In vivo MR imaging of folate-receptor expression with the folate-specific nanospheres in a C6 glioblastoma model

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ABSTRACT

Purpose: To assess the ability of magnetic albumin nanospheres conjugated with folate (FA-MAN) to provide FR-specific enhancement of C6 glioblastoma on magnetic resonance (MR) images.

Procedures: Active targeting effect of magnetic albumin nanospheres conjugated with folate (FA-MAN) was evaluated based on MR images and histopathological analysis. MR imaging of subcutaneously transplanted C6 glioblastomas was performed after intravenous injection of FA-MAN, non-targeted (magnetic albumin nanospheres, MAN) and FA-inhibited (magnetic albumin nanospheres conjugated with folate plus folate, FA-MAN + FA) agents at designated time points. The T2 relaxation times in tumors were compared among different treatment groups and were correlated with histopathological findings. Prussian blue staining and in vivo toxicity assay were also performed simultaneously.

Results: Upon MR imaging in vivo, T2 relaxation time of the tumor sites in the group administered with FA-MAN (T2 is 49 ms, 46 ms and 45 ms at 24 h, 48 h and 72 h, respectively) has statistical difference compared to those in the groups of MAN (T2 is 56 ms, 56 ms and 61 ms at 24 h, 48 h and 72 h, respectively) and FA-MAN + FA nanospheres (T2 is 56 ms, 57 ms and 56 ms at 24 h, 48 h and 72 h, respectively). Prussian blue-stained results demonstrated that more iron particles accumulated in the tumors of the targeted group than those of the other groups. Toxicology assay showed that no noticeable body weight losses were observed after monitoring 31 days, and the results of routine blood parameters, liver and kidney function biomarkers also demonstrated that the nanospheres did not influence the respectively physiological index. Besides, no obvious pathological injuries on the major organs were examined.

Conclusion: Folate-conjugated magnetic albumin nanospheres were more effective in targeting C6 glioblastoma in vivo.

KEYWORDS

Magnetic resonance imaging; targeting imaging; folate; cancer

1. Introduction

Gliomas is a highly chemo-resistant and radio-resistant type of cancer, leading to a poor quality of life and frustrating prognosis. In spite of the recent advances in diagnosis of gliomas, differentiating glioma tissue from normal brain tissue is still difficult in the clinical practice [1]. Consequently, novel and efficient prognostic strategies are in urgent need for surmounting this challenging disease.

Considerable efforts have recently been made in developing easy-to-fabricated probes for magnetic resonance (MR) imaging. MR imaging can realize tumor detection noninvasively due to its superior tissue contrast without radiation exposure [2]. Superparamagnetic iron oxide (SPIO) nanoparticles, as one kinds of MR imaging contrast agents, resulting in signal loss on T2- and T2*-weighted images [3]. Furthermore, due to distinctive properties such as tunable size, shape, and unique physical character over traditional contrast agents, these SPIOs have evoked us to develop a specific molecular imaging probe for the diagnosis of gliomas.

In the current study, we have reported one intriguing probe, bovine serum albumin (BSA)-coated SPIO nanoparticles (MAN), employing for targeted imaging of gliomas in vivo. The BSA nanoparticles offer some promising features such as lower toxicity, fast accumulation and prolonged retention in the tumor region [4–6].

The folate receptor (FR) is strongly up-regulated in many solid tumor cells and endothelial cells of new vasculature in tumor tissues, while it is deficient in most normal organs, suggesting that it is a specific and potential binding target of interest [5,7].
Recent studies from our group suggest that the tumor-targeting nanosphere, composed of folate-SPION-albumin nanospheres, can be employed as one potential contrast agent for active targeting imaging C6 cells in vitro [8].

In this contribution, we take advantage of these active targeted nanospheres for gaining further insight into the usefulness and clinical applicability of these nanospheres. Briefly, upon intravenous injection of the synthesized FA-targeted nanospheres (magnetic albumin nanospheres conjugated with folate, FA-MAN) into C6 tumor-bearing mice, these nanoparticles could accumulate into the tumour sites upon the enhanced permeability and retention (EPR) effect. As folate receptors are overexpressed in C6 glioblastoma cells, specific interaction between folate receptor and folate on FA-MAN will induce the process of ligand-receptor-mediated endocytosis, which plays a critical role in facilitating internalization of the nanospheres into the targeted tumor cells. Besides, systemic administration in vivo, its in vivo long-term toxicity profiles of the FA-MAN were analyzed by hematological and biochemical analyses, to determine a single safe dose for systemic administration in vivo. Therefore, we aim to achieve two major goals: (1) FA-MAN could serve as a MR imaging probe for locating tumors in vivo, and (2) the in vivo long-term toxicity of the obtained FA-MAN is negligible after systemic administration. Detailed experiments were conducted to prove the feasibility of our hypothesis and assess the possibility of FA-MAN for potential clinical applications.

2. Experimental section

2.1 Chemicals and reagents

Bovine serum albumin (BSA, fraction V, pH 7.0), folic acid (FA), N-hydroxysuccinimide (NHS), and N-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich. Isoflurane was purchased from Shandong Keyuan Pharmaceutical Co., Ltd. (China). Superparamagnetic Fe₃O₄ nanoparticles, magnetic albumin nanospheres (MAN) and folate-conjugated magnetic albumin nanospheres (FA-MAN) were obtained as given in previous studies [8–14]. All reagents were of analytical grade and used without any purification unless otherwise stated.

2.2 Cell culture

The rat glioma cell line C6 was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China. The C6 cells (overexpress FR) were seeded and cultured in folate-free RPMI 1640 medium (Invitrogen, IL) and supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, and 10 mg/mL streptomycin at 37 °C under 5% CO₂. Cells were grown to confluence prior to experiments.

2.3 Animal protocol

Female BALB/c nude mice were purchased from the Academy of Military Medical Science (China). All animal experiments were conducted according to the Guidelines of the Animal Care Committee of Southeast University. A C6 tumor model was established by the subcutaneous injection of 200 uL PBS solution containing 2 × 10⁶ H22 cells into the right lower limb. All mice received continuous folate-deficient diet for 3–4 weeks to reduce the serum folate concentration to human physiological levels before the following MR imaging. The tumors were used for diagnostics when they reached a mean size of about 100 mm³.

2.4 Magnetic resonance imaging experiments in vivo

Thirty-two female BALB/c mice were used in this study and then randomly divided into 4 groups (eight mice per group). Mice were anesthetized for imaging with isoflurane (1.5% vol. at 2 L/min) delivered through a nose cone, and breathing rates were detected simultaneously. Then these mice were intravenously administered with a single dose of FA-targeted (FA-MAN), non-FA-targeted (MAN), or FA-inhibited nanoparticles (FA-MAN + FA) (150 μL, 5 mg Fe/kg). Several other mice were used as the controls. Following systemic administration of the nanoparticles, time-dependent biodistribution was performed at 7.0T Micro-MRI (PharmaScan, Brukers, Germany). Scans were performed at 0, 1, 2, 4, 8, 24, 48 and 72 h post-injection. The MRI imaging parameters was set as follows: TR/TE = 408 ms/3.5 ms, slice thickness = 0.8 mm, matrix = 256 × 256, FOV = 35 mm × 35 mm. Images were processed upon the ParaVision software program (PV5.0, Bruker, Germany). T₂ relaxation times were measured by manually drawing a region of interest (ROI) within the tumor areas.

2.5 Histopathological assay

At 72 h after tail vein injection, the tumor tissues were harvested and dehydrated using buffered formalin, different concentrations of ethanol solution, and xylene. Afterwards, liquid paraffin was utilized to trap the dehydrated tumors. The organs and tumor tissues
were sectioned into slices, subsequently Prussian blue iron staining, nuclear fast red staining and hematoxylin and eosin (H&E) staining were performed for histology analysis. The stained slices were analysed by two independent pathologists at Southeast University.

2.6 In vivo toxicology assay

In order to evaluate the toxicity and side effects of FA-MAN nanospheres, fifteen healthy and tumor-free BALB/c mice were randomly assigned to 3 groups, and each group contained 5 mice. On the zeroth day, one group of mice were injected with 150 μL of saline via tail vein, while the other groups were injected with the same dose of FA-MAN/MAN nanospheres (150 μL, 10 mg Fe/kg). After injection, weights and ordinary behaviors of all the mice were scrutinized every other day for the following 30 days. The mice were sacrificed and exfoliated 30 days after i.v. injection, respectively. The serum samples of mice were collected for the following biochemical examinations. Then pathological examinations of the lungs, liver, heart, kidney, and spleen were conducted to investigate whether there was some evidence of metastases or pathological injuries based on hematoxylin and eosin (H&E) staining analysis.

2.7 Statistical analysis

All experiments were carried out in triplicate and the data are expressed as means ± standard deviation (SD). One-way ANOVAs tests are used for multiple comparisons. \( P < 0.05 \) was deemed as statistically significant.

3. Results and discussion

3.1 Magnetic resonance imaging experiments in vivo

To evaluate the active targeting efficacy of the as-prepared nanospheres, the in vivo biodistributions of non-targeted and FA-conjugated nanospheres, as well as FA-conjugated nanospheres co-injection with FA, were determined by \( T_2 \)-weighted MR imaging in C6 tumor-bearing mice. Three series of in vivo MR images were scanned at designed time points postinjection. As shown in Figure 1(a), the \( T_2 \) value of the tumor region became smaller significantly in the group administrated with FA-targeted nanospheres compared to the non-FA-targeted and FA-inhibited groups with the elapse of time. In mice injected with FA-targeted
nanospheres, the signal intensity in the tumor area significantly decreased over time (Figure 1(a)). In mice injected with non-targeted or FA-inhibited nanospheres, the signal intensity of the tumors was also slightly decreased at 1 h and 2 h after injection, but to a less degree than in those given FA-targeted nanospheres (Figures 1(b,c)). Tumor signals in the mice with injection of non-FA-targeted and FA-inhibited nanospheres returned to the baseline 24 h after injection, but remained lower with FA-targeted nanospheres. With the elapse of time, tumor signal in mice given FA-targeted nanospheres eventually reached the baseline. As shown in Figure 2, at the 24 h point, $T_2$ relaxation time of the tumor sites significantly differed between mice treated with FA-targeted, non-FA-targeted and FA-inhibited nanospheres ($P < 0.05$). These results indicate FR-specific retention of the MR contrast agent FA-MAN in C6 glioblastomas. Findings from control experiments indicate that control and FA-inhibited nanospheres were not retained in C6 glioblastomas. Ferric oxide nanoparticles coupled with folate have been recently used for MR imaging studies by Wang et al. for imaging of ovarian tumors [15], Meier et al. for breast tumors [16], and Choi et al. for nasopharyngeal carcinomas [17]. Our exciting data are consistent with these studies, meaning the capability of detecting FR-overexpressing tumors in vivo. Furthermore, the results demonstrate active targeting by FA-MAN since we analyze the uptake of contrast in tumors among targeted (FA-MAN), non-targeted (MAN) and inhibited (FA-MAN + FA) contrast agents, while previous studies did not have these three agents available for comparison. Thus, FA-MAN could potentially be used for noninvasive identification of FR expression in glioblastomas.

3.2 Histopathological assay

To deeper explore the MRI outcomes and validate the existence of FA-MAN in the tumor sites, tumor tissues were harvested at 72 h after injection, dealt with Prussian blue staining and nuclear fast red staining, respectively. Then the slices were observed under light microscopy. As displayed in Figure 3(a), FA-MAN nanospheres in the tumor region was apparent upon Prussian blue staining, which accentuated an inhomogeneous, preferentially intracellular, and partly extracellular distribution of iron particles in FA-MAN-treated C6 glioblastomas. In sharp contrast, little iron deposition was observed in C6 tumor-bearing mice treated with MAN (Figure 3(b)) or FA-MAN + FA (Figure 3(c)). The results are in parallel with the MR imaging analyses.

3.3 In vivo toxicology assay

Low toxicity or even nontoxicity is the prerequisite for biomaterials to be used in the human body [18]. In this regard, 150 µL of FA-MAN/MAN nanospheres (10 mg Fe/ml), which was double the dose of that used for MR imaging, was intravenously injected into healthy mice to examine its toxicity. After administration, body weight changes of mice in the FA-MAN/MAN group and saline group (negative control) were
monitored for 31 days. As shown in Figure 4, no noticeable body weight losses were observed in either group. The mice were subsequently sacrificed to collect blood for biochemical examination, and various organs were harvested for histological examinations. As showcased in Figure 5, the routine blood parameters, liver and kidney function biomarkers were all within normal limits 30 days after intravenous injection. Especially, negligible pathological injuries on the major organs (heart, liver, spleen, lung and kidney) can be examined in the related HE results in Figure 6, which show that the FA-MAN and MAN nanospheres indeed do not result in any significant injuries. Obviously, the above results substantively confirm that the above prepared FA-MAN/MAN/FA-MAN + FA are biocompatible in vivo.

Figure 5. In vivo toxicology and serum sample biochemical examination data acquired from BALB/c mice after 30 days post-injection intravenously with 150 μL physiological saline of FA-MAN/MAN nanospheres (10 mg Fe/ml): Blood routine test contained white blood cells (WBC), red blood cells (RBC) and hemoglobin (HGB); hepatic function biomarkers included alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP); renal function parameters included blood urea nitrogen (BUN) and creatinine (CREA). Untreated healthy mice were used as the negative control. Five mice were included in each group.

Figure 6. H&E staining images of the major organs (heart, liver, spleen, lung, kidney) from different groups. Mice were intravenously injected with 150 μL physiological saline of FA-MAN/MAN nanospheres (10 mg Fe/ml) and dissected after 30 days of post-injection (Scale bar: 50 μm).
Although the targeting ability of nanospheres-FA conjugates may suffer from the adsorption of additional proteins under physiological conditions [19], the successful in vivo MR imaging of the subcutaneous tumor indicated that if the nonspecific adsorption of plasma proteins would occur with respect to the current FA-MAN probe, and realize more accumulation of Fe in the tumor site based on the specific ligand-receptor reaction compared to the routine MR imaging methods.

However, this study has some limitations. One is that only several mice are enrolled in this study, and the other one is that subcutaneous tumor cannot reflect the true glioblastoma in human. Therefore, during the next stage, introducing large animals and establishing orthotopic glioblastoma models are our pursue, so that with more effective strategies on the nanospheres, simultaneous theranostics may come true to enhance the prognosis of glioblastoma.

4. Conclusion
In summary, FR-targeted nanospheres were synthesized for realizing distinctive accumulation and prolonging retention in FR-overexpressed C6 glioblastoma. FA-MAN accumulated to a greater degree in high FR-positive tumors than did MAN or FA-MAN + FA. Our data suggest that FA-coupled MAN has promising potential for monitoring early tumor. Also, intensive studies of FR-targeting contrast agents and enhancement in imaging effect are needed to pave a highly effective way for the diagnosis of FR-positive tumors.

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Disclosure statement
The author reports no conflicts of interest in this work.

Ethical approval
All applicable institutional and/or national guidelines for the care and use of animals were followed.

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