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Non-invasive stem cell tracking in hindlimb ischemia animal model using bio-orthogonal copper-free click chemistry

Si Yeon Lee [a, b, 1], Sangmin Lee [a, 1], Jangwook Lee [a], Ji Young Yhee [c], Hwa In Yoon [a, d], Soon-Jung Park [e], Heebeom Koo [f], Sung-Hwan Moon [e], Hyukjin Lee [c], Yong Woo Cho [d], Sun Woong Kang [g], Sang-Yup Lee [b, **], Kwangmeyung Kim [a, *]

[a] Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Hwarangno 14-gil 6, Seongbuk-gu, Seoul 136-791, Republic of Korea
[b] Department of Chemical and Biomolecular Engineering, Yonsei University, 50 Yonseiro, Seodaemun-gu, Seoul, 120-749 Republic of Korea
[c] College of Pharmacy, Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea
[d] Departments of Chemical Engineering and Bionanotechnology, Hanyang University, Ansan, Gyeonggi-do 426-791, Republic of Korea
[e] Department of Medicine, School of Medicine, Konkuk University, Seoul, Republic of Korea
[f] Department of Medical Lifescience, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 06591, Republic of Korea
[g] Next-generation Pharmaceutical Research Center, Korea Institute of Toxicology, Daejeon, Republic of Korea

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ABSTRACT

Labeling of stem cells aims to distinguish transplanted cells from host cells, understand *in vivo* fate of transplanted cells, particularly important in stem cell therapy. Adipose-derived mesenchymal stem cells (ASCs) are considered as an emerging therapeutic option for tissue regeneration, but much remains to be understood regarding the *in vivo* evidence. In this study, a simple and efficient cell labeling method for labeling and tracking of stem cells was developed based on bio-orthogonal copper-free click chemistry, and it was applied in a mouse hindlimb ischemia model. The human ASCs were treated with tetra-acetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz) to generate glycoprotein with unnatural azide groups on the cell surface, and the generated azide groups were fluorescently labeled by specific binding of dibenzylcyclooctyne-conjugated Cy5 (DBCO-Cy5). The safe and long-term labeling of the hASCs by this method was first investigated *in vitro*. Then the DBCO-Cy5-hASCs were transplanted into the hindlimb ischemia mice model, and we could monitor and track *in vivo* fate of the cells using optical imaging system. We could clearly observe the migration potent of the hASCs toward the ischemic lesion. This approach to design and tailor new method for labeling of stem cells may be useful to provide better understanding on the therapeutic effects of transplanted stem cells into the target diseases.

1. Introduction

Cell therapy, transplantation of therapeutic cells into the living subject, has emerged as an excellent strategy in the treatment of various human diseases. In particular, it has provided enormous therapeutic potency in a variety of ischemic diseases, which is characterized by insufficient supply oxygen and nutrients to the desired tissues. The representative ischemic diseases include cerebral infarction, cardiac infarction, ischemic retinopathy, and hindlimb ischemia. Various cells such as multipotent stem cells and endothelial progenitor cells hold promise for providing therapeutic benefits in the diseases [1]. The *in vivo* fate of the transplanted cells should be carefully investigated for more successful therapy. However, traditional methods for the examination of stem cell therapy were performed relies on postmortem histology.

Many cell-labeling and *in vivo* imaging techniques have been recently developed to understand the fate of cells, and it has provided useful information for their survival, location, distribution, and migration into the transplanted sites [2–4]. They can be classified as direct and indirect methods. The cells can be directly labeled with various imaging probes containing fluorescence dye,

* Corresponding author.
** Corresponding author.
E-mail addresses: leessy@yonsei.ac.kr (S.-Y. Lee), kim@kist.re.kr (K. Kim).
[1] The authors contributed equally to this work.

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radiotracers, gold, and magnetic particles [5–8]. Then, they can be monitored by *in vivo* non-invasive optical, positron emission tomography (PET), computed tomography (CT), and magnetic resonance (MR) imaging. For a typical indirect cell-labeling, the cells have been transfected with reporter gene, providing to more insight about the *in vivo* fate [9]. For example, green fluorescence-expressing protein, lysine-rich protein, and human mitochondrial TK type-2 (hTK2) were utilized for cell monitoring in optical, MR, and PET imaging, respectively [10–12].

Unfortunately, these techniques have limited detection sensitivity, and may not enable to monitor small number of the transplanted cells, leading to barely detectable level of cells. Furthermore, exogenous imaging probes and genes inserted into the cells has often caused significant cytotoxicity, resulting to attenuated their cell viability and therapeutic efficiency. Thus, it is prerequisite of high sensitivity and minimal adverse effects for efficient stem cell therapy. One of critical problems inherent to the cell therapy is the limitation in tracking the movement of transplanted cells. In order to track the stem cells effectually, molecular imaging is required to enable non-invasive and long-term evaluation of the migration, proliferation, and possibly differentiation of stem cell *in vivo*. Although many studies have been devoted to the development of labeling probes for cell therapies, they could not provide the visualization of long-term fate of the cells in clinical trials due to the loss of labeling signals. Therefore, more efficient labeling is necessary in order to allow the determination of the long-term fate *in vivo*, and it should not be lost with time.

Click chemistry, azide-alkyne cycloaddition catalyzed by copper, has received the attention of chemists and biologists for use in biological applications [13]. Recently, bio-orthogonal click chemistry without copper catalyst is being considered for cell labeling method based on its unique specificity and low cytotoxicity to in the living cells [14–16]. It has allowed rapid and high specific chemical reaction in the cells [17,18]. Particularly, this direct chemical reaction is notable in the labeling in combination with metabolic glycoengineering. Based on metabolic glycoengineering, unnatural glycan containing azide groups as bio-orthogonal functional groups are metabolized with less cytotoxicity. As a result, that azide analogs can artificially introduced onto the surface of the cells. It is shown that the combined potential with the cooper-free click chemistry involving ring-strained alkyne groups as the counterparts to the azides. Many reports demonstrated that the click chemistry has the great potential of dynamic *in vivo* imaging [19–21]. In previous study, we also introduced to a novel strategy for targeted delivery of nanoparticles using metabolic glycoengineering and copper-free click chemistry [22].

Herein, our approach for a novel stem cell-labeling and tracking method could provide important evidences for the migration of hASCs in ischemic hindlimb model. First, hASCs were treated with tetra-acetylated *N*-azidoacetyl-D-mannosamine ($Ac_4ManNAz$) as the precursor of multiple glycans with unnatural sialic acids that could be generated onto the surface of cells by metabolic glycoengineering (Fig. 1(a)). The sialic acids contain azide groups ($-N_3$), and consequently the cells expressed artificial azide groups. Based on bio-orthogonal copper-free click chemistry, the azide groups on the cells were next conjugated with near-infrared fluorescent (NIRF) dye-tagged dibenzyl cyclooctyne (DBCO-Cy5), leading to a rapid and simple hASCs labeling with fluorescence (Fig. 1(b)). The DBCO-Cy5-hASCs were finally transplanted and monitored into mouse hindlimb ischemia model (Fig. 1(c)). We performed ischemic injury at inner thigh muscle into the mice, and the cells were intramuscularly injected at outer thigh muscle of them. The injury sites were then monitored in real-time non-invasive whole body NIRF imaging and the three-dimensional optical imaging system for 2 weeks.

2. Materials and methods

2.1. Stem cell labeling by a combination method using $Ac_4ManNAz$ and DBCO-Cy5

The hASCs were exposed with $Ac_4ManNAz$ to generate unnatural azide groups on the cell surface by metabolic glycoengineering. The cells were seeded onto the cell culture plates (3×10^4 cells/35 mm^2 dish), and it was incubated in the culture media containing $Ac_4ManNAz$. Various dose (10–50 μM) and exposure time (24–72 h) of $Ac_4ManNAz$ were treated into the cells in order to determine optimized condition of the azidosugar generation. The cells were washed twice with the DPBS after the each incubation, and it was treated with DBCO-Cy5 (10 μM) to label fluorescently on the cell surface by azide-alkyne click chemistry.

2.2. In vivo cell tracking

The transplanted cells were monitored for 2 weeks using non-invasive *in vivo* fluorescence scanning system (Lumina; Caliper Life Sciences, USA) at the wavelength with a 640 nm of excitation and 710 nm of emission. We detected the NIRF signals of the cells in real time, and the *in vivo* whole body NIRF images were acquired at predetermined times. The regions of interest (ROIs) were drawn over the hindlimb ischemic site for positive signal. To identify the localization and migration of the transplanted cells, three dimensional (3D) reconstructed *in vivo* NIRF imaging were obtained using 360° Fluorescence Tomographic imaging System (FMT; Fluorescence Molecular Tomography, Perkin Elmer, Bedford, MA, USA). Side view and top view of the desired regions were acquired with depth of 13 mm, respectively. A 640 nm pulsed laser diode was used to excite the Cy5 molecules, and the setting for laser power, exposure time and photon count time were fixed in the same condition for each imaging. All other information about materials and methods is provided in Supplementary content.

3. Results

3.1. Development and optimization of a combination method for stem cell labeling

We developed a simple and efficient labeling method for stem cell labeling in order to monitor the *in vivo* behavior of transplanted hASCs into the ischemia hindlimb. Firstly, the hASCs were treated with $Ac_4ManNAz$ as the precursor of unnatural glycan containing bio-orthogonal azide ($-N_3$) groups that could be generated onto the cells by metabolic glycoengineering. Secondly, based on bio-orthogonal copper-free click chemistry, the azide groups onto the cells were conjugated with near-infrared fluorescent (NIRF) dye-conjugated dibenzylcyclooctyne (DBCO-Cy5), leading to a rapid and simple hASCs labeling fluorescently. The stem cell labeling method by bio-orthogonal copper-free click chemistry was optimized to possess a sufficient labeling efficiency by varying the doses or varying incubation time of treated $Ac_4ManNAz$. The hASCs were treated with various dose (0–50 μM) of $Ac_4ManNaz$. The labeling efficiency was determined by confocal fluorescence microscopy after the cells were washed and sequential incubated 10 μM of DBCO-Cy5 for 1 h. The NIRF signal intensity from the hASCs increased in a dose-dependent manner, and particularly the outstanding intensity was observed in those for 50 μM (Fig. 2(a)). In addition, cell labeling via the bio-orthogonal copper-free click chemistry was also estimated in 50 μM of $Ac_4ManNAz$-treated hASCs, varying the incubation time (0–48 h). As shown in Fig. 2(b), fluorescence signal intensity clearly increased in proportion to the incubation time with $Ac_4ManNAz$ up to 48 h. The NIRF intensity of

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