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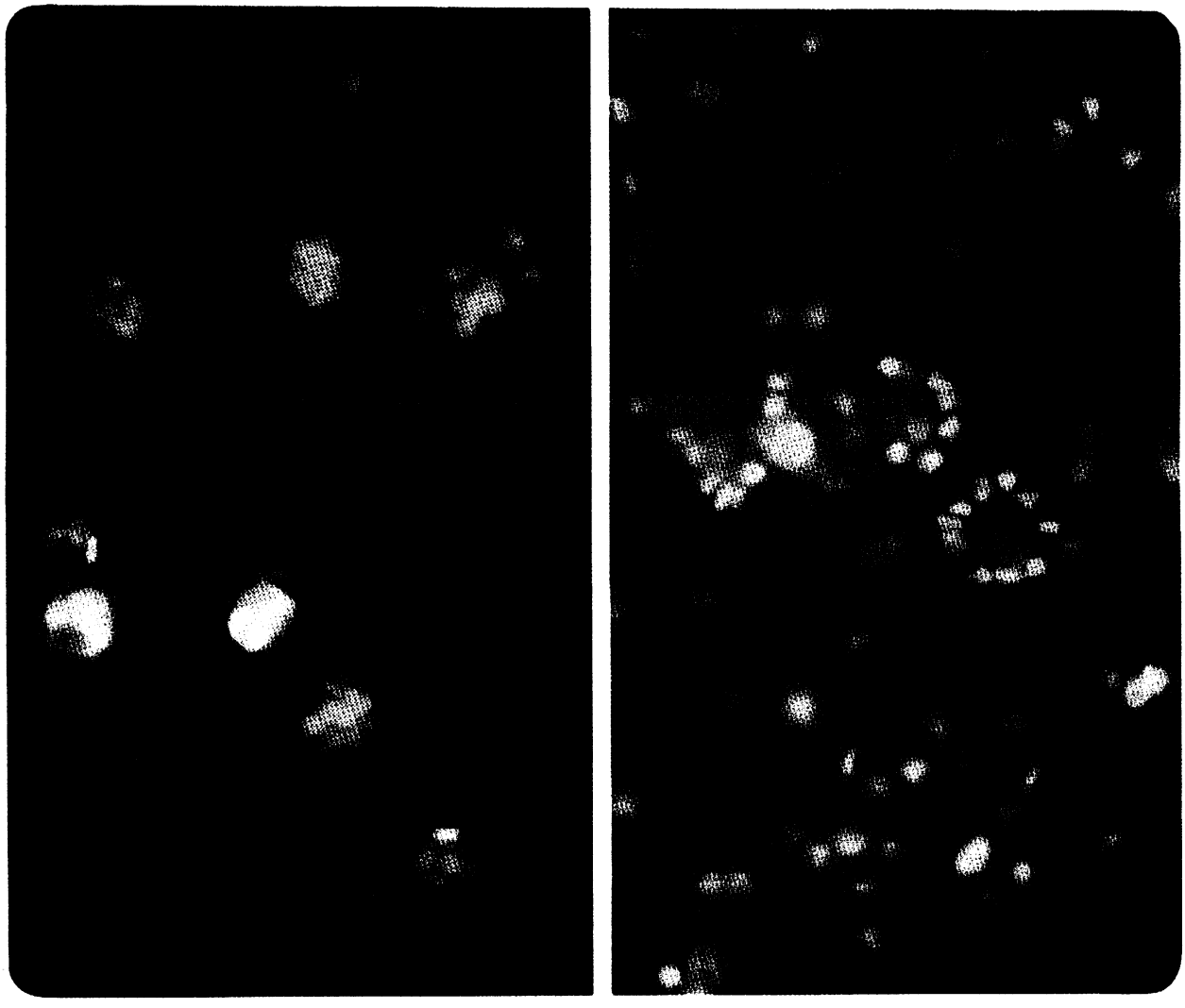

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Cover: Fluorescent micrographs of rabbit pulmonary alveolar macrophages incubated with glutaraldehyde-fixed red blood cells (GRBC) under normoxic (*left panel*) or acute hypoxic (*right panel*) conditions. Fluorescent technique allows differentiation of phagocytosed (green-yellow particles) and nonphagocytosed (red-orange particles) GRBC. Alveolar macrophages exposed to acute hypoxia (*right panel*) phagocytosed fewer GRBC than those in normoxia (*left panel*). For details, see the article by Leeper-Woodford and Mills, beginning on page 326.

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Rapid Communication

Gene Transfer to Respiratory Epithelial Cells via the Receptor-mediated Endocytosis Pathway

David T. Curiel, Santosh Agarwal, Maria Unni Rømer, Ernst Wagner, Matt Cotten, Max L. Birnstiel, and Richard C. Boucher

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Gene-based therapies for a variety of inherited and acquired pulmonary diseases will require the development of vectors capable of safe and efficient transfer of DNA to the respiratory epithelium. The present study examined the feasibility of delivering DNA to respiratory epithelial cells by the receptor-mediated endocytosis pathway. This strategy employs molecular conjugates consisting of a cognate moiety, in this case human transferrin, covalently linked to a DNA-binding moiety, such as a cationic polyamine. Complexes were formed between transferrin-polylysine conjugates (hTfpL) and plasmid DNA carrying the firefly luciferase reporter gene (pRSVL). The conjugate-DNA complexes were added directly to cells in tissue culture and incubated for 24 h, after which cell lysates were analyzed for luciferase enzyme activity by luminometry. An immortalized human respiratory epithelial cell line (HBE1) treated with the transferrin-polylysine-DNA complexes exhibited luciferase enzyme activity significantly augmented over background levels. This respiratory epithelial cell line exhibited greater susceptibility to gene transfer by the transferrin-polylysine conjugates than did non-respiratory epithelial cell lines known to possess high levels of transferrin receptors. Effective gene transfer was shown to require both the DNA-binding moiety and cognate moiety for the cell surface receptor. Specific internalization of the conjugates by the tranferrin pathway was verified by competition for the transferrin receptor. In addition, treatment with agents that either increased transferrin receptor number or decreased lysosomal degradation markedly augmented gene expression mediated by the conjugates. Thus, respiratory epithelial cells possess receptors for transferrin that can be exploited to accomplish gene transfer by the receptor-mediated endocytosis pathway.

A variety of inherited and acquired disorders affecting the cells of the respiratory epithelium may potentially be approached utilizing therapies directed at the level of gene expression. In this regard, the gene mutations etiologic of the inherited pulmonary disorders α_1-antitrypsin deficiency (1) and cystic fibrosis (2) have been defined and phenotypic correction accomplished at the cell or organ level by means of transfer of normal gene counterparts to cells of the respiratory epithelium (3, 4). Acquired pulmonary diseases may also result from genetic lesions as in the subset of pulmonary carcinomas associated with mutations in tumor suppressor genes (5–7). Also in this instance, reversal of the abnormal phenotype has been demonstrated *in vitro* by correction of the defective gene function (8).

Gene-based therapies as an approach to these disorders will require development of gene transfer vectors capable of safe and effective delivery of DNA to the cells of the respiratory epithelium. A variety of DNA-mediated gene transfer vectors have been utilized to accomplish gene transfer to the respiratory epithelium *in vitro* and *in vivo* (9, 10). These vector systems, however, are generally of very low efficiency. In addition, they are associated with significant cellular toxicity. Recombinant viral vectors have also been utilized in this context (3, 11). Although more efficient, these agents suffer from limitations related to the size and design of DNA that can be transferred. In addition, potential safety hazards derive from the obligatory co-transfer of elements of the parent virus genome.

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As an alternative strategy that circumvents both sets of limitations, gene transfer has been accomplished via the receptor-mediated endocytosis pathway utilizing polycation-ligand conjugates (12–14). These synthetic molecules possess two functional domains: a cognate moiety for a cell surface receptor, which is covalently linked to a DNA-binding moiety, such as a cationic polyamine. Interaction of the DNA with the molecular conjugate serves to condense the DNA into compact, ligand-coated particles. When recognized by the appropriate cell surface receptor, the conjugate is internalized by the efficient receptor-mediated pathway, co-transporting the complexed DNA. In the present study, we demonstrate that gene transfer to the respiratory epithelium *in vitro* may be accomplished by the receptor-mediated endocytosis pathway utilizing transferrin-polylysine conjugates. Because this method accomplishes gene transfer by capitalizing on a normal physiologic cellular pathway, it is potentially nontoxic, permitting the potential to administer DNA on a repetitive or continuous basis. Additionally, this strategy offers the potential to achieve cell-specific targeting of gene delivery by virtue of the flexibility of design of the molecular conjugate vector.

Materials and Methods

Formation of Conjugate-DNA Complexes

Human transferrin-polylysine conjugates consisting of human transferrin covalently linked to poly(L-lysine)$_{190}$ were prepared as described (13, 14). The plasmid pRSVL containing the *Photinus pyralis* luciferase gene under the control of the Rous-sarcoma virus long terminal repeat enhancer/promoter was used as a reporter of gene tranfer (15). Purified pRSVL DNA (6 μg) was diluted into 350 μl total volume NaCl 150 mM/Hepes 20 mM, pH 7.3 (HBS). Human transferrin-polylysine conjugate (12 μg) in total volume 150 μl HBS was combined with the DNA and incubated at room temperature for 30 min to form conjugate-DNA complexes.

Cell Culture

The human respiratory epithelial cell line HBE1 was used as a target for gene transfer by transferrin-polylysine conjugates. This cell line is derived from normal human airway epithelium as described (J. R. Yankaskas, personal communication). Cells were maintained in F12-7X medium (16). Control cell lines for transferrin-polylysine conjugate-mediated gene transfer were the non-respiratory epithelial cell lines HeLa and KB. HeLa was maintained in Dulbecco's modified Eagle's medium/5% heat-inactivated fetal calf serum/penicillin at 100 international units/ml//streptomycin at 100 μg/ml//2 mM glutamine. KB cells were grown in Eagle's minimal essential medium/10% heat-inactivated fetal calf serum/penicillin at 100 international units/ml//streptomycin at 100 μg/ml//10 mM nonessential amino acids/2 mM glutamine.

Gene Transfer to Respiratory Epithelial Cells Utilizing Human Transferrin-Polylysine Conjugates

Gene transfer experiments were performed by seeding approximately 5×10^5 cells into 6-cm tissue culture dishes and allowing cells to achieve 50% confluency. For HBE1 cells, 12 h prior to gene transfer experiments, medium was changed to F12-6X, which lacks human transferrin supplement. To evaluate gene transfer mediated by transferrin-polylysine conjugates, conjugate-DNA complexes (500 μl) were added directly to tissue culture dishes containing the various target cells and incubated for 24 h at 37° C, 5% CO_2. For gene transfer experiments to test for the requirement for receptor binding by individual conjugate components, HBE1 cells were preincubated at 4° C prior to addition of complexes formed between pRSVL DNA and individual conjugate components. These were formed by addition of 6 μg plasmid DNA to human transferrin (9 μg) or polylysine (3 μg) in amounts equimolar to transferrin-polylysine conjugate (12 μg). After addition of the complexes, plates were incubated for 2 h at 4° C. This step allowed specific receptor binding of the complexes without cellular internalization. Plates were then washed 3 times with ice-cold F12-7X medium to remove unbound components in the medium, permitting determination of cellular internalization on the basis of specific receptor-mediated mechanisms. After the washing step, plates were gradually warmed up and returned to the incubator (37° C, 5% CO_2) for 24 additional hours. For competition experiments to test for the specificity of the conjugates for the transferrin receptor, cells were treated with hTfpL/pRSVL complexes in the presence of increasing amounts of human transferrin as a specific competitor for the transferrin receptor or bovine serum albumin as a nonspecific competitor. After the addition of complexes and human transferrin or bovine serum albumin competitor, cells were incubated for 24 h (37° C, 5% CO_2). In experiments employing agents to augment cellular availability of transferred DNA, HBE1 cells were treated with 50 μM desferrioxamine (Sigma Chemical Co., St. Louis, MO) or 100 μM chloroquine (Sigma) alone or in combination. For desferrioxamine treatment, cells were pretreated with F12-6X medium containing desferrioxamine for 12 h prior to addition of complexes. For chloroquine treatment, cells were treated with chloroquine for 4 h during exposure to the hTfpL/pRSVL complexes. For both treatments, 4 h after addition the conjugate-DNA complexes were removed, plates washed, and cells incubated for 24 h (37° C, 5% CO_2) in F12-7X medium. Analysis of gene transfer was accomplished by preparation of cellular lysate and evaluation for luciferase gene expression as described (14). Aliquots of cell lysate containing 50 μg of total protein were analyzed. Results are expressed as "Light Units"/50 μg total cellular protein. Experiments were performed 3 to 4 times, and results expressed as mean $\pm$ SEM.

Results

In Vitro Gene Transfer to Respiratory Epithelial Cells Mediated by Transferrin-Polylysine Conjugates

To determine the feasibility of gene transfer via the receptor-mediated endocytosis pathway (Figure 1) in respiratory epithelial cells, the respiratory epithelial cell line HBE1 was used as a target for transduction by transferrin-polylysine conjugates. As a comparison, the non-respiratory epithelial cell lines HeLa and KB were analyzed in parallel (Figure 2). For HBE1 cells, application of conjugate-DNA complexes containing the luciferase reporter gene resulted in levels of reporter gene activity significantly greater than background

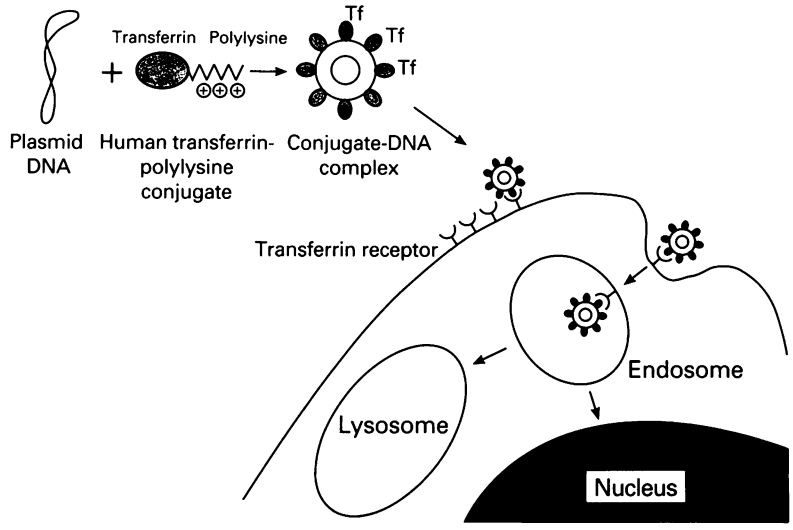

Figure 1. Strategy for gene transfer via the transferrin internalization pathway utilizing transferrin-polylysine conjugates. Interaction of plasmid DNA with human transferrin-polylysine conjugates results in the formation of conjugate-DNA complexes in which the DNA is condensed into compact transferrin-coated particles. The transferrin moiety of the complex binds to cellular receptors for transferrin, allowing the internalization of the complex by the receptor-mediated endocytosis pathway. After internalization, the transferred DNA is localized within the endosomes, whereupon it is either targeted for lysosomal degradation, or alternatively, achieves escape from the endosome to reach the nucleus, where foreign gene expression is effected.

levels in untreated HBE1 cells ($P < 0.005$). This level of gene expression was greater than levels exhibited by non-respiratory epithelial cell lines that are characterized by high numbers of cell surface transferrin receptors (17).

Consistent with the concept that internalized conjugate-DNA complex has different intracellular fates in different target cells, KB cells demonstrated minimal susceptibility to gene transfer by transferrin-polylysine conjugates despite the presence of high levels of transferrin receptors. In this regard, it has been hypothesized that the phenomenon of limited gene transfer by molecular conjugates in the context of appropriate cell surface receptors is due to lysosomal targeting of the DNA (18). Thus, the use of lysosomatropic agents, such as chloroquine, can very significantly augment levels of gene expression mediated by molecular conjugate vectors in these instances (14, 18). For KB cells, augmented gene expression can be demonstrated with the addition of chloroquine (data not shown). In contrast, for the respiratory epithelial cell line HBE1, transferrin-polylysine conjugates alone appear to effect significant levels of foreign gene expression. Thus, gene transfer to respiratory epithelial cells *in vitro* may be accomplished by transferrin-polylysine conjugates. Additionally, the baseline susceptibility of respiratory epithelial cells to gene transfer by this vector appears to be as great or greater than that of cell lines known to possess large numbers of receptors for the conjugate ligand.

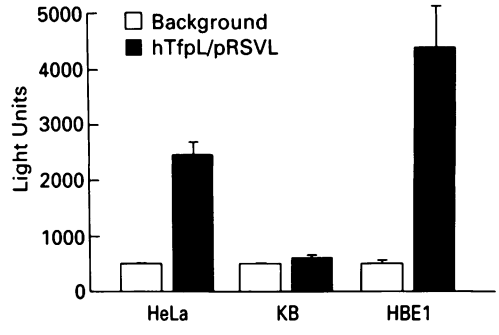

Figure 2. Gene transfer to respiratory epithelial cells *in vitro* mediated by transferrin-polylysine conjugates. The normal respiratory epithelial cell line HBE1 as well as the non-respiratory epithelial cell lines HeLa and KB were treated with conjugate-DNA complexes consisting of human transferrin-polylysine (12 μg) plus the reporter plasmid pRSVL (6 μg), which contains the *Photinus pyralis* luciferase gene. After 24 h of incubation (37° C, 5% CO_2), cell lysates were prepared. Aliquots of cell lysate containing 50 μg total protein were assayed for luciferase gene expression by luminometry. Results are expressed as Light Units/50 μg total cellular protein. Background indicates luciferase activity in untreated control cells. Experiments were performed 3 to 4 times, and results represent mean ± SEM.

Mechanism of Gene Transfer to Respiratory Epithelial Cells Mediated by Transferrin-Polylysine Conjugates

To determine the mechanism of gene transfer to respiratory epithelial cells mediated by transferrin-polylysine conjugates, the contribution of each functional domain of the conjugate molecule in effecting gene transfer was evaluated. Reporter plasmid DNA pRSVL alone, or in combination with polylysine or transferrin, or in combination with transferrin-polylysine conjugate, was evaluated for the ability to accomplish gene transfer after specific binding to the target HBE1 cells (Figure 3A). In this analysis, complexes consisting of the plasmid DNA plus the transferrin-polylysine conjugate mediated levels of reporter gene expression significantly greater than did complexes containing pRSVL in combination with individual complex components, polylysine or transferrin ($P < 0.05$). This result is consistent with the concept that gene transfer mediated by conjugate vectors requires functional domains capable of both DNA binding and cell-surface receptor recognition.

To test whether the cellular internalization mediated by the transferrin-polylysine conjugates was indeed by the transferrin pathway, gene transfer employing the transferrin-polylysine conjugates was carried out in the presence of competition for the transferrin cell surface receptor (Figure 3B). In these experiments, increasing competition for the transferrin receptor by exogenous human transferrin resulted in a marked attenuation of gene transfer mediated by the transferrin-polylysine conjugates ($P < 0.001$). Competition with comparable amounts of an irrelevant protein, bovine se-

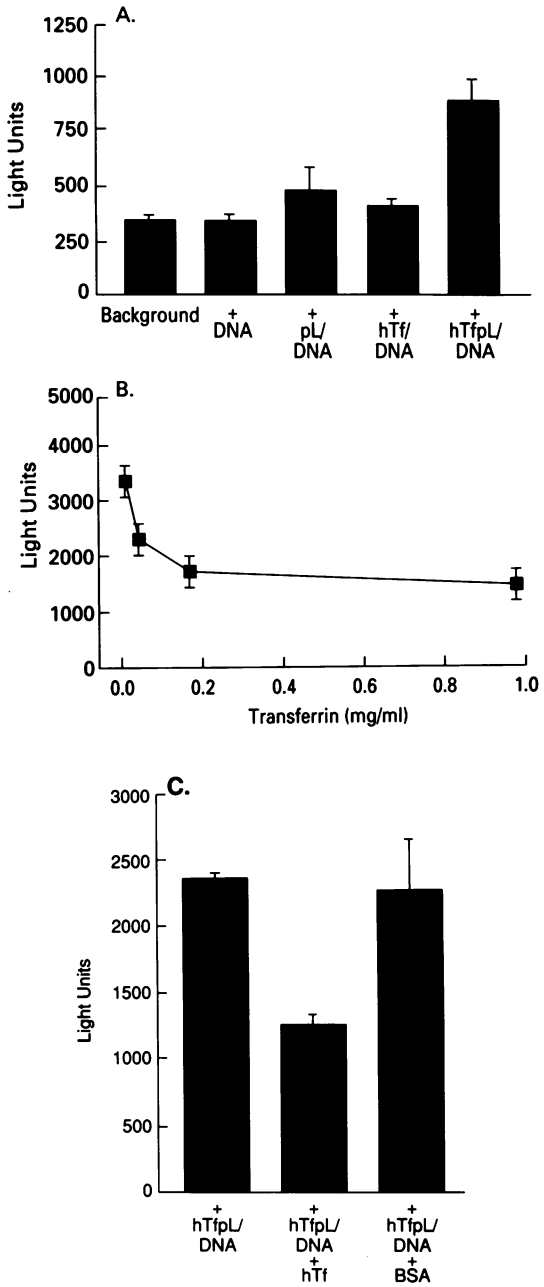

Figure 3. Mechanism of gene transfer to respiratory epithelial cells mediated by transferrin-polylysine conjugates. *Panel A:* Gene transfer mediated by complexes formed with conjugate components. Complexes were formed by combination of 6 μg pRSVL plasmid DNA with 9 μg human transferrin (hTf) or 3 μg polylysine (pL) in amounts equimolar to 12 μg transferrin-polylysine conjugates (hTfpL). Specific binding of the formed complexes was achieved by addition to HBE1 cells with incubation at 4° C for 2 h followed by extensive washing to eliminate unbound, fluid-phase components. Plates were then incubated at 37° C for an additional 24 h. *Panel B:* Effect of competition for transferrin receptor on gene transfer by transferrin-polylysine conjugates. Conjugate-DNA complexes consisting of 6 μg pRSVL DNA and 12 μg human transferrin-polylysine conjugate were added to HBE1 cells in the presence of increasing concentrations of human transferrin. *Panel C:* Effect of specific and nonspecific competition for transferrin receptor on gene transfer by transferrin-polylysine conjugates. Conjugate-DNA complexes consisting of 6 μg pRSVL DNA and 12 μg human transferrin-polylysine conjugate (hTfpL) were added to HBE1 cells in the presence of human transferrin (hTf) (1.0 mg/ml), as a specific competitor for the transferrin receptor, or bovine serum albumin (BSA) (1.0 mg/ml), as a nonspecific competitor.

rum albumin, did not exert a significant effect on gene transfer mediated by the conjugates (Figure 3C). These findings confirm that respiratory epithelial cells possess receptors for transferrin and that gene transfer to these cells may be accomplished by the transferrin internalization pathway utilizing transferrin-polylysine conjugates.

Augmentation of Gene Transfer via the Transferrin Pathway Utilizing Agents that Increase Entry and Survival of DNA

Gene transfer mediated by transferrin-polylysine conjugates may be augmented by agents that enhance DNA availability to target cells. In cells of hematopoietic lineage, enhanced gene transfer via the transferrin pathway has been achieved utilizing agents that either increase cell surface transferrin receptors or decrease the intracellular degradation of internalized DNA (18). Similar strategies were evaluated in cells of the respiratory epithelium (Figure 4). Treatment of HBE1 cells with chloroquine, an agent that inhibits lysosomal degradative processes, resulted in a significant augmentation of gene transfer by the transferrin-polylysine conjugates compared with treatment with the conjugates alone ($P < 0.005$). This is consistent with the concept that increasing the fraction of internalized DNA that avoids lysosomal destruction will allow a corresponding increase in the fraction that survives to effect heterologous gene expression. Treatment of cells with desferrioxamine prior to conjugate addition to augment cell surface transferrin receptor numbers, however, did not augment gene expression levels over conjugate addition at baseline. This suggests that augmented internalization, in the absence of agents that inhibit degradation of the internalized DNA, is not sufficient in itself to enhance effective gene transfer by this pathway. In contrast, however, treatment with both desferrioxamine and chloroquine resulted in levels of reporter gene expression significantly greater than those achieved by treatment with chloroquine alone ($P < 0.005$). This suggests that augmented conjugate entry is effective only in the context whereby lysosomal degradation of the internalized DNA is inhibited.

Discussion

The potential to achieve genetic correction of a variety of inherited and acquired disease processes affecting the cells of the airway epithelium establishes the necessity of developing vectors to accomplish effective gene transfer to these cells. Because of the nature of the pulmonary architecture, however, it is unlikely this can be accomplished by conventional gene therapy strategies (19–22), which involve harvesting of the affected cells followed by extracorporeal genetic modification and reimplantation. Thus, for the cells of the respiratory epithelium, delivery of the therapeutic genetic material will most likely require direct, *in vivo* delivery by the gene transfer vector.

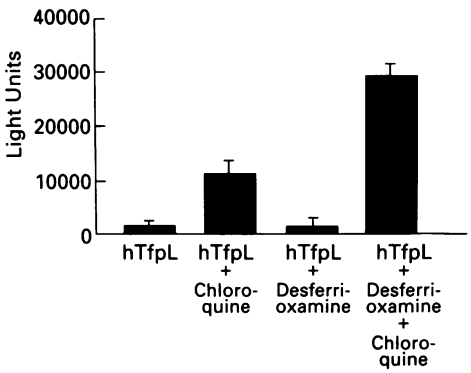

Figure 4. Effect of agents that augment cellular availability of DNA on gene transfer by transferrin-polylysine conjugates. To augment gene transfer mediated by transferrin-polylysine DNA complexes, HBE1 cells were treated with desferrioxamine (50 μM) and/or chloroquine (100 μM).

Presently available gene transfer vectors are not well suited to accomplish direct gene transfer to the respiratory epithelium *in vivo*. The various DNA-mediated and fusion methods are of relatively low efficiency and associated with cell toxicity. Despite these drawbacks, direct, *in vivo* gene transfer to the lung has been reported utilizing cationic liposomes (10, 23). Recombinant viral vectors have been shown to possess higher efficiency gene transfer capacity to airway epithelia in both the *in vitro* and *in vivo* contexts (3, 11). Despite this advantage, however, these agents suffer drawbacks related to the issue of their safety in the context of direct *in vivo* delivery. In this regard, both recombinant adenovirus and retrovirus vectors mediate obligatory co-delivery of viral gene elements with transfer of the heterologous gene.

Gene transfer via the receptor-mediated endocytosis pathway utilizing ligand conjugates offers several advantages for ultimate employment as an *in vivo* gene transfer vector. The cellular internalization of the conjugate is via a physiologic cellular pathway and is thus not likely associated with the cytotoxicity consequent to plasma membrane transition mechanisms of the DNA-mediated and fusion methods (24, 25). As the conjugates interact with DNA in a sequence-independent manner, there are no restrictions on the design of the transferred DNA construct (13, 14). Thus, viral gene elements are not an essential feature, thereby obviating safety hazards deriving from their incorporation. An additional feature of potential utility is that conjugates may be designed that allow the possibility of cell-specific targeting based upon the ligand moiety's specificity. In this regard, cell-specific targeting *in vivo* has been reported utilizing molecular conjugate gene transfer vectors (26, 27).

The design of molecular conjugate vectors capable of achieving cell-specific targeting to the lung will require cell surface receptors unique to the respiratory epithelium. The first step in polycation-ligand conjugate-mediated gene transfer is the recognition of a cell-surface receptor by the ligand portion of the conjugate-DNA complex. For respiratory epithelium, cell-specific receptors have not been delineated, and thus more generalized cell surface receptors were considered in the present study. Transferrin receptors have a wide distribution in a variety of tissue types (17, 28). Thus, this study employed transferrin-polylysine conjugates to evaluate the feasibility of this type of approach for cells of the respiratory epithelium. It is axiomatic that the delineation of respiratory epithelium-specific receptors will allow the design of conjugate vectors capable of exploiting this entry route to achieve gene transfer to selective cell subsets of the respiratory epithelium.

The feasibility of utilizing transferrin-polylysine conjugates for *in vivo* gene transfer to the respiratory epithelium remains to be established. The intracellular fate of internalized conjugate-DNA complexes may be different for immortalized respiratory epithelial cell lines and intact airway epithelium *in situ*, potentially limiting the efficiency of gene transfer by the transferrin pathway. In addition, the distribution of transferrin receptors in airway epithelial cell subsets has not been delineated. Studies of a variety of non-respiratory epithelial tissues have demonstrated transferrin receptor localization within basal cell layers (28), consistent with the concept that transferrin receptor distribution correlates with the state of cellular proliferation (29). Similar localization of transferrin receptors to the airway epithelial basal cell subset would dictate a systemic route of administration of the conjugates to target these cells. Alternatively, localization of transferrin receptors at the apical pole of luminal respiratory epithelial cells would permit airway delivery of the conjugates. In this regard, transferrin levels in the epithelial lining fluid of the lung are enriched relative to serum, suggesting facilitated transport or local production (30). Localization of transferrin to the airway lumen suggests co-localization of the corresponding cell surface receptor, thus potentially allowing the delivery of transferrin-polylysine conjugates by the airway route.

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