Delivery of Condensed DNA by Liposomal Non-viral Gene Delivery System into Nucleus of Dendritic Cells

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In general, foreign substances cannot enter the nucleus of non-dividing cells, in which nuclear membrane does not disappear by cell division, since nuclear transfer is strictly controlled by precise machinery. For example, nuclear proteins are recognized by importin α in the cytoplasm with a nuclear localization signal (NLS) attached to the nuclear proteins, and importin β then combines with the NLS/importin α. The complex binds to the nuclear pore complex (NPC), and passes through the NPC into nucleus. Therefore, it is difficult to transfer foreign plasmid DNA (pDNA) into the nucleus of a non-dividing cell by means of an artificial gene delivery system such as cationic liposomes or cationic polymers.

Many researchers have attempted to artificially transfer pDNA utilizing nuclear transport machinery, i.e., the nuclear transfer of electrostatic complexes between NLS and DNA, NLS-conjugated DNA, etc. We also synthesized NLS conjugated linear pDNA and evaluated its nuclear transfer by a microinjection technique, but, no increase in the nuclear transport of pDNA was observed. This result suggests that a positively charged NLS is not able to function due to electrostatic interactions with the negatively charged DNA. Therefore, it is possible that steric flexibility in the NLS is necessary for an interaction with importin.

In this study, we attempted to transfer pDNA condensed by a cationic peptide protamine into the nucleus of non-dividing mouse bone marrow derived dendritic cells (BMDC) using an NLS-modified liposomal non-viral gene delivery system (NLS-DMEND), of which NLS peptide was presented on the surface to retain its flexibility. We evaluated intracellular trafficking and the gene expression of the NLS-DMEND to evaluate its nuclear transfer capabilities. In addition, we examined the effect of metabolic inhibitors to confirm the energy dependency of the nuclear transfer of pDNA by the NLS-DMEND.

Key words non-viral gene delivery system; non-dividing cell; nuclear localization signal; nuclear transfer

MATERIALS AND METHODS

Materials

Dioleoyl phosphatidylethanolamine (DOPE) and N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-DOPE (NBD-DOPE) were purchased from AVANTI Polar Lipids Inc (Alabaster, AL, U.S.A.). Cholesteryl hemisuccinate (CHEMS) was obtained from SIGMA-Aldrich Co. (St. Louis, MO, U.S.A.). Protamine sulfate salmon milt was purchased from Merck KGaA (Darmstadt, Germany). The pDNA pCMV-luc (7037 bp) encoding luciferase was prepared by EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). The peptides used in this study were synthesized as previously reported. NIH3T3 and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

Preparation of Bone Marrow-Derived Dendritic Cells (BMDC) of Mice

BMDC were prepared based on a previously reported method with some modifications. BM cells were treated with antibodies and rabbit complement to remove natural killer cells, B-lymphocytes, T-lymphocytes and granulocytes. The remaining BM cells were then cultured in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 100 unit/ml penicillin–streptomycin, 10 ng/ml GM-CSF and 10% FCS. On day 2 and day 4, non-adherent cells were removed, and adherent cells were cultured in fresh medium containing GM-CSF. On day 6, non-adherent and loosely adherent cells were used in experiments as immature dendritic cells.

Preparation and Transfection of Octaarginine-Modified Multifunctional Envelope-Type Nano Device (R8-MEND)

The multifunctional envelope-type nano device (MEND) encapsulating protamine-condensed pDNA was prepared by the lipid film hydration method described in a previous report. A stearyl octaarginine (STR-R8) solution (5 mol% of lipids) was added to the suspension of
MEND to attach the R8 peptide to the envelope of the MEND, and the mixture was incubated for 30 min at room temperature. R8-MEND containing 0.4 µg of pDNA suspended in 0.25 ml of DMEM(—) was added to 4×10⁴ NIH3T3 or HeLa cells, followed by incubation for 3 h at 37 °C. Next, 1 ml of DMEM containing 10% fetal calf serum was added to the cells, followed by a further 45 h incubation. The cells were then washed, and solubilized with reporter lysis buffer (Promega, Madison, WI, U.S.A.). The luciferase reaction was initiated by the addition of 50 µl of luciferase assay reagent (Promega, Madison, WI, U.S.A.) to 20 µl of cell lysate, and measured by means of a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (Promega, Madison, WI, U.S.A.). The luciferase activity was measured as mentioned above. Data are reported as the mean±standard deviation (n=3).

### RESULTS AND DISCUSSION

Nuclear membranes are nearly impenetrable barriers, especially in non-dividing cells, to the nuclear transfer of foreign substances, such as pDNA. In fact, the nuclear transfer of pDNA failed in the transfection of condensed luciferase pDNA coated with lipid, the surface of which was modified with R8 (R8-MEND) (Fig. 1), to non-dividing BMDC, although the R8-MEND showed a significantly higher transfection activity in dividing cells (NIH3T3 cells and HeLa cells) by choosing macropinocytosis, which can avoid lysosomal degradation. Results and images are described in the legend of Fig. 2a. The diameters and zeta-potential of the condensed DNA particles and pH-sensitive fusogenic lipid CHEMS (9:2 (molar ratio)) and 1 mol% of stearylated SV40 T antigen-derived NLS (GGP-KKKRRKVPKKKRK) was hydrated with 1 ml of 10 mM HEPES buffer (pH 7.4) for 10 min at room temperature. The hydrated lipid film was then sonicated with a probe-type sonicator. Condensed DNA particles were prepared by mixing a DNA solution (0.1 mg/ml) with a protamine solution (0.1 mg/ml) with vortexing at a N/P ratio of 2.2 as mentioned above. The suspensions of NLS-modified small liposomes and condensed DNA were then mixed at a ratio of 2:1 (v/v) to coat the condensed DNA particle by fusion of the NLS-modified small liposomes with a double lipid membrane as reported previously. The final concentrations of lipid and DNA were 0.367 mM and 0.013 mg/ml, respectively.

### Preparation of NLS-Modified Double Membraneous MEND (NLS-DMEND)

The NLS-modified double membraneous MEND (NLS-DMEND) were prepared by an amended LPD method. First, to prepare NLS-modified small liposomes, lipid film containing unstable lipid DOPE/pH sensitive fusogenic lipid CHEMS (9:2 (molar ratio)) and 1 mol% of stearylated SV40 T antigen-derived NLS (GGP-KKKRRKVPKKKRK) was hydrated with 1 ml of 10 mM HEPES buffer (pH 7.4) for 10 min at room temperature. The hydrated lipid film was then sonicated with a probe-type sonicator. Condensed DNA particles were prepared by mixing a DNA solution (0.1 mg/ml) with a protamine solution (0.1 mg/ml) with vortexing at a N/P ratio of 2.2 as mentioned above. The suspensions of NLS-modified small liposomes and condensed DNA were then mixed at a ratio of 2:1 (v/v) to coat the condensed DNA particle by fusion of the NLS-modified small liposomes with a double lipid membrane as reported previously. The final concentrations of lipid and DNA were 0.367 mM and 0.013 mg/ml, respectively.

### Characterization of NLS-DMEND

Hydrodynamic diameter was measured by a quasi-elastic light scattering method by means of an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka electronics, Japan). A suspension of the NLS-DMEND, which was constructed by a FITC-labeled DNA particle and aqueous rhodamine-entrapped NLS-modified small liposomes, were layered on a discontinuous sucrose density gradient (0 to 30%), and centrifuged at 160000×g for 2 h at 20 °C. A 1 ml aliquot was collected from the top and the fluorescence intensities of FITC and rhodamine were measured. A frozen sample of the double membraneous MEND was observed by cryo transfer observation by transmission electron microscopy (Hitachi H-7650), at a voltage of 120 kV and a magnification of 75000.

### Transfection of NLS-DMEND

A suspension of NLS-DMEND, containing 0.8 µg of pDNA was added to 2×10⁵ BMDC, and the suspension incubated for 1 h at 37 °C in 0.2 ml of 10 mM HEPES buffer (pH 6.5) containing 5% glucose to induce fusion of the NLS-DMEND with the plasma membrane. Then, 0.5 ml of RPMI 1640 containing 10% fetal calf serum and 10 ng/ml of GM-CSF was added to the cells, followed by a further 23 h incubation. In the inhibition experiment, BMDC were treated with a mixture of metabolic inhibitors (final concentrations; 1 µg/ml of Antimyicine A, 0.1% (w/v) of NaN₃, and 10 mM of NaF) for 30 min at 37 °C before addition of NLS-DMEND. The cells were then washed, and solubilized in reporter lysis buffer. Luciferase activity was measured as mentioned above. Data are reported as the mean±standard deviation (n=3). Confocal scanning microscopic analysis of the transfected cells was performed by means of confocal scanning laser microscopy (CLSM) (LSM510 META, Carl Zeiss, Germany) as described previously. Results and images are described in the legend of Fig. 2a. The diameters and zeta-potential of the condensed DNA particles and pH-sensitive fusogenic lipid CHEMS (9:2 (molar ratio)) was hydrated with 1 ml of 10 mM HEPES buffer (pH 7.4) for 10 min at room temperature. The hydrated lipid film was then sonicated with a probe-type sonicator. Condensed DNA particles were prepared by mixing a DNA solution (0.1 mg/ml) with a protamine solution (0.1 mg/ml) with vortexing at a N/P ratio of 2.2 as mentioned above. The suspensions of NLS-modified small liposomes and condensed DNA were then mixed at a ratio of 2:1 (v/v) to coat the condensed DNA particle by fusion of the NLS-modified small liposomes with a double lipid membrane as reported previously. The final concentrations of lipid and DNA were 0.367 mM and 0.013 mg/ml, respectively.

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plasma membrane. After the transfection, DNA particles were observed as numerous pink dots in the nucleus (blue) of the BMDC (Fig. 3a). Furthermore, the transfection activity was enhanced by two orders of magnitude compared to R8-MEND (Fig. 3d). However, in the case of normal transfection of NLS-DMEND, no DNA was observed in the BMDC nucleus (Fig. 3b), and luciferase activity was not improved (Fig. 3d). As shown in Fig. 3b, yellow dots indicating the colocalization of DNA and lipid was observed. This suggests that the NLS-DMEND was not able to escape from endosomes after internalization via endocytosis, although membranes of the NLS-DMEND consist of pH-sensitive fusogenic lipids. In addition, we examined the transfection of NLS-modified MEND by direct fusion method to BMDC. However, no transfection activity was detected (data not shown). Probably, the NLS-modified MEND could not present NLS on its surface after internalization, because direct fusion method significantly changed membrane structure of the MEND. These results suggest that condensed pDNA particles were transferred into the nucleus by direct fusion of the NLS-DMEND, and not only NLS peptide but also double-membrane structure of NLS-DMEND are necessary for transfer of DNA into nucleus of BMDC.

We next examined the effect of metabolic inhibitors (An-timycin A, NaN₃ and NaF) on the nuclear transfer of condensed DNA by NLS-DMEND. In the presence of metabolic inhibitors, the nuclear transfer of condensed DNA was completely prevented, although no effect was observed on the internalization of the MEND by direct fusion method (Fig. 3c). In addition, no significant luciferase activity was detected after the transfection of NLS-DMEND under this condition (data not shown). Thus, the nuclear transfer of DNA particles was an energy dependent event. It is possible that condensed DNA particles pass, in an energy-dependent manner, through the NPC via the nuclear transfer machinery, because we previously clarified the nuclear transfer ability of protamine-condensed pDNA particles by a microinjection technique, and that the protamine-condensed pDNA is highly flexible, although the diameter of the condensed DNA particle (approximately 100 nm) was significantly larger than the NPC pore size.

In conclusion, condensed pDNA particles were transferred into the nucleus of non-dividing cells by NLS-DMEND.

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