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Enhanced plasmid DNA transfection with lysosomotropic agents in cultured fibroblasts

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Abstract

Transfer of plasmid DNA into mammalian cells has posed major challenges for gene therapy. Most non-viral vectors are known to internalize in the cells by endocytosis. Therefore, low transfection efficiency of non-viral vectors may be due to intracellular degradation of input DNA in the endosomes and/or lysosomes. DNA degradation can be inhibited either by inactivating the lysosomal enzymes or obliterating endosome fusion to lysosomes using lysosomotropic agents. We report here the effects of individual lysosomotropic agents such as chloroquine, polyvinylpyrolidone (PVP) and sucrose on β-gal expression in cultured fibroblasts COS, 293 and CHO. Cell viability was influenced by type, exposure time and concentration of lysosomotropic agents. Exposure to chloroquine at high concentration (1000 μM) or more than 4 h at any concentration (10–1000 microM) caused extensive cell death, however, cytotoxicity due to sucrose (5–500 mM) and PVP (0.01–1 mg/ml) was minimal in the cell lines tested. All the agents utilized in this study enhanced the gene expression and the transfection efficiency followed the order of sucrose > chloroquine > PVP at the concentrations used in all cell lines. Results suggest that lysosomotropic agents can enhance transfection efficiency but the degree of transgene expression may be cell- and agent-specific. Of the agents studied, sucrose appears to be an attractive agent in improving gene expression without toxic effect in the cultured fibroblasts. Thus, it can be used as an excipient in the formulation of new gene delivery systems.

Keywords: Plasmid DNA; Gene transfer; Lysosomotropic agents; Cell culture; In vitro; Sucrose; PVP; Chloroquine

1. Introduction

The ability to target functional DNA and to improve efficiency in a specific cell population remains a significant limitation to the development of gene delivery systems. Most methods for DNA delivery involve entry into the cell by endocytosis, which is a cellular process that internalizes macromolecules into the endosomes and

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lysosomes. It has been demonstrated that the acidification of endosomes plays an important role in the uncoating and penetration of viral vectors (Wittels and Spear, 1991). Although, lipid/DNA complexes are known to be internalized by the target cells and transfer into endosomal then lysosomal compartments (Zelphati and Szoka, 1996), the effect of lysosomal pH in the dissociation of DNA/lipid and DNA/polymer complexes is not fully understood. Moreover, inefficient transgene expression of non-viral vectors may be due to a significant fraction of internalized DNA which remains entrapped and degraded in the lysosomes (Luthman and Magnusson, 1983; Lucas et al., 1995). Lysosomes are membrane bound organelles containing a variety of hydrolytic enzymes essential for intracellular digestion. Lysosomal degradation can be partially circumvented either by inactivating lysosomal enzymes, by changing the acidic pH of the lysosomes or obliterating endosome fusion to lysosomes using lysosomotropic agents (Lechardeur et al., 1999). The term 'lysosomotropic' was first used by De Duve to designate all substances that are taken up selectively into lysosomes irrespective of their chemical structure or mechanism of uptake (De Duve et al., 1974). Some of these agents, such as, chloroquine and polyvinylpyrolidone have been used in conjunction with plasmid vectors, viral vectors and in combination with polymeric systems to improve the efficiency of gene delivery (Luthman and Magnusson, 1983; Lucas et al., 1995; Walsh et al., 1996; Legendre and Szoka, 1993; Levy et al., 1994). In the present study, we sought to systematically investigate a series of agents that are known either to inactivate lysosomal enzymes that could be responsible for the degradation of DNA vectors or to accumulate in the lysosomes that inhibits the fusion of lysosomes with incoming vesicles. The rationale for our choice of agents is based on their mechanisms of action. Sucrose was investigated because mammalian cells, in general, lack the intracellular disaccharidase enzyme for metabolizing sucrose (Levy et al., 1994). Thus, sucrose has been noted to cause intracellular cytoplasmic vesicular swelling, within endosomes and lysosomes. Vacuolization could be the consequence of endocytic uptake of sucrose with its subsequent incorporation into lysosomes. In the absence of invertases capable of digesting such sugars within lysosomes, the sequestered sugars would produce an osmotic inflow of water into the vesicles and cause them to become dilated (Wildenthal et al., 1976; Kato et al., 1984; Legendre and Szoka, 1992). Thus, we hypothesize that the simultaneous presence of sucrose and DNA within cytoplasmic vesicles would result in less nuclease-mediated degradation of DNA with more input DNA being available for gene transfection. Similarly, the site of intracellular accumulation of PVP is the vacuolar system due to its resistance to the lysosomotropic enzymes (Munnisma et al., 1980). Studies on PVP uptake and its effect on the intracellular enzymes are very limited. However, it has been demonstrated that PVP is retained within intracellular vesicles after its uptake by fluid phase pinocytosis or related processes (Ose et al., 1980; Michelakakis and Danpure, 1984; Patel et al., 1985). PVP would therefore cause intracellular vesicular swelling and diminish the fusion of lysosomes with incoming internalized DNA. The known effect of chloroquine is to raise pH within lysosomes, thereby also resulting in a suboptimal pH environment for the enzymatic degradation of DNA (Cotton et al., 1992; Wagner et al., 1992). In addition, chloroquine has been shown to increase gene expression of receptors that mediate transfection in liver cells and prevent degradation of proteins absorbed by endocytosis (Fisher and Wilson, 1994). The goals of our study were to: (a) systematically investigate and compare a series of agents (sucrose, PVP and chloroquine) that could hypothetically protect the DNA from intracellular degradation; (b) investigate the effectiveness of these agents in a variety of mammalian cell lines; and (c) understand the advantages and disadvantages of each of these well known agents.

2. Materials and methods

2.1. Materials

Plasmid DNA (pSV-β-galactosidase) (6820 bp), lysis buffer and *O*-nitrophenyl-*b*-*d*-galactopyra-

noside (ONPG) were obtained from Promega (Madison, WI). Qiagen Ultra Pure column (Qiagen) and endotoxin-free buffers were purchased from Qiagen (Chatsworth, CA). The cells (CHO, 293 and COS) were obtained from American Type Culture Collection (ATTC). Minimum essential medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, lipofectamine and antibiotics were purchased from Gibco laboratories (Grand Island, NY). Chloroquine, polyvinylpyrolidone (MW = 10 000 and 40 000 Da) and sucrose were obtained from Sigma (St. Louis, MO). Endotoxin assay kit was purchased from Biowhittaker (Walkersville, MD). All other biologics were purchased from Boehringer Mannheim (Indianapolis, IN).

2.2. Methods

2.2.1. Preparations of plasmid DNA

DNA (pSV-β-gal) was amplified in *E. coli* (DH-5α), extracted by alkaline lysis, purified using a Qiagen column and endotoxin free buffers. The DNA was precipitated in 100% ethanol and rehydrated in sterile Tris-EDTA buffer. Plasmid identity and purity were confirmed by restriction endonuclease digestion (Hind III and EcoR-I) in 0.8% agarose gel and a UV-1201 (Schimadzu, Kyoto, Japan) spectrophotometer at 260/280 nm. Endotoxin level was determined using an endotoxin assay kit (Biowhittaker) according to the manufacturer's protocol. This level was less than 20 unit/mg of DNA.

2.2.2. Cell cultures

The cells were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum and penicillin–streptomycin and maintained at 37°C in a humidified 5% CO_2 incubator. One day prior to the transfection experiment, the cells (10^6/dish) were seeded in a culture dish and allowed to reach 50% confluency.

2.2.3. Osmotic pressure

Lysosomotropic agents were dissolved in opti-MEM-I (reduced serum). The cells were exposed to varying concentrations of sucrose (5, 50 and 500 mM), chloroquine (10, 100 and 1000 μM) or PVP40 (0.01, 0.1 and 1 mg/ml) for 6 h at 37°C in the incubator. The osmotic pressure of the cell medium in the presence of lysosomotropic agents was measured by an Advanced Osmometer (Newton, Highland, MA).

2.2.4. Cytotoxicity studies

The cells were maintained and treated with different concentrations of lysosomotropic agents as explained in the transfection studies unless noted otherwise. After 6 h in the incubator, the cells were first examined under a light microscope and the cytotoxicity of lysosomotropic agents was determined by MTT assays (Mossmann, 1983). This assay is based on the ability of viable cells to convert MTT (3-(4,5 dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide), a soluble tetrazoluim salt, into an insoluble formazan precipitate, which is quantitated spectrophotometrically following solubilization in DMSO. Briefly, the cells were seeded into 96-well microplates at a density of 7×10^4 cells/well. After 24 h, the culture medium was replaced with 100 μl of opti-MEM containing different concentrations of lysosomotropic agents and the cells were incubated for 6 h at 37°C. Thereafter, 0.5 mg/ml of MTT solution was added to each well and incubated for another 4 h and the cell medium was then removed. The formazan crystals were dissolved in 200 μl of DMSO and absorbance was determined using an ELISA reader at wavelengths of 570 nm and 690 nm. In the positive control, cells were incubated with optiMEM-I and treated similarly. Cell survival was expressed as the amount of dye reduction relative to that of the untreated controlled cells.

2.2.5. Transfection experiments

The optimum DNA dose, transfection time and lipid concentration were determined in our previous studies (Ciftci et al., 1997, 1999). The cells were transfected with either plasmid/lysosomotropic agent or lipofectamine/DNA/lysosomotropic agent mixtures. For plasmid transfection, the cells were transfected with plasmid DNA (10 μg/plate) mixed with 2 ml of various concentrations of lysosomotropic agents in optiMEM-I as explained in the positive control

experiments. In positive control experiments, plasmid DNA was mixed with lipofectamine at a 3:1 w/w lipid/DNA ratio in optiMEM-I. After incubation at room temperature for 10 min, 0.1 ml of the DNA–lipid complex was mixed with 2 ml of optiMEM-I containing either sucrose (5, 50 and 500 mM), chloroquine (10, 100 and 1000 μM) or PVP40 (0.01, 0.1 and 1 mg/ml) and added to the cells for transfection. After a 6-h incubation, the cell medium was changed with regular medium containing same concentrations of lysosomotropic agents to eliminate osmotic lysis of lysosomes and the cells were then incubated for an additional 40 h.

The transfection medium was changed with regular medium after 4 h incubation if the cells were transfected in the presence of chloroquine.

2.2.6. Light microscopy studies

The cells were grown on cover slips in a sterile culture dish for phase contrast microscopy studies. They were evaluated after 6-h incubation with transfection reagents (as explained in transfection studies) and at the end of the transfection experiments. In control experiments, cells were not treated with lysosomotropic agents. The cells were then fixed overnight at 4°C in 2.5% gluteraldehyde in 0.1 M phosphate buffer at pH 7.4. The cover slips were then mounted with Crystal/Mount media (Fisher) and evaluated under the Zeiss microscope (Carl Zeiss, West Germany).

2.2.7. β-Gal activity

The β-gal activity in transfected cells was determined spectrophotometrically at 420 nm. The cells were washed with PBS and lysed by adding 400 μl of 1 × lysis buffer (Promega). The cell lysate (50 μl) was mixed with *O*-nitrophenyl-*b*-*d*-galactopyranoside (ONPG) in 2 × assay buffer and incubated at 37°C for 30 min. The reaction was terminated by adding 500 μl of 1 M Na_2CO_3 and absorbance was determined using a spectrophotometer at 420 nm. The β-gal activity was calculated using a β-gal standard curve.

2.2.8. Protein assay

Total protein was assayed by the modified Bradford method (Bradford, 1976). Briefly, 50 μl of cell lysate was mixed with 2 μl of folin reagent and incubated at room temperature for 15 min. The absorbance was determined at 650 or 750 nm using a spectrophotometer.

2.2.9. Statistical methodology

Data were analyzed by analysis of variance (ANOVA) test and were considered statistically significant if $P < 0.05$. Data are shown as mean ± S.D. for each experiment.

3. Results

The cells were transfected with plasmid DNA/lysosomotropic agent mixtures and the results were compared with the lipofectamine transfection method. In preliminary experiments, the optimum amount of DNA and transfection time for an optimal signal in transient transfection was found to be 3 μg/ml and 6 h, respectively (Ciftci et al., 1997, 1999). The highest level of gene expression with minimum cytotoxic effect was obtained with 10 μg/ml lipofectamine. Therefore, these conditions were chosen throughout the transfection experiments.

Preliminary dose ranging experiments for sucrose resulted in choosing the concentrations of interest (5, 50 and 500 mM). Out of the agents studied, sucrose was found to be the most ideal in terms of increased gene expression with no significant cytotoxic effects on cultured fibroblasts when compared to the control (no treatment) during the course of the experiments (Fig. 1).

Transfection efficiency was affected by the concentration of lysosomotropic agents. The marked transfection enhancement was observed when the amount of sucrose increased from 5 to 500 mM and the optimum transfection efficiency was found at 500 mM in all cell lines tested (Fig. 2 a–c). Transgene expression was found to be very low when the cells were transfected with plasmid alone. However, β-gal expression was increased 3–7-fold in the presence of sucrose and the maximum gene expression was observed in the COS cells (Fig. 2 a). Cell viability studies demonstrated that lipofectamine and lipofectamine/DNA did show minimum cytotoxic effects at the concentra-

tion range used. The results are in good agreement with literature findings (Hawley-Nelson etal., 1993; Armeanu et al., 2000). Transfection efficiency was increased approximately 1–3-fold in the presence of sucrose compared to the DNA/lipofectamine complex at the concentration range used in all the cell lines (Fig. 2 a–c).

Examinations of the cells under the light microscope showed that the cultured cells were susceptible to sucrose induced osmotic swelling at the

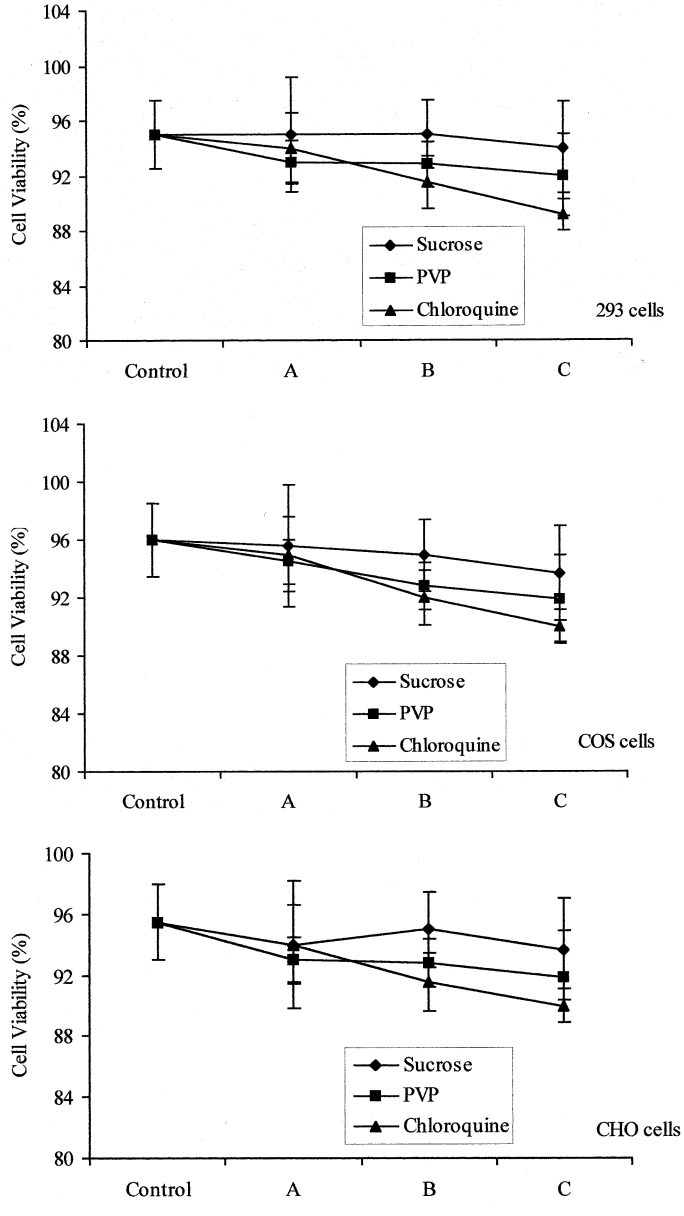

Fig. 1. Dose-dependent cytotoxicity of lysosomotropic agents in cultured fibroblasts. Control: cells were incubated in OptiMEM-I, A: 5 mM, 10 μM and 0.01 mg/ml for sucrose, chloroquine and PVP; B: 50 mM, 100 μM and 0.1 mg/ml for sucrose, chloroquine and PVP; C: 500 mM, 1.0 mg/ml for sucrose and PVP.

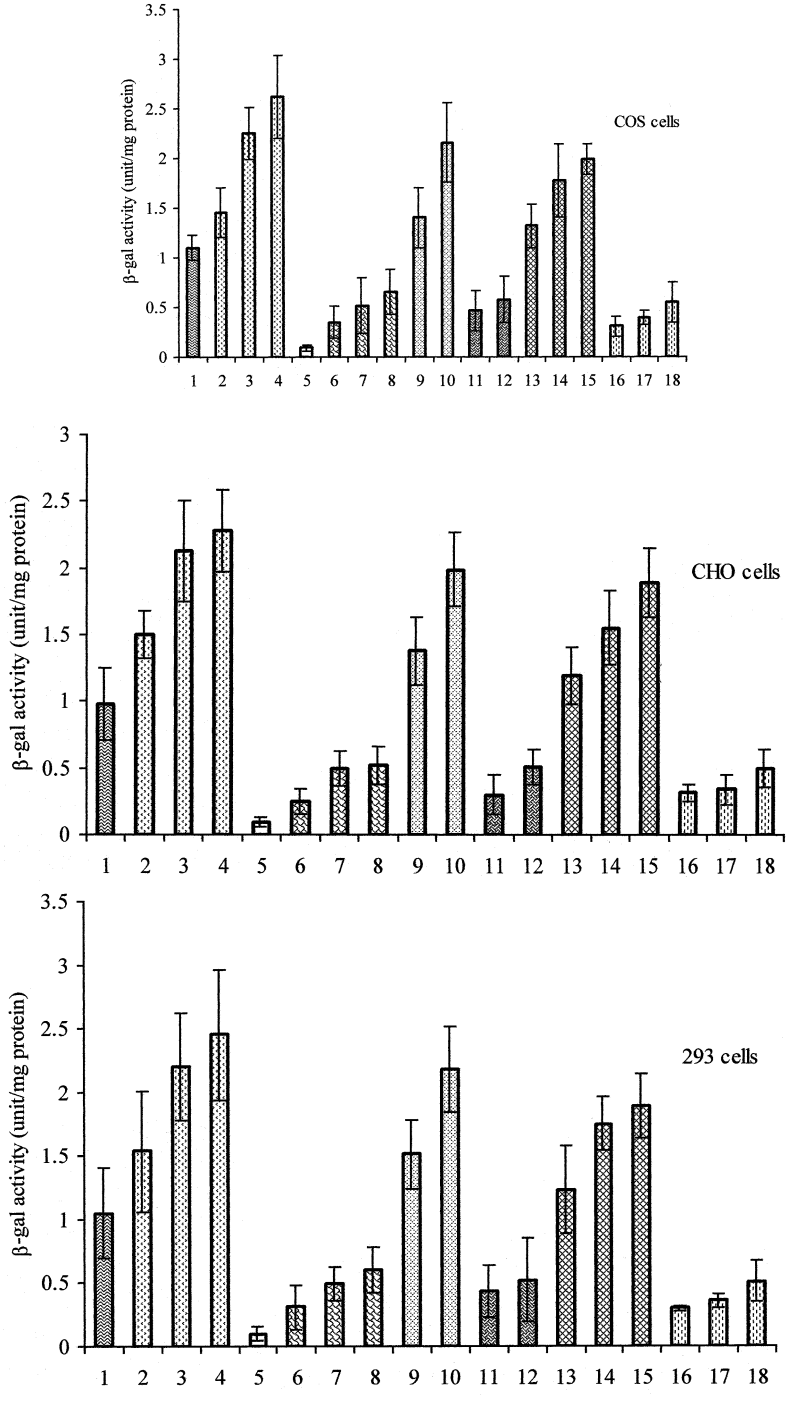

Fig. 2. The effects of lysosomotropic agents on β-gal expression. (1) Lipofectamine alone; (2–4) lipofectamine in the presence of 5, 50 and 500 mM sucrose; (5) plasmid alone; (6–8) plasmid in the presence of 5, 50 and 500 mM sucrose; (9,10) lipofectamine in the presence of 10 and 100 μM chloroquine; (11,12) plasmid in the presence of 10 and 100 μM chloroquine; (13–15) lipofectamine in the presence of 0.01, 0.1 and 1.0 mg/ml PVP; (16–18) plasmid in the presence of 0.01, 0.1 and 1.0 mg/ml PVP.

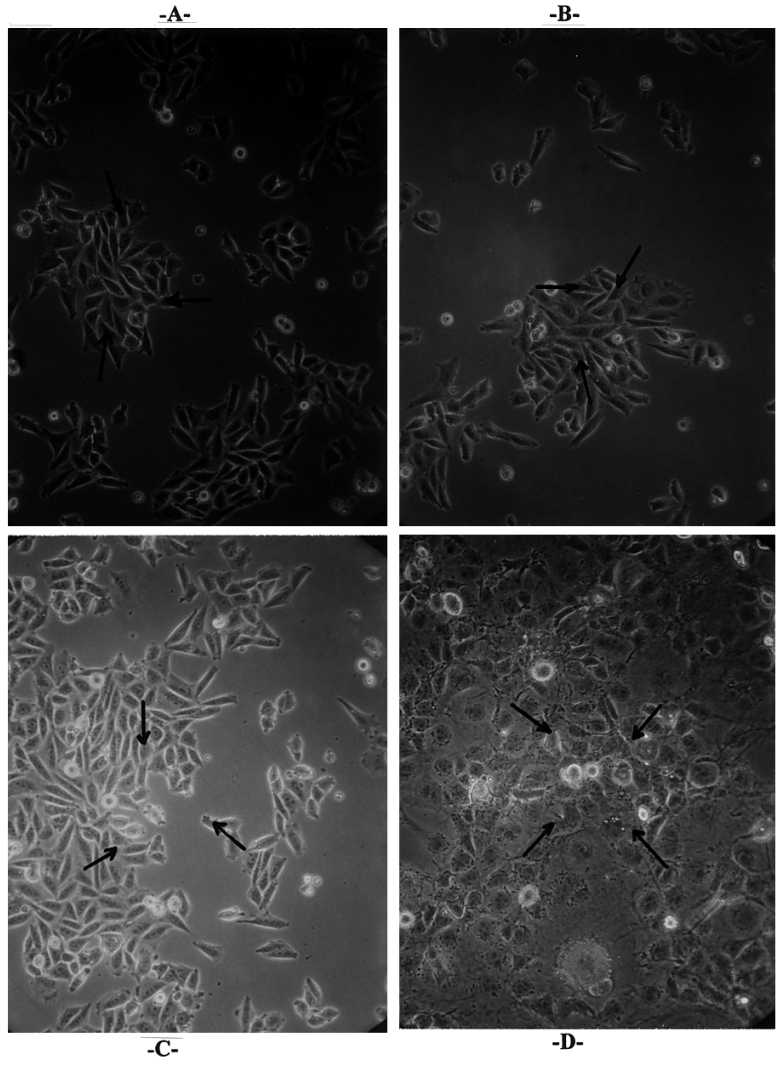

Fig. 3. Examples of phase contrast microscopy pictures of the cultured fibroblasts treated with lysosomotropic agents. Black arrows show lysosomotropic vacuole (140 ×). (A) CHO-sucrose (50 mM); (B) CHO-chloroquine (100 μM); (C) CHO-PVP (1 mg/ml); (D) COS-sucrose (5 mM); (E) COS-PVP (0.1 mg/ml); (F) COS-chloroquine (100 μM); (G) 293-chloroquine (100 μM); (H) 293-PVP (1 mg/ml); (I) 293-sucrose (500 mM).

concentrations tested (Fig. 3). This was confirmed by the osmotic pressure data. The highest osmotic pressure was found in the presence of 500 mM sucrose when compared to the other agents as shown in Table 1.

These results are very similar to those demonstrated by Park et al. (1988) in the cultured fibroblasts exposed to sucrose. Therefore, the sucrose effect was hypothesized to be due to an osmotic pressure within the lysosomes suggesting that indigestible materials reduced the ability of loaded lysosomes to fuse with the endosomes containing newly internalized DNA molecules.

Chloroquine, a drug known to raise lysosomal pH and thus inhibiting lysosomal degradation of macromolecules, was added onto cultured fibroblasts and the results showed that exogenous gene expression could be increased using chloroquine.

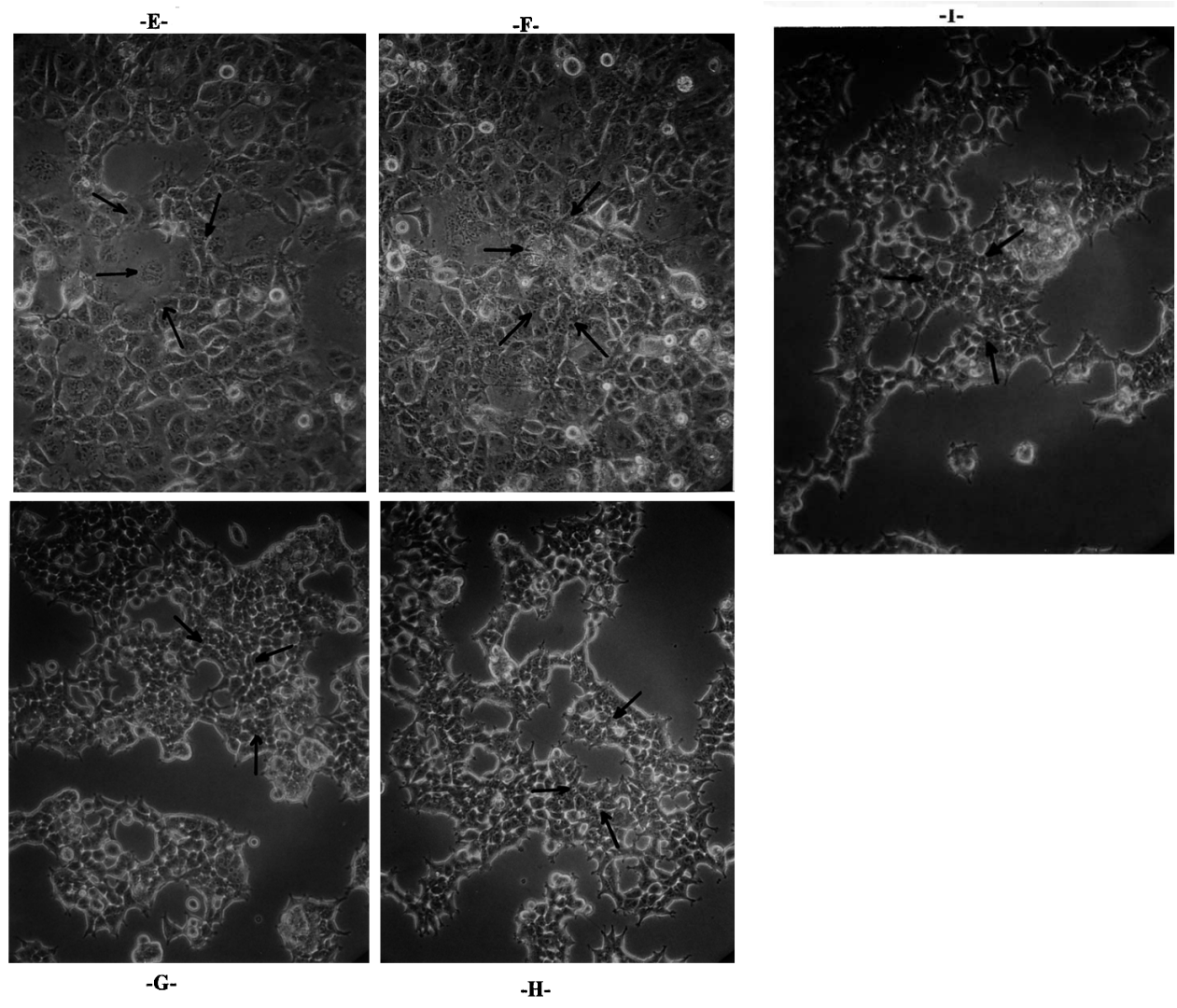

Fig. 3. (*Continued*)

concentrations tested (Fig. 3). This was confirmed by the osmotic pressure data. The highest osmotic have found that chloroquine use was limited with concentration, treatment time and cell type (Fig. 1). Exposure to chloroquine more than 4 h resulted in extensive cell death at any concentration tested. Therefore, the medium was changed with fresh medium containing lysosomotropic agents 4 h after transfection experiments and the cells were incubated for another 40 h. In addition, the exposure to chloroquine at 1000 μM showed significant effects on cell toxicity and the cell viability declined to 10% in all cell lines. Transfection efficiency in fibroblasts showed a linear increase (3–6-fold) as the addition of chloroquine increased from 10 to 100 μM compared to plasmid alone (Fig. 2 a–c). The maximum gene expression (twofold increase compared to lipofectamine alone) was observed in CHO cells when the cells were transfected with lipofectamine in the presence of chloroquine (Fig. 2b). The treatment of fibroblasts at a concentration of 10 and 100 μM

Table 1
Osmotic pressure (mean ± S.D.) of lysosomotropic agents in cell medium ($n = 3$, $P < 0.05$)

	Concentration	Osmotic pressure (mOsm/kg)
Physiologic serum		288 ± 1.5
Cell medium alone		324 ± 3.0
Chloroquine	10 μM	334 ± 2.6
	100 μM	345 ± 1.5
PVP (40 000)	0.01 mg/ml	345 ± 3.5
	0.1 mg/ml	367 ± 2.0
	1.0 mg/ml	390 ± 1.5
Sucrose	5 mM	356 ± 1.5
	50 mM	394 ± 1.0
	500 mM	900 ± 2.0

induced the appearance of numerous clear vacuoles in the cytoplasm of all the cells tested (Fig. 3). The chloroquine enhanced transfection procedure provides a reliable and reproducible assay of released protein and its effect is presumably mediated through its pH elevating effect on the lysosomal compartment, thereby, protecting DNA molecules from nuclease degradation. Chloroquine is also known to bind strongly to DNA and it might therefore protect DNA molecules from nuclease degradation (Wagner et al., 1992).

Of the two different molecular weight PVPs used (10 000 and 40 000 Da) only the PVP at 40 000 Da was suitable for transfection studies. The PVP10 resulted in widespread cell toxicity as evidenced by the light microscope, within a few hours. The reason for this is unknown. Cell viability studies demonstrated that PVP40 did show negligible cytotoxicity on cultured fibroblast at the concentration range used (Fig. 1). The highest gene expression was observed at 1 mg/ml concentration in all cell lines which represents an approximately sixfold increase in COS cells compared to plasmid alone (Fig. 2 a). Transfection of fibroblasts with the lipofectamine in the presence of PVP40 achieved a stronger β-gal level of 1.99 unit/μg protein compared to 1.1 unit/μg observed with lipofectamine in COS cells (Fig. 2 a). The levels of gene expression at low concentrations (0.01 and 0.1 mg/ml) were less compared to 1 mg/ml PVP in lipofectamine and plasmid transfections in all the cell lines (Fig. 2 a–c). PVP40 at all of the concentrations studied exhibited swollen vacuoles in the cytoplasm of fibroblasts (Fig. 3). The osmotic pressure of cell medium in the presence of PVP was higher than cell medium alone at any concentration tested (Table 1). Based on these results the PVP40 effect could be due to the reduced fusion of incoming DNA to the lysosomes, which was caused by the accumulation of a non-digestible macromolecule in the lsysosomes.

4. Discussion

Effective gene delivery into mammalian somatic cells in vitro and in vivo is an essential step in human gene therapy. Once DNA enters the cells, there is limited information concerning how DNA escapes from the endosomal compartment and moves into the cytoplasm or is taken into the nucleus. One of the factors that limits the efficiency of gene delivery to the nucleus after endocytosis is the rapid degradation of DNA within the endosomal and lysosomal compartments. In this study, we have demonstrated cell culture enhancement of the plasmid DNA expression and systematically compared sucrose to other lysosomotropic agents in a broad range of cell types, focusing on concentrations used in other studies. We analyzed the particular advantages of each of the different agents. The widely used lysosomotropic agents in our studies have significant limitations according to our results. This is particularly surprising in view of the widespread investigational use of chloroquine and PVP.

The mechanism of action of sucrose for enhancing gene therapy is most likely related to the inability of cells to degrade sucrose, due to the absence of disaccharide enzymes (Wildenthal et al., 1976; Kato et al., 1984). Sucrose was initially observed by a number of investigators to cause lysosomal swelling, due to the osmotic effects of its continued presence within the endosomal and lysosomal compartments (Storrie et al., 1984; Park et al., 1988). Although no other groups have reported using sucrose to enhance gene transfection in cell cultures, it has been used as a cocipient

in DNA vaccine investigations. The rationale for using sucrose with DNA vaccine injections has been to enhance tissue permeation following the injection of naked DNA vaccines (Lavis et al., 1996).

The implications of our sucrose results are that this agent could be useful to enhance plasmid gene expression in a variety of gene transfer applications. In particular, sucrose may be useful in sustained release systems as excipients with polymeric delivery of DNA. The use of sustained release systems with biodegradable polymers for delivering DNA has been recently reported by our group and by others (Ciftci et al., 1997). In general, this approach has shown relatively low efficiency and perhaps this can be improved through the use of a transfection enhancing agent such as sucrose. Although the mechanism of liposome mediated DNA entry into a cell is not fully understood, it is assumed to be mediated by endocytosis or cell membrane fusion. Based on our observations we think that lipid/DNA complexes are taken up by a similar process to endocytosis and the simultaneous presence of sucrose within endosomes and lysosomes enhanced the escape of internalized DNA therefore resulting in increased gene expression. Sucrose may also be useful if used in conjunction with liposome formulations for gene delivery and viruses. While we observed no cell death or toxicity with sucrose, there is a potential for toxicity with systemic use (Smithsvan Prooije et al., 1990; Gruda et al., 1991). Sucrose intravenously has been reported to cause renal damage (Helmholz, 1933).

Chloroquine has been frequently used in cell culture gene transfection studies (Cotton et al., 1992; Wagner et al., 1992; Lucas et al., 1995), but has not been reported yet to be successful for enabling gene transfection in vivo. As mentioned above, the mechanisms of action of chloroquine seems to be related to elevating the intravesicular pH of lysosomes and endosomes, thereby providing a suboptimal pH environment for the various lysosomal enzymes including those involved in DNA degradation. Co-incorporation of chloroquine within a sustained release chitosan coacervate preparation has been reported to be effective in the DNA transfection of cell cultures. However, efficacy was not reported in vivo (Mao et al., 1996). Although, there has been wide interest in the use of chloroquine in gene transfection studies in vitro, particularly when used in conjunction with various viral vectors (Luthman and Magnusson, 1983; Wagner et al., 1992; Fisher and Wilson, 1994) we have found that chloroquine use is limited by its cytotoxic effect. Chloroquine also causes cytotoxic and side effects in systemic administration and has resulted in 'inclusion body' retinopathy in patients chronically exposed to this agent (Magulake et al., 1993; Mao et al., 1996). Thus, chloroquine in gene therapy investigations must proceed with this potential side effect in mind.

PVP represents an agent that is sequestered in the lysosomal and endosomal compartments (Ose et al., 1980; Patel et al., 1985; Sanchez-Thorin, 1995). In these locations, due to its retention, osmotic related swelling results with diminished functionality of various lysosomal enzyme systems. PVP has never been used in cultured fibroblasts to investigate its effect on gene expression. Surprisingly, we found that low molecular weight PVP10 was toxic in all cells tested. The reason for this is unknown. However, PVP40 has increased gene transfer and the level of gene expression was a function of PVP concentration. PVP40 appears to be taken up into a variety of cell types including macrophages, cultured hepatocytes and kuppfer cells by fluid phase endocytosis (Ose et al., 1980). The total uptake of compound depends on the kinetics of the uptake process and extracellular concentration. We found that the osmotic pressure of PVP40 was higher than both cell medium and physiologic saline. This may be due to altered physicochemical properties of lysosomal membrane caused by the accumulation of non-digestible PVP in the lysosomes. However, further studies are ongoing to confirm this hypothesis. It has been reported that a significant enhancement of gene expression occurs in rat muscle using interactive polyvinyl based delivery systems. The improved tissue dispersion and cellular uptake of plasmid DNA using PVP after direct injection into muscle was explained by the osmotic effects (Mumper et al., 1996). Despite all of this, PVP should also be hypothetically useful as a

cocipient in sustained release formulations as well as enhancing non-sustained release gene transfer methodology.

5. Conclusion

In conclusion, the release of DNA from endosomes can be enhanced using lysosomotropic agents, but the degree of enhancement may be cell- and agent-specific. Sucrose appears to be an attractive agent to improve exogenous gene expression without any toxic effect in cultured fibroblasts. However, caution should be observed with further work using PVP and chloroquine, which had cytotoxic effects not previously reported. Since our long-term goal for this project is to develop a non-viral gene delivery system, sustained action DNA formulation could show synergism with lysosomotropic agents.

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References

Armeanu, S., Pelisek, J., Krausz, E., Fuchs, A., Groth, D., Curth, R., et al., 2000. Optimization of non-viral gene transfer of vascular smooth muscle cells in vitro and in vivo. Mol. Ther. 1 (4), 366–375.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248.

Ciftci, K., Smiley, B., Bonadio, J., Levy, R.J., 1997. Polymer based gene delivery systems: an efficient way to introduce DNA into mammalian cells. Pharm. Res. 13, 1390.

Ciftci, K., Su, J., Trovitch, P., 1999. A promising delivery systems for cancer gene therapy. Pharm. Res. 1 (4), 4124.

Cotton, M., Wagner, E., Zatloukal, K., Phillips, S., Currel, D.T., Birnstiel, M.L., 1992. High-efficiency receptor mediated delivery of small and large (48 kb) gene constructs using the endosome disruption activity of defective or chemically inactivated adenovirus particles. Proc. Natl. Acad. Sci. USA 89, 6094–6098.

De Duve, C., de Barsy, T., Poole, B., Trouet, A. Tulkens, Van Hoof, F., 1974. Lysosomotropic agents. Biochem. Pharmacol. 23, 2495–2531.

Fisher, K.J., Wilson, J.M., 1994. Biochemical and functional analysis of an adenovirus based ligand complex for gene transfer. Biochem. J. 299, 49–58.

Gruda, J., Millette, D., Brother, M., Kabayashi, G.S., Medoff, G., Brajtburg, J., 1991. Structure activity study of inhibition of amphotericin B (fungizone) binding to sterols, toxicity to cells and lethality to mice by ester of sucrose. Antimicrob. Agents Chemother. 35, 24–28.

Hawley-Nelson, P., Ciccarone, V., Jessee, J., Felgner, P.I., 1993. Lipofectamine reagent: a new higher efficiency polycationic liposome transfection reagent. Focus 15, 73–79.

Helmholz, H.F., 1933. Renal changes in the rabbit resulting from intravenous injection of hypetonic solution of sucrose. J. Pediatr. 3, 144–157.

Kato, T., Okada, S., Yutaka, T., Yabuuchi, H., 1984. The effects of loading on lysosomal hydrolases. Mol. Cell. Biochem. 60, 83–94.

Lavis, H.L., McCluskie, M.J., Gerrin, J.L., Purcell, R.H., 1996. DNA vaccine for hepatitis-B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. Proc. Natl. Acad. Sci. USA 93, 7213–7218.

Lechardeur, D., Sohn, K.J., haardt, M., Joshi, P.B., et al., 1999. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther. 6, 482–497.

Legendre, Y.J., Szoka, F.C., 1992. Delivery of plasmid DNA into mammalian cells using pH sensitive liposomes: comparison with cationic liposomes. Pharm. Res. 9, 1235–1242.

Legendre, Y.J., Szoka, F.C., 1993. Cyclic amphipathic peptide–DNA complexes mediate high efficiency transfection of adherent mammalian cells. Proc. Natl. Acad. Sci. USA 90, 893–897.

Levy, L., Meyer, K.B., Barron, L., Szoka, F.C., 1994. Mechanism of gene uptake and expression in adult mouse skeletal muscle. Pharm. Res. 11, 317–321.

Lucas, P., Pouton, J.W., Moss, S., 1995. Influence of formulation factors on gene transfer mediated by cationic polypeptides. Proc. Int. Symp. Control Rel. Bioact. Mater. 22, 468–469.

Luthman, H., Magnusson, G., 1983. High efficiency polymer DNA transfection of chloroquine treated cells. Nucl. Acids Res. 11, 1295–1309.

Magulake, N.O., Ihenecho, H.N., Ike, V.O., 1993. Chloroquine retinopathy in Nigerian patients with heart block. Eye 7, 91–593.

Mao, H.Q., Ro, K., Walsh, H.M., August, J.T., Leong, K.W., 1996. DNA-chitosan nanospheres for gene delivery. Proc. Int. Symp. Control Rel. Bioact. Mater. 23, 401–402.

Michelakakis, H., Danpure, J.C., 1984. The effect of cytochalasin-B on the release of lysosomal enzymes and intralysosomally stored polyvinylpyrolidone in the isolated perfused rat liver. Biochem. Pharmacol. 33, 2047–2053.

Mossmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65, 55–63.

Mumper, R.L., Duguid, J.G., Anwer, K.I., Barron, M.K., Nitta, H., 1996. Polyvinyl derivatives as novel interactive polymers for controlled gene delivery to muscle. Pharm. Res. 13, 701–709.

Munnisma, J., Noteborn, M., Kooistra, T., et al., 1980. Fluid endocytosis by rat liver and spleen. Biochem. J. 192, 613–621.

Ose, L., Ose, T., Reinertse, R., Berg, T., 1980. Fluid endocytosis rat parenchymal and non-parenchymal live cells. Exp. Cell. Res. 126, 109–119.

Park, P., Sullivan, P.C., Storrie, B., 1988. Hypertonic sucrose inhibition of endocytic transport suggests multiple early endocytic compartments. J. Cell. Physiol. 135, 443–450.

Patel, H.M., Tuzel, N.S., Stevenson, R., 1985. Intracellular digestion of saturated and unsaturated phospholipid liposomes by mucosal cells. Possible mechanism of transport of liposomally entrapped macromolecules across the isolated vascularity perfused rabbit illeum. Biochem. Biophys. Acta 839, 40–49.

Sanchez-Thorin, J.C., 1995. The effect of povidone-iodine solution applied at the conclusion of ophthalmic surgery. Am. J. Ophthalmol. 120, 806–808.

Smiths-van Prooije, A.E., De Groot, A.P., Dreef-van derMeulen, H.C., Sinkelman, E.G., 1990. Chronic toxicity and carcinogenicity study of isomalt in rats and mice. Food Chem. Toxicol. 28, 243–251.

Storrie, P., Pool, R.R., Sachdeva, M., Mauret, K.M., Oliver, C., 1984. Evidence for both prelysosomal and lysosomal intermediates in endocytic pathways. J. Cell. Biol. 98, 108–115.

Wagner, E., Zatloukal, K., Cotton, M., Kirloppos, H., Mechter, K., Curel, D.T., et al., 1992. Coupling adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor mediated gene delivery and expression of transfected genes. Proc. Natl. Acad. Sci. USA 89, 6099–6103.

Walsh, S.M., Flotte, T.R., Beck, S., Allen, S., Guggio, W.B., August, T., et al., 1996. Delivery Of CFTR gene to rabbit airways by gelatin DNA microspheres. Proc. Int. Symp. Control Rel. Bioact. Mater. 23, 73–74.

Wildenthal, K., Dees, J.H., Buja, M., 1976. Cardiac lysosomal derangements in mouse heart after long term exposure to non metabolizable sugars. Circ. Res. 40, 26–35.

Wittels, M., Spear, P.G., 1991. Penetration of cells by herpes simplex virus doe not require a low pH-dependent endocytic pathway. Virus Res. 18, 271–290.

Zelphati, O., Szoka, F.C., 1996. Mechanism of oligonucleotide release from cationic liposomes. Proc. Natl. Acad. Sci. USA 93, 11493–11498.