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Tracking and protection of transplanted stem cells using a ferrocenecarboxylic acid-conjugated peptide that mimics hTERT

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ABSTRACT

In vivo tracking of transplanted stem cells has been a central aim of stem cell therapy. Although many tracking systems have been introduced, no method has yet been validated for clinical applications. We developed a novel sophisticated peptide (GV1001) that mimics hTERT (human telomerase reverse transcriptase) and analysed its ability to track and protect stem cells after transplantation. Ferrocenecarboxylic acid-conjugated GV1001 (Fe-GV1001) efficiently penetrated stem cells with no adverse effects. Moreover, Fe-GV1001 improved the viability, proliferation, and migration of stem cells under hypoxia. After Fe-GV1001-labelled stem cells were transplanted into the brains of rats after stroke, the labelled cells were easily tracked by MRI. Our findings indicate that Fe-GV1001 can be used for the in vivo tracking of stem cells after transplantation into the brain and can improve the efficacy of stem cell therapy by sustaining and enhancing stem cell characteristics under disease conditions.

1. Introduction

Stem cell therapy has been used for various intractable diseases. Most animal studies have shown that stem cell therapy has a mild to moderate effect on the targeted diseases. Additionally, the transplantation of various types of stem cells has been suggested to improve neurobehavioural functions in diverse animal models of incurable neurological disorders [1]. Although the exact mechanisms of action of stem cells have not yet been identified, a number of hypotheses have been proposed regarding the mechanisms underlying neuroprotection, regeneration, anti-inflammation, and cell replacement [2]. However, an obstacle hindering the testing of these hypotheses is the difficulty tracking in vivo transplanted stem cells.

Many studies have attempted to overcome this hurdle [3–5]. Numerous technologies, including silica-coated magnetic particles [3], nanoparticles [6,7], and multiphoton luminescent graphene quantum dots [8], have been investigated for their potential to track transplanted stem cells. Despite these efforts, none of these technologies has met the requirements for use in clinical stem cell therapy, and additional efforts are still required to develop technologies for tracking transplanted stem cells in vivo. In addition, the development of tracers that can also enhance desirable stem cell characteristics, such as proliferation, migration, and differentiation, would represent a great advancement in the field since the transplanted stem cells would be present within the lesions caused by a disease.

In our previous study [9], we described a novel peptide, GV1001 (GV1001), that mimics human TERT (telomerase reverse transcriptase; hTERT). We reported that GV1001 penetrated neural

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stem cells (NSCs) efficiently without damaging the cells and effectively protected NSCs from amyloid-β toxicity. Based on these results, we hypothesized that GV1001 conjugated to iron particles would penetrate stem cells for post-transplantation tracking and would also protect stem cells under stressful conditions, such as hypoxia. In the present study, this hypothesis was tested by performing both in vitro and in vivo studies.

2. Materials and methods

2.1. Stem cell culture and labelling of NSCs and MSCs with ferrocenecarboxylic-peptide GV1001 (Fe-GV1001)

All procedures involving animals were performed in accordance with the Hanyang University guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Hanyang University. Every effort was made to minimize the number of animals used and to limit animal suffering. Each animal was used only once.

Neural stem cells (NSCs) were isolated from rodent embryonic brains, cultured, and expanded. NSC culture was performed as previously described [10–13]. Briefly, rat embryos were decapitated at embryonic day 13 (E13). The brains were rapidly removed and placed in a petri dish half full of ice-cold Hank's Balanced Salt Solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, and 2.5 mM HEPES; GIBCO BRL, Grand Island, NY, USA). Single cells were dissociated from the whole cerebral cortex, lateral ganglionic eminence, and ventral midbrain of the foetal rats. The resulting cells were plated at a density of 2×10^4 cells/cm^2 on culture dishes precoated with poly-L-ornithine/fibronectin in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; GIBCO) and cultured in N2 medium (Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12, 25 mg/L insulin, 100 mg/L transferrin, 30 nM selenite, 100 μM putrescine, 20 nM progesterone, 0.2 mM ascorbic acid, 2 nM L-glutamine, 8.6 mM D(+) glucose, and 20 nM $NaHCO_3$; Sigma, St. Louis, MO, USA) supplemented with basic fibroblast growth factor (BFGF; 10 ng/ml, R&D Systems, Minneapolis, MN, USA). Cultures were maintained at 37 °C under a humidified 5% CO_2 atmosphere for 4–6 days.

Human mesenchymal stem cells (hMSCs) (Lonza, Basel, Switzerland; PT-2501) were plated at 5000–6000 cells/cm^2 on culture dishes and cultured in complete medium [DMEM low glucose (GIBCO), 3.7 g/L sodium bicarbonate (Sigma), 1% penicillin-streptomycin, and 10% foetal bovine serum (FBS) (GIBCO)]. Cultures were maintained at 37 °C under a humidified 5% CO_2 atmosphere for 6–7 days.

To label NSCs and MSCs with Fe-GV1001, 1 X 10^6 NSCs and 2 X 10^5 MSCs were seeded on 100-mm dishes, and the cells were treated with various concentrations of Fe-GV1001 (0, 0.01, 0.1, 1, 10, or 100 μM) for 0–48 h.

2.2. Confirmation of penetration of Fe-GV1001 into NSCs and MSCs

NSCs (1 X 10^5) and MSCs (0.5 X 10^5) were seeded on chamber well plates, serum starved for 1 h, and then treated with 10 or 100 μM Fe-GV1001, respectively, for 0–48 h. The cells were then washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were rinsed several times with PBS and mounted on glass slides with mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). The cells were observed under a confocal laser scanning microscope (Leica, Wetzlar, Germany) at the appropriate excitation wavelengths.

Live cell image analysis was also performed as follows: NSCs (4 X 10^5) were seeded on 27-mm Fine-view microplates (InfiniteBio, Inc., Rockaway, NJ, USA) and then exposed to 1 μM FITC-GV1001 for 4 h. To remove non-specific background, the cells were then washed several times with PBS, after which N2 medium was added. Live cells were observed under a DeltaVision microscope (DeltaVision, GE Healthcare, Little Chalfont, UK) for 24 h.

2.3. Prussian blue staining

For Prussian blue staining, cells were fixed in 95% ethanol and then treated with potassium ferrocyanide (1:1 solution of 20% hydrochloric acid and 10% potassium ferrocyanide, Sigma) for 20 min. The cells were then washed with deionized water, treated with nuclear fast red (Sigma) for 5 min, and washed with deionized water. Next, the cells were subjected to 70–100% ethanol (Millipore, Bedford, MA, USA), acetone (Millipore), and xylene (Junsei, Chuo-ku, Tokyo, Japan) treatments and mounted on glass slides with mounting medium (Merck, Kenilworth, NJ, USA). The cells were then observed under an Olympus Bx53 microscope (Olympus, Tokyo, Japan).

2.4. Electron microscopy

NSCs (1 X 10^6) and MSCs (3 X 10^5) were seeded on 35 mm dishes and exposed to 10 and 100 μM Fe-GV1001, respectively, for 8 h. The cells were then washed twice with PBS and fixed with EM fixing buffer for 1 h at room temperature. Twenty-four h after MPP+ (1-methyl-4-phenylpyridinium) treatment, the cells were washed with DMEM and fixed in 0.1 M cacodylate buffer (pH 7.0, TED PELLA INC., Redding, CA, USA) containing 2% paraformaldehyde (Merck) and 0.5% glutaraldehyde (EMS, Hatfield, PA, USA) at 37 °C for 15 min after which fixation was allowed to proceed at room temperature. The cells were washed with the same buffer three times, post-fixed with 1% osmium tetroxide (Heraeus, Hanau, Germany) for 30 min, and EM bloc-stained with 0.2% uranyl acetate (EMS) solution for 1 h. Subsequently, the cells were dehydrated through an ascending ethanol series and embedded with an Epon mixture. Thin sections of 80 nm were obtained using a Reichert-Jung Ultracut E ultramicrotome (Leica) and mounted on a 200-mesh grid. Electron microscopy observation was performed with a Hitachi H-7500 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan) with 80 kV acceleration voltage.

2.5. Flow cytometry analysis

Cells were treated with GV1001 (10 and 100 μM for NSCs and MSCs, respectively) or Fe-GV1001 (10 and 100 μM for NSCs and MSCs, respectively) and incubated for 24 h at 37 °C. The cells were stained with anti-Ki67 (1:200, Abcam, Cambridge, MA, USA), anti-Bax (1:100, Cell Signaling, Beverly, MA, USA), anti-cytochrome *c* (1:100, Cell Signaling), anti-cleaved caspase-3 (Asp 175) (1:100, Cell Signaling), and anti-COX-2 (1:100, Cell Signaling) antibodies. After staining with Alexa 488 anti-mouse IgG1 (BD Biosciences, New Jersey, USA) antibodies for 1 h, Bax, cytochrome *c*, cleaved caspase-3, and COX-2 levels were analysed using an Accuri C6 Flow cytometer (BD Biosciences). Positive cells were analysed with BD Accuri C6 software.

Analysis of penetration of FITC-GV1001 into NSCs was performed as follows: NSCs (1 X 10^6) were seeded on 6-well plates (Thermo Fisher Scientific, Waltham, MA USA) and exposed to 1 μM FITC-GV1001 for 0–48 h. The cells were then rinsed twice with cold PBS and analysed by flow cytometry (Accuri C6 flow cytometer, BD Biosciences). Data were acquired and analysed with BD Accuri C6 software.