Dual-aptamer-based delivery vehicle of doxorubicin to both PSMA (+) and PSMA (–) prostate cancers

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ABSTRACT

We have designed a dual-aptamer complex specific to both prostate-specific membrane antigens (PSMA) (+) and (–) prostate cancer cells. In the complex, an A10 RNA aptamer targeting PSMA (+) cells and a DUP-1 peptide aptamer specific to PSMA (–) cells were conjugated through streptavidin. Doxorubicin loaded onto the stem region of the A10 aptamer was delivered not only to PSMA (+) cells but to PSMA (–) cells, and eventually induced apoptosis in both types of prostate cancer cells. Furthermore, both aptamers were immobilized onto the TCL-SPION for the *in vivo* application of the dual-aptamer-based drug delivery system. Doxorubicin was selectively passed into both types of prostate cancer cells due to the specificity of the dual-aptamer probe. This indicates that targeted drug delivery to prostate cancer cells successfully occurred using the drug-loaded aptamer complex via streptavidin and the probe using nanoparticles. In particular, dual-aptamer nanoparticles can be applied as an active drug delivery vector *in vivo* for the general targeting of both types of prostate cancer, including the PSMA (–) cell line, due to the particles' suitability for use as MR contrast agents.

Keywords: prostate cancer, drug-targeting, A10 PSMA aptamer, DUP-1 aptamer, DPV

1 INTRODUCTION

Various drug-delivery systems have been focused on cancer therapy, including treatments for prostate cancer [1, 2], and aim for efficient transportation resulting in maximizing drug's effectiveness and reduced side effects. These systems need to control the release of drugs, adsorption, site-specific delivery, etc. Drug-delivery to specific targets has especially attrated attention, since it is the most influential factor affecting drug effectiveness [3, 4]. It utilizes some ligands, such as an antibody and an aptamer, which can recognize the specific molecules. Of these, an aptamer has been focused upon as a powerful biological ligand by the virtue of its high binding affinity and selectivity to the target molecules [5-7]. The aptamer also has several advantages for over an antibody as a ligand, such as chemical synthesis *in vitro*, economical production, easy modification, and high stability in various physical and chemical environments.

Prostate cancers are directly related to male deaths [8, 9], and it contains two types of cell lines based on the expression of the PSMA (prostate specific membrane antigen) [10, 11]. For two kinds of prostate cancer cells, an anti-PSMA RNA aptamer (A10) for PSMA (+) cells [12], and a DUP-1 peptide aptamer for PSMA (–) cells [13] were identified. However, researches on imaging and drug targeting for the PSMA (–) prostate cancer have been confined compared to those for the PSMA (+) prostate cancer [14-17], because of retarded identification for its specific ligand, DUP-1 peptide aptamer. Thus, great challenges remain for targeting both types of prostate cancers including PSMA (–) cells.

In this study, we designed an A10 RNA/DUP-1 peptide aptamer conjugate to target both types of prostate cancer cells. Since the A10 RNA aptamer of the conjugate has been known to bind doxorubicin [18, 19], the doxorubicin can be introduced into the prostate cancer cells with the aptamer conjugate. The effects of doxorubicin to the prostate cancer cells were analyzed using a differential pulse voltammetry (DPV) method. Electrochemical methods, including DPV provide a sensitive, selective, and economical analysis [20-27]. DPV method in particular is greatly suitable for analyzing the current changes at an electrode by an oxidation-reduction (redox) reaction in the fields of chemistry and biology [28, 29].

As a drug-loaded, dual-aptamer complex is introduced on prostate cancer cells, apoptosis induced from doxorubicin can be analyzed using DPV method by measuring the oxidation peak current of the guanine which is directly related to apoptosis of cells [30]. In addition, morphologic changes by apoptosis from the doxorubicin were directly observed through a transparent ITO-coated working electrode using an optical microscope, and cell viability was also checked by trypan-blue assay.

2 METHOD

2.1 Preparation of a drug loaded dual-aptamer conjugate

An A10 RNA aptamer was synthesized with *in vitro* transcription, and it was biotinylated with biotin-hydrazide. The drug-loaded dual-aptamer conjugate was prepared via

incubation of a biotin-modified, 4 μM A10 aptamer and 8 μM DUP-1 with 6 μM streptavidin (SA) in culture media (RPMI1640, Hyclone) for two hours on ice, and 2 μM doxorubicin was added in finally for additional two hours. Loading doxorubicin on the dual-aptamer probe, especially the A10 aptamer, was confirmed by fluorescence quenching of doxorubicin.

2.2 DPV measurement of guanine in cells

Electrochemical DPV experiments were performed using KST-P1 (KOSENTECH, Korea) equipment with a conventional three-electrode system with an ITO-coated glass substrate for working electrode. Guanine in various cells, including LNCaP, PC3, PNT-2, HeLa, SW620, and MCF-7, cultured on the working electrode, was detected in 100 mM Tris-HCl (pH 8.0) buffer solution with the potential range of -0.2 to +1.0 V at two minutes equilibration time with a scan rate of 50 mVs^{-1} at room temperature. DPV measurements were performed every 12 hour with the individual electrode.

2.3 Verification of apoptosis using both microscopic method and trypan-blue assay

Each cell, LNCaP, PC3, HeLa, SW620, MCF-7, and PNT-2, was observed three days after treatment of doxorubicin-loaded dual-aptamer conjugate (50 μL of drug-loaded conjugate in 200 μL of culturing media) and only dual-aptamer conjugate on the ITO electrode by optical microscope (Axiovert 40 CFL). After microscopic observation, cells on the electrode were physically detached by pipetting and collected in the eppendorf tube. Suspension of each cell in the tube was combined with an equal volume of 0.1% trypan blue solution for five minutes. Cells were placed in a hemocytometer, which counted the dead and viable cells. Cell numbers were finally obtained by multiplying ($\times 10^4$) to these results. Viability can then be calculated by a below equation.

$$\frac{\text{live cells}}{(\text{dead} + \text{live cells})} \times 100 \qquad (1)$$

3 RESULTS AND DISCUSSION

The conjugate consists of an anti-PSMA RNA aptamer (A10, GGGAGGACGAUGCGGAUCAGCCAUGUUUACGUCACUCCUUGUCAAUCCUCAUCGGC, in which the underlined nucleotide represents the modified pyrimidins of 2'-F UTP and 2'-F CTP) for the PSMA (+) cell line and a DUP-1 peptide aptamer (FRPNRAQDYNTN) for the PSMA (–) cell line. Biotin molecules were introduced at the 3' position of the A10 RNA aptamer and the N-terminal of the DUP-1 peptide aptamer in order to assemble two aptamers with streptavidin. When two aptamers (A10 and DUP-1) formed a bridge via streptavidin, the ratio between the aptamers could be affected for impartial targeting of both types of prostate cancer cells. The critical ratio of 1:2 was decided based on 10 trials for verifying simultaneous binding of the two types of cells (data not shown).

Since it is well known that doxorubicin can bind onto a region of double strand nucleotide, such as stem part in the A10 RNA aptamer, we introduced doxorubicin on the dual-aptamer conjugate to deliver drugs to both prostate cancer cells, including the PSMA (–) cell line. When doxorubicin bound at the dual-aptamer conjugate, especially on the A10 aptamer, its fluorescence was quenched, resulting from intercalating within the A10 aptamer. The binding of doxorubicin was confirmed by fluorescence spectroscopy at 500~700 nm; sequential decreases of intensities of doxorubicin spectra were observed as increasing amounts of the A10 aptamer were added. Based on this result, two equivalent A10 aptamer to doxorubicin were chosen for use, in order to effectively deliver the drug.

As the drug-loaded conjugate treated prostate cancer cells, it could be introduced into the cells by an endocytosis mechanism. After cells were directly cultured onto an ITO working electrode, the effects of doxorubicin can be analyzed by measuring the oxidation peak current of the guanine using DPV method, because its concentration is directly related to apoptosis of the cells. 24 hours after for treatment of the drug-loaded aptamer conjugates, the peak current of the cells' guanine gradually declined, reflecting apoptosis by doxorubicin loaded on the conjugate.

Five cancer cell lines, including prostate cancer cells, were prepared for the controlled experiments: LNCaP from PSMA (+) prostate cancer cells, PC3 from PSMA (–) prostate cancer cells, HeLa from cervical cancer cells, SW620 from colon cancer cells, and MCF-7 from breast cancer cells. Additionally, PNT-2, a non cancerous cell line, was prepared to investigating the effect of weak expression of PSMA in a normal prostate cell line. The cultured cells were trypsinized before preparing suspension state in serum free media. The cells were then directly cultured onto an ITO glass electrode for DPV analysis. When the cell confluency reached approximately 80% (0 day), doxorubicin-bound aptamer conjugate and the only RNA/peptide dual-aptamer probe were respectively applied to each cell cultured on the electrode. The DPV peak currents in both cases were gathered twice daily, over 12-hour intervals, for 2.5 days at nearly 0.8 V.

For prostate cancer cells, the peak current was gradually decreased 24 hours after the addition of the doxorubicin-bound conjugate. Doxorubicin can affect the prostate cancer cells through the interaction between aptamers and prostate cancer; it can introduce into the PSMA (+) cells by A10 RNA aptamer binding onto itself, and doxorubicin can also introduce into PSMA(–) cells by DUP-1 peptide aptamer being bridged to drug-bound A10 RNA aptamer via streptavidin.

On the other hand, other cancer cells (HeLa, SW620, and MCF-7) are not affected by the drug-loaded aptamer complex since the dual-aptamer conjugate is designed only

for prostate cancer. For these non-prostate cancer cells, peak current on DPV plots were not changed by sequential time increasements for 2.5 days. However, weak decreases in peak currents were shown on the PNT2 cell line, reflecting that PNT2 expresses PSMA in small quantities. Although the drug-loaded conjugate affects the normal PNT2 cell lines, its small extent of decline was sufficientily classified from the prostate cancer cells. These DPV results show that the A10/DUP-1 conjugate could synchronously target both types cells.

Without doxorubicin, there were no significant differences between the treatment of the only native RNA/peptide dual-aptamer conjugate and the negative controls, which does not add any materials that can influence the cell growth. This indicates that the dual-aptamer conjugate does not have any toxicity on prostate cancer cell lines.

Generally, usage of an ITO working electrode has the adventage of allowing direct abservation of microscopic images from its transparent property. With this ITO working electrode, morphologic changes of each cell could be also obtained from treatment of the doxorubicin-loaded dual-aptamer conjugate. It is important to note that each cell has its own morphologies and binding appearances. For example, LNCaP grow in colonies, forming flowers combining with several elongated cells, while PC3 grow monotonously with longish seed characters.

The shapes for prostate cancer cells, LNCaP and PC3, did not maintain their own characteristics when detaching from the electrode after treatment of doxuribicn-loaded conjugate. On the other hand, thoese of non-prostatic cancer and prostatic non-cancerous cells (MCF-7, HeLa, SW620, and PNT-2) with the drug-loaded conjugate maintained morphologies similar to healthy cells. This result provides additional evidence that doxorubicin can be actively delivered to the prostate cancer cells with the aptamer conjugate. The cell apoptosis was also confirmed with trypan blue assay, and viability values of each cell were calculated and plotted, with tendencies exactly matched to that of DPV analysis.

All of these confirming methods: DPV analysis, microscopic method, and trypan-blue assay, indicate that targeted drug delivery to prostate cancer cells successfully occurred using the drug-loaded aptamer conjugate.

4 CONCLUSION

In summary, we synchronously delivered doxorubicin to both prostate cancer cells, PSMA (+) and PSMA (–) cell lines, with a single RNA/peptide, dual-aptamer conjugate. Apoptosis induced by doxorubicin was verified using DPV analysis, and its morphologic changes were detected on transparent ITO-coated glass working electrodes. In addition, cell viability, which was calculated from trypan-blue assay, gives evidence for correct targeting. The dual-aptamer conjugate was applied as an active drug-delivery vector for general targeting of prostate cancers, including the PSMA (–) cell line. Although doxorubicin does not directly combine with DUP-1 peptide aptamer, it affects PSMA (–) cells with bridged aptamer conjugate.

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