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Inhalable magnetic nanoparticles for targeted hyperthermia in lung cancer therapy

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ABSTRACT

Lung cancer (specifically, non-small cell lung cancer; NSCLC) is the leading cause of cancer-related deaths in the United States. Poor response rates and survival with current treatments clearly indicate the urgent need for developing an effective means to treat NSCLC. Magnetic hyperthermia is a non-invasive approach for tumor ablation, and is based on heat generation by magnetic materials, such as superparamagnetic iron oxide (SPIO) nanoparticles, when subjected to an alternating magnetic field. However, inadequate delivery of magnetic nanoparticles to tumor cells can result in sub-lethal temperature change and induce resistance while non-targeted delivery of these particles to the healthy tissues can result in toxicity. In our studies, we evaluated the effectiveness of tumor-targeted SPIO nanoparticles for magnetic hyperthermia of lung cancer. EGFR-targeted, inhalable SPIO nanoparticles were synthesized and characterized for targeting lung tumor cells as well as for magnetic hyperthermia-mediated antitumor efficacy in a mouse orthotopic model of NSCLC. Our results show that EGFR targeting enhances tumor retention of SPIO nanoparticles. Further, magnetic hyperthermia treatment using targeted SPIO nanoparticles resulted in significant inhibition of *in vivo* lung tumor growth. Overall, this work demonstrates the potential for developing an effective anticancer treatment modality for the treatment of NSCLC based on targeted magnetic hyperthermia.

1. Introduction

Despite earlier diagnosis and the availability of new molecularly-targeted drugs, lung cancer (specifically, non-small cell lung cancer; NSCLC) is still the leading cause of cancer-related deaths in the United States [1]. Surgical resection is the primary choice of treatment, followed by radiation and/or chemotherapy [2]. Metastatic and locally advanced disease stages are not amenable to surgical resection, and importantly, a majority of patients who undergo surgery eventually experience relapse [3–5]. Poor response rates and survival with current treatments clearly indicate the urgent need for developing an effective means to treat non-small cell lung cancer.

Magnetic hyperthermia is a novel non-invasive approach for tumor ablation and is based on heat generation by magnetic materials, such as superparamagnetic iron oxide (SPIO) nanoparticles, when subjected to an alternating magnetic field (AMF) [6,7]. Depending on the size of SPIO nanoparticles and the frequency of AMF, heat is generated through either Néel or Brownian relaxation. The heat generated dissipates over short distances due to the high thermal conductivity of water and can, therefore, be used for highly focused heating [8,9]. However, inadequate delivery of magnetic nanoparticles to tumor cells can result in sub-lethal temperature change and induction of resistance [10]. Additionally, non-targeted delivery of these particles to the healthy tissues can result in heat damage to normal tissues.

In our studies, we developed epidermal growth factor receptor (EGFR)-targeted, inhalable SPIO nanoparticles for magnetic hyperthermia of non-small cell lung cancer (NSCLC). EGFR overexpression has been observed in as many as 70% of NSCLC patients [11–13], in whom EGFR expression is elevated in epithelial sites within tumors than in sites adjacent to and distant from tumors. We examined the effect of EGFR targeting on accumulation and retention of inhaled SPIO nanoparticles in the tumor tissue and the effect of targeted magnetic hyperthermia therapy on tumor growth in an orthotopic lung tumor model.

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2. Materials and methods

2.1. Materials

Ferrous chloride tetrahydrate, ferric chloride hexahydrate, myristic acid, pluronic f127, ascorbic acid, potassium hydroxide, 1,10 phenanthroline and sodium acetate were purchased from Sigma (St. Louis, MO). Penicillin/streptomycin, fetal bovine serum, RPMI 1640, Dulbecco's phosphate buffered saline, F-12K (Kaighn's modification) and trypsin–EDTA solution were obtained from Invitrogen Corporation (Carlsbad, CA).

2.2. Synthesis of carboxy-terminated pluronic f127 (CTP)

The synthesis of CTP involved the use of an acid anhydride, which is highly susceptible to the presence of moisture. Hence, all the solvents used in the reaction were anhydrous and the reaction environment was maintained as dry as possible. A mass of 2 g of pluronic f127 was dissolved in 40 ml of anhydrous tetrahydrofuran. To the solution, 100 mg of 4-dimethylaminopyridine, 72 μl of triethylamine and 800 mg of succinic anhydride were added, and the flask was sealed immediately. The mixture was stirred at room temperature for 48 h under nitrogen atmosphere. After 2 days, the volatile solvent was removed by rotary evaporation, and the dry residue was dissolved in 40 ml of carbon tetrachloride. The undissolved, unconjugated succinic anhydride was removed by filtration. The remaining polymer solution was concentrated using a rotary evaporator, and CTP was precipitated by drop-wise addition of the solution in cold, dry diethyl ether [14]. The residue was filtered, dried in a vacuum oven at 40 °C overnight and analyzed by proton NMR. The final yield of CTP was 1.74 g.

Completion of the reaction was confirmed by NMR. Around 25 mg of CTP was dissolved in 750 μl of deuterated water and analyzed using a 400 MHz NMR. The NMR spectrum of unmodified pluronic f127 was also obtained for comparison.

2.3. Conjugation of EGFR-targeting peptide or the isotype scrambled peptide to CTP

A mass of 42 mg of CTP was dispersed in 1.8 ml deionized water. To this solution, 10 mg of *N*-(3-Dimethyl aminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and 14 mg of *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (each dissolved in 100 μl deionized water) were added and then stirred for 15 min at room temperature. The pH of the reaction mixture was 6–7. The excess unreacted EDC was quenched by the addition of β-mercaptoethanol at a final concentration of 130 mM. 10 mg of either the EGFR-targeted peptide (YHWYGYTPQNVI) or the scrambled peptide (HWPYAHPTHPSW) [15] was dissolved in 200 μl of deionized water and added to the reaction mixture. 245 μl of 10× PBS was added to buffer the reaction and the pH was maintained around 7–8. The reaction mixture was stirred overnight at room temperature [16]. The solution was dialyzed against water for 48 h using a 3500 Da molecular weight cutoff Slide-a-lyzer® dialysis cassette, and the final solution was lyophilized (Labconco, FreeZone 4.5, Kansas City, MO).

Conjugation of the peptides to CTP was confirmed by NMR. Around 25 mg of the conjugate was dissolved in 750 μl of deuterated water and analyzed by a 400 MHz NMR. NMR spectra of the free peptides were used for identification of the resonances corresponding to the peptide.

2.4. Synthesis of water-dispersible SPIO nanoparticles

SPIO nanoparticles were synthesized from iron chlorides by the addition of a strong base, followed by coating with a fatty acid to prevent oxidation and then with a surfactant to form a stable aqueous dispersion [17]. Specifically, 0.82 g of ferric chloride hexahydrate and 0.33 g of ferrous chloride tetrahydrate were dissolved in 30 ml of degassed and nitrogen-purged water, and 3 ml of 5 M ammonium hydroxide was added drop-wise to this solution, which was then stirred for 30 min. The resulting iron oxide nanoparticles were washed three times with nitrogen-purged water, sonicated in a water-bath sonicator for 2 min, and then heated to 80 °C. About 100 mg of myristic acid was added to the heated mixture and stirred for another 30 min. Excess myristic acid was removed by two washes with ethanol, followed by two additional washes with water to remove excess ethanol. Each wash was followed by magnetic separation of nanoparticles. Myristic acid coated particles were then suspended in 30 ml water using a water-bath sonicator. Targeted or scrambled peptide conjugated pluronic, equivalent to 5% surface coverage of the peptide (5.5 mg and 11.7 mg of targeted and scrambled peptides, respectively), was mixed with pluronic f127 to yield a total mass of 100 mg, which was then added to the suspension and sonicated in a bath sonicator for 1 h. Every step of the synthesis was conducted carefully to minimize exposure to atmospheric oxygen.

2.5. Characterization of SPIO nanoparticles

The average hydrodynamic diameter of SPIO nanoparticles was determined by dynamic light scattering. About 1 mg of SPIO nanoparticles was dispersed in 2 ml of deionized water by sonication and the dispersion was subjected to particle size analysis using a Delsa™ Nano C Particle Analyzer (Beckman, Brea, CA). The measurement was performed at 25 °C and at a 165° scattering angle. Mean hydrodynamic diameter was calculated based on size distribution by weight, assuming a lognormal distribution. Five individual size measurement runs were performed, with each run recording 150 size events.

The iron content of the SPIO nanoparticles was measured using the 1,10 phenanthroline-based iron assay [18]. SPIO nanoparticles were first dissolved in 12 N hydrochloric acid. The solution was then diluted with distilled water to obtain a final acid concentration of 0.2 N. To the acid solution of SPIO nanoparticles, 10 mg/ml ascorbic acid, 1.2 mg/ml 1,10 phenanthroline, 22.4 mg/ml potassium hydroxide and 123 mg/ml sodium acetate were added in a volume ratio of 1:1:1:1:5. Absorbance of the resultant solution was measured at 490 nm using a microplate reader (ELx800 absorbance microplate reader, Biotek, Winooski, VT). Ferric chloride (hexahydrate) solution in 0.2 N hydrochloric acid was used as a standard.

2.6. Magnetic heating rate

SPIO nanoparticles were dispersed in 1 ml of Hank's F-12K medium in 10 mm × 75 mm disposable borosilicate glass cell culture tubes. Magnetic heating was performed using an induction heating system (1 kW Hotshot, Ameritherm Inc., Scottsville, NY) by placing the suspension at the center of a multiturn copper coil that generated AMF (nominal magnetic field strength of 6 kA/m and frequency of 386 kHz). The temperature change was measured using a fluoroptic® probe (Lumasense Technologies, Santa Clara, CA) at five second intervals. Samples were equilibrated to 37 °C using a water-bath before exposure to the field.

2.7. Aerosol generation and characterization

Aerosolization of SPIO nanoparticles was achieved by ultrasonic atomization [19]. A Pyrex glass baffle was constructed in-house and placed in a water-bath, directly over a 1.7 MHz ultrasonic transducer [20]. About 13 ml of SPIO nanoparticle dispersion in 40% ethanol, containing 8 mg magnetite per milliliter was loaded into the baffle. Compressed air directed into the baffle at a flow rate of 0.5 L/min (as measured by an inline flow meter) entrained the aerosol droplets containing the SPIO nanoparticle dispersion and carried the particles into a subsequent drying assembly. The iron oxide output in the aerosol was measured by collecting the aerosolized and dried SPIO particles for a predetermined period of time on Whatman quartz microfiber filters suitable for air sampling [21]. The filters were assayed using the above procedure and the iron oxide output rate was calculated as iron oxide amount collected per unit time.

The aerosol particle size distribution was determined with a Mercer style seven-stage Intox cascade impactor operating at a sample flow rate of 0.5 L/min. The aerosol generated was passed through a heated drying column before passing through the cascade impactor. Aerosol particles deposited at each stage of the cascade impactor were collected and analyzed by iron assay to obtain the particle size distribution. The mass median aerodynamic diameter (MMAD) and associated geometric standard deviation (GSD) were calculated from linear regression of an *X*-probability plot of the cumulative undersized mass as a function of logarithm of the impactor stage cutoff diameter using OriginPro 8 software (OriginLab Corporation, Northampton, MA) [21].

2.8. Cell culture studies

A549 (human lung adenocarcinoma) and A549-luc (luciferase-transfected A549) cells were used in the study. Both cell lines were propagated using F-12K medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution and maintained at 37 °C and in 5% carbon dioxide.

2.8.1. Demonstration of role of EGFR in tumor cell uptake of functionalized nanoparticles

A549 cells were plated in a 6-well plate 4 h before the start of the study. Cells were washed with phosphate buffered saline (PBS) to remove non-adherent cells and 1 mg (magnetite equivalent) of targeted SPIO particles, scrambled peptide-conjugated particles, particles without any peptide, or targeted particles with excess free targeting peptide were added to the cells in a total volume of 2 ml of cell culture medium containing 5% FBS. The plates were incubated on ice for 30 min, washed thrice with PBS and incubated at 37 °C for an additional 45 min. At the end of the incubation, cells were lysed with 400 μl of RIPA buffer and assayed for total cell protein content and iron content [18] (by iron assay procedure described before).

To evaluate EGFR targeting on the effectiveness of magnetic hyperthermia, plated A549 cells were incubated with targeted or non-targeted particles for 30 min at 4 °C, washed three times with PBS, incubated at 37 °C for 45 min and then subjected to AMF (6 kA/m at 386 kHz frequency). Following AMF exposure, 10 μM propidium iodide was added to the cells and observed under a fluorescent microscope immediately and at 24 h after AMF exposure.

2.9. Orthotopic lung tumor model

All animal studies were carried out in compliance with protocol approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Female

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