Research on highly effective and safe lipofection with antioxidant

（抗酸化剤を用いた高効率かつ安全なリポフェクションに関する研究）

長崎大学大学院医歯薬学総合研究科　生命薬科学専攻　Wang Shu

[Purpose]

Gene therapy is a promising approach for curing diseases, especially for genetic disorders and cancers. Among several delivery platforms, cationic liposomes have great potential for clinical use with multiple advantages: i.e., biocompatibility, biodegradability and lower immunogenicity. Although cationic liposomes are useful for gene transfer with ease of preparation and modification compared with viral vectors, the insufficient transfection efficiency (TE) restricts their application. Positive charge of cationic lipid-based non-viral vectors is critical for efficient cellular uptake and endosome escape during gene transfer. However, harmful reactive oxygen species (ROS) also generate during membrane fusion in these processes, which could suppress transgene expression. Here, the author investigated the effects of edaravone, an efficient ROS scavenger, on cationic liposome-mediated gene transfection (lipofection). To achieve effective and safe *in vivo* gene transfer, the author also studied the usefulness of edaravone-loaded liposomes (EDLPs).

[Methods]

Edaravone was co-incubated with cationic liposome/plasmid DNA complexes (lipoplexes) during transfection. The cellular ROS level, TE, and cytotoxicity were analyzed in HepG2 cells. The *in vivo* gene transfer and hepatic injury were examined in male ddY mice. EDLPs, prepared by pH gradient method, were optimized by lipid ratio, incubation time, and temperature.

[Results and discussion]

1. Redox balance is a critical factor determining transfection efficiency in HepG2 cells and mice

1.1 Relationship between TE and ROS levels in HepG2 cells

Edaravone had highest TE promotion ability among six antioxidants (edaravone, all-trans retinoic acid, α-tocopherol, ascorbic acid, *N*-acetylcysteine and *N*-*tert*-butyl-α-phenylnitrone). Then, the author analyzed the relationship between TE and cellular ROS levels and the influence of edaravone application. The number of gene expression-positive cells was increased by co-incubation with 100 μM edaravone (**Fig. 1B**) compared with the control group (**Fig. 1A**) with a significant difference in mean fluorescence intensity (**Fig. 1D**). Lipoplexes enhanced cell ROS levels in comparison with the basal level (**Fig. 1A, C and E**). The cell ROS level was decreased by the addition of edaravone (**Fig. 1A, B and E**). As expected, the gene expression-positive cells in the control and edaravone groups were both centralized in the ROS-negative quadrant (**Fig. 1A and B**).

1.2 Edaravone increased TE in mice

Edaravone application together with lipoplex increased TE in mice with certain dosage; 270 μg and 600 μg edaravone both improved TE in the lungs by around 4 times. For the liver, only 270 μg group increased TE efficiency (3.8 fold), and 600 μg of edaravone might be overdosed.

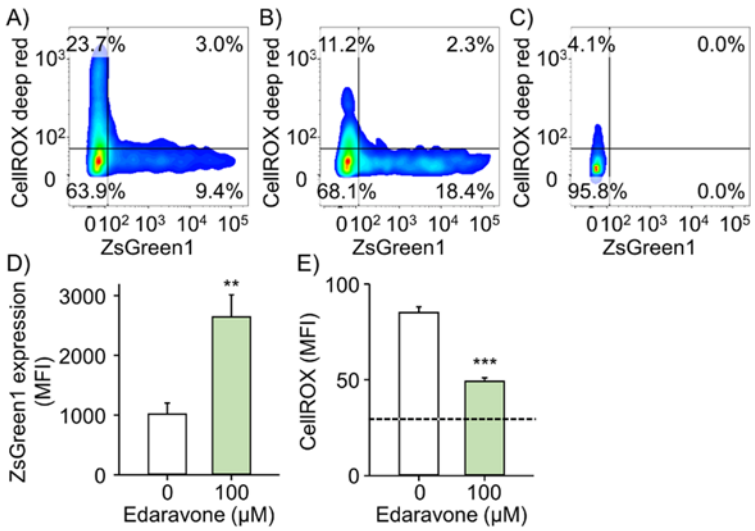

Fig. 1. Relationship between gene expression and cell ROS levels. (A) 0 and (B) 100 μM free edaravone. (C) Cells without lipoplexes to detect basal ROS levels. (D) TE level. (E) ROS level. The dotted line represents the basal ROS level. $^{**}P< 0.01$, $^{***}P< 0.001$, compared with the control.

1.3 Concentration dependency of edaravone on cell metabolic activity, cell death, and TE

The CCK-8 assay showed a lipid concentration-dependent decrease in HepG2 cell metabolic activity, especially at concentrations of >50 μM. Edaravone (100 μM) protected the cells from partial toxicity (**Fig. 2A**). Increasing lipid concentration also resulted in increased cell death measured by propidium iodide (PI). Again, edaravone rescued the cells from death at moderate lipid concentrations between 20 and 50 μM (**Fig. 2B**). TE reached a plateau at a lipid concentration of 50 μM, at which edaravone significantly enhanced the TE (**Fig. 2C**). At a lipid concentration of 100 μM, cell death was obvious with or without edaravone, as a consequence, edaravone no longer worked well for TE enhancement.

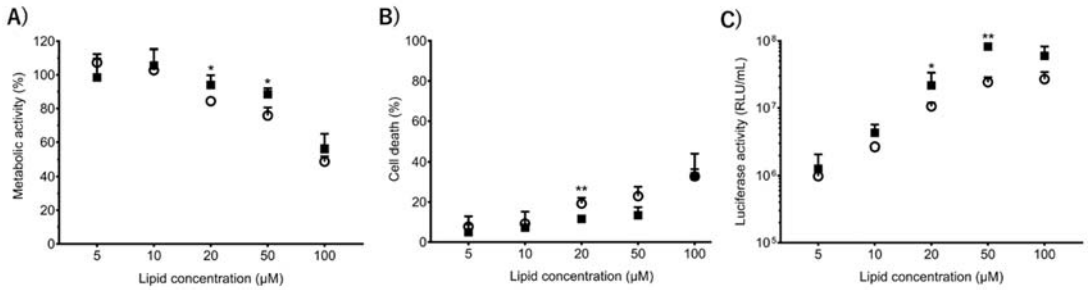

Fig. 2. Metabolic activity, cell death, and TE measurements. Metabolic activity (A), cell death (B), and TE (C) in HepG2 cells treated by lipoplexes with (solid square) or without (open circle) 100 μM edaravone. $^{*}P< 0.05$, $^{**}P< 0.01$.

2. Effective and safe *in vivo* gene transfer by lipoplexes with edaravone-loaded liposomes

2.1 Preparation and optimization of edaravone-loaded liposomes

Edaravone was loaded at hydrophilic phase of liposomes. Lipid molar ratio of DOTAP and cholesterol equal to 8 was optimum as liposome formulation for high encapsulation efficiency of edaravone. Around 67% encapsulation efficiency was obtained by calcium acetate gradient method at 37°C incubation for 10 min. Before and after dialysis, the size, PDI, and ζ potential of liposomes were maintained at around 100 nm, 0.07, and 84 mV, respectively. After loading edaravone, liposomes indicated only slightly decreasing on ζ potential.

2.2 EDLPs strongly enhanced the TE *in vivo*

EDLPs greatly increased TE at certain doses, especially in the 30 μg group, which demonstrated 20 times higher TE than the control group in the lungs (Fig. **3A**). Such

enhancement also occurred in the liver (Fig. **3B**). Concerning the difference in the doses giving the highest TE between the lungs (30 μg) and liver (40 μg), the amount of EDLPs containing 40 μg edaravone in the lungs may have been saturated, providing more edaravone for the liver than the 30 μg dose to scavenge ROS. Optimum doses of EDLPs (30-40 μg) were 10-times lower than free edaravone (270-600 μg), indicating successful delivery of edaravone with lipoplexes.

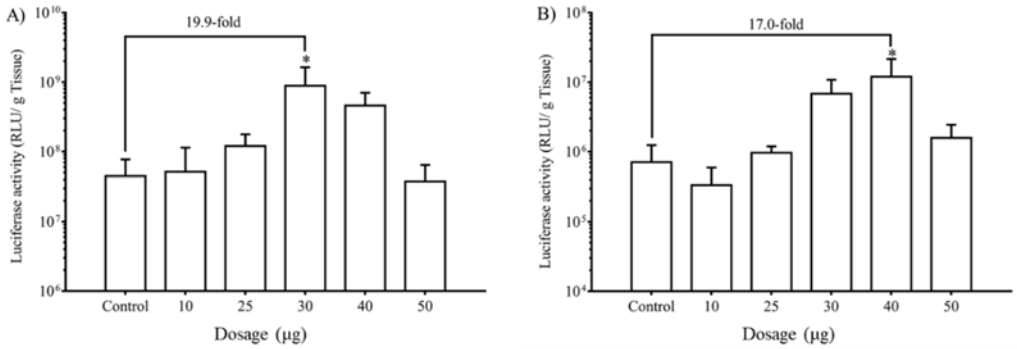

Fig. 3. TE of lipoplexes in murine lungs (A) and liver (B) with various dosages of EDLPs.
Luciferase activity was determined at 6 h after administration. $^{*}P< 0.05$, compared with the control.

2.3 EDLPs ameliorated hepatotoxicity

Similar to free edaravone, the hepatic derivation enzyme (serum AST and ALT) levels significantly decreased after application of EDLPs. Comparing to free edaravone (as high as 600 μg dosage), EDLPs decreased effective dose of edaravone to 30 μg for ameliorating hepatotoxicity as well as enhancing TE.

2.4 Biodistribution of edaravone was altered by liposomes

Five minutes after administration of free edaravone, around 15% of dosage was accumulated in the liver, while accumulation in the lungs was not detectable. Plain cationic liposomes were mostly accumulated in the lungs (over 60%), and accumulated less than 10% in the liver. This high accumulation in the lungs can be due to the high entrapment of lipoplexes in the capillaries of lungs as first-pass organs, as a consequence, induced greatly higher TE in the lungs than that in the liver. The biodistribution property of edaravone in EDLPs was similar to those of liposomes, which was completely different from that of the free edaravone. This altered biodistribution property of EDLPs would be important for improved TE and safety.

[Conclusion]

The author has demonstrated that edaravone enhanced TE in HepG2 cells and mice. Then, formulation of EDLPs decreased effective dose of edaravone. The author also achieved cytoprotection and amelioration of hepatotoxicity by edaravone that scavenged the superfluous ROS to maintain the cell redox balance. EDLPs strengthened the efficiency of edaravone possibly by altering the biodistribution. This thesis would provide the useful information for the development of effective and safe *in vivo* gene transfer systems.

[基礎となった学術論文]

Shu Wang, Shintaro Fumoto, Hirotaka Miyamoto, Masakazu Tanaka, and Koyo Nishida, 2018. Edaravone, a cytoprotective drug, enhances transgene expression mediated by lipoplexes in HepG2 cells and mice. *Int. J. Pharm.*, 548(1), 173-181.