Protamine Enhances the Efficiency of Liposome-Mediated Gene Transfer in a Cultured Human Hepatoma Cell Line

ABSTRACT

Protamine, clinically an antidote for heparin, is a small protein with a high arginine content and is potent in folding DNA. Protamine and DNA can form a compact structure, thus protecting DNA from digestion by intracellular enzymes. Protamine may therefore enhance the efficiency of gene transfer. In this study, we tested the ability of protamine to improve liposome-mediated gene transfer efficiency in a human hepatoma cell line. The results of a preliminary gel retardation assay indicated that 10 μg was the minimal amount of protamine sulfate needed to completely bind 5 μg of a plasmid containing a reporter gene, green fluorescent protein (GFP). For transfection assays, protamine (0, 10, 50, 100, and 500 μg) was added to a DNA-liposome mixture (5μg DNA and 20μg lipofectamine) to transfect cultured Huh7 cells. Transfected cells (those expressing GFP) were counted by means of flow cytometry. The expression index (EI) was calculated as the transfection efficiency (% transfected cells) with protamine divided by the transfection efficiency with DNA and liposome only. The addition of 10, 50, or 100μg of protamine to the liposome-DNA mixture significantly increased the EI, with the greatest increase noted when 10μg was used. The results of this study show that an appropriate amount (10-100 μg) of protamine sulfate increases the transfection efficiency of GFP in Huh7 cells.

Key Words: gene transfection, protamine, liposome, green fluorescent protein

INTRODUCTION

Liposomes are a useful non-viral vector that can carry foreign DNA for gene therapy [1]. They resemble animal cells in that the outer membrane consists of a double layer of lipid molecules. When liposomes fuse with cells, they are taken up by endocytosis, thereby delivering their contents (eg, targeted genes) into the cells[2,3].

Cationic liposomes may be complexed to negatively charged, naked DNA by simply mixing the liposomes and DNA together [4]. The complex is easily taken up by the cells and results in an acceptable level of gene expression [5]. The advantages of this vector are 1) it is non-immunogenic; 2) there is almost no limitation in the size of gene of interest; 3) it can be administered repeatedly; and 4) it is commercially available and ready to use [6,7]. However, its disadvantages are the low transfection efficiency in certain types of cells, lack of tissue targeting ability in standard preparation, and inability to yield a long-term stable expression *in vivo* [8].

For *ex vivo* hepatocyte gene therapy, liposomes may be a good vector because of their availability and lack of limitation in gene size [9]. However, the internal diameter of a liposome is about 0.025 to 0.1 μm, which is much less than the longest dimension of plasmid DNA (about 2 μm) [10]. This means that the expression vector needs to be

compacted inside the liposome.

A possible explanation for the low transfection efficiency of liposome-DNA complex is the relatively linear shape of the DNA in the complex [11]. Linear DNA is highly exposed and is therefore subject to digestion by intracellular DNAase. Thus, methods to deliver DNA in a more compact form might improve transfection efficiency. Protamines are small basic proteins (MW 4000-4250) with a high arginine content, and potent DNA folding ability. In salmon and herring, as well as mammals, sperm DNA is packed in a highly condensed state by protamines. Salmon sperm protamine has been sequenced and shown to contain 32 amino acid residues, 21 of which are arginine [12]. Although the exact mechanism of DNA-protamine complex formation remains unknown, the complex is speculated to acquire a very condensed, almost crytsalline form. In this compact structure, DNA is well protected from enzymatic hydrolysis and may reach the nucleus in an intact form. Indeed, Sorgi et al. demonstrated that protamine enhances liposome-mediated gene transfer to several different types of cells *in vitro* [13], but not specifically in hepatocytes.

Improving gene transfer efficiency may be critical for *ex vivo* hepatocyte gene therapy, especially for autologous hepatocyte transplantation. When hepatocytes are procured from donors and cultured *ex vivo*, a certain fraction of hepatocytes will not survive. A more efficient gene transfer is needed to compensate for the cell loss, and to achieve effective hepatocyte transplantation [14].

In this study, we tested the ability of protamine to enhance the efficiency of liposome-mediated gene transfer in cultured human hepatoma cells. We selected the green fluorescent protein (GFP) gene cloned from jellyfish [15], altered to allow expression of GFP in mammalian cells [16], as the reporter gene. The efficiency of gene transfer could then be estimated by using FACscan to determine the percentage of cells successfully transfected [17].

MATERIALS AND METHODS

Plasmids and Cell Culture The GFP gene was inserted into a commercially available expression vector (pGL-1,Gibco Life Technology, Gaithersburg, MD, USA). This vector is a 5030 bp plasmid containing the cytomegalovirus (CMV) immediate early promoter and an SV40 polyadenylation signal downstream of the GFP gene to direct the processing of mRNA. A conventional large-scale cesium chloride plasmid preparation was performed to amplify and purify the plasmid [18]. Huh7 hepatoma cells (a gift from Dr. Lee PI) [19,20] were cultured and maintained in Dulbecco's modified essential medium (DMEM, Gibco Life Technology) containing 5% fetal bovine serum (FBS, Gibco, Life Technology) in a 37° C incubator with 5% CO_2.

Simplified Gel Retardation Assay to Determine DNA-Protamine Binding Curve Normal saline was used to serially dilute protamine sulfate (Sigma, St. Louis, MO, USA) to 0.01, 0.1, 1, and 10 ½g/½L. Various amounts of normal saline-diluted protamine were mixed with 5 ½g of pGL-1 DNA in a total volume of 10 ½l followed by incubation at room temperature for 15 to 30 minutes. The mixtures then subjected to electrophoresis (100Volts, 30 minutes) through a 1% agarose gel with 1X TBE and stained with ethidium bromide to see if the DNA was retained in the well. The endpoint was the lowest amount of protamine that could bind 5 ½g of DNA completely, such that no DNA ran into the gel: this was used as the starting point for the transfection experiments.

Transfection experiments Forty-eight hours after transfection, the cultured Huh7 cells were harvested and the number of viable cells were counted with a hemacytometer (Reichert, Buffalo, NY, USA) after trypan blue (0.04%) (Gibco) exclusion. LipofectAMINE (Gibco), a mixed formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA)

and the neutral phospholipid dioleoylphosphatidylethanolamine (DOPE)[14] (DOSPA: DOPE=3:1 w/w) was used for the transfection experiments. On the day before transfection, the Huh7 cells were dispensed into 6-well culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) at a density of $6x10^5$ cell per well and incubated overnight at 37¼[in 5% CO_2. On the experiment day, the cultured cells were then rinsed twice with 1ml serum-free DMEM to remove the previous culture media. For the liposome-DNA-protamine mixtures, 10½l LipofectAMINE (L) (2½g/½l) in 100½l serum- free DMEM was mixed with 5½g GFP plasmid DNA (G) (10 ½l, 0.5 ½g/½l) in 100½l serum-free DMEM in 3.5 mm cell culture plates. Protamine sulfate (10 mg/mL) (0, 10, 50, 100, or 500 ½g) was then immediately added to the mixture, followed by incubation at room temperature for 30 minutes. After incubation, 800 ½l serum-free DMEM was added to the transfection mixtures. The final transfection mixtures (total 1 ml for each experiment) were added to the Huh7 cells, followed by incubation at 37°C for 6 to 8 hours. Toxicity (20 ½g lipofectAMINE in 1 mL serum-free DMEM) and negative (5½g GFP plasmid in 1 mL serum-free DMEM without lipofectAMINE) control experiments were also performed. Equal volumes of DMEM+10%FBS were then added to each well after this incubation, without removing the transfection mixture. The culture media was replaced with 2 mL DMEM+5%FBS the next day, and transfection efficiency was analyzed 48 hours later, as described below. All experiments were performed in quadruplicate.

FACscan counting: Cells from culture plate were detached by digestion with trypsin-EDTA 1ml (Gibco) and incubated at 37° C for 5 minutes. Cell suspensions were collected in 1.5 ml microcentrifuge tube and centrifuged 1500 rpm for 2-3 min. Decanted supernatant and then resuspended the cell pellet with 1X phosphate buffer saline (PBS). The cell suspension was then transferred to the counting tube and subjected to the FACscan (Becton Dickinson, Mountain View, CA). The efficiency of transfection was represented by the percentage of number of cells emitting fluorescence. It was calculated by the software CellQuest software (Becton Dickinson). The expression index (EI) is defined below:

$$EI = \frac{\text{\% of cells emitting fluorescence after transfection with x ½g of protamine}}{\text{\% of cells emitting fluorescence after transfection without protamine,}}$$

where x= 0, 10, 50, 100, or 500 ½g of protamine. Data are expressed as the mean» í standard deviation where appropriate. Differences in the GFP EI between experiments were assessed with student's *t* test. *P* value of less than 0.05 were considered statistically significant.

RESULTS

Minimal Amount of Protamine needed to Completely Bind 5 ½g GFP Plasmid. Ten ½g of protamine sulfate could retain almost all DNA in the well (Fig. 1), while smaller amounts could not. Therefore, the minimal amount of protamine used in transfection assay was 10 ½g. We also used larger amounts of protamine (50, 100, or 500 ½g) in other experiments, to check the dose-responsive curve.

Transfection of Huh7 cells with GFP Forty-eight hours after transfection, the cultured Huh7 cells were harvested and the number of viable cells were counted. The numbers of cells after transfection were about the same in each experiment. (G+L: $2.1\pm0.4X10^6$ cells/well; G+L+10 ½g protamine: $1.9\pm0.3X10^6$ cells/well; G+L+50 ½g protamine: $2.1\pm0.3X10^6$ cells/well; G+L+100 ½g protamine:$1.8\pm0.2X10^6$ cells/well;

G+L+500½g protamine: 1.7±0.1X10^6 cells/well; negative control: 2.1±0.3X10^6 cells/well; and toxicity control: 1.9±0.1X10^6 cells/well). There was no statistically significant difference in cell viability among the experimental groups (all p>0.05, student's t test).

Percentage of Cells Emitting Green Fluorescence Counted by FACscan and Expression Index. Forty-eight hours after transfection, the cell culture plates were photographed under fluorescent microscope to check for green light emission (Fig. 2). There was no green fluorescence emission observed or the negative control plate. After each plate was photographed, the cells were harvested and subjected to FACscan cell counting. The results of quadruplicate experiments showed that the tranfection mixture containing G+L + 10½g protamine resulted in the highest EI (EI=3.4» 2.3), followed by G+L+50½g protamine (EI=1.9±0.6), G+L+100½g protamine (EI=1.6±0.9), and G+L+500 ½g protamine (EI= 0.9±0.9). All mixtures except G+L+500½g protamine yielded significantly higher EI than the control (G+L) (Fig. 3).

DISCUSSION

DNA transfection efficiency with liposome vectors is not high. One paper reported only 80 pmol out of 1½mol of oligonucleotide was uptaken in 10^6 cultured HepG2 cells [21]. However, the efficiency can be enhanced by altering the composition, size, and charge of liposome-DNA complex for *in vitro* gene delivery [22]. Not much effort has been directed at investigating the use of molecules to modify DNA and improve transfection efficiency. In a previous study of histone H1, investigators set up a model of positively charged protein that can drastically affect the DNA binding activity of specific transcription factors [23]. Because of its positive charge, protamine sulfate can help to fold DNA and increase the transfection efficiency in the liposome-mediated gene transfer system[13]. In this study, we successfully demonstrated that protamine sulfate can indeed increase gene transfer efficiency in liposome mediated transfection.

The other advantage of using protamine is that it is already used clinically as an antidote for heparin and the adverse drug effects and pharmacology are known. This should facilitate the clinical accessibility of this agent if it is eventually applied in human gene therapy.

The results of the simplified gel retardation assay showed that 10 ½g of protamine is the minimal amount needed to completely bind 5 ½g of GFP plasmid. The minimal amount of protamine also seemed to be the optimal amount for *in vitro* transfection as adding more protamine did not increase the gene transfer efficiency. It is possible that, because of its high affinity for DNA, protamine hinders binding of transcription factors to DNA and thereby inhibits expression when high levels are used for transfection.

In the future, we may apply this method to improve transfection efficiency for primary rat hepatocytes and even human hepatocytes. Cultured cells can be transfected with wild type foreign genes, and stable transfectants can then be selected, and reintroduced back to the hosts. This may provide a good model for gene therapy for treatment of inherited diseases, cancer, and even infectious diseases. Furthermore, protamine can be conjugated with various liver-specific ligands, such as asialoglycoproteins like asialoorosomucoid [24], or asialofetuin [25], and therefore has a potential application in *in vivo* gene therapy. Asialoglycoprotein/polylysine/DNA complexes have been used for targeted in vivo gene delivery in mice[26] and rats[27]. Similarly, protamine may be used to bind foreign DNA and a ligand to target the complex to a specific organ, such as the liver. Once the ligand is taken up by hepatocytes, protamine may help to fold the foreign DNA and transport it to the nucleus for transcription and expression in an intact form. Furhter studies are needed to examine the feasibility of this approach.

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Fig. 1 Green fluorescence emission in (a) G+L , (b) G+L+10p, (c) G+L+50p, (d) G+L+100p,(e) G+L+500p, (f) negative control groups. For the liposome-DNA-protamine mixtures, 10½k LipofectAMINE (L) (2½g/½L) in 100½k serum- free DMEM was mixed with 5½g GFP plasmid DNA (G) (10 ½L, 0.5 ½g/½L) in 100½L serum-free DMEM in 3.5 mm cell culture plates. Protamine sulfate (P) (10 mg/mL) (0, 10, 50, 100, or 500 ½g) was then immediately added to the mixture. (100X magnification)