Effect of Dexamethasone Preincubation on Polymer-Mediated Gene Delivery

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Nuclear membrane is one of the main barriers in intracellular delivery of genetic materials. The previous report showed that glucocorticoid receptor dilated the nuclear pore to 60 nm in the presence of a ligand. It was also suggested that the transport of genetic material to nucleus might be facilitated by glucocorticoid. In this study, the effect of glucocorticoid preincubation in the polymeric gene delivery was investigated. The cells were preincubated with dexamethasone, a potent glucocorticoid, and transfection assays were performed with polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimer. As a result, the transfection efficiency of PEI or PAMAM to the cells in the presence of dexamethasone was enhanced, compared to the cells without dexamethasone. This effect was not observed in the cells preincubated with cholesterol. The polymer/DNA complex was stable in the presence of dexamethasone. In addition, the cytotoxicities of the polymeric carriers to the cells were observed in the presence of dexamethasone. In conclusion, dexamethasone enhances the transfection efficiency of polymeric carriers and may be useful in the development of polymeric gene carriers.

Key Words : Dexamethasone, Gene delivery, Glucocorticoid, Polymer, Transfection

Introduction

Polymeric gene carriers have been developed as an alternative for viral vectors to overcome the problems of viral vectors. Polymeric carriers have some advantages over viral vectors such as non-immunogenecity, low cytotoxicity, and low cost.[1] However, polymeric carriers have suffered from low transfection efficiency compared to viral vectors. Therefore, many efforts have been made to develop efficient polymeric carriers. It was previously suggested that there are three main barriers in the intracellular delivery of genetic materials using polymeric carriers.[2] The three barriers are cellular membrane, endosomal membrane, and nucleus membrane. For gene therapy, a therapeutic gene must be delivered to the nucleus for transcription. In the transfection with polymeric carriers, polymer/DNA complexes are internalized by endocytosis. After endocytosis, the complexes must escape endosome before fusion to lysosome, since the DNA in lysosome would be degraded by nucleases. Cellular uptake and endosomal escape can be facilitated by receptor-mediated endocytosis and endosome-disrupting peptides, respectively.[3-5] For nuclear transport, nuclear localization signal (NLS) peptides have been widely investigated.[6-9] Nuclear membrane allows passive diffusion of small molecules up to 9 nm in diameter due to the size limit of nuclear pore. NLS facilitates energy-dependent transport of large molecules by dilating nuclear pore up to 25 nm in diameter.[10,11] However, the effect of NLS may be limited, because the size of polymer/DNA complex is around 100 nm in diameter.

Glucocorticoid receptor is a nuclear receptor, which is mainly located in cytoplasm in the absence of its ligand. In the presence of ligands, the glucocorticoid receptor binds to the ligand and the receptor-ligand complex is translocated to the nucleus.[12] In the translocation process, the glucocorticoid receptor dilates the nuclear pore to 60 nm, which is favorable for translocation of polymer/DNA complex into nucleus.[13] Therefore, in the presence of glucocorticoid, the transport of polymer/DNA complex into nucleus may be facilitated. To verify this hypothesis, the effect of glucocorticoid on the transfection efficiency of a polymeric carrier was investigated. Dexamethasone, a potent glucocorticoid, was preincubated with the cells and transfection assays were carried out. As a control, cholesterol was incubated with the cells and the transfection efficiency of polymeric carriers was investigated. As prototypes of polymeric carriers, polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimer were used in the transfection experiments. The results suggest that dexamethasone can enhance the transfection efficiency. Therefore, the technique with dexamethasone may be useful for the gene delivery using polymeric carriers and the development of efficient polymeric carriers.

Experimental Sections

Preparation of plasmid. pCMV-Luc was constructed by inserting the HindIII/XbaI firefly luciferase cDNA fragment from pGL3-control vector into the HindIII/XbaI site of pcDNA3.[14] The pCMV-Luc was transformed in *Escherichia Coli* DH5α and amplified in terrific broth media at 37 °C overnight. The amplified pCMV-Luc was purified by using the Maxi plasmid purification kit (Qiagen, Valencia, CA). The purity and concentration of the purified pCMV-Luc were determined by ultraviolet (UV) absorbance at 260 nm. The optical density ratios at 260 to 280 nm of these plasmid preparations were in the range of 1.7-1.8.

Gel retardation assay. The polymer/DNA complex was prepared as described previously.[15] PEI/pCMV-Luc and PAMAM/pCMV-Luc complex was prepared at a 5/1 N/P (nitrogen of polymer/phosphate of DNA) ratio and a 6/1 N/P ratio, respectively. Increasing amounts of dexamethasone were added to polymer/DNA complexes. The final concentrations of dexamethasone were 10 and 20 μg/mL, respectively. The mixtures were incubated at room temperature for 30 min and electrophoresed on 1% (w/v) agarose gel.

***In vitro* transfection.** Human embryonic kidney 293 cells were maintained in DMEM medium supplemented with 10% FBS in a 5% CO_2 incubator. For the transfection studies, 293 cells were seeded at a density of 2.5×10^5 cells/well in 6-well flat-bottomed microassay plates (Falcon Co., Becton Dickenson, Franklin Lakes, NJ) 24 hrs before transfection.

Cholesterol or dexamethasone was dissolved in ethanol. Cholesterol or dexamethasone solution was added to the cells at various concentrations 30 min before transfection. As a control, same volume of ethanol was added to the cells. PEI/pCMV-Luc complexes were prepared at a 5/1 N/P ratio. PAMAM/pCMV-Luc complex was prepared at a 6/1 N/P ratio. The polymer/pCMV-Luc complexes were added to the cells. The amount of pCMV-Luc was fixed at a 2 μg/well. The cells were then incubated for 4 hrs at 37 °C in a 5% CO_2 incubator. After 4 hrs, the transfection mixtures were removed and 2 mL of fresh DMEM medium containing FBS. The cells were incubated for an additional 44 hrs at 37 °C.

Luciferase assay. After transfection, the cells were washed with PBS twice, and 200 μL of reporter lysis buffer (Promega, Madison, WI) was added to each well. After 15 min of incubation at room temperature, the cells were harvested and transferred to microcentrifuge tubes. After 15 sec of vortexing, the cells were centrifuged at 11 k rpm for 3 min. The protein concentrations of the extracts were determined by using Pro-measure protein assay kit (Intron Biotechnology, Seoul, Korea). Luciferase activity was measured in terms of relative light units (RLU) using a Luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). The final values of luciferase were reported in terms of RLU/mg total protein.

Cytotoxicity assay. Evaluation of cytotoxicity was performed by the 3-[4,5-dimetylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay. 293 cells were seeded at a density of 2×10^4 cells/well in 96-well microassay plates (Falcon Co., Becton dickenson, Franklin Lakes, NJ), and incubated for 24 hrs before transfection. Cholesterol or dexamethasone was added to the cells at various concentrations 30 min before transfection. PEI/pCMV-Luc complex was prepared at a 5/1 N/P ratio and PAMAM/pCMV-Luc was formulated at a 6/1 N/P ratio. The amount of pCMV-Luc was fixed at 0.2 μg/well. After the incubation at 37 °C for 4 hrs, the transfection mixture was replaced with 100 μL of fresh DMEM medium supplemented with 10% FBS. The cells were incubated for an additional 44 hrs at 37 °C. After the incubation, 24 μL of 2 mg/mL MTT solution in PBS was added. The cells were incubated for an additional 4 hrs at 37 °C and then MTT-containing medium was aspirated off and 150 μL of DMSO was added to dissolve the formazan crystal formed by live cells. Absorbance was measured at 570 nm. The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = (OD_{570(sample)}/OD_{570(control)}) \times 100$$

where the OD 570 (sample) represents the measurement from the wells incubated with dexamethasone and the OD 570 (control) represents the measurements from the wells treated with ethanol only.

Results

Stability of polymer/DNA complex in the presence of dexamethasone. The stability of polymer/DNA complex in a solution is one of requirements of polymeric gene delivery. To evaluate the stability of polymer/DNA complex in the presence of dexamethasone, a gel retardation assay was carried out. PEI/DNA and PAMAM/DNA complexes were prepared at a 5/1 N/P ratio and a 6/1 N/P ratio, respectively. Dexamethasone was added to the complex solution up to 20 μg/mL concentration. Agarose gel electrophoresis was performed with the polymer/DNA complexes with dexamethasone. As a result, PEI/DNA complex was completely retarded in the presence of dexamethasone (Fig. 1). PAMAM/DNA complexes were not disrupted by dexamethasone and were completely retarded in the presence of dexamethasone (Fig. 1). Therefore, the stability of the polymer/DNA complexes was not disrupted with dexamethasone.

***In vitro* transfection assay with polymer/pCMV-Luc complexes in the presence of dexamethasone.** To verify the effect of dexamethasone on the transfection efficiency of polymer/DNA complex, the transfection assays were performed in the presence of dexamethasone or without dexamethasone. Dexamethasone was added to the cells at various concentrations and the cells were incubated for 30 min. Then, polymer/pCMV-Luc complex was added to the

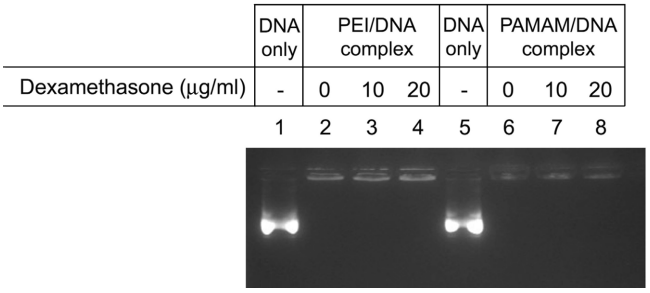

Figure 1. Gel retardation assay. PEI/pCMV-Luc and PAMAM/pCMV-Luc complex was prepared at a 5/1 N/P ratio and a 6/1 N/P ratio, respectively. Increasing amounts of dexamethasone were added to polymer/DNA complexes. The mixtures were incubated at room temperature for 30 min and electrophoresed on 1% (w/v) agarose gel.

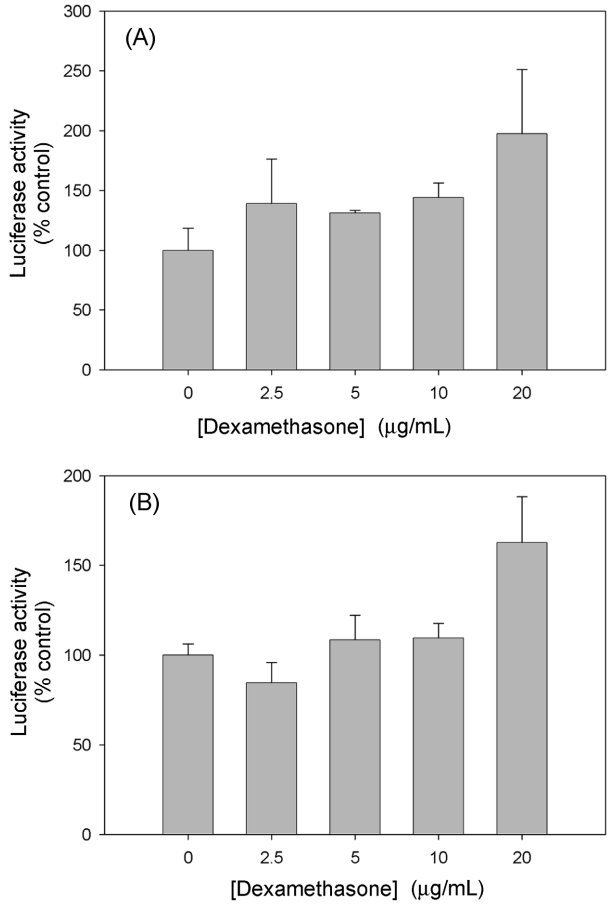

Figure 2. Transfection assay in the presence of dexamethasone. 293 cells were seeded in 6-well microassay plates. 293 cells were incubated with various amount of dexamethasone for 30 min before transfection. After the incubation, PEI/pCMV-Luc complex (A) or PAMAM/pCMV-Luc complex (B) was added to the cells. After the transfection, the cells were harvested and luciferase activity was measured. The data is expressed as mean values (± standard deviation) of four experiments.

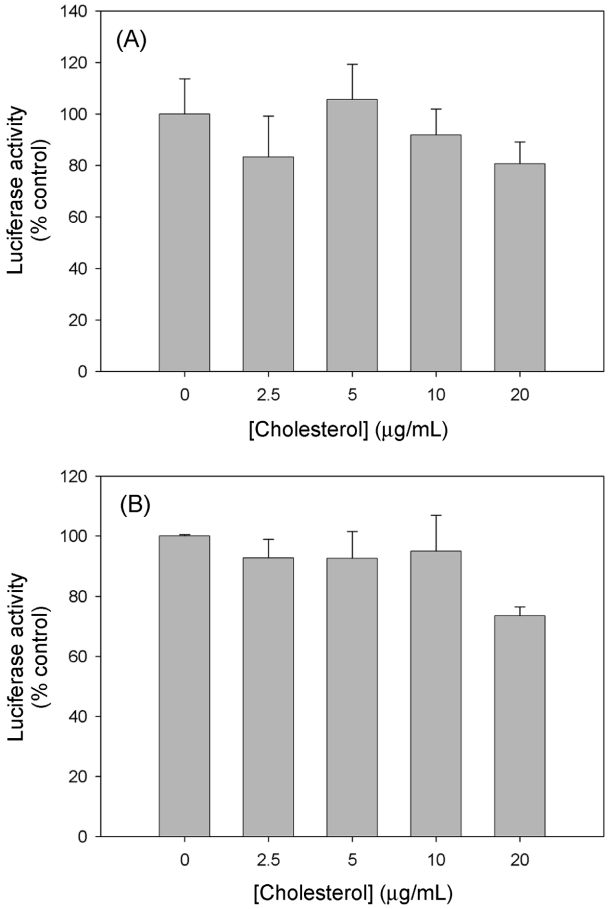

Figure 3. Transfection assay in the presence of cholesterol. 293 cells were seeded in 6-well microassay plates. 293 cells were incubated with various amount of cholesterol for 30 min before transfection. After the incubation, PEI/pCMV-Luc complex (A) or PAMAM/pCMV-Luc complex (B) was added to the cells. After the transfection, the cells were harvested and luciferase activity was measured. The data is expressed as mean values (± standard deviation) of four experiments.

cells. The transgene expression was measured by luciferase assay. The results showed that the transgene expression was increased in the PEI mediated transfection (Fig. 2A) and in the PAMAM mediated transfection (Fig. 2B). These results suggest that dexamethasone increase transfection efficiency of polymeric carriers. This enhancement of transfection may be due to nuclear pore dilation effect of glucocorticoid receptor. Cholesterol, which has similar structure to dexamethasone, was used as a control to verify that transfection enhancement effect is specific to dexamethasone. The cells were preincubated with cholesterol at various concentrations and the transfection assay was performed with PEI/pCMV-Luc and PAMAM/pCMV-Luc complexes. The transfection was performed in a same way as with dexamethasone. The transgene expression was evaluated by luciferase assay. In the PEI mediated transfection, the transfection efficiency did not increase (Fig. 3A). Similarly, the transfection efficiency of PAMAM was not increased by the preincubation with cholesterol (Fig. 3B). Therefore, transfection enhancement effect results from preincubation of the specific steroid, dexamethasone.

Cytotoxicity of dexamethasone. To evaluate the cytotoxicity of dexamethasone to the cells, MTT assay was carried out. Dexamethasone was added to the cells 30 mins before transfection. Then, PEI/DNA or PAMAM/DNA complex was added to cells. After transfection, the cell viability was evaluated by MTT assay. The results showed that dexamethasone did not have any effect on the cytotoxicity of PEI/DNA complexes (Fig. 4A). In addition, the cytotoxicity of PAMAM/DNA complexes was not altered by the addition of dexamethasone (Fig. 4B). These results suggest that the cell viability was not affected by the preincubation of dexamethasone at the indicated concentration range.

Discussion

Glucocorticoid receptor has two favorable effects in the polymer mediated gene delivery. First, the receptor is translocated into nucleus in the presence of its ligand.[12]

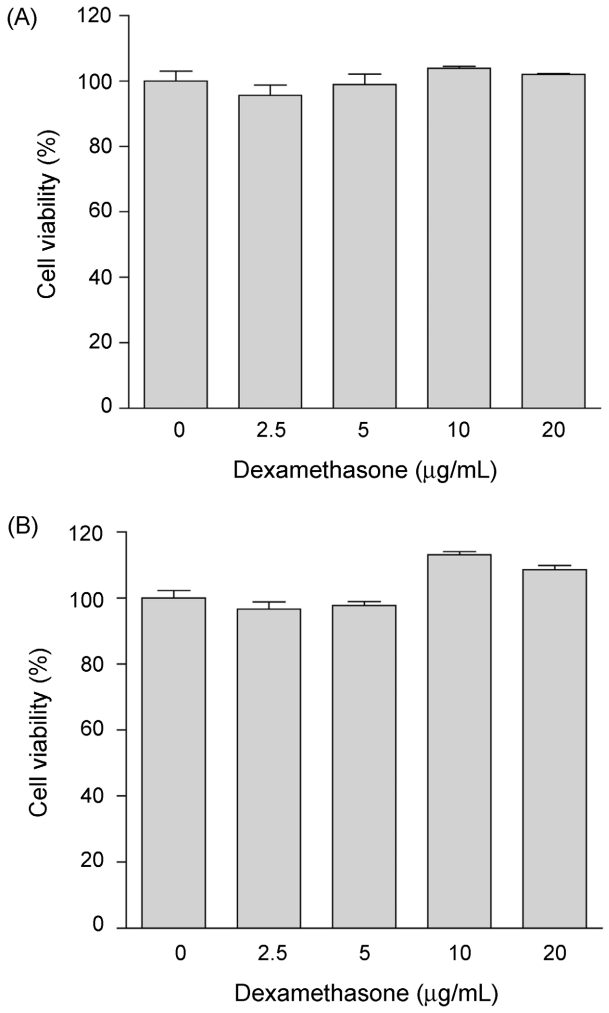

Figure 4. Cytotoxicity of PEI or PAMAM in the presence of dexamethasone. 293 cells were seeded in 96-well microassay plates. Dexamethasone was added to the cells at the concentration of 20 μg/mL 30 min before transfection. PEI/pCMV-Luc complex (A) or PAMAM/pCMV-Luc complex (B) was added to the cells. After transfection, cell viability was measured by MTT assay. The data is expressed as mean values (± standard deviation) of four experiments.

Therefore, it can be used as a nuclear translocation signal. In the previous report, dexamethasone was conjugated to DNA.[16] The dexamethasone-decorated DNA was translocated in a glucocorticoid receptor-dependent manner and accumulated in the nucleus. The nuclear accumulation of the dexamethasone-decorated DNA enhanced the transgene expression. Similarly, dexamethasone-spermine cationic lipid was synthesized for rapid translocation of the delivered gene into nucleus.[17] Dexamethasone-spermine cationic lipid with a neutral lipid, dioleoylphosphatidylethanolamine (DOPE), had potent gene delivery capability, compared to unconjugated mixtures of dexamethasone, spermine, and DOPE. Therefore, the nuclear translocation effect of glucocorticoid receptor in the presence of its ligand is useful for non-viral gene delivery. Second, the glucocorticoid receptor dilates the nuclear pore in the process of translocation into nucleus. The size of nuclear pore is around 9 nm, allowing passive diffusion of small molecules. In the process of translocation of glucocorticoid receptor, the receptor dilates the average NPC channel diameter to 60 nm.[13] The dilation of nuclear pore by glucocorticoid receptor may facilitate the nuclear translocation of DNA. In this study, the nuclear pore dilation effect on the transfection efficiencies of polymeric carriers was investigated in the presence of dexamethasone. The transfection assays showed that dexamethasone enhanced the transfection efficiencies of PEI and PAMAM. In addition, this effect was not observed in the cells preincubated with cholesterol. Therefore, transfection enhancement effect is specific for dexamethasone, suggesting the nuclear translocation of DNA was facilitated by nuclear pore dilation effect of glucocorticoid receptor.

To use this techniques *in vitro* or *in vivo* gene delivery, polymer/DNA complexes should be stable in the presence of dexamethasone and non-toxic to cells. The gel retardation assay suggested that the stability of polymer/DNA complex was not disrupted (Fig. 1). In addition, MTT assay of polymer/DNA complex showed that the cytotoxicity of polymer/DNA complex was not altered in the presence of dexamethasone (Fig. 4A and 4B).

Dexamethasone has pharmacological effect in cells as the most effective anti-inflammatory drug.[12] The glucocorticoid receptor is a transcription regulator, increasing or decreasing the expression of the responsive genes. Decreasing the expression of the pro-inflammatory gene is the main mechanism of glucocorticoid anti-inflammatory effect. This alteration of gene expression may be beneficial to inflammatory disease gene therapy. Dexamethasone may be directly applicable to the local gene delivery with established polymeric gene carriers, which requires anti-inflammatory effect. In this way, dexamethasone has double advantages; anti-inflammatory effect and enhancement effect of therapeutic gene delivery. In addition, dexamethasone may be useful in the development of efficient polymeric gene carriers.

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