VII. 3. Delivery of Na/I Symporter Gene into Skeletal Muscle by Using Nanobubbles and Ultrasound: Visualization of Gene Expression with PET

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Introduction

Gene therapy has been applied to many types of genetic disease such as cancer, muscular dystrophy, and vascular disease. Although virus vectors have been used in gene therapy, various side effects and lower tissue specificity have been reported. Thus, non-virus methods are required to be developed to reduce immunogenicity. In addition, clinical endpoints of gene therapy have to be indicated by monitoring the location, magnitude, and kinetics of transgene and by guiding improvements in the design of efficient formulations in patients1).

The molecular delivery method using nanobubble (NB) and ultrasound (US) is one of non-virus methods. This method is non-invasive and tissue-specific. In the previous study, we demonstrated that the method using US/NB could be used as a potential molecular delivery method for gene therapy2).

Fluorescent and luminescent imaging methods have been used as monitoring of gene expression for animal models. These methods are easy to use; however, they are hardly applied to deep tissues and to clinical trials. Thus it is required to develop human reporter genes for monitoring gene expression to indicate clinical endpoints. The Na/I symporter (NIS) gene are known as radionuclide human reporter genes. It is able to inhibit immune response compared to other reporter genes due to endogenously expression, and accumulates radioiodide because it is a mediator of I transport into the cells. The 124I is
of radiotracers with a longer half-life (4.2 days) comparing to other positron emitting radionuclide. PET is probably the only noninvasive imaging method that currently can be applied in humans for monitoring of gene expression. Recently, Ishii et. al.\textsuperscript{3)} have developed a practical semiconductor animal PET with CdTe detector (Fine-PET). The Fine-PET achieves a high spatial resolution of less than 1mm FWHM.

In the present study, we delivered the human NIS gene into the skeletal muscle of three types of mice by the US/NB method, and visualized hNIS gene expression using \textsuperscript{124}I and the Fine-PET. We demonstrated the methodology could be applicable to human gene therapy.

Materials and Methods

Animal studies were performed in accordance with the ethical guidelines approved by Tohoku University. Three types of mice were used; BALB/c (6-8 weeks, 21-25 g), McH/Lpr-RA1 mice (16 weeks, 32-34 g) showing vascular disease\textsuperscript{4)}, and C57BL/10-mdx Jic mice (5 weeks, 14-15 g) showing muscular dystrophy. Two types of plasmid DNA were used: the luciferase reporter vector, pGL3, which expresses luciferase, and the phNIS vector in which expresses human NIS\textsuperscript{5)}. Acoustic liposomes (ALs) were lipid-based perfluoropropane (C\textsubscript{3}F\textsubscript{8}) filled bubbles (size: 198 ± 30 nm (n = 3), zeta potential: -4.1 ± 0.85 mV (n = 3)). \textsuperscript{124}I was produced by the \textsuperscript{124}Te(p, n)\textsuperscript{124}I reaction, using irradiation of a \textsuperscript{124}TeO\textsubscript{2} target with 14 MeV protons of a 1-4 μA beam current depending on the target production yield of \textsuperscript{124}I\textsuperscript{6)}. Ultrasound exposure conditions were the intensity was 3.0 W/cm\textsuperscript{2}; the duty cycle, 20%; the number of pulses, 200; the PRF, 1000 Hz; and the exposure time, 60 s\textsuperscript{7)}. In order to confirm that hNIS gene was definitely expressed in the tibialis anterior (TA) muscle by the US/NB method, phNIS and pGL3 were cotransfected into the TA muscle. Luciferase activity was measured with \textit{in vivo} imaging system. For the biodistribution, the left TA muscle of BALB/c (n = 14) was cotransfected with pGL3 and phNIS by the US/NB method. Mice were i.v. injected with 370 kBq of Na\textsuperscript{124}I (theoretical specific activity, 8.78 TBq/μmol) dissolved in 100 μL of PBS, and scarified after 30, 60, and 180 min. Immediately after scarification, organs of mice were removed. The radioactivity of each sample was measured by a γ-counter, expressed as the percentage of the injected dose per gram (%ID/g). For the blocking study, cotransfected mice were divided into two groups. One group was i.v. injected with Na\textsuperscript{124}I (1.85 MBq), scarified two hours later. The other was i.p. injected with NaI (57 mM), whose concentration was
one fifth of LD$_{50}$. After 15 min, Na$^{124}$I (1.85 MBq) was i.v. injected, and the TA muscle was removed from both groups for autoradiography (ARG) images. For the PET imaging study, two mice were selected from each 3 types of mice. The mouse was i.v. injected with Na$^{124}$I (74 MBq). Data were acquired in list mode format and binned into 0.6 mm sinograms for image reconstruction. PET images of three types of mice were summed 30-150 min. All measurements are expressed as mean ± S.E.M. (standard error of the mean). An overall difference between the groups was determined by one-way analysis of variance (one-way ANOVA). Simple comparisons of the mean and S.E.M. of the data were performed using Student’s $t$-test. The differences were considered to be significant at $P < 0.05$.

**Results**

In the previous study, we found that a peak of gene expression in muscle of a mouse was 4 days after transfection (data not shown). To obtain guaranteed PET images, we cotransfected the TA muscle with pGL3 and phNIS, selected mice which showed enhanced luciferase activity on day 3, and captured their PET images on day 4 after transfection.

For most tissues, the uptake of the $^{124}$I reached its maximum value at 60 min, and decreased at 180 min (Fig. 1). The TA where transfected NIS showed significant uptake of the radioactive tracer at 60 min ($P < 0.05$) and 180 min ($P < 0.01$), were compared to the control TA (saline injection alone). To confirm that $^{124}$I actually entered the TA muscle due to the expression of NIS, we blocked the uptake of $^{124}$I into the TA muscle by injecting NaI. Figure 2 shows ARG images (A) without and (B) with injection of NaI. The uptake of the radioactive tracer by the TA muscle was indeed blocked by the injection of NaI.

Figure 3 shows bioluminescence, PET and ARG imaging of three types of mice. L is the left TA muscle that was cotransfected with pGL3 and phNIS, and R is the right TA muscle that was injected with saline, as a negative control. Gene expression was shown the same tendency in all mice. All mice were showed same results. Bioluminescence was specifically located at the left TA muscle. The uptake of $^{124}$I in the left TA muscle by NIS was clearly detected in PET imaging compared to that of right TA muscle. The $^{124}$I uptake was confirmed with ARG which shows the same tendency as PET.

**Discussion**

We cotransfected pGL3 and phNIS into the TA muscle using the US/NB method to ensure the hNIS gene expression with the Fine-PET. At day 3 after cotransfection, one
mouse showing an average luciferase activity was selected for each experimental condition. At day 4, the hNIS gene expression in the TA muscle of the selected mice was visualized using the Fine-PET (Fig. 3) successfully.

For biodistribution studies, activity was analyzed in the major organs 30, 60, and 180 min after $^{124}\text{I}$ i.v. injection (Fig. 1). The thyroid gland and stomach have the underlying NIS gene, and the bladder drains iodide. At 60 and 180 min after $^{124}\text{I}$ injection, the TA muscles transfected with the hNIS gene showed accumulation values doubled compared to the control TA. In addition, the uptake of $^{124}\text{I}$ was blocked by i.p. injecting of Nal, indicating that hNIS was actually transfected in the TA muscle (Fig. 2). These results showed that the hNIS gene was transfected to the TA muscle by the US/NB method and the accumulation of $^{124}\text{I}$ was due to its expression.

The intravenous injected activity (74 MBq) of $^{124}\text{I}$ in this study is similar to that of a clinical study. It is the necessary activity to visualize the NIS gene expression. However, with the activity, acute radiation injury from internal exposure is not avoidable in longitudinal studies. In fact, mice injected with $^{124}\text{I}$ (111 MBq) died 1.5 h after the injection (data not shown). Therefore, it is necessary to reduce the injected dose. There are some strategies to reduce injected dose of $^{124}\text{I}$ including 1) development of a new plasmid with a high gene expression efficiency and 2) development of a transfection method to improve the efficiency using NB/US. In addition, improvement of advanced CdTe detectors that can block complex decay scheme of $^{124}\text{I}$, resulting in a better PET picture quality, is also an option to reduce the injected dose.

**Conclusion**

This study provides a new method consisting in non-invasive gene delivery system using the NB/US-hNIS/PET system (to indicate clinical endpoints) for genetic disorders (i.e. vascular disease and muscular dystrophy). These results suggest that the system can be used in clinical studies. In this perspective, we are developing a new plasmid DNA combining therapeutic gene and hNIS gene for visualization of therapeutic effect, and improving the NB/US-hNIS/PET system for advancement of transfection and expression effect.

**References**

Figure 1. Biodistribution of $^{124}$I into TA muscle. The radioactivity of tissues was expressed as the percentage of the injected dose per gram (%ID/g). The bars represent mean ± S.E.M. $P < 0.01$ (**), and $P < 0.05$ (*).
Figure 2. Blocking of $^{124}$I into TA muscle. (A) One group was i.v. injected with Na$^{124}$I (1.85 MBq), scarified two hours later. (B) The other was i.p. injected with NaI (57 mM). After 15 min, Na$^{124}$I (1.85 MBq) was i.v. injected, and the group was scarified two hours later.

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Figure 3. Bioluminescence, PET and ARG images.