaks at 20 h after synchronization indicating that the cell cycles of HTM and HRPE cells are approximately the same.

Uptake of plasmid DNA by HTM and HRPE cells: The first step in transient transfections is transporting plasmid DNA from the extracellular space across the plasma membrane and into the cytosol. To assess whether difficulties in transfecting HTM cells are associated with this step, we used a well documented method of measuring DNA uptake into cells [19,20]. HTM, HRPE, and COS-7 cells were transfected with plasmid DNA that was conjugated with Cy3 and uptake of DNA into the cell cytosol was monitored by immunofluorescence microscopy. Transfection periods (6 h) began 18, 19, and 20 h after synchronization with unsynchronized cells used as controls. Unexpectedly, in all cell types, virtually all cells (about 95%), synchronized and unsynchronized, took plasmid DNA into the cytosol (Figure 2; data not shown for COS-7). Additionally, greater than 90% of cells took up at least some DNA into their nucleus. Thus it appears that the process of DNA uptake by HTM cells is not responsible for low productive transfection rates.

Effect of cell cycle synchronization on productive transfection: Productive transfection requires the plasmid DNA to cross both the plasma membrane and the nuclear membrane. A reliable indicator of productive transfection is the appearance of protein(s) encoded by transfected DNA, in the present case, GFP (Figure 3A). As shown in Figure 3B, we found a three fold increase in productive transfection efficiency in synchronized HTM cells when using a 5 h transfection period.
that began 19 h after synchronization (3.0±1.17% unsynchronized cells compared to 9.0±1.82% in synchronized cells). Productive transfection efficiency in HTM cells was also increased when the transfection period began 18 and 20 h after synchronization although the levels did not attain statistical significance. Conversely, synchronization did not affect the productive transfection rate in HRPE (Figure 3C).

These data suggest that DNA that enters the HTM cell is not likely to be transcribed and translated when it must remain in the cytosol for a prolonged period (as in unsynchronized cells). Alternatively, when the beginning of the transfection period is timed properly, the HTM cell is exposed to DNA during S and M phases when the nuclear envelope has broken down sufficiently to allow significant and relatively rapid plasmid uptake into the nucleus. One explanation for our observation is that DNA is rapidly degraded in HTM cells and that mechanisms of degradation differ between HTM and HRPE.

Expression of DNase I in HTM and HRPE cell cultures: If DNA degradation is playing a role in low baseline transfection efficiencies of HTM cells, it is important to examine the expression of DNA digesting enzymes such as DNase I. To determine DNase I expression in HTM and HRPE cells in culture, fixed cells were probed with anti-DNase IgGs. As shown in Figure 4, DNase I was detected in both HTM and HRPE cells. Labeling was not limited to the nucleus, but was also found cytosolically in both cell types, indicating a possible role for DNase I as a mediator of both nuclear and cytosolic degradation of exogenous DNA.

Effect of DNase I inhibitor on transfection efficiency: To test whether cytosolic DNases were in part responsible for low productive transfection rates in HTM cells, we tested the effects of a DNase I inhibitor on productive transfection efficiency. Unsynchronized HTM and HRPE cell cultures were treated with the DNase inhibitor aurintricarboxylic acid (ATA, 100 nM) during transfection, and productive transfection rates were assessed by expression of GFP. Shown in Figure 5, ATA treated HTM cells exhibited productive transfection rates 2 times higher than untreated cells (control: 3.7±1.1%; with ATA: 7.3±1.2%). Consistent with results from cell synchronization experiments, ATA treatment had no effect on productive transfection rates in HRPE cells. These data suggest that DNase I contributes to low productive transfection rates of HTM cells in culture.

DISCUSSION

We hypothesized that coordinating the time HTM cells begin DNA replication and division with the time cells were exposed to exogenous DNA would greatly increase transfection efficiency. The data indicate that synchronizing HTM cell cycles and timing the beginning of the transfection period with S phase resulted in a 3 fold increase in transfection efficiency. This increase is likely because dividing cells transfect more easily since during division the nuclear membrane breaks down and exogenous DNA can enter the nucleus [21]. Interestingly, plasmid DNA entered the cytosol and nuclei of 90-95% of all synchronized cells, but only about 10% was translated into protein. Since the fluorophore remains conjugated to the DNA even when the plasmid is not intact, there is no way to tell how much of the Cy3 nuclear staining is from functional plasmid and how much is due to nicked DNA or even small oligonucleotides that have been partially degraded before being taken up into the nucleus. These results indicate that the first and third potential barriers to effective heterologous protein expression of DNase I in cultured ocular cells. HTM cells express DNase I. HTM and HRPE cells cultured on coverslips were probed with anti-DNase I IgG and counterstained with DAPI. Control shows background labeling in the absence of anti-DNase I IgG. Shown is a representative experiment (n=3) using two different cell strains for each cell type.

Figure 5. Effect of DNase I inhibitor on transfection efficiency. DNase inhibitor doubles productive transfection efficiency of HTM cells. Cells in log-phase of growth were transfected with plasmid DNA encoding GFP in the presence or absence of a DNase I inhibitor (aurintricarboxylic acid, ATA, 100 nM). Cells were fixed, counterstained with DAPI and visualized using epifluorescence microscopy. Cells expressing GFP protein and total cells (at least 300 cells per condition) were captured digitally and counted to calculate average transfection efficiencies. Statistical differences between ATA-treated and untreated cells were assessed using Student’s t-test (asterisk indicates a p<0.01).
expression (uptake into the cytosol and permeation of the nuclear membrane) are not significant obstacles to efficient transfection in this experimental paradigm. Furthermore, our results suggest that HTM cells have a high capacity to degrade DNA, and that DNA degradation prior to expression may be a limiting factor in efficient protein expression.

Since not reported previously, it was necessary to first characterize the cell cycle of HTM cells in culture so that we could estimate the optimal time to expose cells to plasmid DNA. We reasoned that synchronizing the beginning of the DNA exposure period with S phase would allow the plasmid to reach the nucleus during mitosis-associated nuclear membrane breakdown, increasing the opportunity for plasmid DNA to access HTM cell nuclei and transcription machinery. Using BrdU incorporation into replicating DNA as a marker of S phase, we found that HTM cells began DNA synthesis approximately 18-20 h after reintroduction to serum (Figure 1) and began mitosis four hours later (data not shown). Conveniently, HRPE cell cycle in culture closely parallels that of HTM cells in culture [18]. Thus, the optimum time window for productive transfection efficiency for both cells lines was 18-20 h after cell cycle synchronization.

Chemical transfection reagents have been available since the 1960s, beginning with DEAE dextran [22] and calcium phosphate [23]. However, in the last five to ten years, chemical reagents have improved dramatically, and now the problem of DNA uptake across the plasma membrane has been effectively minimized. Despite this progress, only two other published studies have examined plasmid uptake by cells, independent of exogenous protein expression [15, 24]. Although the first study did not calculate efficiency on a per cell basis, the second reported results similar to ours, approximately 80% of transfected cells took up some exogenous DNA [15]. However, this study was conducted exclusively on transformed cell lines, limiting the applicability of their data to our primary cells. In the present study, plasmid DNA successfully crossed the plasma membrane of about 95% of both primary (Figure 2) and transformed (data not shown) cells using Lipofectamine reagent (a polycationic lipid formulation that prompts the formation of liposomes). Unfortunately such chemical agents do very little to encourage DNA stability in the cytosol and nothing to address the issue of nuclear targeting. With even newer methods including electroporation across the plasma membrane (which is inefficient in our system), and protein based methods, transport through the cytosol to the nucleus can be more directed and efficient; however, these methods have not yet been perfected [24].

In addition to using cell cycle synchronization as a method to increase transfection efficiency, we examined another method. We used a synthetic DNase inhibitor (ATA) to partially inhibit DNases in the cell [25-27]. Since there are many different DNases and since DNases bind readily to actin, our methodology does not presume to completely inhibit DNA digestion. However, even with partial inhibition of DNases, we were able to increase transfection efficiency two fold in HTM cells (Figure 5). Several studies have shown that nuclese inhibitors can successfully increase in vivo and in vitro transfection efficiencies in mammalian tissues [25, 28, 29] leading to our hypothesis that part of the reason HTM cells have such low transfection efficiencies is because of DNA degradation by DNases. In Figure 4, we showed that DNase I, a common DNase, is expressed in the nucleus and cytosol of HTM and HRPE cells (in addition to many other tissues [30]). DNases digest DNA in the nucleus during apoptosis [27] and also digest exogenous DNA phagocytosed by adjacent living cells and immune cells after apoptosis [31]. These normal activities are consistent with the hypothesis that homeostatic phagocytosis and degradation of DNA in HTM cells is mediated by DNases as part of the normal filter function of the conventional outflow pathway. Primarily, inhibition of DNases provides an additional way to increase productive transfection efficiency of HTM cells.

HRPE cells were used as controls in experiments for two main reasons. First, HRPE are primary, nontransformed ocular cells. Second, HRPE cells are active phagocytes, like HTM cells, but with different responsibilities. HRPE cells do not actively degrade exogenous DNA like HTM cells, but ingest lipids and proteins of the shedding outer segments of photoreceptors [32, 33]. These differences in function in vivo are consistent with observed differences in basal transfection rates between the two cells types in vitro, 10.0±1.2% for HRPE compared to 3.0±1.1% for HTM. Interestingly, when HTM cells were treated with the DNase I inhibitor, ATA, transfection rates of HTM cells doubled, and approached rates of HRPE. Since HRPE transfection rates were unaffected by ATA treatment, it seems that DNA degradation plays a less important role in the efficient transfection process in HRPE compared to HTM cells.

In summary, we have identified two methods for increasing transfection efficiency in HTM cells. The first, by synchronizing the cell cycle to time transfection with S-M phase and the second, by partially inhibiting DNA degradation. We have shown that uptake of DNA into the cytosol and nucleus of HTM cells is not the limiting factor in efficient transient transfection and that DNA degradation prior to protein expression may be the main barrier. While the overall transfection rates in HTM cells attained with our methods were still low (about 10%) they are sufficiently high for many common uses of transfected cells including immunocytochemistry and electrophysiology. It is possible that a combination of these or other methods may further increase transfection efficiency.

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