Ternary Complex of Plasmid DNA with Protamine and γ-Polyglutamic Acid for Biocompatible Gene Delivery System

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The purpose of the present study was to investigate the usefulness of the ternary complex with protamine and γ-polyglutamic acid (γ-PGA), which are biodegradable materials for foods and medical products, as a safe gene delivery vector. We formed cationic binary complexes (plasmid DNA (pDNA)/protamine complexes) with high transfection efficiency. The binary complex showed slight toxicity probably related to its total cationic charge. Then, we formed ternary complexes (pDNA/protamine/γ-PGA complexes) by addition of anionic polymer, γ-PGA, and they showed no cytotoxicity. The transfection efficiency of the pDNA/protamine/γ-PGA complexes was as high as that of the pDNA/protamine complexes, although their zeta potentials were different. Inhibition study of the gene expressions in B16-F10 cells suggested that pDNA/protamine complexes were taken up by caveolae-mediated endocytosis and macropinocytosis. On the other hand, pDNA/protamine/γ-PGA complexes were taken up by clathrin-mediated endocytosis and macropinocytosis. Thus, we succeeded in developing the ternary complex as a safe gene delivery vector with biocompatible materials.

Key words protamine; γ-polyglutamic acid; gene delivery; non-viral vector; nanoparticle

Gene therapy is expected to be an effective method to treat cancer, infection, innate immunodeficiency and cardiovascular diseases.[1,2] The success of gene therapy highly depends on the development of effective and secure delivery vectors. Gene delivery vectors are categorized into viral and non-viral vectors. Non-viral vectors have advantages associated with low immunogenic response and can be produced in large-scale manufacture.[3–5] Among non-viral vectors, cationic polymers and cationic liposomes have often been used to form stable cationic complexes with plasmid DNA (pDNA) as polyplexes and lipoplexes, respectively, and have showed high gene expression *in vitro*.[6] Most of polyplexes and lipoplexes shows disadvantages such as poor biodegradability and strong cytotoxicity.

The promising approach for developing safe vector is to construct it with biodegradable materials for foods and medical products although it is challenging. Protamine, which is safe and biodegradable, is used clinically as an antidote to heparin-induced anticoagulation. Protamine/insulin/zinc chloride mixture has also been injected subcutaneously as a sustained release preparation.[7] Protamine is a cationic peptide (molecular weight 4000–4250) with high arginine content and can condense DNA. The nuclear delivery of pDNA condensed by protamine showed high transgene expression after cytoplasmic microinjection because protamine contains a nuclear localization signal.[8] These characteristics of protamine are appropriate for a non-viral vector of pDNA in clinical use. However, protamine was reported to cross-link with the complement protein and induce toxicity clinically such as thrombocytopenia and granulocytopenia responses.[9,10] The toxicity was related to its total cationic charge.[11]

In the previous reports, we successfully constructed the safe ternary complex of pDNA with polyethyleneimine and γ-polyglutamic acid (γ-PGA) with high gene expression.[12,13] The γ-PGA, a naturally occurring peptide, is water-soluble, biodegradable, anionic and nontoxic.[14–16] In the present study, we investigated the usefulness of ternary complex of pDNA with protamine and γ-PGA, which are biodegradable materials for foods and medical products.

MATERIALS AND METHODS

Chemicals and Reagents Protamine (protamine sulfate from salmon) and chlorpromazine (CPZ) were purchased from Nacalai Tesque (Kyoto, Japan). The γ-PGA was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was obtained from Biological Industries Ltd. (Kibbuts Beit Haemek, Israel). RPMI 1640, Opti-MEM I and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). The 2-(4-iodophenyl)-3-(4-nitrophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Dojindo Laboratories (Kumamoto, Japan). Genistein and amiloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Protamine labeled with Texas Red® (TR-protamine) was prepared in our laboratory with Texas Red®-X Protein Labeling Kit obtained from Molecular Probes (Leiden, the Netherlands). All other chemicals were of reagent grade.

Preparation of pDNA Plasmid cytomegalovirus luciferase (pCMV-Luc) was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of the pcDNA vector (Invitrogen, Carlsbad, CA,

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U.S.A.). The pDNA was amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The pDNA was dissolved in 5% dextrose solution as 1 mg/mL and stored at −80°C until analysis.

Preparation and Characterization of Complex For the preparation of binary complexes of pDNA condensed with protamine, pDNA solution and protamine solution were mixed by pipetting thoroughly at various theoretical charge ratios, such as pDNA phosphate/protamine nitrogen=1 : 0.16 (pDNA/protamine0.16 complexes), 1 : 0.8 (pDNA/protamine0.8 complexes), 1 : 1.6 (pDNA/protamine1.6 complexes), 1 : 3.2 (pDNA/protamine3.2 complexes), 1 : 6.4 (pDNA/protamine6.4 complexes) and 1 : 9.6 (pDNA/protamine9.6 complexes) and left for 15 min at room temperature. In the present study, we selected pDNA/protamine6.4 to prepare the ternary complexes.

The γ-PGA solution and pDNA/protamine solution were mixed by pipetting thoroughly at various theoretical charge ratios, such as pDNA phosphate/protamine nitrogen /γ-PGA carboxylate=1 : 6.4 : 2 (pDNA/protamine6.4/γ-PGA2 complexes), 1 : 6.4 : 6 (pDNA/protamine6.4/γ-PGA6 complexes), 1 : 6.4 : 8 (pDNA/protamine6.4/γ-PGA8 complexes), 1 : 6.4 : 12 (pDNA/protamine6.4/γ-PGA12 complexes) and 1 : 6.4 : 16 (pDNA/protamine6.4/γ-PGA16 complexes) and left for 15 min at room temperature.

The particle sizes and zeta potentials of the complexes were measured with a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, U.K.). The number-fractioned mean diameter is shown. The complex formations were evaluated by gel retardation assay. Ten microliter aliquots of complex solution containing 1 μg pDNA were mixed with 2 μL loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel. Electrophoresis (i-Mupid J; Cosmo Bio, Tokyo, Japan) was carried out at 100 V in running buffer solution [40 mM Tris–HCl, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)] for 30 min. The retardation of pDNA was visualized with ethidium bromide staining using a Gel Doc EZ System (Bio-Rad Laboratories, Inc., Tokyo, Japan).

Cell Culture The mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). The B16-F10 cells were maintained in culture medium (RPMI 1640 supplemented with 10% FBS and antibiotics) under a humidified atmosphere of 5% CO_2 in air at 37°C.

***In Vitro* Transfection** The B16-F10 cells were plated on 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of 1.0×10^4 cells/well and cultivated in 500 μL culture medium. In the transfection experiments, after 24 h pre-incubation, the medium was replaced with 500 μL transfection medium (Opti-MEM I) and each complex containing 1 μg pDNA was added to the cells and incubated at 37°C for 2 h. Each complex was also incubated at 4°C for 2 h to determine the low temperature effect. After transfection, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37°C. After 22 h incubation, the cells were washed with phosphate-buffered saline (PBS) and then lysed in 100 μL lysis buffer (pH 7.8 and 0.1 M Tris–HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). Ten microliters of lysate samples were mixed with 50 μL luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). The protein content of lysate was determined by a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) using BSA as a standard. Absorbance was measured using a microplate reader (Sunrise RC-R; Tecan Japan Co., Ltd., Kanagawa, Japan) at 595 nm. Luciferase activity was indicated as relative light units (RLU) per mg protein.

For the inhibition study, the cells were pre-treated with 14 μM CPZ to inhibit clathrin-mediated endocytosis, 200 μM genistein to inhibit caveolae-mediated endocytosis, or 2 mM amiloride to inhibit macropinocytosis for 30 min. After pre-treatment, the complexes were added to the medium containing each inhibitor and incubated for 2 h. After transfection, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37°C, and then the luciferase activities were determined as described above.

To visualize cellular uptake and gene expression of the complexes, B16-F10 cells were transfected by each complex constructed with pEGFP-C1 and TR-protamine as described above. After 22 h incubation, GFP expression and fluorescence distribution of TR-protamine were observed with fluorescent microscopy (400×magnification; BZ-9000; KEYENCE, Osaka, Japan).

WST-1 Assay Cytotoxicity tests of various complexes on B16-F10 cells were carried out using WST-1 commercially available cell proliferation reagent. B16-F10 cells were plated on 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of 3.0×10^3 cells/well in the culture medium. Each complex containing 1 μg pDNA in 100 μL transfection medium were added to each well and incubated for 2 h. After incubation, the medium was replaced with culture medium and incubated for another 22 h at 37°C. The medium was replaced with 100 μL culture medium, and then 10 μL WST-1 reagent (5 mM WST-1 and 0.2 mM 1-methoxy PMS in PBS) was added to each well. The cells were incubated for an additional 2 h at 37°C. The absorbance in each well was measured at a wavelength of 450 nm with a reference wavelength of 630 nm using a microplate reader. The results are shown as a percentage of untreated cells.

Statistical Analysis Results are expressed as the mean with standard deviation (S.D.) of at least three experiments. Statistical analysis was performed using Student's *t*-test. The multiple comparisons test was performed by Dunnett's test. A $p<0.05$ was considered to indicate significance.

RESULTS

Characterization of pDNA/Protamine Complex The particle size and zeta potential of pDNA/protamine complexes are shown in Table 1. The pDNA/protamine complexes were 72.9–132.8 nm in particle size. The addition of protamine to pDNA increased the zeta potential of the complexes. At a charge ratio between 0.8 and 1.6, the zeta potential of pDNA/protamine complexes changed from a negative charge to a positive charge. At a charge ratio of greater than 1.6, the zeta potential of pDNA/protamine complexes reached a plateau and were +25.1 to +26.6 mV.

Complex formations were evaluated by a gel retardation assay (Fig. 1A). Naked pDNA was detected as a band on the agarose gel (lane a). The slight bands of naked pDNA were detected at charge ratios of 0.16 and 0.8 (lanes b and c), al-

Table 1. Particle Size and Zeta Potential of pDNA/Protamine Complex

Complex	Particle size (nm)	Zeta potential (mV)
pDNA/protamine0.16	118.7±33.4	−17.3±3.0
pDNA/protamine0.8	132.8±13.4	−9.3±0.3
pDNA/protamine1.6	83.2±10.6	+25.5±0.7
pDNA/protamine3.2	72.9±3.8	+26.6±0.5
pDNA/protamine6.4	78.7±2.7	+25.7±0.3
pDNA/protamine9.6	88.5±6.5	+25.1±0.5

Each value is the mean±S.D. (n=3).

though no band were observed at charge ratios of 1.6, 3.2, 6.4 and 9.6 (lanes d, e, f and g).

Transfection Efficiency of pDNA/Protamine Complex *In vitro* gene expression efficiency of pDNA/protamine complexes was evaluated with B16-F10 cells (Fig. 2A). The intracellular expression of the luciferase gene was measured as relative light units (RLU) and the results were normalized by protein content. The transfection efficiency of pDNA/protamine complexes depended on the charge ratio. When the charge ratio of protamine was increased, the transfection efficiency improved and the highest levels of gene expression were observed at charge ratios of 6.4 and 9.6.

Characterization of pDNA/Protamine/γ-PGA Complex We used pDNA/protamine complexes at a charge ratio of 6.4, where the complexes showed highest gene expression, to prepare the ternary complex with γ-PGA. Table 2 shows the size and zeta potential of pDNA/protamine/γ-PGA complexes. The pDNA/protamine6.4/γ-PGA complexes were 80.8–111.1 nm in particle size. The addition of γ-PGA decreased the zeta potential of pDNA/protamine6.4/γ-PGA complexes and showed negative charges at charge ratios of 12 and 16. The pDNA/protamine6.4/γ-PGA complexes at charge ratios of 6 and 8 showed aggregation and particle size and zeta potential could not be detected.

Complex formations were examined by a gel retardation assay (Fig. 1B). Naked pDNA was detected as a band on the agarose gel (lane h). The pDNA/protamine6.4 complexes (lane i) and pDNA/protamine6.4/γ-PGA complexes with charge ratios of 2, 12 and 16 (lanes j, k and l) did not show any bands

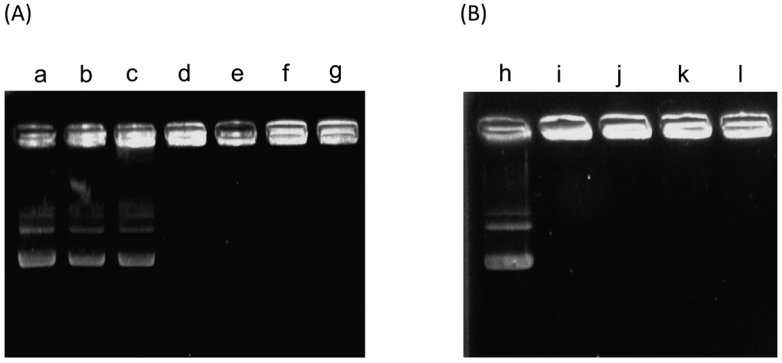

Fig. 1. Gel Retardation Assay of pDNA/Protamine Complex (A) and pDNA/Protamine/γ-PGA (B)

Naked pDNA (a), pDNA/protamine0.16 complexes (b), pDNA/protamine0.8 complexes (c), pDNA/protamine1.6 complexes (d), pDNA/protamine3.2 complexes (e), pDNA/protamine6.4 complexes (f), pDNA/protamine9.6 complexes (g), Naked pDNA (h), pDNA/protamine6.4 complexes (i), pDNA/protamine6.4/γ-PGA2 complexes (j), pDNA/protamine6.4/γ-PGA12 complexes (k), pDNA/protamine6.4/γ-PGA16 complexes (l). Retardation of pDNA was visualized using ethidium bromide.

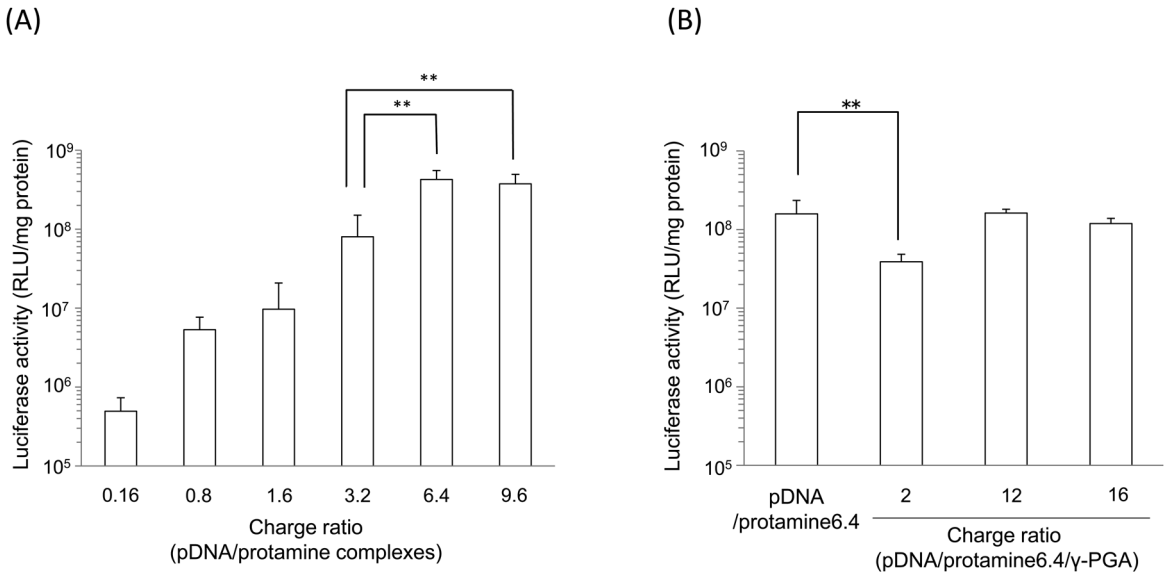

Fig. 2. Transfection Efficiency of pDNA/Protamine Complex (A) and pDNA/Protamine/γ-PGA Complex (B)

B16-F10 cells were transfected with complexes with pCMV-Luc. After 22 h transfection, cells were lysed for quantification of luciferase activity. Values are the means of luciferase activity (RLU/mg protein) with S.D. (n=3). **p<0.01.

Table 2. Particle Size and Zeta Potential of pDNA/Protamine/γ-PGA Complex

Complex	Particle size (nm)	Zeta potential (mV)
pDNA/protamine6.4/γ-PGA2	111.1±0.9	+21.9±0.4
pDNA/protamine6.4/γ-PGA6	N.D.	+5.3±0.1
pDNA/protamine6.4/γ-PGA8	N.D.	−20.1±0.3
pDNA/protamine6.4/γ-PGA12	87.7±1.0	−37.5±0.9
pDNA/protamine6.4/γ-PGA16	80.8±1.8	−41.5±0.6

Each value is the mean with S.D. (n=3). N.D.: not detectable.

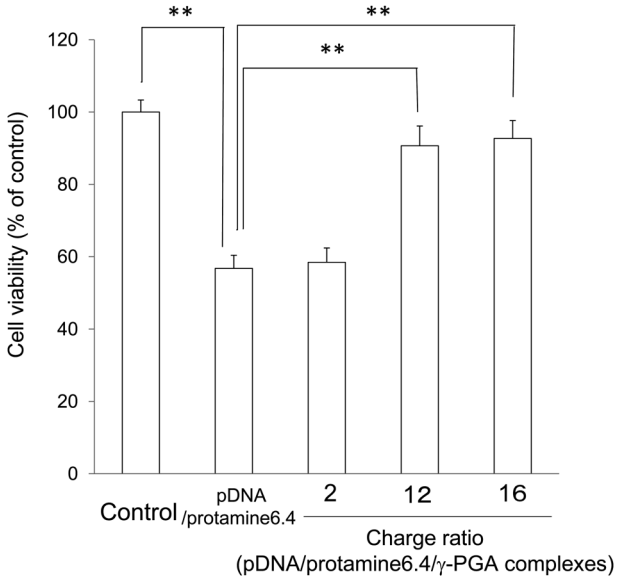

Fig. 3. Cell Viability of B16-F10 Cells Treated with Complexes Was Measured by WST-1 Assay

Cells were incubated with pDNA/protamine6.4 complexes and various charge ratios of and pDNA/protamine6.4/γ-PGA complexes for 2 h, and the cell viabilities were measured at 24 h after treatment. Data are the percentage to untreated cells. Each value is the mean with S.D. (n=8). **p<0.01.

of naked pDNA.

Transfection Efficiency of pDNA/Protamine/γ-PGA Complex To evaluate the transgene efficiencies of the complexes, B16-F10 cells were transfected with pDNA/protamine6.4/γ-PGA complexes and their luciferase activities were determined by the luminescence intensity (Fig. 2B). The pDNA/protamine6.4 complexes had high transgene efficiency, whereas the pDNA/protamine6.4/γ-PGA complexes with a charge ratio of 2 showed significantly lower transgene efficiency than pDNA/protamine6.4 complexes (p<0.01). The pDNA/protamine6.4/γ-PGA complexes with charge ratios of 12 and 16 showed an equivalent gene expression to pDNA/protamine6.4 complexes.

Cellular Toxicity Each complex was added to B16-F10 cells and cell viability was determined with WST-1 assay (Fig. 3). The pDNA/protamine6.4 complexes showed significantly lower cell viability than the control (p<0.01). On the other hand, pDNA/protamine6.4/γ-PGA complexes with charge ratios of 12 and 16 significantly decreased the cytotoxicity of the pDNA/protamine6.4 complexes.

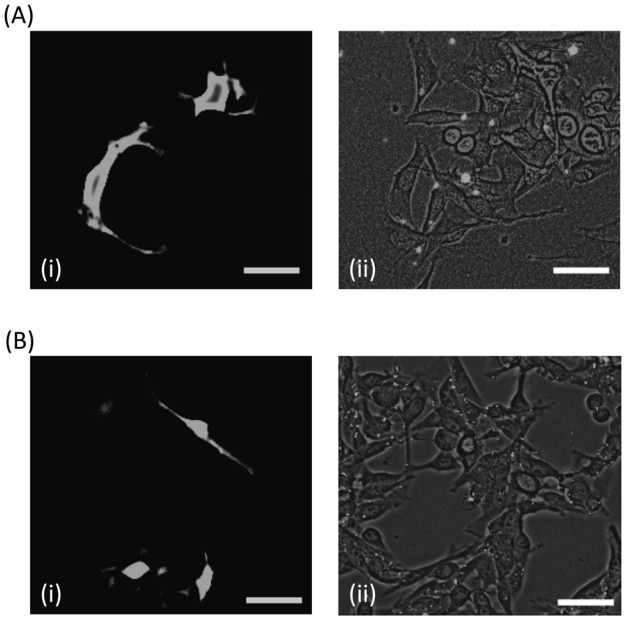

Fig. 4. Fluorescent Microscopy Image of B16-F10 Cells Transfected with pDNA/Protamine6.4 Complexes (A) and pDNA/Protamine6.4/γ-PGA16 Complexes (B) Containing pEGFP-C1 and TR-Protamine

After 22 h transfection, the GFP expression was monitored (400×magnification). Expression of GFP (i) and fluorescent of TR-protamine merged with phase contrast image (ii) are shown. Bar represents 50 nm.

Detailed Study of Gene Transfection of pDNA/Protamine Complex and pDNA/Protamine/γ-PGA Complex The pDNA/protamine complexes and pDNA/protamine/γ-PGA complexes showed high gene expression regardless of their different zeta potentials. The high gene expressions were examined in detail. The cells were transfected with pDNA/protamine6.4 complexes and pDNA/protamine6.4/γ-PGA16 complexes containing pEGFP-C1 and TR-protamine to visualize their cellular uptake and gene expression (Figs. 4Ai, 4Bi). Both pDNA/protamine6.4 complexes and pDNA/protamine6.4/γ-PGA16 complexes clearly showed red fluorescence of TR-protamine and green fluorescence of GFP.

To compare pDNA/protamine6.4 complexes and pDNA/protamine6.4/γ-PGA16 complexes in the endocytosis pathway, we determined the effects of hypothermia or endocytotic inhibitors on transgene efficiency (Figs. 5A, 5B). Both complexes decreased their gene expression with 4°C incubation (p<0.01). The inhibition of caveolae-mediated endocytosis with genistein and the inhibition of macropynocytosis with amiloride significantly decreased the transgene efficiency of the pDNA/protamine6.4 complexes (p<0.05). On the other hand, the transgene efficiency of pDNA/protamine6.4/γ-PGA16 complexes was significantly inhibited by amiloride and CPZ, an inhibitor of clathrin-mediated endocytosis (p<0.01).

DISCUSSION

In the present study, protamine was evaluated as a possible carrier for gene delivery purposes. Protamine is known to be the major component of the sperm nucleus, which can condense and stabilize DNA into a highly compact structure.[17] The pDNA/protamine complexes were formed by electrostatic interactions between the positive arginine-rich domain of

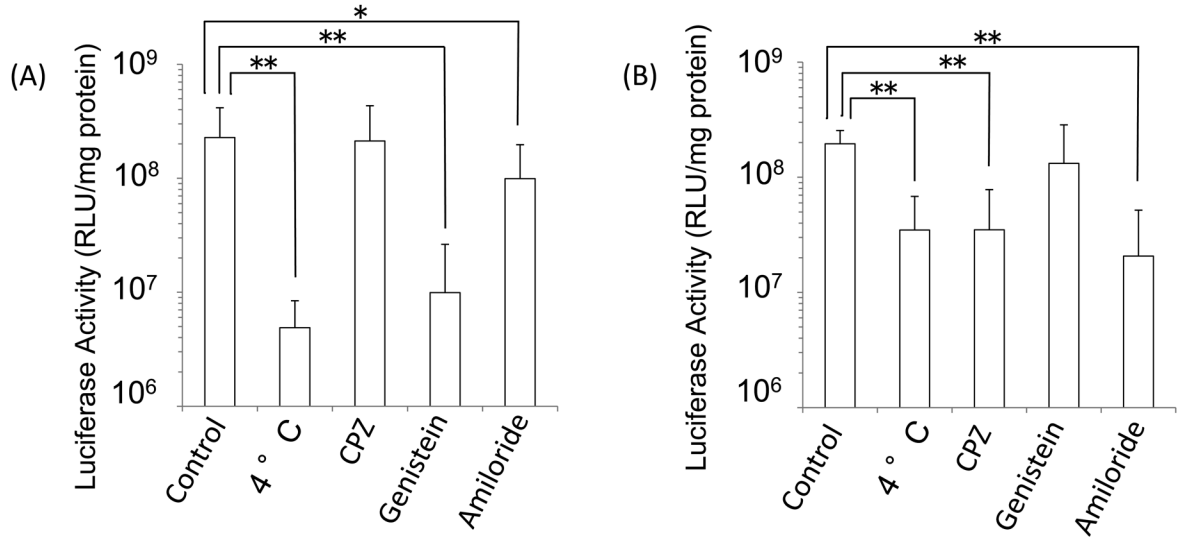

Fig. 5. Influence of Hypothermia and Endocytotic Inhibitors on the Transgene Efficiencies of pDNA/Protamine6.4 Complexes (A) and pDNA/Protamine6.4/γ-PGA16 Complexes (B)

After 22h transfection, luciferase activity was evaluated. Values are the means of luciferase activity (RLU/mg protein) with S.D. (n=16). *p<0.05, **p<0.01 *vs.* control.

protamine and the negative major groove of pDNA.[18] The usefulness of gene delivery containing protamine have been reported in polyplexes and lipoplexes.[19–22]

The stable nanoparticles were formed by addition of protamine to pDNA at various theoretical charge ratios (Table 1). As the charge ratio of protamine was increased, the zeta potential of pDNA/protamine complexes also increased. The zeta potential reached a plateau at a charge ratio of 1.6. The interaction was firm and pDNA contained in the complexes was not released (Fig. 1A).

With increasing charge ratios of protamine, the transfection efficiencies of pDNA/protamine complexes were enhanced and high levels of gene expression of pDNA/protamine6.4 complexes (4.3×10^8 RLU/mg protein) and pDNA/protamine9.6 complexes (3.7×10^8 RLU/mg protein) were observed (Fig. 2A). These transfection efficiencies were as great as the commercially available transfection reagent, lipofectin (1.49×10^8 RLU/mg protein).[23] This high gene expression of pDNA/protamine6.4 must be explained by interaction of cationic protein with a negative charge cellular membrane.[24,25] On the other hand, the morphology of pDNA condensates may be important for gene expression. Because, the various structures of the complexes such as globules, troids, chains and bundles were observed according to preparation procedure.[26]

When the charge ratio of protamine was increased, slight cytotoxicity was observed in the present study (Fig. 3). Although protamine is safe, the large dose of protamine will develop the toxicity because of its cationic charge. In the previous reports, cationic complexes were reported to bind to cellular membrane proteoglycans, destabilizing the membrane and causing severe cytotoxicity.[25,27] We have already reduced the toxicity of the cationic complexes by the addition of anionic polymer without lowering the transfection efficiency.[12,13] We therefore added γ-PGA to the pDNA/protamine complexes. γ-PGA is produced by microbial species typified by *Bacillus subtilis*.[28] Synthesized γ-PGA showed little toxic effect on the human B-cell line EHRB even at high concentration, 100 mg/L. It also showed no toxic effect on mice following the injection of 1 mg γ-PGA and did not cause immunoreactions and inflammatory reactions.[14–16]

We succeeded in forming a small ternary complex with a negative charge by the addition of γ-PGA (Table 2). It was clear that protamine compacted the pDNA completely (Fig. 1B). The *in vitro* gene expression was determined by exposure of pDNA/protamine/γ-PGA complexes to B16-F10 cells (Fig. 2B). The pDNA/protamine6.4/γ-PGA12 complexes and pDNA/protamine6.4/γ-PGA16 complexes showed transfection efficiencies as high as the pDNA/protamine6.4 complexes. The pDNA/protamine6.4/γ-PGA12 complexes and pDNA/protamine6.4/γ-PGA16 complexes showed little cytotoxicity (Fig. 3). The decreased cytotoxicity of complexes must be caused by a negative surface. A negative surface charge may have caused the ternary complexes to increase safety. In fact, the addition of alginic acid and anionic PEG derivatives to the cationic complexes was demonstrated to reduce cytotoxicity.[19,29]

We visualized the cellular uptake and gene expression in B16-F10 cells using the complexes loading pEGFP-C1 and TR-protamine. As a result, the red dots of TR-protamine and GFP expression was observed not only in pDNA/protamine6.4 complexes but also in pDNA/protamine6.4/γ-PGA16 complexes, as shown in Fig. 4. The cells, which had taken much TR-protamine, showed high gene expression. Inhibition studies of gene expressions were carried out by hypothermia and various endocytotic inhibitors, such as CPZ for clathrin-mediated endocytosis, genestein for caveolae-mediated endocytosis, and amiloride for macropinocytosis.[30] The gene expression of pDNA/protamine complexes was inhibited by hypothermia and the addition of genestein and amiloride (Fig. 5A). These results suggest that the pDNA/protamine complexes were taken up by caveolae-mediated endocytosis and macropinocytosis in B16-F10 cells. The gene expression of pDNA/protamine/γ-PGA complexes was inhibited by hypothermia and the addition of CPZ and amiloride (Fig. 5B). The pDNA/protamine/γ-PGA complexes was suggested to be taken up by clathrin-mediated endocytosis and macropinocytosis. pDNA/protamine complexes and pDNA/protamine/γ-PGA complexes may be taken up by a different process.

In the present study, we succeeded in developing a biodegradable gene delivery vector with safety and high transfection efficiency using safe and biocompatible materials, protamine and γ-PGA. This is highly safe and is expected to be utilized clinically.

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