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Full Length Article

Generation and characterization of aptamers targeting factor XIa

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ABSTRACT

Background: The plasma protease factor XIa (FXIa) has become a target of interest for therapeutics designed to prevent or treat thrombotic disorders.
Methods: We used a solution-based, directed evolution approach called systematic evolution of ligands by exponential enrichment (SELEX) to isolate RNA aptamers that target the FXIa catalytic domain.
Results: Two aptamers, designated 11.16 and 12.7, were identified that bound to previously identified anion binding and serpin bindings sites on the FXIa catalytic domain. The aptamers were non-competitive inhibitors of FXIa cleavage of a tripeptide chromogenic substrate and of FXIa activation of factor IX. In normal human plasma, aptamer 12.7 significantly prolonged the aPTT clotting time.
Conclusions: The results show that novel inhibitors of FXIa can be prepared using SELEX techniques. RNA aptamers can bind to distinct sites on the FXIa catalytic domain and noncompetitively inhibit FXIa activity toward its primary macromolecular substrate factor IX with different levels of potency. Such compounds can be developed for use as therapeutic inhibitors.

1. Introduction

Approved antithrombotic drugs target thrombin generation either by inhibiting thrombin and/or factor Xa, or by lowering the plasma levels of the zymogen precursors of these enzymes [1,2]. Because thrombin and factor Xa are central to hemostasis, agents targeting them will inevitably increase bleeding risk. There is interest in developing strategies directed at other enzymes involved in coagulation, with the expectation that they will be safer than inhibitors of thrombin or factor Xa from the standpoint of bleeding. Data from rodent, rabbit and primate models suggest that inhibition of the proteases factor XIa (FXIa) or factor XIIa (FXIIa) reduces occlusive thrombus growth while having relatively small effects on hemostasis [3–7]. Humans lacking factor XII (FXII), the zymogen of FXIIa, do not bleed abnormally, while individuals deficient in factor XI (FXI), the zymogen of FXIa, have a relatively mild bleeding propensity [8]. There are reasons to suspect that FXIa may be preferable to FXIIa as an antithrombotic target. Antibodies that neutralize FXI have a greater effect in a primate thrombosis model than do antibodies inhibiting FXII [9–12]. FXI appears to contribute to stroke and venous thromboembolism (VTE), and perhaps myocardial infarction, in humans, while data supporting a role for FXII in these disorders is weak [3]. A recent phase 2 study demonstrated that reduction of plasma FXI could prevent venous thromboembolism with minimal disturbance of hemostasis in humans undergoing knee replacement [13].

Human FXI is a 160-kDa protease that circulates as a disulfide-linked dimer of identical 80-kDa subunits [14,15]. FXIa contributes to thrombin generation primarily by activating factor IX. Each FXIa subunit contains a 45-kDa heavy chain with four ~90 amino apple domains (designated A1 to A4), and a 35-kDa trypsin-like catalytic domain. Regulation of FXI activation and FXIa activity involves anion-binding sites (ABSs) on the A3 and catalytic domains. Polyphosphates, which are polymers of inorganic phosphate released from activated platelets, bind to FXI ABSs and accelerate zymogen activation [16–18]. Binding of heparin and serpins to the FXIa ABSs results in inhibition of FXIa [19–21].

Anticoagulant aptamers have been developed that target several proteases involved in blood coagulation [22–26]. Aptamers are short, single-stranded oligonucleotides that bind over large surface areas on a target protein. Those that act as anticoagulants usually block specific macromolecular interactions. Here, we describe the isolation and characterization of a library of RNA-based aptamers targeting FXIa. Two aptamers were identified that bind to ABSs on the FXIa catalytic domain, inhibiting FXIa cleavage of a tripeptide chromogenic substrate, and inhibiting FXIa activation of FIX.

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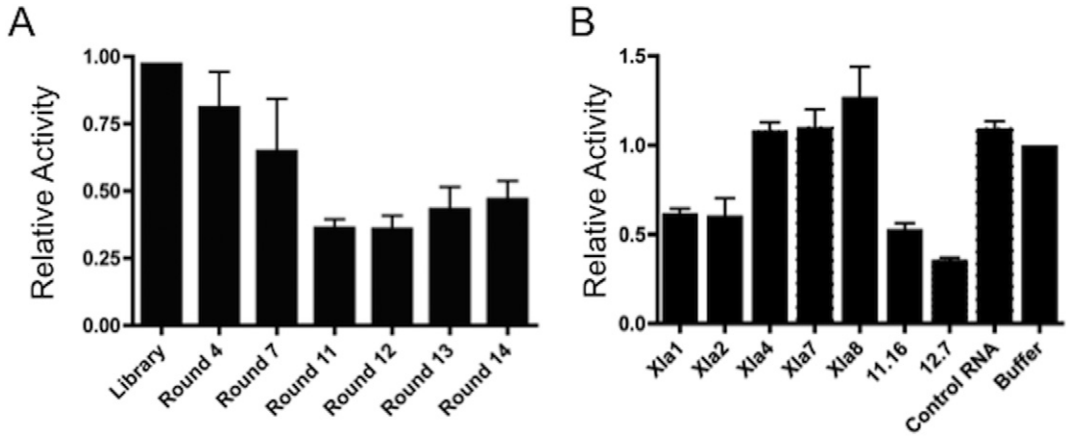

Fig. 1. Anti-FXIa activity of SELEX products. Shown are (**A**) inhibitory activity in aptamer pools from specific SELEX rounds and (**B**) inhibitory activity of specific aptamer species. FXIa (25 nM) was incubated with SELEX RNA (500 nM), and residual FXIa active site activity was measured using a FXIa specific chromogenic substrate. The data were normalized to the rate of substrate cleavage in the absence of RNA (Buffer – arbitrarily assigned a value of 1.00). Shown are means ± SEM of duplicate measures.

2. Materials and methods

2.1. Materials

SELEX DNA templates 5′-TCGGGCGAGTCGTCTG-N_{40}-CCGCATCGT CCTCCCTA-3′ were from Oligos etc. (Wilsonville, OR). 5′ and 3′ SELEX primer sequences, 5′-GGGGGAATTCTAAT-ACGACTCACTATAGGGAGG ACGATGCGG-3′ and 5′-TCGGGCGAGTCGTCTG-3′, respectively, were from Integrated DNA Technologies (Coralville, IA). 2′-flouro modified cytidine and uridine were from Trilink BioTechnology (San Diego). FXI, FXIa, factors IX and IXaβ were from Haematologic Technologies (Essex Junction, VT). FXIIa was from Enzyme Research Lab (South Bend, IN). Normal pooled plasma was from George King Biomed (Overland Park, Kansas). PTT-A Reagent for partial thromboplastin time (aPTT) assays was from Diagnostica Stago (Parsippany, NJ). AMV-RT enzyme was from Roche Applied Science (Indianapolis). Bovine serum albumin (BSA) was from EMD Chemicals (Gibbstown, NJ), ethylene glycol was from Sigma-Aldrich (St. Louis) and lima bean trypsin inhibitor was from USB Corporation (Cleveland). Pefachrome FXIa 3107 was from Centerchem, Inc., (Norwalk, CT) and S-2366 was from DiaPharma Group, Inc. (West Chester Township, OH). Kinetic assays were performed in 96-well flat bottom microtiter plates (Corning, Corning, NY).

2.2. SELEX (systematic evolution of ligands by exponential enrichment)

Solution based SELEX was performed as described [22]. The sequence of the starting RNA template library (termed Sel-3) was 5′-GGGAGGACGAUGCGG-N40-CAGACGACUCGCCCGA-3′, where N40 is a 40 nucleotide randomized region, where equal molar amounts of the four standard nucleobases are included, and C and U are 2′-flourocytidine and 2′-flourouridine ribonucleotides, respectively. This RNA library was transcribed using a modified T7 RNA polymerase (T7 RNA polymerase Y639F) that incorporates 2′-flourocytidine triphosphate and 2′-flourouridine triphosphates [27]. Selection rounds were performed by incubating the starting aptamer library Sel-3 with FXIa and passing the mixture through a 25-mm Protran BA 85 0.45 μm nitrocellulose filter membrane (Whatman, Piscataway, NJ) to separate bound and unbound RNA. Bound RNA was extracted and amplified by RT-PCR. For rounds 1 to 6, 1 nmol RNA was incubated with FXIa in low salt buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, and 0.01% BSA). For rounds 7 to 14, 1 nmol RNA was incubated with FXIa in physiologic salt buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, and 0.01% BSA). Rounds 11, 12 and 14 were cloned and approximately 30 individual aptamers were sequenced per round, as described [28]. Prior to testing, aptamers were diluted into HEPES-based buffers and refolded by heating to 65 °C for 5 min followed by cooling for 3 min at room temperature.

2.3. Recombinant FXIa and FXIa variants

Human FXI was expressed in HEK293 fibroblasts and purified from conditioned media as described [29]. In addition to wild type FXI (FXI-WT), FXI species were prepared with alanine substitutions for (1) Arg^{250}, Lys^{252}, Lys^{253} and Lys255 which form an ABS on the FXI A3 domain (FXI-ABS1) [19]; (2) Lys^{529}, Arg^{530} and Arg^{532} which form a second ABS on the catalytic domain (FXI-ABS2) [20]; or (3) Arg^{504}, Lys^{505}, Arg^{507} and Lys509 (FXI-504-509) in the catalytic domain autolysis loop [21]. Purified proteins were dialyzed against 25 mM Tris-HCl pH 7.4, 100 mM NaCl (TBS). FXIa was generated by incubating FXI (~300 μg/mL) in TBS with 5 μg/mL FXIIa. Complete activation was confirmed by SDS-PAGE. Proteins were stored at −80 °C.

2.4. Screening for aptamer activity

Aptamers were screened for their ability to inhibit FXIa cleavage of a chromogenic substrate and FXIa activation of factor IX. FXIa (25 nM)

Table 1
Frequency of selected RNA aptamer sequences in indicated rounds from FXIa solution-based SELEX. SELEX rounds 11, 12 and 14 were cloned and sequenced to determine individual aptamer sequence frequency in each round. For additional sequences and full DNA template and RNA sequences, refer to Supplemental Tables 1 and 2.

Aptamer	Random region sequence	Round frequency (%)		
		R11	R12	R14
Xla1	ACCGCAUCCGUGAAGAUCCCUCUUCAUCCCCUCCCCCC	17.4	17.4	20.8
Xla2	AAUUACCCGCGUCUGUAGUACACAUGCUAUCCCCUCCCCC	17.4	0	8.3
Xla4	AUCGUGCAUUAUUUCUGGCUACCAGCCAACGGUCCCCCCC	4.3	13.0	0
Xla7	CAAACUAAUUCGGGCCGCGCACUGGGUCCUUCCCCCCC	0	0	25.0
Xla8	GCCGUCCUGAGUCGUAUGAAUUCCGCAUGGUCGUCGUGUG	0	8.7	29.2
11.16	CCAGUCCGCAUCCAUCAUCCCCCUCCCCC	4.3	0	0
12.7	UAACGCCACGCUCGACAACGCGUCGAGUGUCCUCCGCCCC	0	4.3	0