Influence of Autofluorescence Derived From Living Body on In Vivo Fluorescence Imaging Using Quantum Dots

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Quantum dots (QDs) are thought to be a novel inorganic probe for in vivo fluorescence imaging because of their excellent fluorescence properties. Autofluorescence is generally known to be produced from various living bodies including humans, rats, and mice. However, the influence of the autofluorescence on in vivo fluorescence imaging using QDs remains poorly understood. In this article, we assessed the autofluorescence derived from a mouse body and the influence of the autofluorescence on in vivo fluorescence imaging using QDs. The dorsal and ventral autofluorescence derived from a mouse from which the hair was removed were detected under all kinds of excitation/fluorescence filter settings (blue, green, yellow, red, deep red, and NIR) using the Maestro™ in vivo imaging system. The degree of autofluorescence was found to be extremely high in the red filter condition, but transplanted ASCs labeled with QDs on the back of a mouse could be detected in the red filter condition. Moreover, the ASCs labeled with QDs could be traced for at least 5 days. We suggest that fluorescence imaging using QDs can be useful for the detection of transplanted cells.

Key words: Autofluorescence; Quantum dots (QDs); In vivo imaging; Adipose tissue-derived stem cells (ASCs)

INTRODUCTION

Cell transplantation therapy has many advantages, such as easy operation and low invasiveness for patients, in comparison to whole organ transplantation (16). Hepatocyte transplantation has been known to be an effective therapy for treating patients with malignant liver disease. Islet transplantation for diabetic patients has been developed and already applied in clinical practice in many countries (11,15,20). Moreover, cell transplantation therapy using stem cells is an attractive method for the treatment of patients with severe diseases because this method can overcome the major problems associated with the cell source (21). However, the imaging technology available for tracing transplanted cells, including stem cells, is not sufficient for detecting on a cellular level.

Imaging technologies for tracing transplanted cells seem to become more and more important as regenerative medicine has developed. For example, X-ray computed tomography (3), magnetic resonance imaging (17), and ultrasonic sound wave diagnosis (6) have already been applied in a clinical setting; however, these methods cannot detect transplanted cell clusters smaller than

1 cm. Fluorescence imaging technology has been widely used for medical diagnosis and various biological studies and can be analyzed in association with the development of a computer-controlled display camera. Accordingly, fluorescence imaging has been expected to give a new method that can overcome the limitations of the conventional imaging technologies and trace transplanted cells on the cellular level because of the high sensitivity (2,9), although fluorescence imaging is not clinically available at present. However, the sensitivity is largely dependent on the association between the abilities of fluorescence probes (photostability, quantum yield, excitation, and fluorescence wavelength) and autofluorescence derived from a living body.

Quantum dots (QDs) are semiconductor nanocrystals that are composed of CdSe/ZnS-core/shell and are generally known as novel inorganic probes. QDs show many optically unique properties, such as superior photostability, high quantum yields, and narrow fluorescence spectra (8,14,23). QDs with near-infrared region (NIR) emission have received increasing attention as a material suitable for the application of in vivo imaging (4,10). We have

already revealed that QDs could label stem cells efficiently using cationic liposomes such as Lipofectamine® and cell penetrating peptides such as octa-arginine (R8). QDs did not affect the viability and differentiation potential of stem cells (18,19). In addition, QDs have been confirmed to be useful for in vivo fluorescence imaging of transplanted adipose-derived stem cells (ASCs) in mice (22). However, the association between fluorescence properties of QDs and autofluorescence of living bodies for in vivo imaging remains poorly understood.

In this study, we measured the autofluorescence of mice from which the hair was removed and investigated the influence of the autofluorescence on in vivo fluorescence imaging of transplanted ASCs labeled with QDs.

MATERIALS AND METHODS

Materials

QDs (Qdot ITK Carboxyl Quantum Dots; Invitrogen®), Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12; Gibco®), and Hank's balanced salt solution (HBSS; Gibco®) were purchased from Life Technologies™ (Grand Island, NY, USA). The octa-arginine peptide (R8) cell penetrating peptide and phosphate-buffered saline (PBS) were purchased from Sigma-Genosys® (Hokkaido, Japan). Fetal bovine serum (FBS, Biowest) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). BD Matrigel® was purchased from Becton, Dickinson and Company (Tokyo, Japan). Type I collagenase was purchased from Koken Co., Ltd. (Tokyo, Japan). All chemicals were reagent grade and used as received without further purification. Maestro™ (CRI Inc., Woburn, MA, USA) and Clairvivo OPT (Shimadzu, Kyoto, Japan) in vivo imaging systems were employed in this study.

Mice

C57BL/6 mice (7- to 14-month-old females and 6- to 8-month-old males) were purchased from SLC Japan. The mice were housed in a controlled environment (12-h light/dark cycles at 21°C) with free access to water and a standard chow diet before sacrifice. All conditions and handling of animals in this study were conducted with protocols approved by the Nagoya University Committee on Animal Use and Care.

Isolation and Culture of Mouse ASCs

The 7- to 14-month-old female C57BL/6 mice ($n=6$) were killed by cervical dislocation; adipose tissue specimens in the inguinal groove were isolated and washed extensively with HBSS or PBS to remove the blood cells. The isolated adipose tissue specimens were cut finely and digested with 1 ml of 1 mg/ml type I collagenase at 37°C in a shaking water bath for 45 min. The cells were filtrated using 250- μ m nylon cell strainers (BD Biosciences, Tokyo, Japan) and suspended in DMEM/F12 medium containing 20% FBS and 100 U/ml penicillin/streptomycin (Invitrogen Corporation, Auckland, NZ) culture medium. They were centrifuged at $270\times g$ for 5 min at room temperature, and ASCs were obtained from the pellet. They were washed three times by suspension and centrifugation in culture medium and then were incubated overnight in culture medium at 37°C with 5% CO₂. The primary cells were cultured for several days until they reached confluence and were defined as passage 0. The cells were used for the experiments between passages 2 and 5.

Labeling of ASCs Using QDs

QDs 655, 705, or 800 (emission peak at 655, 705, or 800 nm, respectively) and R8 were mixed for 20 min at room temperature at the optimal ratio (QDs/R8=1:10,000), and then R8-QD complexes were prepared. ASCs were incubated with the R8-QD complexes in a transduction medium (DMEM/F12, 2% FBS, 100 U/ml penicillin/streptomycin) at 37°C for 1 h. After 1 h of incubation, the transduction of QDs into ASCs was confirmed by conventional fluorescence microscopy (Olympus Corporation, Tokyo, Japan) and in vivo imaging systems.

Autofluorescence Images and Spectra of Hairless Mice

Hair was removed from 6- to 8-month-old male C57BL/6 mice ($n=3$) using depilatory cream (Reckitt Benckiser Japan Ltd., Tokyo, Japan). Then, the cream was rinsed away completely from the body by a little warm water. The dorsal and ventral autofluorescence derived from the hairless bodies of C57BL/6 mice was then measured under six kinds of excitation/fluorescence filters (blue, green, yellow, red, deep red, and NIR) using the Maestro™ in vivo imaging system (exposure time: 1 s). Excitation and fluorescence wavelengths (nm) of filter settings are shown in Table 1.

Table 1. Excitation and Fluorescence (Emission) Wavelength of Six Kinds of Filters

	Blue	Green	Yellow	Red	Deep red	NIR
Excitation wavelength (nm)	445–490	503–555	575–605	615–665	675–705	710–760
Fluorescence wavelength (nm)	515	580	645	700	750	800

NIR, near infrared.

Fluorescence Spectra of ASCs Labeled With QDs

ASCs (3.3×10^5 cells) labeled with QDs655, QDs705, or QDs800 (0.8 nM) with 100 μ l of transduction medium were prepared in the 1.5-ml tubes (Rikaken, Nagoya, Japan). Fluorescence spectra derived from QDs were measured under six kinds of excitation/fluorescence filters (blue, green, yellow, red, deep red, and NIR) using the MaestroTM in vivo imaging system (exposure time: 1 s).

In Vivo Fluorescence Imaging of ASCs Labeled With QDs

ASCs were labeled with QDs655, QDs755, or QDs800 (0.8 nM) using R8 in the same way. ASCs (1×10^5 cells) labeled with QDs were subcutaneously transplanted with 50 μ l PBS into the backs of the 6- to 8-month-old male C57BL/6 mice ($n=3$). Images were taken using the Clairvivo OPT in vivo imaging system shortly after cell transplantation (excitation: 635 nm at QDs655, 690 nm at QDs705, and 785 nm at QDs800; emission: 670 nm long pass at QDs655, 810 nm long pass at QDs705, and 845 nm long pass at QDs800; exposure time: 3 s).

Next, for the investigation of the effect of BD Matrigel[®] on cell behavior in vivo, ASCs (1×10^5 cells) labeled with QDs800 were subcutaneously transplanted with 50 μ l BD Matrigel[®] on the backs of the hairless mice ($n=3$). Images were taken using the MaestroTM in vivo imaging system in the same way at various times (10 min and 1, 2, 3, and 5 days after cell transplantation). Moreover, the normalized fluorescence intensity was compared between in PBS and BD Matrigel[®].

RESULTS

Dorsal Autofluorescence of Hairless Mice in Various Excitation and Fluorescence Wavelengths

The influence of excitation and fluorescence wavelengths on the dorsal autofluorescence of hairless mice was measured with six kinds of excitation/fluorescence filters using the MaestroTM in vivo imaging system. The dorsal autofluorescence of mice was detected under all filters and especially was detected under a red filter (Fig. 1A).

The autofluorescence intensity was high under blue, green, yellow, and red filters. On the other hand, the intensity was comparatively low under deep red and NIR filters (Fig. 1B).

Ventral Autofluorescence of Hairless Mice in Various Excitation and Fluorescence Wavelengths

The influence of excitation and fluorescence wavelength on ventral autofluorescence of hairless mice was measured with six kinds of excitation/fluorescence filter sets using the MaestroTM in vivo imaging system. The ventral autofluorescence of mouse was also detected in all filters (Fig. 2A).

The autofluorescence intensity was higher than that in dorsal orientation except for under an NIR filter. The

autofluorescence intensity under a red filter became markedly elevated. However, using an NIR filter, the lower level of autofluorescence intensity was maintained (Fig. 2B). These data suggested that the NIR filter was appropriate for in vivo fluorescence imaging.

Fluorescence Spectra of QDs in Various Excitation and Fluorescence Wavelengths

The fluorescence spectra of ASCs labeled with QDs655, 705, and 800 using R8 under six kinds of filters are shown (Fig. 3). The fluorescence intensity of QDs655 was the highest of the three kinds of QDs under the same excitation wavelength. On the other hand, the fluorescence intensity of QDs was higher as the excitation wavelength became shorter. These data suggested that QDs655 was efficient for in vitro fluorescence detection of ASCs among the three kinds of QDs tested.

Influence of Autofluorescence of Mice on Fluorescence In Vivo Imaging

To assess the influence of autofluorescence of mice on fluorescence imaging of ASCs labeled with QDs655, 705, or 800, the labeled ASCs were subcutaneously transplanted on the backs of mice from which all hair was removed (Fig. 4A). The transplanted ASCs labeled with QDs655, 705, and 800 could be detected at the same time (excitation: 635 nm, emission: 670 nm long pass), and almost no autofluorescence derived from mouse bodies could be detected. On the other hand, transplanted ASCs labeled with QDs800 could be detected under both filters (excitation: 690 and 785 nm, emission: 810 and 845 nm long pass) (Fig. 4B). These data suggested that QDs were useful for the labeling and detection of transplanted cells.

Detection of ASCs Labeled With QDs After Subcutaneous Transplantation

To check the effect of BD Matrigel[®] on cell behavior in vivo, ASCs labeled with QDs800 were subcutaneously transplanted with 50 μ l BD Matrigel[®] or PBS on the backs of hairless mice (Fig. 5A-a). The fluorescence derived from ASCs labeled with QDs800 could be detected for at least 5 days in both conditions. However, the fluorescence intensity in the BD Matrigel[®] condition decreased gradually over 5 days (Fig. 5A-b). Moreover, when the normalized fluorescence intensity was measured, the fluorescence intensity in BD Matrigel[®] showed about half the level of that in PBS at 5 days (Fig. 5B). These data suggested that ASCs in BD Matrigel[®] could infiltrate into physiological tissue earlier than in PBS.

DISCUSSION

In vivo fluorescence imaging technology is expected to detect the behavior of transplanted cells and reveal the accumulation of the cells in various organs and tissues

A **B**

Figure 1. Dorsal autofluorescence derived from a hairless mouse. (A) The dorsal autofluorescence images derived from a hairless mouse under six kinds of filters (a: blue, b: green, c: yellow, d: red, e: deep red, and f: NIR). (B) The fluorescence spectra of the dorsal autofluorescence derived from a hairless mouse under six kinds of filters.

for the progress of cell transplantation therapy and regenerative medicine. However, it is well known that almost all living bodies have autofluorescence derived from various biological molecules included in the living bodies (1,7,13). Thus, the influence of the autofluorescence caused by living bodies on in vivo fluorescence imaging technology should be carefully taken into account.

In this study, autofluorescence derived from hairless mice was investigated using the Maestro™ imaging system in various excitation and emission wavelength combinations with six kinds of filters. Dorsal and ventral

autofluorescence derived from hairless mice were detected in all filters, and the degree under a red filter was very high. The autofluorescence derived from liver and large intestine in the living body is thought to be a primary cause of these results. Liver tissue is known to include various biomolecules yielding fluorescence (5,12). Moreover, the normal feed for mice is known to contain fluorescent substances, so the feces in the large intestine caused the strong fluorescence. In fact, little autofluorescence derived from the large intestine of the mouse could be detected, as they were given alfalfa-free feed that did not include any

A **B**

Figure 2. Ventral autofluorescence derived from a hairless mouse. (A) The ventral autofluorescence images derived from a hairless mouse under six kinds of filters (a: blue, b: green, c: yellow, d: red, e: deep red, and f: NIR). (B) The fluorescence spectra of the ventral autofluorescence derived from a hairless mouse under six kinds of filters. The intensity derived from a hairless mouse under a red filter was very high and exceeded the upper limit.

fluorescence substances at all (12). However, the degrees of autofluorescence under deep red and NIR filters were low; thus deep red and NIR filters have been proven to be useful for in vivo fluorescence imaging.

QDs have excellent fluorescence properties, such as superior photostability, high quantum yields, and narrow fluorescence spectra (8,14). In addition, QDs with NIR emission suitable for in vivo imaging have been developed and made commercially available. We assessed the labeling ability of QDs655, QDs705, and QDs800 to ASCs transplanted into the backs of mice. All kinds

of transplanted ASCs labeled with QDs655, QDs705, and QDs800 could be detected efficiently because of the strong fluorescence of QDs, even though the excitation and emission wavelength was in the red filter region. However, the comparatively weak autofluorescence derived from the mice was detected when the excitation wavelength was 635 nm. Therefore, QDs under NIR emission has been confirmed to be the most efficient for in vivo fluorescence imaging.

We investigated whether the invasive difference of transplanted ASCs labeled with QDs800 between in PBS

Figure 3. Fluorescence spectra of QDs under six kinds of filters. (A) Excitation (upper) and fluorescence (lower) wavelength of six kinds of filters (blue, green, yellow, red, deep red, and NIR). (B) The fluorescence spectra of quantum dots 655 (QDs655), 705, and 800 under six kinds of filters.

and BD Matrigel® took place in mice. The transplanted ASCs could be clearly detected without differences in both conditions. However, the fluorescence intensity in the BD Matrigel® condition was markedly decreased in comparison to that in the PBS condition. No differences between two groups could be detected without the transplantation operation (data not shown). Thus, these data suggest

that the ASCs suspended in BD Matrigel® may have the potential to invade into the subcutaneous tissue compared to the ASCs suspended in PBS.

In conclusion, we herein investigated the autofluorescence derived from a mouse body and the influence on in vivo fluorescence imaging by using QDs. The dorsal and ventral autofluorescence derived from a hairless mouse

Figure 4. Influence of autofluorescence of mouse on fluorescence in vivo imaging. (A) The pattern diagram of subcutaneous transplantation of ASCs labeled with QDs655, 705, and 800 on the back of a hairless mouse (upper: ASCs labeled with QDs655, middle: ASCs labeled with QDs705, bottom: ASCs labeled with QDs800). (B) The fluorescence images of ASCs labeled with QDs655, 705, and 800 under three kinds of filters (a: excitation: 635 nm, emission: 670 nm; b: excitation: 690 nm, emission: 810 nm; c: excitation: 785 nm, emission: 845 nm). The fluorescence detected in the left side of the back of the mouse is autofluorescence.

Figure 5. Detection of ASCs labeled with QDs800 after transplantation. (A) The pattern diagram of subcutaneous transplantation of ASCs labeled with QDs800 in PBS or BD Matrigel® on the back of a hairless mouse (a). The fluorescence images of the time course of ASCs labeled with QDs800 in PBS or BD Matrigel® on the back of a hairless mouse (b). (B) The graph of the time course of ASCs labeled with QDs800 in PBS or BD Matrigel® on the back of a hairless mouse.

were detected with all kinds of excitation/fluorescence filters (blue, green, yellow, red, deep red, and NIR) using the Maestro™ in vivo imaging system. The degree of autofluorescence was found to be extremely high under a red filter and extremely low under deep red and NIR filters. The transplanted ASCs labeled with QDs655 showing red fluorescence on the back of a mouse could be detected despite using a red filter. Moreover, the transplanted ASCs could be detected efficiently for at least 5 days using QDs800 showing NIR fluorescence. Our findings suggest that fluorescence imaging using QDs can be useful for labeling and detection of transplanted cells.

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