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Non-invasive monitoring of transplanted endothelial progenitor cells in diabetic ischemic stroke models

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ABSTRACT

Endogenous endothelial progenitor cells (EPCs) are functionally impaired in hyperglycemia through the p38 MAPK signaling pathway. However, the number and function of transplanted exogenous EPCs in diabetic animals remains unclear. The objectives of this study were to establish a non-invasive imaging strategy to monitor the homing of transplanted EPCs in diabetic stroke mice and to assess the effect of RWJ 67657, an inhibitor of p38 MAPK, on the homing ability of exogenous EPCs. Bone marrow-derived EPCs were labeled *in vitro* with a multi-functional nanoprobe modified with paramagnetic chelators and fluorophores before being infused into stroke mice. The signal of the nanoprobe reached its peak on day 5 in both magnetic resonance imaging and near-infrared fluorescence imaging after EPC transplantation in wild-type stroke models. The signal enhancement of diabetic stroke models was significantly lower than that of wild-type controls. However, the signal intensity of diabetic stroke models significantly increased after oral administration of RWJ 67657, indicating that more transplanted EPCs migrated to the ischemic brain. Furthermore, the increased exogenous EPCs induced remarkably greater angiogenesis after stroke. These results suggest that this dual-modal imaging strategy is feasible for non-invasively monitoring transplanted cells *in vivo*.

1. Introduction

Cerebrovascular disease is a common cause of death and disability worldwide, and diabetes is a major risk factor [1]. Ischemic damage is exacerbated in diabetic patients and is associated with poor outcomes [2,3]. Impaired angiogenesis may be one of the possible mechanisms of this damage [4]. Endothelial progenitor cells (EPCs), isolated from bone marrow, spleen, peripheral blood, or umbilical cord blood, have been used for transplantation to promote angiogenesis after cerebral ischemia [5–8]. Transplanted EPCs migrate to the ischemic area to repair the damaged vasculature not only by incorporating into vessels but also by secreting pro-angiogenic factors [3,9]. However, the function of EPCs has been reported to be impaired under hyperglycemic conditions through activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway [10,11]. It has been reported that the inhibitor of p38 MAPK, SB203580, could improve the number and function of EPCs *in vitro* [12,13]. RWJ 67657, a highly selective inhibitor of the p38-α and p38-β MAPK isoforms [14], is more potent *in vitro* than the literature standard, SB 203580 [15]. To date, little is known regarding the effect of RWJ 67657 on the homing ability of transplanted exogenous EPCs in diabetic animals *in vivo*, and the lack of a non-invasive strategy for tracking transplanted cells is a major obstacle.

Recently, the application of nanotechnology in the biomedical field has achieved rapid advancements [16,17]. For example, a large number of promising nanoprobes have been developed and widely used in diagnosis and image-guided therapy [18,19]. Among the available imaging technologies, magnetic resonance imaging (MRI) has high spatial resolution and the ability to visualize tissues without ionizing radiation and without limitations of depth and angle. On the other hand, near-infrared fluorescence imaging (NIRFI) is very sensitive and easily operated. NIRFI shows

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advantages for *in vivo* studies because NIR light with wavelengths in a range of 650–900 nm demonstrates low phototoxicity, minimized absorption and autofluorescence from endogenous tissues, which allows it to visualize biological events in deep tissues. Therefore, the combination of MRI and NIRFI can provide more useful information.

Thus, the present study was performed to non-invasively visualize transplanted EPCs in ischemic brains of wild-type and diabetic mice using MRI and NIRFI with a multi-modal nanoprobe, and to examine whether RWJ 67657 promotes the homing of exogenous EPCs to the ischemic brain in a diabetic mouse model.

2. Materials and methods

2.1. Synthesis of the nanoprobe

The multi-modal imaging probe was synthesized by the conjugation of bacterial cytosine deaminase (bCD) and poly-L-lysine (PLL), as previously described [20]. Briefly, bCD protein, which promotes cell internalization *via* the clathrin-mediated endocytosis pathway [21], was isolated from transformed *Escherichia coli* cultures. PLL was selected as a vector with which to carry both MRI and NIRFI reporters. Gd^{3+}-DOTA was conjugated for use in the *in vivo* T_1-weighted imaging and in the *ex vivo* quantitation of the nanoprobe in the brain. Cy5.5 was used for the NIRFI, whereas rhodamine was used for tissue immunofluorescence staining.

2.2. EPC cultivation

Mononuclear cells were isolated from the bone marrow of C57Bl/6 mice (5 weeks old, male, Academy of Military Medical Science, Beijing, China) *via* ficoll density gradient centrifugation [22]. The cells were cultured in Endothelial Basal Media-2 (EBM-2, Lonza, Basel, Switzerland) supplemented with growth factors, and the culture medium was changed every three days.

2.3. Animal models

All animal experiments were approved by the Institutional Animal Use and Care Committee of the Medical School of Southeast University. Cortical ischemic stroke was induced through a photochemical reaction in wild-type and *db/db* mice (male, 8 weeks old, Academy of Military Medical Science) [23,24]. Mice were anesthetized with 1% isoflurane (Keyue, Shandong, China) using a gas anesthesia mask. Briefly, the brain was illuminated for 15 min after the intraperitoneal injection of Rose Bengal (100 mg/kg, Sigma–Aldrich, St. Louis, MO, USA). For illumination, a fiber optic bundle with a cold light source (Zeiss, Oberkochen, Germany) was centered 2.0 mm to the right of the bregma. Ischemic stroke was confirmed by T_2-weighted imaging 24 h after surgery.

Either labeled or unlabeled EPCs (1×10^6 cells in 100 μL of saline) were infused into wild-type or diabetic ischemic stroke mouse models *via* the ipsilateral internal carotid artery at 24 h post-surgery. To verify whether an inhibitor of p38 MAPK promotes the homing ability of transplanted EPCs in diabetic mice, RWJ 67657 (50 mg/kg/d, Santa Cruz, CA, USA) was given once a day for 5 days *via* intragastric administration, with the first injection occurring 30 min before cortical ischemic stroke induction.

2.4. MRI

MRI was performed using a 7 T small animal MR scanner (Bruker PharmaScan, Ettlingen, Germany). The body temperature and respiratory rate of the mice were monitored using a physiology monitor. A spin echo sequence (repetition time 500 ms, echo time 15 m) and a fast spin echo sequence (repetition time 2000 ms, echo time 50 ms) were used for T_1-and T_2-weighted imaging, respectively. The matrix was 256 × 256; the field of view was 2 × 2 cm; and the slice thickness was 1 mm. The region of interests (ROIs) were manually drawn in the peri-infarct area on the T_1-weighted images. The contrast-to-noise ratio (CNR) was defined as follows: $CNR = (SI_P - SI_C)/SI_N$, where SI_P = signal intensity of the peri-infarct area, SI_C = signal intensity of the contralateral cortex, and SI_N = signal intensity of the background noise. The T_1-weighted images were combined with pseudo-color according to signal intensity using Image J software (NIH, Bethesda, MD, USA). Then, the color-coded images were obtained after removing the signals except those in the peri-infarct area and overlaying them on the original images. The brighter the color-coded image, the higher the signal.

2.5. NIRFI

NIRFI was performed using the Maestro *In vivo* Imaging System (CRi, Woburn, MA, USA). The skull of each mouse was exposed for *in vivo* imaging after being anesthetized. NIRF images were collected at an excitation wavelength of 675 nm and an emission wavelength of 695 nm. Under deep anesthesia, the brain, liver, heart, spleen, lungs, and kidneys of the mice were surgically dissected for *ex vivo* imaging. The imaging parameters were the same as those used for *in vivo* imaging.

The Cy5.5-related fluorescence signal was extracted from the autofluorescence signal on the NIRF images using the multispectral imaging capabilities of the Maestro 2.10.0 software. The signal intensity of the *ex vivo* brains was quantified using the CRi Maestro software. Rectangular ROIs were placed on the left and right hemispheres of the mice. The target-to-background ratio (TBR) was defined as follows: (ROI value from the right hemisphere)/(ROI value from the left hemisphere) [25].

2.6. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Fresh right brain hemispheres of mice in various groups were weighed (approximately 100 mg) and digested as previously described [26]. The gadolinium present in the right hemispheres of the mice was quantified using ICP-MS (PerkinElmer, Waltham, MA, USA).

2.7. Immunostaining

Each mouse was perfused transcardially with phosphate buffer solution (PBS), and some brain tissues were immersed in freshly prepared 4% paraformaldehyde for 24 h. After being dehydrated, the brain tissues were cryostat-sectioned (10 μm) and placed onto poly-L-lysine-coated slides.

The slices were incubated with rabbit anti-mouse CD133 (Santa Cruz), CD34 (Santa Cruz), VEGFR2 (Abcam, Hong Kong, China), and CD31 (Abcam) antibodies followed by staining with Alexa Fluor 488 goat anti-rabbit polyclonal antibody (Life Technologies, Grand Island, NY, USA). Nuclei were counterstained with DAPI. To confirm co-localization of rhodamine and these markers, the sections were scanned by confocal microscopy (Olympus, LEXT, Japan).

To measure the microvessel density (MVD), the slices were stained with rabbit anti-mouse CD31 antibody, followed by horseradish peroxidase-conjugated secondary antibody (Life Technologies). The CD31-positive cells in the peri-infarct region were counted in 4 mice per group in a double-blind manner.

2.8. Statistical analysis

All statistical analyses were performed using the SPSS software (version 18). Numerical data are presented as the mean values ± standard error of the mean (SEM). For the statistical comparisons, an independent-sample *t* test (Figs. 3 and 4) or one-way analysis of variance (ANOVA) with the least significant difference (LSD) post-hoc test (Figs. 5–7) was applied. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Characterization of the nanoprobe

The chemical structure of the nanoprobe is shown in Fig. 1. The hydrodynamic radius of the nanoprobe was measured as 24.4 nm and the molecular weight was 345 kDa. The water proton longitudinal relaxivity was detected as 8.6 $mM^{-1}s^{-1}/Gd^{3+}$ ion. Our previous study showed that this nanoprobe causes little toxicity to EPCs and can be internalized by EPCs. Cellular internalization kinetics indicated that the uptake of the nanoprobe reached its maximum value at 24 h [20].

3.2. EPC cultivation and labeling

Cells were spindle-like on day 7 and exhibited a typical "cobblestone" morphology on approximately day 14 (Fig. 2A). The cultivated cells were examined for the expression of EPC-specific markers, such as CD133, CD34, and VEGFR2 (Fig. 2B). Approximately 90% of these cells were found to not only bind endothelial cell-specific lectin UEA but also take up acLDL (Fig. 2C).

Then, the cultivated cells were incubated with the nanoprobe (2 μM) for 24 h. The labeled cells were detected with an inverted fluorescence microscope (Carl Zeiss, Jena, Germany) by the fluorescence signals from the nanoprobe in the cytoplasm (Fig. 2D).

3.3. Tracking of EPCs in WT ischemic stroke mouse models

The ischemic territories on the T_2-weighted images at the selected time points after transplantation are shown in Fig. 3A. High signal intensity in the peri-infarct area was observed on the T_1-weighted and color-coded images in the probe-labeled EPC