Discovery and characterization of an antibody directed against exosite I of thrombin

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Essentials

- An IgA paraprotein with anti-thrombin activity was not associated with a severe bleeding phenotype.
- This observation challenges the paradigm that anticoagulant therapy necessarily increases bleeding risk.
- Characterization of the antibody showed that it specifically binds to thrombin exosite I.
- A therapeutic drug with the properties of this antibody might be an antithrombotic that doesn’t cause bleeding.

Summary. Background: We report the case of a 54-year-old female who presented with a traumatic subdural hemorrhage. Coagulation tests were markedly prolonged due to the presence of an anti-thrombin IgA paraprotein at 3 g L\(^{-1}\). The patient made a complete recovery and has had no abnormal bleeding during a 7-year follow-up, despite the persistence of the paraprotein. Objectives: To determine how the paraprotein prolonged clotting tests by defining its target and its epitope. Methods: The paraprotein was purified and added to normal pooled plasma for in vitro clotting assays. Binding studies were conducted to determine the affinity of the IgA for thrombin. The Fab was isolated and crystallized with thrombin. Results: The purified IgA was sufficient to confer the patient’s in vitro coagulation profile in normal pooled plasma, and was found to bind specifically and with high affinity to thrombin. A crystal structure of the Fab fragment in complex with thrombin revealed an exosite I interaction involving CDRH3 of the antibody. Conclusions: Although the patient originally presented with a subdural bleed, the hematoma resolved without intervention, and no other bleeding event occurred during the subsequent 7 years. During this period, the patient’s IgA paraprotein levels have remained constant at 3 g L\(^{-1}\), suggesting that the presence of a high-affinity, exosite I-directed antibody is consistent with normal hemostasis. A therapeutic derivative of this antibody might therefore permit antithrombotic dose escalation without an associated increase in the risk of bleeding.

Keywords: anticoagulants; antithrombins; blood coagulation; crystallography; paraproteins; thrombin.

Introduction

Thrombin is the final protease generated by the blood coagulation cascade. Its multiple activities are critical for normal hemostasis but are also the root cause of all forms of thrombosis (Fig. 1) [1–3]. In addition to an active site, thrombin has two basic exosites: exosite I is the fibrinogen recognition site and exosite II is the heparin-binding site. Each site interacts with several cofactors, substrates and inhibitors and plays a part in the expression of thrombin’s activities. Anticoagulant therapies work by either reducing the effective concentrations of the clotting factor precursors (warfarin), by indirectly stimulating protease inhibition by antithrombin (heparins) or directly by targeting factor Xa (e.g. apixaban, edoxaban and rivaroxaban) or thrombin itself (e.g. hirudin, argatroban and dabigatran). All current therapies are associated with a substantial risk of pathologic bleeding [4]. This relationship between anti-thrombotic and anti-hemostatic effects is considered absolute, and results in suboptimal dosing within a narrow therapeutic window. Autoantibody inhibitors of thrombin have been reported, usually associated with exposure to products containing bovine thrombin, and are generally associated with bleed-
Here we describe a randomly acquired anti-thrombin antibody found in a patient who presented with a traumatic subdural hematoma. The patient recovered spontaneously without intervention and has not experienced a further bleeding episode in the 7 years since the initial presentation. The IgA paraprotein was purified and characterized. It binds with high affinity to exosite I of thrombin and is a powerful anticoagulant in vitro. The patient’s low D-dimer levels suggest that the IgA is also acting as a powerful anticoagulant in vivo. However, the lack of bleeding in the patient, despite persistent plasma concentrations of 3 g L⁻¹ over a period of at least 7 years, suggests that the presence of this anti-thrombin antibody is consistent with normal hemostasis.

**Methods**

**Isolation and purification of IgA from patient plasma**

The IgA paraprotein was isolated from patient plasma by affinity chromatography using a column of Jacalin agarose (Sigma, St Louis, MO, USA). After washing the column with phosphate-buffered saline (PBS) total IgA was eluted with melibiose (100 mM in PBS). The eluate was buffer-exchanged into PBS and loaded onto a 1-mL column packed with either PPACK-inhibited thrombin or S195A thrombin (catalytically inert and produced as described previously [7]) conjugated to Sepharose. After washing, the anti-thrombin IgA was eluted with 100 mM glycine pH 3.6, 500 mM NaCl directly into 500 mM Tris pH 7.4, to rapidly neutralize the pH. The flow-through and eluate were examined by electrophoresis using native-blue and SDS-PAGE. The purified thrombin-specific IgA was predominantly monomeric, with only a small amount of higher-order species (Fig. 2A). This material was concentrated and the monomer was purified on a Superdex S200 (GE Heathcare, Uppsala, Sweden). All subsequent studies were conducted with the monomeric IgA fraction. The activity of the purified IgA was confirmed by conducting a thrombin time (TT) test using human plasma thrombin (purchased from Haematologic Technologies, Burlington, VT, USA) in pooled human plasma.

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**Fig. 1.** Central role of thrombin in hemostasis. Normal hemostasis begins with the presentation of tissue factor (TF) to blood proteins. The TF-FVIIa complex forms factor (F) Xa and FIXa and leads to the formation of thrombin (IIa) from prothrombin. Thrombin stimulates its own generation by activating FXI and critical cofactors, FV and FVIII. Thrombin also activates platelets by cleavage of protease-activated receptors (PARs), and cleaves fibrinogen to fibrin polymers. The fibrin polymers are then cross-linked by FXIIIa, which is also activated by thrombin. Most activities of thrombin are partially dependent on its exosites I and II (indicated), with only cleavage of fibrinogen completely reliant on an exosite interaction (I*).

**Fig. 2.** Purification and characterization of the IgA. (A) Native blue gel of the total IgA (first lane) obtained from the patient’s serum after Jacalin agarose purification, the flow-through of the thrombin-Sepharose column (second lane) and the low pH eluate (third lane). The thrombin binding IgA is mostly monomeric, with small amounts of J-chain-linked multimers. (B) The rate of hydrolysis of the small chromogenic substrate S-2238 is saturably increased upon addition of the Fab fragment isolated from the IgA. (C) The thrombin time of pooled plasma is prolonged with increasing IgA concentration.
Biochemical characterization of the antibody

The effect of the antibody Fab fragment (see below) on the activity of thrombin was assessed using the chromogenic substrate S-2238 (0.5 mM human thrombin and 66.5 μM S-2238). A fractional increase in the observed rate of hydrolysis was plotted with respect to Fab concentration. The affinity of the IgA for thrombin was assessed using a FortéBio Octet Red instrument [8], with biotinylated S195A thrombin or biotinylated IgA attached to a streptavidin probe and IgA or human thrombin at various concentrations in the wells. On- and off-rate constants were determined by global fit of the on and off phases, and $K_d$ were either calculated from $k_{off}/k_{on}$ ($K_{d(ecl)}$) or by fitting the steady-state response vs. concentration ($K_d$).

Crystal structure of the IgA Fab in complex with thrombin

The IgA was digested with papain (Sigma) and the Fab fragment was isolated by anion exchange chromatography on Q-Sepharose (GE Healthcare). Excess Fab was incubated with D-Phe-Pro-Arg-chloromethylketone (PPACK)-inhibited human thrombin in 50 mM Tris, 100 mM NaCl, pH 7.4, for 3 h, and the complex was purified on a Superdex S75 (GE Healthcare) size-exclusion column. Fractions were analyzed by SDS-PAGE, pooled and concentrated to 10 mg mL$^{-1}$ in 20 mM Tris pH 7.4. Crystals were obtained from 100 mM sodium phosphate/citrate, pH 4.2, 40% PEG300, and yielded high-quality diffraction data to 1.9 Å resolution using the Diamond Light Source (Didcot, UK). Data were processed using Mosflm [9] and Scala [10], and the structure was solved by molecular replacement using the program Phaser [11] with 2HFF (chains A and B) [12] and 1PPB [13] as search models. The structure was refined using Refmac [14]. Data processing and refinement statistics are given in Table 1. Coordinates and structure factors are deposited in the Protein Databank under accession code 5E8E.

Results and discussion

Case report

A 54-year-old female presented with persistent headache 3–5 weeks after hitting her head on a door. She was not aware of having been unconscious. On examination she had a very mild left-sided weakness, and a CT head scan showed a right subdural hematoma 12 mm in depth. The collection was of high density with foci of reduced density, suggesting subacute onset, and was associated with a 15-mm midline shift. There was no underlying brain contusion or skull abnormality. She was taking glipizide and metformin for type II diabetes. There was no personal history of abnormal bleeding apart from secondary menorrhagia diagnosed 11 years previously. The menorrhagia was attributed to fibroids and a hysterectomy was performed without bleeding complication.

In vitro coagulation tests performed after diagnosis of the subdural hematoma were grossly abnormal. The prothrombin time (PT) and activated partial thromboplastin time (APTT) were prolonged at 40 s (normal < 14 s) and 240 s (normal < 34 s), respectively. The TT with bovine thrombin was 173 s (normal < 20 s) and was unclottable with human thrombin (normal < 20 s). Mixing patient plasma with normal plasma did not correct the prolongation of the PT, APTT or TT assays, with results of 34, 103 and > 60 s, respectively, indicating the presence of a thrombin inhibitor. Heparin contamination was excluded by addition of heparinase to patient plasma.

Further coagulation tests were performed. The antigenic fibrinogen level was 5.0 g L$^{-1}$, and normal fibrin clot formation was confirmed by a reptilase time. Fibrin degradation product D-dimer levels were measured on several occasions, and ranged from 6 to 33 ng mL$^{-1}$, compatible with intense anticoagulation. Thrombin generation in the patient plasma and platelet-rich plasma was measured by calibrated automated thrombography (CAT) [15] with 5 pm tissue factor and 4 μM phospholipid vesi- cles. The CAT using plasma showed an extended lag phase of 30 min (normal 1.7–4.9), in keeping with the

| Table 1 Data processing, refinement and model (5E8E) |
|-----------------------------------------------------|
| **Crystal** |
| Space group | P2$\_1212_1$ |
| Cell dimensions (Å) | $a = 66.77$, $b = 92.23$, $c = 147.49$ |
| Cell dimensions (°) | $\alpha$, $\beta$, $\gamma = 90$ |
| Solvent content (%) | 58.7 |
| Data processing statistics |
| Wavelength (Å) | 1.000 (Diamond, beamline 103) |
| Resolution (Å) | 73.74–1.90, 2.00–1.90 |
| Total reflections | 501 360 |
| Unique reflections | 72 417 |
| Multiplicity | 6.9 |
| Completeness (%) | 99.8 |
| $R_{ave}$ | 0.111 |
| $R_{free}$ | 0.046 |
| Model |
| Number of atoms modeled |
| Protein | 5754 |
| PPACK | 30 |
| Carbohydrate | 123 |
| Phosphate | 25 |
| Citrate | 13 |
| PEG | 21 |
| Sodium ions | 1 |
| Waters | 406 |
| Refinement statistics |
| $R_{factor}$ ($R_{free}$) | 64 730/3744, 4726/233 |
| $R_factor/R_free$ (%) | 20.11/24.41 |
| RMSD of bonds (Å)/angles (°) from ideality | 0.005/1.217 |

RMSD, root-mean-square deviation.
prolonged PT, however, the area under the curve (the endogenous thrombin potential) was normal at 1736 nm.min (normal 851–1759). In platelet-rich plasma the lag time was prolonged at 30 min (normal less than 8.1) and the area under the curve was increased at 2607 nm.min (normal 1317–2009). Platelet aggregation with ADP, collagen, adrenaline, arachidonic acid and ristocetin was normal. The patient’s serum IgA was elevated at 5.2 g L\(^{-1}\) with an IgA kappa monoclonal paraprotein band at 3 g L\(^{-1}\). There was no immune paresis. Urinary light chains were undetectable and serum β2 microglobulin was normal at 2 mg L\(^{-1}\). There was no lymphadenopathy or hepatosplenomegaly.

It was initially planned to surgically evacuate the subdural hematoma, however, upon learning the results of the clotting screen, surgery was postponed in favor of observation. Eight days later a repeat CT head scan showed resolving hematoma with only a 6-mm midline shift. The patient’s headache and mild weakness completely resolved and she was discharged 10 days after admission. No further bleeding occurred. A CT scan 4 years later was normal. The patient has been well and asymptomatic during the 7 years since presentation, and the abnormal clotting tests and the IgA paraprotein levels remain unchanged. Subsequent examination of the patient’s medical records revealed that 5 months before the current presentation she had undergone bilateral endoscopic knee surgery with lateral release of the lateral retinaculum of both patellae. Preoperative clotting tests were not performed because she had no history of bleeding. No abnormal bleeding was noted during or after surgery.

**Biochemical characterization of the IgA**

It was deduced from the initial thrombin generation study that the antibody did not block the active site of thrombin, because CAT utilizes a small fluorogenic substrate of thrombin. To confirm this, the effect of the IgA Fab fragment on cleavage of a small chromogenic substrate (S-2238) was determined. The rate of cleavage of S-2238 was not reduced, but actually enhanced by 30% (Fig. 2B), consistent with the binding of strong exosite I ligands. The IgA also did not substantially affect the rate of thrombin inhibition by antithrombin, in either the absence or presence of activating heparin. In contrast, the addition of purified IgA to pooled human plasma dose-dependently increased clotting time in a TT assay (Fig. 2C), and similarly increased the TT of purified human fibrinogen. This was judged to be due to binding of the antibody in or near the fibrinogen-recognition exosite (exosite I). The antibody also abrogated the effect of thrombomodulin on protein C activation, consistent with an exosite I interaction.

The affinity of the IgA for thrombin was determined using the ForteBio Octet Red instrument (similar to surface plasmon resonance [8]) with S195A human thrombin attached to the tip. A dissociation constant of 1.8 nm was derived from the effect of increasing IgA concentration on the response (Fig. 3A). Analysis of the binding curves for the on and off phases of the reaction (Fig. 3B) yielded a \(k_{on}\) of \(3.3 \times 10^5\) M\(^{-1}\) s\(^{-1}\) and \(k_{off}\) of \(3.7 \times 10^{-4}\) s\(^{-1}\) (\(K_{dcalc} = 1.1\) nm). Similar results were obtained with the antibody coupled to the probe (\(K_d = 2.1\) nm, \(k_{on} = 4.7 \times 10^5\) M\(^{-1}\) s\(^{-1}\), \(k_{off} = 1.9 \times 10^{-3}\) s\(^{-1}\), \(K_{dcalc} = 3.7\) nm). We conclude that the antibody binds to exosite I with a dissociation constant of about 2 nm. Binding of prothrombin to the IgA could not be detected by this method with the IgA on the tip, even when using prothrombin concentrations up to 0.5 μM. Pull-down assays were also unable to detect binding of prothrombin to the IgA using citrated plasma or a purified system. This is consistent with reports showing that exosite I is not functional in prothrombin [16].

**Crystal structure of thrombin in complex with the IgA Fab**

A high-resolution structure of the complex between PPACK-inhibited thrombin and the IgA Fab fragment was obtained from a single crystal (Table 1). We initially...
had no information regarding the sequence of the Fab and used the electron density as a guide to determining amino acid composition. However, full sequence assignment of the Fab required data from N-terminal sequencing of proteolytic fragments. The structure confirmed the exosite I specificity of the antibody (Fig. 4A), with the complementarity determining region 3 from the heavy chain (CDRH3) making the bulk of the contact. In total, 1092 Å² is buried at the interface (half from thrombin and half from the Fab), with 82% contributed by CDRH3. CDRH2 accounts for 14% of the contacts, and CDRH1 and the light chain make 1% and 3% of the contacts, respectively. The Fab interacts with typical exosite I residues [17], and effectively blocks exosite I from interacting with fibrinogen (Fig. 4B). This structure fully explains the effect of the patient’s IgA on the in vitro clotting tests.

**Follow-up**

The initial presentation of the patient with a subdural hematoma and extended clotting times suggested a severe acquired bleeding disorder. We investigated how the paraprotein inhibited coagulation in order to inform treatment if the patient suffered further bleeding. However, the patient made a rapid spontaneous recovery with resolution of the hematoma over a 10-day period, despite persistent gross prolongation of coagulation tests. Seven years later the patient remains well and has experienced no other bleeding event. It is therefore increasingly unlikely that the presenting hematoma was caused by the development of the anti-thrombin IgA paraprotein. Subdural hematoma is reasonably common, especially amongst people over the age of 50, and is usually the result of minor head trauma in patients with normal hemostasis [18–20]. In heavily anticoagulated patients, subdural bleeds do not resolve without reversal of anticoagulation. It is likely that had the patient not presented with a condition that mandated screening of her clotting parameters, this paraprotein would have gone undetected.

Because the paraprotein level has been constant since the initial presentation, it is likely that the IgA was present 5 months earlier when the patient underwent bilateral knee surgery involving dissection of the synovial and joint capsules without any bleeding complications. In addition, the patient recently sliced off the pulp of her left index finger with a mandolin grater, but bleeding stopped spontaneously and she did not require medical attention. It would thus appear that, notwithstanding the original presentation with a subdural hematoma, the presence of the potent anti-thrombin IgA at 3 g L⁻¹ in this patient’s blood does not cause her to bleed, and is therefore compatible with normal hemostasis. In addition, the markedly reduced levels of D-dimer indicate a state of anticoagulation, leading us to conclude that the antibody may have an anti-thrombotic effect.

**Conclusions**

The properties of the anti-thrombin IgA described here challenge the paradigm that antithrombotics necessarily increase the risk of bleeding. The relationship between efficacy and risk imposes a narrow therapeutic window and explains why anti-thrombotic therapy remains suboptimal. The patient appears to be heavily anticoagulated based on the grossly prolonged in vitro clotting times and persistently low D-dimer levels. However, for at least 7 years the patient has led a normal, healthy life in spite of this anticoagulant circulating in her blood at a concentration 10 000-times its $K_d$ for thrombin. We hypothesize therefore, that a therapeutic agent with the properties of this antibody might be an effective antithrombotic without the associated increase in risk of bleeding.

**Addendum**

T. P. Baglin identified the patient, directed the study and wrote the manuscript. J. Langdown conducted the in vitro clotting screens, purified the antibody, produced the Fab fragment, characterized the properties of the antibody, established initial crystallization trials and sequenced the

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Fab variable region. R. Frasson characterized the antibody and obtained the crystals used for determining the crystal structure of the thrombin-Fab complex. J. A. Huntington directed the experiments, solved and refined the crystal structure, analyzed the structure and wrote the manuscript.

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Disclosure of Conflict of Interests

T. P. Baglin and J. A. Huntington were shareholders in XO1 Ltd, a company established to develop a synthetic antibody similar to the IgA described here, acquired by Janssen Pharmaceuticals Inc. in March 2015. T. P. Baglin, J. Langdown and J. A. Huntington are authors of patent WO2013088164A1. J. Langdown and R. Frasson are beneficiaries of an arrangement between XO1 and Cambridge Enterprise.

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