NANOTECHNOLOGIES

Tumor-Specific Contrast Agent Based on Ferric Oxide Superparamagnetic Nanoparticles for Visualization of Gliomas by Magnetic Resonance Tomography

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The aim of this study was to create vector superparamagnetic nanoparticles for tumor cell visualization *in vivo* by magnetic resonance tomography. A method for obtaining superparamagnetic nanoparticles based on ferric oxide with the magnetic nucleus diameter of 12±3 nm coated with BSA and forming stable water dispersions was developed. The structure and size of the nanoparticles were studied by transmissive electron microscopy, dynamic light scattering, and x-ray phase analysis. Their T2 relaxivity was comparable with that of the available commercial analog. Low cytotoxicity of these nanoparticles was demonstrated by MTT test on primary and immortalized cell cultures. The nanoparticles were vectorized by monoclonal antibodies to connexin 43 (Cx43). Specific binding of vectorized nanoparticles to C6 glioma Cx43-positive cell membranes was demonstrated. Hence, vector biocompatible nanoparticles with high relaxivity, fit for use as MRT contrast for the diagnosis of poorly differentiated gliomas, were created.

Key Words: *connexin 43; glioblastoma; magnetic resonance tomography; ferric oxide nanoparticles*

Poorly differentiated gliomas rank first in the structure of neurooncological diseases. Because of their rapid progress and invasive growth, surgical treatment is carried out at the stage when the tumor deeply infiltrates the adjacent tissues. The survival median in this diagnosis is 6 months [12]. This is explained by the absence of a method for early diagnosis, when the tumor is still small and its total removal would not lead to irreversible impairment of brain functions.

The main method for life-time diagnosis of cerebral tumors is magnetic resonance tomography (MRT) with contrast agents [4]. The MRT contrast agents are subdivided into T1 and T2 types by their interactions

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with the magnetic field and modification of the signal intensity in the T1 and T2 mode, respectively.

The only T2 contrast agent widely used in MRT is ferric oxide γ-Fe_2O_3 and Fe_3O_4 nanoparticles with the magnetic nucleus diameter of no more than 20 nm [3]. This size of the nanoparticles explains the superparamagnetism of the phenomenon: in contrast to ferromagnetics, retaining residual magnetism after removal of the external magnetic field, ferric oxide nanoparticles with the nucleus diameters less than 20 nm have zero magnetism after removal of the external magnetic field. Due to this, water suspensions of nanoparticles can be used as MRT contrast agent without risk of nanoparticle conglomeration as a result of magnetic exposure, which can lead to embolism of vital vessels.

In order to reduce the toxicity and prolong the circulation of contrast agents based on superparamagnetic ferric oxide nanoparticles, after synthesis they are as a rule modified by materials reducing their opsonization [6]. This modification increases the hydrodynamic diameter of the particles to 40-60 nm, this ensuring their optimal pharmacokinetics. With this mean diameter, the particles are large enough to be not filtered in the renal glomerular system, but do not yet reach the size at which they are effectively captured by the reticuloendothelial system cells. Accumulation of the particles can be stimulated by their conjugation with vector agents, such as monoclonal antibodies to tumor-specific proteins [9]. Due to this treatment the particles selectively accumulate in the tumor focus, which, in turn, promotes its differentiation from foci of other nature at the early stage of the disease and thus appreciably improves the efficiency of glioma diagnosis.

As for the diagnosis of the borderline of poorly differentiated gliomas, the use of connexin 43 (Cx43), an integral membrane protein hyperexpressed by the peritumoral astroglial roll astrocytes [1], as the target protein seems to be promising. The Cx43 has four transmembrane domains, due to which it is oligomerized into connexones forming pores in cell membrane (hemichannels). Hemichannels of the neighboring cells fuse by means of two extracellular fragments of Cx43 (E1 and E2), forming an integral junction through which the ionic homeostasis is maintained and cell-cell signals are transmitted, regulating fetal cell proliferation, differentiation, apoptosis, and migration in the ontogenesis. The stimulatory effect of Cx43 on invasion of human multiform glioblastoma, including rat experimental glioma C6, has been described [10,8]. Hyperexpression of this marker at the tumor periphery in the zone of active invasion of mobilized glioma cells inspired studies of Cx43 within the framework of the concept of the glioma cell populations resistant to chemo- and radiotherapy. Hence, Cx43-positive cells of poorly differentiated gliomas are a prospective target for life–time diagnosis.

Our aim was the development of a method for creation of vector MRT-contrast agent based on superparamagnetic ferric oxide nanoparticles, specific to glioma cells, and evaluated its biocompatibility for the use in the diagnosis of brain tumors.

MATERIALS AND METHODS

The synthesis of magnetic nanoparticles (MNP) was carried out by thermal degradation of ferric (III) acetylacetonate. Ferric acetylacetonate (2 g) was dissolved in 40 ml absolute benzyl alcohol in a dry flask blown through by nitrogen. The resultant solution was heated to 110°C for 1 h, after which it was boiled with a reflux condenser in nitrogen flow for 40 h. The resultant particles were warmed in acetone and dried till its complete elimination. Water colloid solution of the particles was stabilized with BSA as follows. Distilled water (5 ml) was added to 20 mg particles and pH was brought to 11.1 M with NaOH solution. The resultant dispersion was treated with ultrasound (10 min) and BSA (40 mg) dissolved in 5 ml water was added. The resultant mixture was incubated for 4 h at ambient temperature at continuous stirring, dialyzed in distilled water, and lyophilized.

Iron concentration was measured by colorimetry of iron ions (II) using ferrosine as described previously [11].

The cytotoxicity of C6 glioma cell culture was evaluated by MTT test [7]. The cells were incubated with MNP for 24 h, after which they were washed from the medium and incubated in growth medium (DMEM) for 3 days, and MTT test was then carried out as described previously [7]. Optical density of cell lysate was measured on a VictorX3 plate analyzer (PerkinElmer).

The resultant nanoparticles were analyzed by transmission electron microscopy (TEM) in a Phillips 410LS microscope and by dynamic light scattering (Zetasizer Nanoseries, Malvern). The MNP relaxivity was measured by the nuclear magnetic resonance on a relaxometer (Bruker) at Medical Center of the University of Nebraska.

Conjugation of nanoparticles with antibodies to Cx43. Trout's reagent (2 μl 14 mM solution) was added to 0.5 ml solution of antibodies to Cx43 extracellular fragment, dissolved (50 μg/ml) in 0.1 M carbonate buffer (pH 8.6), obtained at Department of Basic and Applied Neurobiology, V. P. Serbskii Center of Social and Forensic Psychiatry [1], and the mixture was incubated for 1 h at ambient temperature. Antibodies were separated from the reagent excess by gel filtration on Sephadex G-25. Aminopolyethylene glycol-7500-ma-

leimide (NH_2-PEG-MAL) in DMSO at a concentration of 4 mg/ml was then added to the resultant solution and the mixture was incubated for 4 h at 4°C. Carboxyl groups were stimulated by adding 12 µl water-soluble carbodiimide (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbolylimide hydrochloride; 2 mg/ml) and 20 µl water solution of N-hydroxysuccinimide (1.5 mg/ml) to 10 ml nanoparticles solution in PBS (0.45 mg/ml). The solution of modified antibodies was then added to nanoparticles and the preparation was incubated for 12 h at 4°C. Vector nanoparticles were separated from free antibodies by gel chromatography in a column packed with Sepharose CL-4B.

Immunofluorescent analysis of nanoparticles conjugated with monoclonal antibodies to E2Cx43 was carried out on connexin-positive C6 glioma cells fixed with paraformaldehyde. Before adding the nanoparticles, antibody fixation nonspecific centers were blocked by 30-min incubation of cells with 10% goat serum, after which the samples were incubated for 2 h with MNP-BSA conjugated with antibodies at ambient temperature. Second antibodies to mouse immunoglobulins labeled with Alexa Fluor 488 (Invitrogen) were then added. The MNP-BSA conjugated with nonspecific antibodies served as the control. The resultant preparations were analyzed in a DMI 6000 B fluorescent microscope (Leica Microsystems).

RESULTS

Superparamagnetic ferric oxide nanoparticles with the magnetic nucleus 12±3 nm in diameter were synthe-

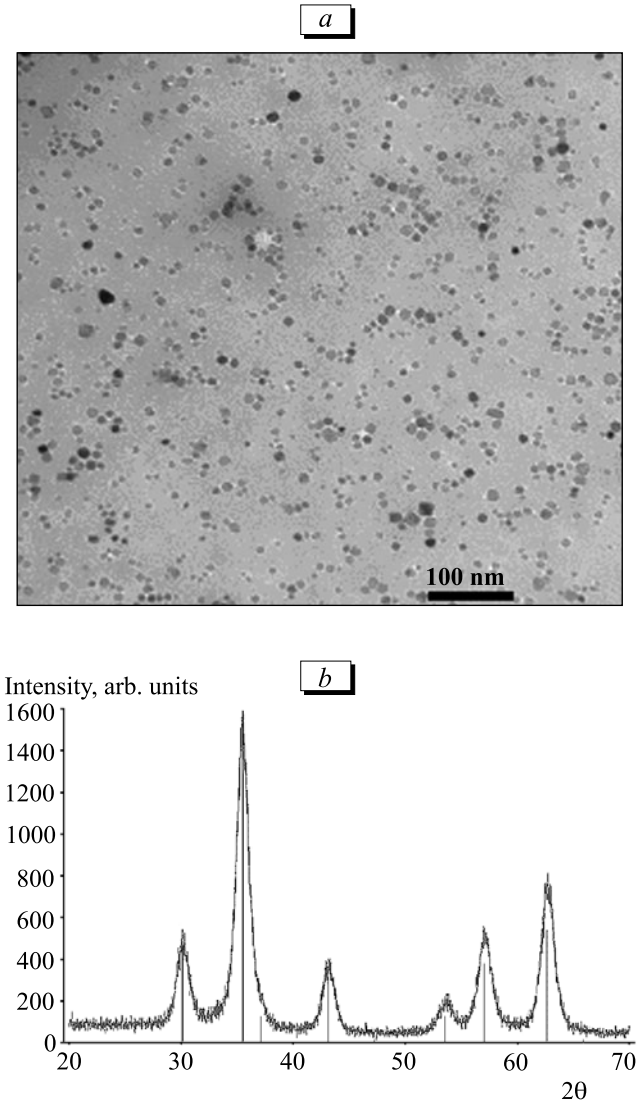

Fig. 1. Ferric oxide nanoparticles obtained by ferric (III) acetylacetonate degradation (electron microscopy, *a*) and x-ray phase analysis of ferric oxide nanoparticles (*b*).

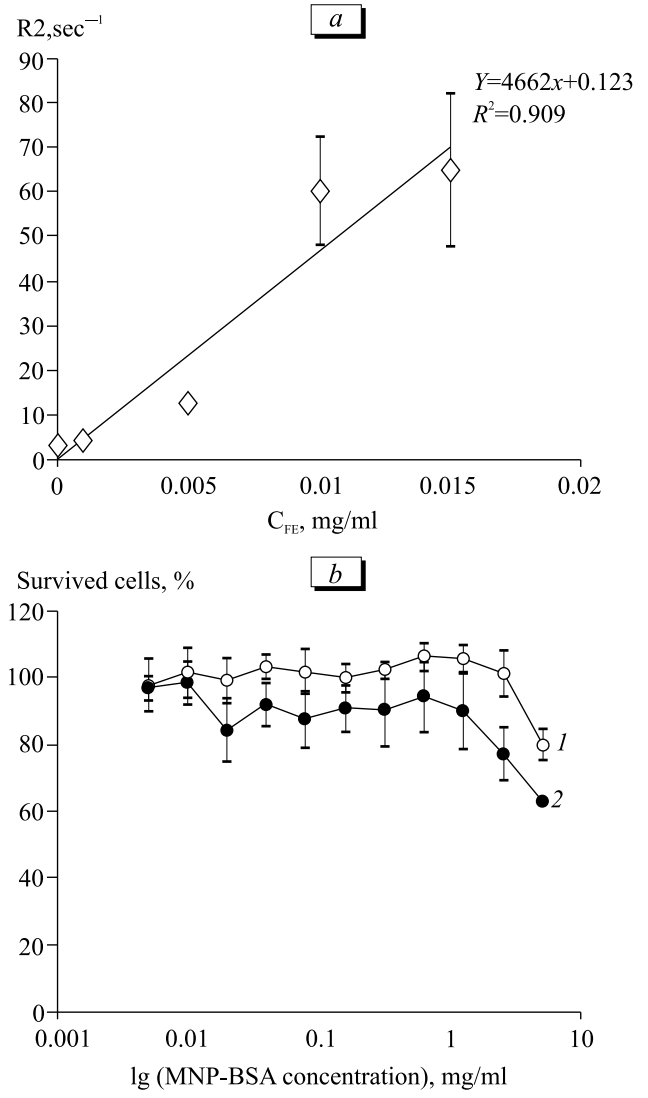

Fig. 2. Relationship between proton relaxation time and concentration of magnetic centers (*a*) and evaluation of cytotoxicity of BSA-coated ferric oxide nanoparticles on C6 glioma cells (*1*) and normal rat astrocytes (*2*) by MTT test (*b*).

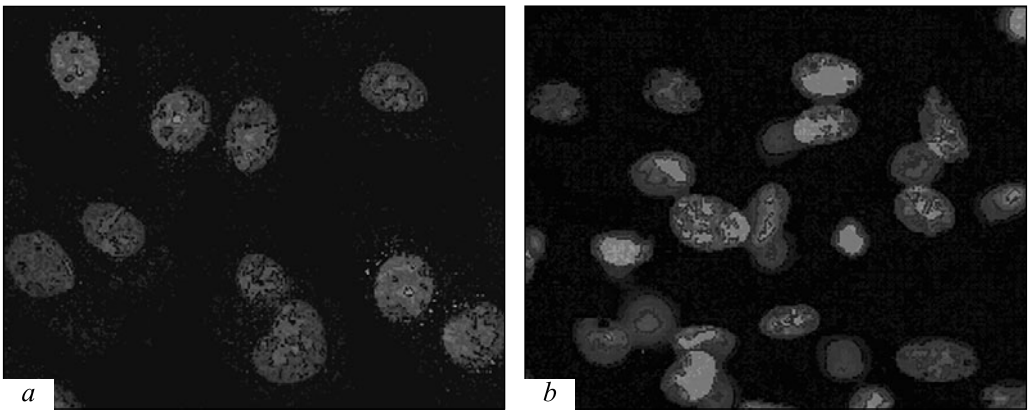

Fig. 3. Immunofluorescent analysis of ferric oxide nanoparticles conjugated with antibodies to Cx43 (*a*) and mouse nonspecific immunoglobulins (*b*) in fixed culture of C6 glioma cells: oil immersion (×1000).

sized (Fig. 1, *a*). After BSA modification of the particles, their size in water colloid solution, measured by dynamic light scattering, was 53±9 nm, their ζ potential -41±6 mV. Polydispersion index of the nanoparticles water suspension was 0.19. The particles retained their size and charge after lyophilization and resuspending in PSB, which confirmed their stability in isotonic solution.

X-Ray structural analysis confirmed that the synthesized nanocrystals had a spinel structure with parameters characteristic of complex ferric oxides Fe_2O_3 and Fe_3O_4 (Fig. 1, *b*).

The proton T2 relaxivity (slope of the curve representing the relationship between inverse T2 relaxation time and concentration of magnetic centers in water solutions of the resultant nanoparticles) measured using a H^1-NMR relaxometer was 4662 $ml \times mg^{-1}sec^{-1}$ (Fig. 2, *a*), which was comparable with the clinically used analog Feridex with T2 relaxivity of 4500 $ml \times mg^{-1}sec^{-1}$.

Evaluation of cytotoxicity on rat glioma C6 cell cultures and normal rat astrocytes showed low cytotoxicity of our nanoparticles. According to MTT test, the maximum allowable nontoxic concentration was 2.5 mg/ml ferric oxide (Fig. 2, *b*).

In order to render vector properties to BSA coated nanoparticles, they were conjugated with monoclonal antibodies to Cx43 extracellular domain. According to the results of immunofluorescent analysis, the minimum concentration at which antiCx43-conjugated nanoparticles specifically interacted with tumor cells was 2 μg/ml, while particles conjugated with mouse nonspecific immunoglobulins exhibited no specificity of this kind (Fig. 3). Immunofluorescent analysis with vector nanoparticles visualized exactly the same placoid structures characteristic of the membrane Cx43 as those visualized with primary antibodies to Cx43. These data attest to high specificity of our vector nanoparticles.

Hence, we have developed a method for the synthesis of highly crystalline low toxic complex ferric oxide (Fe_3O_4) nanoparticles stable in water media and fit for the use in magnetic resonance tomography as the T2 contrast agent and obtained vector nanoparticles specifically binding to Cx43-positive C6 glioma cells. Coating of nanoparticles with BSA makes their water dispersions stable and biocompatible and allows conjugation of the nanoparticles with antibodies by the carbodiimide method with the linker based on heterobifunctional polyethylene glycol (amino-PEG-maleimide). Conjugation of nanoparticles with antibodies to Cx43 rendered them vector characteristics for Cx43-positive C6 glioma target cells. As Cx43 is highly expressed in the peritumoral zone, where glioma invasion is realized, the use of these vector particles can precisely locate the interface of invasion cords and be used for monitoring the treatment efficiency. These nanoparticles can be used for MRT diagnosis and therapy of multiform glioblastoma in patients.

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