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Regulation of fibrin-mediated tumor cell adhesion to the endothelium using anti-thrombin aptamer

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ABSTRACT

Molecular intervention during transient stages of various metastatic pathways may lead to development of promising therapeutic technologies. One of such involves soluble fibrin (sFn) that has been implicated as a cross-linker between circulating blood or tumor cells and endothelial cell receptors, promoting cell arrest on the endothelium during circulation. sFn generation is a result of thrombin-mediated fibrinogen (Fg) cleavage due to either vascular injuries or a tumor microenvironment. For cancer therapy, thrombin-mediated conversions of Fg to sFn thus serve as potential intervention points to decrease circulating tumor cell adhesion to the endothelium and subsequent metastatic events. The purpose of this work was to investigate the function of an anti-thrombin oligonucleotide aptamer in reducing tumor cell arrest. Both molecular and cellular interactions were examined to demonstrate the binding and inhibitory effects of anti-thrombin aptamer. The results show that the aptamer is capable of inhibiting thrombin-mediated Fg conversion, thereby reducing sFn-mediated tumor cell adhesion in a concentration-dependent manner. Notably, the aptamer is able to bind thrombin under dynamic flow conditions and reduce tumor cell adhesive events at various physiological shear rates. This study further indicates that oligonucleotide aptamers hold great promise as therapeutic regulators of tumor cell adhesion, and consequently, metastatic activity.

1. Introduction

Metastasis through the vasculature is a dynamic, multiple-step process by which a population of diseased cells enter newly-formed blood vessels from a primary tumor site, are transported to distant locations, exit the vasculature, and proliferate in these secondary locations [42]. It is the major cause of cancer deaths [6,45]. Thus, development of technologies for intervention during the transient stages of the metastatic pathway would be a promising strategy to inhibit cancer metastasis and to prolong the life-spans of cancer patients [41].

The arrest of tumor cells at the endothelium of blood vessels is a key step during the dynamic process of cancer metastasis. Numerous studies have been performed to define the roles of tumor and endothelial cell receptors in facilitating the attachment of tumor cells [39,44], demonstrating that tumor cell receptors bind to their corresponding ligands on endothelial cells to mitigate the circulatory shear effects that oppose tumor cell adhesion to the endothelium. For instance, both melanoma and endothelial cells express several surface adhesion receptors, such as $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and ICAM-1 [30–32], to serve as cross-linking points for melanoma arrest on the endothelium. In addition to these cell receptors, several lines of highly metastatic tumor cells have proven capable of manipulating the tumor microenvironment via production or overexpression of functional molecules [25,34]. These molecules can directly enhance tumor cell adhesion or induce the synthesis of molecules to cross-link tumor cells to the endothelium. Thrombin is an important molecule that facilitates the arrest of circulating tumor cells to the endothelium, since it catalyzes the conversion of fibrinogen (Fg) to soluble fibrin (sFn). Soluble fibrin has been shown to function as a cross-linker between cell receptors [40] or between receptors on additional tethering cells [1,3,17], such as polymorphonuclear neutrophils [56]. Therefore, the thrombin-mediated conversion of Fg to sFn serves as a potential intervention point for inhibiting tumor cell adhesion to the endothelium.

Using inhibitors to intervene with molecular recognition has been broadly applied for the treatment of human diseases [12,22,29,55]. A variety of inhibitors, such as antibodies, peptides, polysaccharides, and even cells have been studied as molecular interventions [12,16,22,28,33,55]. To avoid side effects, it is important that these inhibitors can bind to target molecules with high affinity and specificity. However, many do not satisfy this critical requirement. For instance, heparin is a routinely used anti-

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thrombin reagent in the clinic. While heparin can increase the inhibition of thrombin as much as 10,000 fold in comparison to endogenous antithrombin [51], heparin can interact with a variety of biomolecules nonspecifically. By contrast, nucleic acid aptamers bind to their target molecules with high specificity due to their rigorous selection process [10,53]. For instance, studies have shown that aptamers can discriminate between targets on the basis of subtle structural differences, such as the presence or absence of a methyl or hydroxyl group in certain proteins [23]. In addition to high specificity, truncated aptamers are small, composed of 15–45 nucleotides. Thus, they can be chemically synthesized with a minimal batch-to-batch variation. Their small size and similarity to endogenous molecules also result in their minimal immunogenicity.

This work was aimed at testing a novel hypothesis that an anti-thrombin aptamer that binds to exosite I of thrombin [5,19] can reduce sFn production and, consequently, tumor cell adhesion to the endothelium under dynamic flow conditions. This hypothesis was tested at both molecular and cellular levels. The binding between anti-thrombin aptamer and thrombin and its effects on Fg–sFn conversion were investigated using surface plasmon resonance and SDS PAGE analysis. The specificity of anti-thrombin aptamer for thrombin was examined using a thrombin coagulation time assay and parallel plate flow chamber adhesion analysis. In the adhesion study, an endothelial monolayer was grown on the glass surface as the base of a parallel plate flow chamber, over which melanoma cells were perfused to examine cell adhesion. The effects of flow parameters on both aptamer-mediated molecular and cellular interactions were analyzed. This study is expected to not only demonstrate a new strategy for reduction of tumor cell adhesion, but also highlight the use of aptamers as novel regulation and inhibition molecules for cellular and molecular interactions.

2. Materials and methods

2.1. Reagents

Potassium chloride, sodium acetate, sodium hydroxide, Tween-20, glycine, sodium dodecyl sulfate, thrombin (human), Trizma base, fibrinogen (human), Gly–Pro–Arg–Pro (GPRP), dithiothreitol, glacial acetic acid, and glucose were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride, hydrogen chloride, magnesium chloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), reagent alcohol, glycerol, acrylamide/bis(acrylamide), ammonium persulfate, tetramethylethylenediamine (TEMED), methanol, bovine serum albumin (BSA), fetal bovine serum (FBS), DMEM/F-12, RPMI 1640, trypsin–EDTA, and DPBS were obtained from Thermo-Fisher Scientific (Waltham, MA). Tris acetate was purchased from Amresco (Solon, OH) and bromophenol blue was purchased from Santa Cruz Biotechnology (Dallas, TX). Stacking gel buffer and resolving gel buffer (4 ×) were obtained from Bio-Rad (Hercules, CA), while Coomassie blue was obtained from Teknova (Hollister, CA). Pooled human plasma was purchased from Innovative Research (Novi, MI) and all DNA sequences were obtained from Integrated DNA Technologies (Coralville, IA). The protein standard was purchased from New England BioLabs (Ipswich, MA) and magnesium chloride was purchased from ATCC (Manassas, VA).

2.2. Surface plasmon resonance

For analysis of anti-thombin aptamer specificity and binding parameters, SPR was performed using a Reichert Technologies SR7500DC spectrometer equipped with an SR8100 autosampler (Depew, NY). Thrombin was cross-linked on the analyte channel of a planar polyethylene glycol/carboxyl sensor chip (Reichert Technologies, Depew, NY): the sensor chip was incubated with a 2:1 molar solution of EDC:NHS in deionized water for 20 min at 20 μL/min and 25 °C. After washing, a 1 μg/mL solution of thrombin in 10 mM sodium acetate (pH: 5.8 with HCl) was injected over the analyte channel for approximately 30 min at 20 μL/min and 25 °C. The SPR studies were run at 25 °C unless otherwise noted and temperature calibrations were performed prior to data collection. All samples were prepared in running buffer: 20 mM tris acetate, 100 mM sodium chloride, 10 mM potassium chloride, and 0.05% Tween-20. Flow rates were maintained above 10 μL/min to establish a stable baseline and minimize effects from potential mass transport limitations [43]. Unless otherwise noted, all aptamers and scrambled sequences were prepared at a concentration of 3 μM and injected at 20 μL/min for 5.5 min, followed by a dissociation period of 5 min. Chip regeneration was performed by injecting 30 mM sodium hydroxide at 100 μL/min for 1 min, followed by a 2 min dissociation period. Data were collected using SPR Autolink software (Reichert Technologies), and plotted as the difference between analyte and reference channels. Kinetic data fitting and analysis were performed using Scrubber 2 (BioLogic Software). Briefly, the concentration series data were zeroed to a point prior to injection and the reference signal was subtracted. Next, data curves were fit according to a simple 1:1 biomolecular interaction model. This modeled fit was then subtracted from the experimental data and adjusted to minimize the resultant χ^2 value [36]. From the error-minimized fit, the association and dissociation rates were obtained.

2.3. SDS-PAGE analysis of fibrinogen conversion

SDS-PAGE was performed to determine the influence of thrombin and anti-thrombin aptamer on fibrinogen (Fg) conversion. Polyacrylamide gels were prepared with a stacking region that began 1 cm below the bottom of the wells. The stacking solution (total volume 3.35 mL) consisted of: 4% acrylamide solution, 1 × stacking gel buffer, 0.1% (*w/v*) SDS, 0.05 % (*w/v*) APS, and 0.1% (*w/v*) TEMED. The resolving solution (total volume 10 mL) consisted of: 5% acrylamide solution, 1 × resolving buffer, 0.1% (*w/v*) SDS, 0.05 % (*w/v*) APS, and 0.1% (*w/v*) TEMED. The gels were run in a buffer of 25 mM Tris, 192 mM glycine, and 0.1% (*w/v*) SDS at a pH of 8.3. For thrombin variation sample preparation, 3 mg/mL Fg and varying concentrations of thrombin were mixed and incubated together for 30 min, followed by the removal of any insoluble fibrin fibers. For anti-thrombin aptamer variation samples, 8 U/mL thrombin was mixed and incubated first with varying concentrations of anti-thrombin aptamer for 1 h. This thrombin concentration was selected since it was capable of high Fg-to-fibrin conversion in the thrombin variation electrophoresis study. To these samples, Fg was added at a concentration of 3 mg/mL and incubated for 30 min, followed by the removal of any insoluble fibrin fibers. Samples were then prepared in a buffer consisting of 1x stacking buffer with 1% (*w/v*) SDS, 10% (*v/v*) glycerol, 0.01% (*w/v*) bromophenol blue, and 1.5% (*w/v*) DTT at a pH of 6.8. Samples were heated in boiling water for 10 min and then placed on ice until loading. The loaded gel was run at 200 V for 30 min. After the gel was run, it was washed and placed in a fixing solution consisting of 50% methanol, 40% deionized water, and 10% acetic acid for 1 h. Next, the gel was stained in a solution of 0.1% Coomassie blue, 50% methanol, 39.9% deionized water, and 10% acetic acid overnight at 4 °C. Gels were destained first in a solution of 40% methanol, 50% deionized water, and 10% acetic acid for 30 min, then in a solution of 5% methanol, 85% deionized water, and 10% acetic acid with solution replacement until the gel background was clear. Immediately after destaining, gels were washed and imaged using a CRI Maestro In-Vivo imaging system (Woburn, MA).