# DESIGN AND CHARACTERIZATION OF POLYSACCHARIDE-BASED NON-VIRAL CARRIERS AIMING AT GENE TRANSFECTION AND GENETIC ENGINEERING FOR CELL THERAPY

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**Chapter 8. Enhanced therapeutic efficacy of dendritic cells genetically engineered by cationized dextran-interleukin 12 plasmid DNA complexes for tumor**

# ABBREVIATIONS

| | |
|---|---|
| αMEM | α-Minimum essential medium |
| AM | Adrenomedullin |
| ASGPR | Asialoglycoprotein receptor |
| BCA | Bicinchonic acid |
| bFGF | Basic fibroblast growth factor |
| BMC | Bone marrow cells |
| CDI | N,N'-carbonyldiimidazole |
| CTL | Cytotoxic T lymphocytes |
| DC | Dendritic cells |
| DLS | Dynamic light scattering |
| DDW | Double-distilled water |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle Medium |
| ELS | Electrophoretic light scattering |
| EtBr | Ethidium bromide |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FCS | Fetal calf serum |
| FITC | Fluorescein isothiocyanate |
| GFP | Green fluorescent protein |
| HGF | Hepatocyte growth factor |
| IFN | Interferon |
| IFN-γ | Interferon-gamma |

| | |
|---|---|
| IL-12 | Interleukin-12 |
| LDH | Lactate dehydrogenase |
| MEM | Minimal Essential Medium |
| MSC | Mesenchymal stem cells |
| Mϕ | Macrophages |
| PBS | Phosphate-buffered saline |
| PET | Polyethylene terephthalate |
| PIC | Polyion complexes |
| RLU | Relative light unit |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| rmGM-CSF | Recombinant mouse-derived granulocyte macrophage colony-stimulating factor |
| TBE | Tris-borate-ethylenediaminetetraacetic acid buffer solution |
| TUNEL | TdT-mediated dUTP nick-endlabeling |
| vWF | von Willebrand factor |
| WST-8 | 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H- tetrazolium |

# GENERAL INTRODUCTION

Cell transplantation has been applicable not only to tumor therapy, but also tissue regeneration therapy. For example, natural killer cells, lymphocytes, macrophages, and dendritic cells have been clinically applied to the tumor therapy based on the natural immune responses for tumor regression. On the other hand, with recent rapid development of cell biology and medicine regarding tissue regeneration, it has been possible to make use of various progenitor and stem cells with high potentials of proliferation and differentiation [1,2] for cell-based tissue regeneration therapy. As one of the most practical and available stem cells, bone marrow-derived mesenchymal stem cells (MSC) have been noted because they are multipotent adult stem cells readily isolatable from the bone marrow of patients themselves [3,4].

It has been demonstrated that cells themselves have good therapeutic potentials in terms of their inherent targetability to the site injured or biological properties [5-7]. However, the therapeutic efficacy of cells transplanted is not always as high as expected, which is one of the largest problems in cell therapy. This is because the grafting rate of cells transplanted is low and consequently the biological functions of cells become low in the body. To tackle the problems, it is indispensable to develop materials, technologies, and methodologies to provide the cells a local environment where the survival and biological functions of cells transplanted can be maintained or enhanced. The therapeutic function of cells in the body cannot always be expected without any environments suitable for cells surviving and functioning. Various approaches have been reported to provide the cell environment [8-13]. For example, induction of angiogenesis in advance around the site transplanted permits sufficient supply of nutrients and oxygen to cells transplanted, resulting in the enhanced grafting rate of cells and the improved biological functions [14-18]. Another approach is to genetically engineer cells. Technology and methodology to genetically engineer cells for the functional activation have been developed by using gene materials, such as plasmid DNA, antisence oligonucleotide or small interfering RNA [19-21]. Genetic engineering is effective in not only artificially modifying the biological functions of cells, such as proliferation, differentiation, and therapeutic abilities applicable for

cell therapy [22-28], but also developing the basic research of cell biology and medicine which is important to identify and investigate genes regulating the biological function of cells. It is highly expected that the biological functions of cells genetically engineered are therapeutically superior to those of the original ones.

Genetic engineering of cells is fundamentally achieved by the transfection procedure of a gene for the cells. Therefore, it is necessary for successful genetic engineering to develop a material, technology, and methodology for safe and efficient gene transfection. The gene transfection system is generally divided into two categories in terms of gene transfection carrier materials: viral [29-32] and non-viral [33-37] systems. For the viral system, the carrier of retrovirus, lentivirus, adenovirus, and adeno-associated virus, has been used to be potentially efficient, although there are several issues to be resolved for the clinical applications, such as the antigenicity and toxicity of virus itself or the possibility of disease transfection. Therefore, efficient technology and methodology of gene transfection without using virus are highly expected.

The non-viral system has several advantages in terms of its safety and no limitation in the molecular size of DNA applied. However, the low transfection efficiency is one major drawback for the research and therapeutic applications. In this circumstance, various experimental trials have been performed to develop the physicochemical and biological properties of non-viral carriers [38-41]. Gene in the naked form is a polyanion of phosphate group-repeated chain and has an expanded molecular structure due to the intermolecular repulsion force of negative charge at the physiological pH. Therefore, it is well recognized that the gene cannot interact with the cell membrane negatively charged due to the electrostatic repulsion and consequently hardly be internalized and subsequently transfected. On the basis of these findings, the non-viral carriers of cationized polymers [38] and cationized liposomes [42-44] have been developed to allow gene to effectively internalize into cells for gene transfection. The cationized materials enable the gene to form a complex with a molecular size and surface charge suitable for the cellular internalization and consequently enhance the complex internalization [37]. However, this method is only to increase the non-specific cellular internalization of gene through the simple electrostatic interaction between the complex and

cell surface. To enhance the specific cellular internalization of complex by making use of biological interactions, there have been several research trials with ligands specific for cell surface receptors, such as folate [45-48], transferrin [49-51], mono- or oligosaccharides [52-57], peptides [58-62], and proteins [63-66]. Incorporation of the ligand will enable gene to internalize into cells in a cell-specific manner. It is also important for effective gene expression to consider the intracellular trafficking of gene-carrier complexes or their stability in the cell. In the case of normal intracellular trafficking, the gene-carrier complex internalized via the endocytosis pathway is carried into the endosomal compartment, followed by lysosomal degradation. Therefore, the carrier should be molecularly designed to allow to effectively escape the gene-carrier complex internalized from the endosomal compartment into the cytoplasm one. For example, when covalently linked with a peptide capable to disrupt the lysosomal membrane under an acidic condition where lysosomal enzymes biologically function, a carrier effectively enhanced the level of gene expression [67]. It is reported that the carrier covalently linked with functional groups having a buffering capacity to accelerate the endosomal escape, so-called “proton sponge effect”, enhanced the gene expression [68]. Furthermore, the carrier covalently linked with a peptide of nuclear localization signal (NLS) enabled a gene to positively deliver to the cellular nucleus [69]. There have been several researches to combine gene-carrier complexes with physical stimuli, such as pressure [70,71], electricity [72,73], ultrasound [74-76], magnetism [77], and light [78], to enhance or regulate the level and pattern of gene expression.

Among the non-viral materials applicable for gene transfection carrier, polysaccharide has some advantages over other carrier materials. Most of them are water-soluble and have a long history of the medical, pharmaceutical, and food applications. The biosafety and bioavailability have been proven from the practical usages. The sample with different physicochemical properties, such as the molecular weight, electric charge, and water solubility, is practically available. Since polysaccharide has reactive groups, the chemical modification can be readily made to change the chemical, physical, and biological properties. Recently, polysaccharides and their derivatives have been paid great attention for drug delivery approaches because of their availability to improve the pharmacokinetical and

pharmacodynamical characteristics of therapeutic agents, such as low molecular weight drugs, bioactive peptides or proteins, enzymes, and plasmid DNA [79]. Chemical or physical conjugation of therapeutic agents with the polysaccharide derivatives increased their biological stability in the blood circulation and consequently prolong the time period of activity [80-83]. On the other hand, polysaccharides have been used to target the agents to the tumor (passive targeting) [84-87] or the liver (active targeting) [88-90]. In addition, paramagnetic substance-loaded or radiolabeled polysacchrides have been applied for diagnostic imaging [91-99]. Another noticable feature of polysaccharide is to compose of different sugars which can be recognized by the corresponding cell receptors of sugar specificity. This biological recognition not only permits the receptor-specific targeting of agents to the cell, but also accelerates their cell internalization via the receptor-mediated endocytosis. Several researches for efficient gene transfection have been carried out with cationized polysaccharides, such as cationized dextran [100-102], schizophyllan [103], and chitosan [104]. However, few researches have been done to systematically investigate and evaluate the effect of physicochemical and biological properties of cationized polysaccharides on the feasibility as a non-viral carrier for gene transfection.

When cells are genetically engineered and used for transplantation therapy, in addition to the enhancement of gene transfection and expression, it is undoubtedly important to consider the physiological and functional conditions of cells transfected from the viewpoint of the practical usage. In the conventional procedure of gene transfection, normally the non-viral carrier is complexed with a plasmid DNA, and then added to the culture medium of cells for transfection. In this case, although the presence of serum is essential to maintain the conditions of cell culture biologically good, the transfection culture is being done generally being carried out without the serum. This is because the plasmid DNA-carrier complex often interacts with serum components. This interaction often reduces the extent of complex internalized into cells, leading to the suppressed level of gene transfection. Taken together, we cannot always say that the culture condition for the conventional transfection is good in terms of cells viability. Basically, there are two approaches to improve the culture conditions of gene transfection. One is the technical modification to perform gene transfection even in the

presence of serum. The other is to improve the methodology of cell culture which enables cells to physiologically proliferate under good conditions in the culture of gene transfection. Since a three-dimensional culture substrate has a large surface area available for cell attachment and the subsequent proliferation compared with a two-dimensional tissue culture plate, cells can be generally proliferated in the three-dimensional substrate at higher rates and for longer time periods than those in the two-dimensional one [105-107]. Moreover, combination with a perfusion culture method can supply nutrients and oxygen to the cells proliferated in the three-dimensional substrate efficiently compared with a static culture method, while harmful metabolic products and wastes generated from cells can be excluded rapidly. It has been previously demonstrated that the proliferation of MSC was greatly influenced by the culture method and significantly enhanced by the perfusion culture method compared with the static method [108]. Therefore, it is highly expected that combination of cationized polysaccharide-based gene transfection with the culture method enhances the level of gene expression to a great extent compared with the conventional method of gene transfection.

The objective of this thesis is to design and prepare various types of non-viral carriers for gene expression from polysaccharides and evaluate the level of gene expression by the plasmid DNA-carrier complexes in terms of their physicochemical and biological properties. The cells are genetically engineered with the carrier complex with a gene coding a therapeutic peptide or protein to assess the therapeutic efficacy of cells genetically engineered with some disease animal models. In Part 1, cationized polysaccharides of non-viral carrier were prepared from different polysaccharides and amine compounds, while the physicochemical and biological characterizations were performed to evaluate the effect of physicochemical properties of cationized polysaccharides on the level of gene expression. In addition, different transfection culture methods combined with the plasmid DNA-cationized polysaccharide complexes were performed to evaluate effect of the transfection method on the level of gene expression and the cells viability.

Chapter 1 describes the effect of polysaccharides type on the level of gene expression. Spermine was chemically introduced to pullulan, dextran, and mannan to prepare respective cationized polysaccharides. The apparent molecular size and zeta potential of cationized

polysaccharide-plasmid DNA complexes were similar, irrespective of the polysaccharide type, but the interaction strength of the complex with a sugar-recognizable lectin depended on the polysaccharide type. The level of gene expression for MSC and HeLa cells by the cationized polysaccharide-plasmid DNA complexes depended on the type of polysaccharide used for cationization as well as cells.

Chapter 2 describes the effect of type of amine compounds introduced to dextran on the level of gene expression. Different amine compounds were chemically introduced to dextran to prepare various cationized dextrans. The buffering capacity of cationized dextran depended on the type of amine compounds, while the highest buffering capacity was observed for the spemine-introduced dextran. The level of gene expression for HeLa cells by the cationized dextran-plasmid DNA complex depended on the type of amine compounds used for cationization. The highest level was observed for the spermine-introduced dextran-plasmid DNA complex.

Chapter 3 describes the effect of molecular weight of pullulan used for cationization on the level of gene expression. Spermine was chemically introduced to pullulan with molecular weights of 5,900, 11,800, 22,800, 47,300, 112,000, and 212,000 to prepare various cationized pullulans. The apparent molecular size, the zeta potential, and the interaction strength of cationized pullulan-plasmid DNA complexes with the lectin depended on the molecular weight of pullulan used for cationization. The level of gene expression for HepG2 cells by the cationized pullulan-plasmid DNA complexes also depended on the pullulan molecular weight and the highest level was observed for the cationized pullulan with a molecular weight of 47,300.

Chapter 4 describes the effect of the extent of spermine introduced to pullulan on the level of gene expression. Various amounts of spermine were chemically introduced to pullulan with molecular weights of 22,800, 47,300, and 112,000 to prepare various cationized pullulans. The apparent molecular size, the zeta potential, and the interaction strength of cationized pullulan-plasmid DNA complexes with the lectin depended on the extent of spemine introduced to pullulan. The level of gene expression for HepG2 cells by the cationized pullulan-plasmid DNA complexes depended on the extent of spermine introduced

to pullulan.

Chapter 5 describes the effect of transfection culture method on the level of gene expression. Pullulan was cationized by the chemical introduction of spermine to prepare cationized pullulan. The spermine-pullulan was complexed with a plasmid DNA and coated on the surface of culture substrate together with Pronectin® of an artificial cell adhesion protein. MSC were cultured and transfected on the complex-coated substrate (reverse transfection method) and the level and duration of gene expression were compared with those of MSC transfected by culturing in the medium containing the plasmid DNA-spermine-pullulan complex (conventional method). The gene expression was significantly enhanced and prolonged by the reverse transfection method compared with that of the conventional method. In addition, the reverse transfection was carried out for a non-woven fabic of polyethylene terephthalate (PET) coated with the complex and Pronectin® by an agitated and stirring culture methods. The level and duration of gene expression for MSC was significantly enhanced by the two methods compared with that of the static method.

In Part 2, cells were genetically engineered by the cationized polysaccharide-gene complexes prepared in Part 1 and applied to several animal disease models to evaluate their therapeutic effects. Chapter 6 describes the genetically engineering of bone marrow-derived mesenchymal stem cells by the cationized dextran-adrenomedullin (AM) plasmid DNA complexes and the enhanced therapeutic efficacy for acute myocardial infarction. Spermine was chemically introduced to dextran to prepare a cationized dextran. MSC were transfected by the conventional method with the complex of cationized dextran and a plasmid DNA coding AM of an anti-apoptotic and angiogenic peptide, to prepare MSC genetically engineered. The MSC genetically engineered secreted a large amount of AM and survived even under the hypoxia condition. Transplantation of MSC genetically engineered significantly improved cardiac function after the myocardial inoculation compared with that of the original MSC.

Chapter 7 describes the enhanced therapeutic efficacy of macrophages (Mϕ) genetically engineered by the cationized dextran-NK4 plasmid DNA complex for tumor.

Spermine was chemically introduced to dextran to prepare a cationized dextran. Mϕ were transfected by the reverse transfection method with the complex of cationized dextran and a plasmid DNA coding NK4 of an anti-tumor activity, to prepare Mϕ genetically engineered. The Mϕ genetically engineered secreted a large amount of NK4 and significantly suppressed the in vitro proliferation of tumor cells. When injected intravenously, the Mϕ genetically engineered accumulated to the tumor tissue and enhanced the in vivo anti-tumor activity to a significantly great extent compared with that of the original Mϕ.

Chapter 8 describes the enhanced therapeutic efficacy of dendritic cells (DC) genetically engineered by the cationized dextran-interleukin 12 plasmid DNA complex for tumor. Spermine was chemically introduced to dextran to prepare a cationized dextran. DC were transfected by the reverse transfection method with the complex of cationized dextran and a plasmid DNA coding IL-12 of an immuno-responsive anti-tumor activity, to prepare DC genetically engineered. The DC genetically engineered secreted a large amount of IL-12. When injected intratumorally, the DC genetically engineered enhanced the in vivo anti-tumor activity to a significantly great extent compared with that of the original DC.

In summary, this thesis describes the feasibility of polysaccharides as a non-viral carrier of plasmid DNA and the enhanced therapeutic efficacy of cells genetically engineered. It is concluded that the cationized polysaccharide is a promising carrier to genetically engineer cells not only to develop the basic research of cell biology and medicine, but also to positively modify and enhance the therapeutic efficacy.

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# PART 1

# EFFECT OF PHYSICOCHEMICAL PROPERTIES OF CATIONIZED POLYSACCHARIDES AND TRANSFECTION CULTURE METHOD ON THE LEVEL OF GENE EXPRESSION

# Chapter 1

## Effect of polysaccharides type on the level of gene expression

## INTRODUCTION

It is of prime importance for successful cell therapy to develop a method of genetic cell engineering aiming at the efficient activation and manipulation of cellular functions. To achieve this genetic engineering of cells, virus carriers have been mainly used because of the high efficiency of gene transfection [1-7]. However, there are several problems to be resolved for the clinical applications, such as the antigenicity and toxicity of virus itself or the possibility of disease transfection. Therefore, efficient technology and methodology of gene transfection without virus vectors are highly expected.

Many types of cationized polymers [8] and cationized liposomes [9-11] have been designed [12-14] as non-viral carrier materials, while complexation with them enabled plasmid DNA to neutralize the anionic charge as well as to reduce the molecular size, which is preferable to enhance the efficiency of plasmid DNA transfection. Among the non-viral carrier materials, polysaccharides have several advantages. They have hydroxyl groups available for simple chemical modification and the susceptibility of cell internalization possibly accelerated by a sugar-recognition receptor of cell surface. However, there is no report where the effect of polysaccharide type on the level of gene expression was systematically investigated.

In this chapter, various polysaccharides, pullulan, dextran, and mannan with the similar molecular weight of around 40,000 were cationized by the chemical introduction of spermine to obtain various cationized polysaccharide derivatives with different extents of spermine introduced. A plasmid DNA was complexed with the cationized polysaccharide derivatives in aqueous solution, and the apparent molecular size and zeta potential of the complexes were measured. The effect of the polysaccharide type on the level of gene expression for various cells, such as Hela cells, HepG2 cells, bone marrow-derived mesenchymal stem cells (MSC), macrophages (Mϕ), and dendritic cells (DC) was investigated.

Chapter 1

# EXPERIMENTAL

## Materials

Pullulan with different weight-average molecular weights of 5,900, 11,800, 22,800, 47,300, 112,000, and 212,000 was purchased from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Dextran and mannan were purchased from Sigma-Aldrich, Inc., St. Louis, MO and Nacalai Tesque. Inc., Kyoto, Japan, respectively. When determined by high performance liquid chromatography (LC-8020 model-II, Tosoh, Tokyo, Japan) using the pullulan with different weight-average molecular weights as standards, the weight-average molecular weights of dextran and mannnan were 57,000 and 36,000, respectively. Spermine was purchased from Sigma Chemical Co., St. Louis, MO. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

## Preparation of cationized polysaccharide derivatives

Spermine was chemically introduced to the hydroxyl groups of polysaccharide by a N,N'-carbonyldiimidazole (CDI) activation method [15]. Briefly, $9.28 \times 10^{-3}$ mole of spermine and various molar amounts of CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of each polysaccharide. Molar ratio of CDI initially added to the hydroxyl groups (OH) of polysaccharide was defined as a [CDI]/[OH] ratio. Following agitation at 35 °C for 20 hr, the reaction mixture was dialyzed against double-distilled water (DDW) for 2 days. The cut-off molecular weights of dialysis membrane used is 12,000-16,000 (Viskase Companies, Inc, Willowbrook, IL). Then, the solution dialyzed was freezed-dried to obtain the samples of spermine-introduced polysaccharide (spermine-polysaccharide). When determined from the conventional elemental analysis, the molar percentages of spermine introduced to the hydroxyl groups of polysaccharides were 12.3, 9.5, and 13.3 mole% for pullulan, dextran, and mannan, respectively.

## Preparation of plasmid DNA

The plasmid DNAs used were the pGL3 plasmid coding a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, Madison, WI) and the pEGFP-N1 plasmid

coding enhanced green fluorescent protein (GFP, Clontech Laboratories Inc., Palo Alto, CA). The plasmid DNA was propagated in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

**Preparation of spermine-polysaccharide-plasmid DNA complexes**

Polyion complexes (PIC) were prepared by mixing an aqueous solution of spermine-polysaccharide with that of plasmid DNA. Briefly, varied amounts of spermine-polysaccharide were dissolved in 50 μl of DDW and mixed with 50 μl of 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 100 μg of plasmid DNA, followed by leaving for 15 min at room temperature to obtain various PIC of spermine-polysaccharide and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of spermine-polysaccharide (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio.

**Electrophoresis measurement spermine-polysaccharide-plasmid DNA complexes**

PIC were prepared in 10 mM PBS solution at an N/P ratio of 3.0. After 15 min of incubation, 10 μl of the complex was added to 3 μl of a loading buffer (0.1% sodium dodecyl sulfate, 5% glycerol, and 0.005% bromophenol blue) and applied on an 1 wt % agarose gel in Tris-borate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.3) containing 0.1 mg/ml ethidium bromide (EtBr). Electrophoretic evaluation of the PIC was carried out in TBE solution at 100 V for 30 min. The gel was imaged with a UV transilluminator (Gel Doc 2000, BIO-RAD laboratories, Segrate, Italy).

**Dynamic light scattering (DLS) and electrophoretic light scattering (ELS) measurements**

PIC were prepared in 10 mM PBS solution at an N/P ratio of 3.0. The DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) equipped with an $Ar^+$ laser at a detection angle of $90^o$ at 25 °C for 15 min. In the present study, the autocorrelation function of samples was analyzed based on the cumulant method and the $R_s$ value was calculated automatically by the equipped computer software and expressed as the apparent molecular size of samples. On the other hand, the zeta potential was measured using ELS-7000AS instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) at 25 °C and an electric field strength of 100 V/cm. From the determined electrophoretic mobility, the zeta potential was automatically calculated using the Smoluchouski equation. Light scattering measurement was done three times for every sample.

**Lectin-induced aggregation of spermine-polysaccharide-plasmid DNA complexes**

PIC were prepared in PBS at an N/P of 3.0, followed by incubation at room temperature for 15 min. To 100 μl of the PIC solution, 100 μl of *Ricinus communis agglutinin* of lectin recognizable to galactose (RCA120, Seikagaku Corporation, Tokyo, Japan, 500 μg/ml) was added, followed by incubation at room temperature. The time profile of solution turbidity change was measured at a wavelength of 500 nm. To confirm the specificity of lectin-sugar interaction, D-galactose was added 40 min after RCA120 addition. The results were expressed as the percentage of the solution absorbance to that of spermine-polysaccharide-plasmid DNA complex alone.

**Cell culture**

HeLa cells were purchased from American Type Culture Collection, Manassas VA, and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 110 mg/ml sodium pyruvate (Invitrogen Corp., Carlsbad, CA), and 10 vol% Fetal calf serum (FCS) (Hyclone laboratories, Inc., South Logan, UT) (DMEM-FCS) at 37 °C in 5% $CO_2$ and 95% air at atmospheric pressure.

HepG2 cells were purchased from American Type Culture Collection, Manassas VA,

USA and maintained in minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution (Invitrogen Corp., Carlsbad, CA, USA), and 10 vol% FCS (MEM-FCS) at 37 °C in 5% $CO_2$ and 95% air at atmospheric pressure.

MSC culture was performed according to the method previously described [16]. Briefly, 3-week-old male Wister rats (Shimizu Laboratory Supplies Co.,Ltd., Kyoto, Japan) were sacrificed to isolate the femur and tibia, and the bone marrow was harvested by flushing the bone with PBS. The suspension of bone marrow cells in PBS was placed into 25 $cm^2$ cell culture flasks (Corning Inc., Corning, NY) and cultured in α-minimum essential medium (αMEM) supplemented with 15 vol% FCS and 1 wt% mixed penicillin and streptomycin solution (Sigma-aldrich Inc., St. Louis, MO, control medium). After incubation at 37 °C in 5% $CO_2$ and 95% air at atmospheric pressure for 3 days, nonadherent cells were removed. Adherent cells were further cultured by exchanging the fresh control medium every 3 days.

Mouse peritoneal Mϕ were isolated from 6-week-old female BALB/c mice (body weight = 20 g, Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan) according to the conventional procedure previously reported [17]. Briefly, mice were injected aseptically with 2 ml of thioglycolate broth (BD Biosciences, San Jose, CA) intraperitoneally. At 4 days after injection, 6.0 ml of DMEM was intraperitoneally injected to the mice, and then the peritoneal cells were collected by syringe aspiration. After centrifugation (1,000 rpm, 5 min, 4°C), pelleted peritoneal cells were resuspended in DMEM supplemented with 15 vol% FCS, penicillin (50 U/ml), and streptomycin (50 U/ml) and seeded on tissue culture plate (Corning, NY) at a density of $1\times10^6$ cells /$cm^2$. After incubating at 37 ° C in a 5% $CO_2$, 95% air atmosphere for 2 hr, the plate was washed thoroughly with DMEM to remove non-adherent cells. The adherent cells obtained were used as Mϕ for the following experiments.

Mouse immature DC were obtained from bone marrow cells (BMC) through the differentiation induction according to the conventional procedure previously reported [18]. Briefly, mouse BMC were harvested from the femur and tibia of 4-week-old female ICR mice or 6-week-old female C57BL/6 mice (body weight = 20 g, Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan). Erythrocytes contaminated in BMC were lysed with ammonium chloride solution and cells were seeded in RPMI-1640 (Sigma-aldrich Inc., St. Louis, MO)

supplemented with penicillin (100 U/ml), streptmycin (100 μg/ml), 10 vol% heat-inactivated FCS and 200 U/ml recombinant mouse-derived granulocyte macrophage colony-stimulating factor (rmGM-CSF, 1 x $10^7$ U/mg, Peprotech/Tebu, Frankfurt, Germany) on day 0. The medium was changed every 2 days thereafter. The cells cultured for 9-14 days were used for all experiments as mouse immature DC.

**In vitro gene transfection experiment**

The transfection experiments were performed independently in triplicate. Cells were seeded on each well of 12 multi-well cluster plate (Corning Inc., Corning, NY) and cultivated in 1 ml of control medium for 24 hr (Hela cells, HepG2 cells, MSC, and DC) or 1 week (Mϕ) . The density seeded was $1.25\times10^5$, $2.0\times10^5$, $5\times10^4$, $5\times10^5$, and $2.5\times10^5$ cells/well for Hela cells, HepG2 cells, MSC, Mϕ, and DC, respectively. Immediately after the medium was exchanged by FCS-free control medium, PIC solution (100 μl) was added, followed by 6 hr incubation for cell gene transfection. Then, the medium was changed to the control medium and cells were incubated further for 24 hr.

Cells were washed with PBS once, lysed in 200 μl of a cell culture lysis reagent (Promega Corp., Madison, WI, USA), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI, USA) was added to 20 μl of supernatant, while the relative light unit (RLU) of the samples was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) Protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturers' instructions in order to normalize the influence of number variance of cells on the luciferase activity.

**Evaluation of plasmid DNA internalization**

Plasmid DNA was labeled with rhodamine by using Label IT® fluorescein isothiocyanate (FITC) Labeling Kit (Mirus®, Madison, WI) according to the manufacturers' instructions. After transfection culture of MSC for 1 day with the free FITC-labeled plasmid

DNA or the complex with spermine-polysaccharide, the cells were treated in cold acetate-buffered solution containing 0.3 vol.% acetic acid, 0.085 M NaCl, and 5.0 mM KCl for 1 min, then the cells were washed with cold PBS to remove the complexes bound onto the cell surface. The cells of three wells were collected by the trypsinization, and the results were considered as an average value of triplicates, while ten thousand cells were measured by the fluorescence activated cell sorter (FACSCalibur, Becton, Dickinson, and Company, Franklin Lakes, NJ) with Cell Quest Pro Software. The percentage of FITC-positive cells was calculated by setting the background population as 98% negative when analyzing control cells [19].

**Microscopic observation of intracellular localization**

Plasmid DNA was labeled with Cy5 by using Label IT® Cy5 Labeling Kit (Mirus®, Madison, WI). After the transfection culture of MSC for 1 day with the complex of Cy5-labeled plasmid DNA and spermine-polysaccharide, cells were washed and fixed with 0.25 wt% glutaraldehyde solution in PBS, followed by staining nucleus with Hoechst 33342 (Molecular Probes, Eugene, OR). Imaging data of cells were collected on an Olympus Fluoview FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan) and processed with Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA).

**Statistical Analysis**

All the data were expressed as the mean±the standard deviation of the mean. Statistical analysis was performed based on the ANOVA, followed by Fisher's PLSD and significance was accepted at $P < 0.05$.

# RESULTS

## Characterization of spermine-polysaccharide-plasmid DNA complexes

**Figure 1** shows the electrophoretic patterns of polyion complexes of plasmid DNA and spermine-polysaccharide prepared at an N/P ratio of 3.0. Migration of plasmid DNA was retarded by complexation with spemine-polysaccharide.

**Table 1** summarizes the physicochemical properties of polyion complexes prepared at an N/P ratio of 3.0. The apparent molecular size and the zeta potential of free plasmid DNA were around 400 nm and -15 mV, respectively. Irrespective of the polysaccharide type, the apparent molecular size and zeta potential was around 160-280 nm and 10-16 mV, respectively.

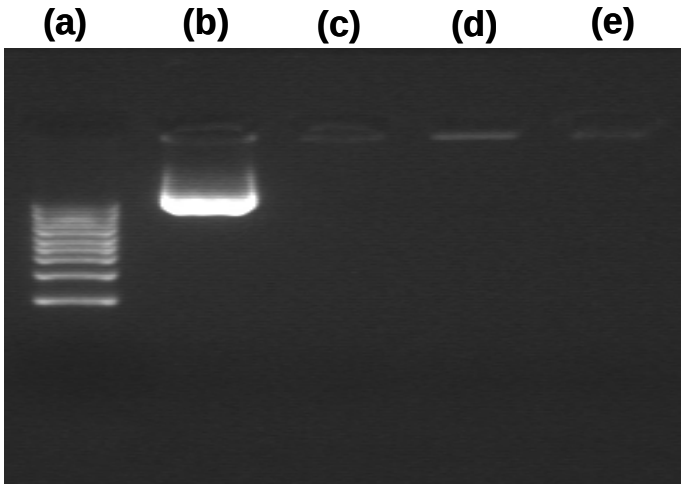



**Figure 1**. Agarose gel electrophoresis of spermine-polysaccharide-plasmid DNA complexes prepared at an N/P ratio of 3.0. The sample applied is DNA marker (a), free plasmid DNA (b) or the complexes of plasmid DNA and spermine derivatives of pullulan (c), dextran (d), and mannan (e). The [CDI]/[OH] ratio of spermine-polysacccharide used for cationization is 1.5.

**Table 1. Apparent molecular size and zeta potential of plasmid DNA complexed with cationized polysaccharides[a)]**

| Polysaccharide | Apparent molecular size (nm) | Zeta potential (mV) |
|---|---|---|
| Pullulan | 226.3 ± 51.9 | +12.2 ± 1.5 |
| Dextran | 157.0 ± 28.3 | +14.0 ± 0.1 |
| Mannan | 293.5 ± 28.5 | +12.0 ± 0.5 |
| Free plasmid DNA | 409.5 ± 61.2 [b)] | -14.7 ± 9.5 |

a) The complex was prepared at an N/P ratio of 3.0.

b) Means ±SD.

**Figure 2** shows the time profile of solution absorbance change of spermine-polysaccharide-plasmid DNA complexes after the addition of RCA120 lectin. The solution absorbance increased time-dependently, although the increment pattern of depended on the polysaccharide type. When galactose was added to the solution, the absorbance decreased.

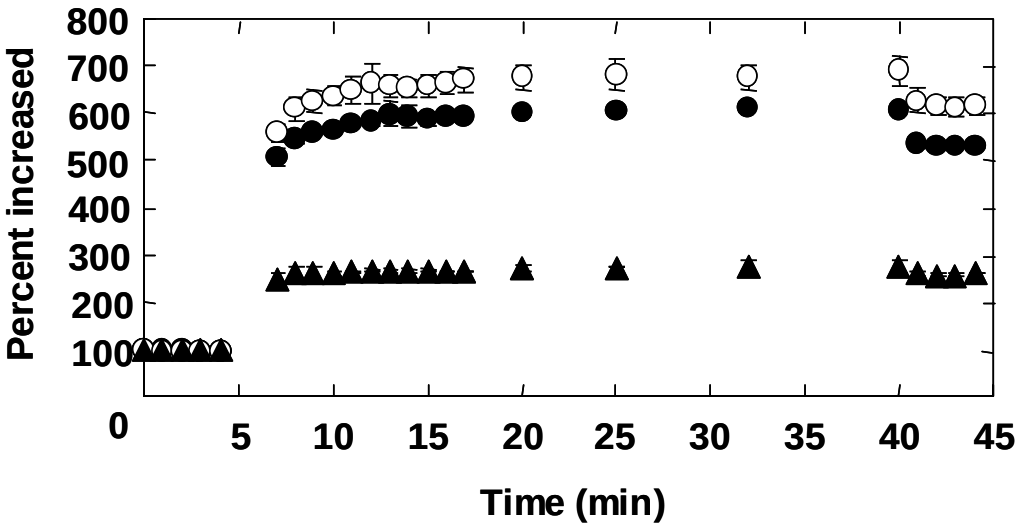



**Figure 2.** Time course of the turbidity change of spermine-polysaccharide-plasmid DNA complexes prepared at an N/P ratio of 3 after RCA 120 addition. Galactose was added 40 min after RCA 120 addition (indicated by an arrow). The type of polysaccharide used for spermine introduction is pullulan (○), dextran (●), and mannan (▲). The [CDI]/[OH] ratio of spermine-polysacccharide used for cationization is 1.5.

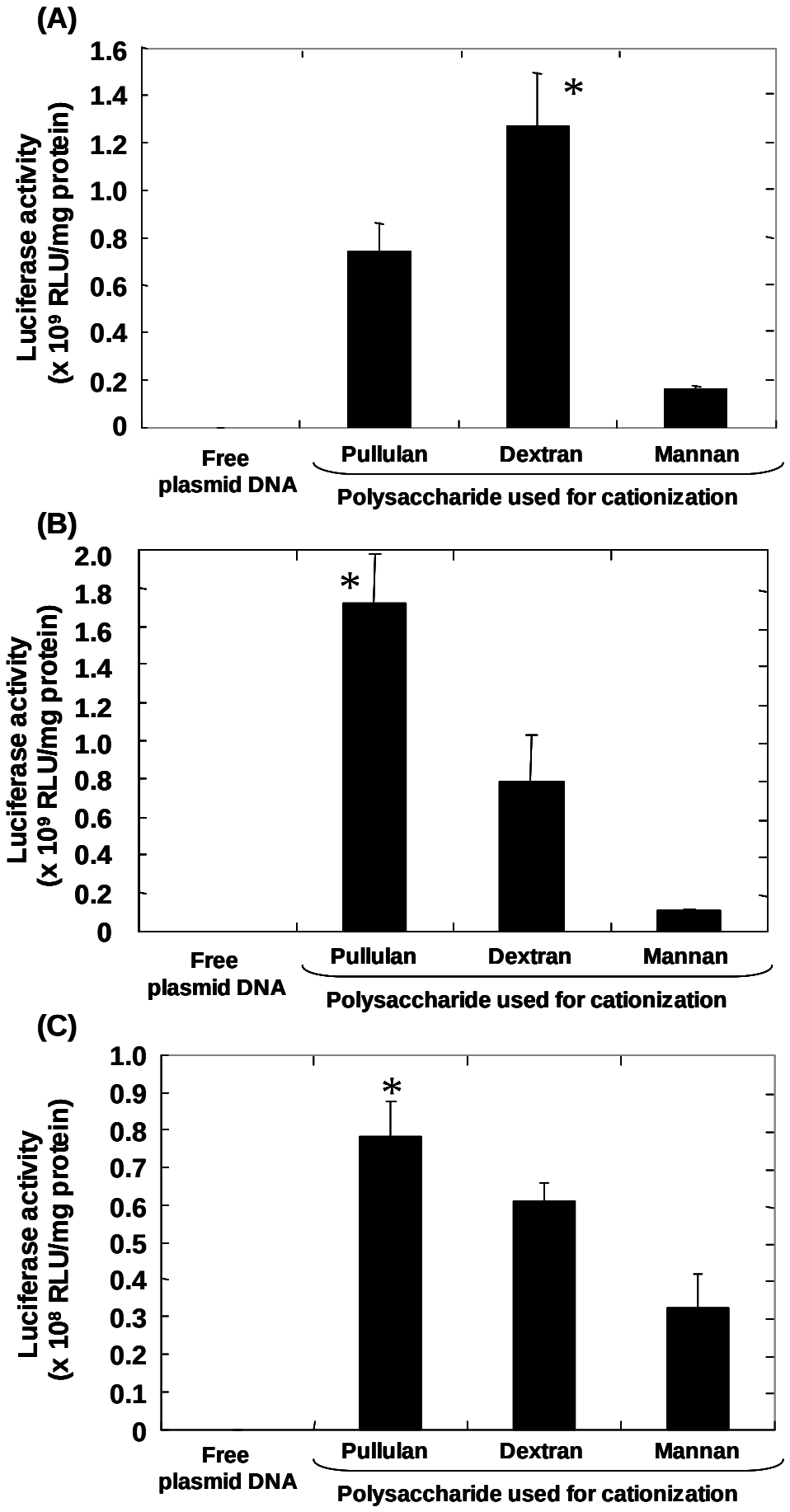
**In vitro gene transfection of spermine-polysaccharide-plasmid DNA complexes**

**Figure 3** shows effect of the type of polysaccharides used for spermine introduction on the luciferase expression of spermine-polysaccharide-plasmid DNA complexes at an N/P ratio of 3.0 for each type of cells. The level of gene expression was enhanced by the spermine-polysaccharide-plasmid DNA complexes, while the level depended on the type of polysaccharide as well as type of cells.

**Figure 4** shows the fluorescence histogram of MSC 1 day after transfection with free FITC-labeled plasmid DNA or the complex with different spermine-polysaccharides. The percentages of plasmid DNA internalized were 3.0, 71.8, 46.5 or 36.3 for MSC transfected with free plasmid DNA, the complex of spermine derivatives of pullulan, dextran, mannan, respectively.

**Figure 5** shows the confocal laser microscopic images of MSC 1 day after transfection with spermine-polysaccharide-plasmid DNA complexes. Most of plasmid DNA were localized around or in the nucleus of MSC. Strong GFP expression was observed in the MSC transfected with spermine-pullulan-plasmid DNA complexes.

Effect of polysaccharide type
on the level gene expression



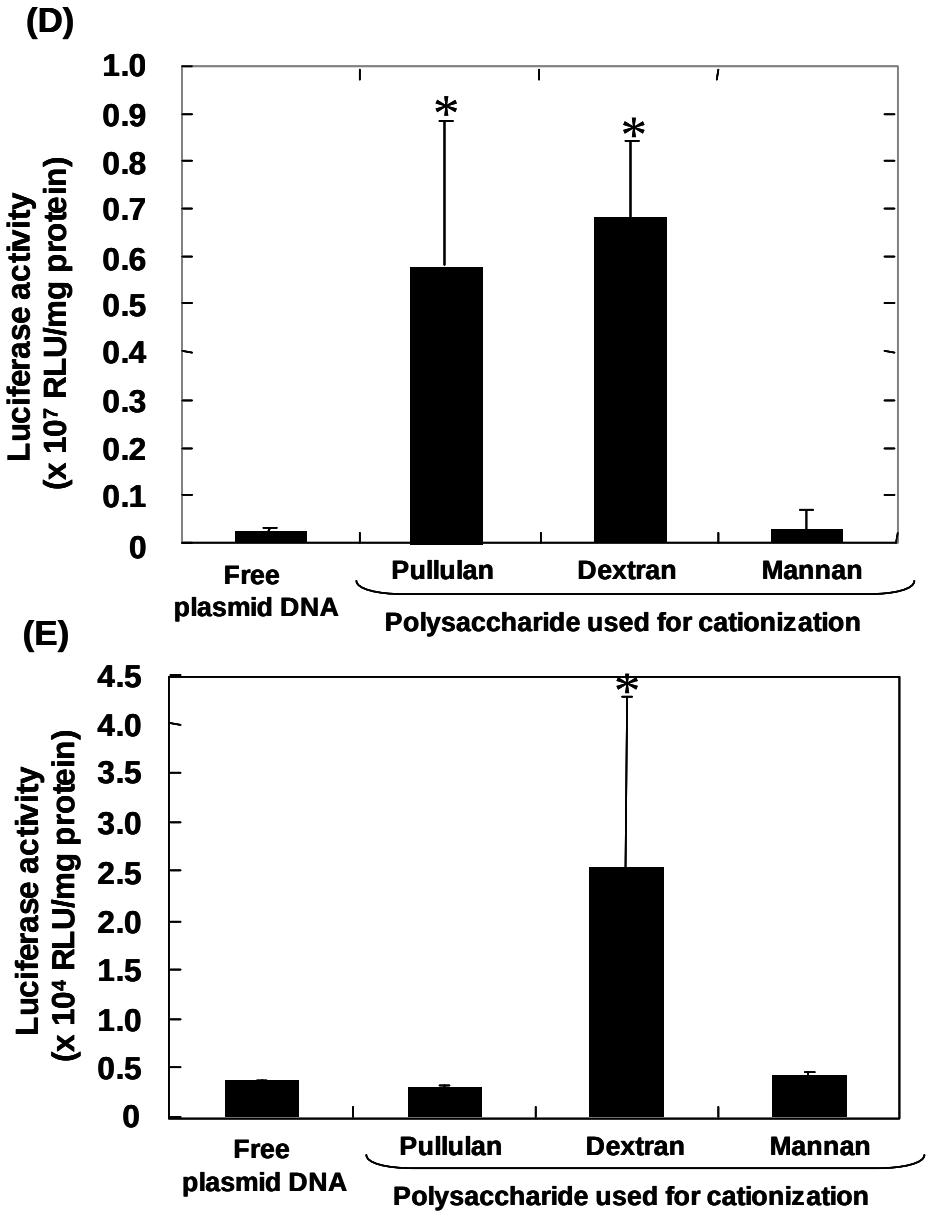



**Figure 3.** Effect of the type of polysaccharide used for spermine introduction on the luciferase expression of spermine-polysaccharide-plasmid DNA complexes for HeLa cells (A), HepG2 cells (B), MSC (C), Mϕ (D), and DC (E). The amount of plasmid DNA applied is 2.5 μg/well and the N/P ratio is 3.0. *, $p < 0.05$; versus the expression level of complexes prepared by other spermine-polysaccharides.

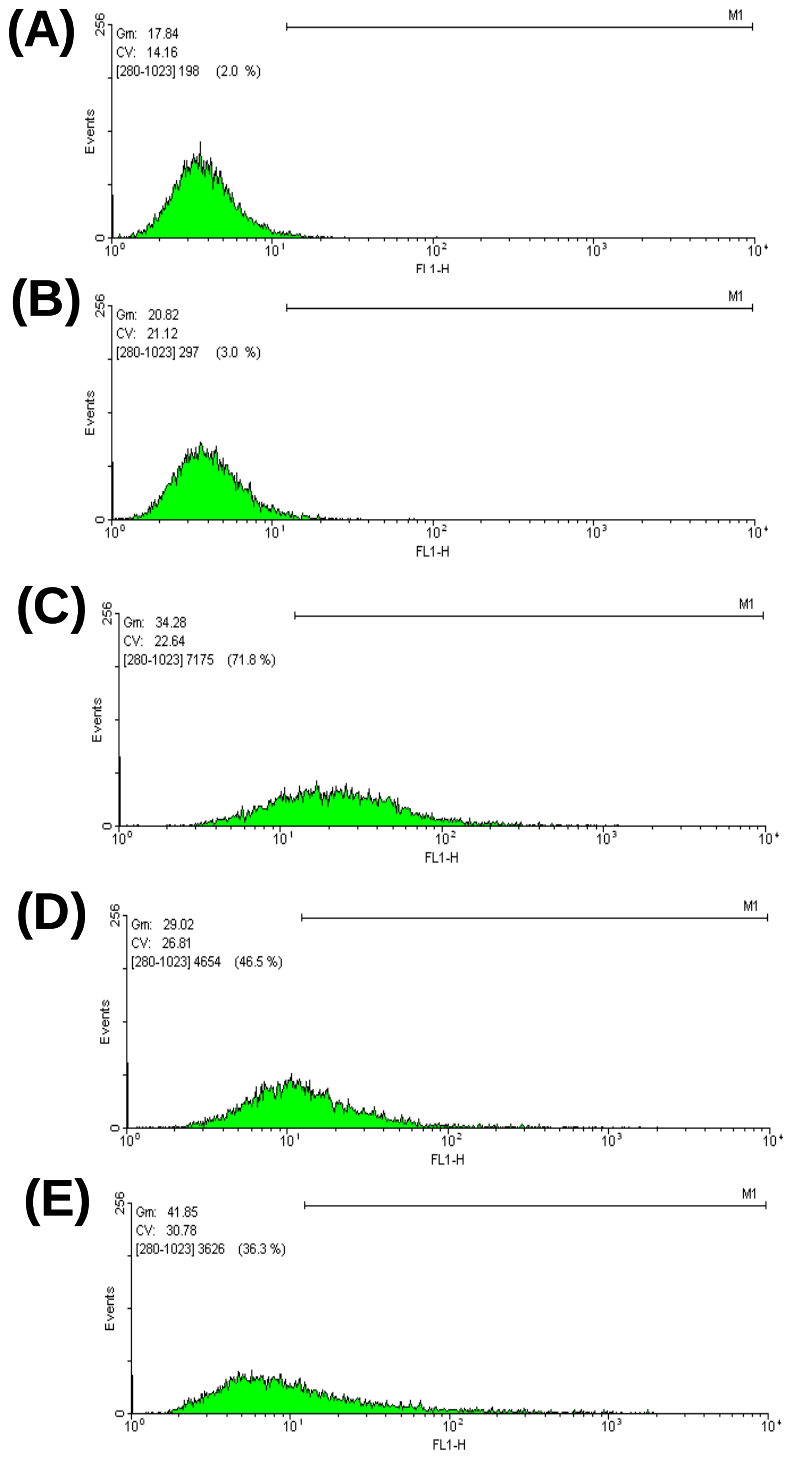



**Figure 4.** Fluorescence histogram of MSC 1 day after non-transfection (A) or transfection with free FITC-labeled plasmid DNA (B) or the complex of spermine derivatives of pullulan (C), dextran (D), and mannan (E). The amount of plasmid DNA applied is 2.5 μg/well and the N/P ratio is 3.0.

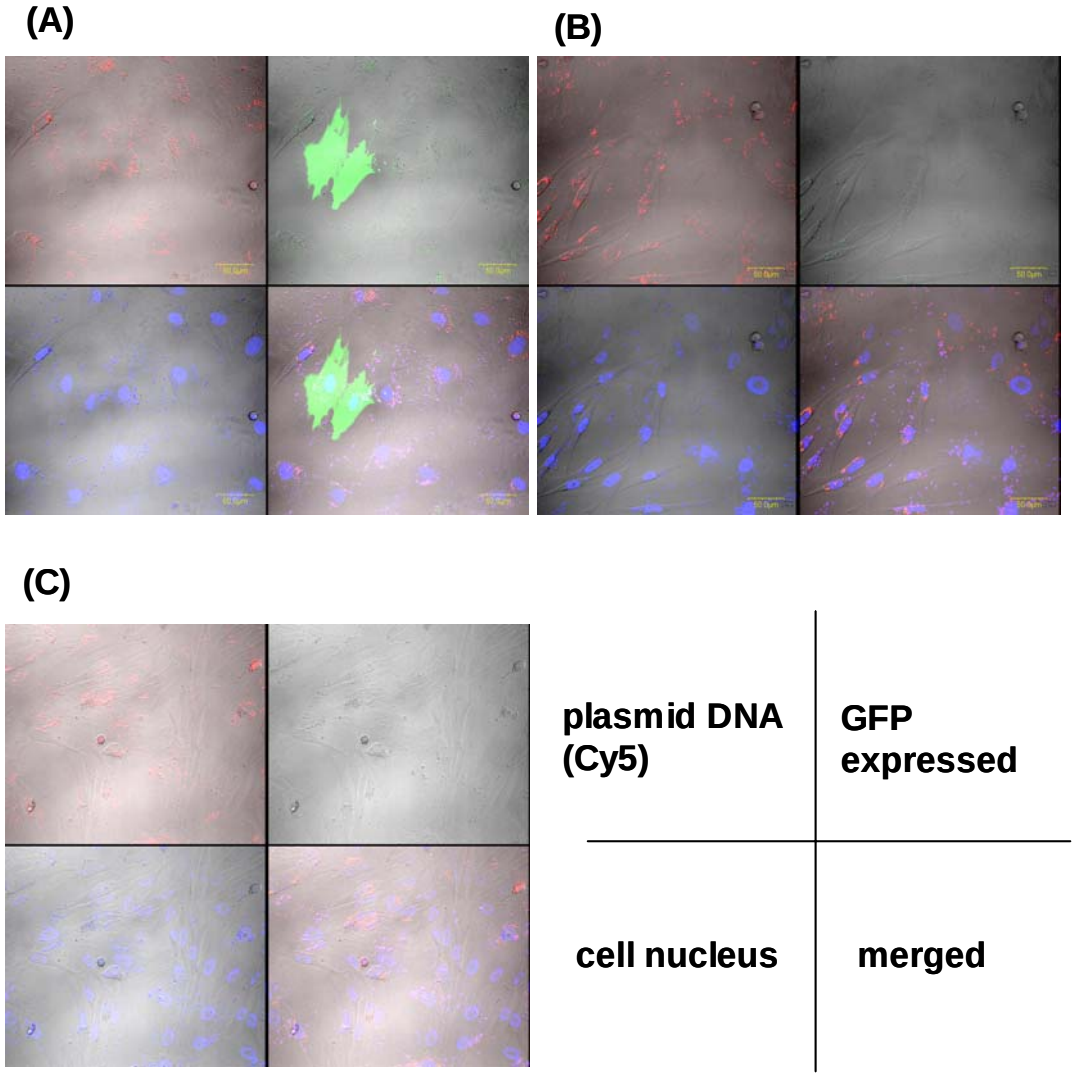



**Figure 5.** Confocal laser microscopic images of MSC 1 day after transfection with GFP plasmid DNA complex of spermine derivatives of pullulan (A), dextran (B), and mannan (C). The plasmid DNA was labeled with Cy5. The amount of plasmid DNA used for transfection is 2.5 μg. The N/P ratio of spermine-polysaccharide-plasmid DNA complex is 3.0. The red, green, and blue points indicate the plasmid DNA, GFP expressed, and cell nucleus, respectively.

## DISCUSSION

The present study demonstrates that the in vitro gene expression of plasmid DNA for various types of cells was greatly enhanced by complexation with the spermine-polysaccharide derivatives. The gene expression level depended on not only the polysaccharide type, but also the cells type.

It is indispensable to develop technology and methodology of gene transfection for genetic engineering of cells. Considering the clinical use, it is practical necessary to develop a non-viral gene carrier. For efficient gene transfection, it is important to design carriers that can electrostatically bind to a genetic material, molecularly condense the material size to a diameter range of several hundred nanometers, which facilitate the cell internalization. In addition, it is also necessary to develop a carrier for cell-specific delivery system, which allows genes to deliver specifically to the target cell and enhance the expression level.

Among the non-viral materials applicable for gene transfection carrier, polysaccharide has an advantage over other substances in terms of the ability to be recognized by the respective cell receptors of sugar specificity. This biological recognition not only permits the receptor-specific targeting of substances to the cell, but also accelerates their cell uptake via the receptor-mediated endocytosis. Therefore, in this study, the effect of polysaccharide type on the level of gene expression was investigated.

Dependence of the polysaccharide type on the level of gene expression (**Figures 3 and 5**) can be explained as follows. The electrophoresis analysis (**Figure 1**) and the DLS and ELS measurements (**Table 2**) revealed that physicochemical properties of plasmid DNA complexes were not changed by the polysaccharide type. On the other hand, the lectin affinity assay (**Figure 2**) revealed that the plasmid DNA complexes with spermine derivatives of pullulan and dextran were strongly recognized by the sugar-recognizable lectin, RCA120, which has the same recognition site of asialoglycoprotein receptor (ASGPR). It is possible that the pullulan and dextran chains present on the complex surface is specifically recognized by the lectin, resulting in the formation of complex-lectin aggregates. Several researcher including us have previously found that the ASGPR was expressed in HepG2 cells as well as MSC [20]. On the other hand, several researchers have been demonstrated that pullulan of a

polysaccharide accumulates in the liver via the ASGPR at significantly higher amounts than other water-soluble polymers [21,22]. Based on these findings, it highly expected that the polysaccharide type affects the cellular internalization of plasmid DNA for HepG2 cells and MSC via ASGPR. This is also supported by the cellular internalization analysis result (**Figure 4**). The level of gene expression for Mϕ was enhanced by the plasmid DNA complex with the spermine derivatives of pullulan and dextran, while the highest gene expression level of DC was observed for the plasmid DNA complex with spermine-dextran (**Figures 3D and 3E**). This phenomenon may be explained in terms of the difference in the type of surface receptors between the cells. It has been reported that both the ASGP [23] and mannose receptors [24] are expressed on the surface of Mϕ. It is recognized that DC possess mannose receptors on the surface[25]. On the other hand, several researchers demonstrated that dextran[26,27] and mannan[28,29] function as a ligand for the mannose receptor. Taken together, it is conceivable that the recognition difference among the polysaccharides affects the extent of cellular internalization, resulting in modifying the level of gene expression. For HeLa cells, there is no report about the surface receptor to recognize sugar residues. Thus, the reason of the highest gene expression by the plasmid DNA complex with spermine-dextran cannot be claimed from the viewpoint of the cell surface receptor.

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# Chapter 2

## Effect of amine compounds introduced to dextran on the level of gene expression

## INTRODUCTION

It has been reported that the level of gene expression is greatly influenced by the physicochemical properties of cationized carriers for complexation including the molecular weight, and the type of amine compounds or the extent for cationization. Among them, amine compounds introduced to the carrier polymer has been known as a factor contributing to not only the cellular internalization, but also the intracellular trafficking, such as the endosomal escape of gene-carrier complex and the travel to the cell nucleus, which regulates the level of gene expression [1-6]. Since a polysaccharide has many reactive groups, different amine compounds can be chemically introduced to it. In Chapter 1, it was demonstrated that dextran of a polysaccharide was the most effective polysaccharide as a non-viral carrier material of transfection for HeLa cells. In this chapter, various amine compounds were chemically introduced to the dextran, and a plasmid DNA was complexed with the cationized dextran derivatives. In addition to the acid-base titration of cationized dextran derivatives, apparent molecular size and zeta potential measurement, gel retardation, and ethidium bromide intercalation assays of the complexes were carried out to evaluate the physicochemical properties of complexes. In vitro gene expression by the complexes was investigated for HeLa cells to evaluate influence of the complex physicochemical properties.

## EXPERIMENTAL

### Materials

Dextran with a weight-average molecular weight of 74,000 and amine compounds (**Figure 1**) of ethylenediamine (N2N), N-ethylethylenediamine (N2N2), N,N-diethylethylenediamine (N2N2,2), spermidine (N3N4N), and spermine (N3N4N3N)

were purchased from Sigma-Aldrich, Inc., St. Louis, MO. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

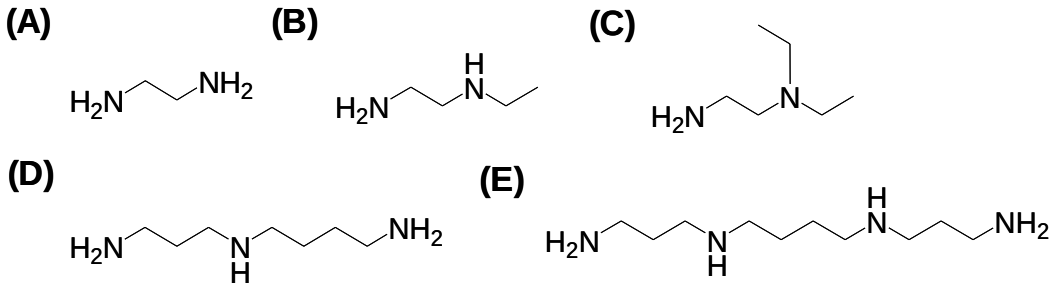


**Figure 1**. Chemical structure of amine compounds used for the cationization of dextran: (A) ethylenediamine (N2N), (B) N-ethylethylenediamine (N2N2), (C) N,N-diethylethylenediamine (N2N2,2), (D) spermidine (N3N4N) or (E) spermine (N3N4N3N).

**Preparation of cationized dextran derivatives**

Each amine compound was chemically introduced to the hydroxyl groups of dextran by a N,N'-carbonyldiimidazole (CDI) activation method [7]. Briefly, $9.28\times10^{-3}$ mole of each amine compound and $4.64\times10^{-3}$ mole of CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of dextran. Following agitation at 35 °C for 20 hr, the reaction mixture was dialyzed against an ultra-pure double distilled water (DDW) for 2 days with a dialysis membrane (the cut-off molecular weight = 12,000-14,000, Viskase Companies, Inc, Willowbrook, IL). Then, the solution dialyzed was freeze-dried to obtain the amine-introduced dextran (cationized dextran). When determined from the conventional elemental analysis, the molar percentages of amine compounds introduced to the hydroxyl groups of dextran were 36.2, 26.3, 34.3, 25.8, and 23.9 mole% for N2N, N2N2, N2N2,2, N3N4N, and N3N4N3N, respectively (**Table 1**).

**Preparation of plasmid DNA**

The plasmid DNA used was the pGL3 vector (5.26 kb) coding a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, USA). The plasmid DNA was propagated in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of the plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

**Acid-base titration of cationized dextran**

Each cationized dextran with the similar molar amount of amino groups ($6.0 \times 10^{-7}$ mole) was dissolved in DDW. To deprotonize all of amino groups in the cationized dextran, 60 μl of 0.01 N NaOH was added. Potentiometric titration was performed with a Horiba F22 pH meter (HORIBA, Ltd., Kyoto, Japan) using 0.001 N HCl. From the titration curve, the pKa values of cationized dextran were determined based on the Henderson-Haselbalch's equation.

**Preparation of polyion complexes (PIC)**

PIC were prepared by mixing an aqueous solution of cationized dextran with that of plasmid DNA. Briefly, varied amounts of cationized dextran were dissolved in 25 μl of DDW and mixed with 25 μl of 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 2.5 μg of plasmid DNA, followed by leaving for 15 min at room temperature to obtain various PIC of cationized dextran and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of cationized dextran (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio.

**Electrophoresis of cationized dextran-plasmid DNA complexes**

PIC were prepared in 10 mM PBS solution at different N/P ratios. After 15 min of incubation, 10 μl of the complex was added to 3 μl of a loading buffer (0.1% sodium dodecyl

sulfate, 5% glycerol, and 0.005% bromophenol blue) and applied on an 1 wt % agarose gel in Tris-borate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.3) containing 0.1 mg/ml ethidium bromide (EtBr). Electrophoretic evaluation of the PIC was carried out in TBE solution at 100 V for 30 min. The gel was imaged with a UV transilluminator (Gel Doc 2000, BIO-RAD laboratories, Segrate, Italy).

**Dynamic light scattering (DLS) and electrophoretic light scattering (ELS) of PIC**

PIC were prepared in 10 mM PBS solution at an N/P ratio of 5.0. the DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) equipped with an $Ar^+$ laser at a detection angle of $90^o$ at 25 °C for 15 min. In the present study, the autocorrelation function of samples was analyzed based on the cumulants method and the $R_s$ value was calculated automatically by the equipped computer software and expressed as the apparent molecular size of samples. On the other hand, the zeta potential was measured using ELS-7000AS instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) at 25 °C and an electric field strength of 100 V/cm. From the determined electrophoretic mobility, the zeta potential was automatically calculated using the Smoluchouski equation. Light scattering measurement was done three times for every sample.

**Ethidium bromide intercalation assay**

A sample containing plasmid DNA (20 μg/ml) and EtBr (0.4 mg/ml) (plasmid DNA-EtBr complex) was used to calibrate to 100% fluorescence. The cationized dextran was added to an aqueous solution of EtBr at different N/P ratios. The fluorescence intensity of the samples (excitation: 510 nm, emission: 590 nm) was measured 15 min later by Gemini EM fluorescent microplate reader (Molecular Devices, sunnyvale CA, USA). The results were expressed as a relative fluorescence intensity (percent decreased against plasmid DNA-EtBr complex).

**In vitro gene transfection experiment**

Transfection experiments were performed in triplicate. HeLa cells were purchased from American Type Culture Collection, Manassas VA, and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 110 mg/ml sodium pyruvate (Invitrogen Corp., Carlsbad, CA), and 10 vol% fetal calf serum (FCS, Hyclone laboratories, Inc., South Logan, UT) (DMEM-FCS) at 37 °C. Cells were seeded on each well of 12 multi-well cluster plate (Corning Inc., Corning, NY) at a density of $1.25\times10^5$ cells/well and cultivated in 1 ml of DMEM-FCS for 24 hr. Immediately after the medium was exchanged by fresh Opti MEM medium (Invitrogen Corp., Carlsbad, CA), the PIC solution (50 μl) was added and incubated for 15 min at room temperature, followed by 6 hr incubation for cell gene transfection. Then, the medium was changed to DMEM-FCS and cells were incubated further for 24 hr.

Cells were washed with PBS twice, lysed in 100 μl of cell culture lysis reagent (Promega Corp., Madison, WI), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI) was added to 20 μl of supernatant, while the relative light unit (RLU) of the samples was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL) according to the manufacturers' instructions in order to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently.

**Evaluation of plasmid DNA internalization**

Plasmid DNA was labeled with rhodamine by using Label IT® CX-Rhodamine Labeling Kit (Mirus®, Madison, WI) according to the manufacturers' instructions. After transfection culture wuth the rhodamine-labeled plasmid DNA, the cells were treated in cold acetate-buffered solution containing 0.3 vol.% acetic acid, 0.085 M NaCl, and 5.0 mM KCl for 1 min, then the cells were washed with cold PBS to remove the complexes bound onto the cell surface. The cells of three wells were collected by the trypsinization, and the results were considered as an average value of triplicates, while ten thousand cells were measured by the fluorescence activated cell sorter (FACSCalibur, Becton, Dickinson, and Company, Franklin

Lakes, NJ) with Cell Quest Pro Software. The percentage of rhodamine-positive cells was calculated by setting the background population as 98% negative when analyzing control cells.

**Cell viability**

Cytotoxicity was assayed using a cell counting kit (Nacalai tesque, Inc., Kyoto, Japan). Cells were seeded on each well of 96 multi-well cluster plate (Corning Inc., Corning, NY) at a density of $1.25 \times 10^4$ cells/well and cultivated in DMEM-FCS for 24 hr. The medium was changed to the fresh Opti MEM medium, and 5 μl of the PIC solution was applied to each well, follow by 6 hr incubation. Then, the medium was changed to DMEM-FCS and 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H- tetrazolium (WST-8) solution was added and the cells were incubated further for 3 hr. The absorbance of samples was measured at 450 nm by VERSAmax microplate reader (Molecular Devices, sunnyvale CA). The percent cell viability was expressed as 100% for control, non-treated cells.

**Statistical Analysis**

All the data were expressed as the mean±the standard deviation of the mean. Statistical analysis was performed based on the ANOVA, followed by Fisher's PLSD and significance was accepted at $P < 0.05$.

## RESULTS

**Characterization of cationized dextrans**

**Figure 2** shows the acid-base titration curves of cationized dextran prepared. The curve profiles depended on the type of amine compounds introduced to dextran. The pKa values of cationized dextran were determined by the curves (**Table 1**). The pKa values of cationized dextran decreased compared with those of original amine compounds used for cationization. **Figure 2B** shows the replotted pH change profiles of cationized dextran as a function of proton addition. When the proton amounts required for pH change from 7.4 to 5.0

were calculated, the orders was N3N4N3N > N2N > N2N2,2 > N3N4N > N2N2.

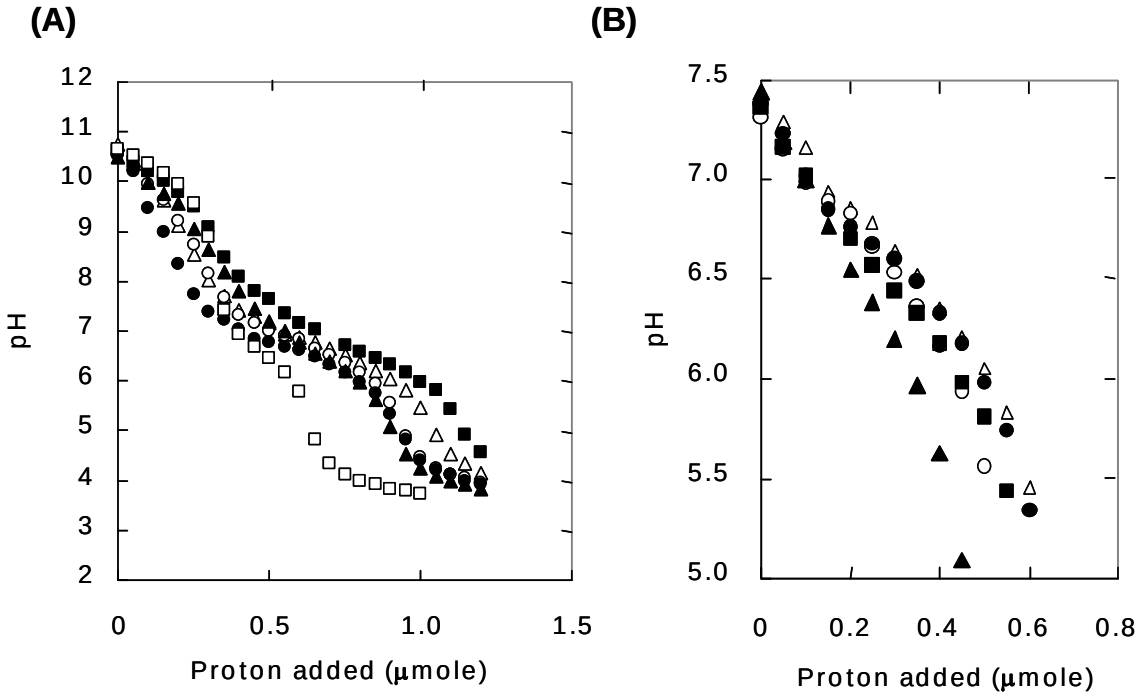


**Figure 2.** (A) Acid-base titration curves of cationized dextran or water (□). The amine compound used for cationization is (●) N2N, (▲) N2N2, (■) N2N2,2, (○) N3N4N or (△) N3N4N3N. (B) A replotted pH change profiles of cationized dextran as a function of the proton addition.

**Table 1. Physicochemical properties and cellular internalization of plasmid DNA and the complexes with dextran cationized by different amine compounds.**

| Cationized dextran | | | Apparent molecular size (nm) | Zeta potential (mV) | Percent internalized |
|---|---|---|---|---|---|
| Amine compound | Percent introduced [a] | pKa [b] | | | |
| N2N | 36.2 | 7.38 | 306 ± 105 [c] | +9.22 ± 1.4 [c] | 9.7 |
| N2N2 | 26.3 | 8.64 | 3330 ±1750 | +9.78 ±1.3 | 5.0 |
| N2N2,2 | 34.3 | 9.08 | 230 ± 33.4 | +11.1 ± 1.0 | 13.5 |
| N3N4N | 25.8 | 9.64, 7.15 | 235 ± 66.7 | +16.3 ± 1.1 | 19.8 |
| N3N4N3N | 23.9 | 9.98, 8.02, 7.16 | 221 ± 77.5 | +16.3± 1.4 | 33.7 |
| | Free plasmid DNA | | 655.8 ± 331.1 | -14.9 ± 4.6 | 2.0 |

a) The extent of amine compounds introduced to the hydroxyl groups of dextran.

b) Determined by the acid-base titration.

c) Means ± S.D.

**Characterization of cationized dextran -plasmid DNA complexes**

**Figure 3** shows the agarose gel electrophoresis image of cationized dextran -plasmid DNA complexes prepared at different N/P ratios. For all the complexes, the migration of plasmid DNA was retarded with an increase in the N/P ratio, and the plasmid DNA disappeared at the N/P ratio higher than a certain value. The extents of retardation in each N/P ratio were different among cationized dextrans used for complexation.

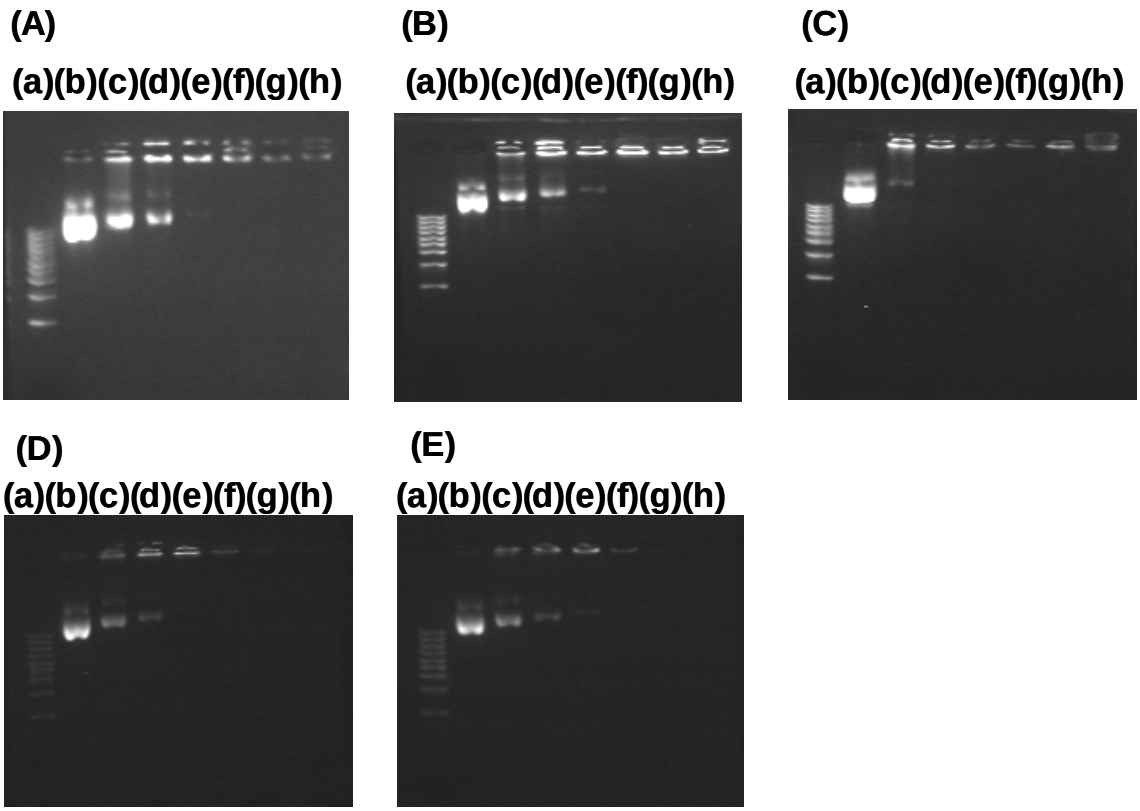


**Figure 3.** Agarose gel electrophoresis of cationized dextran-plasmid DNA complexes prepared at different N/P ratios. The amine compound used for cationization is (A) N2N, (B) N2N2, (C) N2N2,2, (D) N3N4N or (E) N3N4N3N. The sample applied is (a) DNA marker, (b) free plasmid DNA or complexes prepared at N/P ratios of (c) 0.5, (d) 1.0, (e) 1.5, (f) 2.0, (g) 3.0, and (h) 5.0.

The physicochemical properties of polyion complexes prepared at the N/P ratio of 5.0 are summarized in **Table 1**. The apparent molecular size and the zeta potential of free plasmid DNA were around 660 nm and -15 mV, respectively. The polyion complexation of cationized dextran almost reduced the molecular size of plasmid DNA, while the molecular

size oppositely became large for N2N2-introduced dextran. On the other hand, the zeta potential was positive for all the plasmid DNA complexes with cationized dextran, while the values depended on the type of cationized dextrans used for complexation.

**Figure 4** shows the solution fluorescence of cationized dextran-plasmid DNA complexes prepared at different N/P ratios after EtBr addition. The EtBr fluorescence intensity decreased with an increase in the N/P ratio. The pattern of fluorescence intensity decreased depended on the amine compounds introduced to dextran. At the N/P ratio of 5.0, the EtBr fluorescence intensity became low for the PIC prepared by the cationized dextran s except for N2N2-introduced dextran.

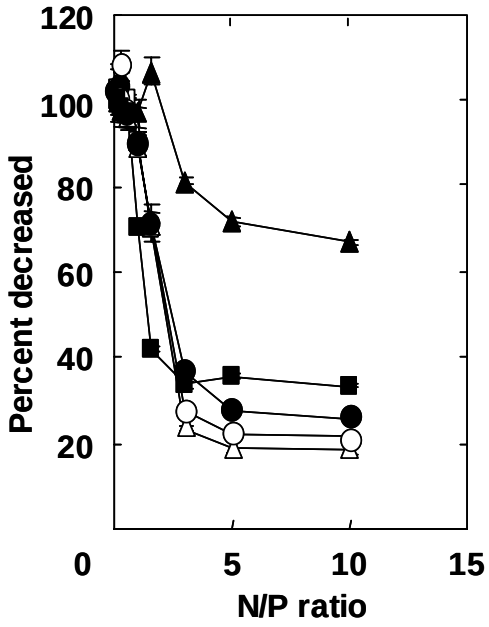


**Figure 4.** Solution fluorescence of cationized dextran-plasmid DNA complexes prepared at different N/P ratios after EtBr addition. The amine compound used for cationization is (●) N2N, (▲) N2N2, (■) N2N2,2, (○) N3N4N or (△) N3N4N3N.

**In vitro gene transfection of cationized dextran-plasmid DNA complexes**

**Figure 5** shows effect of the amine compound introduced on the luciferase expression of cationized dextran-plasmid DNA complexes for HeLa cells. The level of gene expression was different among the cationized dextrans used. The highest level was observed for the complex of cationized dextran with N3N4N3N-introduced dextran.

**Table 1** summarizes the internalization percentages of complexes with cationized dextrans into Hela cells. The highest internalization percentage was observed for the complex of spermine-introduced dextran and plasmid DNA.

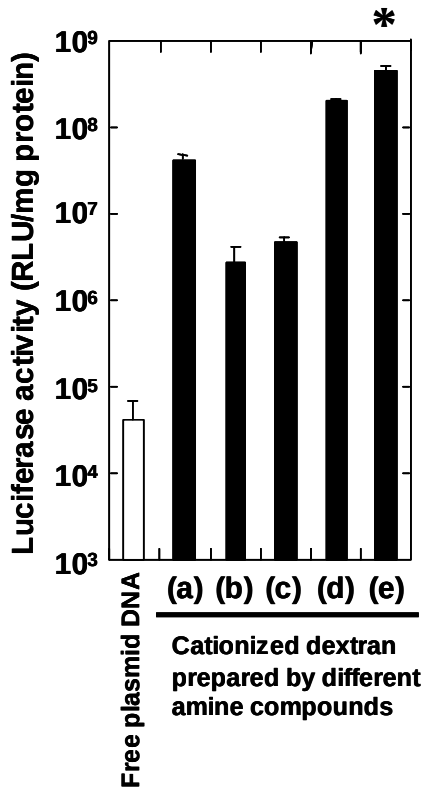


**Figure 5.** Effect of the amine compound introduced on the luciferase expression of cationized dextran-plasmid DNA complexes for HeLa cells. The amine compound used for cationization is (A) N2N, (B) N2N2, (C) N2N2,2, (D) N3N4N or (E) N3N4N3N. The amount of plasmid DNA applied is 2.5 μg/well and the N/P ratio is 5.0. *, $p<0.05$; versus the expression level by complexes prepared with other cationized dextrans.

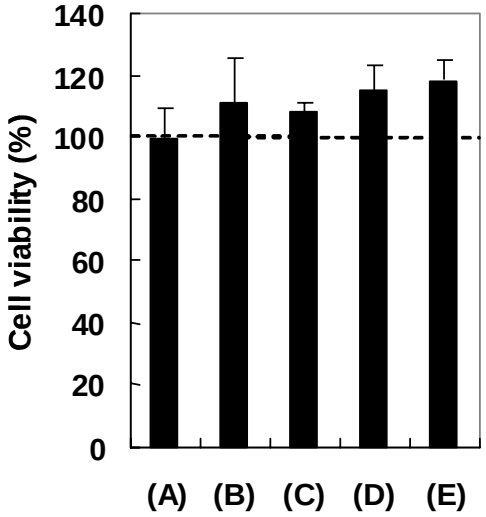


**Figure 6.** Effect of the amine compound introduced on the cell viability of HeLa cells transfected with cationized dextran-plasmid DNA complexes prepared at the N/P ratio of 5.0. The amine compound used for cationization is (A) N2N, (B) N2N2, (C) N2N2,2, (D) N3N4N or (E) N3N4N3N.

**Figure 6** shows the effect of the the amine compound introduced on the cell viability of HeLa cells transfected with cationized dextran-plasmid DNA complexes prepared at the N/P ratio of 5.0. No cytotoxicity was observed for every complex under the condition where the cell transfection experiment is performed.

## DISCUSSION

The present study demonstrates that the in vitro gene expression of plasmid DNA was greatly enhanced by the complexation with the cationized dextran and influenced by the type of amine compounds introduced to dextran. In general, for efficient non-viral gene delivery, it is important to design carriers that can electrostatically bind to a genetic material, molecularly condense the material size to a diameter range of several hundred nanometers, protect the material from enzymatic degradation, enhance the cell internalization, and possess a buffering effect to escape from the endosome. In this chapter, the physicochemical properties of complexes were modified by chemical introduction of different amine compounds used. This derivatization resulted in the different levels of gene expression.

Dextran is a water-soluble, naturally-occurring polysaccharide with multiple hydroxyl groups applicable to chemical modification. It also has low immunogenicity and a long history in clinical use as a plasma expander [8]. In this chapter, the effect of amine compounds introduced to dextran on the physicochemical property of complex and the level of gene expression were investigated.

In this study, five different amine compounds were selected (**Figure 1**). Ethylenediamine (N2N), spermidine (N3N4N), and spermine (N3N4N3N) were selected because the polyamines are present in the body, while N-ethylethylenediamine (N2N2) and N,N-diethylethylenediamine (N2N2,2) were selected to compare the effect of amine structure on the physicochemical property and the gene expression level of cationized dextran complexes. Acid-base titration (**Figure 2A**) revealed that the pKa values of cationized dextran decreased compared with those of original amine compounds used for cationization. This is due to the compound basicity. It is possible that the chemical introduction of amine compounds with the hydroxyl group of dextran produces the amide bond of a electron-withdrawn group, which makes the terminal amino group positive via so-called inductive effect.

DLS and ELS measurements (**Table 1**) and ethidium bromide intercalation assay (**Figure 4**) revealed that the amine compound introduced to dextran greatly affected on the physicochemical properties of plasmid DNA complexes. As shown in **Table 1**, after

complexation with dextran cationized by amine compounds except for N2N2, the apparent molecular size of plasmid DNA became smaller, while the apparent size of complexes using N2N2-introduced dextran was larger in comparison with that of the free plasmid DNA. The EtBr intercalation assay (**Figure 4**) also revealed that the strength of interaction between the plasmid DNA and N2N2-introduced dextran was lower than that of other cationized dextrans. These findings may be due to the steric hindrance of ethylene groups next to the secondary amine the complex could not be formed tightly. The zeta potential (**Table 1**) was positive for all complexes although the values depended on the type of cationized dextrans used for complexation. From the pKa determined, the percentage of amino groups protonized at pH of 7.4 can be calculated, which is 49, 94, 98, 68 or 72 for N2N, N2N2, N2N2,2, N3N4N or N3N4N3N, respectively. The order of zeta potential is in good accordance with that of protonized percentage.

The level of gene expression depended on the amine groups introduced to dextran, while the order was N3N4N3N > N3N4N > N2N > N2N2,2 > N2N2 (**Figure 5**). This is explained by terms of plasmid DNA internalized into the cells, the ability for the complexes to escape from the endosome, and the ability for complex to be dissociated and translated in the nucleus. First, the order of plasmid DNA internalization was N3N4N3N > N3N4N > N2N2,2 > N2N > N2N2 (**Table 1**). It is reported that a cetain range of apparent molecular size and surface state of polyion complexes was effective for the enhancement of plasmid DNA internalization [9,10]. The low percentage of plasmid DNA internalization for N2N2-introduced dextran is due to the large molecular size of complex. Second, it is practically important to consider the buffering capacity at pH ranged from 7.4 to 5.0 for successful gene expression. When pH change profiles of cationized dextran from 7.4 to 5.0 were replotted as a function of proton addition, the order of proton amounts required for pH change was N3N4N3N > N2N > N2N2,2 > N3N4N > N2N2 (**Figure 2B**). Third, the ability of complex for dissociation and translation is also important. When the orders from **Figures 2B** and **4** and **Table 1** are compared with that from **Figure 5**, we can say with certainty that each step, such as plasmid DNA internalization, escape from the endosome, and dissociation and translation of complex, governs the gene expression. However, more detailed investigations, such as the intracellular

fate and stability are required to know the contribution for each step.

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# Chapter 3

## Effect of pullulan molecular weight on the level of gene expression

## INTRODUCTION

Several approaches to the cell-specific gene delivery have been investigated by making use of cell-surface receptors [1] (peptide-recognition receptor [2-5] or sugar chain-recognition receptor [6-10]). Generally the ligand for the receptor is covalently linked to the cationized polymers. For example, the liver targeting of a plasmid DNA is achieved through the covalent conjugation of high molecular weight polylysine with asialoorosomucoid [11,12]. Coupling of galactose residues enabled polyethylenimine to selectively deliver genes to hepatocytes via the asialoglycoprotein receptor-mediated pathway [13,14]. The polysaccharide-based carrier has an advantage over other cationized polymers in terms of the possible cell internalization by a sugar-recognition receptor of cell surface without any ligand coupling.

Pullulan is a water-soluble polysaccharide with a repeated unit of maltotriose condensed through α-1,6 linkage and has an inherent ability to accumulate in the liver [15] at higher amounts via the asialoglycoprotein receptor than other water-soluble polymers [16,17]. In Chapter 1, it was demonstrated that the pullulan was the most effective as a non-viral carrier material of transfection for HepG2 cells of a human hepatoma cell line. In addition, Chapter 2 demonstrated that cationization with spermine converted dextran of a polysaccharide to a non-viral carrier of plasmid DNA with transfection efficiency higher than that with other amine compounds.

It is well recognized that the molecular weight of carrier polymer is one of the factors contributing to the level of gene expression [18-20]. In this chapter, thus, pullulan with different molecular weights was cationized by the chemical introduction of spermine and a plasmid DNA was complexed with the cationized pullulan derivatives. The complexes were characterized in terms of the apparent molecular size and zeta potential, while the complex formation behavior was evaluated by the solution turbidity, gel retardation, and EtBr

intercalation examinations. In addition, and lectin-induced aggregation assays were carried out to examine the susceptibility to sugar recognition. The effect of complex physicochemical properties on the in vitro gene expression by the complexes was investigated for HepG2 cells.

## EXPERIMENTAL

### Materials

Pullulan with different weight-average molecular weights, 5,900, 11,800, 22,800, 47,300, 112,000, and 212,000 was purchased from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Spermine was purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

### Preparation of cationized pullulan derivatives

Spermine was introduced to the hydroxyl groups of pullulan by a N,N'-carbonyldiimidazole (CDI) activation method [21]. Spermine and CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of pullulan (Table 1). Following agitation at 35 °C for 20 hr, the reaction mixture was dialyzed against ultra-pure double distilled water (DDW) for 2 days. (The cut-off molecular weight of dialysis membrane is 1,000 for pullulan with molecular weights of 5,900 and 11,800, Spectrum Laboratories, Inc., Rancho Domiguez, CA, USA. and 12,000-14,000 for pullulan with molecular weights from 22,800 to 212,000, Viskase Companies, Inc, Willowbrook, Illinois.). Then, the solution dialyzed was freeze-dried to obtain the samples of spermine-introduced pullulan (spermine-pullulan). The spermine introduction was determined from the conventional elemental analysis and expressed by the molar extent of spermine introduced to the hydroxyl groups of pullulan.

### Preparation of plasmid DNA

The plasmid DNA used was the pGL3 vector (5.26 kb) coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, USA). The plasmid DNA was propagated

in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of the plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

**Preparation of polyion complexes (PIC)**

PIC were prepared by mixing an aqueous solution of spermine-pullulan with that of plasmid DNA. Briefly, varied amounts of spermine-pullulan were dissolved in 50 μl of DDW and mixed with 50 μl of phosphate-buffered saline (PBS, pH 7.4) containing 100 μg of plasmid DNA, followed by leaving for 15 min at room temperature to obtain various PIC of spermine-pullulan and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio.

**Electrophoresis of spermine-pullulan-plasmid DNA complexes**

PIC were prepared in 10 mM PBS solution at different N/P ratios. After 15 min of incubation, 10 μl of the complex was added to 3 μl of a loading buffer (0.1% sodium dodecyl sulfate, 5% glycerol, and 0.005% bromophenol blue) and applied on an 1 wt % agarose gel in Tris-borate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.3) containing 0.1 mg/ml ethidium bromide (EtBr). Electrophoretic evaluation of the PIC was carried out in TBE solution at 100 V for 30 min. The gel was imaged with a UV transilluminator (Gel Doc 2000, BIO-RAD laboratories, Segrate, Italy).

**Characterization of PIC by dynamic light scattering (DLS) and electrophoretic light scattering (ELS)**

PIC were prepared in 10 mM PBS solution at an N/P ratio of 3.0. DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) equipped with an $Ar^+$ laser at a detection angle of $90^o$ at 25 °C for 15 min. In the

present study, the autocorrelation function of samples was analyzed based on the cumulants method and the $R_s$ value was calculated automatically by the equipped computer software and expressed as the apparent molecular size of samples. On the other hand, the zeta potential was measured using ELS-7000AS instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) at 25 °C and an electric field strength of 100 V/cm. From the determined electrophoretic mobility, the zeta potential was automatically calculated using the Smoluchouski equation. Light scattering measurement was done three times for every sample.

**Ethidium bromide intercalation assay**

A sample containing plasmid DNA (20 μg/ml) and EtBr (0.4 mg/ml) (plasmid DNA-EtBr complex) was used to calibrate to 100% fluorescence. Spermine-pullulan was added to an aqueous solution of EtBr at different N/P ratios. The fluorescence intensity of the samples (excitation: 510 nm, emission: 590 nm) was measured 15 min later by Gemini EM fluorescent microplate reader (Molecular Devices, sunnyvale CA, USA). The results were expressed as a relative fluorescence intensity (percent decreased against plasmid DNA-EtBr complex).

**Lectin-induced aggregation of spermine-pullulan-plasmid DNA complexes**

PIC were prepared in 10 mM PBS solution at an N/P of 3.0, followed by incubation at room temperature for 15 min. To 100 μl of the PIC solution, 100 μl of *Ricinus communis agglutinin* (RCA120, Seikagaku Corporation, Tokyo, Japan, 500 μg/ml) was added and incubated at room temperature. Time profiles of the solution turbidity in the pre-determined time were measured at a wavelength of 500 nm. To confirm the specificity of lectin-sugar interaction, RCA120 containing galactose was added to the PIC solution and then the similar turbidity measurement was performed. The results were expressed as a relative absorbance (percent increased against spermine-pullulan-plasmid DNA complex).

**In vitro gene transfection experiment**

Transfection experiments were performed in triplicate. HepG2 cells were purchased

from American Type Culture Collection, Manassas VA, USA and maintained in Minimal Essential Mediu (MEM) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution (Invitrogen Corp., Carlsbad, CA, USA), and 10 vol% fetal calf serum (FCS, Hyclone laboratories, Inc., Utah, USA) (MEM-FCS) at 37 ° C. Cells were seeded on each well of six-well cluster dish (Corning, NY, USA) at a density of $4\times10^5$ cells/well and cultivated in 2 ml of MEM-FCS for 24 hr. PIC were formed by mixing 50 μl of DDW containing spermine-pullulan and 50 μl of PBS containing 5 μg of pGL3-luciferase plasmid DNA at different N/P ratios. Immediately after the medium was exchanged by fresh Opti MEM medium (Invitrogen Corp., Carlsbad, CA, USA), 100 μl of the PIC solution was added and incubated for 15 min at room temperature, followed by 6 hr incubation for cell transfection. Then, the medium was changed to MEM-FCS and cells were incubated further for 42 hr.

Cells were washed with PBS once, lysed in 200 μl of cell culture lysis reagent (Promega Corp., Madison, WI, USA), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI, USA) was added to 20 μl of supernatant while the relative light unit (RLU) of the samples was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) Protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturers' instructions in order to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently. Asialofetuin (Sigma Chemical Co., St. Louis, MO, USA), which is a natural ligand for the asialoglycoprotein receptor (ASGPR), with a concentration of 1 mg/ml was pre-incubated for 1 hr, and then the similar transfection experiment was performed to evaluate the suppression effect on the level of gene expression.

**Cell viability**

Cytotoxicity was assayed using a cell counting kit (Nacalai tesque, Inc., Kyoto, Japan). Cells were seeded on each well of 96-well cluster dish (Corning, NY, USA) at a

density of $1 \times 10^4$ cells/well and cultivated in MEM-FCS for 24 hr. The medium was changed to the fresh Opti MEM medium, and 10 μl of the PIC solution was applied to each well, follow by 6 hr incubation. Then, the medium was changed to MEM-FCS and 100 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) solution was added and the cells were incubated further for 3 hr. The absorbance of samples was measured at 450 nm by VERSAmax microplate reader (Molecular Devices, sunnyvale CA, USA). The percent cell viability was expressed as 100% for control, non-treated cells.

**Statistical Analysis**

All the data were expressed as the mean±the standard deviation of the mean. Statistical analysis was performed based on the ANOVA, followed by Fisher's PLSD and significance was accepted at $P < 0.05$.

## RESULTS

**Cationization of pullulan**

Spermine was introduced to the hydroxyl groups of pullulan with different molecular weights by the CDI activation method (**Table 1**). The extent of spermine introduced could be changed by altering the amount of CDI added initially (data not shown). Irrespective of the pullulan molecular weight, the extent of spermine introduced was around 10%.

**Characterization of spermine-pullulan-plasmid DNA complexes**

**Figure 1** shows the electrophoretic image of polyion complexes of plasmid DNA and spermine-pullulan prepared at various N/P ratios. Migration of plasmid DNA was retarded with an increase in the N/P ratio, but was not observed at the N/P ratio higher than a certain value.

**Table 1. Preparation and characterization of spermine-pullulan with different molecular weights.**

| Pullulan Molecular weight | [CDI]/[OH] [a)] | [Spe]/[OH] [b)] | Percent introduced] [b)] |
|---|---|---|---|
| 5,800 | 1.5 | 10 | 12.9 |
| 11,800 | 1.5 | 10 | 12.3 |
| 22,800 | 1.5 | 10 | 11.0 |
| 47,300 | 1.5 | 10 | 12.3 |
| 112,000 | 1.5 | 10 | 10.7 |
| 212,000 | 1.5 | 10 | 9.74 |

a) Molar ratio of N,N'-carbonyldi-imidazole (CDI) initially added to the hydroxyl groups (OH) of pullulan.

b) Molar percentage of spermine introduced to the hydroxyl groups of pullulan.

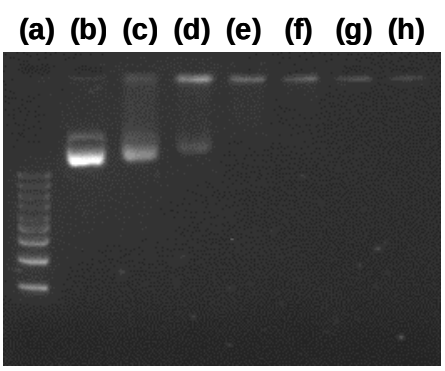


**Figure 1.** Agarose gel electrophoresis of spermine-pullulan-plasmid DNA complexes prepared at different N/P ratios. The molecular weight of pullulan used for spermine introduction is 47,300. The sample applied is (a) DNA marker, (b) free plasmid DNA or complexes prepared at N/P ratios of (c) 0.5, (d) 1.0, (e) 1.5, (f) 2.0, (g) 3.0, and (h) 5.0.

**Table 2. Apparent molecular size and zeta potential of free plasmid DNA and complexes of plasmid DNA and spermine-pullulan with different molecular weights.**

| Pullulan Molecular weight | Percent introduced] [a)] | Apparent molecular size (nm) | Zeta potential (mV) |
|---|---|---|---|
| 5,800 | 12.9 | 1530.4 ± 727.6 [b)] | +11.3 ± 0.73 [b)] |
| 11,800 | 12.3 | 1286.1 ± 0 | +14.4 ± 0.47 |
| 22,800 | 11.0 | 326.5 ± 139.5 | +14.3 ± 0.24 |
| 47,300 | 12.3 | 245.6 ±56.8 | +15.0 ±0.29 |
| 112,000 | 10.7 | 259.3 ± 134.7 | +13.4 ± 0.44 |
| 212,000 | 9.74 | 279.5 ± 127.0 | +14.1 ± 0.68 |
| Free plasmid DNA | | 409.5 ± 61.2 | -14.7 ± 9.5 |

a) The extent of spermine introduced to the hydroxyl groups of pullulan.

b) Means ± S.D.

**Table 2** summarizes the physicochemical properties of polyion complexes prepared at the N/P ratio of 3.0. The apparent molecular size and the zeta potential of free plasmid DNA were around 400 nm and -15 mV, respectively. The polyion complexation of spermine-pullulan with molecular weights of 22,800, 47,300, 112,000, and 212,000 reduced the molecular size of plasmid DNA, but the molecular size oppositely became large for smaller spermine-pullulan. On the other hand, irrespective of the pullulan molecular weight, the zeta potential was around +14 mV.

**Figure 2** shows the relative fluorescence intensity of plasmid DNA intercalated with EtBr after addition of spermine-pullulan. The EtBr fluorescence intensity decreased with an increase in the N/P ratio. The pattern of fluorescence intensity decreased depended on the molecular weights of spermine-pullulan. At the N/P ratios higher than 1.5, the EtBr fluorescence intensity became low for the PIC prepared by the spermine derivatives of pullulan with molecular weights of 22,800, 47,300, 112,000 and 212,000, while that of pullulan with molecular weights of 5,900 and 11,800 exhibited fluorescence intensity decrement at the higher N/P ratio.

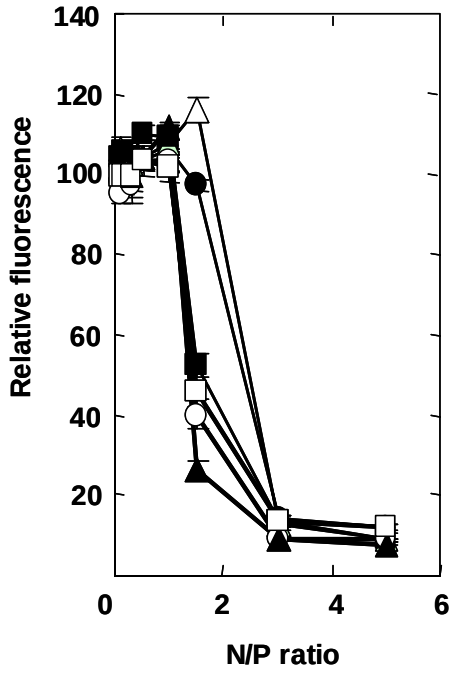


**Figure 2.** Solution fluorescence of spermine-pullulan-plasmid DNA complexes prepared at different N/P ratios after EtBr addition. The molecular weight of pullulan used for spermine introduction is (●) 5,900, (△) 11,800, (■) 22,800, (○) 47,300, (▲) 112,000 or (□) 212,000.

**Figure 3** shows the time profile of relative absorbance of spermine-pullulan-plasmid DNA complexes after addition of RCA120 lectin, which recognizes galactose residues. The relative absorbance increased time-dependently. The pattern of relative absorbance increased depended on the molecular weights of spermine-pullulan (**Figure 3A**). The extent of relative absorbance increased for the PIC prepared by the spermine derivatives of pullulan with a molecular weight of 47,300 was higher than that of pullulan with other molecular weights. In addition, the extent of relative absorbance increased for the PIC prepared by the spermine derivatives of pullulan with a molecular weight of 47,300 decreased by addition of galactose, which was dependent of the concentration of galactose. (**Figure 3B**).

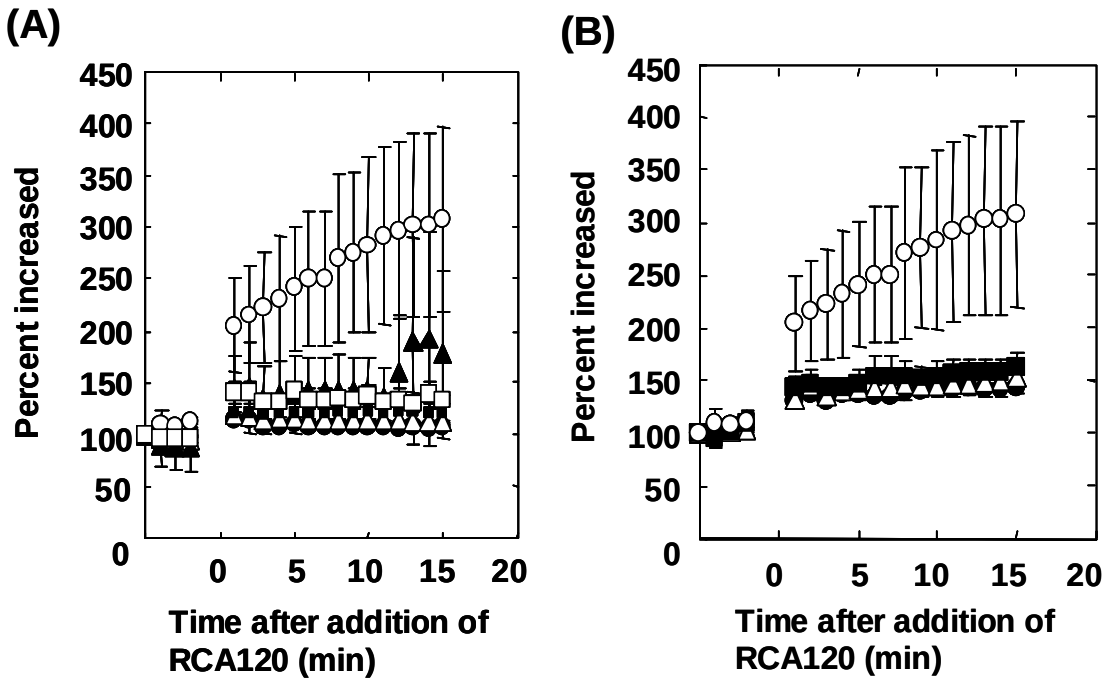


**Figure 3.** (A) Time course of the turbidity change of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3 after RCA 120 addition. The molecular weight of pullulan used for spermine introduction is (●) 5,900, (△) 11,800, (■) 22,800, (○) 47,300, (▲) 112,000 or (□) 212,000. (B) Time course of the turbidity change of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3 after RCA 120 addition in the presence of galactose with different concentrations. The molecular weight of pullulan used for spermine introduction is 47,300. The galactose concentration is (●) 150, (△) 100, (■) 50 or (○) 0 mg/ml.

**In vitro gene transfection of spermine-pullulan-plasmid DNA complexes**

**Figure 4** shows the effect of pullulan molecular weight on the level of gene expression for HepG2 cells. The level of gene expression was different among the spermine-pullulan derivatives used. The highest level was observed for the complex of spermine derivative of pullulan with the molecular weight of 47,300.

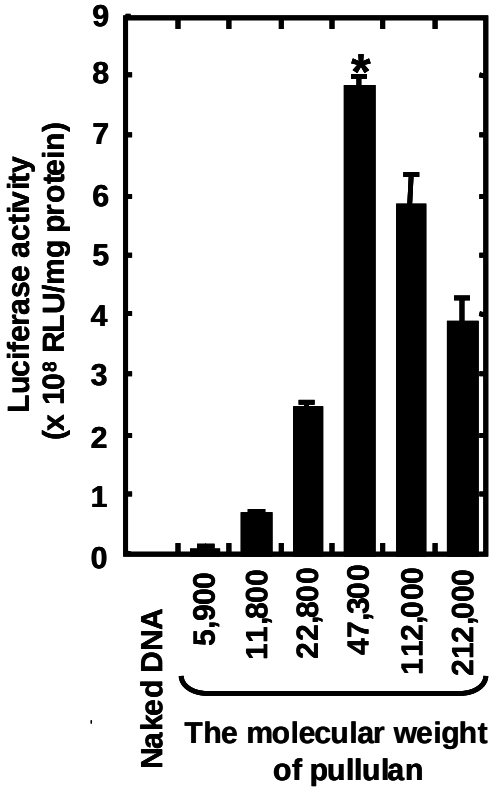


**Figure 4.** Effect of the pullulan molecular weight on the luciferase expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The amount of plasmid DNA applied is 5 μg/well and the N/P ratio is 3.0. *, $p<0.05$; versus the expression level of complexes prepared with other spermine-pullulans.

**Figures 5A** and **B** show the effect of the N/P ratio and the plasmid DNA amount on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells, respectively. The maximum level was observed for the complexes prepared at the N/P ratio of 3.0 (**Figure 5A**), while the level became plateau at the plasmid amounts of 2.5 μg/ ml (**Figure 5B**).

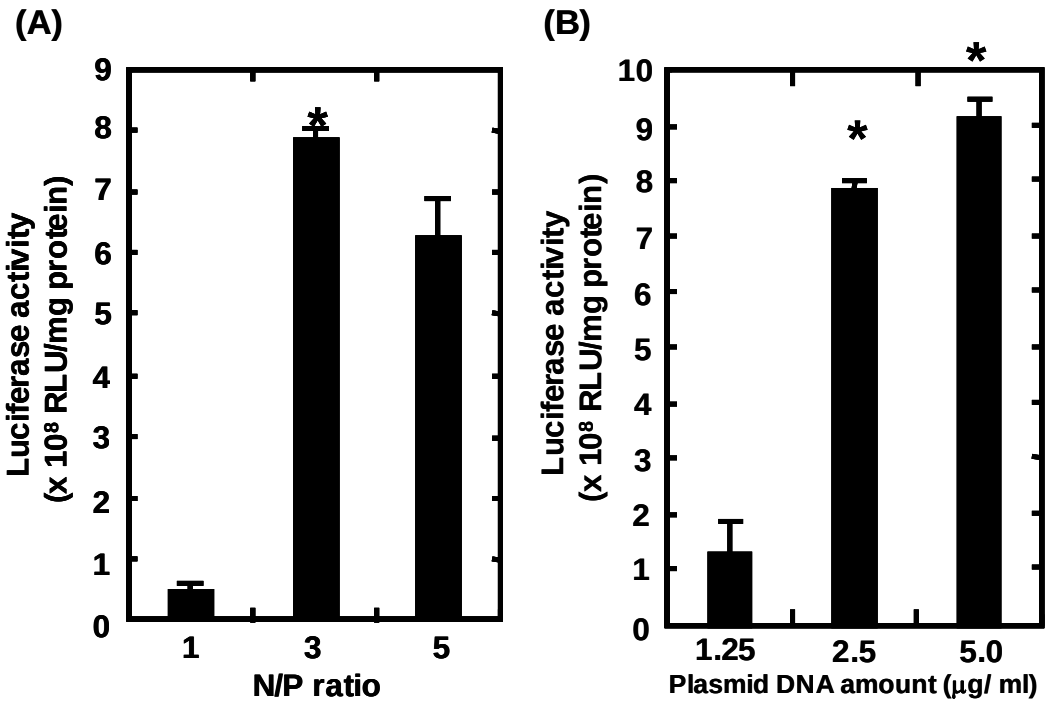


**Figure 5.** (A) Effect of the N/P ratio on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The plasmid DNA amount is 5 μg/well. The molecular weight of pullulan used for spermine introduction is 47,300. *, $p<0.05$; versus the expression level of complexes prepared at other N/P ratios. (B) Effect of the plasmid DNA amount on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The N/P ratio is 3.0. The molecular weight of pullulan used for spermine introduction is 47,300. *, $p<0.05$; versus the expression level of complexes at other plasmid DNA amounts.

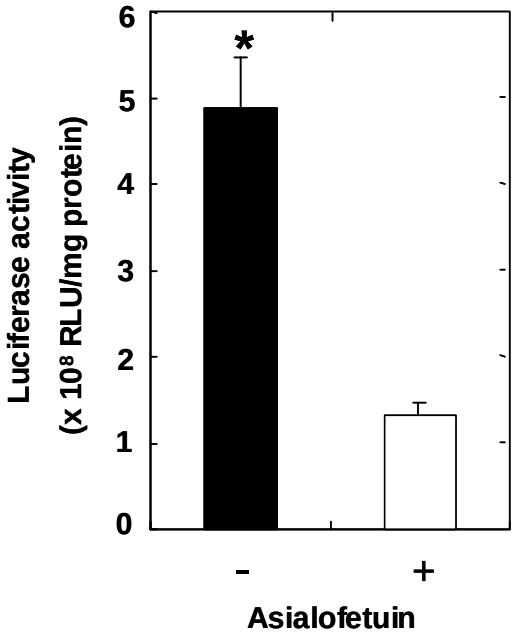


**Figure 6.** Effect of asialofetuin addition on the level of luciferase expression of spermine-pullulan-plasmid DNA complexes prepared at the N/P ratio of 3.0 for HepG2 cells. Cells were pre-treated with asialofetuin before usual gene transfection. The molecular weight of pullulan used for spermine introduction is 47,300, and the plasmid DNA amount is 5 μg/well. *, $p<0.05$; versus the expression level of cells transfected without asialofetuin addition.

**Figure 6** shows the effect of asialofetuin addition on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The level decreased in the presence of asialofetuin pre-treated.

**Cytotoxicity of spermine-pullulan-plasmid DNA complexes**

**Figure 7** shows the viability of cells 3 hr after exposure of PIC prepared at the N/P ratio of 3.0. The cell viability decreased with the increased amount of plasmid DNA added, although it tended to decrease with an increase in the molecular weight of pullulan complexed. However, the similar cytotoxicity was observed for every complex at the concentration of 0.25 μg/ml where the cell transfection experiment is performed.

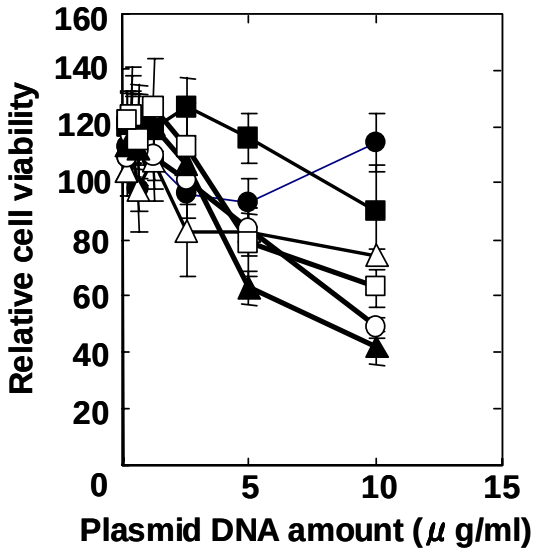


**Figure 7.** Effect of the plasmid DNA amount on the cell viability of HepG2 cells transfected with spermine-pullulan-plasmid DNA complexes prepared at the N/P ratio of 3.0. The molecular weight of pullulan used for spermine introduction is (●) 5,900, (△) 11,800, (■) 22,800, (○) 47,300, (▲) 112,000 or (□) 212,000. The viability of cells without transfection is indicated as 100%.

## DISCUSSION

The present study demonstrates that the in vitro gene expression of plasmid DNA was greatly enhanced by complexation with the spermine-pullulan derivatives and influenced by the molecular weight of pullulan.

Electrophoresis analysis revealed that retarded or no migration of plasmid DNA was observed at an N/P ratio of 1.5 (**Figure 1**). In addition, relative fluorescence of plasmid DNA intercalated with EtBr was reduced by addition of spermine-pullulan at the N/P ratio of 1.5,

except for spermine derivatives of pullulan with molecular weights of 5,900 and 11,200. These findings indicate that the spermine-pullulan formed a polyion complex with the plasmid DNA at the N/P ratio higher than 1.5. Considering the reaction conditions to prepare spermine-pullulan derivatives, it is likely that spermine residues are randomly introduced at equal distance intervals on the sugar main chain although the chain length is different by the molecular weight of pullulan. When a spermine residue on the sugar chain interacts with a plasmid DNA, other residues subsequently interact with the plasmid DNA because the spermine residues are linked on the sugar chain. The longer the sugar chain of spemine-pullulan derivatives or the higher the extent of spermine introduced, it is conceivable that the plasmid DNA interacts to form the complex more easily.

DLS and ELS measurements (**Table 2**) and ethidium bromide intercalation assay (**Figure 2**) revealed that the molecular weight of pullulan greatly affected several physicochemical properties of plasmid DNA complexes with spermine-pullulan. As shown in **Table 2**, after complexation with spermine-pullulan of higher molecular weights ranging from 22,800 to 212,000, the apparent molecular size of plasmid DNA became smaller, while the apparent size of complexes using lower molecular weights of pullulan (5,900 and 11,800) was larger in comparison with that of the free plasmid DNA. The EtBr intercalation assay revealed that the extent of compaction for polyion complexes became smaller for pullulan with lower molecular weights (5,900 and 11,800). It is possible that the molecular chain of spermine-pullulan with higher molecular weights was long enough to condense the plasmid DNA. The short molecular chain of pullulan does not allow the plasmid DNA to condense in size, but form an aggregate structure. On the other hand, the zeta potential of all the complexes was positive value about +14 mV, irrespective of the pullulan molecular weight (**Table 2**). This may be due to the excessive positive charge of spermine-pullulan which is not involved in complexation with plasmid DNA.

The level of gene expression depended on the type of spermine-pullulan derivatives used. This is caused not only by physicochemical properties of polyion complexes, such as the apparent molecular size, the extent of compaction, and the stability of polyion complexes, but also by the interaction of polyion complexes with receptors of cell surface.

It is reported that a cetain range of apparent molecular size of polyion complexes is effective for the enhancement of in vitro gene expression [22,23]. Complexation with spermine-pullulan with molecular weights ranging from 22,800 to 212,000, reduced the molecular size of plasmid DNA, whereas smaller spermine-pullulan did not. It is possible that the compacted complex of reduced size is internalized by cells, resulting in enhanced gene expression. The molecular size of complex will be one of the factors affecting the level of gene expression.

In addition, it is well known that the extent of EtBr intercalated into plasmid DNA is affected by the conformational change of plasmid DNA molecules and the molecular condensation of plasmid DNA [24]. Intercalation with the spermine-pullulan induces the molecular changes, resulting in EtBr release from plasmid DNA which is detected by decrease in the fluorescent intensity. The release will be accelerated as the interaction becomes stronger. The relative fluorescence intensity was not decreased after addition of spermine-pullulan with lower molecular weights at the N/P ratio of 1.5. This suggests that the interaction force with the plasmid DNA was not strong enough to form a complex for efficient gene transfection.

On the other hand, it is recognized that it is necessary for successful gene transfection to dissociate the plasmid DNA and the carrier inside the cells [25]. The dissociation suppression caused by too strong interaction, so-called complex stability too high will oppositely reduces the level of gene expression. A thermodynamic model demonstrates that the dissociation probability decreases with an increase in the amino groups present in a polymer chain [26]. Since the amount of spermine introduced into one pullulan molecule increases with the increasing molecular weight, it may be that the dissociation probability of complex decreases with the molecular weight of pullulan. It is likely that the balance of complex stability governed by the interaction force between the plasmid DNA and spermine-pullulan results in the highest level of gene expression for the complex of spermine derivative of pullulan with the molecular weight of 47,300.

Lectin-induced aggregation assays revealed that the surface property of plasmid DNA complexes with the spermine-pullulan was greatly influenced by the molecular weight of pullulan. As shown in **Figure 3**, the plasmid DNA complex with the spermine derivative of

pullulan with a molecular weight of 47,300 could interact strongly with the RCA120, which recognizes galactose residues. It is well known that the ASGPR also recognizes the galactose residues. Taken together, it is possible that the plasmid DNA complex with the spermine derivative of pullulan with a molecular weight of 47,300 with an inherent affinity for the ASGPR is effectively internalized by cells, resulting in enhanced gene expression. In addition, it is reported that an asialoglycoprotein receptor is expressed on the surface of HepG2 cells. As shown in **Figure 6**, the level of gene expression decreased in the presence of asialofetuin, whereas no decrement effect was observed for one of the transfection regents commercially available, Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) (data not shown). There have been many research papers about gene delivery system with polymers targetable to ASGPR[5-8]. Complexation with a galactosylated polymer or an ASGPR-targeting protein-conjugated polymer enabled plasmid DNAs to selectively deliver to hepatocytes, while the gene expression was suppressed by addition of asialofetuin. Taken together, it is conceivable that the in vitro gene transfection by the spermine-pullulan was facilitated by the ASGPR-mediated uptake.

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# Chapter 4

## Effect of the extent of spermine introduced to pullulan on the level of gene expression

## INTRODUCTION

Complexation with non-viral carrier materials, such as cationized polymers [1] and cationized liposomes [2-4], enable plasmid DNA to neutralize the anionic charge as well as to reduce the molecular size, which is preferable to enhance the efficiency of plasmid DNA transfection. It is well recognized that the extent of cationized residues introduced to the carrier polymer is one factor contributing to the level of gene expression [5-8]. Based on the results in Chapters 1, 2, and 3, it was demonstrated that spermine derivatives of pullulan with several molecular weights had effective gene transfection efficiency for HepG2 cells. In this chapter, pullulan with several molecular weights was cationized by the chemical introduction of spermine to obtain cationized pullulan with different extents of spermine introduced. A plasmid DNA was complexed with the cationized pullulan derivatives in aqueous solution, and the apparent molecular size and zeta potential of the complexes were measured. In addition, their physicochemical properties were evaluated in terms of the solution turbidity, gel retardation, dissociation resistance by heparin, lectin binding, and EtBr intercalation assays. In vitro gene expression by the complexes for HepG2 cells of a human hepatoma cell line was investigated to assess the effect of complex physicochemical properties.

## EXPERIMENTAL

### Materials

Pullulan with different weight-average molecular weights, 22,800, 47,300, and 112,000 was purchased from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Spermine was purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

**Preparation of cationized pullulan derivatives**

Spermine was introduced to the hydroxyl groups of pullulan by a N,N'-carbonyldiimidazole (CDI) activation method [9]. Spermine and CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of pullulan (**Table 1**). Following agitation using magnetic stirrer at 35 °C for 20 hr, the reaction mixture was dialyzed against ultra-pure double distilled water (DDW) for 2 days with a dialysis membrane (the cut-off molecular weight of dialysis membrane is 12,000-14,000, Viskase Companies, Inc, Willowbrook, Illinois.). Then, the solution dialyzed was freeze-dried to obtain the samples of spermine-introduced pullulan (spermine-pullulan). The spermine introduction was determined from the conventional elemental analysis and expressed by the molar percentage of spermine introduced to the hydroxyl groups of pullulan.

**Preparation of plasmid DNA**

The plasmid DNA used was the pGL3 vector (5.26 kb) coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, USA). The plasmid DNA was propagated in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of the plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

**Preparation of polyion complexes (PIC)**

PIC were prepared by mixing an aqueous solution of spermine-pullulan with that of plasmid DNA. Briefly, varied amounts of spermine-pullulan were dissolved in 50 μl of DDW and mixed with 50 μl of phosphate-buffered saline (PBS, pH 7.4) containing 100 μg of plasmid DNA, followed by leaving for 15 min at room temperature to obtain various PIC of spermine-pullulan and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio.

**Turbidity measurement for spermine-pullulan-plasmid DNA complexes**

To evaluate the PIC formation of plasmid DNA with the spermine-pullulan, the solution turbidity was measured at a wavelength of 500 nm in 100 mM sodium phosphate-buffered solution (pH 7.4) at different ionic strengths. The concentration of plasmid DNA solution was fixed at 100 μg/ml for the turbidity measurement.

**Electrophoresis of spermine-pullulan-plasmid DNA complexes**

PIC were prepared in 10 mM PBS solution at different N/P ratios. After 15 min of incubation, 10 μl of the complex was added to 3 μl of a loading buffer (0.1% sodium dodecyl sulfate, 5% glycerol, and 0.005% bromophenol blue) and applied on an 1 wt % agarose gel in Tris-borate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.3) containing 0.1 mg/ml ethidium bromide (EtBr). Electrophoretic evaluation of the PIC was carried out in TBE solution at 100 V for 30 min. The gel was imaged with a UV transilluminator (Gel Doc 2000, BIO-RAD laboratories, Segrate, Italy). To 100 μl of the PIC, 900 μl of heparin at concentrations of 6.25 and 12.5 μg/ml was added, followed by incubation at room temperature for 30 min, and then the similar electrophoresis analysis was performed to evaluate the dissociation resistance of PIC against heparin.

**Characterization of PIC by dynamic light scattering (DLS) and electrophoretic light scattering (ELS)**

PIC were prepared in 10 mM PBS solution at a N/P ratio of 3.0. DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) equipped with an $Ar^+$ laser at a detection angle of $90^o$ at 25 °C for 15 min. In the present study, the autocorrelation function of samples was analyzed based on the cumulant method and the $R_s$ value was calculated automatically by the equipped computer software and expressed as the apparent molecular size of samples. On the other hand, the zeta potential was measured using ELS-7000AS instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) at 25 °C and an electric field strength of 100 V/cm. From the determined electrophoretic mobility, the zeta potential was automatically calculated using the Smoluchouski equation. Light scattering

measurement was done three times for every sample. The PIC used in this measurement was diluted enough to prevent multiple scattering.

**Ethidium bromide intercalation assay**

A sample containing plasmid DNA (20 μg/ml) and EtBr (0.4 mg/ml) (plasmid DNA-EtBr complex) was used to calibrate to 100% fluorescence. Spermine-pullulan was added to an aqueous solution of EtBr at different N/P ratios. The fluorescence intensity of the samples (excitation: 510 nm, emission: 590 nm) was measured 15 min later by Gemini EM fluorescent microplate reader (Molecular Devices, sunnyvale CA, USA). The results were expressed as a relative fluorescence intensity (percent decreased against plasmid DNA-EtBr complex).

**Lectin-induced aggregation of spermine-pullulan-plasmid DNA complexes**

PIC were prepared in PBS at an N/P of 3.0, followed by incubation at room temperature for 15 min. To 100 μl of the PIC solution, 100 μl of *Ricinus communis agglutinin* of lectin recognizable to galactose (RCA120, Seikagaku Corporation, Tokyo, Japan, 500 μg/ml) was added, followed by incubation at room temperature. The time profile of solution turbidity change was measured at a wavelength of 500 nm. To confirm the specificity of lectin-sugar interaction, RCA120 containing galactose was added to the PIC solution and then the similar turbidity measurement was performed. The results were expressed as the percentage of the solution absorbance to that of spermine-pullulan-plasmid DNA complex alone.

**In vitro gene transfection experiment**

Transfection experiments were performed independently in triplicate. HepG2 cells of a human hepatoma cell line were purchased from American Type Culture Collection, Manassas VA, USA and maintained in Minimal Essential Medium (MEM) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution (Invitrogen Corp., Carlsbad, CA, USA), and 10 vol% fetal calf serum (FCS, Hyclone laboratories, Inc., Utah,

USA) (MEM-FCS) at 37 ° C. Cells were seeded on each well of six-well cluster dish (Corning, NY, USA) at a density of $4\times10^5$ cells/well and cultivated in 2 ml of MEM-FCS for 24 hr. PIC were formed by mixing 50 μl of DDW containing the spermine-pullulan and 50 μl of PBS containing 5 μg of the pGL3-luciferase plasmid DNA at different N/P ratios. Immediately after the medium was exchanged by fresh Opti MEM medium (Invitrogen Corp., Carlsbad, CA, USA), 100 μl of the PIC solution was added and incubated for 15 min at room temperature, followed by 6 hr incubation for cell transfection. Then, the medium was changed to MEM-FCS and cells were incubated further for 42 hr.

Cells were washed with PBS once, lysed in 200 μl of a cell culture lysis reagent (Promega Corp., Madison, WI, USA), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI, USA) was added to 20 μl of supernatant while the relative light unit (RLU) of the samples was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) Protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturers' instructions in order to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently. Asialofetuin (Sigma Chemical Co., St. Louis, MO, USA, 1 mg/ml) which is a natural ligand for the asialoglycoprotein receptor (ASGPR), were pre-incubated for 1 hr, and then the similar transfection experiment was performed to evaluate the suppression effect on the level of gene expression.

**Cell viability**

Cytotoxicity was assayed using a cell counting kit (Nacalai tesque, Inc., Kyoto, Japan). Cells were seeded on each well of 96-well cluster dish (Corning, NY, USA) at a density of $1\times10^4$ cells/well and cultivated in MEM-FCS for 24 hr. The medium was changed to the fresh Opti MEM medium, and 10 μl of the PIC solution was applied to each well, follow by 6 hr incubation. Then, the medium was changed to MEM-FCS and 100 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium

(WST-8) solution was added and the cells were incubated further for 3 hr. The absorbance of samples was measured at 450 nm by VERSAmax microplate reader (Molecular Devices, sunnyvale CA, USA). The percent cell viability was expressed as 100% for control, non-treated cells.

**Statistical Analysis**

All the data were expressed as the mean±the standard deviation of the mean. Statistical analysis was performed based on the ANOVA, followed by Fisher's PLSD and significance was accepted at $P < 0.05$.

## RESULTS

**Cationization of pullulan**

Spermine was introduced to the hydroxyl groups of pullulan by the CDI activation method (**Table 1**). The extent of spermine introduced could be changed by altering the amount of CDI added initially, and it was irrespective of the pullulan molecular weight.

**Table 1. Preparation and characterization of spermine-pullulan with different percentages of spermine introduced [c].**

| Pullulan | [CDI]/[OH] [a] | | | | |
|---|---|---|---|---|---|
| Molecular weight | 0.5 | 1.0 | 1.5 | 3.0 | 5.0 |
| 22,800 | 2.69 [b] | 5.60 | 11.0 | 23.0 | 32.5 |
| 47,300 | 1.07 | 5.95 | 12.3 | 20.4 | 32.9 |
| 112,000 | 2.19 | 7.35 | 10.7 | 26.3 | 33.1 |

a) Molar ratio of N,N'-carbonyldiimidazole (CDI) initially added to the hydroxyl groups (OH) of pullulan.

b) Molar percentage of spermine introduced to the hydroxyl groups of pullulan.

c) Each spermine-pullulan with the [CDI]/[OH] ratio of 1.5 is prepared in Chapter 3.

**Characterization of spermine-pullulan-plasmid DNA complexes**

**Figure 1** shows the turbidity result of PIC solution to experimentally confirm the complexation of spermine-pullulan with a molecular weight of 47,300 and different percentages of spermine introduced with the plasmid DNA. In the turbidity experiment, increment or decrement of solution turbidity shows association or dissociation of PIC, respectively. As the ionic strength of solution became higher, the solution turbidity decreased for complexes of spermine-pullulan with percentages of spermine introduced prepared at the molar ratio of CDI initially added to the hydroxyl groups (OH) of pullulan ([CDI]/[OH]) of 1.0, 1.5, 3.0, and 5.0. However, for complexes of spermine-pullulan with the [CDI]/[OH] of 0.5, no solution turbidity was observed for all the ionic strengths studied.

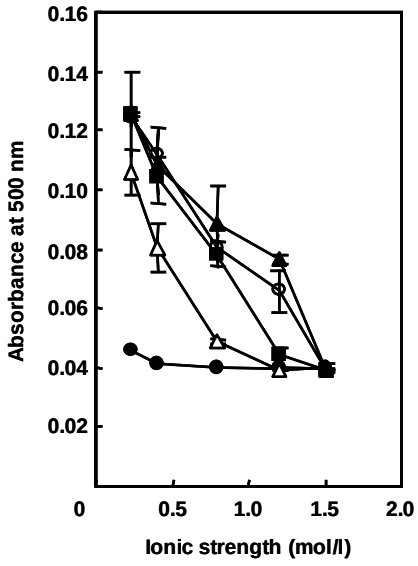


**Figure 1**. Solution turbidity of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3.0 as a function of the solution ionic strength in complex preparation. The molecular weight of pullulan used for spermine introduction is 47,300. The [CDI]/[OH] ratio of spermine-pullulan used for cationization is (●) 0.5, (△) 1.0, (■) 1.5, (○) 3.0 or (▲) 5.0..

**Figure 2** shows the electrophoretic patterns of polyion complexes of plasmid DNA and spermine-pullulan with a molecular weight of 47,300 and different percentages of spermine introduced prepared at a N/P ratio of 3.0. Migration of plasmid DNA was retarded with an increase in the introduction percentage. No retardation was observed for the complex of spemine-pullulan with the [CDI]/[OH] of 0.5.

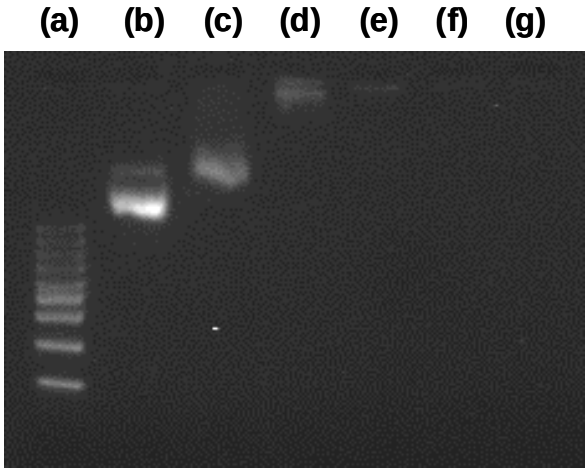


**Figure 2.** Agarose gel electrophoresis of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3.0. The molecular weight of pullulan used for spermine introduction is 47,300. The sample applied is (a) DNA marker, (b) free plasmid DNA or complexes of plasmid DNA and spermine-pullulan with the [CDI]/[OH] ratio of (c) 0.5, (d) 1.0, (e) 1.5, (f) 3.0 or (g) 5.0. The spermine-pullulan with the [CDI]/[OH] ratio of 1.5 is prepared in Chapter 3.

**Figure 3** shows the electrophoretic patterns of polyion complexes of plasmid DNA and spermine-pullulan with different molecular weights and percentages of spermine introduced in the presence of different concentrations of heparin. EtBr exclusion in the presence of heparin showed that the complexes of spermine-pullulan and plasmid DNA were stable and not dissociated. Although heparin addition dissociated the plasmid DNA complexes of spermine-pullulan, the dissociation extent depended on the molecular weight of pullulan and the percent spermine introduced. While the plasmid DNA complexes of spermine-pullulan with lower molecular weights and spermine introduction percentages tended to be dissociated in the presence of heparin at 6.25 μg/ml, (**Figures 3A, (c-e) and 3B and 3C, (c-d)**), whereas those with higher values could not be dissociated even at 12.5 μg/ml of heparin (**Figures 3C, f and g**).

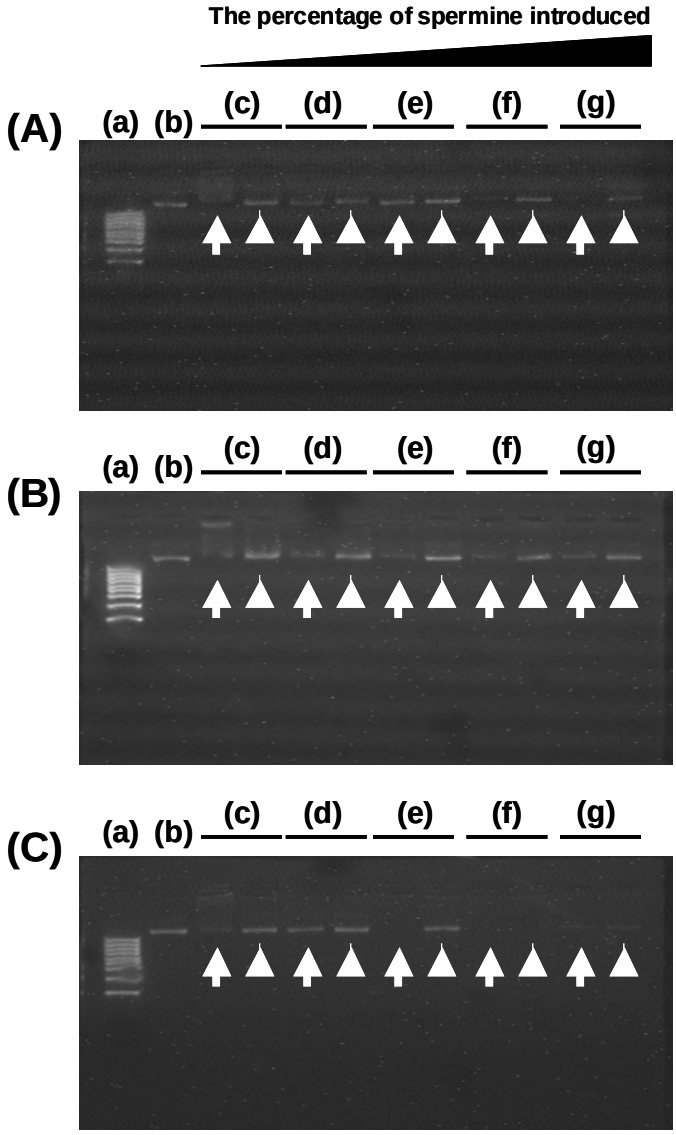


**Figure 3.** Agarose gel electrophoresis of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3.0 in the presence of heparin. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The sample applied is (a) DNA marker, (b) free plasmid DNA or complexes of plasmid DNA and spermine-pullulan with the [CDI]/[OH] ratio of (c) 0.5, (d) 1.0, (e) 1.5, (f) 3.0 or (g) 5.0. Heparin concentration present in the complex is (⇧) 6.25 or (△) 12.5 μg/ml.

**Table 2** summarizes the physicochemical properties of polyion complexes prepared at a N/P ratio of 3.0. The apparent molecular size and the zeta potential of free plasmid DNA were around 400 nm and -15 mV, respectively. The polyion complexation of spermine-pullulan with higher percentages of spermine introduced prepared at the [CDI]/[OH] of 1.5, 3.0 or 5.0 reduced the molecular size of plasmid DNA, irrespective of pullulan molecular weight used for cationization, while the molecular size oppositely became large for smaller spermine-pullulan prepared at the [CDI]/[OH] of 0.5 or 1.0. On the other hand, irrespective of the pullulan molecular weight and percent spermine introduced, the zeta potential was around 10-16 mV.

**Table 2. Apparent molecular size and zeta potential of free plasmid DNA and complexes of plasmid DNA and spermine-pullulan with different percentages of spermine introduced [d].**

| Pullulan | [CDI]/[OH] [a] | | | | |
|---|---|---|---|---|---|
| Molecular weight | 0.5 | 1.0 | 1.5 | 3.0 | 5.0 |
| 22,800 | n.d. | 2200 ± 0 [b] | 330 ± 140 | 290 ± 170 | 200 ± 89 |
| | n.d | +7.0 ± 0.3 [c] | +14 ± 0 | +14 ± 0 | +14 ± 0 |
| 47,300 | n.d. | 1300 ± 0 | 250 ± 57 | 210 ± 57 | 280 ± 180 |
| | n.d | +9.0 ±0.2 | +15 ±0 | +16 ±0 | +17 ±1 |
| 112,000 | n.d. | 1300 ± 0 | 260 ± 130 | 290 ± 180 | 280 ± 180 |
| | n.d | +10 ± 0 | +13 ± 0 | +11 ± 1 | +11 ± 1 |
| Free plasmid DNA | | | 410 ± 61 | | |
| | | | -15 ± 10 | | |

a) Molar ratio of N,N'-carbonyldiimidazole (CDI) initially added to the hydroxyl groups (OH) of pullulan.

b) Apparent particle size (nm). Means ± S.D.

c) Zeta potential (mV). Means ± S.D.

d) Each spermine-pullulan with the [CDI]/[OH] ratio of 1.5 is prepared in Chapter 3.

**Figure 4** shows the relative fluorescence intensity of plasmid DNA intercalated with EtBr after addition of spermine-pullulan. The EtBr fluorescence intensity decreased with an increase in the N/P ratio. The pattern of fluorescence intensity decreased depended on the percent spermine introduced of pullulan. At the N/P ratios of 3.0 or higher, the EtBr fluorescence intensity became low for the complexes of spermine-pullulan with higher introduction percentages, while it did not clearly for the complexes of spermine-pullulan with the lowest percentage.

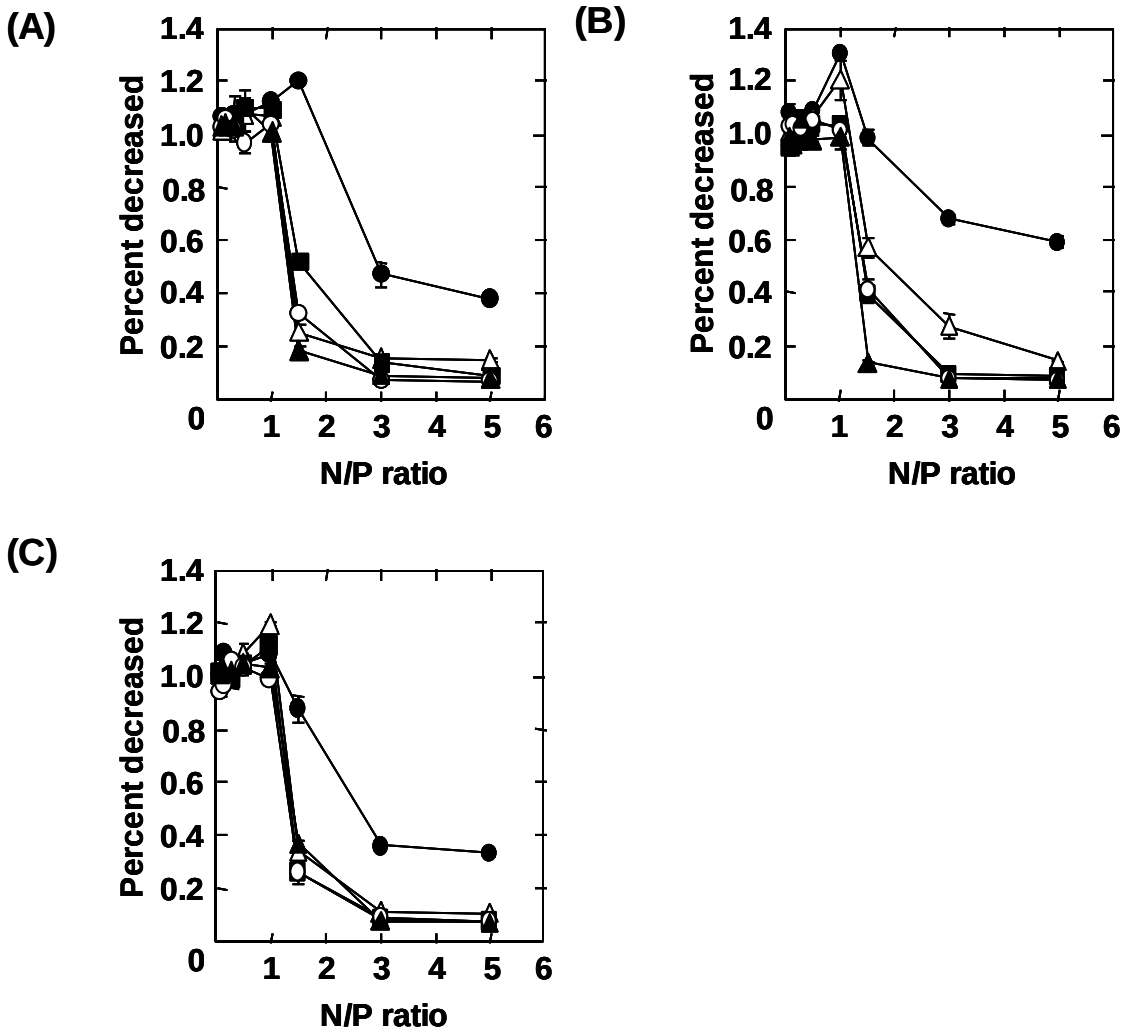


**Figure 4.** Solution fluorescence of spermine-pullulan-plasmid DNA complexes prepared at different N/P ratios after EtBr addition. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The [CDI]/[OH] ratio of spermine-pullulan used for cationization is (●) 0.5, (△) 1.0, (■) 1.5, (○) 3.0 or (▲) 5.0. Each spermine-pullulan with the [CDI]/[OH] ratio of 1.5 is prepared in Chapter 3.

**Figure 5** shows the time profile of solution absorbance change of spermine-pullulan-plasmid DNA complexes after the addition of RCA120 lectin. The solution absorbance increased time-dependently, although the increment pattern of depended on the molecular weight and the percent spermine introduced of pullulan. The extent of absorbance increased was suppressed by addition of galactose in the concentration-dependent manner (data not shown).

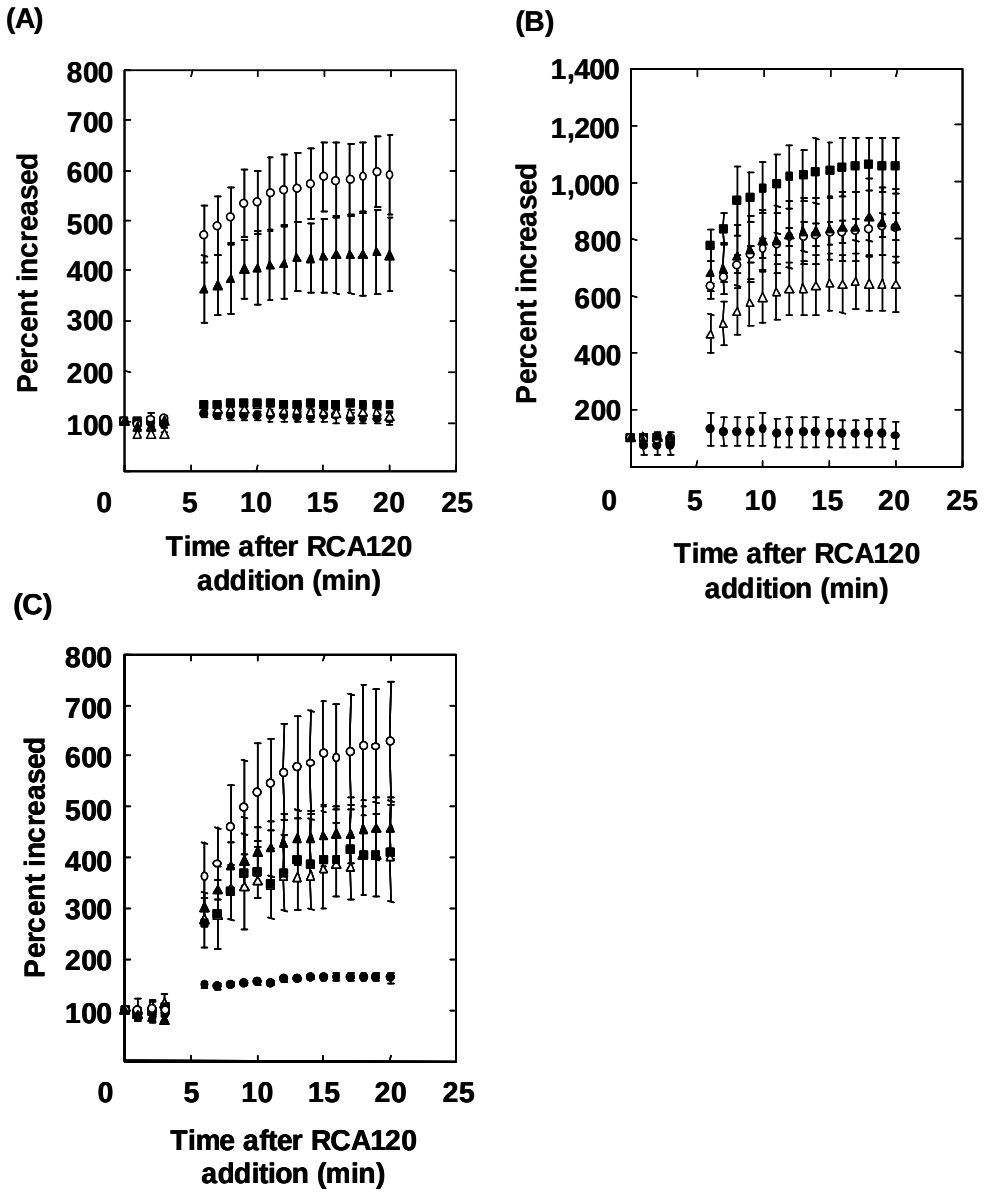


**Figure 5.** Time course of the turbidity change of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3 after RCA 120 addition. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The [CDI]/[OH] ratio of spermine-pullulan used for cationization is (●) 0.5, (△) 1.0, (■) 1.5, (○) 3.0 or (▲) 5.0. Each spermine-pullulan with the [CDI]/[OH] ratio of 1.5 is prepared in Chapter 3.

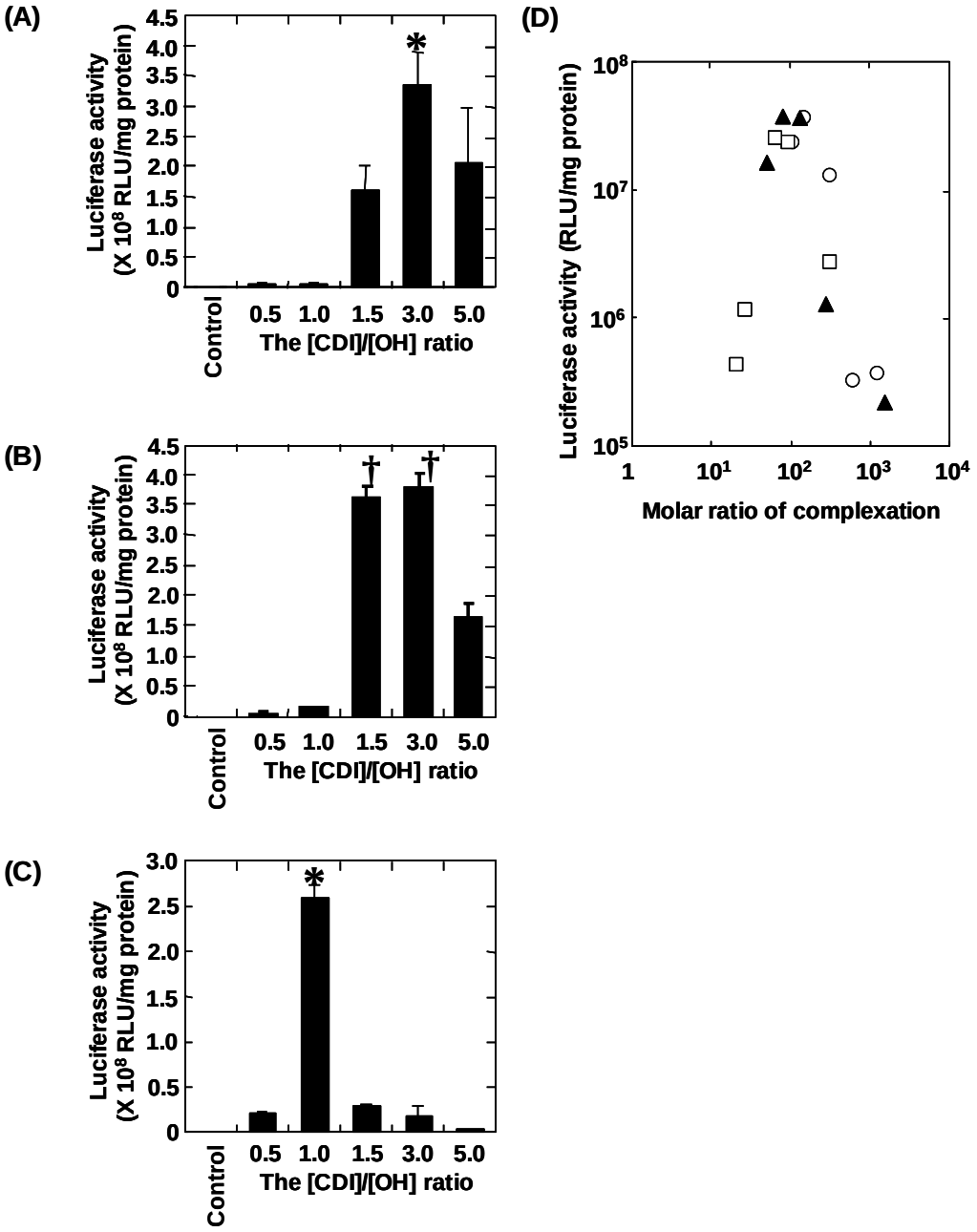


**Figure 6.** Effect of the spermine introduction extent of pullulan on the luciferase expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The amount of plasmid DNA applied is 5 μg/well and the N/P ratio is 3.0. *, $p<0.05$; versus the expression level of complexes prepared by other spermine-pullulans. †, $p<0.05$; versus the expression level of complexes prepared by spermine-pullulans with with the [CDI]/[OH] ratio of 0.5, 1.0, and 5.0. (D) Effect of the molar ratio of spermine-pullulan to plasmid DNA in complexation on the level of gene expression. The molecular weight of pullulan used for complexation is (○) 22,800, (▲) 47,300 or (□) 112,000. Each spermine-pullulan with the [CDI]/[OH] ratio of 1.5 is prepared in Chapter 3.

**In vitro gene transfection of spermine-pullulan-plasmid DNA complexes**

**Figure 6** shows the effect of spermine introduction percentage on the level of gene expression for HepG2 cells. The level of gene expression was different among spermine-pullulan derivatives with different percentages of spermine introduced. In addition, the percentage of spermine introduced where the highest level was observed was different among the pullulan molecular weight used for spermine introduction (**Figures 6A-C**).

**Figure 6D** shows the relationship between the molecular ratio of spermine-pullulan to plasmid DNA and the level of gene expression. Irrespective of the molecular weight of pullulan, a maximum level was observed in the molar ratio of around $10^2$.

**Figure 7** shows the effect of asialofetuin addition on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The level decreased by adding the asialofetuin for any complex.

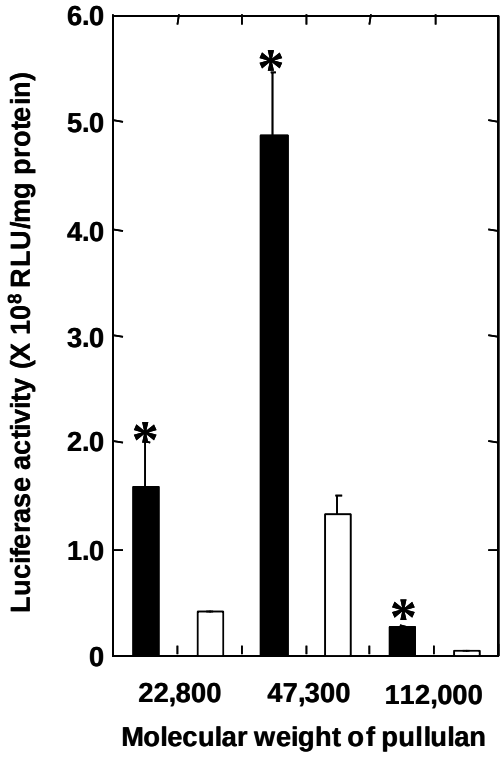


**Figure 7.** Effect of asialofetuin addition on the level of luciferase expression of spermine-pullulan-plasmid DNA complexes prepared at the N/P ratio of 3.0 for HepG2 cells. Cells were pretreated with (solid columns) or without (open columns) asialofetuin before gene transfection. The plasmid DNA amount is 5 μg/well. *, $p<0.05$; versus the expression level of cells transfected without asialofetuin addition at the corresponding molecular weight of pullulan. Each spermine-pullulan with the [CDI]/[OH] ratio of 1.5 is prepared in Chapter 3.

**Cytotoxicity of spermine-pullulan-plasmid DNA complexes**

**Figure 8** shows the viability of cells 3 hr after exposure of PIC prepared at the N/P ratio of 3.0. No cytotoxicity was observed, irrespective of the percent spermine introduced.

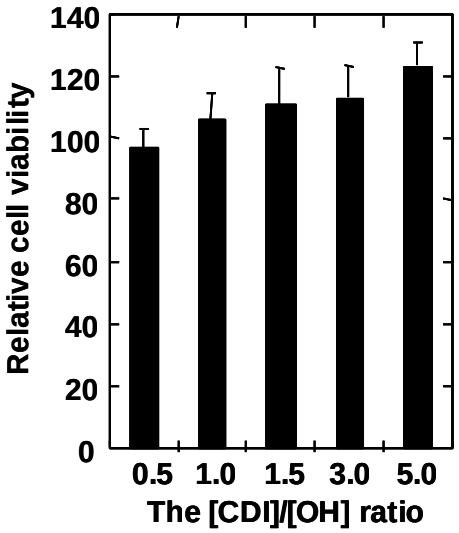


**Figure 8**. Effect of the percent spermine introduced on the cell viability of HepG2 cells transfected with spermine-pullulan-plasmid DNA complexes prepared at the N/P ratio of 3.0. The molecular weight of pullulan used for spermine introduction is 47,300. The viability of cells without transfection is indicated as 100%.

## DISCUSSION

The present study demonstrates that the in vitro gene expression of plasmid DNA was greatly enhanced by complexation with the spermine-pullulan derivatives although it was influenced by the extent of spermine introduced to pullulan.

The extent of spermine introduced could be changed by altering the amount of CDI added initially (**Table 1**). Except for the lowest percentage of spermine introduced, the spermine-pullulan was electrostatically interacted with the plasmid DNA (**Figure 1**). Electrophoresis analysis revealed that retarded or no migration of plasmid DNA was observed at an N/P ratio of 3.0, except for the spermine-pullulan with the lowest spermine introduction (**Figure 2**). From the electrophoresis analysis, plasmid DNA complexes prepared by almost all of spermine-pullulans were formed at an N/P ratio of 3.0. In addition, relative fluorescence of plasmid DNA intercalated with EtBr was reduced by addition of spermine-pullulan with increase in the N/P ratio, although the decrement patterns for spermine-pullulan derivatives with the lowest introduction percentage were not sharp compared with those with higher

introduction percentages (**Figure 4**). These findings indicate that the spermine-pullulan formed a polyion complex with the plasmid DNA, although the complexation was greatly influenced by the percent spermine introduced of pullulan.

From the results of DLS and ELS measurements (**Table 2**) and EtBr intercalation assay (**Figure 4**), it was found that the extent of spermine introduced to pullulan greatly affected the physicochemical properties of plasmid DNA complexes with spermine-pullulan. After complexation with spermine-pullulan of higher spermine introduction percentages prepared at [CDI]/[OH] ratios of 1.5 to 5.0, the apparent molecular size of plasmid DNA became smaller, while the apparent size of complexes at lower introduction percentages at [CDI]/[OH] of 0.5 and 1.0 was large in comparison with that of free plasmid DNA. The EtBr intercalation assay revealed that the extent of compaction for polyion complexes became smaller for spermine-pullulan with lower introduction percentage prepared at [CDI]/[OH] of 0.5 and 1.0. It is possible that the distance interval of spermine residues on the molecular chain of spermine-pullulan with higher introduction percentages was short enough to condense the plasmid DNA. The long distance interval of spermine residues does not allow the plasmid DNA to condense in size, but form an aggregate structure.

From the results of electrophoretic patterns of spermine-pullulan-plasmid DNA complexes in the presence of heparin, it is revealed that the dissociation behavior of the complex depended on the molecular weight and the percent spermine introduced of pullulan. Interestingly, compared with the DLS results (**Table 2**), it is found that complexes with the same molecular sizes (molecular weight of 47,300 or 112,000, and [CDI]/[OH] of 5.0) showed different dissociation behaviors. It is possible that these differences greatly affected the level of gene expression.

Based on not only the apparent molecular size, the extent of compaction, and stability of polyion complexes, but also the interaction of polyion complexes with receptors of cell surface, dependence of the spermine-pullulan derivatives type on the level of gene expression can be explained. Firstly, complexation with more spermine-introduced pullulan prepared at [CDI]/[OH] ratios of 1.5 to 5.0 reduced the molecular size of plasmid DNA, whereas less spermine-introduced pullulan did not, irrespective of the pullulan molecular

weight. It has been recognized that a certain range of apparent molecular size of polyion complexes was effective in enhancing the in vitro gene expression [10,11]. It is possible that the compacted complex of reduced size is more readily internalized by cells, resulting in enhanced gene expression. The molecular size of complex will be one of the factors affecting the level of gene expression.

Next, it is well known that the extent of EtBr intercalated into plasmid DNA is affected by the conformational change of plasmid DNA molecules and the molecular condensation of plasmid DNA [12]. Intercalation with the spermine-pullulan allows to change the molecular structures, resulting in EtBr release from plasmid DNA which is detected by decrease in the fluorescent intensity. The release is accelerated as the interaction becomes stronger. The relative fluorescence intensity was not decreased after addition of spermine-pullulan with the lowest percent introduced. This suggests that the interaction force with the plasmid DNA was not strong enough to form a complete for efficient gene transfection.

It is recognized that appropriate dissociation of the plasmid DNA and the carrier inside cells is necessary for successful translation of gene transfected to [13]. From the result of the electrophoretic patterns of spermine-pullulan-plasmid DNA complexes in the presence of different concentrations of heparin (**Figure 3C**), spermine-pullulans with a molecular weight 112,000 and higher introduction percentages ([CDI]/[OH] of 3.0 or 5.0) complexed strongly with plasmid DNA and the complexes could not be dissociated even in the presence of heparin at the higher concentration. Actually, a thermodynamic model demonstrates that the dissociation probability decreases with an increase in the amino groups present on a polymer chain [14]. Since the amount of spermine introduced into one pullulan molecule increases with an increase in the percentage of spermine introduced, it may be that the dissociation probability of complex decreases with an increase in the percent spermine introduced of pullulan. From the result of gene expression (**Figure 6C**), the levels of gene expression for these complexes were low. These results clearly demonstrate that the dissociation suppression reduced the level of gene expression. It is likely that the balance of complex stability governed by the interaction force between the plasmid DNA and spermine-pullulan results in

the highest level of gene expression.

The percentage of spermine introduced where the highest expression level was observed was different among the molecular weight of pullulan used for spermine introduction (**Figure 6**). The percent spermine introduction for the highest expression level tended to decrease with an increase in the molecular weight of pullulan. At the same N/P ratio, the number of spermine-pullulan complexed with plasmid DNA decreases with an increase in the molecular weight and percent spermine introduced of pullulan. There was an optimal molar number of the spermine-pullulan-plasmid DNA complex for enhanced gene expression at a N/P ratio (**Figure 6D**). This result experimentally indicates that a well-balanced combination of the spermine introduction percentage and molecular weight of pullulan is necessary to enhance the level of gene expression. The complex was characterized in terms of the molecular size, surface charge, lectin recognition, dissociation stability, although it is not always done sufficiently. However, the reason of the optimal number for the maximum gene expression is not clear at present.

Finally, the lectin affinity assay revealed that the spermine-pullulan-plasmid DNA complex was recognized by the sugar-recognizable lectin (**Figure 5**). When the complex was mixed with the lectin, the solution absorbance was increased. The presence of galactose suppressed the absorbance increase in the concentration- dependent manner. This is explained in terms of sugar-specific interaction between the complex and the lectin. It is likely that the pullulan chain present on the complex surface is specifically recognized by the lectin, resulting in the formation of complex-lectin aggregates. The aggregate formation depended on the percent spermine introduced and the molecular weight of pullulan used for complexation. This is because the molecular mobility of surface pullulan chains was different between the complexes. Compared with **Figure 6**, it is apparent that most of complexes strongly interacted with the lectin showed the high gene expression level. Taken together, it is likely that the plasmid DNA complex with the spermine derivative of pullulan with an inherent affinity for the ASGPR is effectively internalized by cells, resulting in enhanced gene expression. In addition, it is reported that an asialoglycoprotein receptor is expressed on the surface of HepG2 cells. As shown in **Figure 7**, the level of gene expression decreased by addition of

asialofetuin, whereas no inhibition effect was observed for a transfection reagent commercially available, Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) (data not shown). Taken together, it is conceivable that the in vitro gene transfection by the spermine-pullulan was facilitated by the ASGPR-mediated uptake.

As shown in **Figure 8**, no cytotoxicity was observed, irrespective of the percent spermine introduced. Since cytotoxicity is one of the drawbacks in cationized polymer-based gene transfection, this result demonstrates that the spermine-pullulan is a feasible gene carrier for enhancement of gene expression.

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# Chapter 5

## Effect of transfection culture methods on the level of gene expression

## INTRODUCTION

It is indispensable for successful cell therapy to develop a method of genetic cell engineering to activate and manipulate cellular functions. To achieve this genetic engineering of cells, several research trials with non-viral gene carriers of cationized polymers and liposomes have been performed. However, the level of gene expression is not as high as that of viral carriers while the time period of expression is generally short. In the conventional procedure of cell culture for gene transfection, serum cannot be generally added to the culture medium. This is because the complex of plasmid DNA and a non-viral carrier often interacts with serum components, leading to the suppressed ability of gene transfection. Since the serum is essential to maintain the biological conditions good for cell culture, the conventional transfection procedure in the absence of serum is not biologically good for cells.

In this chapter, a new transfection method is introduced to permit cell culture under better biological conditions in the presence of serum. In Chapter 1, it was found that spermine derivatives of pullulan is the most effective carrier for bone marrow mesenchymal stem cells (MSC) among other polysaccharide derivatives. The spermine-pullulan complex of a plasmid DNA was coated onto a culture substrate together with a substance of cell adhesion, and then MSC were cultured on the complex-coated substrate by different culture methods for gene transfection. The level and duration period of gene expression by the reverse transfection method were evaluated and compared with that of the conventional transfection method. In addition, the reverse transfection was performed for polyethylene terephthalate (PET) non-woven fabric of a three-dimensional scaffold by an agitated and stirring culture methods. The level and duration period of gene expression for MSC by the two method were evaluated and compared with that of static culture method.

Chapter 5

# EXPERIMENTAL

## Materials

Pullulan with a weight-average molecular weight of 47,300 was purchased from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Spermine was purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

## Preparation of cationized pullulan derivative

Spermine was introduced to the hydroxyl groups of pullulan by a N,N'-carbonyldiimidazole (CDI) activation method [1]. Spermine ($1.87 \times 10^3$ mg) and CDI ($2.25 \times 10^2$ mg) were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of pullulan. Following agitation at 35 °C for 20 hr, the reaction mixture was dialyzed against ultra-pure double distilled water (DDW) for 2 days with a dialysis membrane (the cut-off molecular weight = 12,000-14,000, Viskase Companies, Inc, Willowbrook, Illinois.). Then, the solution dialyzed was freeze-dried to obtain the spermine-introduced pullulan (spermine-pullulan). When determined from the conventional elemental analysis and expressed by the molar extent of spermine introduced to the hydroxyl groups of pullulan, the percentage of spermine introduced was 12.3 mole%.

## Preparation of plasmid DNA

The plasmid DNA used was the pGL3 vector (5.26 kb) coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, USA). The plasmid DNA was propagated in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of the plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

**Preparation of polyion complexes (PIC)**

PIC were prepared by mixing an aqueous solution of spermine-pullulan with that of plasmid DNA. Briefly, varied amounts of spermine-pullulan were dissolved in 50 μl of DDW and mixed with 50 μl of phosphate-buffered saline (PBS, pH 7.4) containing 100 μg of plasmid DNA, followed by leaving for 15 min at room temperature to obtain various PIC of spermine-pullulan and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio.

**Preparation and culture of MSC**

MSC were isolated from the bone shaft of femurs of 3-week-old male Wister rats according to the technique reported by Lennon *et al* [2]. Briefly, both ends of rat femurs were cut away from the epiphysis and the bone marrow was flushed out by a syringe (21-gauge needle) with 1 mL of α-Minimum essential medium (αMEM) supplemented with 15 vol% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 U/ml). The cell suspension (5 ml) was placed into two 25-$cm^2$ flasks (Iwaki Glass, Funabashi, Chiba, Japan) and cultured at 37° C in a 95% air-5% $CO_2$ atmosphere. The medium was changed on day 4 of culture and every 3 days thereafter. When the cells of the first passage became subconfluent, usually 7-10 days after seeding, the cells were detached from the flask by treatment for 5 min at 37° C with PBS solution containing 0.25 wt% trypsin and 0.02 wt% Ethylenediaminetetraacetic acid (EDTA). Cells were normally subcultured at a density of 2 x $10^4$ cells/$cm^2$. Second-passage cells at subconfluence were used for all experiments.

**Conventional transfection of two dimension in the static method**

Cells were seeded on each well of 12-well multi-dish culture plate (Corning, NY, USA) at a density of $5\times10^4$ cells/well and cultured in 1 ml of αMEM medium with 15 vol% FCS for 24 hr. PIC were formed by mixing 50 μl of DDW containing spermine-pullulan and 50 μl of PBS containing 2.5 μg of pGL3-luciferase plasmid DNA at different N/P ratios. Immediately after the medium was exchanged by FCS-free αMEM medium, 100 μl of the

PIC solution was added and transfection culture was performed for 6 hr (the conventional transfection). Following the medium was changed to αMEM with FCS, cells were incubated further for 1, 3, 5, and 7 days.

**Reverse transfection of two dimension in the static method**

Succinic anhydride (90.1 mg) was added to 20 ml of 100 mg/ml gelatin solution in DMSO, followed by agitation at room temperature for 18 hr to allow to introduce the carboxyl groups to the amino groups of gelatin for anionization. When determined as the extent of amino groups decreased by the trinitrobenzene sulfonic acid method [3], the molar amount of carboxylic groups introduced was 100 mole%.

The aqueous solution of the anionized gelatin (100 μg/ml) and Pronectin with different amounts was placed into each well of 12-well multi-well culture plate, followed by leaving at 37° C for 1 hr for coating. Following PBS washing, the well was coated with the plasmid DNA-spermine-pullulan complex containing 2.5 μg of plasmid DNA. Following 30 min incubation, every well was washed with PBS. Then, MSC ($5\times10^4$ cells/well) were seeded on the complex-coated well, followed by cell culture in the αMEM medium with or without 15 vol% FCS for 1, 3, 5, and 7 days.

Cells were washed with PBS once, lysed in 100 μl of cell culture lysis reagent (Promega Corp., Madison, WI, USA), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI, USA) was added to 20 μl of supernatant while the relative light unit (RLU) of the sample was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) Protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturers' instructions. The RLU was divided by the protein amount to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently.

**Reverse transfection of three dimension by different culture methods**

Static, agitated, and stirring methods were used to culture MSC in a non-woven fabric of PET (the fiber diameter = 26 μm, 6 mmΦ x 3 mm) for their plasmid DNA transfection. The similar coating procedure with the complex and Pronectin was performed for the PET non-woven fabric. MSC were seeded into the complex-coated 3-dimentional PET fabric by the agitation method reported previously [4]. Briefly, the non-woven fabric was placed in 0.5 ml of the cell suspension (1 x $10^6$ cells/mL), followed by agitation with an orbital shaker (Bellco Glass, Vineland, NJ) at 300 rpm for 6 h at 37° C. The cell-seeded non-woven fabric was thoroughly washed with the medium to exclude non-adherent cells. The MSC-attached PET fabric was incubated for 2, 5, and 8 days under the conventional static condition (static culture).

For the agitated culture method, the MSC-attached PET fabric prepared was placed in each well of 6-well multi-well culture plate containing 6 ml of αMEM medium with 15 vol% FCS while the culture plate was agitated with the orbital shaker (Bellco Glass, Vineland, NJ) at 50 rpm for 2, 5, and 8 days. On the other hand, the MSC-attached fabric was fixed with a needle immobilized in the spinner flask and 150 ml of medium was stirred at 50 rpm for 2, 5, and 8 days (stirring culture). To measure the level of gene expression for MSC cultured by the static, agitated, and stirring methods, the PET fabrics were collected and the gene expression was assessed by the similar procedure described above.

**Cell viability**

Cytotoxicity was assayed using a cell counting kit (Nacalai tesque, Inc., Kyoto, Japan). MSC were transfected with 0.50 μg of free plasmid DNA or lipofectamine 2000 (0.50 μg) and the spermine-pullulan complexing 0.50 μg of plasmid DNA by the conventional and reverse methods for 2 days. Then, the medium was changed to αMEM medium with FCS and 100 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) solution was added and the cells were incubated further for 3 hr. The absorbance of samples was measured at 450 nm by VERSAmax microplate reader (Molecular Devices, sunnyvale CA, USA). The percent cell viability was expressed as 100% for non-transfected, control cells.

**Statistical Analysis**

Data were expressed as the mean±the standard deviation of the mean. Data were analyzed by two-way ANOVA, and then differences among means were analyzed using Fisher's PLSD multiple comparison test and significance was accepted at $p < 0.05$.

# RESULTS

**Gene expression for MSC by the conventional and reverse transfection methods**

**Table 1** and **Figure 1** show the level and duration period of gene expression after the reverse transfection of MSC together with those of the conventional transfection. For the conventional method where the complex is added into the culture medium, the presence of serum suppressed the level of gene expression. On the contrary, a high expression level of MSC transfected by the reverse method was observed even in the presence of serum. Moreover, the time period of gene expression was prolonged compared with that of the conventional method. When reverse transfected in the absence of FCS, MSC were detached from the culture substrate during the transfection culture (data not shown). Free plasmid DNA did not enhance gene expression, irrespective of the serum presence.

**Table 1. Luciferase expression level of MSC transfected by the conventional and reverse methods in the presence or absence of serum. a)**

| Transfection method | | The level of gene expression (RLU/mg protein) | |
|---|---|---|---|
| | | free plasmid DNA | plasmid DNA-spermine-pullulan complex |
| **Conventional** | FCS (-) | $(3.0 \pm 0.02) \times 10^3$ | $(4.2 \pm 0.62) \times 10^6$ |
| | FCS (+) | $(3.0 \pm 0.98) \times 10^3$ | $(3.3 \pm 0.66) \times 10^5$ |
| **Reverse** | FCS (-) | - | ND c) |
| | FCS (+) | - | $(6.2 \pm 1.4) \times 10^7$ |

The level of gene expression for non-transfected, original MSC was $3.0 \times 10^3$ RLU/mg protein.

a) The MSC were transfected in the static culture.

b) Mean ±S.D.

c) The level could not be measured because of cell death.

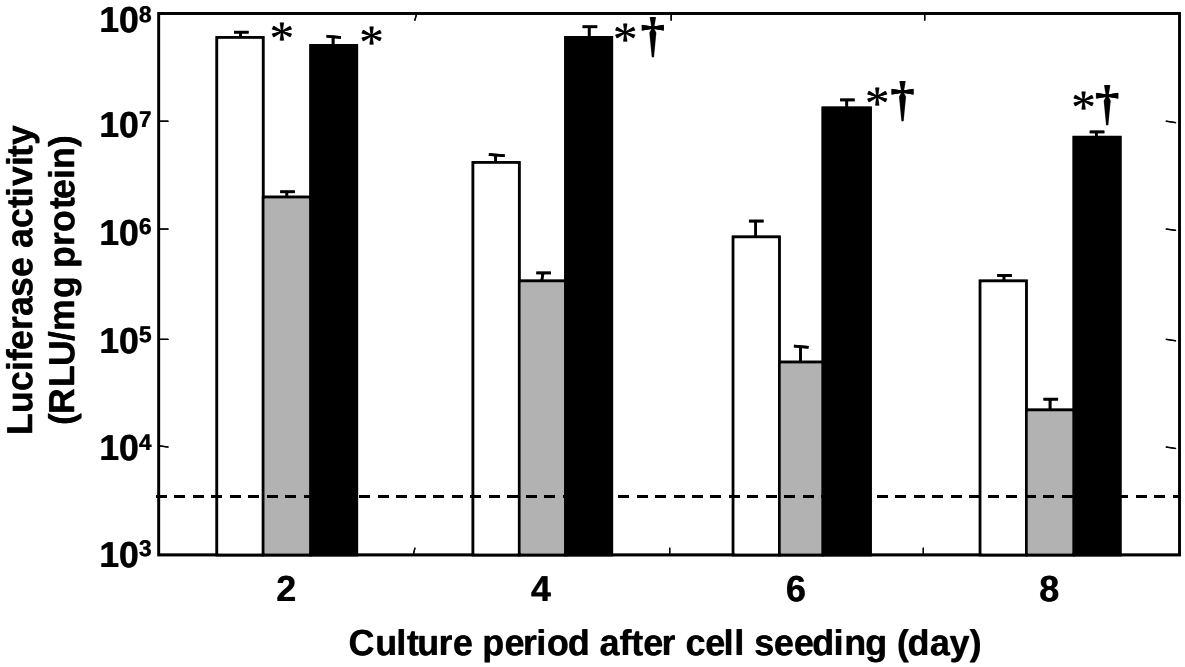


**Figure 1.** Time course of luciferase expression level of MSC transfected by the conventional (open and light gray columns) and reverse methods (solid columns) in the static cuture: (open columns) the plasmid DNA-spermine-pullulan complex in the absence of FCS, (dark gray columns) the plasmid DNA-spermine-pullulan complex in the presence of FCS, and (solid columns) the plasmid DNA-cationized pullulan complex in the presence of FCS. The dotted line indicates the level of non-transfected, original MSC. ***,** $p<0.05$ versus the level in the presence of FCS by the conventional method at the corresponding time. †, $p<0.05$ versus the level in the absence of FCS by the conventional method at the corresponding time.

**Figure 2** shows the level of gene expression of MSC by the reverse transfection method at different coating concentrations of Pronectin®. The expression level was enhanced with the increased coating concentration of Pronectin® of adhesion substance.

**Cell viability**

**Figure 3** shows the cell viability of MSC transfected by the conventional and reverse methods. The cell viability significantly decreased by the conventional transfection culture with both the spermine-pullulan and Lipofectamine 2000 complexing plasmid DNA. On the contrary, the viability of cells after reverse transfection culture was similar to that of non-transfected, original cells.

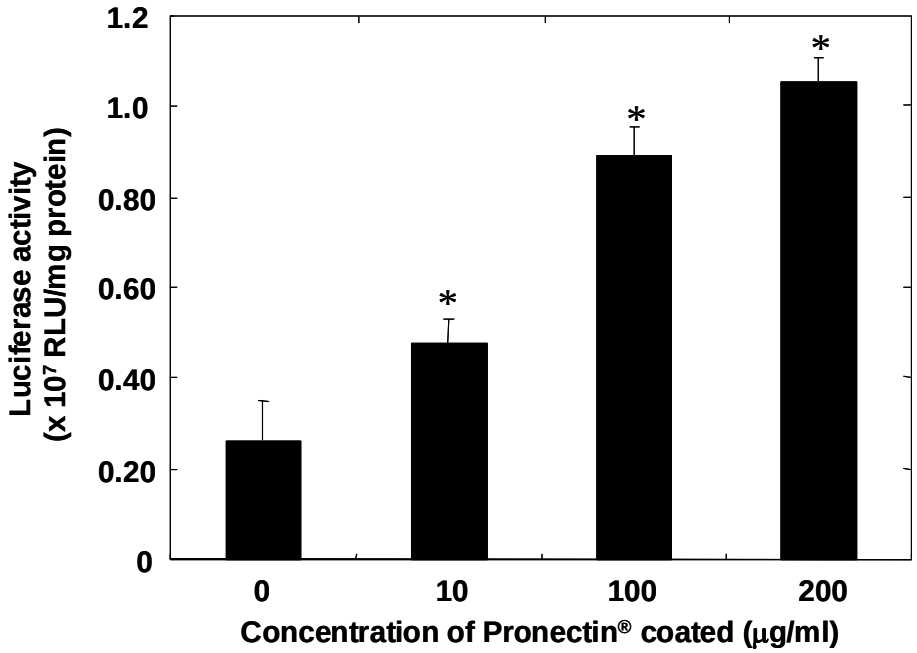


**Figure 2.** Effect of Pronectin® coating concentration on the luciferase expression level of MSC transfected by the reverse method in the static culture. *, $p<0.05$ versus the luciferase activity of MSC in the coating concentration of 0 μg/ml.

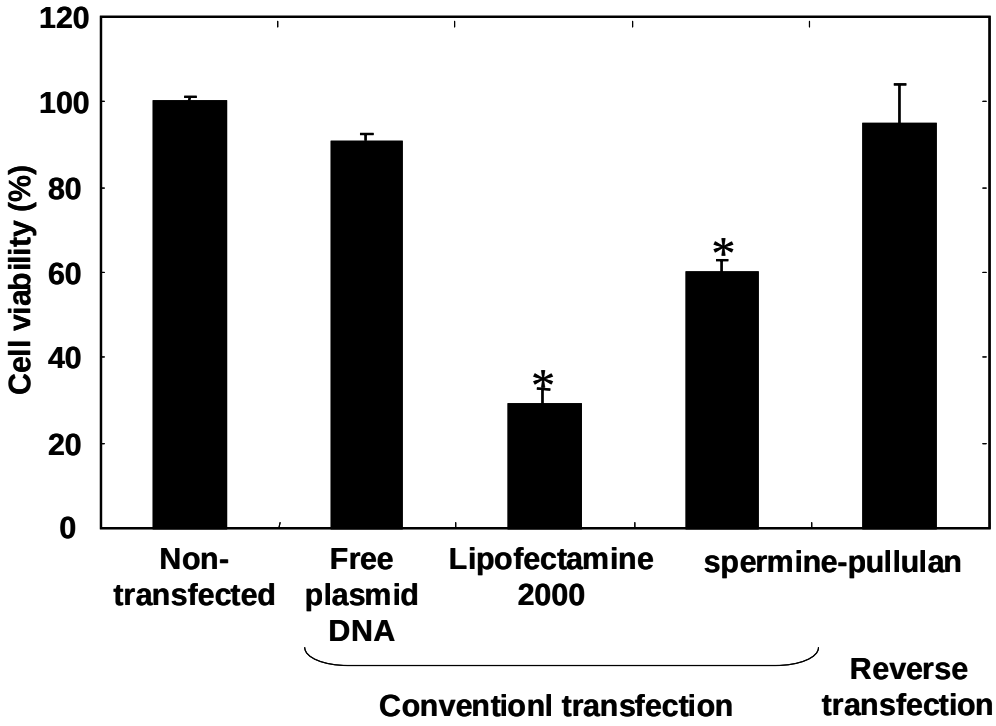


**Figure 3.** Cell viability of MSC 2 days after conventional and reverse transfection cultures. The cells were transfected by the conventional method with free plasmid DNA or that complexed with Lipofectamine 2000 and spermine-pullulan in the absence of serum. The cells were transfected by the reverse method with plasmid DNA-spermine-pullulan complex in the presence of serum. *. $p<0.05$ versus the cell viability of non-transfected, original MSC.

**Gene expression by the reverse transfection of these three dimension in different culture methods**

**Figure 4** shows the time profile of gene expression of MSC after the reverse transfection by different culture methods. Irrespective of the culture method, the level and duration period of gene expression were significantly enhanced by the reverse transfection compared with conventional one. The enhanced and prolonged extents of gene expression depended on the type of culture method, although the time profile of transfected level was similar. The extents increased in the order of the static, agitated, and stirring culture methods. By the reverse transfection culture, the level of gene expression increased by 5 days after cell seeding, but thereafter decreased. This was different from the time profile of gene expression by the conventional transfection culture, where there was a maximum level on the 2nd day for any culture method. The agitated and stirring cultures tended to increase the level of gene expression.

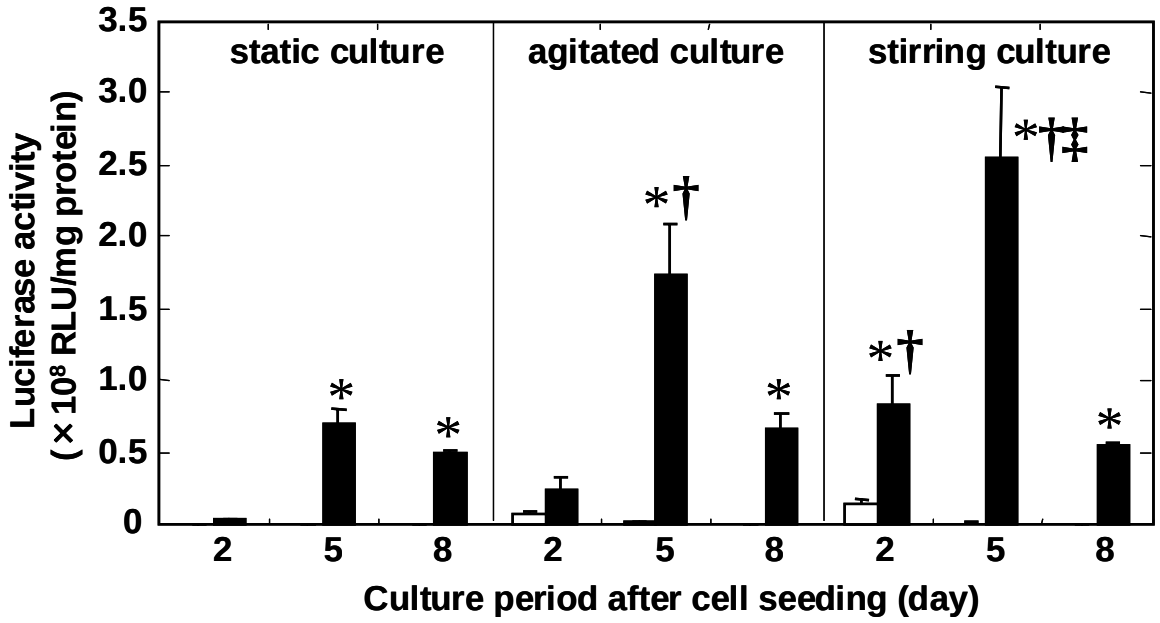


**Figure 4.** Time course of luciferase expression level of MSC transfected by the conventional (open columns) and reverse methods (solid columns) in the static, agitation, and stirring cultures in the PET non-woven fabric: (open columns) the plasmid DNA-cationized pullulan complex in the absence of FCS and (solid columns) the complex in the presence of FCS. *, $p<0.05$ versus the level in the presence of FCS by the conventional method at the corresponding time. †, $p<0.05$ versus the level in the absence of FCS by the reverse method in the static culture at the corresponding time. ‡, $p<0.05$ versus the level in the absence of FCS by the reverse method in the agitation culture at the corresponding time.

**Figure 5** shows the time course of MSC proliferation during the reverse transfection culture. The number of MSC proliferated in the PET fabrics became larger in the order of the static, agitated, and stirring culture methods although little cell proliferation was observed in the static culture.

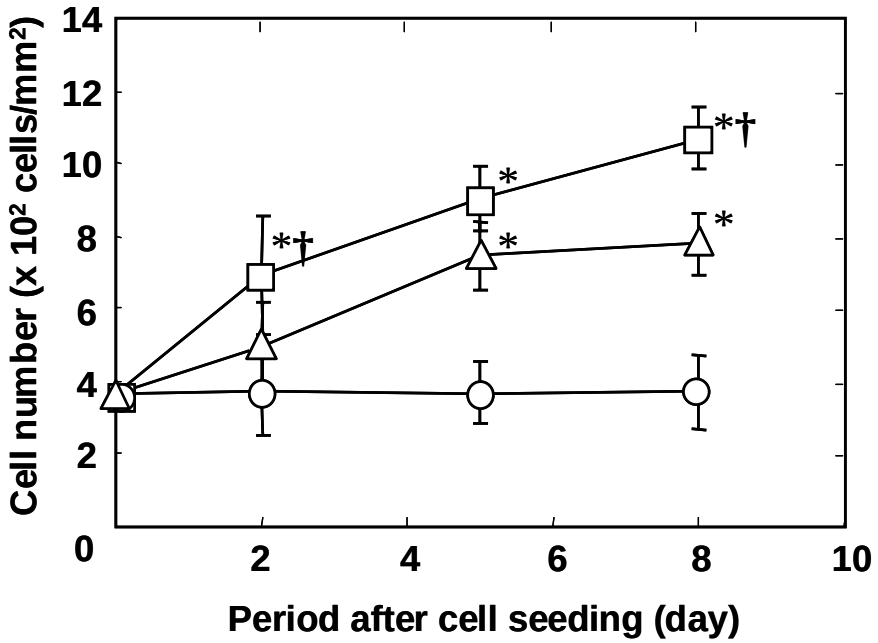


**Figure 5.** Time course of proliferation of MSC transfected by the reverse method in the PET non-woven fabric transfection in the static (○), agitation (△), and stirring cultures (□) . †, $p<0.05$ significant against the cell number in the static culture at the corresponding time. ‡, $p<0.05$ significant against the cell number in the agitation culture at the corresponding time.

## DISCUSSION

The present study clearly demonstrates that the reverse transfection method was more effective in enhancing the level and duration time of gene expression than the conventional method. Considering the positioning of cells and the complex, it is likely that the complex always exists near the cells to be transfected. In addition, Pronectin achieves confirm cell adhesion to the surface of complex-coated substrate. It is highly conceivable that both the continuous exposure of complex to cells and the confirm adhesion between cells and the substrate which minimizes the serum influence on the transfection activity of complex, results in the enhanced and prolonged gene expression.

There are five key steps for expression of plasmid DNA; (1) the attachment of plasmid DNA onto the cell surface, (2) the internalization of plasmid DNA into the cell, (3) the endosomal escape of plasmid DNA, (4) the transfer of plasmid DNA to the nucleus, and (5) the internalization of plasmid DNA into the nucleus. Different from the viral vector, basically the non-viral carrier does not have any specific mechanisms to accelerate and facilitate the above steps. Several trials have been performed to enhance the efficiency of each step. For the steps of (1) and (2), receptor-mediated endocytosis mechanism has been tried to use [5-26]. It is conceivable that the spermine-pullulan-plasmid DNA complex escaped travels in the cytosol by the simple diffusion. Since it does not have any nucleus transfer mechanisms. Once the complex reaches the nuclear membrane, the internalization into the nucleus will be necessary for gene transfection. It has been recognized that non-viral carriers do not have any machineries for nuclear internalization. Therefore, it is possible that when the nuclear membrane disappears in cell division, the complex of non-viral carrier and plasmid DNA eventually enters the inside of nucleus for gene transfection. Based on this mechanism, the transfection efficiency of plasmid DNA by the non-viral carrier will increase as the proliferation of cells to be transfected promotes. In the reverse transfection method, cells are cultured in the presence of serum. It is highly possible that the serum presence enables cells to proliferate more efficiently under better culture conditions, resulting in enhanced gene transfection. The cell viability after the reverse transfection culture was significantly higher than that of the conventional transfection culture in the absence of FCS. This strongly indicates that the reverse transfection is performed under culture conditions good for cell activities. It should be noted that cells always contact with the complex in the reverse transfection culture, which is quite different from the contact time for 6 hr in the conventional transfection culture.

To allow cells to proliferate under in vitro culture conditions, it is necessary to contrive the local environment of cells, including the medium, the substrate of cells attachment and proliferation, and the oxygen nutrients supply or wastes exclusion. Some trials have been performed to accelerate the in vitro proliferation of MSC. MSC proliferated more efficiently by the addition of basic fibroblast growth factor (bFGF) into the culture medium

[27,28] and the surface modification of culture substrates [28]. Since the MSC proliferation is substrate-dependent, it is preferable to increase the surface area of culture substrate. Several three-dimensional substrate, so-called scaffold, have been designed to demonstrate their feasibility in the proliferation enhancement [29-31]. Additionally, from the viewpoint of oxygen and nutrients supply and wastes exclusion, the culture methods have been designed. Stirring and perfusion culture methods were effective in enhancing the rate of MSC proliferation compared with the static culture method [4,32].

For the agitated and stirring culture methods, since the culture medium is circulated, oxygen and nutrients are supplied to MSC and cellular wastes are excluded more efficiently than the static culture method without active medium circulation. The plasmid DNA-carrier complex does not have any inherent potentials to allow the plasmid DNA to positively integrate into the genome of cells. Considering the gene transfection mechanism, it is likely that the plasmid DNA has a chance to internalize into the nucleus of cells for gene expression only when the nuclear membrane of cells disappears in cell division. The medium-circulated culture method promoted cell proliferation (**Figure 5**). Taken together, it is highly conceivable that more efficient proliferation of MSC under better biological conditions promotes the internalization of complex without any lethal damages to cells, resulting in enhanced gene expression. In conclusion, the reverse transfection method combined with the stirring cell culture method in the presence of the adhesion substance is a promising technology to enhance the efficiency of gene expression for stem cells. This technology is applicable for any type of cells. It is expected that this transfection method with the non-viral pullulan carrier can be applied to the genetic engineering for cell therapy as well as basic research of stem cell biology and medicine.

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# PART 2

# ENHANCED IN VIVO THERAPEUTIC EFFICACY OF CELLS GENETICALLY ENGINEERED BY CATIONIZED POLYSACCHARIDE-PLASMID DNA COMPLEXES

# Chapter 6

## Enhanced therapeutic efficacy of bone marrow-derived mesenchymal stem cells genetically engineered by cationized dextran-adrenomedullin plasmid DNA complexes for acute myocardial infarction

## INTRODUCTION

Despite the recent remarkable progress in medical and surgical treatment for ischemic heart disease,[1] it is still a major cause of death worldwide.[2] Recently, cell therapy has been paid great attention as a promising approach for ischemic heart disease.[3-6] One of the most promising cells is bone marrow-derived mesenchymal stem cells (MSC), which are multipotent adult stem cells readily isolatable from the bone marrow of patients themselves.[7,8] It is well recognized that MSC can differentiate into not only osteoblasts, chondrocytes, neurons, and skeletal muscle cells, but also vascular endothelial cells [9] and cardiomyocytes.[10,11] In fact, transplantation of MSC has been demonstrated to induce angiogenesis and improve cardiac functions for animal models of myocardial infarction or dilated cardiomyopathy.[12-15] However, the therapeutic potential of MSC is often hindered by their poor viability at the transplanted site.[10] Thus, it is of prime importance to develop technology and methodology to survive MSC transplanted and enhance the biological functions.

Recently, the technology to genetically manipulate adult stem cells has increasingly become important for basic biological and medical researches on stem cells as well as cell therapy with genetically engineered cells. To this end, viruses have been commonly used for gene expression of stem cells because of the high expression efficiency. Although adenoviruses and retroviruses have high transfection efficiency, they have several clinical limitations, such as the mutagenesis, cacinogenesis, and induction of immune responses**.** Many researches have been reported on the superior therapeutic effects of stem cells genetically engineered by viral vectors,[16-22] but the strategy is not available for clinical cell therapy because of the usage of viral vectors. Therefore, it is highly desirable to develop an efficient and safe non-viral carrier for the gene transfection of MSC. In Chapter 1, it was

found that complexation with the cationized dextran allows a plasmid DNA to prepare a size-condensed structure with a positive charge which efficiently allows plasmid DNA to internalize into cells. The cationized dextran can interact with a sugar-chain recognizable receptor present at the cell surface.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[23] In addition, AM has antiapoptotic and angiogenic properties which may be effective for the treatment of ischemic heart disease.[24-30] Taken together, MSC genetically engineered by AM will be a promising and effective cell source for cell therapy.

The purposes of this chapter were 1) to investigate whether transplantation of MSC transfected by the AM plasmid DNA with the spermine-dextran (AM-MSC) enhances the therapeutic efficacy of MSC for a rat model of myocardial infarction and 2) to examine the underlying mechanisms of the therapeutic effects induced by AM-MSC.

## EXPERIMENTAL

### MSC Culture

MSC culture was performed according to the method previously described.[7] In brief, 3-week-old male Lewis rats were sacrificed, and the bone marrow was harvested by flushing the cavity of the femur and tibia with 10 mM of phosphate-buffered saline (PBS, pH 7.4). The suspension of bone marrow cells in PBS was placed into 100-mm dishes (Corning, NY) and cultured in α-Minimal essential medium (α-MEM) supplemented with 15% bovine fetal calf serum (FCS) and 1% penicillin streptomycin solution (control medium). After incubation at 37 °C in 5% $CO_2$ and 95% air at atmospheric pressure for 3 days, nonadherent cells were removed. Adherent cells were further cultured by exchanging the fresh control medium every 3 days. The cells were detached with 0.25 wt% trypsin-containing 0.8 mM Ethylenediaminetetraacetic acid (EDTA) solution in PBS, subcultured in 225-$cm^2$ cell culture flasks, and 80% confluent cells were used as MSC in the following experiments.

### Preparation of Cationized Dextran and Plasmid DNA

To prepare cationized dextran, spermine was chemically introduced to the hydroxyl

groups of dextran by a N,N'-carbonyldiimidazole (CDI) activation method.[31] Briefly, 15 and 1.5 excess molar amounts of spermine and CDI against the hydroxyl group of the dextran, were added to 1 mg/ml dextran solution in dehydrated dimethyl sulfoxide. The reaction solution was agitated at 35 °C for 20 hr to introduce spermine residues to the hydroxyl groups of dextran, followed by dialysis against ultra pure double distilled water (DDW) for 2 days and freeze-dried to obtain a spermine-introduced dextran (spermine-dextran). A pcDNA1.1-CMV vector (Invitrogen, CA) encoding human AM or luciferase cDNA was constructed. The spermine-dextran aqueous solution (50 μl, 260 μg/ml) was added to the plasmid DNA PBS solution (100 μg/ml) at the same volume, followed by 15-min leaving at room temperature to obtain a spermine-dextran-plasmid DNA complex.

**RT-PCR Analysis**

To investigate whether MSCs express the asialoglycoprotein receptor (ASGPR), reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. In brief, total RNA of MSCs was extracted with guanidine isothiocyanate (RNeasy Mini Kit, Qiagen). Then, reverse-transcribed single-stranded cDNA was subjected to PCR (PCR Amplification Kit, Takara) using primer sets for ASGPR (Proligo, forward, 5'-CGAAGCTTGAGCTGCCAGATGGCC-3'; reverse, 5'-GGAACGGGTTGAGACAGAGTGT-3') and GAPDH (Clonetech Laboratories, Inc., forward, 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3'; reverse, 5'-CATGTAGGCCATGAGGTCCACCAC-3').

**Ex Vivo Gene Transfection of MSC**

MSC ($1\times10^4$ cells/cm$^2$) were cultured with the spermine-dextran-plasmid DNA complex at the plasmid DNA dose of 0.5 μg/cm$^2$ at 37 °C for 6 hr in serum-free medium. The level of human AM expressed in the culture medium (n = 6) was measured by radioimmunoassay. The culture medium was acidified with acetic acid, boiled to inactivate intrinsic proteases, and lyophilized. The AM level in the culture medium was measured with a radioimmunoassay kit (Shionogi, Osaka, Japan).[32] To investigate the mechanism of gene

transfection, MSC were cultured with spermine-dextran-luciferase plasmid DNA complexes for 6 hr in the serum-free medium with or without a polyclonal goat anti-ASGPR1 antibody (Santa Cruz Biotechnology Inc., CA, USA) and in serum-free medium at 4 ° C. Luciferase gene expression was quantified using a commercial kit (Luciferase Assay System, Promega, WI) and the relative right unit (RLU) was determined by a luminometer (Micro. Luminat. Plus LB 96V, Berthold, Germany). The total protein of each well was determined by bicinchonic acid (BCA) Protein Assay Reagent (Pierce, IL) in order to normalize the influence of number variance of cells on the luciferase activity.

**Animal Model**

Myocardial infarction was produced in male Lewis rats weighing 180 to 220 g by left coronary artery ligation, as described previously.[33] Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg body wt) and artificially ventilated using a volume-regulated respirator. Hearts were exposed via a left thoracotomy and the left coronary artery was ligated 2-3 mm from its origin between the pulmonary artery conus and the left atrium, using a 6-0 Prolene suture. Following MSC transplantation, the heart was restored to its normal position and the chest was closed. A sham operation consisting of thoracotomy and cardiac exposure but without coronary artery ligation was also performed. The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

**Therapeutic Transplantation of Genetically Engineered MSC**

Transplantation of AM gene-transfected MSC, non-transfected MSC, or saline was performed immediately after coronary ligation. AM gene-transfected MSC (AM-MSC) and non-transfected MSC (MSC) ($5 \times 10^6$ cells in 100 µl PBS) or saline (control) was injected into the myocardium at five points in the border zone surrounding the infarction site, with a 27-gauge needle. Saline was injected into the myocardium of normal rats (sham).

**Echocardiographic Studies**

Echocardiographic studies were performed by an investigator blinded to treatment allocation 4 weeks after coronary ligation. Two-dimensional targeted M-mode tracings were obtained at the level of the papillary muscles using an echocardiographic system equipped with a 7.5-MHz phased-array transducer (HP SONOS 5500; Hewlett Packard Company, Andover, MA), in order to analyze demension and function of left ventricular (LV). Anterior and posterior end-diastolic wall thickness and LV end-diastolic and end-systolic dimensions were measured by the American Society for Echocardiology leading-edge method from at least three consecutive cardiac cycles. LV fractional shortening was calculated as (LVDd-LVDs)/LVDd x 100 where LVDd = LV diastolic dimension and LVDs = LV systolic dimension.

**Hemodynamic Studies**

Hemodynamic studies were performed 4 weeks after coronary ligation. A polyethylene catheter (PE-50) was inserted in the right carotid artery for measurement of mean arterial pressure. Next, the catheter was advanced into the LV for measurement of LV pressure. Hemodynamic variables were measured using a pressure transducer (model P 23 ID, Gould) connected to a polygraph. After completion of these measurements, the left and right ventricles were excised and weighed. Infarction size was determined as a percentage of the entire LV area, as reported previously.[34] In brief, incisions were made in the LV so that the tissue could be pressed flat. The circumference of the entire flat LV and the visualized infarcted area, as judged from both the epicardial and endocardial sides, was outlined on a clear plastic sheet. The difference in weight between the two marked areas on the sheet was used to determine infarction size and was expressed as a percentage of LV surface area.

**Histological Examination**

To measure fibrosis in cardiac muscle, the LV myocardium (n = 5 each group) was fixed in 10% formalin, cut transversely, embedded in paraffin, and stained with Masson’s trichrome. To detect capillaries in the myocardium, samples of harvested muscle (n =5 each

group) were embedded in paraffin,and immunohistologically stained using polyclonal rabbit anti- von Willebrand factor (vWF, Dako Cytomation, Glostrup, Denmark). Transverse sections were randomly obtained, and 10 randomly selected fields of infarct region were analyzed. The number of capillaries was counted by light microscopy at a magnification of x 200. The number of capillaries in each field was averaged and expressed as the number of capillary vessels. An additional 10 rats were used to examine whether AM-MSC or MSC transplanted differentiated into cardiomyocytes or vascular endothelial cells. Suspended AM-MSC and MSC were fluorescently labeled with a PKH26 Red Fluorescent Cell Linker Kit (Sigma Chemical Co., St. Louis, MO) before transplantation, as reported previously.[35] The fluorescent-labeled MSC were injected into the myocardium immediately after coronary ligation. This subgroup of rats was killed four weeks after coronary ligation. After LV excision, muscle samples were embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), snap-frozen in liquid nitrogen, and cut into sections. Immunofluorescent staining for cardiac and endothelial cell markers was performed using monoclonal mouse anti-desmin (Dako Cytomation, Glostrup, Denmark), anti-cardiac troponin T (NeoMarkers, Fremont, CA), and polyclonal rabbit anti-vWF (Dako Cytomation, Glostrup, Denmark). Fluorescein isothiocyanate (FITC)-conjugated IgG antibody (BD Pharmingen, CA) was used as a secondary antibody**.**

**TUNEL Staining**

To examine whether AM gene transfer has antiapoptotic effects on MSCs in vitro, in situ labeling of fragmented DNA was performed with TdT-mediated dUTP nick-endlabeling (TUNEL). MSCs were seeded on slides in 12-well plates ($2 \times 10^4$ cells/well) and cultured in regular growth medium under normoxic conditions for 24 hours. Gene transfection for AM was performed as described above and the cells were incubated in serum-free medium under hypoxic conditions (1% $O_2$, 5% $CO_2$, and 94% $N_2$) at 37 °C for 36 hrs. Finally, cells were fixed with 4% buffered formalin. TUNEL staining was performed with a commercially available kit (Apop Tag Plus, Chemicon, Canada) according to the manufacturer’s instructions, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Ten randomly

selected microscopic fields were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

The antiapoptotic effect of AM gene transfer on MSCs *in vivo* was also evaluated by TUNEL assay. Twenty-four hours after intramyocardial injection of PKH26-labelled transfected or non-transfected MSCs, cardiac muscle was embedded in OCT compound and snap-frozen in liquid nitrogen. Cardiac muscle from base to apex was cut transversely into 5 μm-slices in order to calculate the numbers of transplanted MSCs present within the heart (n = 5 each). TUNEL staining was performed with a commercially available kit (Apop Tag Plus, Chemicon, Canada). Slides were mounted with Vector Shield (Vector Laboratories, CA) containing an antifade reagent. The number of TUNEL/PKH 26 double-positive cells was counted in 10 fields for each rat.

**Statistical Analysis**

Numerical values were expressed as mean ± SEM. unless otherwise indicated. Comparisons of parameters among the four groups were made using one-way analysis of variance (ANOVA), followed by the Newman-Keul's multiple comparison test. The $P$ value < 0.05 was considered to be significant.

# RESULTS

## Gene Expression of MSC by the Spermine-dextran of Non-Viral Carrier

The spermine-dextran of cationized polysaccharide was successfully prepared by the CDI activation method (**Figure 1A**). The percentage of spermine introduced to the hydroxyl group of dextran was approximately 10% by an elemental analysis (data not shown). The plasmid DNA of negative charge was easily complexed with the positively-charged spermine-dextran via the electrostatic interaction. When measured by dynamic light scattering and electrophoretic light scattering, the apparent molecular size and zeta potential of spermine-dextran-plasmid DNA complexes prepared were approximately 200 nm and +12 mV, respectively. Complex formation with the spermine-dextran enabled the plasmid DNA to significantly enhance the expression level of MSC compared with that of naked plasmid DNA or a transfection reagent commercially available, Lipofectamine$^{TM}$ 2000 (**Figure 1B**). RT-PCR demonstrated the presence of the ASGPR, a specific receptor for asialoglycoproteins, in MSC (**Figure 1C**). The level of gene expression of MSC by the spermine-dextran-plasmid DNA complex was significantly decreased in the presence of anti-ASGPR antibody or at 4 ° C (**Figure 1D**). These results suggest that the spermine-dextran-plasmid DNA complexes are internalized into the MSC at least in part via the receptor-mediated endocytosis. **Figure 1E** illustrates the time course of AM secretion into the culture medium by AM-MSC and MSC. The level of AM secretion increased with the time up to 3 days after gene transfection. The release of AM from MSC was not influenced by the presence of spermine-dextran alone (data not shown).

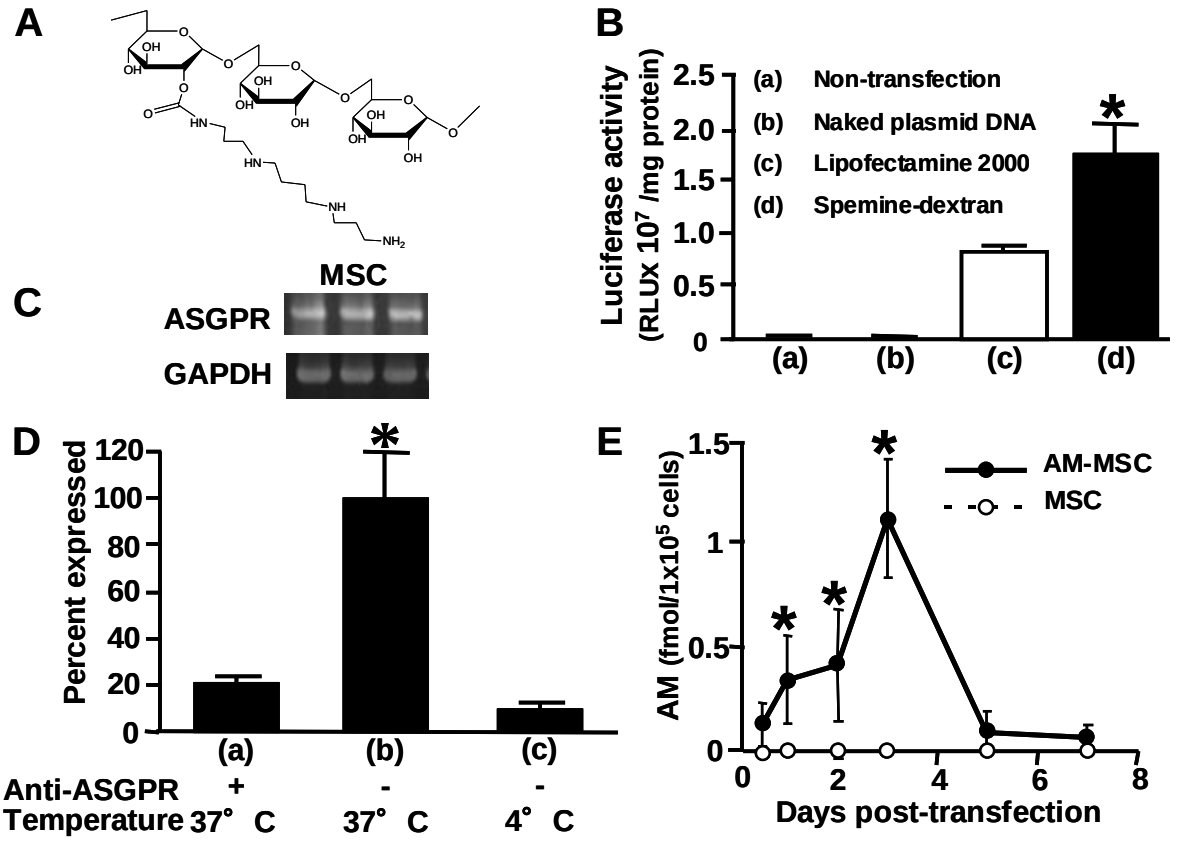


**Figure 1.** Gene expression profiles of MSC by spermine-dextran. (A) Schematic chemical structure of spermine-dextran used as a non-viral carrier for gene transfection. (B) Gene expression of MSC transfected with spermine-dextran-luciferase plasmid DNA complexes. MSC were not transfected (a) and done with naked luciferase plasmid DNA (b), Lipofectamine 2000-luciferase plasmid DNA complexes (c), and spermine-dextran-luciferase plasmid DNA complexes (d) at 37 ° C for 6 hr. *, $P < 0.05$; versus the expression level of Lipofectamine 2000. Complex formation with the spermine-dextran enabled the plasmid DNA to significantly enhance the expression level of MSC compared with that of naked plasmid DNA or a transfection reagent commercially available, Lipofectamine$^{TM}$ 2000. (C) Expression of the asialoglycoprotein receptor (ASGPR) on MSC. (D) Effects of anti-ASGPR antibody and temperature on the gene expression of MSC transfected with spermine-dextran-luciferase plasmid DNA complexes. MSC were transfected by spermine-dextran-luciferase plasmid DNA complexes at 37 ° C for 6 hr (normal transfection) with (a) or without an anti-ASGPR antibody (b) and at 4 ° C for 6 hr without the antibody (c). The gene expression level of MSC normal-transfected is expressed as 100%. *, $P < 0.05$ versus the value of other groups. The level of gene expression of MSC by the spermine-dextran-plasmid DNA complex was significantly decreased in the presence of anti-ASGPR antibody or at 4 ° C. (E) Time course of AM secreted from MSC following the transfection by spermine-dextran-AM plasmid DNA complexes. *, $P < 0.05$ versus the level of normal MSC at the corresponding time period. The level of AM secretion increased after 1-day incubation are the high level lasted for at least 3 days after gene transfection.

**Antiapoptotic Effect of MSC Transfected with the Spermine-Dextran-AM Plasmid DNA Complex**

Hypoxia and serum withdrawal induced apoptosis of MSC in vitro (**Figures 2A and B**). However, AM gene transfection markedly decreased the number of apoptotic MSC compared with the original MSC. These results suggest that AM produced by gene transfection acts as a survival factor of MSC. Some of non-transfected MSC were in vivo positive for TUNEL staining 24 hr after transplantation (**Figures 2C and D**). On the other hand, AM-MSC were rarely positive by TUNEL staining. These results suggest that AM gene transfection significantly decreased the number of apoptotic MSC in vivo.

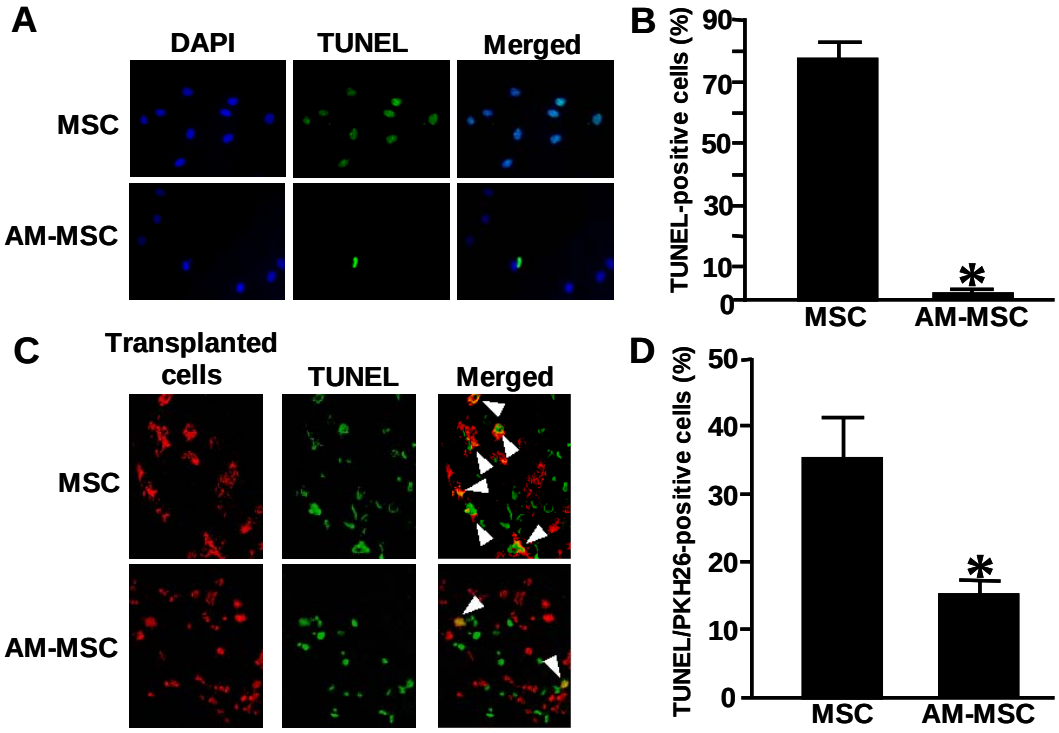


**Figure 2.** *In vitro* and *in vivo* anti-apoptotic effects of MSC transfected with spermine-dextran-AM plasmid DNA complexes. **(A)** Immuno-fluorescent photographs of TUNEL staining in vitro. Nuclei were stained with DAPI. Green fluorescence indicates TUNEL-positive cells. Hypoxia and serum withdrawal induced apoptosis of MSC. However, apoptosis was rarely detected in AM-transfected MSC (AM-MSC). **(B)** Quantitative analysis of *in vitro* TUNEL assay for AM-MSC and MSC. AM gene transfection markedly decreased the number of apoptotic MSC compared with the original MSC. **(C)** Immuno-fluorescent photographs of TUNEL staining in vivo. Red fluorescence marks transplanted MSC; green fluorescence indicates TUNEL-positive cells (arrows). Magnification, x400. **(D)** Quantitative analysis of *in vivo* TUNEL assay for AM-MSC and MSC. AM-MSC were rarely positive by TUNEL staining. *, $P < 0.05$ versus the MSC.

**Angiogenesis and Myogenesis Induced by AM-MSC**

Immunohistochemical analysis demonstrated that the number of vWF-positive microvessels in the AM-MSC group was significantly higher than that in the MSC and control groups (**Figures 3A and B**). These results suggest that AM-MSC had angiogenic potency stronger than MSC alone. Immunofluorescence also demonstrated that some of AM-MSC formed vascular structures and were positive for vWF 4 weeks after transplantation (**Figure 3C**). A small fraction of AM-MSC were positive for cardiac markers desmin and cardiac troponin T (**Figures 4A and B**), although the percentage of desmin- or troponin T-positive cells did not significantly differ between the AM-MSC and MSC groups (data not shown).

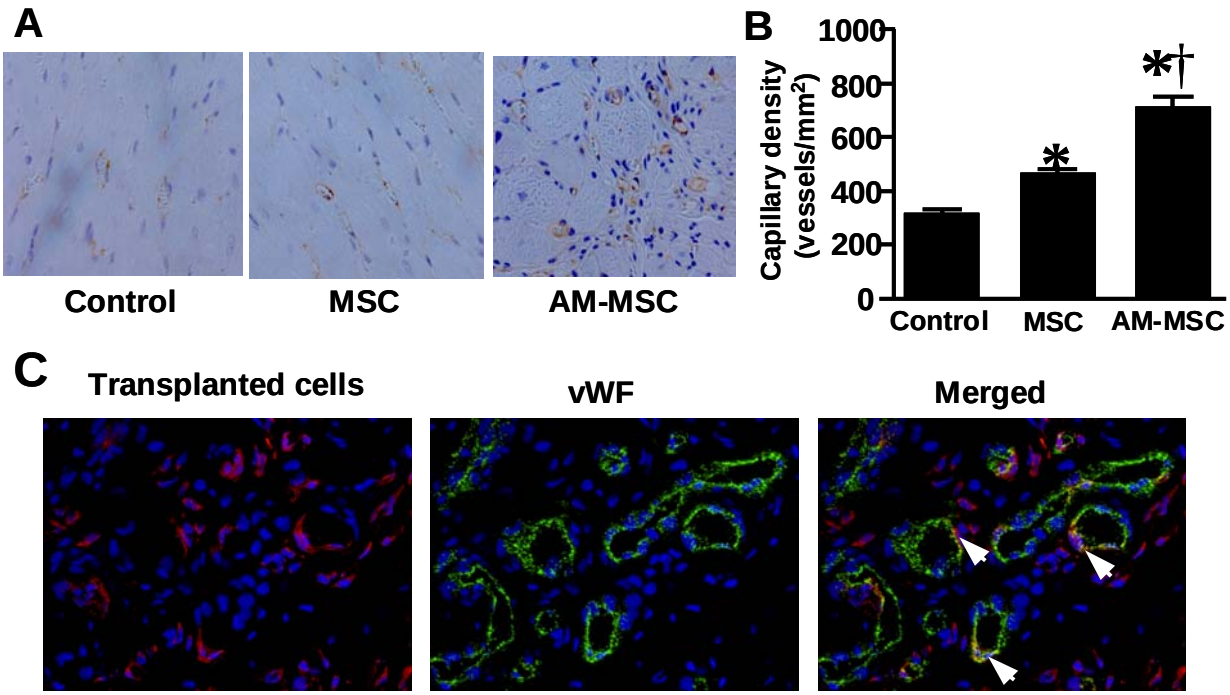


**Figure 3.** Angiogenic profiles of AM-MSC. **(A)** Representative photographs of immunohistochemical staining for vWF in peri-infarct area. Magnification x 200. **(B)** Quantitative analysis of capillary density in peri-infarct area. The number of vWF-positive microvessels in the AM-MSC group was significantly higher than that of the MSC and Control groups**.** Control, myocardial infarction rats given vehicle; MSC, those given MSCs, and those given AM-MSC. *, $P < 0.05$ versus the Control group. †, $P < 0.05$ versus the MSC group. **(C)** Some of AM-MSC (red) were positive for vWF (green) and formed vascular structures in the myocardium. Magnification, x 400.

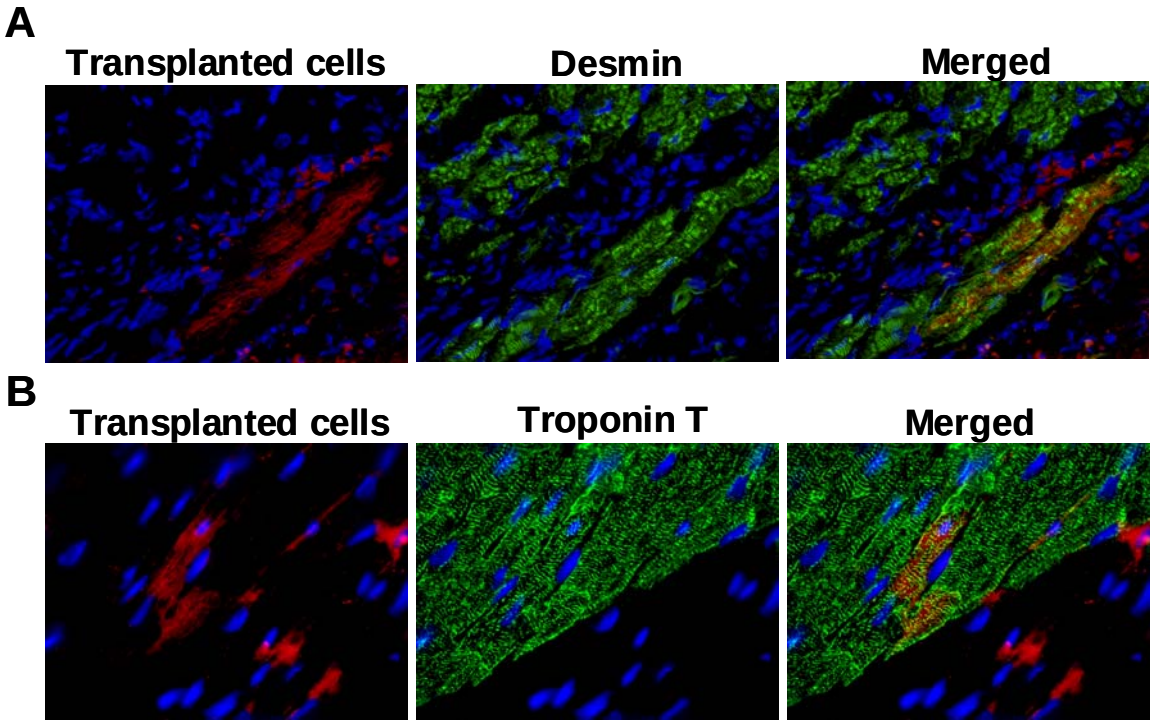


**Figure 4**. Myogenic profiles of AM-MSC. Red fluorescence marks transplanted cells; green fluorescence indicates desmin **(A)** and troponin T **(B)**. Magnification, x 400.

**Therapeutic Effects of AM-MSC on Myocardial Infarction**

Compared to the control group, transplantation of AM-MSC or MSC significantly decreased the infarct size (**Figure 5A**). Semi-quantitative analysis demonstrated that rats transplanted with AM-MSC showed the smallest infarct size (**Figure 5B**). Body weight was significantly higher in the AM-MSC group than in the control and MSC groups (**Table 1**). Right ventricular weight in the AM-MSC group was significantly lower than that in the control or MSC group, although left ventricular (LV) weight did not differ among the three groups. The lowest LV end-diastolic pressure was observed for the AM-MSC group (**Figure 5C**), while the LV fractional shortening (%FS) was the highest among the all groups (**Figure 5D**). The AM-MSC and MSC groups showed the small LV dimension in end-diastole and end-systole compared with the control group (**Table 2**). The anterior wall of the AM-MSC and MSC groups was thicker than that of the control group.

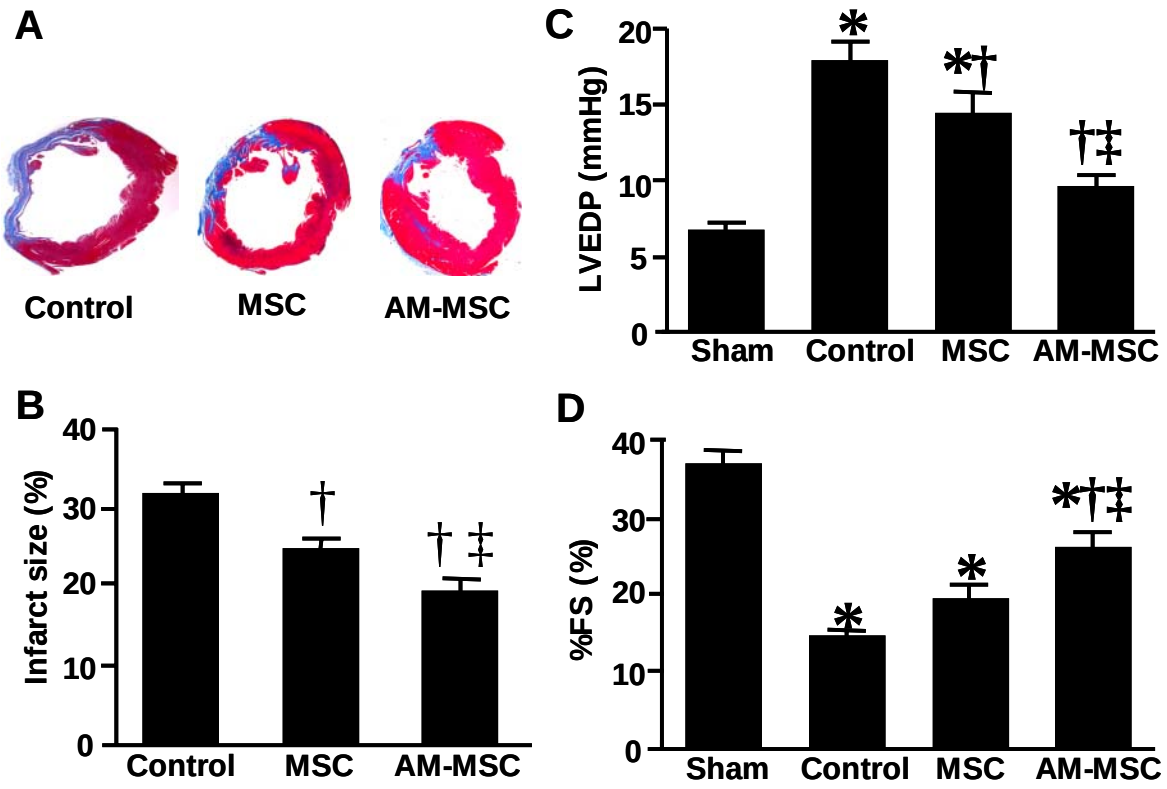


**Figure 5.** Therapeutic effects of AM-MSC transplantation on the myocardial infarct size and hemodynamics 4 weeks after coronary ligation. **(A)** Representative photographs of myocardial sections stained with Masson trichrome. **(B)** Semi-quantitative analysis of myocardial infarct size. Rats transplanted with AM-MSC showed the smallest infarct size. **(C, D)** Effects of MSC transplantation on LV end-diastolic pressure (LVEDP) and LV fractional shortening (%FS). The lowest LVEDP was observed for the AM-MSC group and the %FS was the highest among the all groups. *, $P < 0.05$ versus the Sham group. †, $P < 0.05$ versus the Control group. ‡, $P < 0.05$ versus the MSC group.

**Table 1. Physical characterization of animals used for each experimental group**

| | Sham | Control | MSC | AM-MSC |
|---|---|---|---|---|
| **Number** | 13 | 13 | 13 | 13 |
| **Body weight, g** | 307±3 a) | 290±4 * | 293±4 * | 308±3 † ‡ |
| **LV wt/body wt, g/kg body wt** | 2.25±0.05 | 2.53±0.06* | 2.39±0.05 * | 2.59±0.08* |
| **RV wt/body wt, g/kg body wt** | 0.54±0.02 | 1.05±0.09* | 0.80±0.06*† | 0.66±0.02 † |
| **Heart rate, bpm** | 412±5 | 372±8* | 395±10 † | 402±3 † |
| **Mean arterial pressure, mmHg** | 139±4 | 110±7* | 117±3* | 118±4* |

a) Means ± SEM. *, p<0.05; vs. Sham, †, p<0.05 vs. Control, ‡, p<0.05 vs. MSC.

**Table 2. Echocardiographic data of animals treated**

| | Sham | Control | MSC | AM-MSC |
|---|---|---|---|---|
| **LVDd, mm** | 6.4±0.1[a)] | 9.3±0.2* | 8.3±0.3* † | 8.3±0.3* † |
| **LVDs, mm** | 4.0±0.1 | 8.0±0.3* | 6.8±0.4*† | 6.2±0.4*† |
| **%FS, %** | 36±2 | 14±1* | 19±2* | 25±2* † ‡ |
| **AWT diastole, mm** | 1.6±0.0 | 1.1±0.0* | 1.3±0.0* † | 1.3±0.0* † |
| **PWT diastole, mm** | 1.7±0.3 | 1.8±0.1 | 1.8±0.1 | 1.7±0.0 |

a) Means ± SEM. *, $p<0.05$; vs. Sham, †, $p<0.05$ vs. Control, ‡, $p<0.05$ vs. MSC.

## DISCUSSION

The present study demonstrated that 1) transplantation of AM-MSC showed enhanced therapeutic potency for a rat model of myocardial infarction and 2) the beneficial effect was mediated by the promoted anti-apoptotic and angiogenic effects of AM-MSC.

Complexation with the spermine-dextran of non-viral vector enabled the plasmid DNA to decrease the apparent size small enough to internalize into cells and to have a positive surface charge which can interact with the cell surface of negative charge. The features promoted the transfection of plasmid DNA, enhancing the level of gene expression. The level of gene expression of MSC by the spermine-dextran-plasmid DNA complexes was significantly decreased in the presence of anti-ASGPR1 antibody. Expectedly, the ASGPR, a specific receptor for asialoglycoproteins, is expressed in MSC. Taken together, it is highly possible that the spermine-dextran-plasmid DNA complexes were internalized into the MSC at least in part via the receptor-mediated endocytosis. It should be noted that the level of gene expression of MSC by the spermine-dextran-plasmid DNA complexes was significantly higher than that of a commercially available transfection reagent, Lipofectamine$^{TM}$ 2000 (**Figure 1B**). Considering these points, the spermine-dextran is a promising non-viral vector to enhance gene expression of MSC through enhanced receptor-mediated endocytosis.

AM has multiple bioactivities to inhibit the apoptosis of cardiomyocytes and endothelial cells and to induce angiogenesis.[24,26,28-30] Transfection with the spermine-dextran-AM DNA complexes allowed MSC to secret a large amount of AM. As the

secretion mechanism, it is conceivable that the AM-MSC stimulated themselves in an autocrine fashion or the surrounding cells in a paracrine one. In fact, decreased apoptosis of AM-MSC in vitro and in vivo compared with MSC indicates that AM-MSC was relatively resistant to myocardial ischemia. Transplantation of AM-MSC greatly improved cardiac functions for rats with myocardial infarction compared with that of MSC. Considering that the conventional cell therapy is often hindered by the poor viability of the transplanted cells, this improvement in cardiac function may be mediated by increased viability of genetically engineered AM-MSC. In addition, AM-MSC markedly increased the number of capillaries in the myocardium while they significantly formed vascular structure and vWF-positive for 4 weeks after transplantation. It seemed that a very small fraction of transplanted MSC differentiated into cardiomyocytes. Taken together, AM gene-transfected MSC may have potent therapeutic effects on ischemic myocardium at least in part through anti-apoptotic and angiogenic effects of AM produced by the transplanted MSC.

There are several researches on the superior therapeutic effects of stem cells genetically engineered by viral vectors.[16-22] Mangi et al. have reported that rat MSC were genetically engineered by an ex vivo retroviral transduction to overexpress the prosurvival gene Akt1. The Akt-engineered MSC were more resistant to apoptosis and could enhance cardiac repairing after transplantation into an ischemic rat heart.[20] Intratumoral injection of epidermal growth factor receptor-MSC adenovirally engineered to secrete interferon-alpha to intracranial GL261 gliomas resulted in significantly prolonged survival in comparison with that of MSC alone.[21] These reports strongly demonstrate that genetic engineering is one of the promising technologies to positively improve the efficacy of cell therapy although the viral approach may be practically limited for clinical application. In the present study, we developed a novel genetic engineering technology for MSC by a non-viral carrier. Gene-modified MSC with the spermine-dextran showed a great therapeutic potential for myocardial infarction as compared with MSC alone. This therapeutic approach will be applicable to any gene construct to treat a variety of intractable diseases in the future.

In conclusion, a novel efficient gene delivery into MSC was developed by using a nonviral vector, the spermine-dextran. Transplantation of AM gene-engineered MSC

enhanced the therapeutic potency of MSC for a rat model of myocardial infarction, and the beneficial effect was mediated by the promoted anti-apoptotic and angiogenic effects of AM gene-modified MSC. Thus, gene-modified MSC with the spermine-dextran may have a great therapeutic potential for myocardial infarction.

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# Chapter 7

## Enhanced therapeutic efficacy of macrophages genetically engineered by cationized dextran-NK4 plasmid DNA complexes for tumor

## INTRODUCTION

Macrophages (Mϕ) play an important role in the natural surveillance system for tumor. Mϕ have an inherent ability to actively recognize the occurrence of tumor and prevent or suppress the tumor growth [1]. However, the Mϕ anti-tumor activity is not always strong enough to suppress the tumor growth. As one trial to resolve this issue, Mϕ should be activated for their anti-tumor function by any method. Since Mϕ themselves possess the inherent nature of tumor recognition, it is possible that they work as the natural carrier to target the tumor tissue, upon injecting intravenously. To provide Mϕ with an anti-tumor activity, it is one of the promising strategies to allow an anti-tumor gene to transfect. Therefore, it is necessary to develop the efficient technology of gene transfection.

Hepatocyte growth factor (HGF) has been noted as a signal molecule that plays an important role in development, morphogenesis, and regeneration of living systems [2-5]. On the other hand, for malignant tumors, HGF plays a definitive role in invasive, angiogenic, and metastatic behavior of tumor cells by way of the c-Met receptor [6-10]. Therefore, it is highly expected that the inhibition of interaction between HGF and the c-Met receptor effectively suppresses the malignant activity of tumors. Based on this concept, Date et al. [11] prepared an antagonist for HGF. The antagonist (NK4) is composed of the $NH_2$-terminal hairpin domain and the subsequent four kringle domains of α-subunit of HGF. The NK4 binds to the c-Met/HGF receptor, but does not induce the tyrosine phosphorylation of c-Met [11]. NK4 competitively inhibits some biological events driven by the HGF-Met receptor binding, including the invasion and metastasis of distinct types of tumor cells [11,12]. Moreover, NK4 has an anti-angiogenic activity which is independent of the activity as an HGF antagonist [13]. The recombinant protein of NK4 has been used for tumor animal models to demonstrate the in vivo therapeutic feasibility and the blocking effect on HGF functions [12-14]. In addition, desired antitumor effects have been reported through the stable expression of NK4 gene in tumor cells

with recombinant adenoviruses [15-19].

In this study, Mϕ were transfected by the NK4 plasmid DNA to give them a nature to secret an anti-tumor NK4 protein. Genetic engineering of Mϕ was performed by the reverse transfection with the complex of a cationized dextran and NK4 plasmid DNA. In vitro inhibition of tumor cells growth was investigated by co-culture with Mϕ genetically engineered. Following the intravenous injection into tumor-bearing mice, the in vivo anti-tumor activity of Mϕ engineered was evaluated. We examine feasibility of the reverse transfection method in the gene expression for Mϕ comparing with the conventional transfection of plasmid DNA complexed with the cationized dextran and Lipofectamine 2000 commercially available.

## EXPERIMENTAL

### Materials

Dextran with a weight-average molecular weight of 74,000 and spermine were purchased from Sigma Chemical Co., St. Louis, MO. Pronectin® (Lot #: R1-7K002-A) was kindly supplied from Sanyo Chemical Industries, Kyoto, Japan. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

### Preparation of cationized dextran derivative

Spermine was introduced to the hydroxyl groups of dextran by a N,N'-carbonyldiimidazole (CDI) activation method [20]. Spermine ($1.87 \times 10^3$ mg) and CDI ($2.25 \times 10^2$ mg) were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of dextran. Following agitation at 35 °C for 20 hr, the reaction mixture was dialyzed against ultra-pure double distilled water (DDW) for 2 days with a dialysis membrane (the cut-off molecular weight = 12,000-14,000, Viskase Companies, Inc, Willowbrook, IL). Then, the solution dialyzed was freeze-dried to obtain the spermine-introduced dextran (spermine-dextran). When determined from the conventional elemental analysis, the molar percentage of spermine introduced to the hydroxyl groups of dextran was 17.9 mole%.

**Preparation of plasmid DNA**

The plasmid DNAs used were a pCMV vector [21] coding for a firefly luciferase gene (pCMV-luciferase), a NK4 gene (pCMV-NK4), and a green fluorescent protein gene (pCMV-GFP). The plasmid DNA was propagated in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of the plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

**Preparation of polyion complexes (PIC)**

PIC were prepared by mixing an aqueous solution of spermine-dextran with that of plasmid DNA. Briefly, 4.0 μg of spermine-dextran was dissolved in 50 μl of DDW and mixed with 50 μl of phosphate-buffered saline (PBS, pH 7.4)) containing 2.5 μg of plasmid DNA, followed by leaving for 15 min at room temperature to obtain the PIC of spermine-dextran and plasmid DNA at a nitrogen number of spermine-dextran / the phosphorus number of plasmid DNA ratio of 3.0. To complex the plasmid DNA with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA), 50 μl of Opti-MEM (Invitrogen Corp., Carlsbad, CA) containing 5.0 μg of Lipofectamine was mixed with 50 μl of Opti-MEM containing 2.5 μg of plasmid DNA, followed by leaving for 15 min at room temperature.

**Preparation and culture of Mϕ**

Mouse peritoneal Mϕ were isolated from 6-week-old female BALB/c mice (body weight = 20 g, Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan) according to the conventional procedure previously reported [22]. Briefly, mice were injected aseptically with 2 ml of thioglycolate broth (BD Biosciences, San Jose, CA) intraperitoneally. At 4 days after injection, 6.0 ml of Dulbecco's modified Eagle medium (DMEM) was intraperitoneally injected to the mice, and then the peritoneal cells were collected by syringe aspiration. After centrifugation (1,000 rpm, 5 min, 4 C), pelleted peritoneal cells were resuspended in DMEM

supplemented with 15 vol% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 U/ml) and seeded on tissue culture plate (Corning, NY) at a density of $1\times10^6$ cells / $cm^2$. After incubating at 37 °C in a 5% $CO_2$, 95% air atmosphere for 2 hr, the plate was washed thoroughly with DMEM to remove non-adherent cells. The adherent cells obtained were used as Mϕ for the following experiments.

**Conventional gene transfection**

Mϕ were seeded on each well of 12-well multi-dish culture plate (Corning, NY, USA) at a density of $5\times10^5$ cells/well and cultured in 1 ml of DMEM medium with 15 vol% FCS for a week. Immediately after the medium was exchanged by the FCS-free DMEM medium, 100 μl of solution of the plasmid DNA complex with the cationized dextran or Lipofectamine 2000 or free plasmid DNA was added while the 3 hr culture was performed for gene transfection in the presence or absence of FCS. Following the complete rinsing of cells with the FCS-free medium, cells were incubated in the DMEM medium with FCS further for 1, 3, and 5 days.

**Reverse gene transfection**

Succinic anhydride (90.1 mg) was added to 20 ml of 100 mg/ml gelatin solution in DMSO, followed by agitation at room temperature for 18 hr to allow to introduce the carboxyl groups to the amino groups of gelatin for anionization. When determined as the extent of amino groups decreased by the trinitrobenzene sulfonic acid method [23], the molar amount of carboxylic groups introduced was 100 mole%.

The aqueous solution (200 μl) of the anionized gelatin (100 μg/ml) and Pronectin (200 μg/ml) was placed into each well of 12-well multi-well culture plate, followed by leaving at 37° C for 1 hr to allow them to adsorb. Following PBS washing, the well was coated with the plasmid DNA-spermine-dextran complex containing 2.5 μg of plasmid DNA. Following incubation for 30 min, every well was washed with PBS. Then, Mϕ ($5\times10^4$ cells/well) were seeded on the complex-coated well, followed by cell culture in the DMEM medium with or without 15 vol% FCS for 1, 3, and 5 days.

**Assay of gene expression**

To assay the level of gene transfection, cells were washed with PBS once, lysed in 100 μl of cell culture lysis reagent (Promega Corp., Madison, WI), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI) was added to 20 μl of supernatant while the relative light unit (RLU) of the sample was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) Protein assay kit (Pierce, Rockford, IL) according to the manufacturers' instructions. The RLU was divided by the protein amount to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently. After the reverse transfection culture with the spermine-dextran-NK4 plasmid DNA complex for different time periods, the amount of NK4 protein secreted was measured by an IMMUNIS ® HGF EIA kit (Institute of immunology Co. LTD., Tokyo, Japan) according to the manufacturers' instructions.

**Microscopic observation of intracellular localization**

To investigate the intracellular localization of plasmid DNA, the pCMV-luciferase was fluorescently labeled by using Label IT® CX-Rhodamine Labeling Kit (Mirus®, Madison, WI, USA) according to the manufacturers' instructions. After the conventional and reverse transfection cultures for different time periods with the complex of rhodamine-labeled plasmid DNA and the spermine-dextran, cells were washed and fixed with 0.25 wt% glutaraldehyde solution in PBS, followed by staining nucleus with Hoechst 33342 (Molecular Probes, Eugene, OR). Imaging data of cells were collected on an Olympus Fluoview FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan) and processed with Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA).

**In vitro anti-tumor activity of Mϕ genetically engineered**

The in vitro anti-tumor activity of Mϕ was evaluated in terms of their suppression activity of tumor cells growth. Briefly, Mϕ ($1\times10^6$ cells) were genetically engineered by the reverse transfection culture with the complex of spermine-dextran-NK4 plasmid DNA in 6 well multi-culture plate (COSTAR®, Corning Inc, NY) as described above. Meth-A cells ($1\times10^5$ cells) were added to the Mϕ culture 3 hr later. After co-culture for 1, 3, and 6 days, the number of cells grown was counted. The experiment of each sample was performed for three wells. As controls, tumor cells were cultured with non-transfected, original Mϕ ($1\times10^6$ cells) or without Mϕ and the number was counted similarly.

**In vivo therapeutic assay of Mϕ genetically engineered**

Meth-A cells ($1\times10^6$cells/mouse) suspended in 200 μl of PBS were subcutaneously inoculated into the back of BALB/C mice. Mϕ were genetically engineered by the reverse transfection with the complex of spermine-dextran-NK4 plasmid DNA as described previously. When the tumor was grown to be the longest and the widest diameter of about 10 mm, tumor therapy with Mϕ genetically engineered was started. Mϕ genetically engineered were taken off with a rubber policeman, washed twice, and resuspended in PBS. Then, 100 μl of Mϕ ($5\times10^6$ cells) suspended in PBS was intravenously injected via the tail vein of tumor-bearing mice. At different time periods after intravenous injection, the longest and widest diameter of tumor mass were measured with a caliper and the tumor volume was calculated by using the formula of (length) ×(width) $^2\times1/2$ $^{24}$. As controls, saline or non-transfected, original Mϕ was injected similarly.

**Statistical Analysis**

Data were expressed as the mean±the standard deviation of the mean. Data were analyzed by two-way ANOVA, and then differences among means were analyzed using Fisher's PLSD multiple comparison test and significance was accepted at $p < 0.05$.

# RESULTS

## Gene expression by spermine-dextran-plasmid DNA complexes

**Figure 1** shows the level of gene expression of spermine-dextran-luciferase plasmid DNA complexes for Mϕ by the conventional and reverse methods. For the conventional transfection method, the expression level by the spermine-dextran-plasmid DNA complex was significantly higher than that of plasmid DNA with or without the Lipofectamine 2000 complex. In the presence of FCS, the level by the reverse transfection method was significantly higher than that by the conventional one.

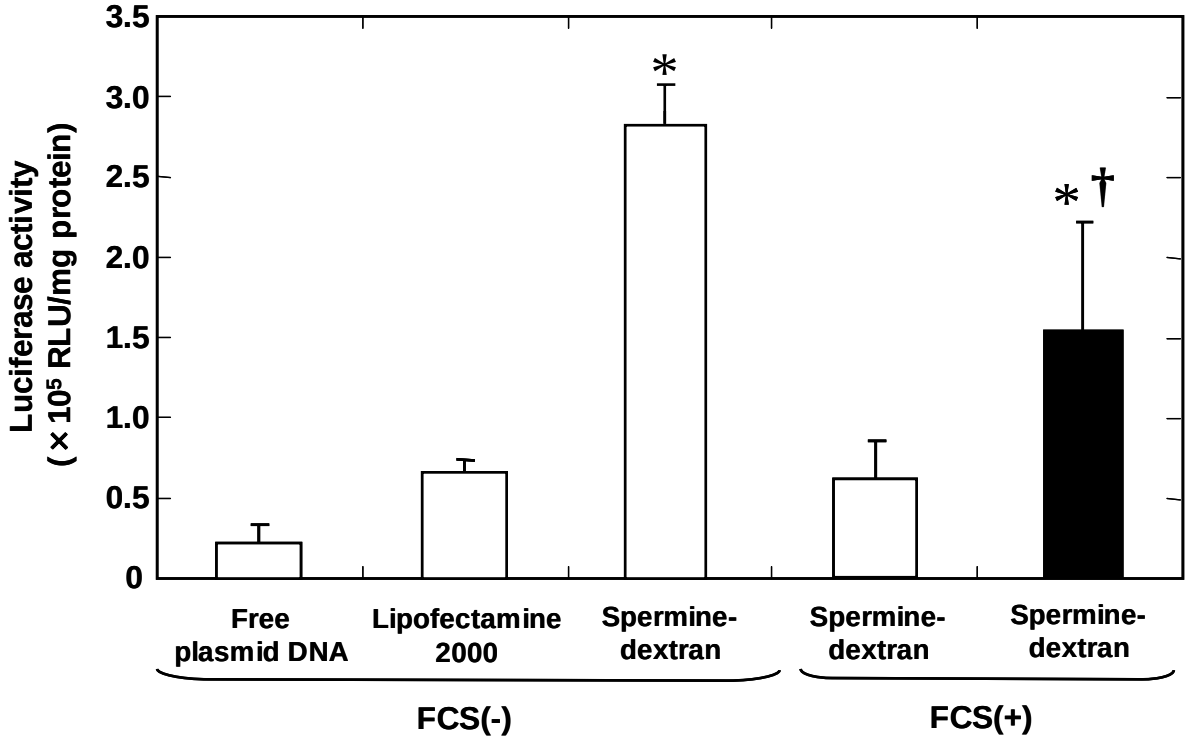


**Figure 1.** The level of luciferase expression for Mϕ 2 days after transfection of luciferase plasmid DNA with the spermine-pullulan by the conventional (open bars) and reverse methods (closed bars). The cells were cultured in the presence (FCS(+)) or absence of FCS (FCS(-)). *, $p<0.05$; versus the expression level of free plasmid DNA.
†, $p<0.05$; versus the expression level of spermine-dextran-plasmid DNA complexes by the conventional method (FCS(+)).

**Figure 2** shows the time course of gene expression level of spermine-dextran-luciferase plasmid DNA complexes for Mϕ by the conventional and reverse methods. The level of gene expression by the reverse method of spermine-dextran complexes was high and lasted for a long time period compared with that by the conventional one.

**Figure 3** shows the confocal microscopic images of Mϕ at different time points after transfection with spermine-dextran-plasmid DNA complexes by the conventional and reverse methods. When transfected by the reverse method, the plasmid DNA was localized in the cell nucleus at higher amounts even at a shorter time point than that by the conventional method while the nucleus localization lasted for longer time periods.

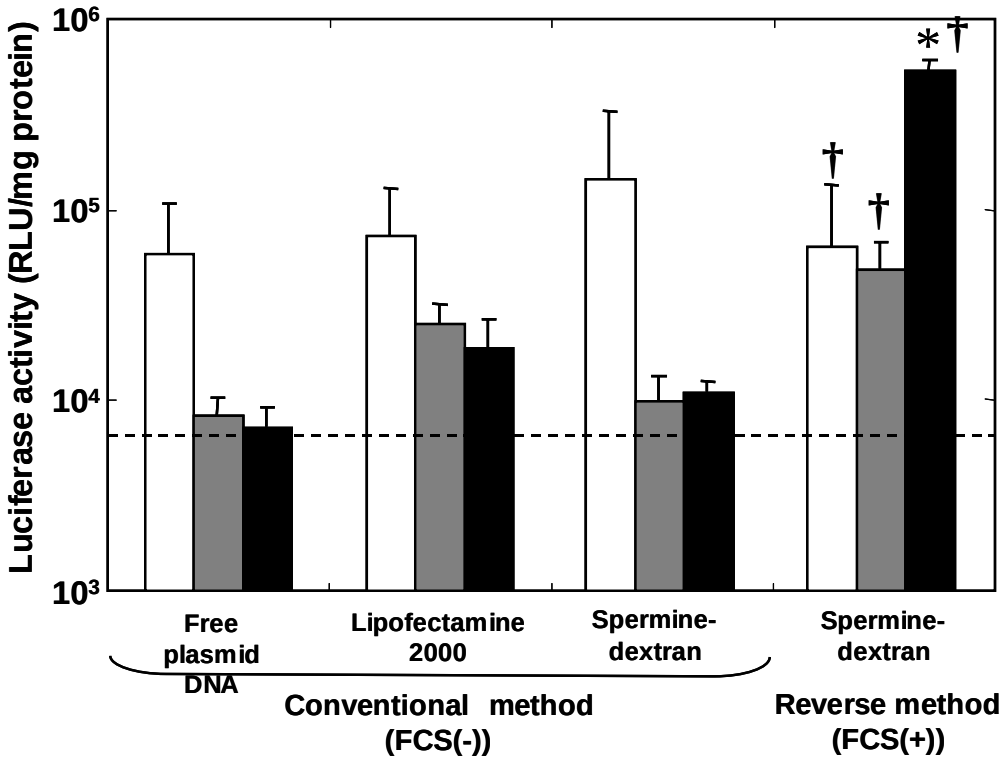


**Figure 2.** Time course of luciferase expression level for Mϕ after transfection of luciferase plasmid DNA with the spermine-dextran by the conventional and reverse methods. The cells were cultured for 2 (open bars), 4 (light gray bars), and 6 (solid bars) days in the presence (FCS(+)) or absence of FCS (FCS(-)). The dotted line indicates the level of non-transfected, original Mϕ. ***,** $p<0.05$; versus the expression level of spermine-dextran-plasmid DNA complexes by the conventional method at the corresponding time. †, $p<0.05$; versus the expression level of free plasmid DNA at the corresponding time.

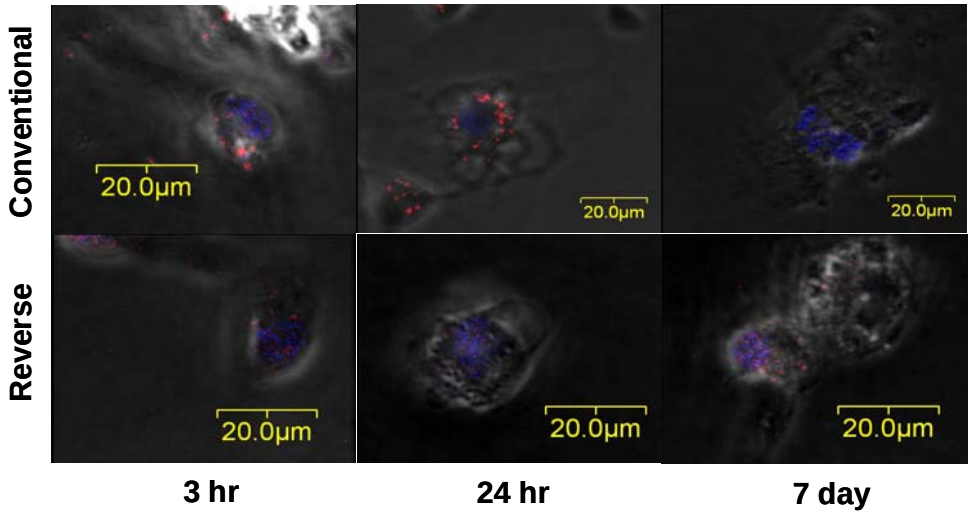


**Figure 3.** Confocal laser microscopic images of Mϕ 3 and 24 hr or 7 days after transfection of luciferase plasmid DNA with the spermine-dextran by the conventional and reverse methods. The red and blue points indicate the plasmid DNA and cell nucleus, respectively.

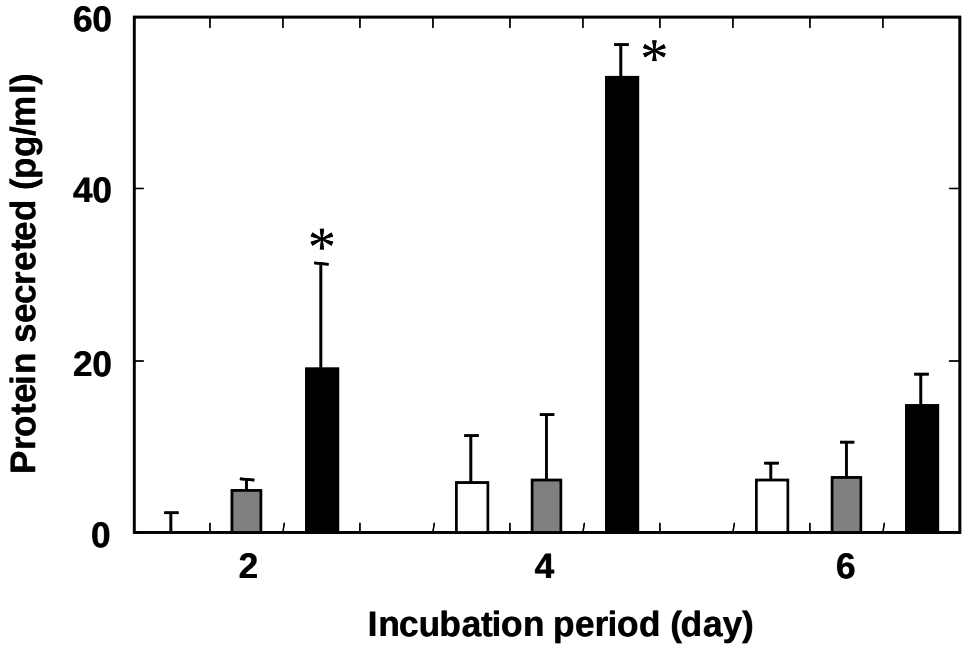


**Figure 4.** Time course of NK4 secretion of non-transfected, control Mϕ (open bars) or those transfected with free NK4 plasmid DNA by the conventional method (light gray bars), and the spermine-dextran-NK4 plasmid DNA complex by the reverse method (solid bars). *, $p<0.05$; versus the expression level of other groups at the corresponding time.

**In vitro anti-tumor properties of Mϕ genetically engineered by spermine-dextran-NK4 plasmid DNA complexes**

**Figure 4** shows the time course of NK4 secretion of Mϕ transfected with spermine-dextran-plasmid DNA complexes by the conventional and reverse methods. When transfected with the spermine-dextran-plasmid DNA complexes by the reverse method, Mϕ secreted NK4 protein at significantly higher amounts even 2 days after transfection than those of non-transfected or transfected with free NK4 plasmid DNA. Moreover, the NK4 protein secretion lasted for longer time periods.

**Figure 5** shows the in vitro anti-tumor activity of Mϕ transfected by the reverse method of spermine-dextran-NK4 plasmid DNA complexes. Mϕ genetically engineered by the complex suppressed the in vitro proliferation of Meth-A cells to a significantly great extent compared with non-engineered naïve cells.

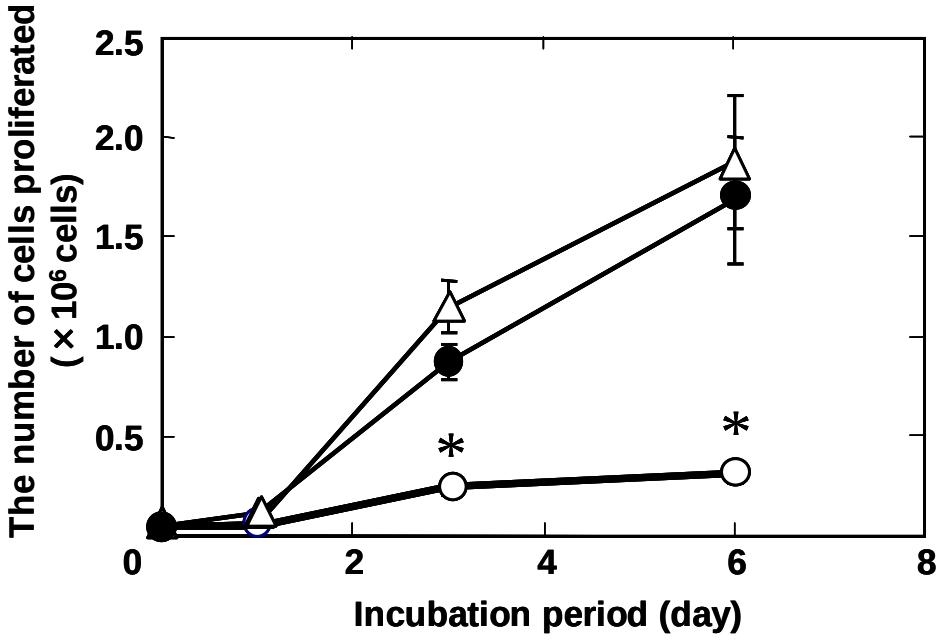


**Figure 5.** Proliferation profiles of Meth-A cells cultured with (open marks) or without Mϕ (solid marks). The cells were transfected with the spermine-dextran-NK4 plasmid DNA complex by the reverse method (○) and not transfected (△). *****, $p<0.05$; versus the cell number of other groups at the corresponding time.

**In vivo anti-tumor activities of Mϕ genetically engineered by the reverse transfection method with spermine-dextran-NK4 plasmid DNA complexes**

**Figure 6** shows the fluorescent microscopic image of tumor tissue 1 day after intravenous injection of GFP-transfected Mϕ to tumor-bearing mice. It is apparent that Mϕ intravenously injected accumulated in the tumor tissue.

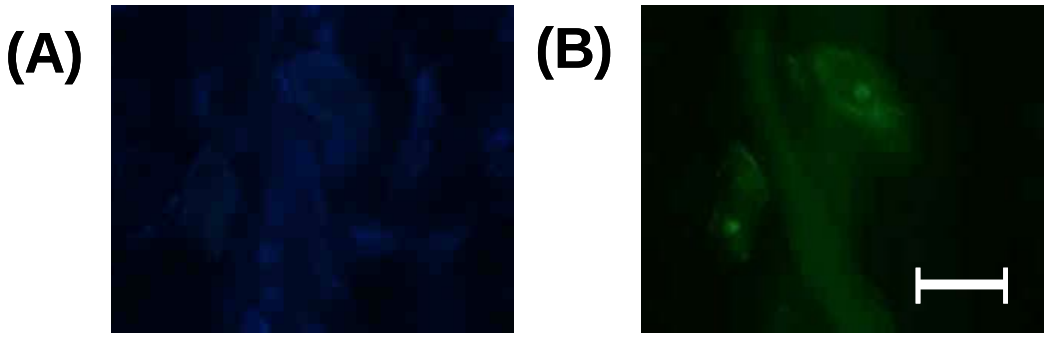


**Figure 6.** Fluorescence microscopic images of tumor tissue 1 day after intravenous injection of GFP-transfected Mϕ to tumor-bearing mice: (A) nucleus staining and (B) Mϕ GFP. The bar length is 20 μm.

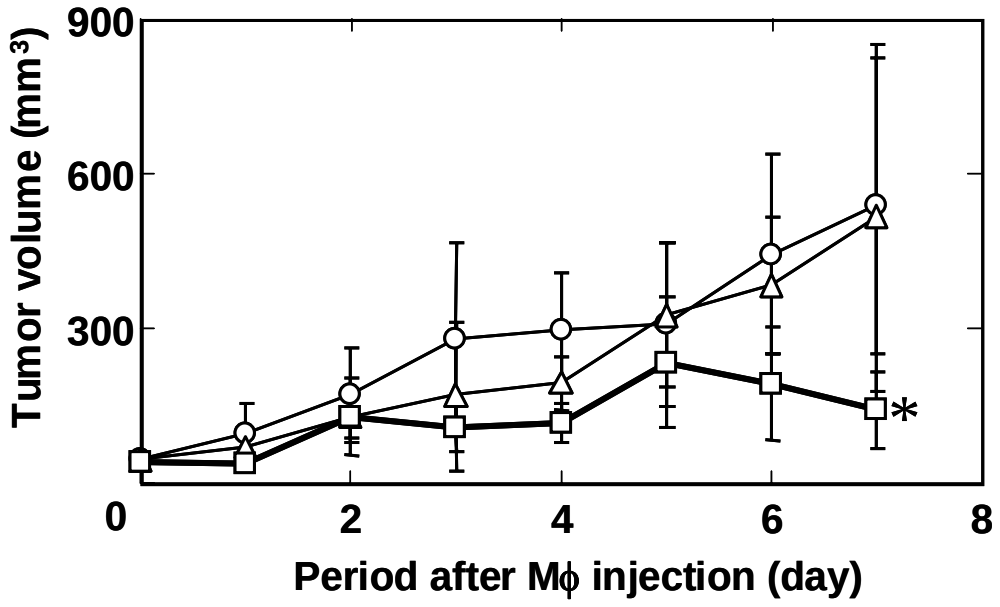


**Figure 7.** Time course of tumor volume change after intravenous injection of saline (○), non-transfected, original Mϕ (△), and Mϕ transfected with the spermine-dextran-NK4 plasmid DNA by the reverse method (□). *****, $p<0.05$ versus the tumor volume of other groups at the corresponding time.

**Figure 7** shows the in vivo anti-tumor activity of Mϕ transfected by the reverse method of spermine-dextran-NK4 plasmid DNA complexes. The intravenous injection of Mϕ transfected with the spermine-dextran-NK4 plasmid DNA complexes by the reverse method tended to suppress the in vivo growth of tumor cells even at the early time period and exhibited significantly higher anti-tumor effect in vivo than that of non-transfected Mϕ.

## DISCUSSION

The present study clearly demonstrates that the delivery of NK4 plasmid DNA based on the natural potential of Mϕ to recognize the tumor tissue was effective in the in vivo anti-tumor therapy. This is the first report to show the tumor targetable therapy of Mϕ genetically engineered. This idea to therapeutically treat diseases by making use of the natural homing ability of cells, so-called gene-cell hybrid therapy, has been reported to demonstrate their therapeutic efficacy [25-33]. With the recent advent of biological and medical researches regarding cells, various types of cells have been available, and consequently cell therapy will get more and more popular.

The efficacy of reverse transfection in gene expression was also confirmed in this study for the luciferase (**Figures 1 and 2**) and NK4 plasmid DNAs (**Figure 4**). The higher efficiency of gene transfection can be explained in terms of the unique feature of reverse method. Mϕ are always cultured on the complex of plasmid DNA and spermine-dextran of non-viral carrier. It is likely that the localization of plasmid DNA close to cells enhances the possibility of cellular internalization of plasmid DNA, resulting in increased efficiency of gene transfection. In addition, as described in Chapter 5, the transfection culture can be performed in the presence of serum, which is better conditions for cell culture. It is apparent from **Figure 3** that the plasmid DNA was localized in the nucleus of cells at higher amounts and for longer time periods. This may be also due to the efficient and continuous supply of plasmid DNA to cells.

Mϕ were targeted to the tumor tissue following their intravenous injection into mice carrying a tumor mass (**Figure 6**). This is due to their natural tumor targetability. Mϕ genetically engineered showed the anti-tumor activity (**Figure 5**). It is possible to consider

several mechanisms of macrophages to kill tumor cells. First, macrophages transfected secreted anti-tumor NK4 proteins to directly kill Meth-A cells. Secondly, it is well known that macrophages naturally function as an effector cell to injure tumor cells by the secretion of various anti-tumor cytokines and the direct cell contact [34]. This inherent nature of macrophages would result in the anti-tumor effect. Taken together, it is likely that after intravenously injected, Mϕ genetically activated by the NK4 plasmid DNA of anti-tumor effect are naturally homed to the tumor tissue, resulting in the local and continuous secretion of NK4 protein at the tumor tissue to kill tumor cells. The amount of NK4 protein secreted increased with time up to 4 days. Thus, it is conceivable that this delayed secretion did not suppress the in vivo tumor growth at the early time period after cells injection.

Since Mϕ have an inherent ability to recognize and home to the site of inflammation and diseases other than the tumor tissue, the natural targeting by Mϕ as a drug carrier will be therapeutically available to various applications. This approach will be promising cell therapy of next generation which can overcome the present problems to be resolved.

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# Chapter 8

# Enhanced therapeutic efficacy of dendritic cells genetically engineered by cationized dextran-interleukin 12 plasmid DNA complexes for tumor

## INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells, which possess a capacity to generate primary immune responses [1]. After the antigen uptake, DC process these antigens into small peptides and move into secondary lymphoid organs to present the antigenic peptides to lymphocyte for their immunological activation [2-4]. For the activation, DC are biologically matured and obtain immunological responsibility, which express high levels of cell-surface major histocompatibility complex antigen complexes and costimulatory molecules. The responding lymphocytes include naïve T cells, helper $CD4^+$ T cells, cytolytic $CD8^+$ T cells, naïve B cells, memory B cells, natural killer cells, and natural killer T cells, all of which are critical elements of immunological responses. DC play a central role in the natural immunization. Therefore, because of the function of DC, DC generated *ex vivo* are highly expected to be a promising cell suitable for vaccination in cancer and infectious diseases [5]. However, the immune response of DC is not strong enough for vaccination in vigorous tumors. It is necessary for successful DC-based tumor vaccination to activate the immunological function for anti-tumor response.

Interleukin-12 (IL-12) is a heterodimeric cytokine produced by DC, macrophages, polymorphonuclear leukocytes, and keratinocytes [6]. The anti-tumor effect of DC genetically modified with an IL-12 gene was examined in mouse tumor models [7]. IL-12 enhances the anti-tumor effect of natural killer cells and CTL activities, plays a key role in the induction of Th1-type immune responses including interferon gamma (IFN-γ) production, and has IFN-γ/IFN-inducible protein 10-dependent antiangiogenic effects [8]. It has been reported that following intratumoral injection, bone marrow-derived DC genetically modified with an IL-12 gene enhanced their uptaking tumor antigens at the tumor site and moved into secondary lymphoid organs to induce an immune response against the tumor [9]. Intratumoral

injection of IL-12-engineeded DC suppressed the in vivo growth of tumors established and induced a strong anti-tumor T-cell response [9]. However, in the researches reported, retrovirus or adenovirus vectors have been used for genetic engineering of DC.

The objective of this chapter is to investigate the gene transfection of DC for the genetic engineering by the spermine-dextran of non-viral carrier and compared with that of other transfection reagents commercially available. Moreover, gene transfection by the reverse transfection method with the spermine-dextran-plasmid DNA complex was carried out to enhance gene transfection efficiency [10]. DC genetically engineered by the complex of mouse interleukin 12 (mIL-12) plasmid DNA and spermine-dextran were intratumorally injected to tumor-bearing mice to evaluate the in vivo anti-tumor effect. The IFNγ production of spleen cells isolated from tumor-bearing mice injected with DC engineered and the anti-tumor activity were investigated.

## EXPERIMENTAL

### Materials

Dextran with a weight-average molecular weight of 74,000 and spermine were purchased from Sigma Chemical Co., St. Louis, MO. Pronectin® (Lot #: R1-7K002-A) was kindly supplied from Sanyo Chemical Industries, Kyoto, Japan. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification

### Preparation of cationized dextran derivatives

Spermine was introduced to the hydroxyl groups of dextran by a N,N'-carbonyldiimidazole (CDI) activation method [11]. Spermine (1870 mg) and CDI (225 mg) were added to 50 ml of dehydrated dimethyl sulfoxide (DMSO) containing 50 mg of dextran. Following agitation at 35 °C for 20 hr, the reaction mixture was dialyzed against ultra-pure double distilled water (DDW) for 2 days with a dialysis membrane (the cut-off molecular weight = 12,000-14,000, Viskase Companies, Inc, Willowbrook, IL). Then, the solution dialyzed was freeze-dried to obtain the spermine-introduced dextran (spermine-dextran). When determined from the conventional elemental analysis, the molar

percentage of spermine introduced to the hydroxyl groups of dextran was 12.7 mole%.

**Preparation of plasmid DNA**

The plasmid DNAs used were the pGL3 plasmid coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, Madison, WI), the pEGFP-N1 plasmid coding enhanced green fluorescent protein (GFP, Clontech Laboratory Inc., Shiga, Japan), and the pGEG. mIL-12 plasmid coding for a bicistronic expression cassette for murine IL-12 p35 and p40 genes [12], which was kindly supplied by Dr. Mazda, Kyoto Prefectural University of Medicine, Kyoto. The plasmid DNA was propagated in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

**Preparation of polyion complexes (PIC)**

PIC were prepared by mixing an aqueous solution of spermine-dextran with that of plasmid DNA. Briefly, various amounts of spermine-dextran were dissolved in 50 μl of DDW and mixed with 50 μl of phosphate-buffered saline (PBS, pH 7.4) containing 2.5 μg of plasmid DNA, followed by leaving for 15 min at room temperature to obtain the PIC of spermine-dextran and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio. To complex the plasmid DNA with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA), 50 μl of Opti-MEM (Invitrogen Corp., Carlsbad, CA) containing 5.0 μg of Lipofectamine was mixed with 50 μl of Opti-MEM containing 2.5 μg of plasmid DNA, followed by leaving for 15 min at room temperature.

**Preparation and culture of DC**

Mouse immature DC were obtained from bone marrow cells (BMC) through the differentiation induction according to the conventional procedure previously reported [13]. Briefly, mouse BMC were harvested from the femur and tibia of 4-week-old female ICR mice or 6-week-old female C57BL/6 mice (body weight = 20 g, Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan). Erythrocytes contaminated in BMC were lysed with ammonium chloride solution and cells were seeded in RPMI-1640 (Sigma-aldrich Inc., St. Louis, MO) supplemented with penicillin (100 U/ml, Sigma-aldrich Inc., St. Louis, MO), streptmycin (100 ug/ml, Sigma-aldrich Inc., St. Louis, MO), 10 vol% heat-inactivated fetal calf serum (FCS) (Hyclone laboratories, Inc., Utah, USA) and 200 U/ml recombinant mouse-derived granulocyte macrophage colony-stimulating factor (rmGM-CSF, 1 x $10^7$ U/mg, Peprotech/Tebu, Frankfurt, Germany) on day 0. The medium was changed every 2 days thereafter. The cells cultured for 9-14 days were used for all experiments as mouse immature DC.

**Conventional gene transfection**

DC were seeded on each well of a 12 well multi-dish culture plate (Corning Inc., Corning, NY) at a density of 2.5 x $10^5$ cells/well and cultured in 1 ml of RPMI-1640 medium with 10 vol% FCS and 50 U/ml rmGM-CSF for 24 hr. Immediately after the medium was exchanged by FCS and rmGM-CSF-free RPMI-1640 medium, 100 μl of PIC solution containing 2.5 μg of plasmid DNA at different N/P ratios was added, and transfection culture was performed for 3hr. After the medium was changed to RPMI-1640 with 10 vol% FCS and rmGM-CSF, cells were incubated further for 1, 4, and 7 days.

**Reverse gene transfection**

In vitro gene transfection by the reverse method was performed according to the method described previously [10]. Briefly, succinic anhydride (90.1 mg) was added to 20 ml of 100 mg/ml gelatin solution in DMSO, followed by agitation at room temperature for 18 hr to allow to introduce the carboxyl groups to the amino groups of gelatin for anionization. When

determined as the extent of amino groups decreased by the trinitrobenzene sulfonic acid method [14], the molar amount of carboxylic groups introduced was 100 mole%. The aqueous solution of anionized gelatin (100 μg/ml) and Pronectin (200 μg/ml) was placed into each well of 12-well multi dish culture plate and left at 37 °C for 1 hr for coating. After PBS washing, the well was coated with the polyion complex solution containing 2.5 μg of plasmid DNA at an N/P ratio of 3. After 30 min incubation, the wells were washed with PBS. Then, DC (2.5 x $10^5$ cells/well) were seeded on the complex-coated well, followed by cell culture in the RPMI-1640 medium with 10 vol% FCS and 50 U/ml rmGM-CSF for 2, 5, and 8 days.

**Assay of gene expression**

To assay the level of luciferase expression, cells transfected were washed with PBS once, lysed in 100 μl of cell culture lysis reagent (Promega Corp., Madison, WI), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI) was added to 20 μl of supernatant while the RLU of the sample was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) Protein assay kit (Pierce, Rockford, IL) according to the manufacturers' instructions. The relative light unit (RLU) was divided by the protein amount to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently.

To assay GFP expression efficacy, cells transfected were washed with PBS twice and detached by trypsinization. After centrifugation (1,000 rpm, 5 min), the cells were resuspended and washed with PBS twice. Cells (1 x $10^4$) were measured by fluorescence activated cell sorter Calibur with Cell Quest Pro Software (FACSCalibur, Becton, Dickinson & Co. Biosciences, San Jose, CA). The percentage of positive cells was calculated by setting the background population as 98 % negative when analyzing control cells[15].

After the reverse transfection culture with the spermine-dextran-mIL-12 plasmid DNA complex for different time periods, the amount of mIL-12 produced were measured by the mIL-12 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturers'

instructions.

**Evaluation of plasmid DNA internalization into cells**

Plasmid DNA was labeled with Cy5 by using Label IT® Cy5 Labeling Kit (Mirus Bio Co., Madison, WI). The Cy5-labeled plasmid DNA was complexed with spemine-dextran. The cells were treated in cold acetate buffer containing 0.3 vol% acetic acid, 0.085 M NaCl, and 5.0 mM KCl for 1 min, washed with cold PBS to remove the complexes bound to the cell surface. The cells were collected by the trypsinization, then ten thousand cells were measured by FACSCalibur flow cytometer. The percentage of Cy5-positive cells was calculated by setting the background population as 98% negative when analyzing control cells.

**Microscopic observation of intracellular localization**

After the conventional and reverse transfection cultures for 1 day with the complex of Cy5-labeled plasmid DNA and the spermine-dextran, cells were washed and fixed with 0.25 wt% glutaraldehyde solution in PBS, followed by staining nucleus with Hoechst 33342 (Molecular Probes, Eugene, OR). Imaging data of cells were collected on an Olympus Fluoview FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan) and processed with Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA).

**Cell viability**

Cytotoxicity after gene transfection was assayed using a cell counting kit (Nacalai tesque Inc., Kyoto, Japan). The medium was changed to RPMI-1640 with FCS and rmGM-CSF, and 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) solution was added and the cells incubated for another 4 hr. The absorbance of samples was measured at 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale CA). The percentage cell viability was expressed as 100 % for non-transfected, control cells.

**Evaluation of anti-tumor effect**

B16 melanoma cells ($1\times10^5$cells/mouse) suspended in 200 μl of PBS were subcutaneously inoculated into the right flank of C57BL/6 mice on day 0. DC were genetically engineered by the reverse transfection with the complexes of spermine-dextran-mIL-12 plasmid DNA as described previously. On day 9, when the tumor size reached approximately 0.10–0.05 $cm^3$, tumor therapy with DC genetically engineered was started. DC genetically engineered were taken off with a rubber policeman, washed twice, and resuspended in PBS. Then, 100 μl of DC ($1\times10^6$ cells) suspended in PBS was intratumorally injected. At different time periods after intratumoral injection, the longest and widest diameter of tumor mass were measured with a caliper and the tumor volume was calculated by using the formula of (length) ×(width) $^2\times1/2$ $^{16}$. As controls, PBS, non-transfected, original DC or those transfected with spermine-dextran-GFP plasmid DNA complex by the reverse method was injected similarly.

**In vitro cytokine release assays**

Splenocytes were obtained from the spleen harvested from tumor-bearing mice 7 days after intratumoral injection with PBS, original DC or those transfected with the complex of spermine-dextran and GFP or mIL-12 plasmid DNA by the reverse method. Briefly, the spleen taken was pressed through a sterile gauze, rinsed twice. These cells (1 x $10^6$ cells) were co-cultured in 100 mm-dish (Corning, Inc., Corning, NY) with mytomycin C (Sigma)-treated B16 cells (1 x $10^6$ cells) in culture medium containing 25 U/ml recombinant mouse IL-2 (2.5 x $10^6$ U/mg; Prospec, Rehovot, Israel) for 6 days. Then, the culture medium was collected and measured the amount of interferon-γ (IFN-γ) by mouse IFN-γ (R & D Systems) ELISA kits according to the manufacturers' instructions.

**Measurement of cytotoxic T-lymphocyte (CTL) activity**

Splenocytes harvested as described above (1 x $10^6$ cells) were stimulated in 100 mm-dish with mytomycin C-treated B16 cells (1 x $10^6$ cells) in culture medium containing 25 U/ml recombinant mouse IL-2 for 2 days. Then, the cells stimulated were used as effectors for

measurement of CTL activity. B16 (target) cells (5 x $10^4$ cells each) were cultured in 96 well multi-dish culture plate (Corning Inc., Corning, NY) for 24h. After washing twice, various number of effector cells were added to the plate. After 4 hr co-culture, the supernatant (50 μl) was collected, and the LDH activity was measured by LDH-Cytotxic Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The CTL activity was expressed as the number percentage of target cells injured by splenocytes to those completely injured by adding 0.2 vol% tween 20.

**Evaluation of in vivo tumor immunity**

B16 cells ($1\times10^5$cells/mouse) suspended in 200 μl of PBS were subcutaneously inoculated into the both flanks of C57BL/6 mice on day 0. DC were genetically engineered by the reverse transfection with the complexes of spermine-dextran-mIL-12 plasmid DNA as described previously. On day 9, 100 μl of DC ($1\times10^6$ cells) suspended in PBS was injected into the tumor mass grown in the right flank of the mice. At 29 days after intratumoral injection, each tumor volume in the both flanks was calculated as described above. As controls, PBS, non-transfected, original DC or those transfected with spermine-dextran-GFP plasmid DNA complex by the reverse method was injected similarly

**Statistical analysis**

Numerical values were expressed as means±standard errors of the mean unless otherwise indicated. Comparisons of parameters between the 4 groups were made using one-way analysis of variance, followed by the Newman-Keul's multiple comparison test. $P<0.05$ was considered to be significant.

# RESULTS

## Gene expression by spermine-dextran-plasmid DNA complexes

**Figure 1** shows the level of luciferase expression for DC 1 day after transfection of luciferase plasmid DNA complexed with the spermine-dextran at different N/P ratios by the conventional and reverse method. In the conventional method, the level of gene expression by the spermine-dextran-plasmid DNA complexes at an N/P ratio of 3.0 was significantly higher than those of free plasmid DNA and the plasmid DNA complexes of Lipofectamine 2000 or the spermine-dextrans at other N/P ratios (**Figure 1A**). On the other hand, the expression level by the reverse transfection method was significantly higher than that of the conventional one (**Figure 1B**).

**Figure 2** shows the time course of percent internalized of GFP plasmid DNA and GFP expression for DC transfected with free plasmid DNA and spermine-dextran-plasmid DNA complexes by the conventional and reverse methods. The plasmid DNA in the free form could be hardly internalized while little gene expression was observed. For the plasmid DNA in the complex form, there was no difference in the time course of plasmid DNA internalized into DC different between the conventional and reverse methods, while the percentage of plasmid DNA internalized was significantly higher for the reverse method than for the conventional one (**Figure 2A**). The level of GFP expressed by the conventional method decreased with culture time, whereas it was retained at a high value for the reverse method (**Figure 2B**).

**Figure 3** shows the confocal laser microscopic images of DC 1 day after transfection with free plasmid DNA and spermine-dextran-plasmid DNA complexes by the conventional and reverse methods. The plasmid DNA in the free form was hardly internalized into DC. The number of plasmid DNA internalized by DC for the reverse method tended to be larger than that of the conventional one.

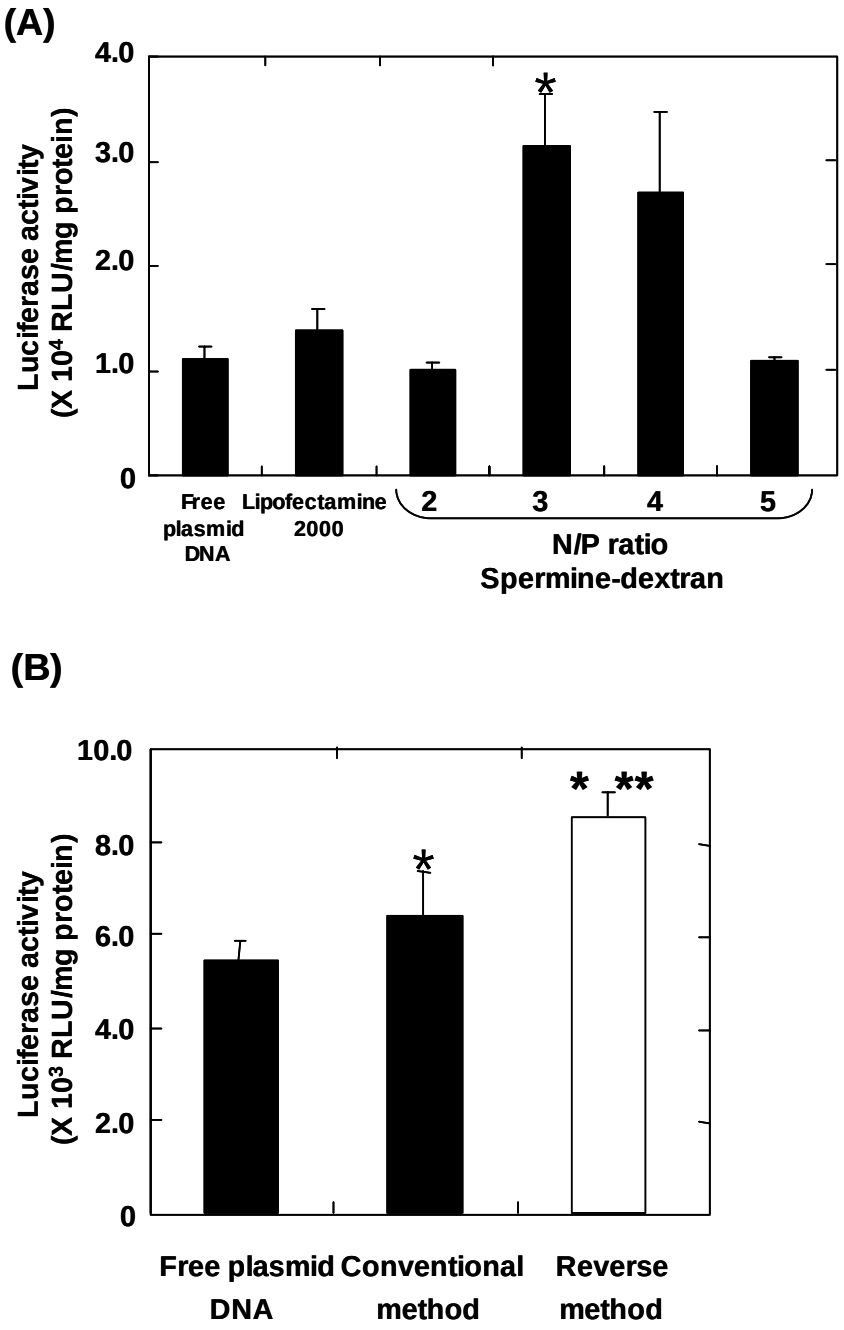



**Figure 1.** (A) The level of luciferase expression for DC 1 day after transfection of luciferase plasmid DNA complexed with the spermine-dextran at different N/P ratios by the conventional method. As controls, free plasmid DNA and plasmid DNA complexes with Lipofectamine 2000 are used. The amount of plasmid DNA used for transfection is 2.5 μg. (B) The level of luciferase expression for DC 1 day after transfection of luciferase plasmid DNA complexed with the spermine-dextran at a N/P ratio of 3.0 by the conventional (open column) and reverse methods (black columns). As control, free plasmid DNA is used. The amount of plasmid used for transfection is 2.5 μg. *, $p < 0.05$; versus the expression level of DC transfected with free plasmid DNA. **, $p < 0.05$; versus the expression level of DC transfected with the spermine-dextran-plasmid DNA complex by the conventional method.

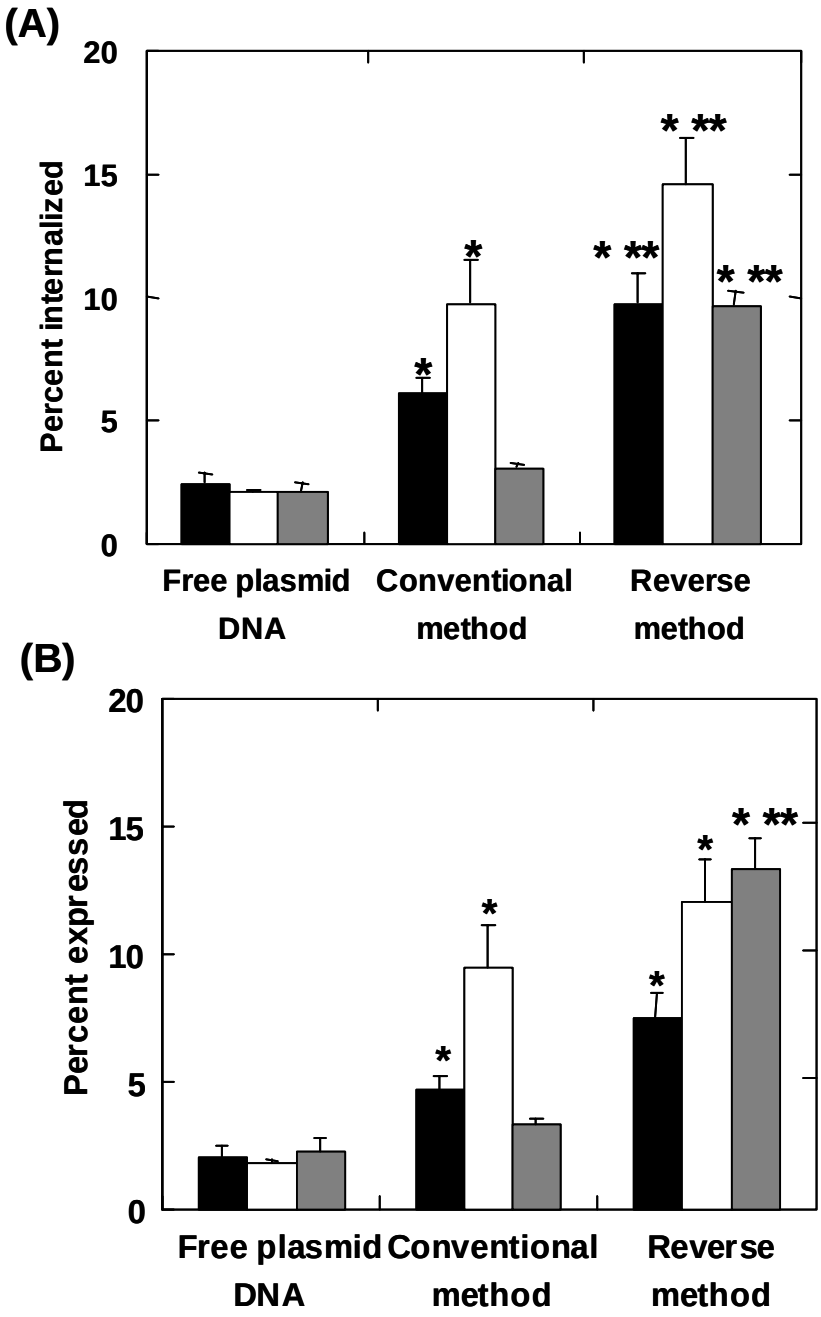



**Figure 2.** Time course of percent GFP plasmid DNA internalized (A) and GFP expression for DC (B) transfected with free plasmid DNA and spermine-dextran-plasmid DNA complexes by the conventional and reverse methods. The cells were cultured for 1 (black columns), 4 (open columns), and 7 days (gray columns). The amount of plasmid DNA used for transfection is 2.5 μg. The N/P ratio of spermine-dextran-plasmid DNA complex is 3.0. *, $p < 0.05$; versus the percent GFP plasmid DNA internalized or GFP expression for DC transfected with free plasmid DNA at the corresponding time. **, $p < 0.05$; versus the percent GFP plasmid DNA internalized or GFP expression for DC transfected with with the spermine-dextran-plasmid DNA complex by the conventional method at the corresponding time.

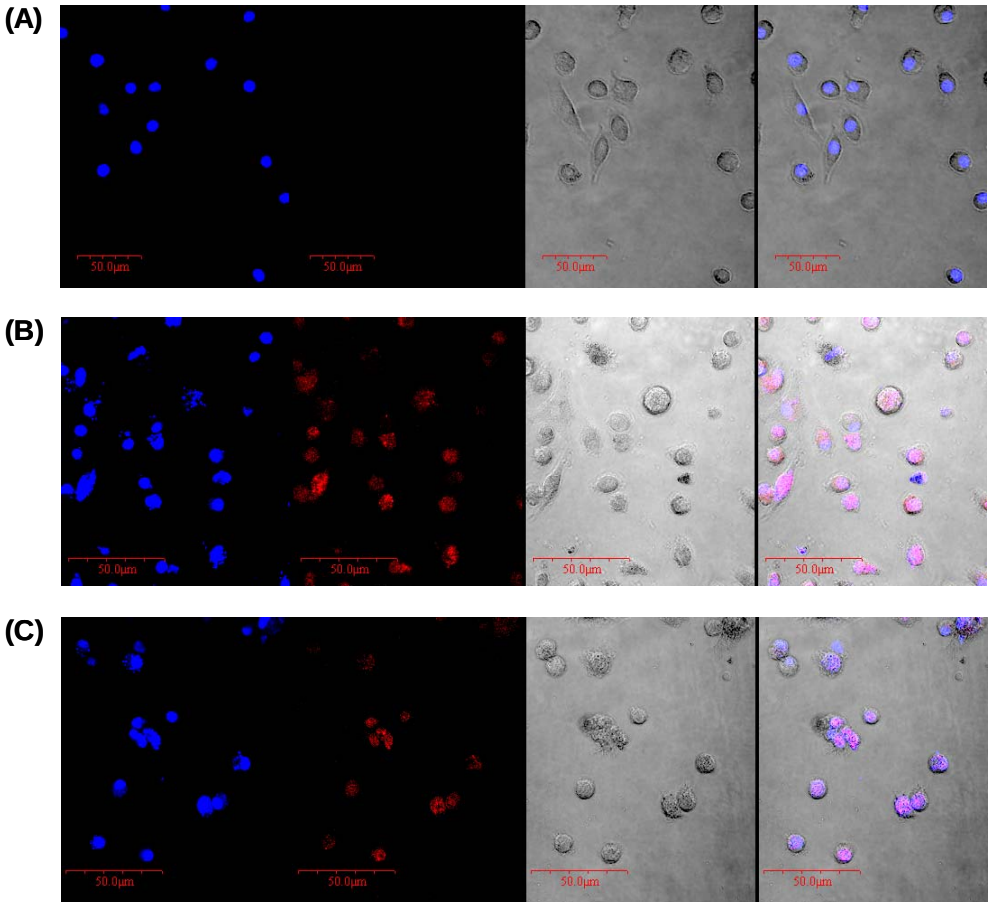



**Figure 3.** Confocal laser microscopic images of DC 1 day after transfection with free plasmid DNA (A) and spermine-dextran-plasmid DNA complexes by the conventional (B) and reverse methods (C). The plasmid DNA was labeled with Cy5. The amount of plasmid DNA used for transfection is 2.5 μg. The N/P ratio of spermine-dextran-plasmid DNA complex is 3.0. The red and blue points indicate the plasmid DNA and cell nucleus, respectively.

**Viability of DC transfected by the conventional and reverse methods**

**Figure 4** shows the viability of DC 1 day after transfection with free plasmid DNA, plasmid DNA-Lipofectamine 2000 complexes, and spermine-dextran-plasmid DNA complexes by the conventional and reverse methods. The cell viability decreased significantly by the conventional transfection method with the spermine-dextran and Lipofectamine 2000 complex with plasmid DNA. On the contrary, the viability after the reverse transfection culture was similar to that of non-transfected, original cells.

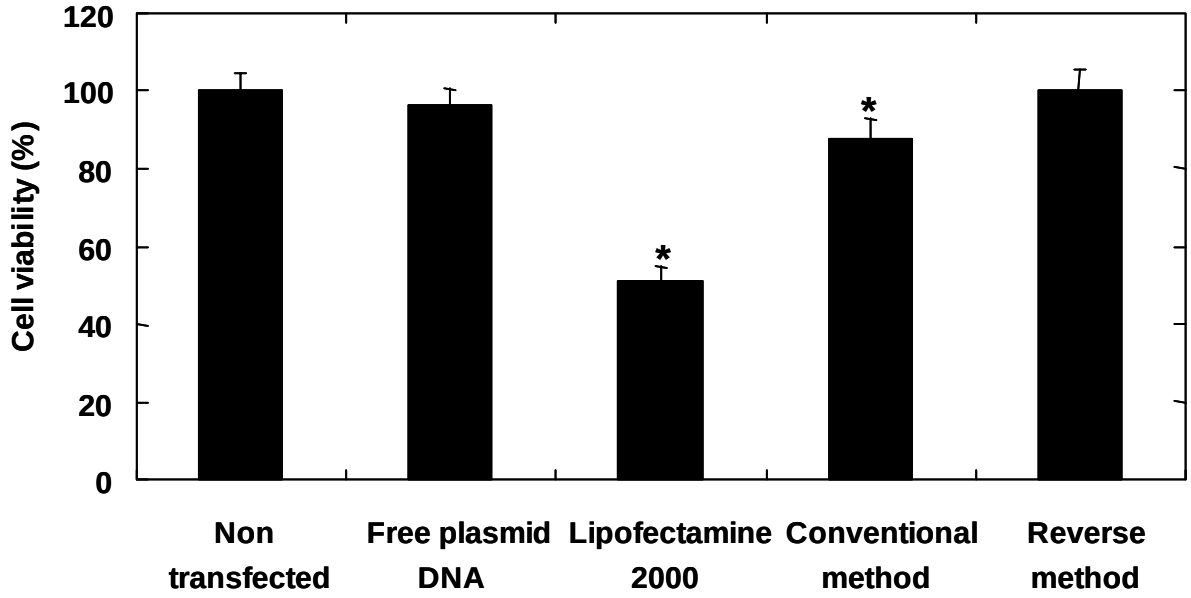



**Figure 4.** Viability of DC 1 day after transfection with free plasmid DNA, plasmid DNA-Lipofectamine 2000 complexes, and spermine-dextran-plasmid DNA complexes by the conventional and reverse methods. The amount of plasmid DNA used for transfection is 2.5 μg. The N/P ratio of spermine-dextran-plasmid DNA complex is 3.0. *, $p < 0.05$; versus the viability of DC without transfection.

**IL-12 expression of DC trasfected by spermine-dextran-mIL-12 plasmid DNA complex**

**Figure 5** shows the time course of mIL-12 production of DC transfected with PBS, complexes of spermine-dextran and GFP or mIL-12 plasmid DNAs by the reverse method. The amount of mIL-12 produced by DC transfected with the spermine-dextran-mIL-12 plasmid DNA complexes was significantly higher than that of non-transfected, original DC until at least 14 days after transfection. The amount of mIL-12 produced by DC transfected with spermine-dextran-GFP plasmid DNA complexes increased with culture time.

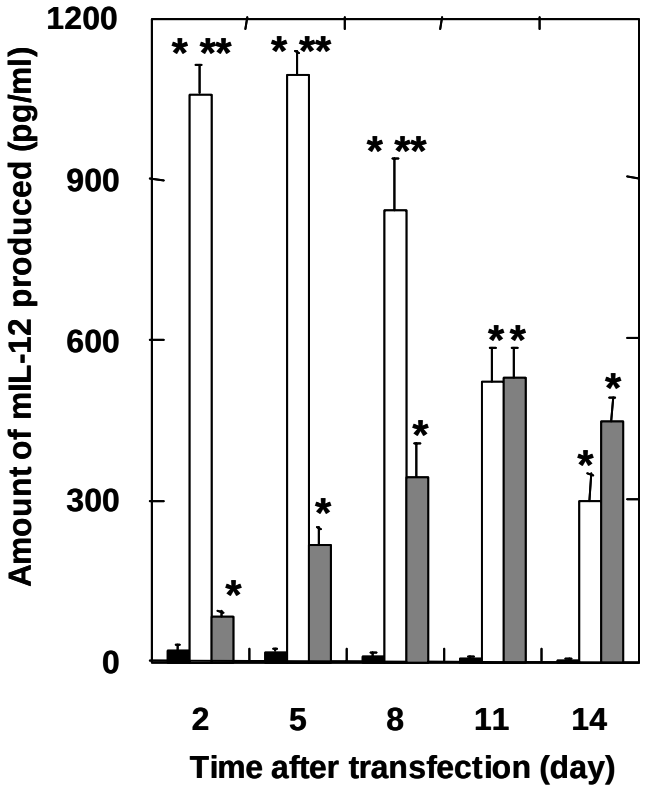
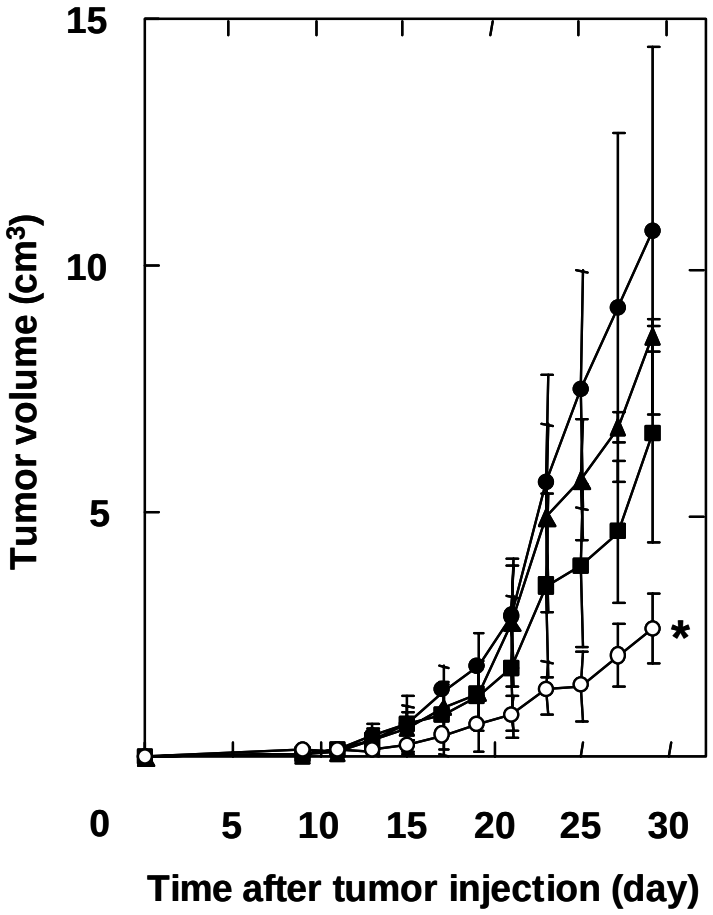



**Figure 5.** Time course of mIL-12 production of DC transfected with PBS (closed bars), complexes of spermine-dextran and GFP (gray bars) or mIL-12 plasmid DNAs (open bars) by the reverse method. The amount of plasmid DNA used for transfection is 2.5 μg. The N/P ratio of spermine-dextran-plasmid DNA complex is 3.0. *, $p < 0.05$; versus the amount of mIL-12 produced by DC transfected with PBS at the corresponding time. **, $p < 0.05$; versus the amount of IL-12 produced of DC transfected with spemine-dextran-GFP plasmid DNA complex at the corresponding time.

**In vivo anti-tumor effect of DC genetically engineered with spermine-dextran-mIL-12 plasmid DNA by the reverse transfection method**

**Figure 6** shows the time course of tumor volume change after the intratumoral injection of PBS, non-transfected, original DC, and DC transfected with complexes of spermine-dextran and GFP or mIL-12 plasmid DNAs by the reverse method. The intratumoral injection of DC transfected with the spermine-dextran-mIL-12 plasmid DNA complexes by the reverse method tended to suppress the in vivo growth of tumor cells even at the early time period and exhibited significantly higher anti-tumor effect in vivo than that of PBS, original DC, and DC transfected with spermine-dextran-GFP-plasmid DNA complex.




**Fugure 6.** Time course of tumor volume change after intratumoral injection of PBS (●), non-transfected, original DC (▲), and DC transfected with complexes of spermine-dextran and GFP (■) or mIL-12 plasmid DNAs (○) by the reverse method. *, $p < 0.05$; versus the tumor volume of other groups at the corresponding time.

**IFN production and CTL activity of DC genetically engineered with spermine-dextran-mIL-12 plasmid DNA by the reverse transfection method**

**Figure 7** shows the IFN-γ secretion and CTL activity by the splenocytes of tumor-bearing mice 7 days after intratumoral injection of PBS, non-transfected, original DC, and DC transfected with complexes of spermine-dextran and GFP or mIL-12 plasmid DNAs by the reverse method. Intratumoral injection of DC genetically engineered with the spermine-dextran-mIL-12 plasmid DNA complex by the reverse method enhanced IFN-γ production of splenocytes to a significantly great extent compared with that of original DC or those genetically engineered with spermine-dextran-mIL-12 plasmid DNA complex by the

reverse method (**Figure 7A**). For DC transfected with the spermine-dextran-mIL-12 plasmid DNA complex, as the effector/target ratio increased, the CTL activity of splenocytes harvested became high to a significantly great extent compared with that of original DC or those genetically engineered with spermine-dextran-mIL-12 plasmid DNA complex by the reverse method (**Figure 7B**).

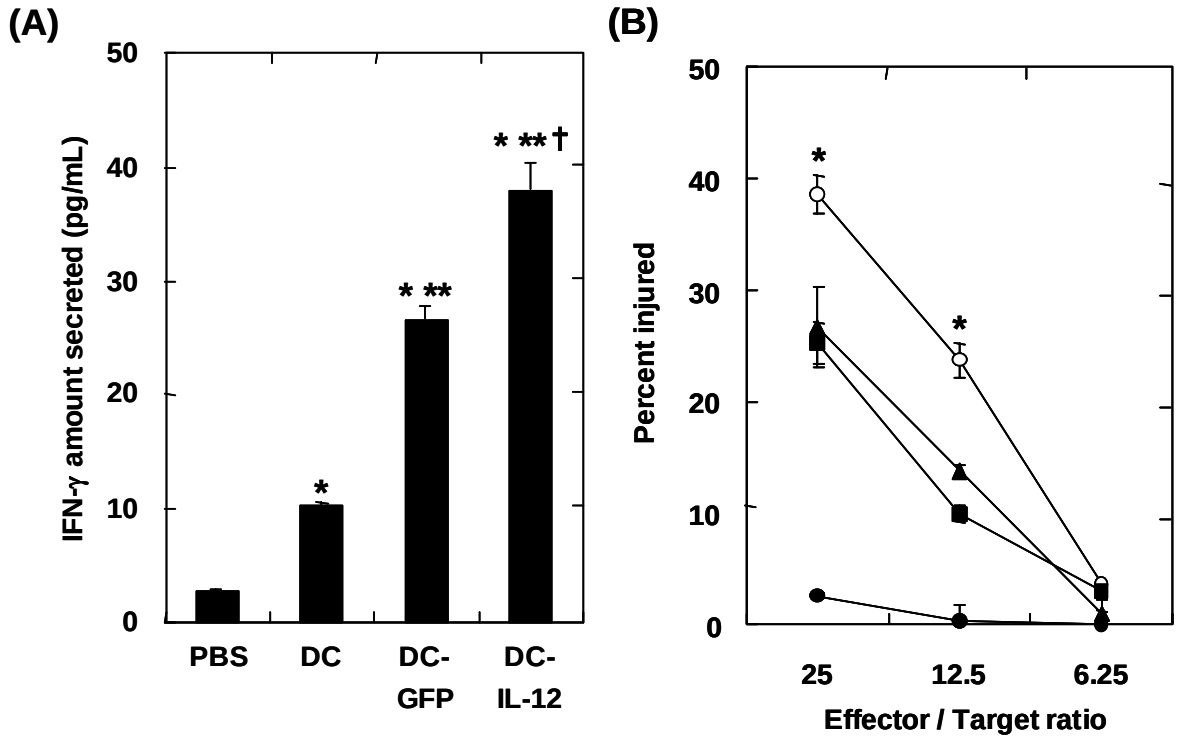



**Figure 7.** (A) IFN-γ secretion by splenocytes of tumor-bearing mice 7 days after intratumoral injection of PBS, non-transfected, original DC (DC), and DC transfected with complexes of spermine-dextran and GFP (DC-GFP) or mIL-12 plasmid DNAs (DC-IL-12) by the reverse method. *, $p < 0.05$; versus the amount of IFN-γ secreted for the PBS group. **, $p < 0.05$; versus the amount of IFN-γ secreted for the DC group. †, $p < 0.05$; versus the amount of IFN-γ secreted for the DC-GFP group. (B) CTL activity as a function of effector/target ratio. CTL activity was expressed as the percentage of B16 (target) cells injured by co-culture with splenocytes (effector) harvested from tumor-bearing mice 7 days after the intratumoral injection of PBS (●), non-transfected, original DC (▲), and DC transfected with complexes of spermine-dextran and GFP (■) or mIL-12 plasmid DNAs (○) by the reverse method. *, $p < 0.05$; versus the percentage of B16 cells injured by other groups at the corresponding effector/target ratio.

**Evaluation of in vivo tumor immunity**

To confirm the induction of systemic and therapeutic immunity, the growth of contralateral non-treated tumor, which is distant from the tumor mass injected with DC genetically engineered by the spermine-dextran-mIL-12 plasmid DNA complexes, was evaluated (**Table 1**). Intratumoral injection of DC genetically engineered significantly suppressed the growth of the tumor mass, irrespective of the direct injection.

**Table 1. Tumor volume in both the flanks of tumor- bearing mice with or without intratumoral injection of DC genetically engineered**

| | Tumor volume [a] ($cm^3$) | |
|---|---|---|
| | Injected | Non-injected |
| PBS | 9.9 +/- 1.4 | 9.6 +/- 1.9 |
| DC | 8.0 +/- 0.4 | 7.8 +/- 0.9 |
| DC-GFP | 7.3 +/- 0.0 | 8.5 +/- 0.0 |
| DC-IL 12 | 5.6 +/- 0.9 * | 5.9 +/- 0.1 * |

[a] The tumor volume was measured 29 days after tumor inoculation.
*, $p < 0.05$; versus the tumor volume of other groups.

## DISCUSSION

The present study clearly demonstrates that DC genetically engineered by IL-12 plasmid DNA complexed with the spermine-dextran enhances the anti-tumor effect through the immune surveillance potential. The in vivo growth of tumor cells was significantly suppressed by injection of DC engineered (**Figure 6**). The splenocytes isolated from tumor-bearing mice following the injection of DC engineered showed the IFN production and CTL activity (**Figure 7**). The tumor growth suppression was observed for the tumor mass without the DC injection (**Table 1**). This is the first report to show the immuno-responsive anti-tumor effect of DC genetically engineered by a non-viral carrier.

In this study, the spermine-dextran was used for gene transfection. Complexation with the spermine-dextran of a non-viral vector enabled the plasmid DNA to decrease the apparent size of spermine-dextran plasmid DNA complexes small enough to allow their cellular internalization and to have a positive surface charge which can interact with the cell surface of negative charge. Moreover, it has been reported that many types of sugar-recognizable receptor are expressed on DC [17]. Taken together, it is highly possible that the spermine-dextran-plasmid DNA complexes were internalized into the DC via the receptor-mediated endocytosis. It is highly conceivable that the features promoted the transfection of plasmid DNA for DC, enhancing the level of gene expression.

The level of gene expression of DC by the spermine-dextran-plasmid DNA complex at an N/P ratio of 3.0 was significantly higher than that by Lipofectamine 2000 of a commercially available transfection reagent (**Figure 1A**). It has been previously reported that the N/P ratio affects not only the physicochemical properties of the complex, but also the gene transfection efficiency [18,19]. At low N/P ratios, the complexes formed are unstable and have too large molecular size to be internalized into cells, resulting in low gene transfection efficiency. On the other hand, at high N/P ratios, the complexes are too stable to dissociate and have high cytotoxicity due to the excessive amount of cationic spermine-dextran, resulting in low gene transfection efficiency. Therefore, it is possible that the complexes at an N/P ratio of 3.0 have a condition suitable for gene transfection of DC. Considering these points, the spermine-dextran is a promising non-viral vector to enhance the gene expression of DC through the enhanced receptor-mediated endocytosis.

Chapter 5 demonstrated that the reverse transfection method gives cells good culture conditions, resulting in enhanced and prolonged gene expression with less cytotoxicity. The efficacy of reverse transfection in gene expression was also confirmed in this study for the luciferase (**Figures 1B**), GFP (**Figure 2**) and mIL-12 plasmid DNAs (**Figure 5**). The higher efficiency of gene transfection can be explained in terms of the unique feature of reverse method. DC are always cultured on the complex of plasmid DNA and spermine-dextran of non-viral carrier. It is likely that the localization of plasmid DNA close to cells enhances the possibility of cellular internalization of plasmid DNA, resulting in increased efficiency of

gene transfection. In addition, the transfection culture can be performed in the presence of serum, which is a condition better for cell culture. It is apparent from **Figure 3** that the plasmid DNA was localized in the nucleus of cells at higher amounts. This may be also due to the efficient and continuous supply of plasmid DNA to cells (**Figure 2**). Different behaviors between the time course of percent internalized of GFP plasmid DNA (**Figure 2A**) and that of GFP expression (**Figure 2B)** for DC transfected with spermine-dextran-plasmid DNA complexes by the reverse method were observed. It will need some time to express protein after gene transfection. Thus, the time rag between the plasmid DNA internalization and gene expression would be observed. When cells internalize the complex too much, it is conceivable that their capacity to take up decreases. Therefore, the percent internalized would decrease at day 7.

Although less cytotoxicity was observed (**Figure 4**), transfection of the spermine-dextran-GFP plasmid DNA complex affects not only the amount of mIL-12 produced in DC (**Figure 5**), but also that of IFN-γ secreted by splenocytes (**Figure 7A**). It is well known that DC themselves tend to produce IL-12 by various stimuli [20,21]. Therefore it is possible that only the stimulus of plasmid DNA transfection allowed DC to produce IL-12. Enhancement of IFN production by spermine-dextran-GFP plasmid DNA complex may be explained in terms of interaction of DC and IL-12. It is reported that IFN production from DC is influenced by IL-12 action [22,23]. The roles of DC and T cells in the tumor immunity are as follows [1]. DC phagocytoses a tumor antigen in the tumor tissue. Then, DC process these antigens into small peptides and move into the lymphoid organs, such as the spleen. During these steps, DC are biologically matured and obtain immunological responsibility, which express high levels of cell-surface major histocompatibility complex antigen complexes and costimulatory molecules. Receptors of helper $CD4^+$ T cells (Th0) in the lymphoid organs interact with the MHC class II of the DC, followed by the differentiation into the T helper 1 (Th1) or 2 (Th2) cells and IFN production. On the other hand, it has been reported that IL-12 promotes the differentiation of Th0 cells into Th1 cells [24-26], enhances CTL activity by $CD8^+$ T cells [27], and infiltration of T cells into the tumor tissue [28]. In the present study, the intratumoral injection of DC genetically engineered with spemrine-dextran-mIL-12 plasmid

complexes by the reverse method suppressed tumor growth (**Figure 6**) and induced a strong anti-tumor T-cell response (**Figure 7**). These results suggest that mIL-12 over-expression at the tumor site or, alternatively, in the secondary lymphoid sites could be important for the anti-tumor response. Chemokine production induced by mIL-12/IFN-γ elaboration at the tumor site would be responsible for these effects, at least in part, through enhanced recruitment of cytolytic effector cells into tumors as well as possibly anti-angiogenic effects. It is apparent from Figure 6 that the tumor growth was gradually suppressed by the intratumoral injection of DC genetically engineered with spemrine-dextran-mIL-12 plasmid complexes by the reverse method (**Figure 6**). It may be due to that there are many steps in tumor immunity as described above, consequently it takes some time to express the tumor suppression. Systemic immune responses, as demonstrated by IFN-γ production and CTL activity (**Figure 7**), were also significantly higher and tumor specific when injected DC genetically engineered. These findings well correspond to those of research where DC genetically engineered with IL-12 gene by retrovirus were intratumorally injected [9]. These results indicate that high cytotoxic activity in association with a Th1-type response could indeed contribute to the profound *in vivo* anti-tumor effects. Experiments involving a subsequent re-challenge with additional tumors (**Table 1**) further confirmed systemic anti-tumor immunity after intratumoral injection of DC genetically engineered.

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# SUMMARY

**Chapter 1.**

The objective of this chapter is to prepare the non-viral carrier of gene transfection from various polysaccharides and evaluate the feasibility in gene expression for various cells , such as HeLa cells, HepG2 cells, MSC, Mϕ, and DC. Various amounts of spermine were chemically introduced into pullulan, dextran, and mannan with a molecular weight of around 40,000 to prepare cationized polysaccharides (spermine-polysaccharide). Each cationized polysaccharide was complexed with a plasmid DNA at various ratios and the vitro gene transfection was investigated for HeLa cells, HepG2 cells, MSC, Mϕ, and DC. The level of gene expression depended on the type of cationized polysaccharides as well as the type of cells. These findings indicate that the cationized polysaccharide derivative is a promising non-viral carrier of plasmid DNA which is internalized in a receptor-mediated fashion.

**Chapter 2.**

The objective of this chapter is to prepare a non-viral carrier of gene expression from dextran of a polysaccharide and evaluate the effect of amine compounds introduced to dextran on the level of gene expression. Dextran with a molecular weight of 74,000 was cationized by the chemical introduction of different amine compounds. The cationized dextran was complexed with a plasmid DNA and the vitro gene transfection was investigated for HeLa cells. The level of gene expression depended on the amine compound introduced to dextran. The highest level was observed for the complex of spermine-introduced dextran and plasmid DNA. The highest cellular internalization and the best buffering effect were observed among every cationized dextran. Every complex did not show any cytotoxicity. It is concluded that the superior properties of spermine-introduced dextran enabled the plasmid DNA to enhance the expression level to a great extent compared with other cationized dextrans. The cationized dextran is a promising non-viral carrier of plasmid DNA.

**Chapter 3.**

The objective of this chapter is to prepare a novel gene carrier from pullulan, a polysaccharide with an inherent affinity for the liver, and evaluate the feasibility in gene transfection. Pullulan with different molecular weights was cationized by chemical introduction of spermine. The cationized pullulan derivative was complexed with a plasmid DNA and applied to HepG2 cells for in vitro gene transfection. The level of gene expression was depended on the molecular weight of cationized pullulan derivatives and the highest level was observed for the cationized pullulan derivative with a molecular weight of 47,300. Pre-treatment of cells with asialofetuin decreased the level of gene expression by the complexes. These findings indicate that the cationized pullulan derivative is a promising non-viral carrier of plasmid DNA which is internalized in a receptor-mediated fashion.

**Chapter 4.**

The objective of this chapter is to prepare a novel gene carrier from pullulan, a polysaccharide with an inherent affinity for the liver, and evaluate the feasibility in gene transfection. Various amounts of spermine were chemically introduced into pullulan with molecular weights of 22,800, 47,300, and 112,000 to prepare cationized pullulan derivatives with different percentages of spermine introduced. Each cationized pullulan derivative was complexed with a plasmid DNA at various ratios and applied to HepG2 cells for in vitro gene transfection. The level of gene expression depended on the percent spermine introduced of cationized pullulan derivatives and the molecular weight of pullulan. However, when compared at the complexation molar ratio of pullulan derivative to the plasmid DNA, the expression level became maximum around the ratio of $10^2$, irrespective of the pullulan molecular weight. Pre-treatment of cells with asialofetuin of asialoglycoprotein receptor ligand decreased the level of gene expression by the complexes. The cationized pullulan derivative with an appropriate physicochemical character is a promising non-viral carrier which promotes the receptor-mediated internalization of plasmid DNA and consequently enhances the expression level.

**Chapter 5.**

A new non-viral method of gene transfection was designed to enhance the level of gene expression for rats mesenchymal stem cells (MSC). Pullulan was cationized by chemical introduction of spermine to prepare cationized pullulan of non-viral carrier (spermine-pullulan). The spermine-pullulan was complexed with a plasmid DNA of luciferase and coated on the surface of culture substrate together with Pronectin® of artificial cell adhesion protein. MSC were cultured and transfected on the complex-coated substrate (reverse transfection) and the level and duration of gene expression were compared with those of MSC transfected by culturing in the medium containing the plasmid DNA-spermine-pullulan complex (conventional method). The gene expression was significantly enhanced and prolonged by the reverse transfection method compared with that of the conventional method. The reverse method permitted the transfection culture of MSC in the presence of serum, in contrast to the conventional method, which gave cells a good culture condition to lower cytotoxicity. The reverse transfection was carried out for a non-woven fabic of polyethylene terephtalate (PET) coated with the complex and Pronectin by an agitated and stirring culture methods. The level and duration of gene expression for MSC was significantly enhanced by the two methods compared with that of the static method. It is possible that the medium circulation improves the culture conditions of cells in terms of oxygen and nutrition supply and wastes excretion, resulting in enhanced gene expression.

**Chapter 6.**

Mesenchymal stem cells (MSC) are being expected as one cell source for cardiac reconstruction because of their differentiation potential and ability to supply growth factors. However, the therapeutic potential of MSC is often hindered by the poor viability at the transplanted site. Here, as one trial to overcome this issue, a non-viral carrier of cationized polysaccharide is introduced for genetic engineering of MSC. Spermine-introduced dextran of cationized polysaccharide (spermine-dextran) was internalized into MSC by way of a sugar-recognizable receptor to enhance the expression level of plasmid DNA. When genetically engineered by the spermine-dextran complex with plasmid DNA of

adrenomedullin (AM), MSC secreted a large amount of AM, an anti-apoptotic and angiogenic peptide. Transplantation of AM gene-engineered MSC significantly improved cardiac function after myocardial infarction compared with that of MSC alone. Thus, this genetic engineering technology by the non-viral spermine-dextran is a promising strategy of MSC therapy for ischemic heart disease.

**Chapter 7.**

The objective of this chapter is to genetically engineer macrophages (Mϕ) for biological activation and evaluate their anti-tumor activity in a tumor-bearing mouse model. Mouse peritoneal Mϕ were incubated on the surface of culture dish which had been coated with the complex of a cationized dextran and luciferase plasmid DNA complex plus a cell adhesion protein, Pronectin® for gene transfection (reverse transfection). When compared with the conventional transfection where Mϕ were transfected in the medium containing the complex, the level of gene expression by the reverse method was significantly high and the time period of gene expression was prolonged. Confocal microscopic observation revealed that the plasmid DNA was localized in the cell nucleus to a higher extent by the reverse transfection method. Following the reverse transfection of Mϕ by the plasmid DNA of a hepatocyte growth factor antagonist (NK4) complexed with the cationized dextran, the NK4 protein was secreted at a higher amount for a longer time period in contrast to the conventional transfection of free plasmid DNA. The NK4-transfected Mϕ exhibited a stronger inhibition activity for in vitro growth of Meth-A fibrosarcoma cells. When injected intravenously into mice carrying a mass of Meth-A tumor cells, the Mϕ engineered were accumulated in the tumor tissue and showed significant anti-tumor activity. It is concluded that Mϕ injected functioned as the natural carrier of tumor targeting for anti-tumor NK4 molecules, resulting in enhanced suppression of tumor growth at a high selectivity.

**Chapter 8.**

The objective of this chapter is to genetically engineer dendritic cells (DC) for biological activation and evaluate their anti-tumor activity in a tumor-bearing mouse model. Mouse DC were incubated on the surface of culture dish which had been coated with the complex of a cationized dextran and luciferase plasmid DNA complex plus a cell adhesion protein, Pronectin® for gene transfection (reverse transfection). When compared with the conventional transfection where DC were transfected in the medium containing the complex, the level of gene expression by the reverse method was significantly high and the time period of gene expression was prolonged. Following the reverse transfection of DC by a plasmid DNA of mouse interleukin-12 (mIL-12) complexed with the cationized dextran, the mIL-12 protein was secreted at higher amounts for a longer time period. When injected intratumorally into mice carrying a mass of B16 tumor cells, the DC genetically activated showed significant anti-tumor activity.

Summary

# LIST OF PUBLICATIONS

**Chapter 1.**

**Jun-ichiro Jo**, Arimichi Okazaki, Kentaro Nagane, Masaya Yamamoto, and Yasuhiko Tabata. Preparation of cationized polysaccharides as gene transfection carrier for bone marrow-derived mesenchymal stem cells, submitted.

**Chapter 2.**

**Jun-ichiro Jo**, Kentaro Nagane, Masaya Yamamoto, and Yasuhiko Tabata. Effect of amine type on the expression of plasmid DNA by cationized dextran, submitted.

**Chapter 3.**

**Jun-ichiro Jo**, Tomonori Ikai, Arimichi Okazaki, Masaya Yamamoto, Yoshiaki Hirano, and Yasuhiko Tabata. Expression profile of plasmid DNA by spermine derivatives of pullulan with different molecular weights. Journal of Biomaterials Science Polymer Edition, 18(7), 883-99, 2007.

**Chapter 4.**

**Jun-ichiro Jo**, Tomonori Ikai, Arimichi Okazaki, Masaya Yamamoto, Yoshiaki Hirano, and Yasuhiko Tabata. Expression profile of plasmid DNA by spermine derivatives of pullulan with different extents of spermine introduced. Journal of Controlled Release, 118, 389-98, 2007.

**Chapter 5.**

Arimichi Okazaki, **Jun-ichiro Jo**, and Yasuhiko Tabata. A reverse transfection technology to genetically engineer adult stem cells. Tissue Engineering, 13 (2), 245-51, 2007.

**Chapter 6.**

**Jun-ichiro Jo**, Noritoshi Nagaya, Yoshinori Miyahara, Masaharu Kataoka, Mariko Shiba-Harada, Kenji Kangawa, and Yasuhiko Tabata. Transplantation of Genetically Engineered Mesenchymal Stem Cells Improves Cardiac Function in Rats With Myocardial Infarction: Benefit of a Novel Nonviral Vector, Cationized Dextran. Tissue Engineering, 13 (2), 313-22, 2007.

**Chapter 7.**

Takahiro Okasora, **Jun-ichiro Jo**, and Yasuhiko Tabata. Augmented anti-tumor therapy through natural targetability of macrophages genetically engineered by NK4 plasmid DNA. Gene Therapy, 15, 524-30, 2008.

**Chapter 8.**

Masataka Yoshida, **Jun-ichiro Jo**, and Yasuhiko Tabata. Augmented anti-tumor effect of dendritic cells genetically engineered by interleukin-12 plasmid DNA, submitted.

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Naoki Nishishita, **Jun-ichiro Jo**, Masaya Yamamoto, Yasuhiko Tabata, and Yoshiaki Hirano. The gene transfection using cell attachment peptides for gene therapy. Peptide Science, 2007, 443-46, 2008

**Jun-ichiro Jo** and Yasuhiko Tabata. Gene delivery system based on cationized polysaccharide carriers. Chapter 10, Soft Nanomaterials Edited by Hari Singh Nalwa, in press.

Akinaga Sonoda, Norihisa Nitta, Shinichi Ohta, Ayumi Seko, **Jun-ichiro Jo**, Shigehiro Morikawa, Yasuhiko Tabata**,** Masashi Takahashi, Kiyoshi Murata. Development of a conjugated gadolinium and cisplatin–gelatin possessing properties as an intravascular contrast agent for MR imaging. European Journal of Radiology, in press.

Akinaga Sonoda, Norihisa Nitta, Ayumi Seko, Shinich Ohta, Shigeyuki Takamatsu, Yoshio Ikehata, Isamu Nagano, **Jun-ichiro Jo**, Yasuhiko Tabata, Masashi Takahashi, Osamu Matsui, and Kiyoshi Murata. Development of dextran magnetite complex-conjugated cisplatin having selective hyperthermic and magnet-controlled potential. Molecular Medicine Reports, in press.

List of publications

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