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Investigating Cytokine Binding Using a Previously Reported TNF-Specific Aptamer

James D. Fisher[1,2], Morgan V. DiLeo[1,3], William J. Federspiel[1,2,3,4*]
[1]McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, USA
[2]Departments of Chemical Engineering, University of Pittsburgh, Pittsburgh, USA
[3]Departments of Bioengineering, University of Pittsburgh, Pittsburgh, USA
[4]Departments of Surgery, University of Pittsburgh, Pittsburgh, USA
Email: fisherj4@upmc.edu, mod8@pitt.edu, *federspielwj@upmc.edu

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ABSTRACT

Cytokines are of chief importance in the pathophysiology of sepsis and other systemic inflammatory response syndromes. We are designing and testing an extracorporeal cytokine adsorption device (CAD) that can remove cytokines via adsorption on biocompatible, microporous beads. The goal of this study was to determine whether a previously reported TNF binding DNA aptamer, 5'-GCGGCCGATA AGGTCTTTCC AAGCGAACGA ATTGAACCGC-3', could be immobilized our hemoadsorption polymer surface to increase the removal rate of TNF. A reservoir consisting of horse serum spiked with a known concentration of TNF was perfused through our CAD packed with aptamer modified or unmodified (control) polymer beads. The binding affinity of the TNF aptamer was characterized using an enzyme-linked oligonucleotide assay (ELONA). As a positive control a well-established DNA aptamer that binds PDGF BB was also subjected to the same ELONA to validate the assay. TNF capture using the CAD showed no TNF removal over four hours for both the aptamer modified and unmodified control beads. Additionally, the results of the ELONA showed no binding of TNF to the reported aptamer; however the PDGF BB aptamer did bind PDGF BB. Based on these results we are able to conclude that the reported TNF specific aptamer does not bind TNF. These results will be of importance to other studies exploring aptamers for specific binding of TNF.

Keywords: Aptamers; Sepsis; Hemoadsorption; TNF

1. Introduction

Sepsis is defined as systemic inflammation in the presence of infection. The worldwide prevalence of this disease and the lack of efficient treatment options have made sepsis one of the leading causes of death in the world and the most common cause of death in adult, noncoronary intensive care units [1]. Systemic inflammation results in the excessive production of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-6 (IL-6) and anti-inflammatory cytokines such as interleukin-10 (IL-10). Overproduction of cytokines along with their interactions with one another contribute to the pathological process of sepsis [2].

One emerging therapy aimed at treating sepsis is hemofiltration. The aim of this therapy is to remove pro- and anti-inflammatory cytokines from circulation using convection, thus reestablishing a physiological balance. Hemofiltration studies with sepsis have shown an initial decrease in cytokine removal followed by a gradual decrease as the filter becomes saturated [3]. However, Kellum and Dishart (2002) were able to show evidence that the primary mechanism responsible for interleukin-6 (IL-6) removal during hemofiltration is most likely due to adsorption onto the membrane rather than filtration from plasma [4]. Thus, newer therapies now focus on hemoadsorption in addition to hemofiltration. Our group is developing a cytokine capture device that consists of a column packed with microporous polymer beads through which whole blood is perfused [5]. The polymer beads used in the cytokine adsorption device (CAD) have a high surface area (850 m^2/g), making them ideal for adsorption-based therapy.

Our device removes a variety of middle-molecular weight proteins in the 10-30 kDa range including cytokines generally considered of clinical relevance to sepsis such as IL-6, IL-10, and TNF. While our device has been effective at removing both IL-6 and IL-10 in both *in vitro* and *ex vivo* animal studies, the removal rate of TNF has been considerably slower than that of IL-6 and IL-10 [6]. To address this issue, we have begun studying specific capture of TNF by immobilized ligands on the outer surface of the polymer beads currently used in the CAD as a

*Corresponding author.

means to increase the removal rate of TNF. An ongoing focus in our group has been immobilizing antibodies as specific capture ligands for TNF. Antibodies have several drawbacks, including the substantial cost associated with coating several grams of polymer beads with TNF antibodies, in addition to their limited shelf life. Another concern is the potentially harmful immune response that may occur if antibodies leach off of the beads.

A novel alternative to antibodies are aptamers, short strands of oligonucleotides. Aptamers fold into a unique three dimensional structure (similar to antibodies) which allows them to specifically bind to a variety of biomolecules with affinity constants comparable to that of antibodies (~10^{-9} M). These nucleic acid oligomers are synthesized via an iterative *in vitro* selection process called SELEX (systematic evolution of ligands by exponential enrichment) [7,8]. Aptamers possess several advantages over their antibody counterparts as they are less expensive, synthesized *in vitro*, have longer shelf lives and are less likely to elicit immunogenicity than antibodies [9,10]. Moreover, various chemical functionalities can be added to the 5' and 3' ends of the aptamer to allow for easier conjugation to a surface.

In this work, our group investigated the TNF specific aptamer sequence, 5'-GCGGCCGATA AGGTCTTTCC AAGCGAACGA ATTGAACCGC-3', reported in the patent submitted by Zhang *et al.* [11]. We immobilized this aptamer on the surface of porelesspolystyrene-divinylbenzene (PSDVB) beads and tested its ability to bind TNF in the CAD and subsequently deplete it from the circulating serum solution. Poreless PSDVB beads were used since the aptamers, unlike antibodies, are small enough to diffuse into the porous network of our standard porous PSDVB beads. In addition, the specificity of this aptamer sequence to human TNF alpha was evaluated using the enzyme-linked oligonucleotide assay (ELONA) methodologies of Yan *et al.* [12].

2. Methods

Carboxyl groups were incorporated onto the surface of the poreless PSDVB beads to provide a functionalized surface for aptamer coupling, using a modified polystyrene oxidation protocol [13]. Batches of 2 grams of poreless PSDVB beads were incubated in manganese (VII) oxide in H_2SO_4 at 65˚C for 1 hour. The surface concentration of carboxyl groups was measured to be 24 nmol/g polymer using a para-nitrophenol colorimetric assay [14]. Beads were washed with 6 N hydrochloric acid and DI water. Functional groups were activated by incubating the beads with a 1 mg/ml solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 4.5) for 1 hour. The beads were washed with MES buffer followed by DI water. Aptamers were coupled to the beads by adding 11 μg/ml aptamer solution in sodium phosphate buffer (pH 7.0) and incubating at room temperature for 2 hours. Aptamers used in this step were functionalized with an amine group at the 5' end for coupling. Beads were washed with a .05% Tween solution followed by DI water.

For TNF capture experiments, 1.5 grams of aptamer-immobilized beads or unmodified beads were packed in an unused CAD and connected in series with a peristaltic pump. Inlet and outlet tubes were connected to an 8 ml reservoir of horse serum spiked with TNF at a concentration of ~1200 pg/ml. The reservoir was perfused through the CAD at a flow rate of 0.8 ml/min and samples were taken at t = 0, 15, 30, 60, 90, 120, 180, 240 min. TNF concentrations were measured using a Biosource enzyme-linked immunosorbent assay (ELISA) (Invitrogen) according to the manufacturer's instructions.

The ELONA technique was performed as follows. Recombinant human TNF (ThermoFisher) was diluted to 5 μg/ml using coating buffer (0.05 M Sodium Carbonate pH 9.76), and 100 μl of this solution was incubated overnight at 4˚C in a polystyrene microplate. As a negative control, recombinant human IL-6 (Thermo) at the same concentration was used. The plate was washed with a 0.15 M NaCl buffer, pH 7.4, containing 0.1% Tween 20, and remaining adsorption sites were then blocked with 100 μl of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 h at 37˚C. Wells were once again washed followed by addition of 100 μl of TNF-specific DNA aptamers at concentrations of 1.10×10^7, 1.10×10^5, 1104, 552, and 276 pg/ml. 100 μl of the 1104 pg/ml aptamer solution was added to the IL-6 coated well as a negative control, and 100 μl of biotinylated TNF antibody (Biosource) was used a positive control. The aptamer/antibody solutions were incubated at 37˚C for 1 h and then washed. 100 μl of streptavidin-conjugated horseradish peroxidase (Biosource) was added to each well and incubated at 37˚C for 1h. The wells were washed for the final time, after which 100 μl of tetramethylbenzidine (TMB) substrate solution was added to each well. After 20 min, the optical density was measured at 450 nm on a MultiSkan Plus microplate reader (ThermoFisher).

We also evaluated the affinity of a well-established aptamer for its target ligand using ELONA to ensure that the technique was being done correctly. Green *et al*. published a DNA aptamer sequence, 5'-CAGGCTACGG-CACGTAGAGCATCACCATGATCCTG-3', which exhibited high binding affinity towards platelet-derived growth factor BB (PDGF-BB) [15]. The methods used in the PDGF-BB ELONA were the same as those used in the TNF ELONA. Wells were coated with PDGF-BB and the PDGF-BB aptamer was the target analyte.

3. Results

Figure 1 shows the results of TNF capture for horse serum perfused through the CAD packed with aptamer-

immobilized poreless PSDVB beads and unmodified poreless PSDVB beads (control).

Neither the aptamer-immobilized nor the unmodified beads were able to significantly decrease the circulating concentration of TNF.

The results of the human TNF and PDGF-BB ELONAs are shown in **Figures 2** and **3**, respectively. The TNF ELONA data indicates that the TNF aptamer exhibited no binding affinity toward recombinant TNF. The positive control, a biotinylated human TNF antibody, showed a significant amount of binding affinity toward TNF relative to the negative control and test wells. The PDGF-BB ELONA, however, demonstrated that the PDGF-BB aptamer did have significant binding affinity to PDGF-BB relative to the control wells. The wells of the PDGF-BB ELONA corresponding to concentrations 4.51×10^8, 4.51×10^6, and 4.5×10^4 pg/ml did not show a decrease in signal as these concentrations were beyond the detection limit of the plate reader.

However, the subsequent concentrations showed a decrease in signal with a decrease in concentration of aptamer, ruling out the possibility of non-specific binding.

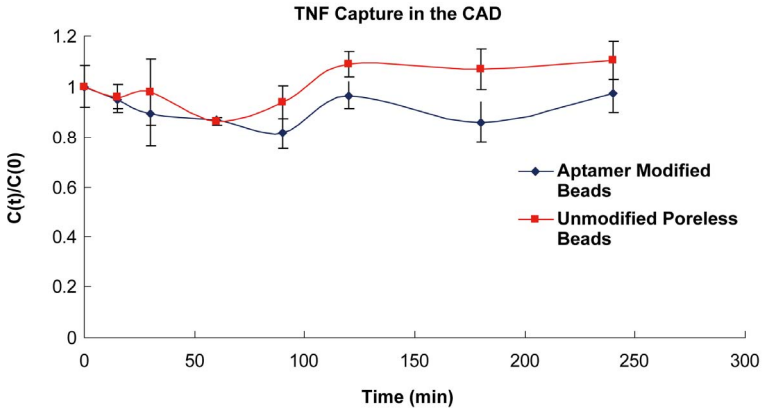

Figure 1. TNF capture with aptamer-immobilized and unmodified PSDVB beads.

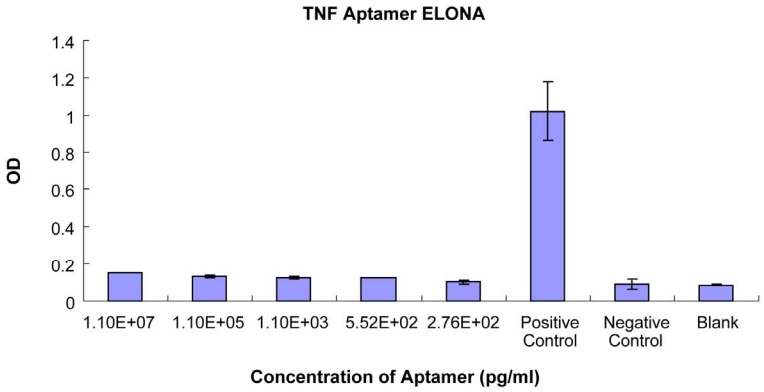

Figure 2. TNF aptamer ELONA.

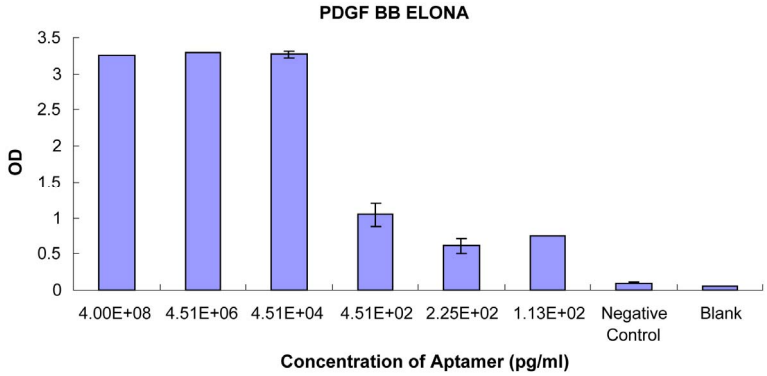

Figure 3. Platelet derived growth factor BB ELONA.

4. Discussion

The ability of CADs packed with aptamer-immobilized or unmodified beads to capture TNF from horse serum was tested. The results in **Figure 1** show that TNF capture with unmodified and aptamer-immobilized beads was negligible. The aptamer-immobilized on the surface of the PSDVB beads was reported to specifically bind TNF, therefore we expected that the aptamer-immobilized beads would display a significantly higher ability to capture TNF than the control beads. Based on the surface density of carboxyl groups on the beads, we calculated that if successfully coupled there would be at least a 10 molar excess of aptamer to TNF. Therefore, one possible explanation is that the TNF aptamer was not successfully coupled to the surface of the PSDVB beads. This is unlikely however, as we have successfully coupled antibodies to the PSDVB beads using the same chemistry. Another possible explanation was that the reported aptamer did not bind to TNF. To characterize the affinity of the published aptamer for TNF, we utilized a previously reported enzyme-linked oligonucleotide assay (ELONA) [12].

From the ELONA data we are able to conclude that the TNF aptamer sequence does not specifically bind TNF. There are several possible explanations for this finding. The discrepancies in data could be a result of differences in the protein at which the aptamer was targeted. Our group used commercially available recombinant human TNF from ThermoFisher Scientific, but the recombinant protein used by Zhang *et al*. was produced in their laboratory. The target proteins were synthesized in different environments, which may suggest that the three dimensional structure of the proteins may have differed enough to impact the aptamer's affinity toward TNF. The TNF used in our work was in its correct three-dimensional shape, as evidenced by our positive control, a TNF antibody, being able to bind TNF in the ELONA. A TNF antibody was not used as a positive control in the group's patent or published description of the RNA aptamer [11,12].

The aptamer sequence, 5'-GCGGCCGATA AGGTC-TTTCC AAGCGAACGA ATTGAACCGC-3', reported by Zhang and coworkers does not appear to bind commercially available recombinant TNF. While this result is a negative finding, we believe that this correspondence provides important data to other investigators who may be studying specific ligands for TNF.

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