Intravital Monitoring of Intact siRNA Using Fluorescence Resonance Energy Transfer

Kazuko Toh1, Takahiro Nomoto1, Yu Matsumoto1, Sumiyo Watanabe1, Shigeto Fukushima1, Hiroyuki Chaya2, R. James Christie2, Kanjiro Miyata2, Nobuhiro Nishiyama2, Kazunori Kataoka1

1The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
2Tokyo Institute of Technology, R11-11, 4259 Nagatsuta, Midori-ku, Yokohama 226-0033, Japan

toh@bmw.t.u-tokyo.ac.jp

ABSTRACT SUMMARY

In small interfering RNA (siRNA) therapy, it is necessary to deliver an adequate amount of intact siRNA to the target tissues and cells to achieve the desired therapeutic effect. Monitoring not only the distribution but also the intactness of siRNA in a living body is essential for developing efficient nucleic acid delivery systems. Here, we describe a new method to evaluate the intravital distribution and intactness of siRNA using a combination of fluorescence resonance energy transfer (FRET) and intravital imaging techniques. To demonstrate the usefulness of this technique, naked siRNA and polyion complexes were intravenously injected to mice, and siRNA intactness was evaluated. Naked siRNA showed rapid degradation and eventual extravasation into the surrounding tissues while the polyion complexes exhibited protective effect in the bloodstream, sustaining siRNA intactness. Our new method may illuminate new facets in nucleic acid delivery systems.

INTRODUCTION

Nucleic acid medicine attracts increasing attention as a new therapeutic drug for intractable diseases. However, the nucleic acid is subject to enzymatic digestion in the harsh in vivo condition, hampering its practical application [1]. Hence, an effective nucleic acid delivery system is necessary for successful therapeutic outcome. The amount of the nucleic acid delivered to the targeted tissues and cells has been evaluated by methods such as radioisotope or fluorescence labeling of the nucleic acids [2-4]. However, these methods cannot distinguish between the intact and degraded nucleic acids. In this regard, monitoring the intactness of siRNA has been previously described in in vitro studies using FRET techniques [5, 6]. The FRET between the donor-acceptor fluorescent dyes located on both 5’ and 3’ ends of the siRNA strand could be used as the indicator for the intactness.

We have recently developed the intravital real-time confocal laser scanning microscopy (IVRTCLSM) to explore the behavior of administered drugs in situ [7]. The IVRTCLSM provides instant histopathology of a living animal including bloodstream, kidney, liver, and tumor in simultaneous multicolor fluorescence. In this study, we propose a new method to evaluate the distribution and the intactness of siRNA in vivo by combining FRET and IVRTCLSM.

EXPERIMENTAL METHODS

1. Preparation of polyion complexes

TAMRA- and Cy5-labelled siRNA was used as a FRET pair. In vivo-jetPEI was prepared according to the manufacturer’s protocol. Poly(ethylene glycol)-b-poly(lysine) (PEG-PLys; MW of PEG: 42,000; polymerization degree of PLys segment: 18) and siRNA were dissolved separately in 10mM HEPES buffer (pH 7.3). PEG-PLys was added to siRNA solution. PEG-PLys/siRNA was prepared at the N/P ratio (molar ratio of amino groups in PLys to phosphate groups in siRNA) of 5.

2. In vitro study

siRNA was degraded thoroughly by RNase A. After the RNase A was inactivated with RNase inhibitor, intact siRNA was added at various mixing ratios. The fluorescence spectra of the mixtures were measured using a spectral detector equipped to the Nikon A1R confocal laser scanning microscope system. Fluorescent ratio of Cy5 to TAMRA (C/T ratio) was calculated to establish a calibration curve for assessing the degradation state of siRNA.

3. In vivo study

Naked siRNA and polyion complexes were intravenously injected, and the distribution and intactness of the siRNA were determined by observing the ear lobe dermis of mice using intravital spectral imaging. Cy5-labelled siRNA was used for the blood circulation study, and the TAMRA-Cy5-labelled siRNA was used for FRET study.

RESULTS AND DISCUSSION

1. C/T ratio as an indicator for the intactness of siRNA

Theoretical correlation between the C/T ratio (F) and the quantitative ratio of intact siRNA to the total amount (x) was estimated with the following equation:

\[ x = (a_d - b_d F)/(b_t + b_d F + a_d - a_t) \]

where \( a_t, a_d, b_t, b_d \) are defined as follows:

- \( a_t \): TAMRA intensity of intact siRNA
- \( a_d \): TAMRA intensity of degraded siRNA
- \( b_t \): Cy5 intensity of intact siRNA
- \( b_d \): Cy5 intensity of degraded siRNA

To confirm whether the actual values accorded with this theory, we measured the actual C/T ratio by mixing intact and disintegrated siRNA. As shown in Fig. 1, the theoretical values were in agreement with the actual values. This result suggests that the C/T ratio can represent the intactness of siRNA.
normalized blood circulation and extravasation values.

siRNA was computed by multiplying the ratio of intact siRNA (TAMRA) to the total siRNA. Here, the decay in the vasculature (Fig. 3) after injection demonstrates partial protection of siRNA (red lines in Fig. 3). The calculation allowed us to distinguish the intact and degraded siRNA, demonstrating no extravasation of intact siRNA. These results suggest that siRNA quickly degraded and the degraded siRNA extravasated into the surrounding tissue. Although in vivo-jetPEI also exhibited low C/T ratio in the extravascular tissue (Fig. 3E), in vivo-jetPEI showed longer circulating time with high C/T ratio, suggesting some improvements in siRNA protection (Fig. 3B). In contrast, the PEG-PLys polymer complexes displayed excellent retention of intact siRNA (Fig. 3C) with minimal extravasation (Fig. 3F), indicating sustained siRNA intactness and prolonged circulation time.

2. Intravital imaging of TAMRA-Cy5-labelled siRNA

Intravenously injected TAMRA-Cy5-labelled siRNA was imaged by IVRTCLSM, and the resulting C/T ratios were calculated in each pixel and expressed in rainbow scale (Fig. 2). At 15 min post injection, naked siRNA showed low C/T ratios at extravascular area, suggesting that siRNA was degraded and extravasated into the surrounding tissue. C/T ratios of In vivo-jetPEI were also low at extravascular area while there remained high C/T ratios in the vasculature. This suggests that In vivo-jetPEI partially protected siRNA in the circulation. PEG-PLys retained in the vasculature at high C/T ratios with minimal extravasation, demonstrating outstanding protection of siRNA.

Figure 2. C/T ratio pictures at 15 min post injection. C/T ratios are expressed in rainbow scale from blue (low) to pink (high). Arteries (narrow) and veins (wide) are traced with white dotted lines for better visualization of the extravasation.

3. Blood circulation and intact siRNA retention study

Fluorescent intensities of the Cy5-labelled siRNA in the veins and surrounding tissues were measured to obtain the blood circulation and extravasation curve (blue lines in Fig. 3). Blood circulation and extravasation values were normalized using the maximum fluorescent intensity in the vein of each injected sample (typically 30 seconds after injection). Cy5-labelled naked siRNA showed rapid decay in the vasculature (Fig. 3A) and the gradual rise in the extravascular tissue (Fig. 3D); however, these values cannot represent the amount of intact siRNA. Here, TAMRA-Cy5 labelled siRNA was used to calculate the ratio of intact siRNA (x). The relative amount of intact siRNA was computed by multiplying the x values to the normalized blood circulation and extravasation values.

Figure 3. Blood circulation and extravasation curves. (Blue line: sum of intact and degraded siRNA, red line: intact siRNA)

CONCLUSION

We have developed the new methodology that can distinguish the intact and degraded siRNA in the living animal, using FRET and IVRTCLSM. This technique revealed that the PEG-PLys polymer complexes efficiently protected intact siRNA and retained in the bloodstream, demonstrating the usefulness of this method for the siRNA delivery research.

REFERENCES


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