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Ferritin reporter used for gene expression imaging by magnetic resonance

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ABSTRACT

Magnetic resonance imaging (MRI) is a minimally invasive way to provide high spatial resolution tomograms. However, MRI has been considered to be useless for gene expression imaging compared to optical imaging. In this study, we used a ferritin reporter, binding with biogenic iron, to make it a powerful tool for gene expression imaging in MRI studies. GL261 mouse glioma cells were over-expressed with dual-reporter ferritin–DsRed under β-actin promoter, then gene expression was observed by optical imaging and MRI in a brain tumor model. GL261 cells expressing ferritin–DsRed fusion protein showed enhanced visualizing effect by reducing T2-weighted signal intensity for *in vitro* and *in vivo* MRI studies, as well as DsRed fluorescence for optical imaging. Furthermore, a higher contrast was achieved on T2-weighted images when permeating the plasma membrane of ferritin–DsRed-expressing GL261. Thus, a ferritin expression vector can be used as an MRI reporter to monitor *in vivo* gene expression.

Introduction

Magnetic resonance imaging (MRI) is one of the minimally invasive imaging techniques for high spatial resolution tomograms providing anatomical and functional information. Other tomographic imaging techniques like computed tomography (CT) and positron emission tomography (PET) generally entails radiation exposure, unlike MRI, which is free from radiation exposure. Therefore, MRI is accepted as a safer imaging technique compared to others, though it is unsuitable for visualization of gene expression. As a result, optical imaging is generally used for this purpose because several fluorescent and chemiluminescent reporters such as GFP and DsRed and luciferase enzyme systems are available [1]. The availability of gene products capable of altering local signals of MRI for contrast, will be a useful MRI reporter for gene expression.

Iron derivatives are common MRI contrast agents that decrease signal intensity on T2-weighted images as a result of the magnetic susceptibility effect [2]. Thus, localized accumulation of iron derivates are detected by MRI [3]. In fact, iron derivates such as ferumoxides and ferric ammonium citrate are useful in clinical diagnosis of liver neoplasm [4] and gastrointestinal tract [5], respectively. Recent studies suggest use of genes encoding iron-binding proteins as a potential candidate of MRI reporter for *in vivo* MRI detection of gene expression [6].

Ferritin, an endogenous iron storage metalloprotein, consists of 24 light and heavy polypeptide chains encapsulating an iron oxide core with up to 4500 iron atoms [7]. Heavy chain of ferritin mainly binds to iron oxide [8]. Ferritin further creates magnetic fields that affect relaxation time of water protons diffusing through the magnetic field [9]. This makes heavy chain of ferritin an ideal MRI reporter for *in vivo* gene expression in MRI studies.

In this study, we designed a vector capable of expressing heavy chain of ferritin and DsRed fusion protein (ferritin–DsRed) under a β-actin promoter, and made ferritin–DsRed-overexpressing GL261 cell mouse clonal glioma cells, to detect the cells by *in vivo* and *in vitro* magnetic resonance imaging. Results of this study suggest that this ferritin reporter system was useful to detect *in vivo* gene expression by MRI.

Materials and methods

Cell lines. Murine GL261 glioma cells including DsRed expressing and ferritin–DsRed-expressing derivatives were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma–Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum and Penicillin–Streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were regularly photographed under a fluorescent microscope (IX-70, Olympus, Tokyo, Japan).

Plasmids and electroporation. pferritin–DsRed plasmids were made from a pDsRed-N1 vector (CLONTECH Laboratories, Inc., Mountain View, CA, USA) by insertion of a mouse heavy chain of ferritin sequence (NM_010239). The mouse heavy chain of ferritin sequence was amplified by polymerase chain reaction, then PCR products were treated with restriction enzymes, HindIII and BamHI, and ligated into the pDsRed-N1 vector at the multi-cloning site. GL261 cells (1×10^6 cells/400 μl) were mixed with 10 μg of the

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plasmids in a 4 mm gap cuvette. The cuvette was set in an ECM830 electroporator (BTX Instrument Division Harvard Apparatus, Inc., Holliston, MA, USA) and electroporation was performed under the following condition (Choose mode: LV mode, Set Voltage: 170 V, Set Pulse Length: 70 ms, Set Number of Pulses: 1). Electroporated cells were cultured in the medium with 400 μg/ml of G418 for selection of mixed clone expressed ferritin–DsRed for 7 days.

RNA extraction and RT-PCR. Total RNA was extracted from cells using RNeasy Mini kit and RNase-free DNase set (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. RNA (1 μg) was reverse transcribed at 37 °C for 90 min in a mixture containing 100 U of recombinant M-MLV reverse transcriptase, 0.1 μg DNA random hexamers, 40 U RNase inhibitor and 1.4 mM dNTPs, in a final volume of 50 μl. The cDNA was amplified with *Taq* DNA polymerase (Takara, Tokyo, Japan), using primer pairs specific to DsRed (sense primer: TTC CAG TAC GGC TCC AAG GT; antisense primer: GAG GAG TCC TGG GTC ACG GT) and ferritin–DsRed (sense primer: CGA GAT GAT GTG GCT CTG AA; antisense primer: GAG GAG TCC TGG GTC ACG GT) for 35 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) and glyceraldehyde 3 phosphate dehydrogenase, GAPDH (sense primer: TGC ACC ACC AAC TGC TTA G; antisense primer: GAT GCA GGG ATG ATG TTC) for 30 cycles, respectively. The PCR products were resolved by electrophoresis on 2% agarose gels stained with ethidium bromide, then photographed using Light Capture (ATTO Corporation, Tokyo, Japan).

Western blotting. Electroporated G261 cells (1×10^6 cells) were lysed on ice in 100 μl TNE buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml approtinin, 10 μg/ml leupeptin), and sonicated before BCA protein quantification (Pierce, Rockford, IL, USA). Samples of equal protein quantity were separated on 12.5% SDS–PAGE gels and transferred to nitrocellulose membranes for Western blotting according to the iBlot gel transfer system (Invitrogen). To detect DsRed, we used anti-DsRed polyclonal rabbit antibody (CLONTECH Laboratories, Inc., 1:1000 dilution) and ECL plus detection system (GE Healthcare UK Ltd., Buckinghamshire, England). The same membrane was re-probed by anti-GAPDH monoclonal antibody and ECL plus detection took place after detection of DsRed.

FACS analysis. Fluorescence of DsRed in electroporated GL261 cells was analyzed using a FACSCalibur cell sorter (BD Bioscience, San Jose, CA, USA) equipped with a 530 nm filter (bandwidth ± 15 nm) and a 585 nm filter (bandwidth ± 21 nm) and CellQuest software (BD Bioscience).

Brain tumor model. Viable cells were counted using a hemocytometer, and the concentration was adjusted to 5×10^5 cells/5 μl of PBS. Each C57BL/6 mouse was anesthetized and placed in a stereotactic frame, then the skull was exposed. One millimeter of burr holes were drilled and the following coordinates were used to position the 10 μl Hamilton syringe, placing the needle 4.0 mm anterior to the bregma, 4.0 mm lateral to the midline, and 3.5 mm ventral to the cortical surface to deliver cells into the striatum. At each site, an injection volume of 5 μl was delivered at a rate of 1 μl/min, and the needle was withdrawn after an additional 5 min. At 3 weeks after injection, the hearts of anesthetized mice were perfused with about 100 ml isotonic saline, and then each brain was isolated, frozen in liquid nitrogen, and embedded in an OCT compound (Tissue Tek; Miles, Elkhart, IN, USA). In some cases, permeated cells by sonication for 1 min on ice were injected stereotactically into the brain, and the brain was similarly embedded after *in vivo* imaging.

Magnetic resonance imaging (MRI). Mice transplanted GL261 cells were anesthetized with 1.0% isoflurane and held in an MRI coil. It was set on MRI equipment (MRTechnology, Inc., Tsukuba, Japan) and T1 and T2-weighted images were acquired according to the manufacturer's procedure. Recombinant ferritin–DsRed from *Escherichia coli* or GL261 cells were collected in 1.5 ml tubes, and T1 and T2-weighted images were acquired by similar procedure.

Optical imaging. Anesthetized mice were observed using a Maestro (Cambridge Research & Instrumentation, Inc., MA, USA) *in vivo* imaging system equipped with LCTF (Liquid Crystal Tunable Filter), which was useful for spectral analysis between DsRed and autofluorescence. Coronal brain sections were also observed using Maestro, and DsRed-specific fluorescence was detected.

Immunohistochemical staining and Berlin blue staining. Coronal sections (8 μm) of the brains were cut with a cryostatmicrotome, transferred to a MAS coated slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan) and immediately air-dried. Following fixation with 4% paraformaldehyde in PBS (pH 7.2) at room temperature for 10 min, sections were incubated in a blocking buffer (1% bovine serum albumine, 10% normal goat serum, and 0.01% sodium azide) for 30 min at room temperature and labeled with polyclonal antibodies against DsRed at a dilution of 1:500 for 1 h at room temperature. Then sections were visualized with Cy2-conjugated goat anti-rabbit IgG (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) and counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA). Several sections were stained with Berlin blue staining. In brief, sections were fixed with ethanol at room temperature for 10 min and washed three times with PBS. The sections were incubated in a mixture of 2% potassium ferrocyanide solution with 1% HCl in equal amounts for 20 min at room temperature and counterstained with Kernechtrot solution (Muto Pure Chemicals Co. Ltd., Tokyo, Japan). Photographs of these sections were then taken under a fluorescent microscope (BX-50, Olympus, Tokyo, Japan).

Image analysis. Images acquired from optical imaging and from MRI were analyzed using Maestro software (Cambridge Research & Instrumentation, Inc.) and Image-Pro Plus software (Roper Industries, Inc., Sarasota, FL, USA), respectively.

Results

Some GL261 cells expressed DsRed at 24 h after electroporation, and the transformants expressing ferritin–DsRed were concentrated by G418 selection (Fig. 1A). FACS analysis showed that ferritin–DsRed-expressing GL261 cells had lower fluorescence compared to DsRed only expressing cells (Fig. 1B). To confirm transgene expression, mRNA expression of DsRed and ferritin–DsRed was analyzed by RT-PCR (Fig. 1C). GL261 cells, electroporated with pferritin–DsRed vectors, were positive for mRNA of both heavy chain of ferritin and DsRed. In addition, analysis by Western blotting indicated existence of ferritin–DsRed fusion protein in lysates from GL261 cells electroporated with pferritin–DsRed vectors (Fig. 1D). To determine whether ferritin–DsRed fusion protein in GL261 cells served as an MRI reporter, the cell pellet (Fig. 2A) was analyzed by T1-weighted images and T2-weighted images (Fig. 2B), since T1-weighted images are generally useful in investigating anatomical structures and T2-weighted images are effective in finding lesion sites in tissues by increasing the signal intensity. T2-weighted images are also highly effective in visualizing existence of iron in high T2-weighted signal intensity in animal tissues. As the first step to examination, we examined recombinant ferritin–DsRed fusion protein from *E. coli* using MRI. In general, T2-weighted images from proteinous pellets (data not shown) or cell pellets (Fig. 2B; middle) in aqueous liquid showed high intensity, however, we found that T2-weighted images from recombinant ferritin–DsRed indicated enhanced visualizing effect by reduction of T2-weighted signal intensity; dense deposits were observed at a site of a pellet (Fig. 2B; top). As the following step, cell pellets from ferritin–DsRed-expressing GL261 cells were observed using