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Novel redox nanomedicine improves gene expression of polyion complex vector

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Abstract

Gene therapy has generated worldwide attention as a new medical technology. While non-viral gene vectors are promising candidates as gene carriers, they have several issues such as toxicity and low transfection efficiency. We have hypothesized that the generation of reactive oxygen species (ROS) affects gene expression in polyplex supported gene delivery systems. The effect of ROS on the gene expression of polyplex was evaluated using a nitroxide radical-containing nanoparticle (RNP) as an ROS scavenger. When polyethyleneimine (PEI)/pGL3 or PEI alone was added to the HeLa cells, ROS levels increased significantly. In contrast, when (PEI)/pGL3 or PEI was added with RNP, the ROS levels were suppressed. The luciferase expression was increased by the treatment with RNP in a dose-dependent manner and the cellular uptake of pDNA was also increased. Inflammatory cytokines play an important role in ROS generation *in vivo*. In particular, tumor necrosis factor (TNF)-α caused intracellular ROS generation in HeLa cells and decreased gene expression. RNP treatment suppressed ROS production even in the presence of TNF-α and increased gene expression. This anti-inflammatory property of RNP suggests that it may be used as an effective adjuvant for non-viral gene delivery systems.

Keywords: non-viral gene delivery system, anti-inflammation, reactive oxygen species, nitroxide radical-containing nanoparticle, polyplex

1. Introduction

Recent developments in molecular targeted therapy have markedly changed the concept of drug design. Drugs have evolved from traditional low-molecular-weight synthetic compounds to high-molecular-weight compounds such as enzymes and antibodies. Gene therapy has generated considerable worldwide attention as a forthcoming technology with a high efficiency in targeting genetic diseases and cancers [1, 2].

One of the most serious issues concerning effective gene therapy is the *in vivo* delivery of genes. Viral vectors have been studied and evaluated as effective gene carriers. However, exogenous viral vectors can cause side effects such as inflammation, antigenicity and carcinogenicity. High safety and performance are thus required for novel gene carriers [3–5]. Non-viral gene vectors are promising candidates as gene carriers that lack the many adverse effects of viral vectors. The polyion complex with a polycation is a representative non-viral vector that electrostatically interacts with DNA [6]. However, in contrast to viral vectors, these synthetic vectors have several drawbacks such as toxicity and low transfection efficiency. A representative example is polyplex, a major gene carrier and a polyion complex between negatively charged DNA and polycations, such as polyethyleneimine (PEI), poly(L-lysine), chitosan and cationic dendrimer. Polyplex is relatively stable against enzymatic degradation owing to the compaction of the

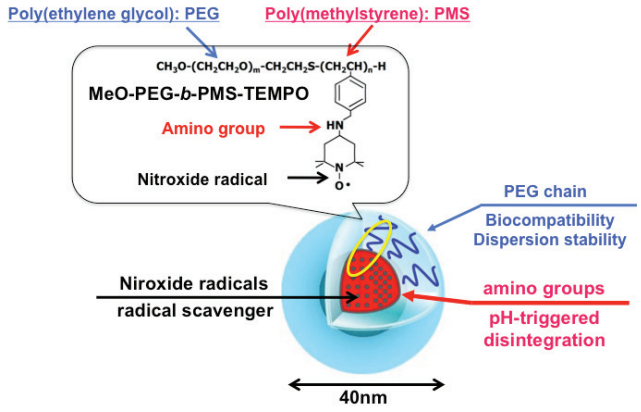

Figure 1. Schematic of RNP.

DNA molecule in the complex [7–12]. However, although the high-molecular-weight polyamine in polyplex possesses strong cationic charges, it shows significant cellular toxicity because of electrostatic interactions with negatively charged cell membranes. For instance, when PEI is mixed with cultured leukemic monocyte macrophage cells, it induces the generation of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [13]. These cytokines in turn activate the key regulators of the immune system, nuclear factor-κB (NF-κB) [14], followed by an induction of downstream cytokines. This causes the generation of reactive oxygen species (ROS), resulting in the amplification of the inflammation reaction [15–17]. Yang *et al* clearly demonstrated that the ROS generation was increased with increasing in the molecular weight of PEI [16]. We hypothesized that ROS generation and inflammation reaction strongly correlate with the inefficient gene expression in the case of the polyamine-based non-viral gene delivery system.

Nitroxide radical is known to catalytically react with ROS owing to its redox characteristics. For example, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), which is a stable nitroxide radical, is a known ROS scavenger that efficiently scavenges superoxide and hydroxyl radicals *in vitro* [18]. The ROS scavenging property of the nitroxide radical plays a role in suppressing the immunoresponse and inflammation *in vitro* by inhibiting the activation of NF-κB [19].

We have developed a novel nitroxide radical-containing nanoparticle (RNP) that effectively scavenges ROS because it contains 3000 to 5000 nitroxide radicals in the core (figure 1) [20, 21]. The RNP was prepared from self-assembling amphiphilic block copolymers (MeO-PEG-b-PMS-TEMPO) composed of a hydrophilic poly(ethylene glycol) (PEG) segment and a hydrophobic poly(methylstyrene) (PMS) segment possessing TEMPO moieties. This nanoparticle design makes it feasible for *in vivo* use because of the prevention of rapid renal excretion of low-molecular-weight TEMPOL and reduction under cellular conditions. During the course of our study, RNP decreased the acute toxicity caused by ROS [22, 23]. If the ROS scavenging property of the nitroxide radical effectively enhances gene expression in polyplex, the non-viral gene therapy would become more feasible.

In this report, RNP was examined as an adjuvant for *in vitro* gene expression. Branched PEI (25 kDa) was employed as a complexing agent for the pGL3 gene. HeLa cells were treated with PEI/pDNA with or without RNP, and the ROS generation and transfection efficiency were examined.

2. Materials and methods

2.1. Chemicals

PEI (Branched, 25 kDa) and methoxyl-poly(ethylene glycol)-sulfanyl (MeO-PEG-SH) were purchased from Aldrich (Milwaukee, WI, USA) and NOF Corporation (Tokyo, Japan), respectively. Chloromethylstyrene (CMS) was kindly provided by Seimi Chemical Co., Ltd. (Kanagawa, Japan). 4-Amino-2,2,6,6-tetramethylpiperidinyl-1-oxyl (4-amino-TEMPO) was purchased from Aldrich Chemical Co, Inc, USA, and TNF-α was purchased from WAKO Pure Chemical Industries, Ltd (Osaka, Japan).

2.2. Synthesis of RNP

RNP was prepared following the methods of Yoshitomi *et al* [20]. An amphiphilic block copolymer possessing a poly(ethylene glycol) (PEG) chain as a hydrophilic segment and a polystyrene chain as a hydrophobic segment with TEMPO repeating units was prepared using two-step reactions. After the PEG-b-poly(chloromethylstyrene) (PEG-b-PCMS) was prepared by the radical telomerization reaction of CMS using MeO-PEG-SH as a telogen, 4-amino-TEMPO was immobilized as a side chain of the PCMS segment by the amination reaction. RNP was prepared from the synthesized polymer (MeO-PEG-b-PMS-TEMPO) by the dialysis method.

2.3. Cell culture

Human cervical cancer cells (HeLa) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen) at 37 °C under 5% CO_2.

2.4. Plasmid DNA and pDNA/PEI complex

The plasmid pGL3-control pDNA coding for the firefly luciferase gene (Promega, Madison, WI, USA) was amplified in *Escherichia coli.* The plasmid was isolated and purified using a QIAGEN HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). PEI/ pDNA complex was prepared by mixing indicated amounts of plasmid DNA with PEI at N/P ratios of 10 (N/P = molar ratio of PEI amino group to DNA phosphate).

2.5. Transfection of HeLa cells

HeLa cells were seeded in a 24-well plate (1×10^5 cells per well). After 24 h, PEI/pDNA complex (DNA = 0.75 μg per

well, N/P = 10), RNP (final polymer concentrations were 0.033, 0.33, 3.3 and 33 μM), and TNF-α (final concentrations were 0.06, 0.6 and 6 nM) were added to each well. The RNP concentration which is low enough not to induce cytotoxicity was used according to our previous paper [20]. After a 24 h incubation, the transfection medium was changed to fresh DMEM with 10% FBS, and the cells were further incubated for 24 h. After the incubation, the cells were washed with phosphate-buffered saline (PBS) twice. The cells were lysed in reporter lysis buffer (Promega), and luciferase activities were measured using the Luciferase Assay Kit (Promega). The results were expressed as light units per milligram of cell protein determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.6. Cellular uptake of pDNA

Fluorescein isothiocyanate (FITC)-labeled pDNA was prepared using a Label IT Nucleic Acid Labeling Kit (Mirus Bio LLC, Madison, WI, USA). HeLa cells were seeded at a density of 1×10^5 cells per well in a 24-well dish and kept overnight at 37 °C in 5% CO_2 atmosphere. The PEI/pDNA polyplex micelles (N/P = 10) were added at a pDNA concentration of 0.75 μg/well and incubated at 37 °C in 5% CO_2 atmosphere in the presence or absence of RNP (33 μM) for 18 h. The cells were washed with PBS twice. FITC fluorescence intensities of cells were detected using a flow cytometer (Guava EasyCyte Mini System; Millipore).

2.7. Intracellular ROS measurement

HeLa cells were seeded in a 24-well plate (1×10^5 cells/well). After 24 h, RNP (final concentration was 33 μM), PEI/pDNA complex (DNA = 0.75 μg/well, N/P = 10), PEI (same concentration with PEI/pDNA), and TNF-α (final concentrations were 0.06, 0.6 and 6 nM) were added to each well. After 48 h incubation, the cells were washed once in 1 ml of PBS. One milliliter of a 10 μM solution of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; WAKO) for hydroxyl radical determination, or dehydroethidine (HE; WAKO) for superoxide anion determination was added to each well, followed by incubation for 15 min at 37 °C in the dark. The dyes were then removed and the cells were washed once with 1 ml of PBS. The cells were harvested by scraping and placed in 1.5 ml tubes containing 500 μl of PBS. The production of intracellular ROS was measured by flow cytometry. The mean fluorescence intensity of 5000 cells was quantified using the Guava EasyCyte Mini System.

3. Results and discussion

3.1. ROS scavenging effect of RNP on PEI/pDNA gene delivery system

The ROS scavenging effect of RNP on gene expression was measured by adding RNP into a cell culture model. RNP and polyplex were added simultaneously to the culture dish because the ROS generation has been reported to occur

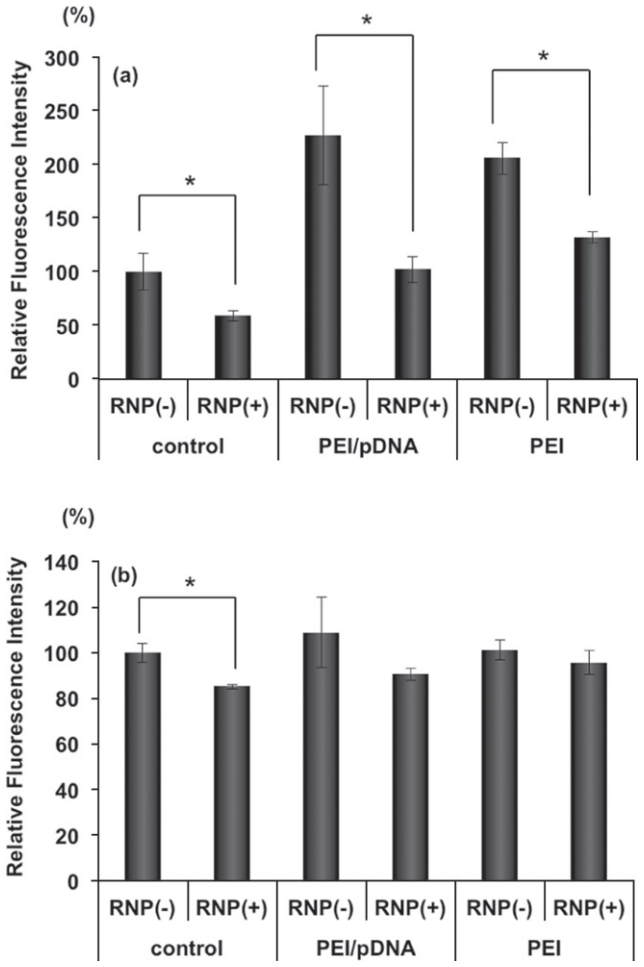

Figure 2. Effects of RNP on ROS levels in HeLa cells. ROS levels in HeLa cells were measured by flow cytometry. Graphs indicate relative fluorescence intensities (%) derived from (a) DCF (hydroxyl radical) and (b) HE (superoxide anion) of HeLa cells treated with polyplex (PEI/pDNA) or polymer (PEI) compared with untreated control cells. pGL3 (0.75 μg per well) was transfected as a PEI/pDNA complex ($N/P = 10$). PEI was added at the same concentration as the PEI of polyplex. The experiments were repeated twice ($n = 3$, $^*P < 0.01$).

immediately [13]. We investigated whether RNP suppressed ROS levels during the transfection experiment, and used two fluorescent dyes for detecting ROS: DCFH-DA for hydroxyl radicals and HE for superoxide anion. As shown in figure 2(a), the addition of PEI alone or PEI/pDNA polyplex significantly increased the concentration of the hydroxyl radical due to the stimulation of HeLa cells by cationic polyamines. When RNP was added to the culture medium, the ROS levels decreased significantly, indicating that the nitroxide radical in RNP worked as an effective hydroxyl radical scavenger, as anticipated. A similar tendency was observed in the case of superoxide radicals (figure 2(b)), although the difference in the concentration of superoxide radicals, with and without RNP, was unremarkable.

Before the effect of RNP on the transfection efficiency was evaluated, the interaction between RNP and the PEI/pDNA polyplex was investigated by electron spin resonance (ESR). We have already confirmed that the ESR

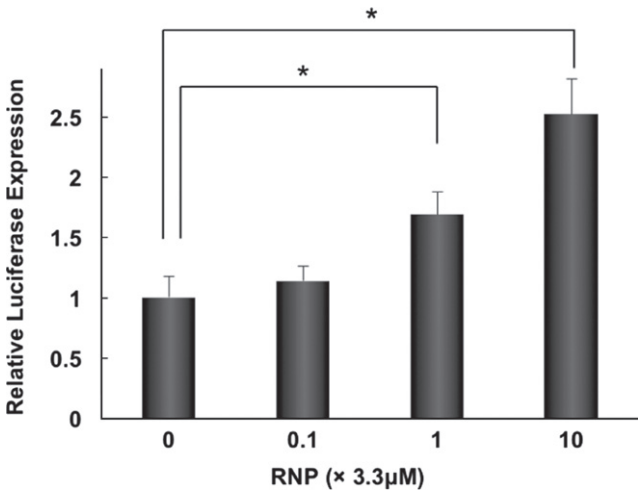

Figure 3. Luciferase gene expression in Hela cells transfected with PEI/pDNA with RNP. PEI/pDNA complex ($N/P = 10$) was transfected into HeLa cells. The luciferase expression ratio was normalized to the expression level at RNP (0 mM) = 1. The experiments were repeated 4 times ($n = 5$, $^{*}P < 0.01$).

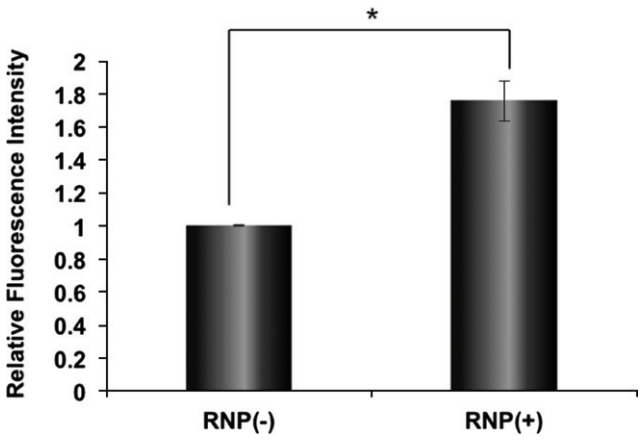

Figure 4. Uptake of FITC-labeled pDNA into HeLa cells. The figure shows relative fluorescence intensities for HeLa cells treated with FITC-labeled PEI/pDNA with or without RNP (33 μM). The experiments were repeated twice ($n = 3$, $^{*}P < 0.01$).

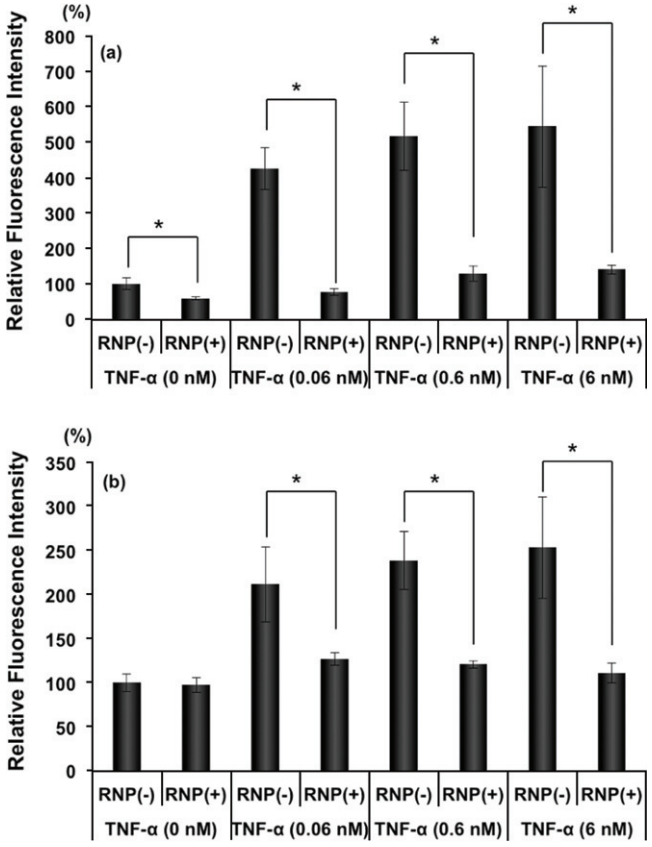

Figure 5. Effects of RNP on ROS levels in HeLa cells treated with TNF-α. ROS levels in HeLa cells were measured by flow cytometry. The figure shows relative fluorescence intensities (%) from (a) DCF (hydroxyl radical) and (b) HE (superoxide anion) of HeLa cells treated with TNF-α (0, 0.06, 0.6 and 6 nM) with or without RNP (33 μM) compared with untreated control cells. The experiments were repeated twice ($n = 3$, $^{*}P < 0.01$, $^{**}P < 0.05$).

spectra of RNP markedly change from a broad to sharp triplet due to the disintegration of RNP [20]. If RNP interacts with the PEI/pDNA polyplex, a change in the ESR spectra of RNP must be observed owing to the morphology change in RNP. When RNP was mixed with the PEI/DNA polyplex, no change in the ESR spectra of RNP was detected, namely, a broad signal was observed regardless of the RNP/polyplex ratio (data not shown); this result indicates no interaction between RNP and the PEI/pDNA polyplex under the present conditions. The effect of RNP on the transfection efficiency of the PEI/pDNA polyplex was then evaluated *in vitro* using HeLa cells. As shown in figure 3, the gene expression increased significantly with increasing amount of RNP ($\geqslant 3.3\,\mu$M). At 33.3 μM of RNP concentration, the expression of PEI/pDNA was 2.5 times higher than that without RNP addition. No significant change in protein content was found regardless of the RNP concentrations indicating no significant difference in the cell viability for these experiments.

To elucidate the mechanism of the increased expression of the PEI/pDNA polyplex in the presence of RNP, the cellular uptake of FITC-labeled PEI/pDNA to HeLa cells was evaluated with and without RNP. The uptake of the FITC-labeled PEI/pDNA was investigated by flow cytometry. When transfection of the PEI/pDNA polyplex was carried out in the presence of RNP, the uptake of pDNA almost doubled, as shown in figure 4. Although other steps during the gene delivery and expression might be improved by RNP, RNP definitely enhanced the cellular uptake of plasmid DNA.

3.2. Effect of inflammatory cytokine

Since polyamines are positively charged, they strongly interact with the anionic cellular membrane, enhancing the levels of inflammatory cytokines such as TNF-α and IL-6 from macrophages and/or neutrophils. These cytokines can stimulate transcriptional factors such as NF-κB and cause inflammation. TNF-α is also reported to induce mitochondrial ROS generation [15], which stimulates NF-κB. Thus, both cytokines and ROS amplify inflammation. ROS generation strongly correlates with the cellular uptake of the PEI/pDNA polyplex as shown in figure 4, and we hypothesized that

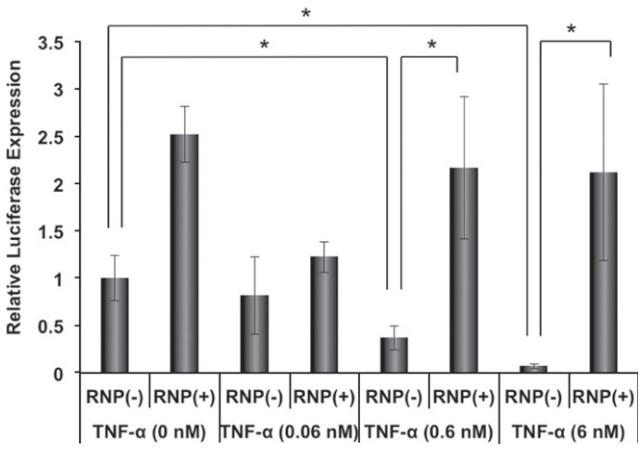

Figure 6. Relative luciferase gene expression in HeLa cells treated with TNF-α. HeLa cells treated with TNF-α (0, 0.06, 0.6 and 6 nM) and RNP [(−): 0 μM; (+): 33 μM]. pGL3 (0.75 μg per well) was transfected as PEI/pDNA complex ($N/P = 10$). The luciferase expression ratio was normalized to that of untreated cells [TNF-$\alpha = 0$, and RNP(−) = 1]. The experiments were repeated 4 times ($n = 5$, $^{*}P < 0.01$).

increased inflammation plays an important role in the gene expression efficiency. Therefore, *in vitro* gene expression efficiency of the polyplex was examined in the presence of TNF-α, and the ROS scavenging effect of RNP on gene expression was also evaluated.

The treatment with TNF-α was confirmed to increase the production of both types of ROS, whereas the addition of RNP effectively suppressed intracellular ROS production as shown in figures 5(a) and (b). Accordingly, it was reconfirmed that RNP effectively scavenged ROS produced by inflammatory cytokine. Figure 6 shows the luciferase gene expression in HeLa cells stimulated with TNF-α. The level of gene expression decreased with increasing TNF-α concentration. By the addition of RNP, however, gene expression was restored even under the high concentration of TNF-α. Because the total protein amounts in the cells remained unchanged regardless of the TNF-α concentration, there was no significant effect of TNF-α on cell viability. Accordingly, the gene expression was restored by the addition of RNP due to the ROS-scavenging effect of RNP. From these results, we conclude that ROS scavenging is a rational and valid strategy to improve the expression of genes in a gene delivery system.

4. Conclusions

We have investigated the gene expression efficiency of polyplex in the presence and absence of RNP, which is a novel ROS scavenger that we developed. Suppression of ROS generation reduced the inflammation caused by polyamines and increased the cellular uptake of polyplex; this resulted in increased gene expression efficiency. Although the detailed mechanism of action is now being investigated, we consider that the suppression of ROS and inflammation can provide a new strategy to the field of gene delivery.

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