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Cancer stem cell labeling using poly(L-lysine)-modified iron oxide nanoparticles

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ABSTRACT

Cell labeling using magnetic nanoparticles is an increasingly used approach in noninvasive behavior tracking, *in vitro* separation of cancer stem cells (CSCs), and CSC-based research in cancer therapy. However, the impact of magnetic labeling on the biological properties of targeted CSCs, such as self-renewal, proliferation, multi-differentiation, cell cycle, and apoptosis, remains elusive. The present study sought to explore the potential effects on biological behavior when CSCs are labeled with superparamagnetic iron oxide (SPIO) nanoparticles *in vitro*. The glioblastoma CSCs derived from U251 glioblastoma multiforme were labeled with poly(L-lysine) (PLL)-modified γ-Fe_2O_3 nanoparticles. The iron uptake of glioblastoma CSCs was confirmed through prussian blue staining, and was further quantified using atomic absorption spectrometry. The cellular viability of the SPIO-labeled glioblastoma CSCs was assessed using a fluorescein diacetate and propidium iodide double-staining protocol. The expressed specific markers and multi-differentiation of SPIO-labeled glioblastoma CSCs were comparatively assessed by immunocytochemistry and semi-quantitative RT-PCR. The effects of magnetic labeling on cell cycle and apoptosis rate of glioblastoma CSCs and their differentiated progenies were assayed using a flow cytometer. The results demonstrated that the cell viability and proliferation capacity of glioblastoma CSCs and their differentiated progenies were not affected by SPIO labeling compared with their unlabeled counterparts. Moreover, the magnetically labeled CSCs displayed an intact multi-differentiation potential, and could be sub-cultured to form new tumor spheres, which indicates the CSCs capacity for self-renewal. In addition, cell cycle distribution, apoptosis rate of the magnetically labeled glioblastoma CSCs, and their differentiated progenies were not impaired. Therefore, the SPIO-labeled CSCs could be a feasible approach in conducting further functional analysis of targeted CSCs.

1. Introduction

Cell labeling using superparamagnetic iron oxide (SPIO) nanoparticles (mostly maghemite, γ-Fe_2O_3, or magnetite, Fe_3O_4) has gained increasing interest in various biological and medical applications, such as cell separation [1], resonance imaging (MRI) [2,3], tissue engineering [4,5], and magnetofection [6], due to their outstanding biocompatibility and superparamagnetic properties. More significantly, an efficient magnetic labeling offers promising new approaches for cell-based therapy [7,8], in which magnetic labeling can non-invasively track the delivery of various therapeutic cells (e.g., T cells and stem cells) to the tumor site. Moreover, magnetic labeling can track subsequent differentiation/proliferation of these therapeutic cells, allowing a better understanding of cancer development and intervention mechanisms.

Two strategies are currently utilized in conducting cell labeling using SPIO nanoparticles. The first strategy is to directly attach the magnetic particles onto cell surfaces [9]. The second is based on a receptor-mediated or transfection agent-mediated endocytosis pathway, which can internalize magnetic particles [10]. However, nanoparticles cannot efficiently label the desired cells due to the repulsive electric interaction between the cell membrane and the nanoparticles [11] because both the cell membrane and SPIO nanoparticles have a negative charge surface. Thus, a complex made up of transfection agents and SPIO nanoparticles was often utilized to enhance the labeling efficiency of the SPIO nanoparticles. The use of transfection agents is helpful in transporting SPIO nanoparticles to the target cells. The currently used transfection agents, such as lipofectamine [12], poly(L-lysine) (PLL) [13,14], protamine sulphate [15,16], and polyethylenimine [17], are mostly cationic. Among these transfection agents, PLL is commonly used to

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enhance cell adhesion to the culture dish surface via *in vitro* cell cultivation, which is a method used to transport iron oxide nanoparticle to the cells. The use of positively charged PLL, which coats the negatively charged SPIO nanoparticles, leads to an enhanced SPIO-cell binding via electrostatic interactions and enhanced cellular uptake [18].

Aside from common cell labeling, stem and progenitor cells have also been magnetically labeled in recent years to track their biodistribution and migration *in vivo* [19]. The labeling of various embryonic stem cells (ESCs) [20,21], neural stem cells [22,23], mesenchymal stem cells [24,25], and hematopoietic and non-hematopoietic stem cells with SPIO nanoparticles has been previously conducted [26,27]. However, the influence of SPIO nanoparticle labeling on the behaviors of various stem cells was considered controversial. The mesenchymal stem cells labeled with SPIO nanoparticles demonstrated a marked inhibition of chondrogenesis despite preserved adipogenic and osteogenic differentiation [28]. Moreover, the labeling of human mesenchymal stem cells with SPIO nanoparticles and transfection agents led to a decrease in cell migration capacity *in vitro* and colony formation ability [29]. Krejci et al. [20] have reported that the procedure resulted in the partial inhibition of cavitation of embryoid body during ESC differentiation and enabled neuronal differentiation from ESCs although SPIO labeling had no effect on cell viability and self-renewal. However, other reports have suggested that SPIO labeling does not result in any observable effects on the *in vitro* cardiac differentiation potential of ESCs [30]. Wang et al. [31] have concluded that SPIO nanoparticles did not affect the viability and transdifferentiation potential or cell-factor secretion of adipose-derived stem cells. Arbab et al. [32] have reported that stem cell labeling using ferumoxides-protamine sulfate complexes does not inhibit the function or differentiation capacity of $CD34^+$ hematopoietic stem cells and mesenchymal stem cells.

Glioblastoma is the most common malignant tumor in the central nervous system in adults, and can arise as either primary glioblastoma or as a result of the malignant progression of a low-grade glioma [33]. Malignant gliomas have a poor prognosis and remain difficult to cure despite aggressive treatment through surgery, radiotherapy, and chemotherapy [34,35]. The median survival for patients with glioblastomas is only 12 months–15 months and 2 years–5 years for patients with anaplastic gliomas [36]. Conventional brain tumor therapies currently focus on bulk population reduction of tumor cells. However, increasing evidences have shown that only a small fraction of cells is capable of initiating and maintaining brain tumor growth [37]. Thus far, considerable efforts have been devoted to the characterization of these cells because they might be a better therapeutic target in brain tumor therapy. Studies have suggested that cancer stem cells (CSCs) play an important role in carcinogenesis and tumor progression [38]. CSCs, referred to as tumor-initiating cells, are thought to constitute a small subset of cells within a tumor that both initiate the primary disease and its recurrence because of their capacity for self-renewal and inherent chemo- and radio-resistance, and give rise to an increase in non-tumorigenic cancer cell progeny population through differentiation [39,40]. Nonetheless, the effects of SPIO labeling on CSC behaviors, as well as the functionality of CSC-derived progenies remain unclear.

The objective of the present study is to investigate if the biological properties, such as cell viability and proliferative capacity, self-renewal capacity, multi-differentiation potentials, cell cycle distribution, and apoptosis rate, of CSCs are affected by PLL-modified magnetic nanoparticle labeling. To realize these purposes, PLL-modified SPIO nanoparticles were first utilized to label the CSCs derived from U251 glioblastoma multiforme. The effects of magnetic labeling on glioblastoma CSCs and their differentiated progenies were then investigated. This study would facilitate the MRI-assisted tracking of CSCs and monitor their survival, migration and transformation *in vivo*, and would be further used to treat targeted glioblastoma cells.

2. Materials and methods

2.1. Synthesis of γ-Fe_2O_3 magnetic nanoparticles and their surface modification with PLL

The γ-Fe_2O_3 magnetic nanoparticles used in the present study were prepared from magnetite (Fe_3O_4) [41,42]. First, 3 mL of $FeCl_3$ (2 mol/L dissolved in 2 mol/L HCl) was added to 10.33 mL of deionized (DI) water, and then 2 mL of Na_2SO_3 (1 mol/L) was added dropwise into the mixture for 1 min with stirring. When the color of the solution changed from red to light yellow, it was added to 80 mL of NH_4OH solution (0.85 mol/L) with vigorous stirring. A black precipitate quickly formed, and was allowed to completely crystallize for another 40 min. After washing with deoxygenated water, the black precipitate was diluted to 168 mL (with a mass concentration of 3 mg/mL) and was adjusted to a pH of 3.0 with HCl (0.1 mol/L). The suspension was then heated at 90 °C for 5 min, and was stirred under aeration (with air) for 90 min at 110 °C. The color of the suspension slowly changed from black to reddish-brown. After washing with DI water via magnetic decantation, the reddish-brown precipitate was dried to form the γ-Fe_2O_3 powder.

To modify the surface of γ-Fe_2O_3 nanoparticles with PLL, 1.8 mL of γ-Fe_2O_3 nanoparticles (2 mg/mL) was first added into 1.8 mL of serum-free RPMI-1640 medium (Gibco Invitrogen, Paisley, Scotland, UK). Subsequently, 0.4 mL of PLL (0.45 mg/mL) was added after mixing the solution using a pipette. After incubation for 2 h in a shaking incubator, the prepared PLL-modified γ-Fe_2O_3 nanoparticles were then stored in a refrigerator at 4 °C.

2.2. Characterization of γ-Fe_2O_3 nanoparticles and PLL-modified γ-Fe_2O_3 nanoparticles

The prepared γ-Fe_2O_3 nanoparticles and the PLL-modified γ-Fe_2O_3 nanoparticles were morphologically characterized by transmission electron microscopy (TEM) (Hitachi H-600, Japan). The γ-Fe_2O_3 nanoparticles crystal structure was characterized by an X-ray diffractometer (XRD) (Philips D/Max-2500, Holland) using a monochromatized X-ray beam with nickel-filtered Cu-Ka radiation. Magnetic measurements on the γ-Fe_2O_3 nanoparticles were carried out on a vibrating sample magnetometer (VSM) (Lakeshore-7304, USA) by changing H between +1375 and −1375 Oe. The Fourier transform infrared (FT-IR) spectra of the γ-Fe_2O_3 nanoparticles and PLL-modified γ-Fe_2O_3 nanoparticles were obtained using FT-IR spectroscopy (Nicolet NEXUS 670, USA).

2.3. Primary glioblastoma sphere culture

The human glioblastoma cell line U251 was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were routinely cultured with an RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, CA, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin, then placed in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were normally passaged at a ratio of 1:3 every three days to maintain their exponential growth phase. During the exponential growth phase, the U251 glioblastoma cells were harvested using a treatment of 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.4) for 3 min at 37 °C. The prepared glioblastoma cell suspension was centrifuged at 1000 rpm for 5 min, and re-suspended in a serum-free neural stem cell medium (SFM) containing Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Gibco, Grand Island, NY), B27 (1×, Invitrogen, Carlsbad, CA, USA), recombinant human epidermal growth factor (20 ng/mL; Peprotech Inc., Rocky Hill, NJ, USA), basic fibroblast growth factor (20 ng/mL; Peprotech Inc.), and leukemia inhibitory factor (10 ng/mL; Chemicon, USA) with/without 8 ng/mL of vincristine (Haizheng Pharmaceutical Co., Ltd., Zhejiang, China). The number of cells was then counted using a regular hemacytometer, and the cells were seeded in 24-well plates with 2×10^4 cells/well. Subsequently, one-third of the culture medium was replaced with an equal volume of fresh SFM after the cells were cultured for 24 h. After 3-d culture, all culture medium was discarded and the wells were filled with 600 μL of fresh SFM. To passage the spheres, the glioblastoma spheres were collected through gentle centrifugation at 800 rpm for 5 min and mechanically dissociated by pipetting the mixture up and down. Thereafter, the cell suspension was filtered through a 200 mesh filter to remove the remaining aggregates. Single-cell suspensions were then seeded with the desired density in SFM supplemented with growth factors, and were continuously cultured to once again form the glioblastoma spheres.

2.4. Labeling of glioblastoma CSCs with PLL-modified γ-Fe_2O_3 nanoparticles

After reaching the size of approximately 100 cells/sphere to 150 cells/sphere, the primary glioblastoma spheres were dissociated to prepare a single-cell suspension.