Biochimica et Biophysica Acta 1858 (2016) 3017–3023

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Enhancement of liposome mediated gene transfer by adding cholesterol and cholesterol modulating drugs

Yun-Ui Bae [a,1], Jae-Wan Huh [b,1], Bieong-Kil Kim [c], Hyeon Young Park [a], Young-Bae Seu [c], Kyung-Oh Doh [a,*]

[a] Department of Physiology, College of Medicine, Yeungnam University, Daegu 42415, Republic of Korea
[b] Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 05505, Republic of Korea
[c] School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 41566, Republic of Korea

ARTICLE INFO

Article history:
Received 5 May 2016
Received in revised form 3 August 2016
Accepted 19 September 2016
Available online 21 September 2016

Keywords:
Liposomes
Transfection
Cholesterol
Gene therapy

ABSTRACT

Cholesterol is an important cell membrane component and has been used as co-lipid for cationic liposome to enhance gene delivery. However, the role of cholesterol in transfection efficiency has not been fully understood. In this study, transfection efficiency of liposome was measured after cholesterol was added to the cell culture medium. As a result, addition of cholesterol increased transfection efficiency of several liposomes consisting of different lipid components in various cells (AGS, CHO, COS7 and, MCF7). Furthermore, treatment of cells with cholesterol modulating drugs, imipramine and U18666A, also increased transfection efficiency of liposomes. To elucidate the role of added cholesterol in gene transfer, endocytotic mechanism was studied and also revealed that adding cholesterol in culture media induced participation of caveolae-mediated endocytosis and micropinocytosis in CHO cell. Therefore, the results of this work suggest that modulation of intracellular cholesterol can be an important method to enhance gene delivery.

1. Introduction

Gene therapy has been expected to provide novel and powerful approaches for treating and preventing diseases such as cancer [1] and heart failure [2]. One of the primary objects in the gene therapy is to develop efficient and nontoxic gene carriers that can effectively deliver foreign genetic materials such as plasmid, antisense, decoy, oligonucleotide, and siRNA into the target cells [3]. Although understanding the exact mechanism of transfection is essential for the rational design of more advanced nano-carriers [4], there are limited studies until now. Among many factors, recent studies including ours [5–7] confirmed transfection efficiency depends on the cell-type and one of most important factors determining efficiency of gene delivery might be the characteristic and condition of cells. Therefore, changing condition of cell could be a new way to facilitate gene therapy. The recent study actually demonstrated that stable overexpression of Niemann-Pick C1 (NPC1) in CHO cells increased the transfection efficiency [4]. Interestingly, a previous study reported this NPC1 overexpressing CHO cell had a 1.5-fold increase in total cellular cholesterol [8]. It is concordant with our previous report which showed enhanced gene delivery by addition of cholesterol to the media before transfection [9].

Cholesterols are distributed unevenly among the membranes of animal cells [10] and plasma membrane is relatively rich in cholesterol; cholesterol/phospholipids mole ratio of plasma membrane is known to be 0.7–0.8 [11]. Although cholesterol is essential for cell membrane and tightly regulated, cellular cholesterol is known to move between organelles [12]. In recent study, doubling cholesterol in culture media of human fibroblast showed movement of cholesterol from plasma membrane to intracellular compartments implying that active trafficking of cellular cholesterol [13]. On the other hand, cholesterol have been used as the major lipid component of liposome for gene delivery because of its less toxicity [14] and resistance to serum [15]. As a co-lipid, cholesterol was shown to have a superior performance compared to DOPE [16,17] and has been used for in vivo gene delivery study [18]. In addition, lipoplexes were known to be internalized by cholesterol-dependent endocytosis [19]. We also found that cholesterol-derived liposome had a resistance to depletion of membrane cholesterol, and the addition of cholesterol or cholesterol-derived liposome to cell culture media increased the transfection efficiency of DOTAP liposome in COS-7 cells [9]. Although cholesterol-derived lipids induce more fusion of cell membrane and liposome, the increased transfection by cholesterol addition to culture media was not elucidated.

Therefore, we investigated the underlying mechanisms of transfection related to cholesterol. We measured the effect of cholesterol addition in several cell lines, and the effect of cholesterol modulating drugs such as imipramine and U18666A on transfection efficiency was investigated in this experiment. Imipramine is known to inhibit cholesterol trafficking and accumulate cholesterol in late endosome and lysosome

* Corresponding author.
E-mail address: kodoh@ynu.ac.kr (K.-O. Doh).
[1] These two authors contributed equally.

http://dx.doi.org/10.1016/j.bbamem.2016.09.013
0005-2736/

[20]. U18666A was shown to decrease intracellular trafficking of cholesterol out of lysosome [21] and the effect of U18666A was different between low dose and high dose [21]. In a recent study, high-dose U18666A decreased transfection efficiency [4], whereas our result shows that low-dose U18666A increased transfection efficiency of DOTAP.

2. Materials and methods

2.1. Cell line and materials

AGS (human stomach adenocarcinoma), CHO (Chinese hamster ovary), COS7 (African green monkey kidney), and MCF7 (human breast carcinoma) cell lines were obtained from the Korean Cell Line Bank. Cells were grown at 37 °C in DMEM for COS7, F-12 K for CHO and RPMI 1640 for MCF7 and AGS. Media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in a humidified 5% CO_2 incubator at 37 °C. Cell culture media and reagents, including FBS, were purchased from Welgene (Korea). Imipramine and U18666A were purchased from Sigma (USA).

2.2. Liposome preparation

DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride) and Lipofectamine (LFA) were purchased from Roche (Germany) and Invitrogen (USA), respectively. CHOL-E having an amine head, ether linker and cholesterol tail was synthesized, and its liposome preparation was carried out according to the method previously reported [9]. Four liposome formulations with different DOTAP:DOPE weight ratios, for example, 1:0 (T1P0), 3:1 (T3P1), 1:1 (T1P1), and 1:3 (T1P3) were prepared according to a method previously reported [5].

2.3. Preparation of plasmid DNA

pcDNA-Luc, a plasmid of 5.149 kb containing the firefly luciferase reporter gene sequence, was obtained from Welgene (Korea). DNA plasmids were amplified in *Escherichia coli* XL1-Blue strain and purified using a maxi-kit (Qiagen Inc., USA), according to the manufacturer's instructions. DNA purity was determined by agarose gel electrophoresis and measuring optical density (OD). DNA with an OD_{260}/OD_{280} ratio of ≥1.8 was used in this study.

2.4. In vitro transfection and treatment of cholesterol and cholesterol modulating drugs

pcDNA-Luc was used at a concentration of 0.5 μg/well unless otherwise specified. A liposome solution was separately prepared by diluting 2.1 μg of the initial liposomal stock solution with transfection optimizing medium (TOM, Welgene, Korea) to a final volume of 50 μl. Then, the liposome solution was added to 50 μl of the DNA stock solution. This lipoplex solution was incubated for 15 min at room temperature. The lipoplexes were tested for their ability to transfer DNA into AGS, CHO, COS7 and MCF7 cells. 24 h prior to transfection, the cells were transferred to 24-well culture plates at a density of 30,000 cells/well for COS7, 40,000 cells/well for CHO and MCF7, or 50,000 cells/well for AGS. 30 min before transfection, the medium was removed and the cells from each well were briefly washed with 100 μl of sterile phosphate-buffered saline (PBS); then 150 μl of TOM was added to each well. Lipoplex solution (100 μl) was then added to each well, and plates were incubated for 4 h. After 4 h incubation with lipoplex, media was removed and changed with fresh media. Water soluble cholesterol (Sigma, USA) was added to culture media at the indicated concentration for 1.5 h before transfection and the treatment of imipramine or U18666A at indicated case. Imipramine and U18666A were treated for 24 h before transfection. After the treatment of cholesterol or cholesterol modulating drugs, cells were briefly washed with TOM.

2.5. Luciferase assay

24 h after transfection, medium was aspirated and wells were washed twice with 200 μl of ice-cold PBS. 1X reporter lysis buffer (100 μl; Promega Corp., USA) was then added to each well to lyse cells for 1 h on an ice tray. Lysates were completely collected into Eppendorf tubes and centrifuged (15,000*g*, 4 °C) for 5 min. Supernatants were transferred to Eppendorf tubes on ice, which were used for luciferase and protein assays. For luciferase assays, 20 μl of cell lysate was transferred to a white opaque 96-well plate and luciferase activity was measured using LMax II 384 luminometer (Molecular Devices Corp., USA) and the luciferase assay kit (Promega Corp., USA). Protein contents were quantified by the bicinchoninic acid (BCA) assay (PIERCE, USA) according to the manufacturer's instructions. Luciferase efficiency was normalized to the total protein content and expressed as relative luminescence units per μg of protein (RLU/μg protein).

2.6. Chemical inhibition of the endocytic pathway

COS-7 cells were seeded at a density of 6×10^4 per well into 24-well plates 2 days before the treatment. Cells were treated with endocytic pathway inhibitors for different period of time before lipoplexs were added: chlorpromazine (10 μg/ml) or genistein (50 μM) for 1 h, amiloride (50 μM) and methyl-β-cyclodextrin (MBC, 3 mM) for 15 min at 37 °C in fresh serum free medium. All reagents were purchased from Sigma-Aldrich (USA).

2.7. Staining and quantitative measurement of cellular cholesterol

Cells were washed twice with PBS and then treated with filipin (0.25 mg/ml, Sigma, USA) for 2 h to stain free cholesterol inside the cells. After staining, intracellular cholesterol was examined under a fluorescence microscope (Nikon ECLIPSE TE300, Japan). Quantitative cellular cholesterol was measured by cholesterol detection kit (abcam, UK) according to manufacturer's instruction.

2.8. Statistical analysis

The statistically significant differences between the groups were evaluated by one-way ANOVA and Tukey's post hoc test. Asterisks indicate statistically significant differences ($p < 0.05$).

3. Results and discussion

3.1. Effects of cholesterol addition on the transfection efficiency of DOTAP in various cells

To determine the effect of adding cholesterol to the culture media on transfection efficiency, different concentrations of water-soluble cholesterol were added into MCF7 and CHO cells before transfection with plasmid carrying luciferase gene by DOTAP liposome (Fig. 1A). In both cells, addition of cholesterol increased transfection efficiency of DOTAP in a dose- dependent manner. In MCF7 cells, the addition of 4 mg/ml cholesterol induced 218% increase in luciferase activity compared with control. The same concentration of cholesterol in CHO cells enhanced transfection efficiency of DOTAP more than 6 times compared to control. When the effects of cholesterol addition on transfection efficiency of DOTAP were compared between four different cells, the enhancement in CHO cells was most prominent (Fig. 1B). Although the transfection efficiencies were increased in all tested cells, lipid composition was also important factor determining transfection efficiency [22,23]. Therefore, we analyzed the effect of cholesterol addition in the culture media with different compositions of DOTAP liposomes. Intriguingly, cholesterol based