Aptamer-Conjugated Nanorods for Targeted Photothermal Therapy of Prostate Cancer Stem Cells


Abstract: Prostate cancer results in about 30,000 deaths annually in the United States, making it the second leading cause of cancer mortality in men in the Western world. Therefore, it is of great significance to capture and kill prostate cancer cells. It is well known that cancer stem cells are responsible for the maintenance and metastasis of tumors. This concept offers the possibility of developing a selective therapeutic approach in which cancer stem cells are directly targeted and killed. In this work, aptamers selected against DU145 prostate cancer cells (aptamer CSC1) and their subpopulation of cancer stem cells (aptamer CSC13) were linked to the surfaces of gold nanorods (AuNRs), and the resulting conjugates were successfully used to target and kill both cancer cells and cancer stem cells by near-infrared (NIR) laser irradiation. Even though cancer stem cells represent only a small population among all cancer cells, the entire cell viability was very low after laser irradiation, suggesting that tumorigenesis could be successfully controlled by this aptamer-based method, thus paving the way for early diagnosis and targeted therapy.

Introduction

Prostate cancer is the second leading cause of cancer mortality in men in the Western world,[1] accounting for about 30,000 deaths annually in the United States alone. It is estimated that 238,590 new cases and 29,720 deaths from prostate cancer will occur in the United States during 2013.[2] About 97% of patients are aged 50 and older, and about 62% are 65 years of age and older.[3] Since prostate cancer is common in aging men, it is highly desirable to develop most effective therapeutic approaches with the fewest side effects. Currently, prostate cancer is generally diagnosed by a finding of elevated prostate-specific antigen (PSA) followed by a biopsy.[4] However, the major limitation of PSA testing is the low specificity and high prevalence of detecting benign prostatic hyperplasia, especially in older men.[5] Most men with prostate cancer benefit from hormone therapy in the short term, such as androgen ablation therapy,[5] but, unfortunately, few are cured, as the tumor eventually returns, which presumably results from the growth of cancer stem cells (CSCs).[6]

The CSC hypothesis was first proposed 50 years ago to explain the observed functional heterogeneity within tumors.[7] However, the first actual cancer stem cells were found in leukemia cells by Dick and colleagues in 1997.[8] This discovery revealed that a defined subset of cells was solely responsible for development of the disease. To date, CSCs have been observed in acute and chronic myeloid leukemia,[9] as well as in various solid tumors, including those of the breast, brain, lung, and prostate gland.[10]

The function of normal stem cells in the adult organism is to renew and repair aged or damaged tissue, as a unique proliferative characteristic in each cell division.[10] Similarly, some tumor cells have the capacity of infinite self-renewal to drive tumor cell proliferation. As Scheme 1A shows, these infinitely self-renewing tumor cells, termed cancer stem cells, represent only a small population of cells within a given tumor mass, but they have the potential to develop disease.[11] Otherwise, the bulk of tumor mass is composed of more differentiated cells that lack such self-renewal capacity and are subject to apoptosis during tumor growth.[10] In addition to their self-renewal property, CSCs appear to be relatively resistant to commonly used cancer therapies, such as radiation and chemotherapy.[5] Thus, despite the small quantity of CSCs resident in the tumor mass, they may cause recurrence of the tumor many years after treatment.
Current therapies, including surgery, radiotherapy, and chemotherapy, aim to eradicate or kill every cancer cell. The CSCs concept, however, suggests a fundamentally different approach because each tumor contains a small fraction of stem cells responsible for the maintenance and propagation of the disease. The development of new therapies that specifically target and kill CSCs may therefore provide less toxic and more long-lasting methods to kill the entire tumor.

The metastatic prostate cancer cell line DU145 is a model of a particularly pernicious prostate cancer, which is hormone-insensitive and does not express PSA on the surface, making it difficult to target by standard antigen–antibody biorecognition strategies. Aptamers comprise a promising class of targeting molecules. Aptamers are single-stranded DNA or RNA molecules that have been evolved in a test tube to bind to a specific target and offer many advantages over the more commonly used targeting moieties such as antibodies. As such, aptamers have been extensively used in drug delivery and cancer therapy. For example, aptamer–gold nanorod (AuNR) conjugates, which show excellent longitudinal plasmon resonance absorption in the near-infrared (NIR) range and can efficiently transfer NIR light to localized heating, have been used for photothermal therapy for leukemia.

Our laboratory has selected several aptamers that recognize different subsets of DU145 cells through a SELEX (systematic evolution of ligands by exponential enrichment) process. One aptamer, designated CSC1, recognizes all DU145 cells, while another, designated CSC13, recognizes only prostate cancer stem cells. In this work, the DU145 cell line was used as a model to study selective photothermal therapy for CSCs using aptamer–AuNRs conjugates as probes. By covalent linkage of aptamers with AuNRs, both specific cell targeting and selective photothermal destruction of cancer cells can be achieved. The therapeutic result showed that AuNRs, as hyperthermia reagents, could selectively kill prostate cancer cells, including cancer stem cells, by virtue of the high specificity of aptamers. Since CSC13 recognizes only prostate cancer stem cells, it showed high efficacy in killing them, thus limiting the self-renewal potential of CSCs and supplying, in turn, a broad-spectrum approach to the targeting and killing of prostate cancer.

Results and Discussion

Characterization of Aptamer–AuNR Conjugates

In this work, aptamers were conjugated to AuNR surfaces through thiol–Au covalent linkages. The thiol-modified aptamers are composed of four important parts: 1) a thiol alkane linking the aptamers to the gold surface by formation of thiol–Au covalent bonds; 2) a hydrophilic 36 unit poly(-ethylene glycol) (PEG) as a linker to separate the alkane thiol from the aptamer so that signal quenching by the gold surfaces can be avoided; 3) an aptamer segment to specifically bind target cells; and 4) 3'-biotin with dye-labeled streptavidin used as the signal reporter of aptamer to demonstrate the specific binding of aptamer with target cancer cells.

Transmission electron microscopy (TEM) imaging of AuNRs showed that the nanorods have an average width of 13 nm and an average length of 45 nm, resulting in two resonance absorption bands at 516 nm and 780 nm, respectively. In order to reduce AuNR cytotoxicity and aggregation, thiol-terminated methoxypoly(ethylene glycol) (mPEG-SH) was introduced to coat the surface of AuNRs. The absorption spectra revealed that the absorptions bands changed very little after modification with aptamers (Figure 1), suggesting the successful functionalization of AuNRs with aptamer and mPEG-SH.

Aptamer Specifically Targets DU145 Cells

As shown in Scheme 1B, aptamer CSC1 recognizes all prostate cancer cells, while aptamer CSC13 binds only a portion of the cells, that is, the prostate cancer stem cells. After co-
valent functionalization with AuNRs, the aptamer–AuNR conjugates proved to be highly promising for cell-specific targeting with enhanced signaling, as well as increased binding affinity. In addition, the conjugates could be used for targeted hyperthermia therapy. According to the CSC concept, CSC1–AuNRs were expected to kill all cancer cells, while CSC13–AuNRs would kill only CSCs. However, both aptamer conjugates were demonstrated to induce the apoptosis of prostate cancer cells because of the killing of cancer stem cells.

The specific binding of the aptamers and aptamer–AuNR conjugates was demonstrated by flow cytometric analysis (Figure 2). A random DNA library (Lib) and Lib–NR conjugates showed only very weak fluorescence signals in flow cytometry, thus suggesting that random sequences were unable to specifically bind cancer cells. Aptamer CSC13, by contrast, resulted in a strong fluorescence signal, thereby indicating a higher binding affinity of CSC13 to DU145 cells as compared to Lib. Since CSCs comprise only a portion of prostate cancer cells, CSC1, which binds the larger population of prostate cancer cells, led to a stronger signal than CSC13.

Compared to individual aptamers, aptamer–AuNR conjugates showed a >6-fold enhancement in fluorescence intensity owing to the presence of multiple aptamers on the gold surface. The results obtained after conjugation with NRs indicate that the aptamer probes maintained their binding capability and that the conjugates have an enhanced binding affinity in cancer cell recognition. However, no significant enhancement in fluorescence intensity was detected for Ramos cells, a control cell line which does not bind with either CSC1 or CSC13 aptamers, further confirming the specific recognition of the target cells by the aptamer–NR conjugates.

Confocal microscopy also showed the specific binding of the aptamer to prostate cancer cells (Figure 3). After incubation with DU145 cells, aptamers emitted greater fluorescence on the cell surface compared with Lib because of their specific biorecognition of the membrane protein of cancer cells. In addition, aptamer CSC1 showed a stronger red fluorescence than aptamer CSC13, indicating that more CSC1 aptamer bound to the prostate cancer cells. However, an even more intense red fluorescence could be observed when aptamers were linked onto the gold surface, supplying an enhanced fluorescence signal for imaging and significantly improving the binding affinity with cancer cells. By contrast, even though the Lib sequences was modified with gold nanorods, the fluorescence was too weak to detect by confocal imaging, suggesting the selective binding of aptamers CSC1 and CSC13 to target cancer cells.

Unconjugated AuNRs are Nontoxic to Cells

Some concern has been raised that free cetyltrimethylammonium bromide (CTAB) used in the preparation of CTAB-capped nanorods can be cytotoxic, such nonspecific toxicity would be unwelcome. In this work, AuNRs were prepared using CTAB as a soft template. To reduce the cytotoxicity of CTAB in the solution, centrifugation was performed twice to remove excess CTAB before and after DNA–SH loading, respectively. Furthermore, the polymer of mPEG–SH was introduced to stabilize the solution and minimize the cytotoxicity. To check the cytotoxicity of the probes, we added different concentrations of AuNRs to cells alone, without the application of laser irradiation, and an MTS assay was performed on the cell solution. At a low concentration of AuNRs, the cell viability assay (Figure S1

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Figure 1. Absorption spectra and TEM image of Au NRs. Concentrations: AuNRs, 0.8 nm; aptamer, 200 nm. Scale bar: 50 nm.

Figure 2. Flow cytometric assay to monitor the specific binding of aptamers with a) DU145 cells (target cells) and b) Ramos cells (control cells).

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in the Supporting Information) showed a low percentage of dead cells. When the concentration of AuNRs reached the nanomolar level after enrichment by centrifugation, the cell viability was more than 84% for both DU145 and Ramos cells, respectively, supporting the notion that the AuNRs themselves are of low toxicity to the cells.

**Photothermal Activation of AuNRs**

AuNRs are especially attractive candidates for photothermal therapy based on their facile synthesis with tunable absorption and high absorption cross-section in the NIR region.\(^23\) These photonic properties can convert the absorbed photon energy into thermal energy to induce cellular hyperthermia for cells in close proximity, providing an opportunity for the clinical application of highly efficient and tumor-specific photothermal therapy.\(^23,24\) We tested the hyperthermia effect by measuring the temperature as a function of time in real time (Figure 4). For 0.4 nm AuNRs, the temperature sharply increased from 25°C to 55°C because AuNRs absorbed sufficient energy to generate heat.\(^25\) The temperature of CSC1–AuNRs and CSC13–AuNRs increased from 37°C to 50°C and 47°C after binding to the DU145 cell line, respectively. The final temperature was slightly lower because of the removal of unbound NRs. However, after incubation with control Ramos cells and removal of unbound aptamer–AuNRs, the temperatures were as low as those of the cells only, suggesting no binding of aptamer–AuNR conjugates with the Ramos cell line, thus avoiding the harmful effects of the NIR laser. Therefore, the proposed aptamer–AuNRs supply a selective photothermal therapy for prostate cancer.

**Aptamer–AuNRs Specifically Kill Cancer Cells**

Confocal imaging (Figure 5) and MTS assay (Figure 6) were used to monitor the therapeutic results after NIR laser irradiation. The confocal imaging of cells was performed immediately after exposure to the NIR laser and staining with propidium iodide (PI), an intercalating agent and a fluorescent molecule that can be used as a stain to identify dead cells in a population.\(^18\) Figure 5 demonstrates that the direct irradiation of cells by the NIR laser maintained a high cell viability due to the low light absorption of natural endogenous cytochromes of cells in the NIR region.\(^26\) However, after incubation with aptamer–AuNR conjugates and irradiation by the NIR laser, prostate cancer cells showed strong PI red fluorescence, thus suggesting the death of cancer cells by photothermal therapy. The photothermal properties can be attributed to the light–heat conversion mechanism, whereby AuNRs absorb and convert laser light in the NIR range into heat. This heat is released to the immediate surrounding medium, increasing the surrounding temperature and resulting in the destruction of adjacent cells. A previous investigation by our group showed that the destructive effect was exerted by means of heat stress on the cells, rather than by mechanical perforation of their membranes.\(^18\) To confirm the specific killing of target cancer cells, Ramos cells were used as a control. Under conditions identical to those with the prostate...
cancer cells, Ramos cells showed no PI signal. Thus, the results indicate that the aptamer–AuNRs conjugates possess a high binding specificity and are, therefore, highly promising for selective cell recognition and targeted cancer cell therapy.

MTS assays (Figure 6) of cancer cells were performed after 10 minutes of NIR treatment, followed by incubation at 37°C for two days. The data are expressed as the mean ± standard deviation, and the statistical differences were assessed by the Student’s t-test. After NIR laser irradiation (812 nm) with CSC1- and CSC13-modified AuNRs, the cell viability decreased to 36% (p < 0.001) and 47% (p < 0.05), respectively, by the photothermal killing of cancer cells. Importantly, in confocal imaging (Figure 5), the red fluorescence of DU145 cells incubated with CSC1–AuNRs was much weaker than seen with CSC1–AuNRs. The CSC1–AuNRs conjugates bound and killed only a small subset of CSCs, resulting in a correspondingly weaker PI signal. Here, MTS assay showed an approximate therapeutic result between CSC1–AuNRs and CSC13–AuNRs conjugates based on the killing of stem cells, which apparently could not self-renew even after two days following photothermal treatment, thereby indicating that the tumor could be controlled by the targeting and killing of CSCs. The control cell assay showed that the aptamer–AuNR conjugates were not as phototoxic (cell viability is more than 91%) to the nontarget Ramos cells, illustrating the selective photothermal therapy of aptamer–NR conjugates based on the stem cell therapy concept. After NIR laser treatment, both CSC1–NRs and CSC13–NRs could effectively kill cancer cells. Even though cancer stem cells account for a small fraction of the cancer cell population, the tumor can be controlled by targeting and killing the cancer stem cells because of the loss of the self-renewal ability of the entire tumor, thus leading to its apoptosis.

Conclusions

In this work, the metastatic prostate cancer cell line DU145 was used as a model to study the cancer stem cell therapy concept. DU145 cells are hormone-insensitive and do not express PSA, making this cell line an excellent model for hard-to-treat prostate cancers. To specifically target DU145 cells, especially the stem cell fraction, we selected aptamers specific for these cells and attached these aptamers to AuNRs. Incubation of cells with these conjugates followed by NIR laser treatment caused photothermal destruction of cancer cells. In addition, the high specificity of aptamer–AuNR conjugates allowed the selective destruction of the cancer cells by NIR irradiation, thus avoiding harmful exposure of the surrounding normal tissue and, in turn, supplying promising candidates for use in phototherapy modalities. Importantly, the therapeutic effect suggested that the cancer stem cell concept can be used as a basis for effective and safe applications in early diagnosis and targeted therapy. It is fully anticipated that aptamer-based cancer stem cell therapy will be used to target more types of intractable cancers.
10 min, DU145 and Ramos cells were incubated in DMEM and RPMI 1640 culture medium, respectively, at 37°C under 5% CO₂ atmosphere for an additional 48 h. To measure the cytotoxicity, 120 μL MTS reagent (diluted 1:4 with medium) were added to each well and incubated for 2 h. The absorbance was recorded at 490 nm using a Tecan Safire microplate reader.

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**FULL PAPER**

**Photothermal Therapy**

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**A more apt method:** Aptamers CSC1 and CSC-13 previously selected against cancer stem cells (CSCs), respectively, were attached to the surface of gold nanorods (NRs) to target and kill prostate cancer cells (DU145 cell line) by photothermal therapy. Although CSCs represent only a small population among all cancer cells, the whole cell viability was very low after laser irradiation, suggesting that tumorigenesis could be successfully controlled by this aptamer-based method.