Development of peptide conjugated Superparamagnetic Iron Oxide (SPIO) Nanoparticles for Targeted MR Imaging and Therapy of Pancreatic Cancer

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ABSTRACT

Development of multifunctional nanoparticles that selectively target to human tumors for *in vivo* tumor imaging as well as inhibition of tumor growth holds a great promise for improving survival rate of cancer patients. In this study, we have engineered peptide conjugated superparamagnetic iron oxide (SPIO) nanoparticles targeting to urokinase plasminogen activator receptor (uPAR) or a single chain antibody to epidermal growth factor receptor (ScFvEGFR), which are highly expressed in human pancreatic cancer tissues. We demonstrated that the SPIO nanoparticles bind to and are internalized by pancreatic cancer cells *in vitro,* resulting a significant shortened T2 detected by MRI scan and positive Prussian blue staining in the tumor cells. Furthermore, the targeted-SPIO nanoparticles markedly inhibited the growth of pancreatic cancer cells *in vitro*. Using an orthotopic human pancreatic cancer xenograft model in nude mice, *in vivo* MRI demonstrated that systemic delivery of the targeted SPIO nanoparticles leads to accumulation of the IO nanoparticles in intra-pancreatic tumors causing a significant signal drop in those areas. Examination of tissue distribution of the target-IO nanoparticles by Prussian blue staining of frozen tissue sections showed high levels of iron staining in pancreatic cancer lesions but not in adjacent normal pancreas. Normal liver and spleen also displayed high levels of iron staining, while normal lung tissue had a low level of iron staining. Both kidney and heart tissues lacked iron staining. Therefore, those multifunctional nanoparticles have potential for the development of tumor-targeted imaging probes and drug delivery particles for the detection and treatment of pancreatic cancer.

Keywords: **Tumor targeted nanoparticles, pancreatic cancer, MRI, uPAR, EGFR.**

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the US because of its extremely difficult to diagnosis and poor prognosis. There are about 29,000 new cases of pancreatic cancer each year and 28,000 deaths [1,2]. Novel approaches are in urgent need for improving the diagnosis and treatment of pancreatic cancer patients. Recent advances in nanotechnology have shown an exciting frontier in the effort to apply novel approaches for detection and treatment of human cancer [3, 4, 5]. However, a major obstacle limiting its clinical application is that non-specific nanoparticles are unable to reach sufficient concentrations in the tumor site to either produce a detectable signal for tumor imaging or to carry optimal amounts of therapeutic agents into tumor cells. One approach to overcome this problem is to develop tumor–targeted nanoparticles that can specifically accumulate in the tumor areas sufficient enough to allow sensitive detection of the tumor mass.

EGFR signaling pathway plays a key role in regulation of cell proliferation, survival and differentiation. uPA is a serine protease that regulates multiple pathways involved in matrix degradation, cell motility, metastasis and angiogenesis. uPAR are highly expressed in many human tumor cells, intratumoral fibroblasts and tumor endothelial cells. However, uPAR is undetectable in the majority of normal tissues except for low levels in macrophages, granulocytes, thymus, kidney and spleen [6]. It has been shown that EGFR and uPAR are highly expressed in human pancreatic cancer tissues [7-8]. Delivery of a non-catalytic amino-terminal fragment (ATF) of uPA using an adenoviral vector inhibited tumor growth and angiogenesis [9]. Prior preclinical studies have shown that blocking EGFR signaling inhibits the growth of pancreatic cancers [10]. Therefore, peptides or antibodies that bind to uPAR or EGFR and block the receptor function should be ideal for the production of multifunctional nanoparticles.

Superparamagentic iron oxide (SPIO or IO) nanoparticles and their derivatives have been tested as magnetic resonance imaging (MRI) contrast agents and can be used in human. Their long blood retention time, low toxicity and biodegradability are attractive properties for developing target specific imaging [11, 12]. SPIO nanoparticles possess unique paramagnetic properties resulting change of relaxivitiesof water for MRI contrast. Recent studies have demonstrated that SPIO nanoparticles can be internalized by cells, generating significant susceptibility changes resulting in strong T_2 and T_2 contrast for MR detection of magnetically labeled cells [13]. By modifying its coating material and surface chemistry, selected molecules can be conjugated to SPIO, providing functionalities and specificity.

In this study, we have developed a novel method to produce uniformly sized SPIO nanoparticles with functionalized surface to conjugate targeted peptides and single chain antibodies. Using this approach, we engineered SPIO nanoparticles targeting uPAR and EGFR. Our results demonstrated that this novel SPIO agent exhibits specificity in pancreatic cancer cells *in vitro* and target specific contrast in MR imaging of a human pancreatic xenograft cancer model in nude mice. This tumor targeted MRI probe has the potential for detection of primary and metastatic pancreatic cancer.

EXPERIMENTAL METHODS

Preparation of SPIO nanoparticles with functionalized surface. SPIO was prepared by heating iron oxide powder and oleic acid in octadecene over 315 °C. The size of SPIO was tuned through changes such as heating time, temperature, and concentration of the iron oxide and oleic acid [14]. SPIO nanoparticles with highly uniformed core sizes of 9 or 10 nm were used for this study. Amphiphilic polymers were coated to the surface to convert hydrophobic SPIO nanoparticles to stable, water soluble and biocompatible nanoparticles adopting the approach for quantum dots developed by Dr. Gao [3]. The hydrocarbon chains of the polymer intercalate into the inner hydrophobic layer that stabilize SPIO nanoparticle surface while carboxylic acid groups in the out-layer make the SPIO nanoparticles hydrophilic and reactive for conjugating proteins, peptides or small molecules (Figure 1).

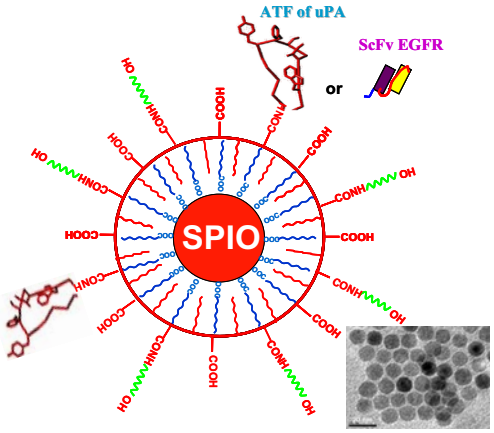

Figure 1. Schematic illustration of targeted SPIO nano-particles. An amphiphilic polymer coated SPIO-nanoparticle conjugated with ATF of uPA peptide or ScFvEGFR single chain antibody. An EM photo of SPIO nanoparticles is inserted.

Engineering tumor targeted-SPIO nanoparticles. A 135-amino acids of the amino-terminal fragment of mouse uPA (ATF), which contains a receptor binding domain but lacks a catalytic domain of uPA, was produced in a bacteria-expressing system using a plasmid construct with an ATF gene and 6x his-tag (cloned in our laboratory). A plasmid construct containing a ScFvEGFR single chain antibody gene was produced by Dr. Adams and the protein was expressed in bacteria-expressing system. Recombinant proteins were purified using Ni-columns. 17 KD ATF peptides or 25 KD ScFvEGFR single chain antibodies were conjugated to the SPIO nanoparticles via carboxyl groups after activation with EDAC to form ATF-IO or ScFvEGFR-IO. An estimated ratio of 10 protein molecules per IO nanoparticle was used for the conjugation.

Examination of specificity of the targeted-IO nanoparticles using MRI scan or Prussian blue staining. Human Pancreatic cancer cell line MIA PaCa-2 (from ATCC) was incubated with unconjugated, ATF-, ScFvEGFR- or control GFP-IOs for 2 hrs. After washing, the cells were examined by Prussian blue staining or embedded in 1% agorse gel plate for MRI T2 measurement to determine specificity of the SPIO nanoparticles. T2 measurements of SPIO-labeled cells was accomplished using T2 weighted fast spin echo sequence with variable echo times (TEs). T2 values of cell plates were calculated from images collected at various TEs to obtain maps of T2.

Cell proliferation assay. To determine the effect of blocking uPAR by ATF or EGFR by ScFvEGFR on proliferation of pancreatic cancer cells, we plated MIA PaCa-2 cells in 96-well plates and incubated with unconjugated, ATF-, or ScFvEGFR-IO for 48 hrs. The percentage of viable cells was determined by MTS Cell Proliferation Assay. Cell images were taken using an inverted microscope.

Examination of specificity and tissue distribution of the targeted-SPIO nanoparticles. An orthotopic pancreatic tumor xenograft model was established in athymic nude mice by surgically implanting MIA PaCa-2 cells into pancreas. Under anesthesia, $5x10^6$ of the cells were injected directly into pancreas. Four weeks after the cell implantation, MRI scan was performed on each mouse to obtain images prior contrast administration as control. Unconjugated control agents, ScFvEGFR- or ATF-IO nanoparticles (about 200 μg each) were then injected through the tail vein and MRI scan was performed on mice using a 3T scanner and a 5-cm wrist coil. Typical image parameters were: filed of view (FOV) of 110 mm, imaging matrix of 256 x 146 and 40 slices with 1.2 mm slice thickness without slice gap in the coronal section, TE of 10 ms, TR of 350 ms for T1 weighted spin echo imaging or TE of 2.6 ms. To examine the retention of contrast agent, mice were scanned at the different time points post IO-nanoparticle administration.

RESULT AND DISCUSSION

Targeted SPIO nanoparticles exhibit specific binding to pancreatic cancer cells. Both ATF- and ScFvEGFR-IO nanoparticles were able to bind and be internalized by human pancreatic cancer cells. After incubating with MIA PaCa-2 cells, ATF- or EGFR-IO nanoparticles specifically bound to MIA PaCa-2 cells evidenced by significantly shortened T2 in ATF-IO or ScFvEGFR-treated cells but not in IO nanoparticle or GFP-IO nanoparticle-treated cells (Figure 2A). Specific binding and internalization of ATF- or ScFvEGFR-IO nanoparticles were further confirmed by Prussian blue staining (Figure 2B). Although previous reports suggested that there is a species specificity in binding uPA to its receptor, we found that recombinant mouse ATF peptides produced in our lab were able to bind to uPAR positive mouse and human cancer cells.

ATF and SvFcEGFR Inhibit proliferation of human pancreatic cancer cells. To determine whether ATF and ScFvEGFR can block function of their receptors and inhibit growth of tumor cells, we examined the effect of ATF- or ScFvEGFR-IO nanoparticles on MIA PaCa-2 cells *in vitro*. We found that incubation of the cells with ATF- or ScFvEGFR-IO nanoparticles significantly inhibited proliferation of the tumor cells (Figure 3A). Induction of cell death was also found after treatment, especially in ScFvEGFR treated cells (Figure 3B). Therefore, ATF and ScFvEGFR have potential to serve as tumor targeting peptides as well as therapeutic reagents.

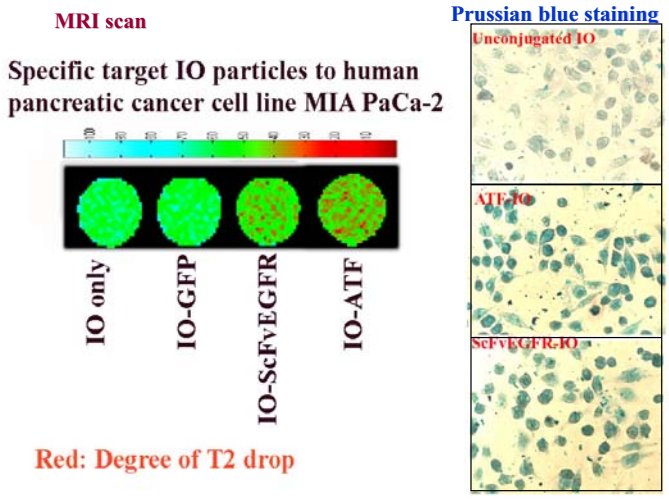

Figure 2. Specific binding of the targeted-IO nanocrystals to pancreatic cancer cells detected by MRI scan or Prussian blue staining.
For MRI scan, MIA PaCa-2 cells were incubated with various IO nanoparticles for 2 hrs. After washing, the cells were embedded in 1% agrose and analyzed by MRI scan as described in the Method. Red colored signals represent cell clusters bound to the targeted-IO particles and displayed reduced T2 signals. Prussian blue staining on cells growing in chamber slides was performed according to a standard protocol. A strong blue iron staining was found inside the cells incubated with ATF- or ScFvEGFR-IO while unconjugated–IO treated cells only showed a weak non-specific uptake of IO particles.

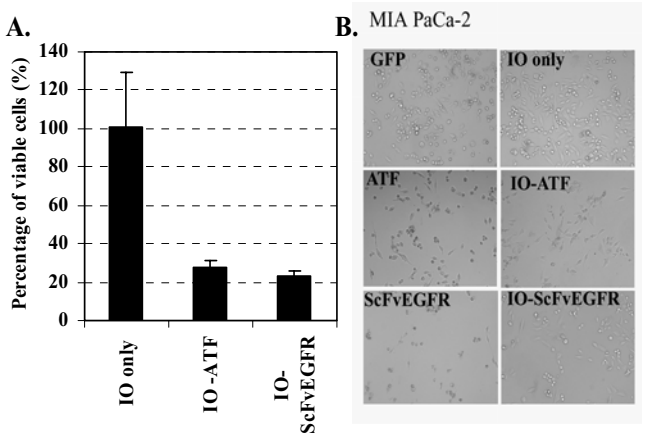

Figure 3. Inhibition of cell proliferation and/or induction of cell death after treating human pancreatic cancer cells with free ATF and ScFvEGFR peptides or IO-conjugated peptides. A. MTS cell proliferation assay. B. Representative areas of cell images 48 hrs after treatment.

***In vivo* imaging of orthotopic human pancreatic tumor xengrafts after systemic delivery of ATF- or ScFvEGFR-IO nanoparticles.** To determine MRI specificity and tissue distrubution of the tageted IO nanoparticles, we eastablished a human pancreatic tumor model in nude mice. MR images of the tumor bearing mice before and after adminstration of different SPIO nanoparticles via. the tail vein of each mouse. Post contrast MRI scans were performed at different time pionts from 5 minutes to 30 hours.

MRI scan of animals bearing orthotopic pancreatic tumors showed bright signals in the tumor areas in T1 weighted imaging before the IO nanoparticle injection (Figure 4A, **Pre**). However, 5 hours after systemic delivery of ATF- or ScFvEGFR-IO nanoparticles, a significant signal drop in those areas was observed (Figure 4A, **5 hrs**), suggesting a T2 shortening effect from the accumulation of the targeted-SPIO nanoparticles in orthotopic pancreatic cancers. As expected, we also observed a strong T2 effect in liver and spleen of the mice due to high liver and spleen uptake of IO reported previously. At 30 hours after administration of the contrast

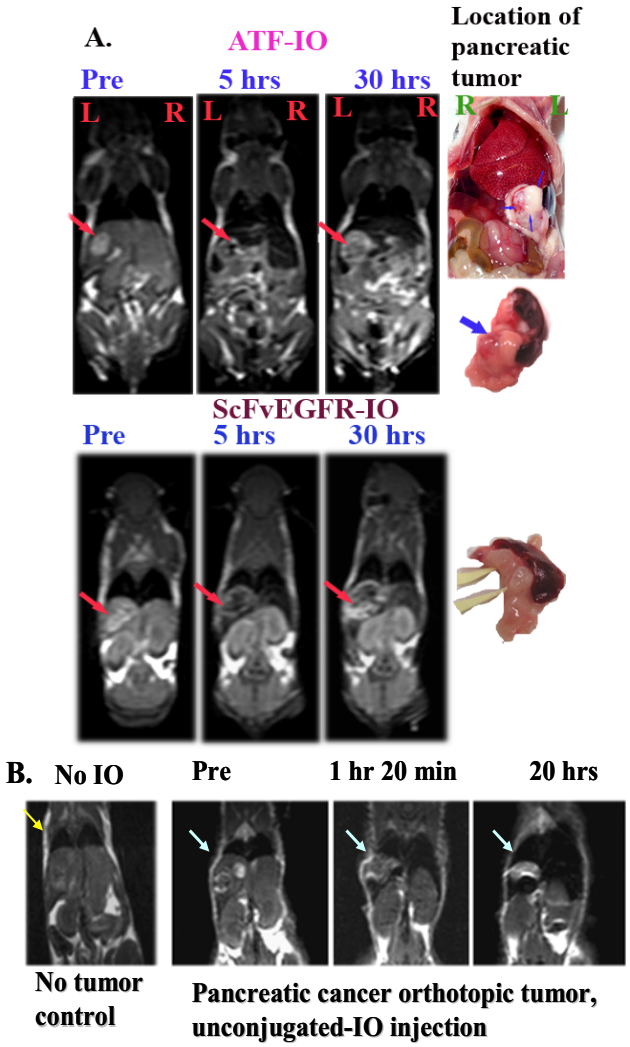

Figure 4. MRI detection of pancreatic cancer lesions xenografted inside pancreas of nude mice using ATF- or ScFvEGFR-IO nanoparticles.
A. MRI images of the tumor-bearing mice after ATF- or ScFvEGFR-IO particle injection. Arrows indicate the tumor areas. Images showing are representative images from one of five mice in each group.
B. MRI image of a normal mouse did not show bright signal in pancreatic areas. Moreover, T2 effect was not detected in the tumor-bearing mice after injection of unconjugated-IO particles.

agent, signals in some tumor areas were slightly higher than that observed at 5 hours, suggesting dynamic changes in amount of the SPIO-nanoparticles in the tumors (Figure 4A, **30hrs**).

In the postmortem examination of the mice after completion of MRI scans, we found that the location of orthotopic pancreatic tumors observed in the mice correlates well with the tumor images obtained from MRI scan. For example, we found a tumor mass (5 mm) located inside pancreas and under the spleen (Figure 4A) in a tumor-bearing mouse injected with ATF-IO. MRI scan also detected a similar sized area with hyper-intense signal in pre-contrast imaging but displaying hypo-intensity due to the strong T2 effect (Figure 4A) introduced by the presence of SPIO. However, images from a tumor-bearing mouse injected with unconjugated IO nanoparticles did not show such significant T2 signal change in the tumor area (Figure 4B).

To determine the tissue distribution of the targeted-IO nanoparticles, we collected tissues from pancreatic tumors and several normal organs of a mouse injected with ATF-IO nanoparticles for 30 hours. Frozen tissue sections from the tumor and normal organs were examined by Prussian blue staining. Interestedly, we found a strong iron staining in pancreatic tumor lesions but not in surround normal pancreas (Figure 5A). We also detected a high level of iron staining in liver and spleen. A low level of iron staining was seen in lung tissue. However, kidney, pancreas and heart tissues were consistently negative for the iron staining (Figure 5B).

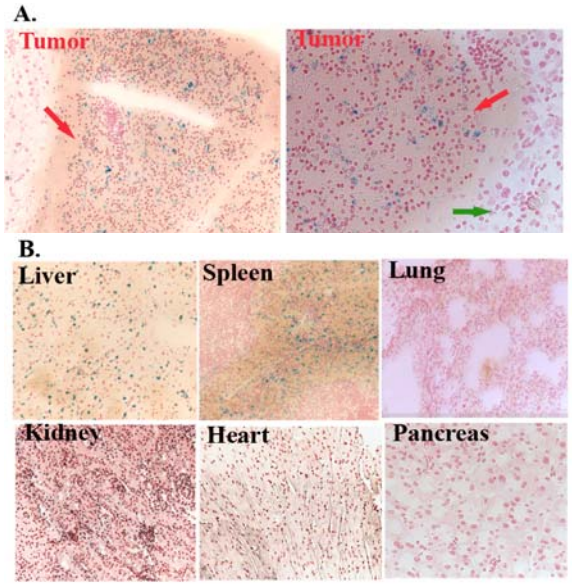

Figure 5. Examination of tissue distribution of ATF-IO nanoparticles after the tail vein delivery.
A. Selective accumulation of the IO particles in pancreatic tumor (red arrows) but not in normal pancreas adjacent to tumor areas (green arrow). B. In normal tissue, blue iron staining was detected in liver and spleen but not in normal pancreas, heart and kidney. A low level of blue staining was detected in lung. Red: counter staining with nuclear red. All tissues were collected from the same mouse 30 hrs after ATF-IO nanoparticle injection.

In summary, we have developed a novel approach to conjugate targeting peptide ligands to SPIO nanoparticles. Using uPAR or EGFR-targeted SPIO nanoparticles, specific binding of the IO nanoparticles to human pancreatic tumor cells can be achieved. Systemic delivery of the targeted SPIO nanoparticles leads to accumulation of the targeted IO nanoparticles and produced significant MRI signal change in orthotopic pancreatic tumor lesions in nude mice as the result of strong T2 effect from SPIO nanoparticles. Since both EGFR and uPAR can be internalized by cells, this may facilitate the accumulation of SPIO nanoparticles in the tumor. Upregulation of uPAR in intra-tumoral fibroblasts and endothelial cells should lead the further increase of the concentration of the imaging probes in the tumor to improve the sensitivity of MRI-detection. Additionally, ability of ATF- and ScFvEGFR-IO nanoparticles to internalize into tumor cells may allow those nanoparticles as vehicles for delivering therapeutic reagents into tumor cells. Therefore, our results suggested that ATF- or ScFvEGFR-IO nanoparticles have great potential for target specific *in vivo* MR imaging and therapy of pancreatic cancer.

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