Protamine Sulfate Enhances the Transduction Efficiency of Recombinant Adeno-Associated Virus-Mediated Gene Delivery

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Purpose. The purpose of this study was to evaluate glucose responsiveness in HepG2 human hepatoma cells transduced by a recombinant adeno-associated virus (rAAV) vector containing the insulin gene promoter, and to investigate the effect of protamine sulfate on rAAV-mediated gene delivery.

Methods. Recombinant AAV vector, AAV.Ins.Luc.Δ EGFP, was employed to transduce HepG2 hepatoma cells. Virus infection was carried out either in the absence or presence of protamine sulfate, followed by fluorescence microscopic examination, luciferase activity assay, and flow cytometric analysis. Electrokinetic measurements were carried out to determine the effect of protamine sulfate on zeta potential of the cells and the virus.

Results. Glucose-responsive luciferase gene expression was obtained in rAAV-transduced HepG2 cells. Addition of 5 μg/ml protamine reversed the zeta potential of the cells and the virus particles, leading to enhanced transgene expression in rAAV-transduced HepG2 cells. Enhancement of protamine sulfate on rAAV-mediated gene transfer was dose-dependent. Addition of more than 5 μg/ml protamine resulted in a reduction of infectability of the virus.

Conclusions. Glucose responsiveness in the millimolar concentration range can be obtained in rAAV-transduced HepG2 cells. Protamine sulfate, up to 5 μg/ml, enhanced the rAAV transduction efficiency in HepG2 cells. The enhancement was correlated with zeta potential of the cells and the virus.

KEY WORDS: recombinant adeno-associated virus; protamine sulfate; gene delivery; hepatoma cells; zeta potential.

INTRODUCTION

Adeno-associated virus (AAV), a nonpathogenic single-stranded DNA virus of the parvovirus family, is currently being developed as an efficient gene delivery vector (1,2). The virus offers several interesting features for gene therapy, such as wide host range, lack of immune reaction, and transduction of both dividing and nondividing cells (3,4). In the absence of the helper virus, AAV integrates into the host chromosome 19 (5). The recombinant adeno-associated virus (rAAV) vector is derived from defective AAV plasmid in which all the genomic DNA except the inverted terminal repeat (ITR), the only *cis*-acting sequences necessary for replication, encapsidation, and integration into host chromosome, is replaced by the desired gene of interest (6–8).

Insulin-dependent diabetes mellitus (IDDM), or type 1 diabetes, results from autoimmune destruction of insulin producing pancreatic islet beta-cells, leading to insulin deficiency (9,10). Patients with type 1 diabetes rely on frequent administration of insulin, which is associated with several undesired effects due to fluctuation of circulatory insulin levels. Recent development of genetic engineering raised the possibility of maintaining normoglycemia by coupling sensing of blood glucose levels to the release of insulin (11). Scientists therefore have turned toward the use of surrogate cells to deliver insulin. Several attempts by genetic manipulation of islet beta cells, muscle cells, or hepatocytes have been made to generate insulin-producing cells (12–18). In the previous study, a rAAV vector containing the insulin gene promoter-driven luciferase gene was constructed and used to transduce pancreatic islet beta cells *in vitro*. Glucose-responsive reporter gene expression was demonstrated (19). Gene therapy for diabetes mellitus, however, would be most desirable if glucose responsiveness in the millimolar or physiological concentration range could be obtained from extrapancreatic sources, such that "artificial" beta cells can be obtained by genetic manipulation of the patient's own cells. Among the bioengineered cells investigated, hepatocytes have been of major interest due to their synthetic capability of several enzymes involved in glucose homeostasis.

Despite the relatively high efficiency of rAAV and its ability to transfer the foreign genes to the nondividing cells, rAAV-mediated transgene expression in liver cells appeared to be relatively low and required high multiplicity of infection (MOI), which is the major obstacle to clinical application of hepatic gene therapy (20). It is thus desired to improve the efficiency of rAAV-mediated transduction, such that the amount of viral vectors being administered can be reduced. Recent studies on adenovirus-mediated gene delivery have demonstrated that gene transfer can be improved by the addition of polycations such as polybrene, poly-L-lysine, DEAE-dextran, and protamine (21–23). However, the underlying mechanism for the augmentation of gene transfer by these substances was unclear. It was hypothesized that the use of polycations brings about neutralization of membrane charges or bridging of virus particles and the cell surfaces, and thus results in the enhancement of gene transfer (23). The results obtained from adenovirus studies suggested that a similar effect may be obtained in the recombinant adeno-associated virus system. It is speculated that the addition of polycations such as protamine during virus adsorption would increase the efficiency of rAAV-mediated gene transfer.

In the present study, a previously constructed AAV-based vector, containing the luciferase gene under control of the rat insulin I gene promoter and the enhanced green fluorescent protein (EGFP) gene driven by the cytomegalovirus (CMV) immediate early promoter (19), was utilized to examine rAAV-mediated gene delivery in the human hepatocytes. We attempted to evaluate whether glucose responsiveness in the millimolar concentration range can be obtained in HepG2 human hepatoma cells transduced by rAAV. In addition, we hypothesized that addition of polycations during adsorption of rAAV would augment the transduction efficiency. The fo-

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ABBREVIATIONS: CMV, cytomegalovirus; EGFP, enhanced green fluorescence protein; FACS, fluorescence-activated cell sorter; ITR, inverted terminal repeat; MOI, multiplicity of infection; PBS, phosphate-buffered saline; rAAV, recombinant adeno-associated virus.

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cus on the use of protamine sulfate was based on its safety and previously established effect on adenovirus. Here in this study we present the first experimental evidence showing that protamine sulfate enhances the transduction efficiency of rAAV-mediated gene delivery.

MATERIALS AND METHODS

Cell Culture

HepG2 cells, a human hepatocellular carcinoma cell line, were cultured and maintained in Eagle's minimum essential medium (MEM), supplemented with nonessential amino acids, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg amphotericin B/ml, in an atmosphere of 5% CO_2 at 37°C. All tissue culture media were obtained from Gibco/BRL Life Technologies (Gaithersburg, MD).

Recombinant Adeno-Associated Virus Vector

The construction of recombinant adeno-associated virus vector, pAAV.Ins.Luc.ΔEGFP, has been described (19). Briefly, the vector was constructed from an adeno-associated virus (AAV2) backbone lacking the *cap* and *rep* sequences (24). The expression cassette, containing the luciferase gene driven by the rat insulin I gene promoter and the EGFP gene driven by the CMV immediate early gene promoter, was inserted between the ITR sequences.

The production of rAAV was carried out as previously described (19,25) except that packaging of AAV vectors with the helper plasmid was carried out by calcium phosphate-mediated transfection of HeLa cells. The AAV *rep* and *cap* genes were provided in *trans* by the packaging plasmid. Twenty-four hours later, cells were infected with helper wild-type adenovirus 5 at a ratio of approximately 10 infectious adenovirus particles per cell. At 60–72 h postinfection, cells were harvested and the virus was purified by double CsCl gradient ultracentrifugation. Genomic titers of rAAV in each fraction were quantified by DNA dot blot hybridization. [α-^{32}P]-dCTP labeled DNA probes were prepared by the random primer labeling system (Life Technologies, Gaithersburg, MD), and the signal intensities in the autoradiograph were compared with that of the standard plasmid DNA on the same blot.

Transduction of HepG2 Cells with Recombinant Adeno-Associated Virus

HepG2 cells were grown in 12-well culture plates. Approximately 1×10^5 cells were plated in each well 1 day before viral infection. Cells in each well were infected with rAAV at the desired MOI, diluted in the serum-free medium to a final volume of 100 μl, and incubated at 37°C in 5% CO_2. For those experiments performed in the presence of protamine, rAAV was diluted in the serum-free medium containing varying concentrations of protamine (50 mg/ml stock), ranging from 0.5 μg/ml to 500 μg/ml, and then added to the culture medium. Two hours later, virus was removed and replaced with fresh medium containing 10% fetal bovine serum. For those experiments on glucose-stimulation, the medium was removed 24 h later and replaced with glucose-free medium containing the desired concentrations of glucose. At 48 h posttransduction, rAAV-transduced HepG2 cells, treated with or without protamine, were examined under a Zeiss Axiovert 100TV fluorescence microscope/MC80DX, followed by luciferase activity assays and flow cytometric analysis.

Luciferase Activity Assays

Forty-eight hours after rAAV transduction, cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μl cell lysis buffer (Promega Corp., Madison, WI). Twenty microliters of cell lysate were combined with 100 μl luciferase assay reagent (Luciferase Assay System, Promega Corp.), and luciferase activity assays were performed. Emitted light was measured for 6 s at ambient temperature in a Packard Microplate Scintillation & Luminescence Counter (Meriden, CT). Total protein concentration of the cell lysate was determined with the Bio-Rad DC Protein Assay Reagents (Bio-Rad Laboratories, Hercules, CA). Luciferase activity was expressed as counts per second normalized to total protein concentration.

FACS Analysis of EGFP Expression in rAAV-Transduced HepG2 Cells

The percentage of the EGFP-expressing cells was quantified by flow cytometric analysis. At 48 h posttransduction, cells were rinsed with PBS, trypsinized, and centrifuged at 1000 rpm for 5 min. Flow cytometric analysis was performed by a FACSCalibur (Becton Dickinson Immunocytometry System, San Jose, CA) using 488 nm excitation source and 530/30 nm band-pass filter set. The percentage of EGFP-expressing cells was determined after compensating for autofluorescence using untransduced cells as a negative control. Totals of 20,000 cells were counted. Data acquisition and analysis were performed using the CellQuest program.

Electrokinetic Measurements

Approximately 5×10^6 HepG2 cells per 10 cm culture dish were rinsed with PBS, trypsinized, and centrifuged at 1000 rpm. The cell pellet was resuspended in a 10 ml solution containing 300 mM glucose and 0.011 M NaCl (pH 7.3) (26). In the same solution , 10 ml rAAV samples were prepared at 10^7 virus particles/ml. Various amounts of protamine were then added to aliquots of 10 ml solution containing either the cells or the virus, followed by incubation at 37°C for 2 h with gentle rocking every 15–20 min.

Zeta potential of the cells and the virus at various concentrations of protamine were determined from triplicate samples at 25°C by a Malvern Zetasizer3000 (Malvern Instruments Limited, UK), operated at 150 volts. Total five measurements were taken for each sample.

In Vitro Cytotoxicity Assay

To evaluate the cytotoxic effect of protamine, HepG2 cells were plated in 96-well plates at a density of 1×10^4 cells per well in 100 μl media containing 10% fetal bovine serum. Twenty-four hours later, cells were infected with rAAV at an MOI of 500, diluted in 30 μl culture medium containing various concentrations of protamine. Cells were then incubated at 37°C for 2 h, after which the medium was removed and replaced with 100 μl fresh medium containing 10% fetal bovine

serum. Cell survival rate was assessed by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Reagent (Promega Corp., Madison, WI). The absorbance at 490 nm was recorded with a SPECTRAmax PLUS microplate reader (Molecular Devices Corp., Sunnyvale, CA) and the results were analyzed by the SOFTmax PRO program. All samples were measured from triplicate wells and compared with the control cells without protamine treatment.

RESULTS

In the present study, we evaluate glucose responsiveness in rAAV transduced hepatocytes and explore the possible enhancement effect of protamine on rAAV-mediated gene transfer.

Fluorescence Microscopy

To determine the efficiency of rAAV vectors to transduce human hepatoma cells, HepG2 cells were cultured and transduced with rAAV at an MOI of 500. The use of EGFP gene allows the transgene expression visible under the fluorescence microscope. As shown in Fig. 1A, at an MOI of 500, HepG2 cells were efficiently transduced by rAAV. Transduction of HepG2 hepatoma cells by rAAV.Ins.Luc.ΔEGFP showed that green fluorescence was produced as early as 12 h postinfection, and the expression was stable up to 60–72 h after virus transduction. The percentage of the EGFP-expressing cells increased with time after rAAV infection. The fluorescence photomicrographs shown in Fig. 1 demonstrated the enhancement effect of protamine on rAAV transduction efficiency. At 48 h posttransduction, the percentage of fluorescent cells was significantly increased in those cells treated with 5 μg/ml protamine as compared to those without protamine treatment (Fig. 1B).

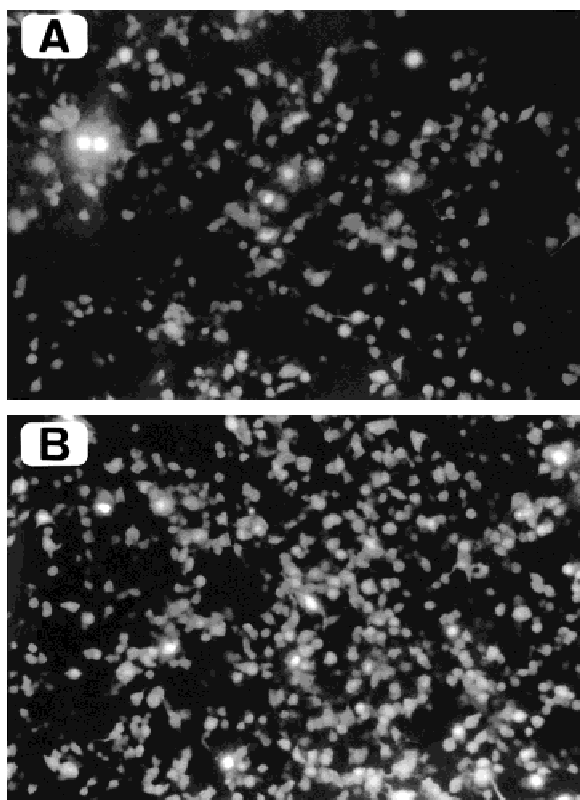

Fig. 1. Fluorescence photomicrographs of HepG2 cells at 48 h posttransduction with rAAV.Ins.Luc.ΔEGFP at an MOI of 500, (A) in the absence of protamine, (B) in the presence of 5 μg/ml protamine sulfate.

Glucose-Responsiveness in rAAV-Transduced HepG2 Cells

At 48 h after rAAV transduction (MOI = 500), HepG2 cells were lysed and luciferase activity assays were performed. Fig. 2 shows the glucose-responsive luciferase activities in rAAV-transduced HepG2 cells at 48 h posttransduction. Increasing glucose concentrations induced a progressive increase in normalized luciferase activities. Exposing cells to 9 mM (equivalent to 162 mg/dl) glucose stimulated an increase in insulin promoter-driven luciferase activity approximately threefold higher than those grown in 1 mM (equivalent to 18 mg/dl) glucose. Luciferase expression remained relatively stable for glucose concentrations greater than 9 mM, and the glucose responsiveness obtained was within the physiological range of glucose.

Effect of Protamine on the Efficiency of rAAV-Mediated Gene Transfer *In Vitro*

To evaluate the relationship between the number of transducing rAAV particles and the transduction efficiency, HepG2 cells were transduced with increasing quantities of rAAV, from an MOI of 100 up to 1000, either in the absence or presence of 5 μg/ml protamine. After 2 h of incubation at 37°C, the supernatant containing the virus and protamine was removed and replaced with fresh medium. Cells were further cultivated in the 37°C incubator. At 48 h posttransduction, cells were harvested, followed by luciferase activity assays and flow cytometric analysis. As expected, the transduction efficiency increased with the number of transducing rAAV particles during infection (Fig. 3). Transduction of HepG2 cells with rAAV at an MOI of 500 in the absence of protamine resulted in approximately 37% EGFP-expressing cells,

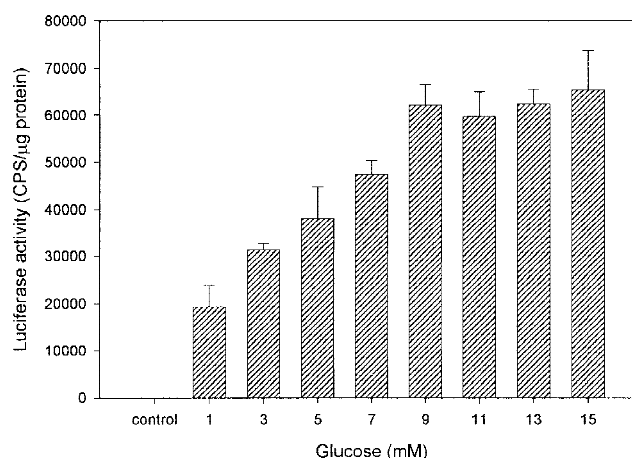

Fig. 2. Glucose-responsive luciferase activities in rAAV transduced HepG2 cells. HepG2 cells were transduced with rAAV at an MOI of 500. At 24 h posttransduction, cells were subjected to stimulation of various concentrations of glucose. At 48 h posttransduction, cells were washed and lysed, followed by luciferase activity assays. The untransduced cells served as the control. The data presented are the means ± standard deviations from triplicate wells.

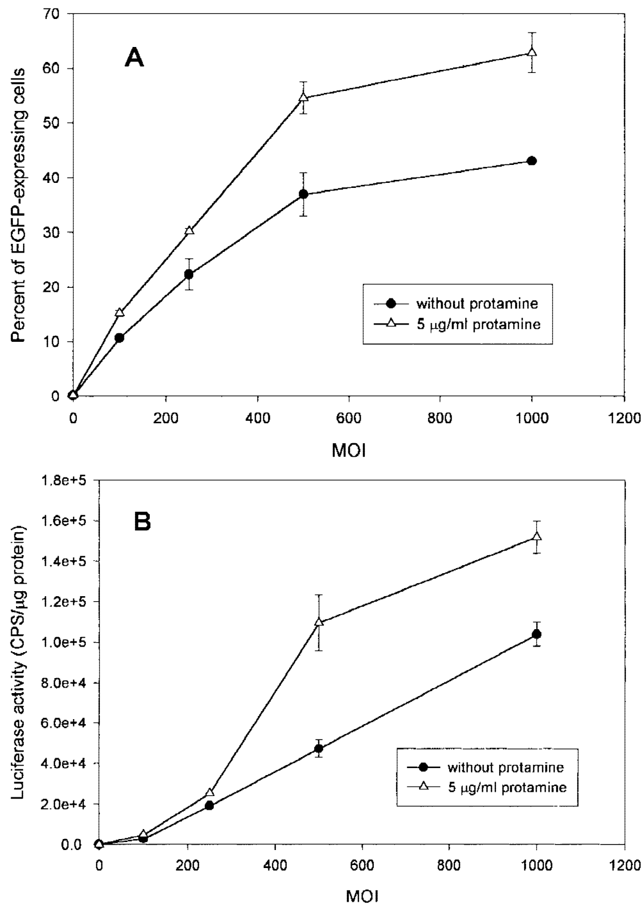

Fig. 3. Dependence of the rAAV-mediated transgene expression on the number of transducing rAAV particles. Approximately 1×10^5 HepG2 cells were plated in each well in 12-well plates and transduced with varying amounts (MOI) of rAAV.Ins.Luc.ΔEGFP particles either in the absence or presence of 5 μg/ml protamine. Flow cytometric analysis and luciferase activity measurements were performed at 48 h posttransduction. (A) The percentage of the EGFP-expressing cells was calculated by dividing the number of EGFP-expressing cells, obtained from flow cytometry, by the total number of cells counted. (B) Luciferase activities of rAAV transduced HepG2 cells, expressed as counts per second (CPS) per μg of protein. The data presented are the means ± standard deviations from triplicate wells.

as determined from flow cytometric analysis (Fig. 3A). Addition of 5 μg/ml protamine during rAAV infection increased the transduction efficiency, resulted in more than 54% of the HepG2 cells expressed EGFP.

The luciferase activities obtained from rAAV-transduced HepG2 cells showed that increasing rAAV doses during infection significantly increased the insulin promoter-driven luciferase activities (Fig. 3B). At an MOI of 500, treatment of HepG2 cells with 5 μg/ml protamine increased the luciferase activity approximately twofold higher than those in the absence of protamine.

To determine the effect of protamine concentration on rAAV-mediated gene transfer, HepG2 cells were infected with rAAV at an MOI of 500 in the presence of incremental doses of protamine (0, 0.5, 1, 5, 10, 50, 100, 500 μg/ml). At 48 h posttransduction, cells were harvested, and flow cytometric analysis and luciferase activity assays were performed. Fig. 4 shows that cell infectability by rAAV, as determined from flow cytometric analysis (Fig. 4A) and the luciferase activities (Fig. 4B), increased with the concentration of protamine up to 5 μg/ml. At an MOI of 500, the maximum increase in the reporter gene expression in the presence of 5 μg/ml protamine was approsimately 1.5-fold (EGFP) to 2.3-fold (luciferase) higher than those cells without protamine treatment. Further addition of protamine decreased the rAAV transduction efficiency and the transgene expression. In the presence of greater than 50 μg/ml protamine, both EGFP expression and luciferase activities reduced significantly.

Zeta (ζ) Potential Measurements

To determine the effect of protamine on the surface potential, cells and virus were incubated with various concentrations of protamine for 2 h, followed by electrokinetic measurements. The effects of protamine concentration on the ζ-potential of HepG2 cells and the rAAV particles are demonstrated in Fig. 5. Addition of protamine increased the surface charge of the virus particles from –8.6 mV, in the absence

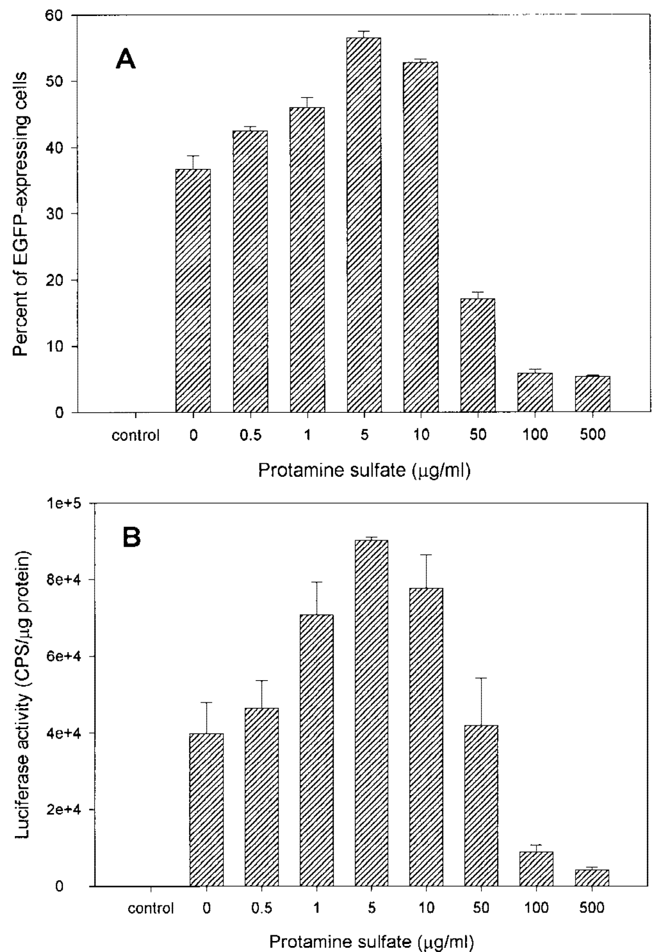

Fig. 4. Effect of protamine concentration on the transduction efficiency of rAAV. Approximately 1×10^5 HepG2 cells were plated in each well in 12-well plates and transduced with rAAV at an MOI of 500 in the presence of varying concentrations of protamine. At 48 h posttransduction, cells were harvested, followed by flow cytometric analysis and luciferase activity assays. The untransduced cells served as the control. Results shown are (A) the percentage of EGFP-expressing cells and (B) luciferase activities (CPS/μg protein), from triplicate wells, presented as means ± standard deviations.

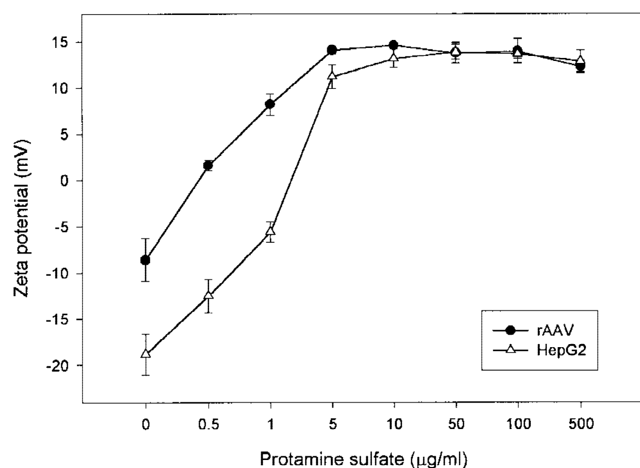

Fig. 5. Effect of protamine concentration on the zeta potential of the rAAV particles (●) and HepG2 cells (△). Total five measurements were taken for each sample. The data presented are the means ± standard deviations from triplicate wells.

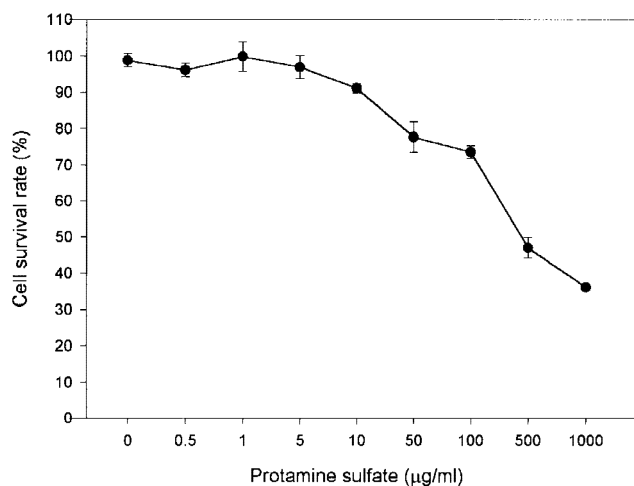

Fig. 6. Cytotoxic effect of protamine on HepG2 cells. Approximately 1×10^4 cells were plated in 96-well plates, and infected with rAAV at an MOI of 500 in the presence of varying concentrations of protamine. After 2 h incubation the medium containing the virus and protamine was removed and replaced with 100 μl fresh medium. Cell survival rate was determined using the CellTiter 96 Aqueous One solution Cell Proliferation Assay Reagent. The data presented are the means ± standard deviations from triplicate wells.

of protamine, up to +14.1 mV, in the presence of 5 μg/ml protamine, with the charge reversal occurred at 0.42 μg/ml protamine. Addition of increment of protamine also progressively elevated the ζ -potential values of the cells from −18.8 mV, in the absence of protamine, up to +11.2 mV, in the presence of 5 μg/ml protamine, with the charge reversal occurred at 2.33 μg/ml protamine. The results presented here indicated that protamine was adsorbed both to the negatively charged virus particles and the cell surfaces. During the experiments the virus particles were first mixed with protamine in the culture medium before being added to the cells; therefore, the surface charge of the virus was reversed by the adsorption of protamine, which made the negatively charged cells more accessible to the virus particles, leading to greater adsorption of virus onto the cell surfaces, and thus increasing the transduction efficiency of rAAV. Addition of more than 5 μg/ml protamine does not seem to change the ζ-potentials of the cells and the virus any further.

In Vitro Cytotoxic Effect of Protamine

To determine whether protamine exerts any cytotoxic effect, HepG2 cells in 96-well plates were transduced with rAAV at an MOI of 500 in the presence of varying amounts of protamine. Two hours later the medium containing the virus and protamine was removed and replaced with fresh medium. Cell survival rate was assessed by the colorimetric cell proliferation assay. Fig. 6 shows the cell survival rate after protamine treatment, illustrating that protamine does not exhibit any significant cytotoxic effect below 10 μg/ml. The survival rate of HepG2 cells was decreased to 78% in the presence of 50 μg/ml of protamine, which was further reduced to 47% in the presence of 500 μg/ml of protamine, indicating that protamine exerts significant cytotoxic effect at concentrations greater than 10 μg/ml.

DISCUSSION

Diabetes mellitus is a metabolic disease characterized with chronic hyperglycemia. Gene therapy for diabetes mellitus can be approached by introduction of the insulin gene to the somatic cells. The ideal situation would be to couple the magnitude of insulin secretion with environmental stimulation such that inducibility of the system can reach the physiological effective level. In previous studies a rAAV vector carrying the luciferase gene under control of the insulin gene promoter was shown to be able to transduce pancreatic islet cells, leading to glucose-responsive expression of the reporter gene (19). Due to limited availability of the pancreatic islet cells and the potential problems associated with immune responses by transplantation, the use of nonislet surrogate cells for gene therapy of diabetes has been of great interest. In the present study we attempted to determine whether glucose responsiveness can be obtained in rAAV-transduced human hepatoma cells. Results presented in this study demonstrated that HepG2 hepatoma cells can be efficiently transduced by rAAV and that exposing rAAV-transduced HepG2 cells to glucose produced a concentration-dependent increase in the reporter gene expression. Glucose responsiveness was obtained in the millimolar concentration, corresponding to the physiological glucose range.

Polycations were previously shown to enhance the infectivity of several viral vectors (21–23). It was hypothesized that polycations enhance the adsorption of viral vectors to the cells and thus improve the transduction efficiency of rAAV-mediated gene transfer. Our results demonstrated that protamine enhanced the rAAV transduction efficiency *in vitro*. At 48 h posttransduction, a significant increase in the percentage of EGFP expressing cells was observed in those cells treated with protamine (Fig. 1). Quantitative analysis by flow cytometry indicated that transduction efficiency increased with doses of rAAV applied to the cells during infection (Fig. 3), and the enhancement of protamine on rAAV transduction was concentration-dependent (Fig. 4). The percentage of EGFP-expressing cells was significantly increased in the presence of 5 μg/ml protamine, suggesting that the presence of protamine may enhance adsorption of rAAV particles to the

cells. The optimal dose of 5 μg/ml is consistent with the previously reported value for adenovirus (23). However, the underlying mechanism for this phenomenon was unclear. It was first demonstrated in this study that addition of protamine up to 5 μg/ml neutralized the negatively charged moieties present on the surfaces of both the cells and the virus particles, reversed their surface potentials, such that adsorption and binding of rAAV to the cells increased, leading to augmented transduction efficiency. This observation was well supported by the experimental evidence showing excellent correlation between the maximum plateau value of ζ-potential at 5 μg/ml protamine, both for the cells and the virus (Fig. 5), and the maximum transduction efficiency at the same concentration (Fig. 4). On the other hand, protamine was found to be toxic to HepG2 cells at concentrations greater than 10 μg/ml. The cell survival rate was reduced to 78% in the presence of 50 μg/ml protamine. Further increase in protamine concentration resulted in decreased cell viability. The dramatic decrease in rAAV transduction at protamine concentrations greater than 5 μg/ml, however, does not correlate with the magnitude of change of the cell survival rate. Lanutti *et al.* reported similar observation in adenovirus-mediated gene transfer (23). It is speculated that the charge effect brought about by the polymers was abrogated by addition of protamine greater than 5 μg/ml, changing the cell susceptibility to rAAV due to steric hindrance caused by the presence of high concentrations of protamine, and thus decreased rAAV adsorption onto the cells.

In summary, we have demonstrated in this study that glucose-responsive reporter gene expression in the millimolar glucose concentration was obtained in rAAV-transduced HepG2 human hepatocytes. The presence of protamine sulfate increased zeta potential of the cells and the virus, and thus induced an enhancement of rAAV-mediated gene transfer. Maximum transduction efficiency was obtained at 5 μg/ml protamine. Further addition of protamine decreased the rAAV transduction efficiency due to steric hindrance and the cytotoxicity of the polymer.

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