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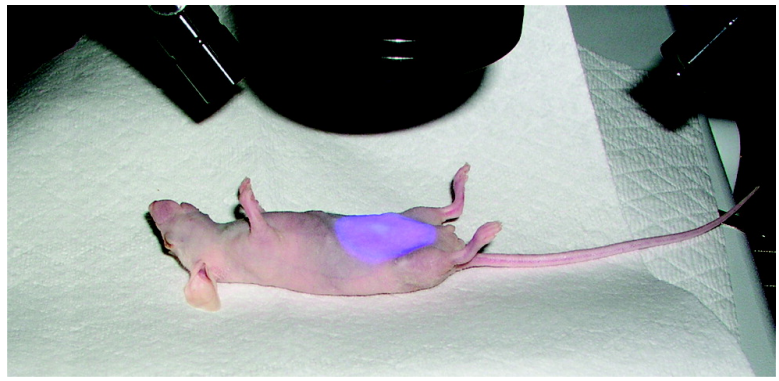

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Magnetic Nanoparticle−Peptide Conjugates for in Vitro and in Vivo Targeting and Extraction of Cancer Cells

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Abstract: Magnetic cobalt spinel ferrite nanoparticles coated with biocompatible polygalacturonic acid were functionalized with ligands specific for targeting expressed EphA2 receptors on ovarian cancer cells. By using such magnetic nanoparticle−peptide conjugates, targeting and extraction of malignant cells were achieved with a magnetic field. Targeting ovarian cancer cells with receptor specific peptide-modified magnetic nanoparticles resulted in cell capture from a flow stream *in vitro* and from the peritoneal cavity of mice *in vivo*. Successful removal of metastatic cancer cells from the abdominal cavity and circulation using magnetic nanoparticle conjugates indicate the feasibility of a dialysis-like treatment and may improve long-term survival rates of ovarian cancer patients. This approach can be applied for fighting other cancers, such as leukemia, once the receptors on malignant cells are identified and the efficacy of targeting ligands is established.

Introduction

Magnetic nanoparticles have promising potentials in biomedical applications because of the unique abilities of magnetic interactions over space and physical barriers.[1,2] Over the past several years, they have shown very promising applications as *in vitro* medical diagnostic tools. In these studies, magnetic magnetite, Fe_3O_4 nanoparticles were used for ultrasensitive detection of prostate-specific antigen (PSA) and for detecting amyloid-β-derived diffusible ligands (ADDLs), which is a potential Alzheimer's disease (AD) marker.[3,4] Fe_3O_4 nanoparticles have also been employed in the *in vitro* detection of certain leukemia and lymphoma cells.[5,6]

We have been exploring possible *in vivo* approaches aimed at deterrence of the metastatic spread of cancer cells from primary carcinomas. The lethality of cancer is often not due to tumorigenesis at the primary locus but due to metastasis of the disease. To this end, we developed biologically modified magnetic nanoparticles as part of a therapeutic approach to capture and extract cancer cells from the body. For cell capture, using nanoparticles with stronger magnetic properties than Fe_3O_4 would be required. Cobalt spinel ferrite, $CoFe_2O_4$ nanoparticles belong to the same spinel ferrite materials family as magnetite. However, they have displayed much stronger magnetic responses at ambient temperatures.[7,8] We chose ovarian cancer as a model to develop our extraction technique. Ovarian cancer is one of the most lethal gynecological malignancies. The survival rate for patients with late stage disease sits at around 20%. Because of the difficulty in early diagnosis of ovarian cancer, 81% of all cases are detected in late stages with metastatic spread of malignant cells.[9] The most significant pathway of ovarian tumor spread occurs via exfoliation of malignant cells from primary tumor sites, leading to dissemination of cancer cells throughout the peritoneal cavity[10] and worsening the prognoses for cancer patients. [11,12] In addition, some cancer cells may escape during primary tumor excision, and the development of resistance in these cells to current chemotherapies can lead to regrowth of a tumor cell population. Intraoperative rupture of malignant epithelial ovarian neoplasms has also been shown to worsen the prognosis of patients with early stage ovarian cancer.[13] Thus, combining the extraction of residual tumor cells to limit the metastatic spread as part of routine treatment procedures could be a strategy to improve long-term survival for cancer patients.

We report here our study on capture of cancer cells from a flow stream *in vitro* and from the peritoneal cavity of mice *in vivo* using magnetic $CoFe_2O_4$ nanoparticles functionalized with a receptor-specific ligand. A polypeptide with a sequence of

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GGGGYSAYPDSVPMMSK was used as a targeting ligand for ovarian cancer cells. The core of the peptide, YSAYPDSVPMMS (YSA), was reported as an ephrin mimetic peptide that binds specifically to the receptor tyrosine kinase (RTK), EphA2 using the YPDSVP region.[14] Because EphA2 is more highly expressed by ovarian carcinoma cells when compared with normal ovarian surface epithelium,[15,16]magnetic nanoparticle−YSA peptide conjugates should be able to selectively bind to the ovarian cancer cells and enable them for magnetic attraction.

Experimental Section

Preparation of $CoFe_2O_4$ Nanoparticles with a Biocompatible Polymer Coating and with YSA Peptide Conjugation. The superparamagnetic $CoFe_2O_4$ nanoparticles were synthesized with a micelle method, and the mean diameter was 8 nm with a size distribution of less than 15%. The detailed experimental procedures have been reported elsewhere.[7] The nanoparticles (200 mg) and polygalacturonic acid (600 mg, Alfa Aesar) were added into 80 mL of 5 M NaOH solution at ambient temperature. After sonication for 5 h with a Model 60 Sonic Dismembrator (Fisher Scientific), the coated nanoparticles were separated from the solution using a magnet. After being washed a few times with water, the coated nanoparticles were resuspended in distilled water. Glucuronic acid was also tested as the biocompatible coating with similar procedures. 1.9 mg of peptide having a sequence of GGGGYSAYPDSVPMMSK were added to 10 mL of an aqueous suspension of the nanoparticles with polygalacturonic acid coating ($\sim 1.7 \times 10^{15}$ particles/mL). The mixture was sonicated for a few minutes. The solution was protected from light and stored at 4 °C overnight to complete the formation of amide bonds between carboxyl groups on the polymer coating and the primary amine on the C-terminal lysine residue.

The YSA peptide was synthesized using standard Fmoc chemistry as reported in the literature.[17] A Rhodamine tag was conjugated on the N-terminus, and the four N-terminal glycine residues were used to distance the Rhodamine from the binding region and prevent steric hindrance to receptor binding.

Cell Growth. The BG-1 cell line was provided by Julie M. Hall and Kenneth S. Korach, Receptor Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NIH, Division of Intramural Research, Environmental Disease and Medicine Program, Research Triangle Park, NC. The BG-1 cells were cultured in DMEM:F12/50:50 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Mediatech, Inc., Herndon, VA) at 37 °C, in a 5% CO_2 atmosphere. The Hey cell line was provided by Gordon Mills, Department of Molecular Therapeutics, The University of Texas, MD Anderson Cancer Center. The Hey cells were propagated in RPMI 1640 (Mediatech) supplemented with 2 mmol of L-glutamine-1 (Sigma), penicillin, streptomycin, and 10% heat-inactivated FBS at 37 °C, in a 5% CO_2 atmosphere.

Cell Staining. Cells were incubated overnight with 20 mg/mL of fluorescein diacetate (FDA) (Research Organics) or 20 mg/mL 5(6)-carboxyeosin diacetate (Research Organics) at 37 °C, 5% CO_2. Cells were removed from the cell culture flask with trypsin+EDTA, washed once with PBS, and resuspended to 2.8×10^6 cells mL^{-1}. For confocal imaging, cells were incubated overnight on chamber slides (Laboratory Tek) and washed the next day with PBS.

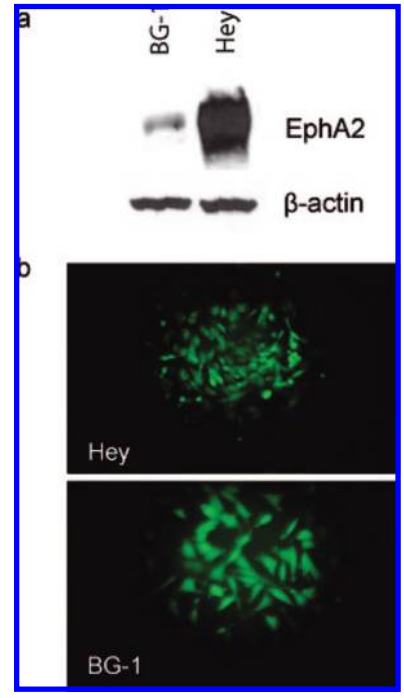

Figure 1. Expression EphA2 receptor in the Hey and BG-1 cell lines. (a) Immunoblot demonstrating the low expression of EphA2 by the BG-1 cell line and the high expression of the receptor in the Hey cell line. β-Actin was used to show equal loading of the samples. (b) Equivalent uptake of the fluorescien fluorophore by the Hey cells and the BG-1 cells.

Rhodamine labeled nanoparticles with or without conjugated YSA peptide were added to the cells and incubated for 1 h at 37 °C, in a 5% CO_2 atmosphere. Cells were washed followed by fixation in 4% paraformaldehyde and coverslipped for imaging analysis.

Mouse Studies. Female *nu/nu* mice were obtained from Taconic (Hudson, NY) and Balb/c mice were from Harlan (Indianapolis, IN). All experiments were conducted with the approval of the Institutional Animal Care and Use Committee at the Georgia Institute of Technology (Atlanta, GA).

Microscopy. In vitro studies were conducted using a 40× objective on an Olympus IX71 inverted microscope with green and red filters and a mercury short arc HBO lamp. Images and video were taken using an Olympus DP71 12.5 million pixel digital camera. Confocal images were obtained with a 40× objective using a Zeiss LSM 510 laser scanning confocal microscope.

Results and Discussion

The magnetic $CoFe_2O_4$ nanoparticles were coated with biocompatible polygalacturonic acid to diminish the adverse immune response[18] and also to facilitate the surface modification. After coating of the polymer, the particles became irregular in shape and with a dimension in the range 100−200 nm. Glucuronic acid also worked very well as a biocompatible coating, which formed a shell around each nanoparticle with a thickness of 5−10 nm.

Hey and BG-1 ovarian carcinoma cell lines were used in our studies. While both Hey and BG-1 lines showed expression of EphA2, the expression was several fold higher in the Hey cell line (Figure 1a). For testing the cell targeting and magnetic attraction *in vitro*, these cancer cell lines were incubated with fluorescein diacetate (FDA) (Figure 1b) with the green emission

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at 515 nm, which can be distinguished from the nanoparticulate conjugates with the Rhodamine tag emitting red at 610 nm. The labeled Hey cells were introduced into a circulating system driven by a peristaltic pump to determine if EphA2 expressing cells could be extracted from a flow stream. A capillary tube, with a flow rate of ~1.22 mL/min inside, was centered in the circuit and placed above a microscope objective. The continual flow of the green fluorescent Hey cells was observed through the tube. Approximately 2 min after the introduction of Rhodamine-tagged magnetic nanoparticle−YSA peptide conjugates, a magnet, with a field strength of ~2600 Gauss, was placed on one side of the capillary tube, and the Hey cells accumulated on the tube wall closest to the magnet. When the magnet was removed, the accumulated Hey cell aggregates dispersed rapidly back into the circulating stream (supplementary video). The cells did not show any response to the magnet if the same magnetic nanoparticles were used but without the YSA peptide ligand. The capture of the cancer cells by the magnet demonstrated the peptide-functionalized nanoparticles caused the cells to become magnetically attractable. Therefore, it may be feasible to effectively remove disseminated cancer cells from the circulation or peritoneal cavity using a dialysis-based approach.

The specific binding of the YSA-conjugated magnetic nanoparticles to Hey cells was verified by using confocal microscopy studies. Hey cells were incubated in chamber slides and allowed to adhere to the slides overnight. The next day, cells were washed and incubated with Rhodamine-tagged magnetic nanoparticles or with the conjugates of the Rhodamine-tagged magnetic nanoparticle and YSA peptide. Cells were then fixed, and the binding of the magnetic nanoparticles to the cells was examined under fluorescence. Cells incubated with Rhodamine-tagged nanoparticles showed little or no binding to the particles (Figure 2a) while cells incubated with Rhodamine-tagged nanoparticle−YSA peptide conjugates showed binding of the particles over a large amount of the cell surface area (Figure 2b). A higher magnification of one of the Hey cells with Rhodamine-tagged nanoparticle−YSA peptide conjugates is shown in Figure 2c. This result indicates that the magnetic nanoparticles bind specifically to the Hey cells through the YSA peptide/EphA2 interaction and that nonspecific binding of non-YSA-tagged particles approached background levels.

For testing cell capture *in vivo*, approximately 1.4×10^6 FDA-loaded Hey cells in 500 μL of PBS were introduced by injection into the peritoneum of an anesthetized female *nu/nu* mouse and allowed to disperse for 5 min with gentle abdominal massage to facilitate cell diffusion. The abdomens of the mice were exposed to 488 nm light under a stereo microscope, and there was no visible fluorescent signal at or around the injection site. This was followed by the injection of 500 μL of Rhodamine-tagged magnetic nanoparticle−YSA peptide conjugates. After an additional 5 min of incubation with abdominal massage, the abdomens of the mice were examined under the microscope and no visible fluorescent signal was observed. After a 2600 Gauss magnet with a size of ~1 cm^3 was placed on the skin of the abdomen for 30 s and then removed, the mouse was exposed to 488 nm light again (Figure 3a). A green emission from the FDA-loaded Hey cells was clearly visible through the skin at the site of magnet placement (Figure 3a and 3b), which indicated a large accumulation of Hey cells at this site. When the excitation wavelength was switched to 530 nm to excite the Rhodamine tag, a red emission was visible through the skin at the same spot indicating the existence of Rhodamine-tagged nanoparticulate conjugates (Figure 3c), which was consistent with the presence of the dark mass under the bright field (Figure 3d). When the magnet was moved over the region and then pulled about 1 cm away from the original aggregation site, the green and red fluorescent spots shifted to the new location. The lack of any visible fluorescent signal prior to applying a magnet onto the mice suggested the dispersion of cells and nanoconjugates. The results obtained with a magnet applied indicate that the Hey cells were captured by the magnetic nanoparticulate conjugates in the peritoneal cavity of the mouse via YSA peptide/EphA2 recognition and then were consolidated onto the topside of the cavity by the magnet.

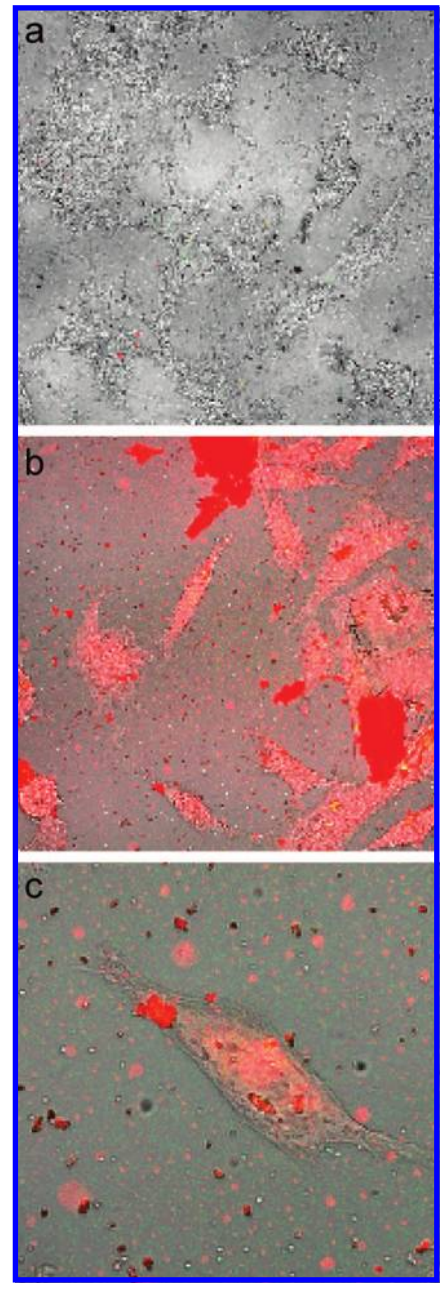

Figure 2. Confocal microscopic images of *in vitro* targeting of Hey cells. (a) Hey cells incubated with rhodamine-tagged magnetic nanoparticles (200X). (b) Hey cells incubated with rhodamine-tagged nanoparticle−YSA peptide conjugates (200×). (c) Higher magnification of Hey cells labeled with rhodamine-tagged nanoparticle−YSA peptide conjugates (400×).

The same study was conducted on FDA-loaded BG-1 ovarian cells. Although the fluorescence of BG-1 cells *in vitro* was as

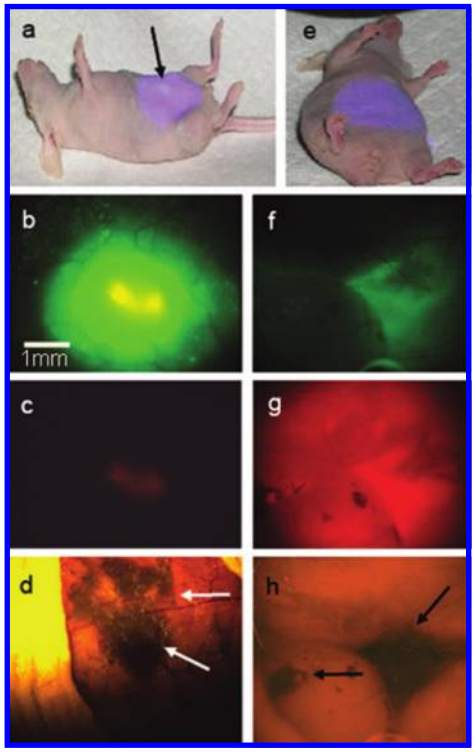

Figure 3. In vivo peritoneal targeting of Hey and BG-1 cells with magnetic nanoparticle−YSA peptide conjugates. (a) Green fluorescence of FDA-loaded Hey cells in the center of illumination through the abdominal skin of an anesthetized mouse. The cells were pulled to the cavity surface by the magnet via the nanoparticulate conjugates. (b) Close view of the FDA-loaded Hey cells emitting green through the skin at the site of the magnet. (c) The nanoparticulate conjugates emitting red through the skin at the site of the Hey cells shown in image b. (d) Magnetic nanoparticle−YSA peptide conjugates observed through the peritoneum under a bright field at the site of the magnet. (e) No visible fluorescence of FDA-loaded BG-1 cells through the abdominal skin of an anesthetized mouse under the illumination after the magnet was removed. (f) Close view of the green fluorescing BG-1 cells after the abdominal skin was removed. (g) The nanoparticulate conjugates emitting red at the site of the BG-1 cells shown in image f. (h) Magnetic nanoparticle−YSA peptide conjugates observed under a bright field at the site of the BG-1 cells. Images were taken using a Canon C5050Z digital camera on an Olympus SZX12 stereo microscope with green and red filters, a mercury short arc HBO lamp, and using a 1.6× objective.

intense as that of the Hey cells, no visible fluorescent emission through the skin was observed upon exposure of the abdomen of the mouse to the 488 nm excitation light (Figure 3e). Fluorescence was visible through the peritoneum once the outer abdominal skin was removed (Figure 3f). The emission was much weaker compared to the one produced by the same number of Hey cells. Intense red fluorescence was clearly seen when the excitation wavelength was switched to the 530 nm range (Figure 3g), and the nanoparticles were easily seen under a bright field (Figure 3h). This indicated that the lack of BG-1 cell aggregates was not due to a shortage of Rhodamine-tagged magnetic particle conjugates. Thus, the low intensity of the fluorescent emissions from the BG-1 cells could be attributed to a smaller number of cells being sequestered by the nanoparticulate conjugates because of relatively low EphA2 receptor expression by the BG-1 cell line. A total of four trials were run for each cell line producing similar results.

The difference in extraction efficiencies of the Hey and BG-1 cells implies the specificity of YSA peptide, which was confirmed by *in vivo* experiments on magnetic extraction of a mixed population of Hey and BG-1 cells within the peritoneal

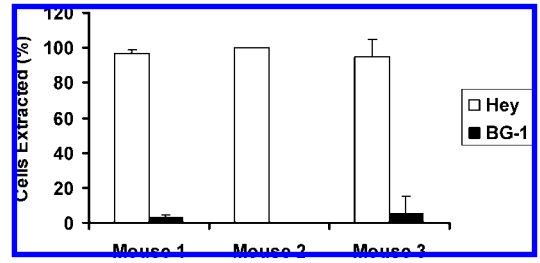

Figure 4. Extraction efficiencies of the Hey and BG-1 cells. Compositions of Hey and BG-1 cells in the cell populations extracted from the peritoneum of three Balb/C female mice. The ratios were averaged from five counts performed on each of three mice. Error bars show the standard deviations.

cavity. The Hey cells were incubated with FDA, and the BG-1 cells were incubated with 5(6)-Carboxyeosin diacetate (CDA) with a 560 nm emission. An equal number of cells from each cell line were mixed and introduced into the peritoneal cavity of three Balb/c female mice. After 5 min of cell incubation and abdominal massage, magnetic nanoparticulate conjugates were injected into the peritoneal cavity and incubated for 5 min. The peritoneal fluid was extracted and filtered magnetically before being examined using a hemocytometer to determine the number of green fluorescent (Hey) and red fluorescent (BG-1) cells. Although the initially mixed cell populations contained 50% Hey and 50% BG-1 cells, Hey cells accounted for 95−100% of extracted cell populations on average from the three trials (Figure 4). The scarcity of BG-1 cells in extracted cell populations was consistent with the specificity of YSA peptide−magnetic nanoconjugates. The highly specific binding of the YSA peptide to the EphA2 receptor enabled the magnetic conjugates to differentiate EphA2-rich ovarian carcinoma cells from EphA2-poor cells.

Our initial findings demonstrate that magnetic nanoparticle−YSA peptide conjugates can target and remove metastatic cancer cells from the fluid of the abdominal cavity or circulatory system. Such results suggest the feasibility of a dialysis-like system for the extraction of cancer cells, which, combined with surgery and chemotherapy, may improve the long-term survival rates for cancer patients. In ovarian cancer cases, significant removal of disseminated cancer cells from the abdominal cavity could lead to reduction of the malignant cell population and reduce the odds of metastatic spread. Additional studies, including evaluation of toxic effects from magnetic nanoparticles, are needed before this method can advance to clinic trial stage. Further improvement of this concept may include refinement of the extraction process with an array of peptides using patient specific tumor protein expression profiles. Since small peptides have been reported to prevent tumor cell adherence onto tissues in a murine model using a bladder tumor cell line,[19] they might also be incorporated into the magnetic cell extraction technique to reduce the possibility of tumor implants and therefore greatly enhance the efficiency of preventing metastatic spread of cancer. Since EphA2 is also highly expressed in other types of cancers, applications of the YSA peptide−magnetic nanoconjugates could be expanded beyond ovarian cancers. Furthermore, this *in vivo* extraction approach utilizing magnetic nanoparticles may also be used in principle for the treatment of viral diseases by targeting and removing viruses and virus-infected cells and therefore bolster the immune system to fight infections.

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Supporting Information Available: A 30 s video clip to show the capture of ovarian Hey cancer cells in the capillary tube by a magnet. This material is available free of charge via the Internet at http://pubs.acs.org.

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