In Vivo Fluorescence Visualization of Anterior Chamber Injected Human Corneal Endothelial Cells Labeled With Quantum Dots

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Purpose. The injection of cultured human corneal endothelial cells (cHCECs) into the anterior chamber (AC) is a newly developed modality for the successful treatment of corneal endothelium dysfunction. Here, we investigated whether or not cHCECs could be labeled using quantum dots (QDs) composed of semiconductor nanoparticle octa-arginine (R8) to trace injected cHCECs and examined the utility of in vivo fluorescence imaging to analyze the dynamics and accumulation of QD-labeled injected cHCECs in a corneal endothelial dysfunction mouse model.

Methods. The cHCECs, either of high quality or with cell-state transition, were labeled by adding a mixture of QD655 and R8. The labeling efficiency and the unchanging of the cell phenotypes by the labeling was confirmed by flow cytometry. The labeled cHCECs were injected into the AC of either healthy mice or mice with corneal endothelium damaged by cryogenic treatment. The kinetics of the injected cHCECs was traced quantitatively via multiphoton confocal laser microscopy.

Results. QD labeling induced no morphologic change in the cHCECs or in the expression of the functional markers of cHCECs (i.e., Na+/K+-ATPase and zonula occludens-1). The injected cHCECs-QDs were quantitatively detected, and the retention of cHCECs-QDs was evident, from 3 to 48 hours post cell injection on the posterior surface in the cryogenically injured corneal endothelial mouse model eyes, yet not in the noninjured healthy control eyes.

Conclusions. The findings of this study show that in the field of regenerative medicine, QD labeling of cells presents a convenient and sensitive method of finely monitoring the fate of injected cells in vivo.

Keywords: quantum dots (QDs), in vivo fluorescence imaging, cultured human corneal endothelial cells (cHCECs)

It is widely known that hexagonal, cobblestone-shaped corneal endothelial cells (CECs) exist in the corneal endothelium, and that they play an essential role in the maintenance of corneal transparency. Moreover, human CECs (HCECs) reportedly have several functions in the maintenance of corneal transparency, such as acting as a barrier between the aqueous humor and corneal stroma and exerting Na+/K+-ATPase activities that regulate corneal hydration.1,2 Since the proliferative potential of HCECs is limited,3–5 severe damage to the corneal endothelium due to pathological conditions such as Fuchs endothelial corneal dystrophy (FECD) leads to corneal endothelial dysfunction and the loss of corneal transparency, and can ultimately lead to loss of vision.6–8 Reportedly, corneal endothelial dysfunction is mainly caused by various pathologic conditions, as well as by surgeries such as laser iridotomy and those for the treatment of cataracts and glaucoma.9 When corneal endothelium damage is severe, keratoplasty is performed to restore the function of the damaged tissue.

The direct injection of cultured HCECs (cHCECs) onto the posterior cornea via cell injection into the anterior chamber (AC) has been considered an ideal surgical method for reconstituting the corneal endothelial layer in patients afflicted with severe endothelial dysfunction. Our group as well as others have been investigating the possibility of cHCEC injection in the form of a cell suspension.10–16 Koizumi et al.13 developed a new corneal cell injection method involving
the intraocular injection of substrate-free cHCECs, and the efficacy of those cells has been confirmed in a cell injection study using a cynomolgus monkey model of corneal endothelial dysfunction. In a recent study by Kinoshita et al.,17 the authors reported the successful in vitro expansion of cHCECs and the restoration of functional corneas via the injection of cHCECs in suspension, and the findings in that study revealed that at 24 weeks post cell injection, the ECD density exceeded 1000 cells/mm² in 10 of the 11 treated eyes; there was also an improvement in the best-corrected visual acuity of more than two lines in 9 of the 11 treated eyes.

However, it should be noted that the dynamics and accumulation of the transplanted cHCECs remain poorly understood. At present, several imaging technologies are used to examine and monitor the structures of the cornea, and it has been reported that specular microscopy, optical coherence tomography (OCT), and in vivo confocal microscopy (IVCM) are frequently used to monitor and diagnose corneal endothelial dystrophy.18–22 Fourier-domain OCT (FD-OCT) is reportedly useful for characterizing and measuring the Descemet’s membrane and cornea guttae for the pre- and postoperative monitoring of patients with FECD.23,24 Moreover, specular microscopy and IVCM are commonly used by ophthalmologists to monitor and diagnose the CEC density and morphology in patients with FECD.25 In addition, ex vivo imaging methods, such as staining with Alizarin Red S and trypan blue,26 organic fluorescence dyes,27 and scanning electron microscopy,28,29 have also been used. However, it is very difficult to confirm the dynamics and the state of the accumulation of cHCECs injected into the AC via the use of these methods.

Quantum dots (QDs) composed of semiconductor nanoparticles are inorganic fluorescence probes with many distinctive fluorescence properties, such as high luminescence, high quantum yields, superior photostability (long-term labeling), and wide excitation wavelengths.30,31 Due to these optical characteristics, QDs have received a great deal of attention as fluorescence probes for not only biomolecules, but also the cells in living organisms, including stem cells. We developed a QD labeling method for living cells using octaarginine (R8), a cell-penetrating peptide,32 and confirmed its utility for the in vivo imaging of injected cells labeled with QDs in mice.

In this present study, to overcome issues with tracing injected cHCECs, we investigated whether or not cHCECs could be labeled by QDs using R8 and assessed the influence of QD labeling on the phenotype of cHCECs. In addition, we investigated the utility of in vivo and ex vivo fluorescence imaging to analyze the dynamics and accumulation of injected cHCECs labeled with QDs in a corneal endothelial dysfunction mouse model.

## MATERIALS AND METHODS

### Isolation of HCECs

The human tissue used in this study was handled in accordance with the tenets set forth in the Declaration of Helsinki. HCECs were obtained from two human donor corneas and were cultured prior to undergoing karyotyping analysis. Human donor corneas were obtained from CorneaGen, Inc. (Seattle, WA, USA). Informed written consent for eye donation for deceased donors was obtained. All tissues were recovered under the tenets of the Uniform Anatomical Gift Act (UAGA) of the particular state in which the donor consent was obtained and the tissue was recovered. The age of the donors was 22 and 26 years, and the endothelial cell density (ECD) of the obtained donor corneas was 3528 and 3202 cells/mm², respectively.

The two donor corneas were preserved in Optisol-GS storage medium (Chiron Vision, Inc., Irvine, CA, USA) and imported via international air transport for research purposes. Donor information accompanying the donor corneas showed that they were considered healthy and free of any corneal disease, with no history of chromosomal abnormality.

### Cell Culture of HCECs

HCECs obtained from two donor corneas were cultured according to the previously published protocols, yet with some modifications.33 Briefly, Descemet’s membranes with CECs were stripped from the donor corneas and digested for 2 hours at 37°C with 1 mg/mL collagenase A (Roche Applied Science, Penzberg, Germany). The stripped HCECs were then seeded in one well of a type 1 collagen–coated six-well plate (Corning, Inc., Corning, NY, USA). Culture medium was then prepared according to the previously published protocols. The HCECs were then cultured at 37°C in a humidified atmosphere containing 5% CO₂ and the culture medium was changed twice per week. Once the chCECs had reached confluence, they were passaged at a density of 800 cells/mm² after treatment with 10× TrypLE Select (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 12 minutes at 37°C. The chCECs at passage 2 were used for all experiments.

### Flow Cytometry Analyses of the HCECs

The chCECs were collected from the culture dish via the use of TrypLE Select treatment, as described above, and then suspended at 4 × 10⁶ cells/mL in flow cytometry (FACS) buffer (phosphate-buffered saline [PBS] containing 0.5% bovine serum albumin [BSA] and 0.05% NaN₃). Next, an equal volume of antibody solution was added, and then incubated for 2 hours at 4°C. The antibody solutions were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-human CD26 mAb, PE-conjugated anti-human CD166 mAb, PerCP-Cy 5.5-conjugated anti-human CD24 mAb, and PE-Cy 7-conjugated anti-human CD44 (all from BD Biosciences, San Jose, CA, USA), and APC-conjugated anti-human CD105 (eBioscience, Inc., San Diego, CA, USA). After washing with FACS buffer, the chCECs were analyzed via the use of the BD FACS Canto II Flow Cytometry System (BD Biosciences).

### QD Labeling of chCECs Using R8

We previously reported a method for transducing QDs with R8 into stem cells. Briefly, QD655 (Qdot 655 Carboxylate Quantum Dots with emission at 655 nm; Thermo Fisher Scientific, Inc.) (8.0 nM) and R8 (Sigma Aldrich Japan, Tokyo, Japan) (80 μM) were mixed in the transduction medium (Opti-MEM I; Thermo Fisher Scientific, Inc.), 2% fetal bovine serum, 5 ng/mL epidermal growth factor, 20 μg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, and 50 μg/mL gentamicin for 4 hours at 37°C, followed by the medium then being added into the chCEC culture dish. Next, the chCECs were cultured for 24 hours at 37°C, and then washed three times with the transduction medium. The chCECs labeled with QD655 (chCECs-QDs655) were then counted and prepared for their functional analysis or in vivo fluorescence imaging of the transplanted chCECs. The chCECs-QDs655 were observed via fluorescence microscopy (BZ-X700; Keyence Corporation, Osaka, Japan) and high-speed multiphoton confocal laser microscopy (A1MP/A1RMP; Nikon Corporation, Tokyo, Japan). The labeling efficiency and fluorescence intensity were evaluated using flow cytometry.
Quantum Dots Labeling Visualize the Fate of cHCEC

Cytotoxicity of QDs to cHCECs

Cultured HCECs (1 × 10^4 cells) were seeded in a 96-well plate (BD Falcon; BD Biosciences, Tokyo, Japan) with 100 μl culture medium, and were then cultured for 24 hours. The cells were labeled with QDs655 (cHCECs-QDs655; 0.8, 2.0, 4.0, and 8.0 nM) using R8 (0.8, 2.0, 4.0, and 8.0 μM). After a 4-hour transduction, the cHCECs were washed twice with a transduction medium and then incubated for 24 hours. The viable cells were then counted using a cell counting kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan). CCK-8 reagent (Dojindo Laboratories) (10 μL) was added to each well, and the reaction was then allowed to proceed for 2 hours. The absorbance of the sample at 450 nm was measured against a background control using a microplate reader (PolarStar OPTIMA; BMG Labtech GmbH, Ortenberg, Germany).

Immunocytochemical Staining

For immunocytochemical staining, the cHCECs and cHCECs-QDs655 were fixed with ice-cold methanol for 10 minutes, and then permeabilized with PBS containing 0.1% Triton X-100 surfactant (Sigma-Aldrich Corp., St. Louis, MO, USA) at room temperature for 15 minutes, respectively. After the blocking of nonspecific reactivity with 1% BSA in PBS for 1 hour at room temperature, Na/K-ATPase and zonula occludens-1 (ZO-1) staining was performed with 2 μg/mL mouse anti-Na/K-ATPase Ab (EMD Millipore Corporation, Temecula, CA, USA) and rabbit anti-human ZO-1 Ab (Thermo Fisher Scientific, Inc.) followed by Alexa Fluor 594-conjugated anti-mouse IgG Ab and Alexa Fluor 488-conjugated anti-rabbit IgG Ab (Thermo Fisher Scientific, Inc.). After washing with PBS, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories) and observed under a fluorescence microscope (BZ-X700 or BZ-9000; Keyence Corporation).

Animals Used in the Experiments

Male C57BL/6 (H-2b) mice (SLC, Osaka, Japan) between 8 and 12 weeks of age were used in the experiments. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine, Kyoto, Japan. Prior to any surgical procedures being performed, all animals were deeply anesthetized with an intraperitoneal injection of ketamine (3 mg) and xylazine (0.15 mg).

Cryogenic Treatment and HCEC Injection Into the AC of the Eye

In the right eye of each mouse, cryogenic treatment was performed to destroy the majority of the CECs. Briefly, after the topical application of a mydriatic agent (Mydrin P; Santen Pharmaceutical Co., Ltd., Osaka, Japan), transcorneal freezing was performed by gently placing a stainless steel cryoprobe (2 mm in diameter) precooled to −196°C with liquid nitrogen on the central cornea (cryogenic treatment). To avoid any damage to the adjacent tissue, including the lens and the trabecular meshwork, no physical pressure was applied. The cryoprobe was then kept on the corneal surface until an ice ball formed on the cornea and covered the entire corneal surface (i.e., approximately 10 seconds), as mentioned elsewhere. Immediately after freezing, the cryoprobe was freed from the corneal surface via irrigation with balanced salt solution (BSS) and the cornea was allowed to thaw naturally. No topical medication was applied during the study period.

After destruction of the CECs via a cryogenic treatment, an oblique incision at the paracentral cornea of host C57BL/6 mice was made using a microknife, and 3 μL aqueous humor was extracted using a glass needle. BSS (3 μL) was then injected, and the same amount was extracted to wash the AC. HCECs (2 × 10^4 cells) in 5 μL glucose bicarbonate intraocular irrigation solution (Opeguard MA; Senju Pharmaceutical Co., Ltd., Osaka, Japan) supplemented with 0.024% human serum albumin, 1.06 mM ascorbic acid, 4.5 mM lactic acid, and 100 μM Rho-associated protein kinase (ROCK) inhibitor Y-27632 were then injected into the AC of the right eyes of the mice with or without cryogenic treatment. The same volume of the vehicle without HCECs was also injected into the AC of the right eyes of the control mice with or without cryogenic treatment without any leakage. Post injection, the following four groups were then examined: vehicle injected into the normal mice (i) and into the cryogenically treated mice (ii), and cHCECs-QDs655 injected into the normal mice (iii) and into the cryogenically treated mice (iv). It should be noted that Opeguard MA is widely used in the clinical setting as a perfusate, and that the three above-described supplements that were used, that is, albumin, ascorbic acid, and lactic acid, are the primary components of the aqueous humor.

The cell numbers were calculated based on the number of cells in 300 μL solution containing cHCECs (0.5–1 × 10^6 cells) injected into human cases. The mice were then kept in a cornea side-down position (i.e., similar to the facedown [prone] position used in the human cases) for 3 hours, with an additional 1 mg ketamine being injected once every hour to encourage the precipitation of HCECs onto the Descemet’s membrane at the posterior surface of the cornea.

In Vivo and Ex Vivo Fluorescence Imaging of cHCECs-QDs in the Mice With Cryogenic Treatment

For the in vivo fluorescence imaging studies, we prepared C57BL/6 mice given nonfluorescence feed without alfalfa (alfalfa-free feed) (Japan SLIC, Inc., Hamamatsu, Japan) for 1 week in order to diminish the influence of the autogenic fluorescence in the mice. The right eyes of the mice were then subjected to cryogenic treatment (i.e., −196°C in liquid nitrogen). After 2 hours, cHCECs-QDs655 (2 × 10^4 cells/3 μL) were injected into the AC of the right eye. The mice were then anesthetized and monitored at multiple time points (i.e., at 3, 24, and 48 hours) using the IVIS Lumina K Series III imaging system (PerkinElmer, Inc., Waltham, MA, USA) with an excitation filter of 640 ± 15 nm and an emission filter of 680 ± 10 nm.

For the ex vivo fluorescence imaging investigations, both eyes and five major organs (heart, lungs, kidneys, spleen, and liver) were harvested from each mouse, and then immediately subjected to fluorescence imaging using the IVIS Lumina K Series III imaging system. The measurement conditions used were the same as those used for the in vivo imaging described above. The region of interest (ROI) was measured with the assistance of the IVIS Lumina K Series III imaging system. The IVIS System, including IVIS Spectrum Lumina K Series III, is an expandable, sensitive imaging system that is easy to use for fluorescence and bioluminescence imaging in vivo at video-speed rate. This system includes a highly sensitive charge-coupled device (CCD) camera, light-tight imaging chamber, and complete automation and analysis capabilities. As the leading optical imaging platform for in vivo analysis, IVIS Systems include a range of practical accessories developed through experience in research laboratories worldwide.
Quantum Dots Labeling Visualize the Fate of cHCEC

The data were represented as the ratio of fluorescence intensity (RFI), which equals the fluorescence intensity of the eye after each injection of cHCECs-QDs655 divided by the fluorescence intensity of the eye at 3, 24, and 48 hours post injection of cHCECs-QDs655.

Examination of the cHCECs-QDs in the Tissues and Organs

The tissues and organs (eyes, heart, lungs, kidneys, spleen, and liver) extracted from the body of each euthanized mouse were dipped in PBS (1 mL) with Hoechst33342 (1 μL) overnight in order to dye the cell nuclei. These tissues and organs were then placed on a 35-μm glass-bottom dish and observed using high-speed multiphoton confocal laser microscopy (A1MP²/A1RMP²) (Hoechst33342: excitation: 402.5 nm, emission: 425–475 nm; QDs655: excitation: 402.5 nm, emission: 663–738 nm). The eyes were then observed from two different directions (front and side view). In addition, the lungs and liver were sliced at a thickness of approximately 1 cm, with all areas of each slice then being observed.

Statistical Analyses

In regard to the statistical analyses, the numerical values are presented as the mean ± standard deviation (SD). Each experiment was repeated in three different animals. Statistical significance was evaluated using an unpaired Student’s t-test for comparisons between two groups; P values of <0.05 were considered statistically significant. Statistical Package for the Social Sciences (SPSS) (IBM SPSS Statistics; IBM Corporation, Armonk, NY, USA) software was used for all of the statistical analyses.

To confirm quantitatively the different accumulation in the eyes with or without cryogenic treatment, a statistical analysis was performed using the SPSS version 14.0 software package for Windows. For the multiple-group analysis, the homogeneity of variance was assessed by Levene’s test for equality of variances. Parametric comparisons were made via the use of analysis of variance (ANOVA). If the results of the ANOVA were significant, Student’s t-test was used to evaluate the significance of the individual differences.

RESULTS

Isolation and Identification of HCECs

For a convenient visible understanding of the methods used for the isolation and culture methods of the HCECs, a schema is illustrated in Figure 1a. Both sets of cHCECs exhibited hexagonal morphology showing a fully differentiated mature HCEC subpopulation (SP) under phase-contrast microscopy examination (Figs. 1b, 1c). In our previous reports, we defined the cHCEC SP with CD166⁺CD24⁺CD44⁰/lowCD105⁰CD26⁻ as effector cells that ensure a safe and stable cell injection therapy as an innovative and novel “regenerative medicine” treatment modality.³⁻⁷⁻⁵³ Cultured HCEC lots A and B showed high expression (approximately 90% and 80%, respectively) of CD166⁺, CD24⁺, CD105⁺, CD44⁻/low, and CD26⁻, and low expression (approximately 10% and 20%, respectively) of the CD44⁻/low SP (Figs. 1d-g). In this study, HCEC lot A was used for the subsequent experiments.

Examination of cHCECs Labeled With QDs

A schema of the experimental protocol for the labeling of the cHCECs by QDs is shown in Figure 2a. Red fluorescence derived from QDs655 in cHCECs was strongly observed for at least 2 days after labeling by a fluorescence microscope (BZ-X700) (Fig. 2b). To identify the location of QDs655 in the cHCECs, cHCECs were observed via high-speed multiphoton confocal laser microscopy (A1MP²/A1RMP²). QDs655 was confirmed to have transduced into almost all of the cHCECs, and was located in the cytoplasm (Figs. 2c–e). The labeling efficiency of QDs655 was 97.4% at day 0 and 99.4% at day 2 after 4-hour transduction (Figs. 2f, 2g).

In addition, to investigate the effect of the transduction time of QDs on the efficiency of cHCEC labeling, the labeling efficiency of QDs655 after 24 hours of transduction was estimated. The labeling efficiency at day 0 after 4- and 24-hour transduction was 96.0% and 94.1%, respectively (Supplementary Figs. S1a–c).

Cytotoxicity of QDs to cHCECs

To investigate the cytotoxicity of QDs655 to cHCECs, cHCECs labeled with QDs655 (cHCECs-QDs655; 0.8, 2.0, 4.0, and 8.0 nM) using R8 (0.8, 2.0, 4.0, and 8.0 μM) with 4-hour transduction were incubated for 24 hours at 37°C. No remarkable cytotoxicity was observed with <8.0 nM QDs655 (Fig. 2h). Similarly, no cytotoxicity was confirmed with 24-hour transduction (Supplementary Fig. S1d), and the morphology and fluorescence images were confirmed to have not changed by fluorescence microscopy (BZ-X700) (data not shown). These findings suggest that cHCECs can be labeled with QDs655 at high efficiency without cytotoxicity.

Properties of cHCECs Labeled With QDs

To investigate the influence of QD labeling on the function of cHCECs, the expression of CD44, Na⁺/K⁺-ATPase, and ZO-1, well-known functional markers of cHCECs,³⁻¹⁻³⁻¹ was evaluated using flow cytometry analyses and immunocytochemical staining. The mean fluorescence intensity of CD44 expression was 1549 in the nonlabeled cHCECs and 1688 in the QDs655-labeled cHCECs. No significant changes in the CD44 expression were observed due to QDs655 labeling (Fig. 3a). In addition, the expression of Na⁺/K⁺-ATPase (red fluorescence) and ZO-1 (green fluorescence) in the cHCECs labeled with QDs655 was also observed by fluorescence microscopy (BZ-X700) (Fig. 3b), whereas no fluorescence was detected in isotype controls of Na⁺/K⁺-ATPase and ZO-1 (Fig. 3c). These findings suggest that QD labeling did not affect the properties of the cHCECs.

In Vivo Fluorescence Imaging of Injected cHCECs-QDs

The experimental design of the in vivo/ex vivo fluorescence imaging used in this study is shown in Figure 4a, and the four groupings described above are illustrated in Figure 4b. No fluorescence was detected in the right eyes of the control mice (i.e., with cryogenic treatment only) (Fig. 4c). These findings suggest that the injected cHCECs-QDs655 were clearly detected by an in vivo fluorescence imaging system (i.e., the IVIS Lumina K Series III imaging system).

Time Course of Transplanted cHCECs in the Right Eye

To investigate the time course of injected cHCECs, the fluorescence derived from cHCECs-QDs655 in mice with or without cryogenic treatment was observed at 5, 24, and 48 hours post injection. The fluorescence of cHCECs-QDs655 in the enlarged view of the eye region (Fig. 4c, white-dotted
rectangle) of the mice was detected for at least 48 hours post injection, regardless of the cryogenic treatment. However, decreases in the fluorescence intensity were observed in both cell-injected groups with and without cryogenic treatment (Figs. 4d iii, 4d iv).

To assess the fluorescence intensity derived from cHCECs-QDs655, the obtained eyes were observed. The fluorescence of the obtained right eyes of mice with cryogenic treatment at 48 hours post cell injection was stronger than that of the mice without cryogenic treatment (Fig. 4e). The fluorescence intensity on ex vivo fluorescence imaging appeared to be almost equal to the real reflection of the QDs655 retained inside the eyes, due to the high optical transparency and the low autofluorescence of the eyes. Therefore, an ROI analysis was performed on the ex vivo fluorescence images in order to semiquantitatively analyze the accumulation rate of QDs655. The fluorescence intensity of mice without cryogenic treatment was lower than that of the mice with cryogenic treatment (Fig. 4f). The RFI ([fluorescence intensity at each hour / fluorescence intensity at 3 hours] × 100%) of the injected cHCECs in the mice without cryogenic treatment was approximately 15% at 24 hours and approximately 8% at 48 hours. In contrast, the RFI of the injected cHCECs in the mice with cryogenic treatment was approximately 34% at 24 hours and approximately 21% at 48 hours (Fig. 4g). These findings suggest that injected cHCECs accumulated in the eyes with cryogenic treatment at a higher efficiency than in the eyes without cryogenic treatment.

**Examination of the cHCECs-QDs in Right Eyes**

The above-described method used for the examination of the cHCECs-QDs in the right eyes of the mice is illustrated in Figures 5a through 5c. When the eyes obtained from the mice cryogenically treated at 3 hours post cHCEC injection were observed from the front, the blue fluorescence derived from the corneal cells stained with Hoechst33342 was confirmed (Figs. 5d i, 5d ii), and the red fluorescence derived from the injected cHCECs-QDs655 was confirmed in deeper parts than in the corneal cells (Figs. 5d iii, 5d iv). To verify the state of the
FIGURE 2. The labeling of cHCECs by quantum dots. Schematic diagram of the experimental protocol for the labeling of cHCECs by QDs and the analysis of the labeling efficiency (a). The phase and fluorescence images of nonlabeled and labeled cHCECs by QDs655 (8.0 nM) at day 0 and day 2 after the labeling (b). Confocal microscopy images of cHCECs labeled with QDs655 (cHCECs-QDs655) (c). The cell nuclei were labeled with Hoechst33342 (blue fluorescence). A 3-D confocal image of cHCECs-QDs655 (d). High-magnification 3-D image of the cHCECs-QDs655 shown in (d, e). Flow cytometry analysis of the labeling efficiency of cHCECs-QDs655 at day 0 (f) and day 2 (g) after the labeling. Cytotoxicity of QDs655 to cHCECs at 24 hours after 4-hour labeling (h). Data shown as the mean ± SD.
accumulation of cHCECs-QDs655 injected into the AC, the eyes were observed from the side. The red fluorescence derived from cHCECs-QDs655 was observed along the inside of the blue fluorescence derived from corneal cells uniformly (Figs. 5e, 5f), whereas no fluorescence derived from cHCECs-QDs655 was observed in the vitreous body, retina, lower choroid, or sclera area (Figs. 5g–i). No red fluorescence was observed in the eyes obtained from the mice cryogenically treated without cHCECs-QDs655 injection (Fig. 5j). These findings suggest that the injected cHCECs attached to the cryogenically treated posterior corneal surface (i.e., damaged corneal endothelium) within 3 hours.

Next, we investigated the time course of the accumulation state of the injected cHCECs. The red fluorescence derived from cHCECs-QDs655 gradually decreased at 24 and 48 hours, yet the fluorescence was detectable for at least 48 hours post injection and the degree of fluorescence was significantly higher than that in the normal mice (Figs. 5k, 5l). In addition, the accumulation of injected cHCECs-QDs655 was observed from the front of the boundary between the cornea and the AC at 30 μm intervals (Supplementary Fig. S2) as well as in three-dimensional (3-D) confocal images of corneal cells and injected cHCECs-QDs655 (Figs. 6a–d). These findings suggest that the cHCECs injected into the AC of the eye in cryogenically treated mice accumulated in the area of the damaged corneal endothelium.

Examination of the cHCECs Labeled With QDs in Organs

Five organs (lungs, heart, liver, spleen, kidneys) in which cHCECs not fixed to corneal endothelium were predicted to accumulate were isolated and examined. In the cryogenically treated mice, the red fluorescence derived from cHCECs-QDs655 was observed only in the lungs (Fig. 7a) (observation data for the kidney and heart are not shown). In contrast, in the normal mice, the red fluorescence derived from cHCECs-QDs655 was observed not only in the lungs but also in the liver, while no fluorescence was observed in the other three
FIGURE 4. In vivo fluorescence imaging of the mice with or without cryogenic treatment post injection of cHCECs-QDs655. Schematic diagram of the experimental protocol for in vivo fluorescence imaging of mice in four groups at 3, 24, and 48 hours post injection of cHCECs-QDs655 (a). The four groups (i–iv) are shown as follows: (i) normal, (ii) cryogenic treatment, (iii) normal + injection, and (iv) cryogenic treatment + injection. The location of the anterior chamber in a sectioned diagram of an eye (b). Cultured HCECs-QDs655 were injected into the AC in the right eye of the mice. In vivo fluorescence imaging of mice with cryogenic treatment post injection of cHCECs-QDs655 (c). In vivo imaging of the right eye of mice in the four groups at 3, 24, and 48 hours post injection of cHCECs-QDs655 (d). Ex vivo imaging of both eyes of
mice in the four groups at 48 hours post injection of chHCECs-QDs655 (e). The time course of fluorescence intensity of each right eye obtained from mice in the four groups at 3, 24, and 48 hours post injection of chHCECs-QDs655 (f). The time course of the ratio of fluorescence intensity of each right eye obtained from mice in two groups (iii and iv) at 3, 24, and 48 hours post injection of chHCECs-QDs655 (g). The data are shown as the mean ± SD values. *P < 0.05.

organisms (spleen, kidney, heart) (Fig. 7b). These findings suggest that unattached chHCECs did travel through the AC of the eye and subsequently accumulated in the lungs and liver.

DISCUSSION

We previously reported our newly developed methods for expanding chHCECs highly expressing CD166⁺, CD24⁻/⁺, CD105⁺, CD44⁻/⁺/⁶, and CD26⁻ SPs with hexagonal morphology and no signs of cell-state transition into the senescence phenotype, epithelial–mesenchymal transition, or fibroblastic cell morphology. This SP of chHCECs comparable to fully matured differentiated HCECs in fresh tissues is expected to function as the best cell source for regenerative medicine to reconstitute the corneal endothelial layer of patients with endothelial dysfunction. The combination of the CD markers

Figure 5. Confocal images of the right eyes post injection of chHCECs-QDs655. Diagram of a cross-sectional mouse eye and high-speed multiphoton confocal laser microscopy images (a). Images of a glass-bottom dish including a mouse eye on the stage of a multiphoton excitation fluorescence microscope (b, c). Enlarged figure of (b) (d). Confocal images (i–iv) of the right eye from the front (d). Eye obtained from the cryogenically treated mice at 3 hours post cHCEC-QDs655 injection (d). Confocal images of the right eye from the side of the cornea and AC area (e), the cornea and ciliary body area (f), the vitreous body area (g), and the retina and sclera area (h, i). Confocal images of the right eye obtained from cryogenically treated mice (j). Confocal images of the right eye obtained from cryogenically treated mice at 3, 24, and 48 hours post cHCEC injection (k). Confocal images of the right eye obtained from normal mice at 48 hours post cHCEC injection (l). Eyes obtained from three mice were set on glass-bottom dishes, and then different eye tissues and different areas of the each tissue were observed.
FIGURE 6. Three-dimensional confocal images of the right eye at 48 hours post cHCEC-QD injection into cryogenically treated mice. Three-dimensional confocal images of the right eye in a normal mouse (a, b). Three-dimensional confocal images of the right eye after cHCEC-QD injection into cryogenically treated mice (c, d). The corneal cells were stained with Hoechst33342 (blue) and isolectin conjugated with FITC (green). Damaged corneal endothelium was stained with isolectin conjugated with FITC.

FIGURE 7. Confocal images of five major organs post injection of cHCECs-QDs655. Confocal images of major organs (lungs, liver, spleen) obtained from cryogenic mice at 48 hours post cHCEC injection (a). Confocal images of major organs (lungs, liver, spleen, kidneys, heart) obtained from normal mice at 48 hours post cHCEC injection (b). The corneal cells were stained by Hoechst33343 (blue fluorescence) and the injected cHCECs were labeled with QDs655 (red fluorescence).
Quantum Dots Labeling Visualize the Fate of cHCEC

In this present study, we investigated the accumulation of injected cHCECs using high-speed multiphoton confocal laser microscopy, and found that the conditions and time course in normal eyes differed from those in the cryogenically treated eyes. The cHCECs injected into the AC of the cryogenically treated eyes accumulated in the corneal endothelium within 3 hours post cHCEC injection. Our findings confirmed the differences between the accumulation of cHCECs-QDs at the corneal endothelium in endothelial dysfunction model mice and in normal mice. In addition, we obtained 3-D images of corneal cells and transplanted cHCECs-QDs655. Moreover, the findings in this study suggest that QD-labeled cells might be safe for human application. Hence, a future application of QD-labeled cHCECs into patients after strict safety confirmation under governmental regulation may provide a new tool for the in vivo fluorescence imaging system and confocal laser microscopy. We discovered that the labeling efficiency of QDs was more than 95% with 4-hour transduction, and confirmed no cytotoxicity at <8 nM QDs. Moreover, our findings confirmed that QD labeling has no influence on the morphology or expression of CD44, Na⁺/K⁺-ATPase, and ZO-1, which are well-known functional markers of cHCECs. Injected cHCECs-QDs were semiquantitatively detected in the eyes of mice using the IVIS Lumina K Series III system, and were found to accumulate at the corneal endothelium within 3 hours post cHCEC injection. Thus, our findings confirmed the differences between the accumulation of cHCECs-QDs at the corneal endothelium in endothelial dysfunction model mice and in normal mice. In addition, we obtained 3-D images of corneal cells and transplanted cHCECs-QDs655. Moreover, the findings in this study suggest that QD-labeled cells might be safe for human application. Hence, a future application of QD-labeled cHCECs into patients after strict safety confirmation under governmental regulation may provide a new tool for detailed studies on the in vivo function of not only cHCECs, but a wide range of cells planned for future application in cell-based regenerative medicine therapy.

In conclusion, the findings in this study indicate that QDs can be utilized for the in vivo fluorescence monitoring of injected cHCECs, and these results may also help further advance research on the clinical application of QDs in the field of regenerative medicine.

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