An approach to mitochondrial gene silencing by the mitochondrial delivery of anti-sense RNA

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ABSTRACT SUMMARY
Mitochondrial gene therapy would be expected to have great medical benefits. To achieve such a strategy, it will be necessary to deliver therapeutic agents to mitochondria in living cells. In the present study, we report on an approach for controlling mitochondrial gene expression by delivering nucleic acids to mitochondria via membrane fusion using a MITO-Porter, a liposome-based mitochondrial delivery system. Intracellular observations verified that the MITO-Porter delivered nucleic acids to mitochondria. Moreover, mitochondrial delivery of antisense RNA using a MITO-Porter resulted in mitochondrial RNA knockdown, followed by a decrease in mitochondrial membrane potential. This provides a demonstration of its potential use of such delivery system in mitochondrial gene therapies.

INTRODUCTION
In recent years, genetic mutations of mitochondrial DNA (mtDNA) have been implicated in a variety of diseases. It is now well accepted that mutations and defects in the mitochondrial genome, mtDNA, form the basis of a variety of mitochondrial diseases. Therefore, mitochondrial gene therapy would be expected to have great medical benefits. We previously constructed a MITO-Porter, a liposome-based mitochondrial delivery system that functions via membrane fusion.

EXPERIMENTAL METHODS
Complexed oligo DNA (ODN) particles were prepared with protamine in 10 mM HEPES buffer (pH 7.4). MITO-Porter encapsulating ODN particles was constructed by the lipid film hydration method, as previously reported. When mitochondrial RNA knockdown was evaluated, antisense 2’Ome-RNA targeted to mitochondria-encoded cytochrome c oxidase subunit II (COX2) was packaged. Particle diameters were measured using a quasi-elastic light scattering method, and z potentials were determined electrophoretically using laser doppler velocimetry.

For the quantification of COX2-encoding mRNA, qRT-PCR was performed. At 24 hr after transfection, total RNA was extracted from the collected cells using a GenElute Mammalian Total RNA Kit and the first-strand cDNA was then synthesized using a PrimeScript RT reagent Kit and an oligo dT primer. The amount of COX2-encoding mRNA was normalized by that of mitochondria-encoded NADH dehydrogenase subunit I (ND1)-encoding mRNA.

Membrane potentials were evaluated using a JC-1 system (Invitrogen Corp., Carlsbad, California). JC-1 emits a red fluorescence in mitochondria when the mitochondria have a high membrane potential, while the fluorescence changes to green and the dye is distributed to the cytosol when the potential is low. At 48 hr after transfection, the cells were treated with JC-1, and Hoechst33342 was added to the medium for staining nuclei. After incubation, the cells were observed by CLSM.
RESULTS AND DISCUSSION

The MITO-Porter encapsulating nucleic acids was constructed as described in our previous report\(^4\). Particle diameters were approximately 200 nm and the $\zeta$-potentials were around +60 mV. We next evaluated the cellular uptake of ODN by carriers using flow cytometry (Figure 2A). When the ODN was packaged in the MITO-Porter, the cellular uptake efficiency was strongly enhanced. We also observed the intracellular trafficking of ODN delivered by the carrier using CLSM. As shown in Figure 2B, the fluorescent labeled ODNs were co-localized with mitochondria, and are observed as yellow signals indicated by allows.

To validate mitochondrial gene silencing by the mitochondrial delivery of antisense RNA, after transfection, the mRNA levels of the target, COX2 (mitochondrial endogenous protein) were measured. Figure 3 provides information on the quantification of relative mRNA-levels coding COX2 normalized by ND1 (mitochondrial endogenous protein).

The mRNA levels of COX2 were decreased as the result of the mitochondrial delivery of antisense RNA. On the other hand, the mtDNA-levels were comparable in all samples (data not shown). The results show that the mitochondrial delivery of antisense RNA by MITO-Porter contributed to mitochondrial RNA knockdown.

We then evaluated mitochondrial membrane potential using JC-1. In the case of non-treated cells and cells treated with mock RNA (Figure 4A, B), red colored mitochondria were observed, indicating that the mitochondria have a high membrane potential. On the other hand, we observed a green color in the cytosol after the transfection of antisense RNA (Figure 4C). This result shows that the mitochondrial membrane potential was decreased by mitochondrial delivery of antisense RNA using the MITO-Porter.

CONCLUSION

In this study, we attempted to control mitochondrial gene expression by delivering nucleic acids directly to mitochondria using a MITO-Porter system. The findings show that the MITO-Porter delivered nucleic acids to mitochondria in living cells. In addition, the findings demonstrated that the mitochondrial delivery of antisense RNA by the MITO-Porter contributed to mitochondrial RNA knockdown. It is also noteworthy that mitochondrial function was decreased as the result of the delivery of antisense RNA to mitochondria using the MITO-Porter. These results suggest that the MITO-Porter holds promise as a delivery system for mitochondrial gene therapy.

REFERENCES


ACKNOWLEDGMENTS

This work was supported, in part by, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, Japan (NIBIO), a Grant-in-Aid for Young Scientists (A) and a Grant-in-aid for Scientific Research (S) from the Ministry of Education, Culture, Sports, Science and Technology of Japanese Government (MEXT).