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Synthesis and Characterization of Dexamethasone-Conjugated Linear Polyethylenimine as a Gene Carrier

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ABSTRACT

Linear polyethylenimine (25 kDa, LPEI25k) has been shown to be an effective non-viral gene carrier with higher transfection and lower toxicity than branched polyethylenimine (BPEI) of comparable molecular weight. In this study, dexamethasone was conjugated to LPEI25k to improve the efficiency of gene delivery. Dexamethasone is a synthetic glucocorticoid receptor ligand. Dexamethasone-conjugated LPEI25k (LPEI–Dexa) was evaluated as a gene carrier in various cells. Gel retardation assays showed that LPEI–Dexa completely retarded plasmid DNA (pDNA) at a 0.75:1 weight ratio (LPEI/pDNA). LPEI–Dexa had the highest transfection efficiency at a 2:1 weight ratio (LPEI–Dexa/DNA). At this ratio, the size of the LPEI–Dexa/pDNA complex was approximately 125 nm and the zeta potential was 35 mV. LPEI–Dexa had higher transfection efficiency than LPEI and Lipofectamine 2000. In addition, the cytotoxicity of LPEI–Dexa was much lower than that of BPEI (25 kDa, BPEI25k). In conclusion, LPEI–Dexa has a high transfection efficiency and low toxicity and can therefore be used for non-viral gene delivery. J. Cell. Biochem. 110: 743–751, 2010.

KEY WORDS: BRANCHED POLYETHYLENIMINE (BPEI); DEXAMETHASONE; GENE DELIVERY; LINEAR POLYETHYLENIMINE (PEI); TRANSFECTION

Polyethylenimine (PEI) is one of the most widely used polymeric gene carriers [Han et al., 2000; Lee and Kim, 2002, 2005; Kang et al., 2005]. PEI can condense DNA efficiently and has high transfection activity compared with other polymeric carriers such as poly-L-lysine [Boussif et al., 1995]. There are two types of PEIs: branched and linear PEIs. PEIs have different characteristics in vitro and in vivo, depending on their structures [Wightman et al., 2001; Bertschinger et al., 2006]. In a salt-containing condition, the transfection efficiency of linear PEI (25 kDa, LPEI25k)/DNA complexes was higher than that of branched PEI (25 kDa, PEI25k)/DNA complexes, although LPEI25k/DNA complexes formed larger particles [Wightman et al., 2001]. In addition, LPEI25k and BPEI25k have different physical characteristics. LPEI25k forms complex with DNA and the complex size is around 100 nm in salt-free conditions. However, the gradual addition of salt increases the size of LPEI25k/DNA complex until the complex ultimately forms aggregates. BPEI forms complex of constant size with DNA even in the presence of salt.

BPEI25k has relatively high transfection efficiency due to its high DNA affinity and proton-buffering effect [Boussif et al., 1995; Lee et al., 2003b; Akinc et al., 2005; Kang et al., 2005]. However, BPEI25k has a high cytotoxicity and, therefore, clinical application of BPEI25k has been limited [Fischer et al., 1999; Benns et al., 2001; Petersen et al., 2002; Lee and Kim, 2005]. High cytotoxicity of BPEI25k may be due to the large number of positively charged primary amines. It has previously been shown that PEI with less branches and smaller molecular weight is less toxic to cells [Fischer et al., 1999; Godbey et al., 1999]. Furthermore, it has been shown that LPEI25k is an effective non-viral gene carrier with a higher gene expression and lower toxicity than BPEI of comparable molecular weight in certain types of cells [Wightman et al., 2001; Brissault et al., 2006]. In addition, LPEI25k has over 520 secondary amines, which may have proton-buffering effect. Therefore, LPEI25k may enhance the endosomal escape of polymer/DNA complexes.

The glucocorticoid receptor is an intracellular receptor [Yu et al., 1992]. Dexamethasone, a potent synthetic glucocorticoid, binds to the glucocorticoid receptor after cellular entry, and the receptor/dexamethasone complex translocates into the nucleus [Strubing and Clapham, 1999; Rebuffat et al., 2001]. Therefore, dexamethasone-conjugated polymer/DNA complexes are efficiently delivered into

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the nucleus with the glucocorticoid receptor, resulting in increased transgene expression [Choi et al., 2006; Bae et al., 2007; Kim et al., 2009]. Furthermore, during translocation of the glucocorticoid receptor into the nucleus, nuclear pores are dilated, which may aid nuclear entry of polymer/DNA complexes [Shahin et al., 2005].

In general, improvement in the transfection efficiency of LPEI25k would be useful to non-viral gene therapy, since higher transfection rates can reduce the dose of the drug or gene required, thereby increasing the treatment efficacy. In this study, dexamethasone was conjugated to LPEI25k to enhance the transfection efficiency of LPEI25k. Dexamethasone-conjugated LPEI25k (LPEI–Dexa) was characterized and evaluated as a gene carrier in various cells. Our results suggest that LPEI–Dexa is a useful gene carrier with high transfection efficiency and low cytotoxicity.

MATERIALS AND METHODS

SYNTHESIS OF LINEAR PEI–DEXA

LPEI25k was dissolved in 1.8 ml of anhydrous dimethyl sulfoxide (DMSO) with a 25-fold molar excess of Traut's reagent and dexamethasone-21-mesylate as described previously, with some modifications [Choi et al., 2006]. To minimize cross-linking side reactions, anhydrous DMSO was used, and humidity was mitigated during the reaction. The reaction was allowed to proceed for 4 h at room temperature and was then quenched by the addition of an excess amount of cold ethyl acetate. The precipitated product was solubilized in water and dialyzed for 1 day against pure water using a dialysis membrane (MWCO, 1,000). The mixture was further freeze-dried, and a white product was obtained (50% yield). The product was solubilized D_2O for ^1H NMR analysis (300 MHz, Korea Basic Science Institute).

PREPARATION OF PLASMID DNA (PDNA)

pSV-Luc was purchased from Promega (pGL3-promoter, Madison, WI). pEGFP-N1 was purchased from Clontech (Mountain View, CA). pCMV-Luc and pSV-VEGF were constructed previously [Han et al., 2001; Lee et al., 2003a]. pDNAs were transformed in *Escherichia coli* DH5α and amplified in Terrific Broth media at 37°C overnight at 230 rpm. pDNAs were purified using the Maxi plasmid purification kit (Qiagen, Valencia, CA). Purified pDNA was dissolved in Tris–EDTA (TE) buffer. The purity and concentration of pDNA were determined by ultraviolet (UV) absorbance at 260 nm. The optical density ratios at 260–280 nm were in the range of 1.7–1.8.

AGAROSE GEL RETARDATION ASSAY

LPEI–Dexa/pSV-Luc and LPEI25k/pSV-Luc complexes were prepared at various weight ratios ranging from 0 to 5 in PBS. After 30 min of incubation at room temperature, the samples were subjected to electrophoresis on a 1% agarose gel containing ethidium bromide. After electrophoresis, the gel was analyzed on a UV illuminator to determine the location of the pDNA.

PARTICLE SIZE AND POTENTIAL MEASUREMENTS

LPEI–Dexa/pSV-Luc complexes were prepared at several weight ratios to measure particle size and zeta potential. As a control, LPEI/pSV-Luc complexes were prepared at various weight ratios. After 30 min of incubation at room temperature for complex formation, the particle sizes and zeta potentials of the complexes were determined using the Zetasizer Nano ZS system (Malvern Instruments, UK) at 25°C.

CELL CULTURES AND TRANSFECTIONS

Rat cardiomyocytes H9C2, rat smooth muscle A7R5, human embryonic kidney (HEK) 293, NIH3T3, and mouse leukemic monocyte macrophage Raw264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in DMEM supplemented with 10% FBS in a 5% CO_2 incubator. For transfection assays, the cells were seeded at a density of 1×10^5 cells/well in six-well flat-bottomed microassay plates (Falcon Co., Becton Dickinson, Franklin Lakes, NJ) 24 h before transfection. LPEI–Dexa/pSV-Luc complexes were prepared at various weight ratios in 5% glucose solution. LPEI/pSV-Luc complexes were prepared at a 2:1 weight ratio, determined based on optimized transfection assays. BPEI25k/pSV-Luc complexes were prepared at a 5:1 N (nitrogen atom of polymer)/P (phosphate group of DNA) ratio based on previous reports [Abdallah et al., 1996; Lemkine et al., 1999; Turunen et al., 1999; Nguyen et al., 2000]. Lipofectamine 2000 (Invitrogen, Carlsbad, CA)/pSV-Luc complexes were prepared at a 2:1 weight ratio as suggested by the manufacturer. Dexamethasone-conjugated small molecular weight BPEI (2 kDa, PEI2k-Dexa)/pSV-Luc complexes were prepared at an 8:1 weight ratio as described in the previous report [Bae et al., 2007; Kim et al., 2009]. Before transfection, the medium was replaced with 2 ml of fresh DMEM without FBS. Then, the polymer/pSV-Luc complexes were added to the cells. The amount of pSV-Luc was fixed at 1 μg/well. The cells were then incubated for 4 h at 37°C in a 5% CO_2 incubator. After 4 h, the transfection mixtures were removed, and 2 ml of fresh DMEM medium containing FBS was added. The cells were incubated for an additional 20 h at 37°C.

LUCIFERASE ASSAYS

After transfection, the cells were washed twice with PBS, and 200 μl of reporter lysis buffer (Promega) was added to each well. After 15 min of incubation at room temperature, the cells were harvested and transferred to microcentrifuge tubes. After 15 s of vortexing, the cells were centrifuged at 13,000 rpm for 5 min. The extracts were transferred to fresh tubes and stored at −70°C until use. The protein concentration of the extract was determined with a BCA protein assay kit (Pierce, Iselin, NJ). Luciferase activity was measured in terms of relative light units (RLU) using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Luciferase activity was monitored and integrated over a period of 20 s. Final luciferase values were reported in terms of RLU/mg total protein.

TUMOR NECROSIS FACTOR (TNF)-α ASSAY

Raw264.7 cells were seeded at a density of 1×10^5 cells/well in six-well plates 24 h before transfection. For the inflammatory activation, Raw264.7 cells were treated with 20 ng/ml lipopolysaccharide (LPS, *E. coli* 055:B5; Sigma, St. Louis, MO). Polymer/pSV-Luc complexes were prepared at optimized weight ratios. Transfection was performed as described above. The TNF-α level

was measured by enzyme-linked immunosorbent assay (ELISA) using a mouse TNF-α ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's instruction.

FLOW CYTOMETRY

H9C2 and A7R5 cells were seeded at a density of 1×10^5 cells/well in six-well flat-bottomed microassay plates (Falcon Co., Becton Dickinson) 24 h before transfection. Polymer/pEGFP-N1 complexes were prepared at optimized weight ratios. Transfection was performed as described above. After transfection, the cells were harvested to fresh tubes. The cells were washed with PBS and then the suspended cells were centrifuged at 2,000 rpm for 5 min. The supernatant was removed and the cells were resuspended in FACS buffer (PBS with 0.02% NaN_3, 0.2% FBS). After centrifugation at 2,000 rpm for 5 min, the supernatant was removed and the cells were resuspended in fixing buffer (FACS buffer with 1% formaldehyde). The cells were transferred to FACS tubes. Flow cytometry was performed using the BD FACSCalibur™ (BD Biosciences Immunocytometry Systems, San Jose, CA).

CONFOCAL IMAGES

HeLa cells were plated at 5×10^3 cells/well in an eight-well Lab-Tek chambered coverglass the day before transfection. After 16–20 h incubation, the cells were treated with the Alexa 647 labeled pCMV-Luc and LPEI, LPEI–Dexa complexes in DMEM containing 10% FBS (250 ml/well). The cells were then incubated further for 20 h. Next day, cells were rinsed twice with PBS, fixed with 10% formalin for 5 min and nuclei were stained with 10 μg/ml Hoechst33342 for 5 min. The slide from media chamber was detached and the cells were covered with mounting solution (Vectashield mounting medium for fluorescence, Vector Labs, Burlingame, CA) and a coverslip. The distribution of fluorescence was analyzed on a Zeiss LSM5 LIVE microscope.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) ASSAY

H9C2 cells were seeded at a density of 1×10^5 cells/well in six-well plates 24 h before transfection. Polymer/pSV-VEGF complexes were prepared at optimized weight ratios. Transfection was performed as described above. The VEGF level was measured by ELISA using a human VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

CYTOTOXICITY ASSAY

Evaluation of cytotoxicity was performed by the MTT assay. H9C2 and A7R5 cells were seeded at a density of 1×10^4 per well in 24-well plates and incubated for 24 h before transfection. LPEI25k/pSV-Luc and LPEI-Dexa/pSV-Luc were prepared at a 2:1 weight ratio (Polymer/pSV-Luc) in a 5% glucose solution. The amount of pSV-Luc was fixed at 0.3 μg/well. The medium was replaced with fresh DMEM without FBS before transfection, and polymer/pSV-Luc complexes were added to the cells. After incubation at 37°C for 4 h, the transfection mixture was replaced with 500 μl of fresh DMEM supplemented with 10% FBS, and the cells were incubated for an additional 24 h at 37°C. After incubation, MTT solution in PBS was added. The cells were incubated for an additional 4 h at 37°C. After the incubation, MTT-containing medium was aspirated off and 750 ml of DMSO was added to dissolve the formazan crystals formed by live cells. Absorbance was measured at 570 nm. The cell viability (%) was calculated according to the following equation:

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

where the $OD_{570(\text{sample})}$ represents the measurement from the well treated with polymer/plasmid DNA complex and the $OD_{570(\text{control})}$ represents the measurements from the well treated with 5% glucose.

STATISTICAL ANALYSIS

The one-way analysis of variance (ANOVA) was used for comparisons involving more than two groups. The comparisons with two groups were made by Student's *t*-test. *P*-value under 0.05 was thought to be statistically significant.

RESULTS

SYNTHESIS AND CHARACTERIZATION OF LPEI–DEXA

Dexamethasone was conjugated to LPEI25k using Traut's reagent (Fig. 1). The synthesis of LPEI–Dexa was confirmed by ^{1}H NMR. From the NMR spectrum, it was calculated that 23 dexamethasone units were conjugated per LPEI. Formation of PEI-Dexa/pSV-Luc complexes was confirmed by a gel retardation assay. LPEI–Dexa/pSV-Luc complexes were prepared at various weight ratios, and LPEI/pSV-Luc complexes were prepared as controls. In Figure 2A, LPEI25k retarded pSV-Luc completely at a 5:1 weight ratio. However, LPEI–Dexa retarded pSV-Luc at a 0.75:1 weight ratio, suggesting that LPEI–Dexa has a greater ability to form complexes with pDNA than does LPEI25k (Fig. 2B).

The particle size and surface charge of LPEI–Dexa/DNA complex were measured by dynamic light scattering and zeta-potential measurements. LPEI–Dexa/pSV-Luc complexes were prepared at various weight ratios. As a control, LPEI25k/pSV-Luc complexes were prepared at a 2:1 weight ratio, since the LPEI25k/pSV-Luc complexes showed the highest transfection efficiency at a 2:1 weight ratio (data not shown). The size of LPEI–Dexa/pSV-Luc complex was decreased up to a 1.5:1 weight ratio and the size at or above a 1.5:1 weight ratio was stable with a particle size of approximately 125 nm (Fig. 3A). The size of the LPEI–Dexa/pSV-Luc complex was smaller than that of the LPEI25k/pSV-Luc complex. The zeta potential of LPEI–Dexa/pSV-Luc complex increased with increasing weight ratios and was saturated at a 2:1 weight ratio (Fig. 3B).

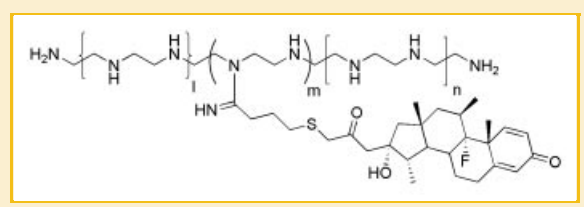

Fig. 1. Structure of LPEI–Dexa.

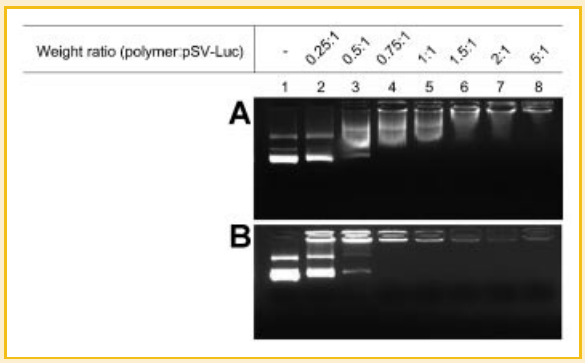

Fig. 2. Gel retardation assay. PEI/pSV-Luc (A) and LPEI–Dexa/pSV-Luc (B) complexes were prepared at various weight ratios. The mixtures were incubated at room temperature for 30 min and then electrophoresed on a 1% (w/v) agarose gel.

TRANSFECTION EFFICIENCY OF LPEI–DEXA

To evaluate the transfection efficiency of LPEI–Dexa, in vitro transfection assays were performed. To optimize the transfection conditions for LPEI–Dexa, LPEI–Dexa/pSV-Luc complexes were prepared at various weight ratios and transfected into H9C2 or A7R5 cells. In H9C2 cells, the transfection efficiency of LPEI–Dexa was saturated at a 2:1 weight ratio (LPEI–Dexa/pSV-Luc) (Fig. 4A). These results were also obtained in transfection assays to A7R5 cells (Fig. 4B). Therefore, LPEI–Dexa/pSV-Luc complexes were prepared at a 2:1 weight ratio for all subsequent experiments.

To compare LPEI–Dexa with other non-viral gene carriers, transfection assays were performed with LPEI25k, LPEI–Dexa, BPEI25k, and Lipofectamine 2000, and the complexes were transfected into H9C2 cells. LPEI–Dexa had higher transfection efficiency than LPEI25k, BPEI25k, and Lipofectamine 2000 (Fig. 5A). LPEI–Dexa also showed the highest transfection efficiency among the carriers in A7R5 cells (Fig. 5B). These results indicate that

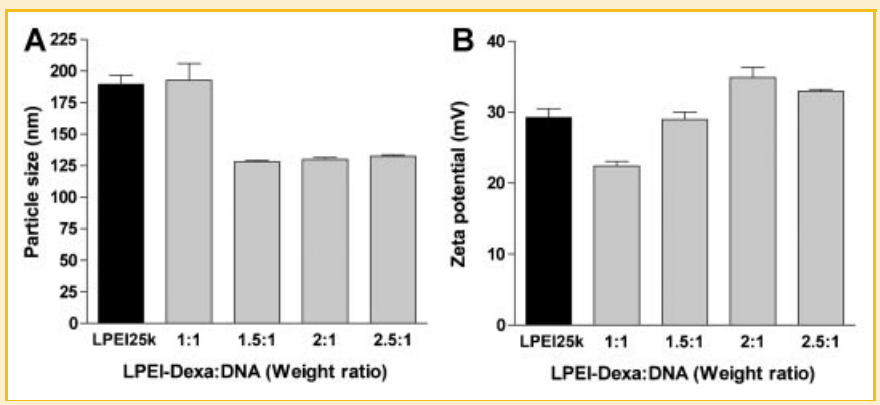

Fig. 3. Particle size (A) and zeta potential (B) of LPEI–Dexa/DNA according to various weight ratios. The data are expressed as the mean values (±standard deviation) of three different experiments.

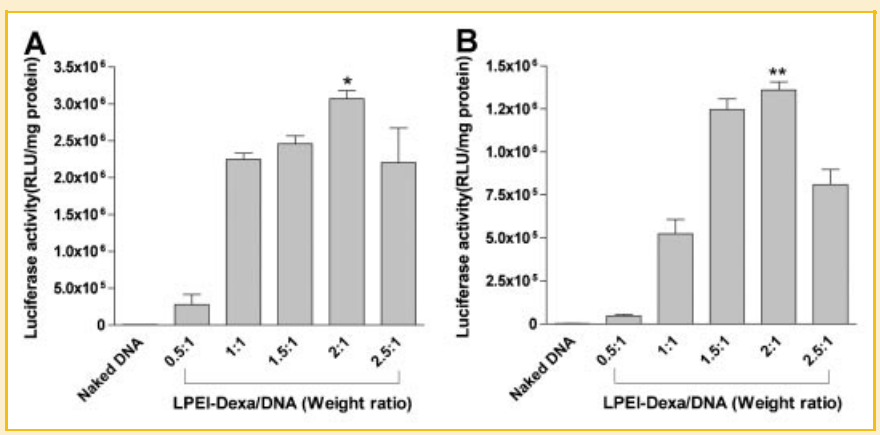

Fig. 4. Transfection efficiency of LPEI–Dexa according to weight ratio. LPEI–Dexa/pSV-Luc complexes were prepared at various weight ratios and transfected into H9C2 (A) and A7R5 (B) cells. Transfection efficiency was measured by luciferase assays. The data are expressed as the mean values (±standard deviation) of quadruplicate experiments. Significance was determined by one-way analysis of variance (ANOVA). $^{*}P < 0.01$ as compared with naked DNA, 0.5:1, and 1:1 weight ratios. $^{**}P < 0.01$ as compared with naked DNA, 0.5:1, 1:1, and 2.5:1.

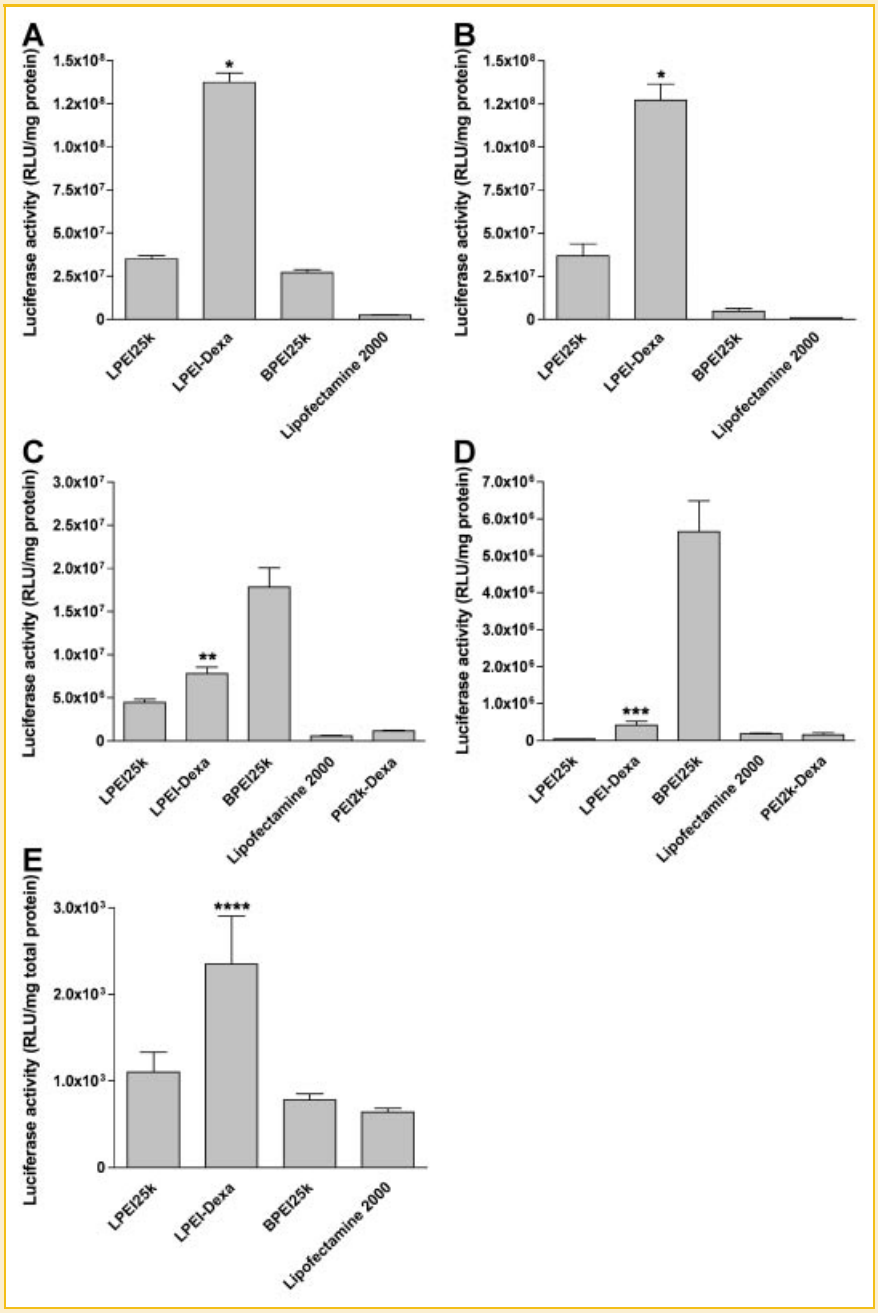

Fig. 5. Transfection efficiency of LPEI–Dexa. Polymer/pSV-Luc complexes were prepared as described in the Materials and Methods Section and transfected into H9C2 (A), A7R5 (B), HEK293 (C), NIH3T3 (D), and Raw264.7 (E) cells. The transfection efficiency of each complex was measured by luciferase assay. The data are expressed as the mean values (±standard deviation) of quadruplicate experiments. Significance was determined by one-way analysis of variance (ANOVA). $^{*}P < 0.01$ as compared with LPEI25k, BPEI25k, and Lipofectamine 2000. $^{**}P < 0.01$ as compared with BPEI25k, Lipofectamine 2000, and PEI2k-Dexa. $^{***}P < 0.01$ as compared with BPEI25k, $^{****}P < 0.05$ as compared with BPEI25k and Lipofectamine 2000.

dexamethasone conjugation improves the transfection efficiency of LPEI. In HEK293 or NIH3T3 cells, BPEI25k had higher transfection than LPEI25k and LPEI–Dexa. However, the transfection efficiency of LPEI–Dexa was higher than that of LPEI25k, suggesting that conjugation of dexamethasone improved the transfection efficiency of LPEI252k (Fig. 5C,D). In addition, LPEI–Dexa showed higher transfection efficiency than PEI2k-Dexa in HEK293 and NIH3T3 cells. In Raw264.7 cells, the transfection efficiencies of the carriers were comparatively low (Fig. 5E). The luciferase activities of the transfected Raw264.7 cells are lower than those in other cells. However, LPEI–Dexa showed the highest efficiency among the carriers in Raw264.7 cells (Fig. 5E).

Raw264.7 cells secreted inflammatory cytokines such as TNF-α in response to activation by LPS. Since dexamethasone is an anti-inflammatory drug, LPEI–Dexa might reduce the TNF-α secretion. To identify this anti-inflammatory effect of LPEI–Dexa, the TNF-α

level was measured by ELISA after transfection of LPEI–Dexa/pDNA complex. Raw264.7 cells were activated with LPS and then, polymer/pDNA complexes were added to the cells. The ELISA results showed that LPEI–Dexa did not reduce the TNF-α level (Fig. 6). It seems that the concentration of intracellular LPEI–Dexa was not enough to reduce TNF-α level.

To evaluate the rate of transfected cells with each carrier, the cells were transfected with polymer/pEGFP-N1 complexes and analyzed by flow cytometry. In transfection assay to H9C2 cells, LPEI–Dexa showed higher number of GFP positive cells than LPEI25k, BPEI25k, and Lipofectamine 2000 (Fig. 7A). LPEI–Dexa also showed the highest number of GFP positive cells among the carriers in A7R5 cells (Fig. 7B).

NUCLEAR TRANSLOCATION OF LPEI–DEXA/PDNA COMPLEX

The higher transfection efficiency of LPEI–Dexa than LPEI25k may be due to its efficient nuclear translocation. Therefore, confocal microscope analysis was performed with LPEI–Dexa and LPEI25k. HeLa cells were transfected with LPEI–Dexa/pDNA or LPEI25k/pDNA complexes. In the LPEI–Dexa/DNA complex transfected cells, more fluorescently labeled DNAs were identified in the nucleus than in the LPEI25k/DNA-transfected cells (Fig. 8).

VEGF GENE DELIVERY EFFICIENCY OF LPEI–DEXA

To evaluate the therapeutic gene delivery efficiency of LPEI–Dexa, pSV-VEGF was transfected into H9C2 cells with LPEI25k, LPEI–Dexa, BPEI25k, and Lipofectamine 2000. VEGF is one of the therapeutic genes for ischemic disease gene therapy [Isner et al., 1996; Kutryk and Stewart, 2003; Lee et al., 2003a]. VEGF induces angiogenesis to increase blood supply to the ischemic region. In vitro transfection of pSV-VEGF with the carriers showed that LPEI–Dexa had the highest VEGF gene delivery efficiency (Fig. 9). The results confirmed that LPEI–Dexa had higher efficiency than LPEI25k and Lipofectamine 2000 in the delivery of therapeutic genes as well as reporter genes.

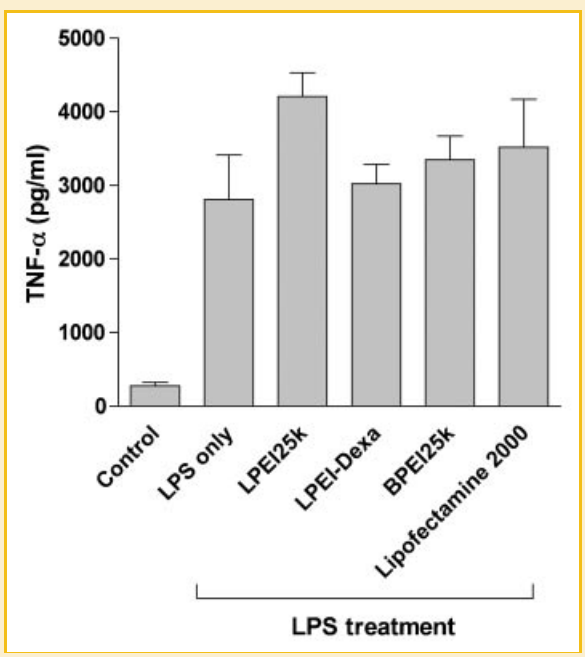

Fig. 6. TNF-α assay after transfection of LPEI–Dexa/pSV-Luc complex to the activated Raw264.7 cells. Raw264.7 cells were activated with LPS. Polymer/pSV-Luc complexes were added to the activated Raw264.7 cells. After 24 h of incubation, the culture media were harvested and the TNF-α level was measured by ELISA. The data are expressed as the mean values (±standard deviation) of quadruplicate experiments.

CYTOTOXICITY OF LPEI–DEXA

To evaluate the cytotoxicity of LPEI–Dexa, LPEI–Dexa/pSV-Luc complexes were transfected into H9C2 cells and A7R5 cells. The cytotoxicity of LPEI–Dexa was compared with those of LPEI25k, BPEI25K, and Lipofectamine 2000. Polymer/pSV-Luc complexes were transfected into H9C2 or A7R5 cells and the cytotoxicity was measured using the MTT assay. In H9C2 cells, the cytotoxicity of LPEI–Dexa was slightly higher than that of LPEI, suggesting that the conjugation of dexamethasone increased the toxicity of the polymer. However, LPEI–Dexa was less toxic than BPEI25k in H9C2 cells (Fig. 10A). In A7R5 cells, LPEI–Dexa showed slightly higher toxicity than LPEI, but much less toxicity than BPEI25k (Fig. 10B).

DISCUSSION

In this study, dexamethasone was conjugated to LPEI25k to improve the transfection efficiency of LPEI25k. Physical characterization of LPEI–Dexa/pDNA complex was performed. In addition, in vitro transfection and cytotoxicity assays were performed to evaluate LPEI–Dexa as a gene carrier. LPEI–Dexa had higher transfection efficiency than LPEI25k and Lipofectamine 2000 in all tested cells. In some types of cells, LPEI–Dexa had higher transfection efficiency than BPEI25k (Fig. 5). Furthermore, the cytotoxicity of LPEI–Dexa was lower than that of BPEI25k (Fig. 10). Considering that BPEI25k and Lipofectamine 2000 are widely used non-viral gene carriers, LPEI–Dexa may be useful for non-viral gene delivery with low toxicity and high transfection efficiency.

LPEI–Dexa showed higher transfection efficiency than LPEI25k, and Lipofectamine 2000 in various cells. This higher transfection efficiency may be due to two effects. First, conjugation of dexamethasone to LPEI25k may facilitate nuclear trafficking of LPEI–Dexa/pSV-Luc complexes. Dexamethasone is a potent glucocorticoid, and it has been reported that conjugation of dexamethasone to DNA, liposomes, or polymers increases gene delivery efficiency. In the presence of ligand, the glucocorticoid receptor changes its conformation and translocates into the nucleus. Therefore, dexamethasone-conjugated polymers may translocate into the nucleus due to binding to the glucocorticoid receptor, which may increase the nuclear transport of DNA bound to the polymer [Choi et al., 2006; Bae et al., 2007]. Dexamethasone has previously been conjugated to polyamidoamine (PAMAM) dendrimers and BPEI2k [Bae et al., 2007; Kim et al., 2009]. Confocal microscope studies using fluorescently labeled DNA have shown that dexamethasone-conjugated polymers

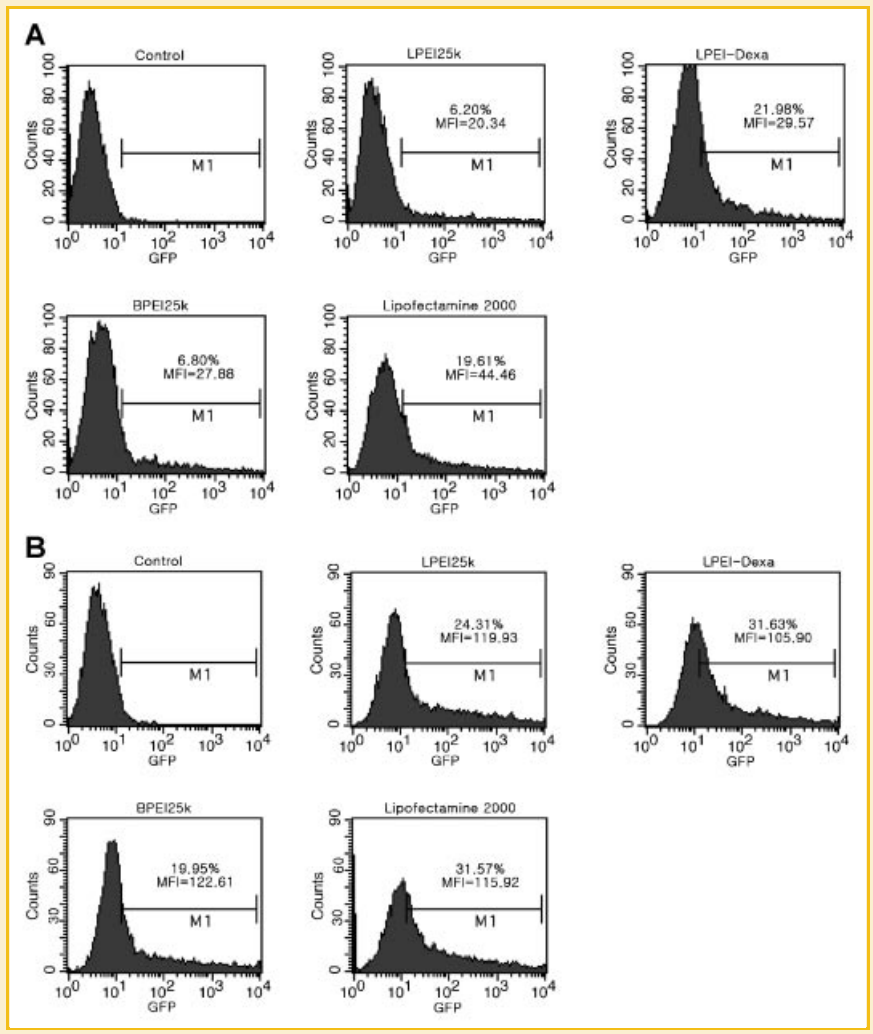

Fig. 7. Flow cytometry after transfection of LPEI–Dexa/pEGFP-N1 complex to H9C2 (A) and A7R5 (B) cells. Polymer/pEGFP-N1 complexes were prepared as described in the Materials and Methods section and transfected into H9C2 (A) and A7R5 (B) cells. The GFP-positive cells were analyzed by flow cytometry.

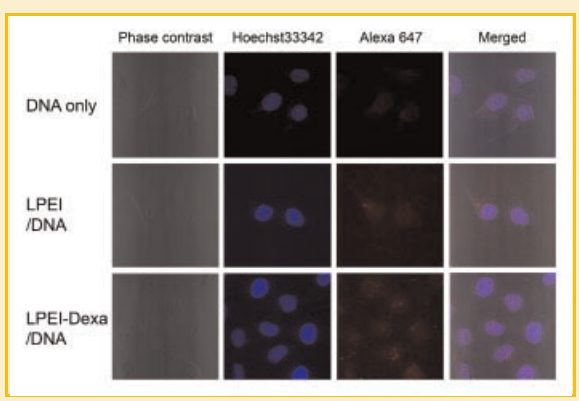

Fig. 8. Confocal microscope analysis after transfection of LPEI–Dexa/pEGFP-N1 complex. Alexa 647 fluorescently labeled pDNA was complexed with LPEI–Dexa or LPEI25k. Polymer/pDNA complexes were transfected into HeLa cells. Naked pDNA was transfected to the cells as a control. After 20 h, the cells were analyzed with confocal microscope. Nuclei were stained with Hoeschst33342.

have higher nuclear transport efficiency than non-conjugated polymers [Choi et al., 2006; Bae et al., 2007], most likely due to the characteristics of the glucocorticoid receptor. The glucocorticoid receptor is a nuclear receptor that is localized in the cytosol in the absence of ligand [Yu et al., 1992]. Another positive effect of dexamethasone in gene delivery is dilation of nuclear pores [Shahin et al., 2005]. When the ligand-bound glucocorticoid receptor enters the nucleus, it dilates the nuclear pores up to 60 nm, which may facilitate DNA trafficking into the nucleus.

Second, LPEI–Dexa may form micelle-like structures in aqueous solution and behave like higher molecular weight LPEI. The dexamethasone of LPEI–Dexa may form a core and induce the formation of a micelle structure with hydrophilic LPEI on the surface. A micelle structure would lead to a higher charge density on the surface. Therefore, LPEI–Dexa in aqueous solution may form tight and stable polymer/pDNA complexes, increasing transfection efficiency. The gel retardation assay and particle size measurements suggested that LPEI–Dexa might form tighter complexes with DNA than does LPEI25k. LPEI–Dexa retarded

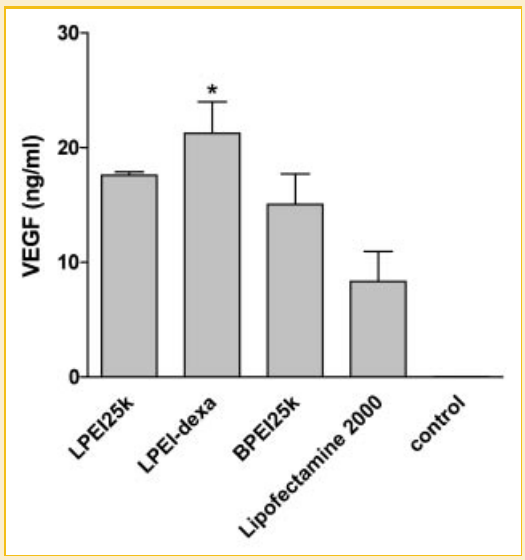

Fig. 9. VEGF assay after transfection of LPEI–Dexa/pSV-VEGF complex to H9C2 cells. Polymer/pSV-VEGF complexes were prepared as described in the Materials and Methods Section and transfected into H9C2 cells. After 24 h incubation, the cell culture media were harvested and the VEGF concentration was measured by ELISA. Significance was determined by one-way analysis of variance (ANOVA). $^{*}P < 0.05$ as compared with control and Lipofectamine 2000.

DNA completely at a lower weight ratio than did LPEI25k (Fig. 2). This effect may also increase the cytotoxicity of the carrier; indeed, LPEI–Dexa is more cytotoxic than LPEI25k (Fig. 10).

In the previous article, PEI2k-Dexa was synthesized by conjugation of dexamethasone to BPEI2k [Bae et al., 2007; Kim et al., 2009]. BPEI2k has different characteristics from LPEI25k or BPEI25k. Due to low molecular weight, BPEI2k has lower transfection efficiency than LPEI25k and BPEI25k. Conjugation of dexamethasone increased the transfection efficiency of BPEI2k remarkably. However, LPEI25k has already high transfection efficiency, which are comparable to that of BPEI25k. Therefore, it was useful to determine whether the transfection efficiency of LPEI25k could be further improved by conjugation of dexamethasone. Indeed, transfection assays showed that conjugation of dexamethasone increased the transfection efficiency of LPEI25k (Fig. 5).

Luciferase assay showed that LPEI–Dexa had higher transfection efficiency than Lipofectamine 2000 in A7R5 and H9C2 cells (Fig. 5A,B). However, DNA delivery efficiency of LPEI–Dexa was almost equal to that of Lipofectamine 2000 on the percentage of GFP-expressing cells (Fig. 7A,B). In LPEI–Dexa mediated transfection, DNA may be transported into the nucleus more efficiently than Lipofectamine-2000-mediated transfection. This may be due to the nuclear translocation effect of glucocorticoid receptor when it binds to its ligand. Therefore, the expression level of the luciferase gene was higher in LPEI–Dexa-mediated transfection than that in Lipofectamine-2000-mediated transfection, although LPEI–Dexa and Lipofectamine 2000 delivered similar level of DNA into the cells.

Dexamethasone is a widely used anti-inflammatory drug. Thus, LPEI–Dexa might have an anti-inflammatory effect. To determine if LPEI–Dexa had an anti-inflammatory effect, LPEI–Dexa/pDNA complexes were added to LPS-activated macrophage cells. LPEI–Dexa, however, did not down-regulate levels of the pro-inflammatory cytokine, TNF-α (Fig. 6). This indicates that the concentration of dexamethasone in the transfection mixture was not high enough to have a physiological effect.

In conclusion, LPEI–Dexa formed stable complexes with pDNA more efficiently than LPEI25k. In addition, LPEI–Dexa had higher transfection efficiency than LPEI25k and Lipofectamine 2000. Therefore, LPEI–Dexa may be useful for non-viral gene delivery.

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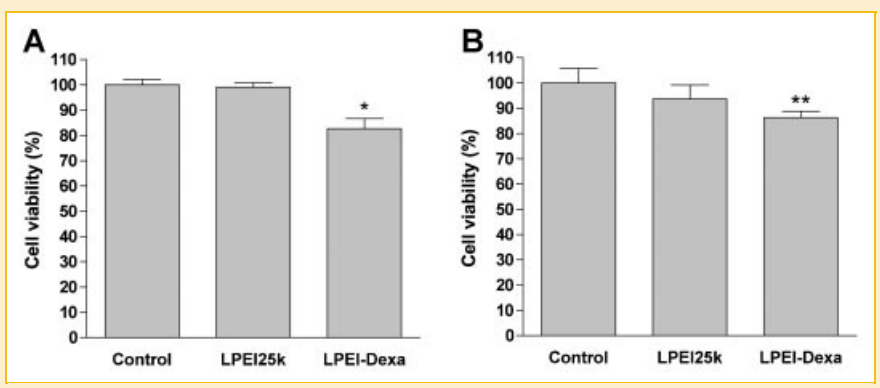

Fig. 10. Cytotoxicities of LPEI25k, LPEI–Dexa, BPEI25k, and Lipofectamine 2000. Polymer/pSV-Luc complexes were prepared as described in the Materials and Methods Section and transfected into H9C2 (A) and A7R5 (B) cells. After transfection, cell viability was measured by the MTT assay. The data are expressed as mean values (±standard deviation) of quadruplicate experiments. Significance was determined by one-way analysis of variance (ANOVA). $^{*}P < 0.01$ as compared with control and LPEI25k. $^{**}P$ is not significant as compared with control and LPEI25k.

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