Urokinase Plasminogen Activator Receptor (uPAR) Targeted Multifunctional Magnetic Nanoparticles for in vivo Molecular MRI of Pancreatic Cancers

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Introduction
Novel nanoparticles, including magnetic nanoparticles, have become increasingly attractive for the development of “molecular imaging” probes that promise high specificity and sensitivity to biomarkers or biological processes in vivo. Magnetic nanoparticles such as iron oxide nanoparticles not only offer high relevancies for stronger contrast effect but also allow for robust surface chemistry for loading multiple target specific ligands to improve the specificity and affinity to the targeted cancer cells. Furthermore, nanoparticles exhibit unique pharmacokinetics, such as long blood retention time, providing favorable properties for cell surface receptor targeted imaging. In this study, we have developed a receptor targeted nanoparticle probe for imaging of the xenograft pancreatic tumor model, using an amino terminal fragment (ATF) peptide of urokinase plasminogen activator (uPA) conjugated to the size highly uniformed iron oxide nanoparticle (IONP). This new nanoparticle probe has a high specificity to the pancreatic tumor that over-expresses urokinase plasminogen activator receptor (uPAR) which regulates multiple pathways in the cancer development such as matrix degradation, cell motility, metastasis and angiogenesis. High affinity and successful delivery of this nanoprobe to the uPAR expressing tumor allow for the sufficient T2 contrast for imaging of the tumor in animal models.

Materials and Methods

Mouse ATF Peptides. A cDNA fragment encoding amino acids of 1 to 135 residues of mouse uPA, isolated by PCR amplification using a PCR primer pair containing was cloned into pET101/D-TOPO expression vector (Invitrogen, Carlsbad, CA, USA). The cDNA sequences were confirmed by DNA sequencing. Recombinant ATF peptides were expressed in E. coli BL21 (Invitrogen) and purified from bacterial extracts under native conditions using a Ni2+NTA-agarose column (Qiagen, Valencia, CA). To generate a near infrared (NIR) fluorescent dye, Cy5.5, labeled peptide, Cy5.5™ maleimide (GE Healthcare Bio-Sciences Corp.), was conjugated to ATF peptides using the manufacturer’s protocol.

Engineering uPAR-targeted ATF-IO and Cy5.5-ATF-IO or control Cy5.5-IO nanoparticles. Paramagnetic IONP with a uniform 10 nm core size were prepared, stabilized and functionalized with amphiphilic polymers with our patented method. ATF peptides were then conjugated to the surface of the IONP, via carbonyl cyanide m-chlorophenylhydrazone (CCMCH)-mediated coupling of ATF peptides to amino side groups of the peptides (Figure 1). Conjugation efficiency of ATF peptides to the IO nanoparticles was determined using Cy5.5-ATF-IO particles by fluorescence spectroscopy and then compared the values to a linear standard curve prepared using various concentrations of Cy5.5-ATF peptides. To prepare the Cy5.5-IO control nanoparticles, the Cy5.5-mono NHS ester was reacted with the amine groups of the polymer on nanoparticle’s surface in PBS buffer, pH 7.4, for overnight.

Orthotopic human pancreatic cancer xenograft model. Pancreatic tumor model was established using a human pancreatic cancer cell line, MIA PaCa-2. 1x107 of MIA PaCa-2 cells were injected directly into the pancreas of the 6 to 8 week old nude mice (Taconic, Hudson, NY) using a surgical procedure approved by IACUC of the institution. Orthotopically xenografted pancreatic tumors reached to 5 mm in diameter and ready for experiments in about 3 to 4 weeks.

In vivo MRI and NIR imaging. Tumor bearing mice were scanned using a 3T MRI scanner with a rodent coil for pre- and post-IO contrast MR images, fer the mice were injected with 0.2-0.3 nmol of ATF-IO, Cy5.5-ATF-IO, or IO nanoparticles in 100 µl of PBS through the tail vein, they were imaged at different time points. The imaging sequences included: T1-weighted spin echo or gradient echo method. TR/TE = 31.2 msec/8 msec; flip angle = 30° with 512 frequency encoding steps and 256 phase encoding steps, and 40 slices at 0.5 mm slice thickness were used in the three-dimensional T1 weighted FLASH imaging. A multi-echo T2; weighted fast spin echo sequence was used to obtain T2 relaxometry of the whole mouse. Region of interest (ROI) method was used to evaluate and quantify the contrast agent induced signal or T2 value changes in the tumor and other organs. Before and at different time points after the injection of Cy5.5-ATF-IO, non-targeted IO, or Cy5.5-IO nanoparticles, NIR imaging of the tumor-bearing mice was performed using Kodak in vivo FX imaging system

Immunofluorescence labeling and immunohistochemical staining. Frozen pancreatic normal and cancer tissue sections were obtained after the imaging experiments. Frozen tissue sections were fixed with ice-cold acetone for 10 min and blocked with 10% (wt/vol) goat serum for 10 min. The slides were incubated with 5 µg/ml of an anti-uPAR polyclonal rabbit antibody, Alexa Fluor 555-goat anti-rabbit IgG and counterstained with Hoechst 33342. The slides were examined under a fluorescence microscope. Human or mouse endothelial cells were identified using a monoclonal anti-human-CD31 antibody (BD Biosciences). Mouse macrophage was identified using an Alexa fluor 488-labeled rat anti-mouse CD68 antibody. Prussian blue staining was used to confirm the presence of IONP in the tissues and cells.

Results and Discussions
We have successfully produced a recombinant ATF of uPA and conjugated ATF peptides on the surface of IONP with 8 to 10 of ATF peptides to each nanoparticle. From in vitro cell binding studies, we found that ATF-IONP bind to and were subsequently internalized by uPAR-expressing cancer cells and endothelial cells. Our preparation of IONP has blood circulation T1/2 of 10 hours typically. Systemic delivery of ATF-IO nanoparticles leads to the selective accumulation of the nanoparticles in tumors of an orthotopic human pancreatic cancer xenograft model, as confirmed by both optical and MRI imaging. IONP induced contrast was observed in T2 weighted MRI and MR imaging of pancreatic cancer. For example, we observed a stronger contrast effect but also allow for robust surface chemistry for loading multiple target specific ligands to improve the specificity and affinity to the targeted cancer cells. Furthermore, nanoparticles exhibit unique pharmacokinetics, such as long blood retention time, providing favorable properties for cell surface receptor targeted imaging. In this study, we have developed a receptor targeted nanoparticle probe for imaging of the xenograft pancreatic tumor model, using an amino terminal fragment (ATF) peptide of urokinase plasminogen activator (uPA) conjugated to the size highly uniformed iron oxide nanoparticle (IONP). This new nanoparticle probe has a high specificity to the pancreatic tumor that over-expresses urokinase plasminogen activator receptor (uPAR) which regulates multiple pathways in the cancer development such as matrix degradation, cell motility, metastasis and angiogenesis. High affinity and successful delivery of this nanoprobe to the uPAR expressing tumor allow for the sufficient T2 contrast for imaging of the tumor in animal models.

Fig. 1. uPAR-targeted IO nanoparticles for optical and MR imaging of pancreatic cancer. (A) Micromorphology and MR imaging of the orthotopic human pancreatic tumor xenograft model. Target specificity and biodistribution of IONP nanoparticles were evaluated in vivo by NIR imaging with Cy5.5-ATF-IONP (Fig. 1). Prussian blue, immunofluorescence and immunohistochemical staining of the tissues collected from the animals after imaging experiments showed that positive iron stain cells were found to be co-localized in the tumor areas that are positive for uPAR antibody staining. Interestingly, blue positive iron cells were abundantly found in the tumor and stromal immediately adjacent to tumor cells, but not in surrounding normal pancreatic tissue. CD31 positive endothelial cells in tumor vessels and CD68 positive macrophage in the tumor were also positive for iron staining, suggesting that MRI signal produced from the tumor may be due to the uptake of the ATF-IO nanoparticles in both tumor cells and tumor associated macrophages. Interestingly, both NIR imaging and T2 weighted MRI showed transient signal changes in the kidney and bladder after using ATF-IONP for imaging of mice, which is not seen when using non-conjugated particles, indicating there is possible altered biodistribution of ATF-IONP. Our experimental evidences of binding of uPA to uPAR leading to the internalization of the ligand-receptor complex and in vivo uPAR targeted delivery of ATF-IONP suggest that uPAR is an excellent molecular target for in vivo receptor targeted tumor imaging and drug delivery and uPAR targeted ATF-IONP MRI probe has a great potential for imaging of the pancreatic cancer.