Aptamer:Toxin Conjugates that Specifically Target Prostate Tumor Cells

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Abstract

We have used RNA aptamer:gelonin conjugates to target and specifically destroy cells overexpressing the known cancer biomarker prostate-specific membrane antigen (PSMA). Aptamer:toxin conjugates have an IC_{50} of 27 nmol/L and display an increased potency of at least 600-fold relative to cells that do not express PSMA. The aptamer not only promotes uptake into target cells but also decreases the toxicity of gelonin in non-target cells. These results validate the notion that "escort aptamers" may be useful for the treatment of specific tumors expressing unique antigen targets. (Cancer Res 2006; 66(12): 5989-92)

Introduction

The prostate-specific membrane antigen (PSMA) is a type 2 integral membrane glycoprotein expressed on the surface of prostate carcinoma and the neovasculature of most other solid tumors. The antigen is abundantly expressed at all stages of the cancer (1) and is therefore an attractive target for cancer immunotherapy and imaging.

Previous efforts to selectively destroy cancer cells have generally focused on the use of antibodies to deliver toxic payloads (2). In particular, the ribosomal toxin gelonin, a small N-glycosidase protein with a molecular weight of 28 kDa, causes cell death by cleaving a specific glycosidic bond in rRNA and thereby disrupting protein synthesis. However, unlike other ribosomal toxins, such as ricin and abrin, gelonin lacks a translocation domain and thus does not efficiently get into cells at significant concentrations ($IC_{50} \sim 2$ μmol/L; ref. 3).

Gelonin is also useful because it can be expressed as a recombinant protein (rGel) in bacteria. Like gelonin, rGel is not efficiently internalized into cells and has very little nascent toxicity (3). rGel can be chemically conjugated or genetically fused to targeting and delivery moieties at either its NH_2- or COOH-terminal ends or via introduced cysteine residues (4, 5). For example, cytokine vascular endothelial growth factor (VEGF) has been coupled to gelonin, and the conjugate has been shown to specifically kill cancer cells overexpressing the VEGF receptor FLT-1 (6).

Aptamers have previously been selected to bind a variety of targets ranging from small molecules (7) to proteins (8) and whole cells (9, 10). The nucleic acid binding species offer a number of significant advantages over antibodies, including greater stability, ease of synthesis, and lower production costs, making them attractive alternatives for use in both diagnostic and therapeutic applications (8, 11). In the current study, we have used a RNA aptamer that specifically binds PSMA to escort gelonin into prostate tumor cells that express PSMA on their surfaces. The toxin conjugate specifically destroys PSMA-positive prostate cancer cells with an IC_{50} of 27 nmol/L and displays an increase in toxicity of at least 600-fold when compared with cells that do not express PSMA.

Materials and Methods

RNA synthesis. The selection and characterization of anti-PSMA aptamers has previously been described (12). All RNAs were synthesized by runoff transcription from double-stranded DNA templates bearing a T7 RNA polymerase promoter. The sequence of the RNA aptamer (A9) used was 5′-GGGAGGACGAUGCGGACCGAAAAAGACCUGACUUCUAUACUAAGUCUACGUUCCCAGACGACUCGCCCGA. All transcription reactions were carried out using the Y639F mutant T7 RNA polymerase. Typical reactions (20 μL) were carried out for 8 hours at 37°C and contained 1× transcription buffer [40 mmol/L Tris (pH 8), 30 mmol/L $MgCl_2$, and 5 mmol/L spermidine], 1 mmol/L ATP and GTP, 2 mmol/L of 2′ F dCTP and 2′ F dUTP (TriLink Biotech, CA), and 2 μg double-stranded DNA (dsDNA) template. Following transcription, samples were treated with DNase for 10 minutes at 37°C, and the RNA was purified on a denaturing (7 mol/L urea) 8% polyacrylamide gel. Gel slices containing the RNA product of the appropriate size were eluted overnight in H_2O, and the RNA was recovered by ethanol precipitation.

Toxin conjugation. To a 1.6 mL solution of PBS containing (129 μmol/L) anti-PSMA aptamer, a 5-fold molar excess of the cross-linker *N*-succinimidyl-3-(2-pyridylodithio)propionate (SPDP; Pierce, Rockford, IL) was added and allowed to react for 30 minutes at room temperature. Excess, unreacted SPDP was removed by gel filtration using Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden) gel chromatography. Recombinant gelonin (5-fold molar excess versus aptamer) was reduced by adding 2 mmol/L DTT (Sigma, St. Louis, MO) and stirring for 30 minutes at room temperature. Excess, unreacted DTT was removed by Sephadex G-25 gel chromatography. Aptamer-SPDP was slowly added to the rGelonin-DTT, with stirring, and the conjugation was allowed to proceed overnight (20 hours) at 4°C, under N_2 gas. Iodoacetamide (Sigma) was then added to a concentration of 2 mmol/L to block any remaining, unconjugated aptamer. The concentration of NaCl was reduced to <10 mmol/L by dilution, and the conjugate was applied to a Blue Sepharose (Amersham) column. Unconjugated aptamer was eluted by washing with PBS [10 mmol/L sodium phosphate, 150 mmol/L NaCl (pH 7.2)], and the conjugate was eluted with 10 mmol/L sodium phosphate, 1,000 mmol/L NaCl. Unconjugated rGelonin was removed by passage through a Superose S-75 fast protein liquid chromatography column (Amersham). Purified conjugate was concentrated using an Amicon Ultra filter (Millipore, Billerica, MA).

Although conjugation using SPDP typically proceeds via a primary amine, we found that the unmodified anti-PSMA aptamer still conjugated to rGel. The link between the aptamer and rGel was confirmed by electrophoretic analysis (see Supplementary Data).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-05-4583

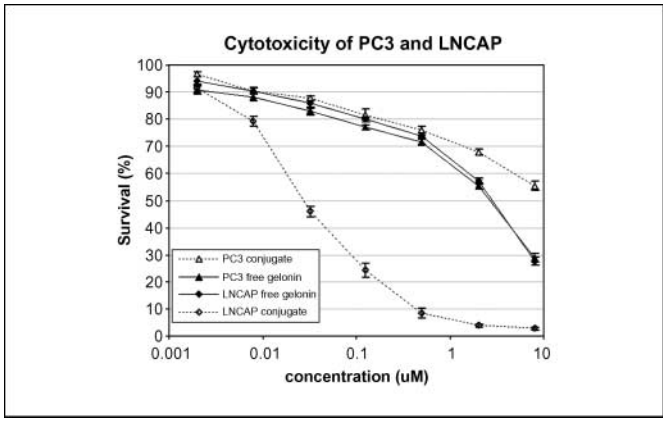

Figure 1. Cytotoxicity assays with LNCaP (PSMA positive) and PC3 (PSMA positive) prostate cancer cell lines. Assays were done using either free gelonin or the aptamer:gelonin conjugate. With LNCaP cells, the aptamer:gelonin conjugate had an IC_{50} of 27 nmol/L and showed a toxicity increase of >600-fold when compared with PC3 cells (IC_{50} ~ 15 μmol/L). The toxin conjugate also showed a 180-fold increase in toxicity when compared with free gelonin (IC_{50} = 5 μmol/L).

Cytotoxicity assays. LNCaP (PSMA positive) or PC3 (PSMA negative) cells were grown to 70% confluency in 96-well culture plates. Cells were then incubated in 100 μL culture media that contained from 8 to 0 μmol/L (8, 2, 0.5, 0.125, 0.032, 0.008, 0.002, 0.0005, 0.00013, or 0 μmol/L) free gelonin or aptamer:gelonin conjugate for 72 hours. The viability of cells was then examined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (CellTiter 96 Nonradioactive Cell Proliferation Assay, Promega, Madison WI). The color change was measured at 490 nm after 1 hour of incubation. Each data point represents the mean $A_{490\ nm} \pm$ SE.

Western blot analysis. LNCAP and PC3 cells were grown to 80% confluence, after which the medium was replaced with PBS, and the cells were incubated with free aptamer, aptamer:rGel conjugate, or free rGel at 5 nmol/L for 24 hours. Cells were then harvested and lysed by the addition of 0.5 mL of CHAPS buffer [0.5% CHAPS, 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 5 mmol/L β-mercaptoethanol, and 0.1 mmol/L AEBSF]. The lysate (20 μL) was run on a polyacrylamide 4% to 20% gradient gel and transferred to a nitrocellulose membrane, and the membrane was probed with an anti-gelonin antibody (rabbit origin, Rosenblum Lab) or an anti-PSMA antibody (Zymed Laboratories, Inc., San Francisco, CA). An anti-β-actin antibody (clone AC-15, Sigma-Aldrich, St. Louis, MO) served as an internal control. Staining was carried out with secondary IgG-AP conjugates.

Immunostaining of cells. LNCaP cells were grown to 80% confluence in chambered slides and then incubated with 1 nmol/L aptamer:gelonin conjugate in media for 1 hour at room temperature. The cells were fixed with 4% formaldehyde for 10 minutes followed by washing thrice with PBS. The RNA portion of the aptamer conjugate was detected using a quantum dot-labeled antisense oligonucleotide (B-CGCATCGTCCTCCC, where B is a 5′ biotin; IDT, Coralville, IA). Streptavidin-coated quantum dots (Qdot 605, Qdot Corp., Hayward, CA) were preincubated with the biotinylated oligonucleotide for 30 minutes at a molar ratio of 1:1 before cell labeling. Fluorescent labeling of the gelonin portion of the conjugate was achieved using the rabbit anti-rGel antibody and anti-rabbit FITC-labeled IgG.

Confocal microscopy. Single images and image z-stacks were collected using a Leica TCS SP2 AOBS confocal system (Leica Integrated Systems Division, Malvern, PA) with a ×63 oil lens (HCX PL APO 1.4-.60 NA Blau CS). The image shown represents a single section taken at a depth of 7 μm, roughly halfway through the cell. 4′,6-Diamidino-2-phenylindole staining was imaged by excitation at 351 nm (AR/UV), and emission was monitored at 440 to 480 nm. FITC labeling was imaged by excitation at 488 nm (Ar/HeNe), and emission was monitored from 505 to 530 nm. The Qdot 605 was excited at 543 nm, and the emission was monitored from 590 to 620 nm.

Results

Anti-PSMA aptamers that contained the modified, nuclease-resistant pyrimidines 2′ F UTP and 2′ F CTP were synthesized from dsDNA templates by runoff transcription. Following purification, the aptamers were reacted with the bifunctional linker SPDP, which contains an amine-reactive NHS ester and a thiol-reactive pyridyldisulfide group.

The RNA/linker conjugates were subsequently incubated with purified rGel, and reaction with surface cysteines yielded aptamer:gelonin conjugates as previously described (4).

Cytotoxicity assays were done using a PSMA-expressing prostate tumor cell line (LNCaP) and a non–PSMA-expressing prostate tumor cell line (PC3; Fig. 1). The aptamer conjugate had an IC_{50} of 27 nmol/L with LNCaP cells and showed a 600-fold increase in toxicity when compared with PC3 cells (IC_{50} of ~15 μmol/L; Fig. 1, △ and◇). The toxin conjugate also showed a ~180-fold increase in toxicity with LNCaP cells when compared with free gelonin. Interestingly, when the aptamer conjugate was assayed with PC3 cells, it was found to be ~3-fold less toxic than rGel alone, suggesting that the aptamer may actually inhibit nonspecific uptake or presentation of the toxin. Moreover, the efficacy of the aptamer conjugates may in fact be larger than 600-fold because toxicity in PC3 cells essentially leveled off at the highest concentrations tested with no indications that further increases would lead to additional toxicity. By fitting the data without assuming additional increases in toxicity to PC3 cells, aptamers show a 10,000-fold increase in LNCaP cells relative to PC3 cells. Additional experiments were conducted in which LNCaP cells were treated with either aptamer alone, a combination of free aptamer and free gelonin, or the aptamer:rGelonin conjugate pretreated with DTT before the addition to cells. DTT should reduce the disulfide bond in the SPDP linker and thus release gelonin from the aptamer. Only the intact aptamer:rGelonin conjugate displayed significant cytotoxicity (see Supplementary Data). Toxin targeting

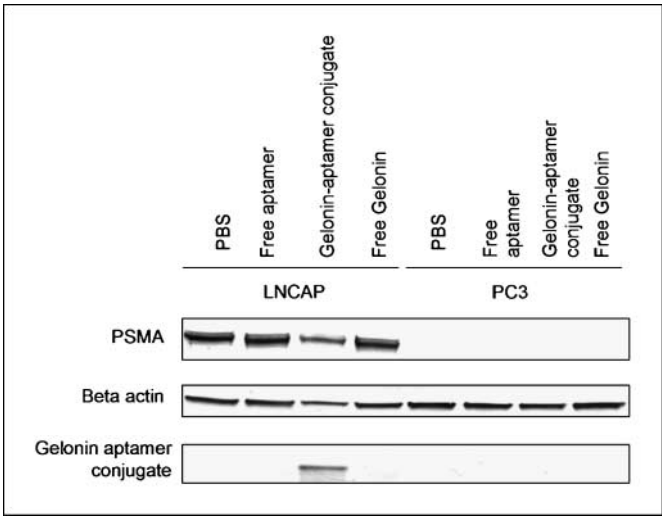

Figure 2. Western blots of cell extracts following aptamer treatments. Gelonin was only detected in LNCaP (PSMA positive) cells after treatment with the aptamer:toxin conjugate. *Lane 1,* LNCaP cells incubated with PBS only; *lane 2,* LNCaP cells incubated with free aptamer; *lane 3,* LNCaP cells incubated with aptamer:rGel conjugate; *lane 4,* LNCaP cells incubated with free rGel; *lane 5,* PC3 (PSMA negative) cells incubated with PBS only; *lane 6,* PC3 (PSMA negative) cells incubated with free aptamer; *lane 7,* PC3 (PSMA negative) cells incubated with aptamer:rGel conjugate; *lane 8,* PC3 (PSMA negative) cells incubated with free rGel.

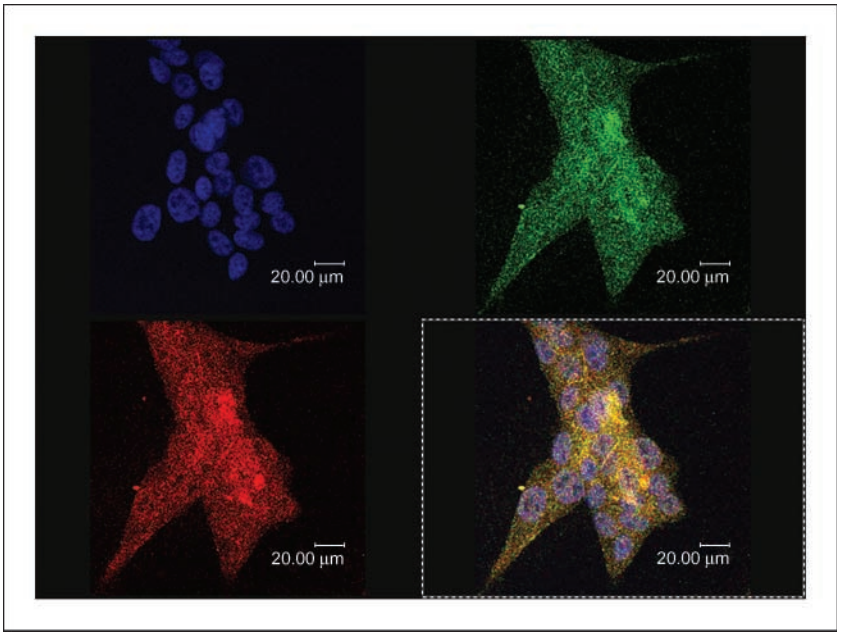

Figure 3. Internalization of aptamer conjugates. Fixed LNCaP cells were stained using either a quantum dot-labeled antisense oligonucleotide complementary to the aptamer portion of the toxin/conjugate or an anti-gelonin antibody (followed by a FITC-labeled secondary antibody; similar results were observed with phycoerythrin-labeled secondary antibodies). Following merging of the images, the observed fluorescence from these separate stains was found coincident throughout the cell. Image represents a single section taken at a depth of 7 μm. *Blue, top left,* 4′,6-diamidino-2-phenylindole–stained nuclei of LNCaP cells. *Green, top right,* treatment with anti-gelonin antibody followed by a secondary antibody-phycoerythrin conjugate. *Red, bottom left,* treatment with anti-aptamer antisense oligonucleotide conjugated to Qdot 605. *Merged, bottom right,* colocalized areas appear yellow.

with aptamers is at least as good as that previously observed with antibodies (4), whereas the chemical or enzymatic synthesis of aptamers should allow for much better quality control of reagent production.

To confirm that the observed cell death was indeed due to the uptake of the aptamer:gelonin conjugates, we preformed both Western blot and immunofluorescence analyses on both LNCaP and PC3 cells treated with the aptamer:gelonin conjugate. Consistent with the cytotoxicity results in Fig. 1, only the PSMA-positive LNCaP cells treated with the aptamer:gelonin conjugate yielded detectable gelonin (Fig. 2). No gelonin signal was observed for LNCaP cells treated with same concentration of free rGel. Additionally, no gelonin signals were observed in any of the PSMA-negative PC3 cells.

Further confirmation of internalization was obtained by *in situ* immunofluorescence microscopy. Assays were done with LNCaP cells grown to 80% confluence in chambered slides and incubated with or without 1 nmol/L of the aptamer:rGel conjugate. A relatively low concentration of aptamer conjugate was used in these experiments because higher concentrations led to a loss of cells from the slide during the wash steps before fixation. Cells were permeabilized and probed with either an anti-gelonin antibody or a biotinylated oligonucleotide complementary to a portion of the anti-PSMA aptamer. The biotinylated oligonucleotide was fluorescently labeled by conjugation to a streptavidin-coated quantum dot.

As shown in Fig. 3 (see also Supplementary Figs. S5 and S6), both the aptamer and gelonin portions of the conjugate were observed and coincident within the cell. The positions of the fluorescent signals within cells were further confirmed by taking a series of images along the *z*-axis (Supplementary Fig. S7). Cells treated with gelonin alone showed no detectable internalization of the toxin (data not shown).

Discussion

Aptamers selected to bind isolated prostate specific membrane antigen have also been shown to bind PSMA-expressing prostate tumor cells. For example, Lupold et al. used a truncated variant of the anti-PSMA-aptamer A10 to specifically label LNCaP cells (12). Additionally, Farokhzad et al. have shown that multiple A10 aptamers can specifically deliver polylactate/polyethylene glycol nanoparticles to LNCaP cells (13). More recently, we generated quantum dot conjugates with a slightly different anti-PSMA aptamer (A9, the same aptamer used in the current studies) and used these conjugates to label both LNCaP cells grown in tissue culture and LNCaP cells grown in a tissue phantom (collagen matrix; ref. 14). We have now significantly extended this work by showing that a single A9 anti-PSMA aptamer can specifically and therapeutically deliver a toxin payload.

Although the development of modern *in vitro* selection methods took place >10 years ago, it is only recently that the therapeutic use of aptamers has begun to be shown (for a review, see ref. 15). For example, the efficacy of an aptamer that binds and inhibits the coagulation factor IXa has been shown in animal models (16). More recently, the first aptamer-based drug Macugen has been approved by the Food and Drug Administration. These demonstrations are likely the first of many, as it seems that aptamers are largely nonimmunogenic and nontoxic (17). Thus, our demonstration that aptamers can act in an "escort" capacity for therapeutics is timely and significant (18), especially because aptamers can be directly selected against tumor cells and tissues (9, 19, 20) and have binding specificities on par with antibodies. That said, the cost of goods for the production of nucleic acid therapeutics remains relatively high, especially for long nucleic acids, such as aptamers. Improvements in chemical synthesis that should lead to the introduction of small interfering RNA therapeutics will also prove relevant to the development of aptamer therapeutics (11).

Acknowledgments

Received 12/21/2005; revised 4/21/2006; accepted 5/5/2006.

Grant support: This work was supported by a grant from the Foundation for Research and the National Institutes of Health.

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