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Immunotherapy of CD30-expressing lymphoma using a highly stable ssDNA aptamer

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ABSTRACT

CD30 is highly expressed on Hodgkins lymphoma and anaplastic large cell lymphoma, making it an attractive target for therapy. We describe the generation of serum-stabilized ssDNA aptamers that bind CD30 via a hybrid SELEX methodology. The selected aptamer bound CD30 with high affinity and specificity. Further optimization of the aptamer led to a short, truncated variant with a 50-fold higher affinity than its longer counterpart. The multivalent aptamer was able to induce oligomerization of CD30 receptors and, in effect, activate downstream signaling, which led to apoptosis of ALCL cells. Immunotherapy using aptamer-based co-stimulation provides an alternative to antibodies, and has potential to transform cancer treatment.

1. Introduction

Aptamers are small oligonucleotide molecule ligands, RNA or single-stranded DNA (ssDNA), and have high affinity for target binding [1–3]. In contrast to protein antibodies, aptamers can be easily generated through chemical synthesis and manufactured at much less cost. In addition, as a short oligonucleotide biomaterial, the aptamers show little or no activation of the immune response *in vivo*. These features of aptamers allow numerous possibilities for their use in medical applications. While clinical applications of aptamers have not yet been well investigated, they have demonstrated an ability to specifically target certain biomarkers on cancer cells, including CD30 protein, which has been detected in some hematological malignancies [4,5]. Expression of CD30 has been considered as a specific diagnostic biomarker of anaplastic large cell lymphoma (ALCL) and classical Hodgkin lymphoma (cHL) [6–11]. CD30 is also a biomarker used for targeted therapy by an antibody–drug conjugate, brentuximab, which was recently approved by the FDA [12–14]. Moreover, studies have shown that trimerization of CD30 receptors induced by the CD30 ligands can activate cellular signaling, and subsequently regulate functions of the targeted cells [15]. Although CD30 receptors lack the death domain, the ligand-induced activation could induce apoptosis of the ALCL tumor cells [16]. These observations suggest a potential immunotherapy approach to treat ALCL by selectively targeting and inducing trimerization of CD30, activating the cellular signaling pathway and triggering tumor cell apoptosis through a natural biological process. To advance aptamer technology for clinical use, we previously tested a reported RNA-based aptamer sequence for both flow cytometry analysis of CD30-expressing lymphoma cells and immunostaining of formalin-fixed and paraffin-embedded tumor tissues [17,18]. Although the RNA-based aptamers are widely studied, their clinical applications are largely limited due to poor stability under biological and physiological conditions [19]. Modified RNA nucleotides have been incorporated into the aptamers to enhance their stability, however, chemical modification of RNA nucleotides has demonstrated minimal improvement in prolonging the half-life of aptamers *in vivo* [19–21]. Another potential solution to overcome this technical obstacle is to exploit the inherent stability of ssDNA (compared to RNA) in biological environments, and develop ssDNA-based aptamers. In this study, an ssDNA-based aptamer specific for CD30 was developed and its physical and biological properties were investigated.

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2. Materials and methods

2.1. Cell lines and reagents

Karpas 299 (K299, T-cell lymphoma), Jurkat, Molt-4, SupT1 (T-cell leukemia), U937 (histiocytic lymphoma), HDLM2, KM-H2, (Hodgkin lymphoma), K562 (chronic myeloid leukemia), HL60 (acute promyelocytic leukemia), HEL (erythroleukemia), Jeko-1 (B-cell lymphoma), Maver-1 (mantle cell lymphoma), CA46 (Burkitts lymphoma), SKBR3 (breast adenocarcinoma), and LNCAP (prostate carcinoma) cell-lines were used and obtained from American Type Culture Collection (Manassas, VA). All cell lines were cultured in recommended medium supplemented with heat-inactivated Fetal Bovine Serum (FBS) (GIBCO, Grand Island, NY), and 100 IU/mL penicillin–streptomycin. The washing buffer used during aptamer enrichment contained 4.5 g/L glucose and 5 mM $MgCl_2$ in Dulbecco's PBS (Sigma, St. Louis, MO). One mg/mL BSA (Fisher, Waltham, MA) with 0.1 mg/mL t-RNA were added to reduce nonspecific background binding, and to make binding buffer from the wash buffer. Trypsin was purchased from Fisher, and PCR reagents and Taq polymerase were purchased from Takara Bio (Mountain View, CA).

2.2. Development of ssDNA aptamers using hybrid systematic evolution of ligands by exponential enrichment (hybrid SELEX) approach

The library for SELEX contained a random core of 30 mer with an 18 mer primer binding region on both sides. Biotin reverse primer was used to generate single-stranded DNA, and a forward primer labeled with either FITC or Cy5 was used to monitor aptamer selection. OligAnalyzer® software from IDT Technologies was used to optimize primers. The aptamer pools were PCR amplified with Fwd Primer: 5′-TAC CAG TGC GAT GCT CAG-3′ and Rev Primer: 5′-GTC AAC CGA ATG CGT CAG-3′.

For SELEX, approximately two million CD30-positive K299 cells were washed with PBS and centrifuged at 270 *g*. The cells were incubated with a DNA library which was rapidly cooled on ice after heating at 95 °C for 5 min. Selection was initiated with a 20 nmol ssDNA library and gradually reduced as the selection progressed. The selection stringency was also increased by reducing the incubation time from 60 min in the first round to 20 min at the end of selection. Unbound DNA was removed by centrifugation, and the target-bound DNA eluted by heating the cells at 95 °C for 5 min. The eluted DNA was PCR amplified by Taq DNA polymerase, and PCR conditions were optimized to yield a clear, single band after each round of SELEX. Single-stranded DNA was generated from the PCR product using high-affinity streptavidin-sepharose beads which acted as binding sites for the biotin-labeled anti-sense strand. The sense strand with the fluorophore was eluted using 200 mM NaOH. This ssDNA was used for the next round and the process was repeated iteratively until significant affinity toward target CD30+ cells was observed using a flow cytometer. To reduce the number of probable aptamers, a counter selection with Jurkat cells was performed after 10 rounds of SELEX, and then performed for every alternate round of SELEX.

In addition, protein selection with purified His-tagged CD30 protein was carried out similarly, with minor changes: 1) the binding buffer did not contain glucose and included 2 mg/mL BSA; 2) His-tagged CD30 protein was conjugated to TALON magnetic beads and incubated with DNA aptamer pools; 3) bound and unbound DNA was separated on a magnetic stand; and 4) aptamer pools were further refined by performing a negative selection specifically against the TALON beads.

The identification of ssDNA aptamers with higher nuclease stability and *in-vivo* use were generated by modifying the cell-SELEX protocol, and terming the protocol "hybrid SELEX." Hybrid SELEX involves the use of cell surface markers to isolate aptamers binding the target of interest (e.g., CD30) expressed on tumor cells via cell-SELEX and through the use of purified protein. We started the selection with $\sim 10^{14}$ unique DNA sequences contained in 20 nmol of a ssDNA library containing a 30 mer random region. The stringency of selection was increased by reducing the concentration of the amplified ssDNA pool and the time of incubation, and increasing the washing time and volume. Also, a negative selection was performed with Jurkat cells, which do not express the CD30 protein, to remove aptamers binding the commonly expressed proteins and other molecular markers on the surface of cells. After 15 rounds of selection, the resulting aptamer pool not only possesses binding specificity for the target CD30 biomarkers, but for any other unique surface makers expressed on the target CD30-expressing K299 cell line. This results in a family of aptamers which may also include a few off-target aptamers specific to the target cells. We then used purified His-tagged CD30 protein to further focus the aptamer pool specifically toward CD30 and remove aptamers targeting other markers. The His-tagged CD30 was then immobilized on TALON magnetic beads. Three rounds of selection were performed with increasing stringency in which we reduced the concentration of the DNA pool by 100-fold, and increased the washing time from 10 min to 30 min, and the washing volume from 100 uL to 1000 uL. The pool was then incubated with TALON beads to remove the sequences binding the beads. We tested the selection progress with flow cytometry using Cy5-labeled primer to generate a fluorescently-labeled pool with CD30-positive and -negative cells. The selection was stopped when no further progress was observed for 3 rounds of selection.

The enriched pool from the last round of hybrid SELEX was cloned using a T7 vector and sequenced at the sequencing core of the Baylor College of Medicine, Houston. Pools 20–23 were tagged with multiplex identifier primers for next-generation sequencing, and sequenced using the Ion-Torrent sequencing platform. Analysis of NGS data was performed using MAFFT alignment software [22,23].

2.3. Flow cytometric analysis

One-half million cells were washed with PBS and then incubated with 100 uL of a 250 nM Cy5- or FITC-labeled DNA pool, then re-suspended in 250 μL binding buffer. Ten thousand cells were counted on a BD LSR II flow cytometer (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ). The fluorescence readouts represented the affinity of the fluorophore-labeled pools toward the CD30-positive cells.

After the generation of aptamers, FITC-labeled aptamers were used for characterization using CD30-positive and CD30-negative cells. The apparent dissociation constants (K_ds) were measured by flow cytometry using a series dilution of the aptamers that were incubated and analyzed on the flow cytometer.

2.4. Competition experiments

Competition was performed by incubating a fluorophore-labeled aptamer with an unlabeled aptamer sequentially, in reverse order, and simultaneously. The experiments were performed using a 1:10 ratio of labeled aptamer to unlabeled aptamers.

2.5. Biostability assays

One microgram of DNA and RNA CD30 aptamer was incubated with human serum for 24 h, and samples were collected at different times using phenol–chloroform extraction. The amount of digested aptamer was visualized on a 5% agarose gel.

2.6. Aptamers in complex media

Target K299 cells were mixed with non-target U937 cells and incubated with 10 nM Cy3-labeled aptamer for 30 min, washed with washing buffer 5×, and tested on a flow cytometer. Non-specific binding of the aptamer was tested by addition of K299 cells with leukocytes after lysing red blood cells from the whole blood. Similarly, K299 cells were also added directly to whole blood and tested for specific binding of aptamers. Flow cytometry was performed with PerCP CD45 and FAM CD30 antibody, along with the Cy-3-CD30 aptamer.

2.7. Fluorescence microscopy

Imaging was performed using an Olympus epifluorescence microscope (Olympus America, Center Valley, PA). The mixture of CellTrace CFSE-stained U937 control cells and unstained K299 cells was incubated with 100 nM of the Cy3-labeled aptamer C2NP for 30 min, then washed with washing buffer 5×.

2.8. Biotherapy assay

K299 and HDLM2 cells were incubated with the CD30 aptamer and the ratio of dead/live cells was measured using a dual-staining assay after 24, 48, 72, and 96 h. The dual-staining assay used propidium iodide, which stains for dead cells, and Hoechst 33362 dye, which stains for live cells. Absolute cell counts were measured using flow count beads in the assay. For immunotherapy, the experiments were performed with biotin-labeled 1 μM DNA and 250 nM streptavidin to form a multimeric aptamer, and CD30 signaling was initiated by trimerization of CD30 antigens. A sample control with streptavidin was also included in the experiments.

2.9. Statistics

The binding affinity and immunotherapy co-stimulation experiments were performed in duplicate and repeated two times. The average of the readings was used, and the error bars represent the standard deviation. The graphs were plotted using MS-Excel 2010.

3. Results

3.1. Development of ssDNA aptamers specific for CD30

The resultant ssDNA pool was cloned after 23 rounds of enrichment and selection using the hybrid SELEX approach (Supplementary Fig. S1). Among the sequenced clones, sequence C2 (Fig. 1A) showed the highest frequency and was selected for further analysis. Simultaneously, next-generation sequencing analysis