CHARACTERIZATION OF AN ALPHA2-ANTIPLASMIN ANTIBODY

CHARACTERIZATION OF AN ALPHA2-ANTIPLASMIN ANTIBODY

By

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Abstract

Thrombotic disorders include myocardial infarction (MI), acute ischemic stroke (AIS) and venous thromboembolism (VTE), which encompasses pulmonary embolism (PE), and deep vein thrombosis (DVT). To prevent further complications or mortality in patients with MI and AIS, rapid restoration of blood flow is needed to minimize organ damage. Such treatment also is needed in patients with massive PE. Blood flow can be restored mechanically via percutaneous coronary intervention with stent implantation for MI and by thrombectomy in patients with AIS or PE. Alternatively, pharmacological reperfusion can be achieved by systemic administration of plasminogen activators (PAs). PAs convert plasminogen to the fibrinolytic enzyme, plasmin. Plasmin then degrades the clot into soluble fragments. Streptokinase (SK) and urokinase (UK) were the first therapeutic clot dissolving drugs but both lead to excessive bleeding complications because of non-specific effects. Current therapy focuses on clot specific agents such as recombinant tissue-PA (rt-PA) or tenecteplase (TNK), a rt-PA variant. However, there is a risk of intracranial bleeding in at least 1% of patients, which can be fatal or disabling. Thus, a need exists for new strategies to enable safer reperfusion that are not associated with potentially fatal side effects.

This study focuses on the therapeutic role of alpha2-antiplasmin (α2AP). α2AP is the primary inhibitor of plasmin. One approach to thrombolysis is to attenuate α2AP with an inhibitory antibody (A2AP IgG). Inhibition of α2AP would enable clot lysis with lower doses of PAs, thereby reducing the risk of bleeding and serving as a safer approach to thrombolytic therapy. We aimed to characterize A2AP IgG and evaluate its effect on fibrinolysis *in vitro* and *in vivo*.

A2AP IgG1 was selected and developed using phage display and an antibody gene library with human and rabbit α2AP as the antigen. Affinity maturation was performed and the Fc portion of the A2AP IgG1 was subsequently changed to the IgG4 isotype which yielded A2AP IgG4. A2AP IgG4 binds α2AP with 63-fold higher affinity than A2AP IgG1 as determined using surface plasmon resonance (SPR). SDS-PAGE and western blot analysis reveals that both antibodies bind to the plasmin-α2AP (PAP) complex, fibrinogen, and fragment X but not to α2AP; results confirmed by ELISA.

In functional studies, A2AP IgG1 significantly reduced plasmin inhibition by α2AP by 5.5-fold. Both A2AP IgG1 and A2AP IgG4 shortened tissue-PA (t-PA)-mediated clot lysis in a concentration dependent manner. A2AP IgG4 was 2.2-fold more potent than A2AP IgG1 in human plasma and 1.4-fold more potent in rabbit plasma. Compared with t-PA or TNK alone, addition of either antibody enhanced the lysis of preformed plasma clots. Combining A2AP IgG4 with 10% of the highest t-PA or TNK dose produced more clot lysis than the highest dose of t-PA or TNK alone.

In a rabbit jugular vein thrombosis model, A2AP IgG4 alone produced 20% lysis. When combined with a low dose of TNK, 40% clot lysis resulted, which was significantly greater than the 30% clot lysis observed with a higher dose of TNK. A2AP IgG4 alone or in combination with a lower dose of TNK did not cause significantly more bleeding than the higher dose of TNK alone and did not degrade circulating fibrinogen. Thus, we have shown that by inactivating α2AP, A2AP IgG attenuates α2AP activity, and accelerates clot lysis *in vitro* and *in vivo*. This demonstrates that antibody-mediated inhibition of α2AP, enhances thrombolysis and enables use of lower doses of PAs.

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List of Abbreviations

α2AP-	alpha2-antiplasmin
α2-MG-	alpha2-macroglobulin
αC-	C-terminus of fibrinogen Aα chain
AIS-	acute ischemic stroke
Ala-	alanine
AP-	activation peptide
APC-	activated protein C
Arg-	arginine
ASO-	antisense oligonucleotide
Asn-	asparagine
Asp-	aspartic acid
AT-	antithrombin
BSA-	bovine serum albumin
CD-	cluster of differentiation
DOACs-	direct oral anticoagulants
DVT-	deep vein thrombosis
EPCR-	endothelial protein C receptor
F-	clotting factor
FITC-	fluorescein isothiocyanate
Fp-	fibrinopeptides
Gln-	glutamine

Glu-	glutamic acid
GP-	glycoprotein
HRP-	horseradish peroxidase
HSA-	human serum albumin
IVC-	inferior vena cava
Leu-	leucine
Lys-	lysine
MAb-	monoclonal antibody
Met-	methionine
MI-	myocardial infarction
MPs-	microparticles
OPD-	o-phenylenediamine dihydrochloride
PAs-	plasminogen activators
PAI-	plasminogen activator inhibitor
PAP-	plasmin-α2AP
PE-	pulmonary embolism
PLs-	phospholipids
PPP-	platelet poor plasma
Pro-	proline
rt-PA-	recombinant tissue plasminogen activator
RU-	response units
sc-tPA-	single-chain tissue plasminogen activator

sc-uPA-	single-chain urokinase plasminogen activator
Ser-	serine
SK-	streptokinase
SP-	serine protease
TAFI-	thrombin activatable fibrinolysis inhibitor
tc-tPA-	two-chain tissue plasminogen activator
tc-uPA-	two chain urokinase plasminogen activator
TF-	tissue factor
Thr-	threonine
TM-	thrombomodulin
t-PA-	tissue-plasminogen activator
UK-	urokinase
u-PA	urokinase-plasminogen activator
TFPI-	tissue factor pathway inhibitor
TNK-	tenecteplase
Val-	valine
VTE	venous thromboembolism
vWF-	von Willebrand factor

Chapter 1: Introduction

1.1 Overview of hemostasis

Hemostasis refers to the stoppage of bleeding. It is essential to prevent blood loss from a vessel in the event of a breach to the vascular system while also maintaining the fluid state of blood within the body. Disruption of this delicate process may result in bleeding or thrombosis, with the latter manifesting as a local blockage due to a clot or an embolism if the clot breaks free and travels in the circulation to lodge in another vessel. Hemostasis can be divided into three stages, with the first and second heavily relying on platelet function (Boon, 1993).

Platelets coordinate the primary stage of hemostasis by forming the platelet plug that is generated after a breach to the vasculature. Normally, platelet activation is inhibited by vasodilators such as nitric oxide, cluster of differentiation (CD) 39 and prostacyclin. Platelets quickly adhere to exposed sub-endothelium and are able to form a platelet plug within minutes. Collagen, a protein present in the sub-endothelium is exposed to the blood following an injury and plays a role in platelet adherence (Fredenburgh and Weitz, 2018, Boon, 1993). Adherence of platelets also depends on plasma fibronectin (Wang *et al*., 2014) and von Willebrand factor (vWF), a plasma protein synthesized by megakaryocytes and endothelial cells. Platelets bind vWF through their glycoprotein (GP) Ibα and GPIIb/IIIa receptors and collagen through their GPVI receptors (Fredenburgh and Weitz, 2018). Once the platelets have bound to the endothelium, thromboxane A_2 is synthesized and ADP is released from storage granules which both are then able to bind different G-protein-coupled receptors on the surface of the platelets. Thromboxane A_2 binds to the thromboxane receptor and ADP binds to $P2Y_{12}$ and $P2Y_1$ receptors to further activate and recruit more platelets to the site of injury. Once activated, the thromboxane and ADP receptors on platelets cause an increase in calcium concentration within

the platelets, a cytoskeleton rearrangement and the exposure of anionic phospholipids to their surface (Fredenburgh and Weitz, 2018). These processes are necessary for the acceleration of the second stage of hemostasis, the clotting cascade, as activation and assembly of clotting factor complexes occurs on the surface of activated platelets (Boon, 1993). Localization of these coagulation factors to the site of injury is necessary to prevent dissemination of coagulation throughout the vascular system. The third stage of hemostasis consists of clot lysis, also referred to as fibrinolysis (Hoffman, 2003).

The cell-based model of coagulation (Figure 1) explains how coagulation depends on tissue factor (TF) bearing cells and platelets, all in three distinct steps: initiation, amplification and propagation (Smith, 2009). The initiation step takes place on the surface of TF-bearing cells such as monocytes, smooth muscle cells, or fibroblasts (Monroe and Hoffman, 2001, Hoffman, 2003, Ovanesov *et al.*, 2005). During initiation, TF combines with clotting factor (F) VIIa to form the FVIIa-TF complex which requires calcium ions to form the extrinsic tenase complex, which activates FX. TF can also bind FVII. The FVIIa-TF complex produces small amounts of FIXa and FXa which are able to produce small amounts of thrombin (Mann and Ziedins, 2004). During the amplification phase, FV is released from platelet alpha-granules onto the surface of platelets and is activated to FVa by thrombin. Thrombin also cleaves FVIII in the circulating vWF/FVIII complex to activate it to FVIIIa. In addition to activating FV and FVIII, thrombin also activates small amounts of FXI to FXIa (Hoffman, 2003, Monroe and Hoffman, 2001). During the propagation phase, FIXa binds its cofactor, FVIIIa on the surface of a negatively charged phospholipid such as phosphatidylserine and with calcium ions, form the intrinsic tenase complex. This complex is then able to activate FX to FXa. FXa can then bind to its cofactor, FVa to form the prothrombinase complex, which in the presence of calcium ions and platelet-derived

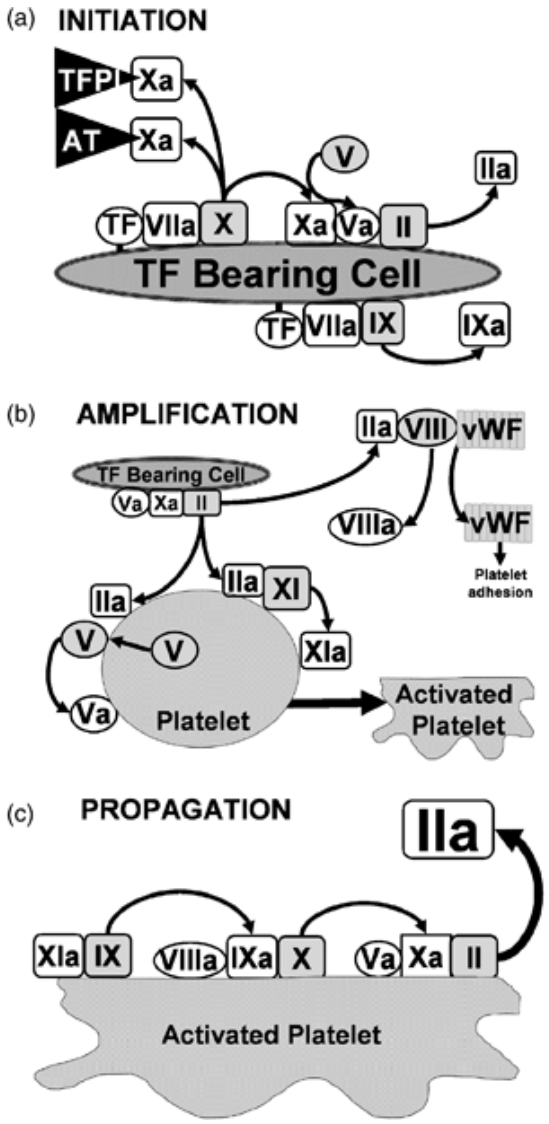


Figure 1: The cell-based model of coagulation.

The three phases of coagulation occur on three different types of surfaces. Initiation happens on the surface of a TF bearing cell such as the fibroblast. During this phase, small amounts of Factor IXa, Factor Xa, and thrombin are produced. Amplification occurs on the surface of the platelets and is marked by further thrombin production resulting in activation of the platelets. Finally, the propagation phase on the surface of activated platelets is marked by the formation of the intrinsic tenase and prothrombinase complexes which result in a burst of thrombin production (Smith, 2009).

phosphatidylserine converts prothrombin to thrombin. This results in a burst of thrombin production (Mann and Ziedins, 2004).

1.2 The coagulation cascade

The coagulation cascade (Figure 2) is an essential mechanism in place to prevent or limit blood loss from a vessel in the event of damage to the vasculature through the formation of a clot in the area of damage. Several coagulation proteins cleave and activate subsequent proteins in the cascade. The aim of blood coagulation is to form thrombin, which functions to cleave soluble fibrinogen to insoluble fibrin to form a clot (O'Donnell *et al*., 2019, Gailani and Renné, 2007, Monroe and Hoffman, 2001, Loof *et al*., 2014).

1.2.1 The extrinsic pathway of coagulation

The extrinsic pathway of coagulation was named as such because the initiator TF, is located outside of the vasculature. In the event of a breach to the vasculature as a result of a disruption to the endothelium, platelets are exposed to collagen that is present in the vessel wall and circulating vWF. Circulating FVII within the plasma is exposed to TF from extravascular cells (Monroe and Hoffman, 2001). The TF/FVIIa complex initiates the extrinsic pathway of coagulation. The TF/FVIIa complex with calcium activates FX to FXa and FIX to FIXa which furthers production of FXa (Boon, 1993, Crowther and Kelton, 2003). TF is expressed on cell membranes in the sub-endothelial layers of blood vessels. TF is expressed constitutively in a variety of tissues such as smooth muscle cells, pericytes, and adventitial fibroblasts. Under pathological conditions, TF can also be expressed by endothelial cells. TF's wide pattern of expression in various tissues of the body including the brain, epithelial cells of the lung, cardiomyocytes of the heart, and the placenta, signify its important role in hemostasis (Mackman *et al*., 2007, Witkowski *et al*., 2016). TF is an integral membrane protein and is therefore

localized to the membrane of the cell where it was synthesized. TF is not found in or expressed in circulating resting platelets (Monroe and Hoffman, 2001). TF has also been found in plasma, with the majority being present in the form of microparticles (MPs) which are small fragments released from activated vascular cells. MPs are elevated in a variety of diseases such as cardiovascular disease and cancer which suggests that TF-positive MPs play a role in thrombosis in individuals with these diseases. Various cells have been hypothesized to possess the ability to form TF-positive MPs, such as vascular smooth muscle cells and monocytes (Mackman *et al.*, 2007).

1.2.2 The intrinsic contact pathway of coagulation

The contact or intrinsic pathway of coagulation was named as such because all the coagulation factors necessary for this pathway are present within the blood. However, the names were given 50 years ago, so the context is different now (Boon, 1993). The pathway is initiated when FXII undergoes auto activation due to contact with a negatively charged surface such as collagen, nucleic acids, or polyphosphates (Gailani and Renné, 2007). FXIIa can then activate FXI to FXIa and prekallikrein to kallikrein. Kallikrein can then feedback positively to activate more FXII to FXIIa. All of these reactions require the presence of high molecular weight kininogen as a cofactor, with the exception of FXII autoactivation. FXIa activates FIX in the presence of ionized calcium. FIXa, its cofactor FVIIIa, ionized calcium, and platelet-derived phosphatidylserine form the intrinsic tenase complex and activate the substrate FX to FXa (Boon, 1993, Gailani and Renné, 2007). FXa, its cofactor FVa, ionized calcium, and phosphatidylserine form the prothrombinase complex, which can then activate prothrombin to thrombin to generate the fibrin clot. FVIIIa, FIXa, and FXIa are some of the clotting factors that

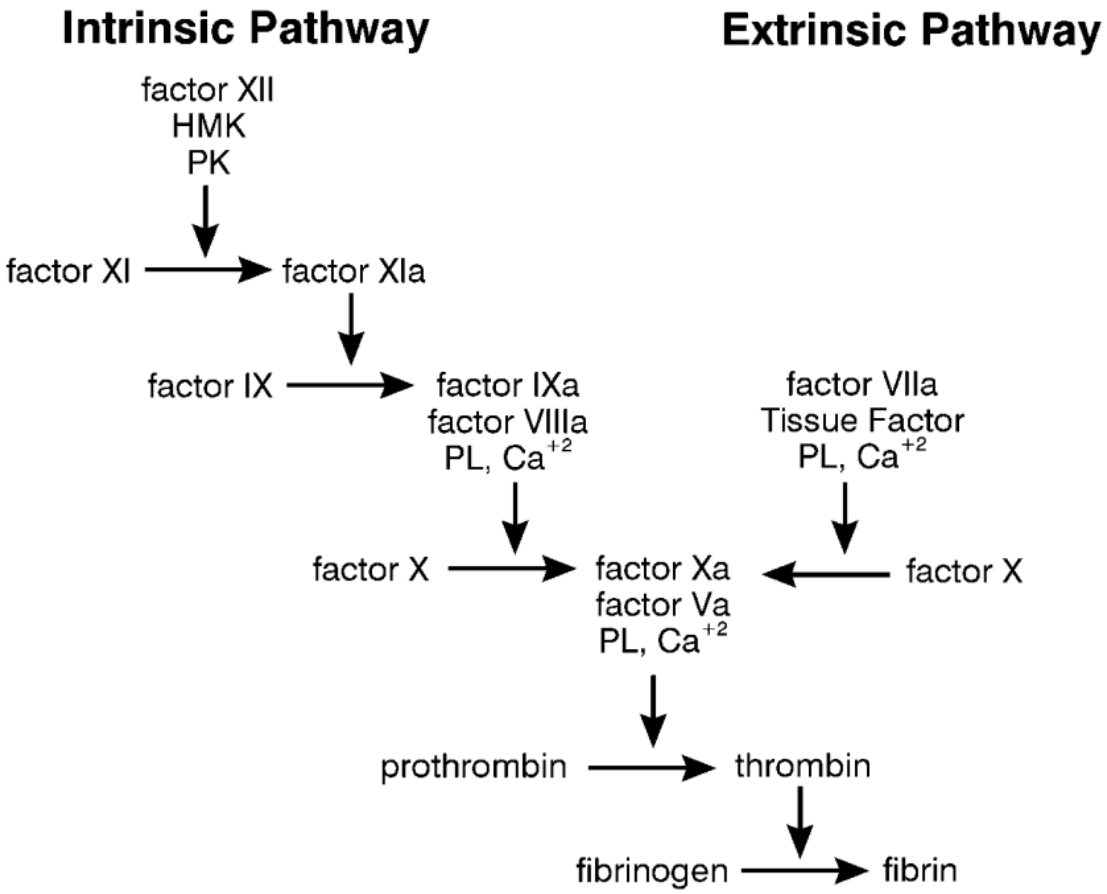


Figure 2: The coagulation cascade.

The intrinsic pathway is initiated when FXII is activated which results in sequential activation of other clotting factors. The extrinsic pathway is initiated upon damage to the vasculature where TF binds to FVIIa to activate downstream clotting factors. Both pathways converge at the production of FXa. In the presence of the cofactor FVa, calcium and phospholipids (PLs), thrombin is produced which is then able to convert fibrinogen into fibrin to form a clot (Monroe and Hoffman, 2001).

are important for sustained thrombin generation and dysregulation of these factors would contribute to thrombotic or bleeding disorders (Gailani and Renné, 2007).

1.2.3 The common pathway of coagulation

The common pathway is defined as where the intrinsic and extrinsic pathways of coagulation converge. The FXa generated from these pathways complexes with FVa and ionized calcium on the surface of activated platelets to form the prothrombinase complex (Boon, 1993). The substrate prothrombin is cleaved to thrombin which then converts soluble fibrinogen into insoluble fibrin. Thrombin also activates FXIII to FXIIIa, which is mainly responsible for cross-linking the generated fibrin clots and also cross-links α2AP to fibrin (Crowther and Kelton, 2003).

In animals in response to injury, the formation of fibrin initiates at the base of the thrombus (near TF site) and the fibrin strands develop upward and outward. At low concentrations of thrombin, thicker fibrin fibers form and at higher concentrations of thrombin, thinner fibrin fibers form. The thinner fibres are able to pack into a tighter network which renders them more resistant to clot lysis (Swieringa *et al*., 2018). The fibrin network formed with the help of thrombin is then able to stabilize the platelet thrombus. Also, an extensive network of fibrin is present on the platelets and has the ability to extend from the platelet and thrombus area to recruit and activate additional platelets (Swieringa *et al*., 2018).

Fibrin polymerization is initiated by thrombin cleavage of fibrinopeptides (Fp) A and FpB from the N-termini of the Aα and Bβ chains of fibrinogen which forms the fibrin monomer. FpA is cleaved more rapidly than FpB, but the rate of release of FpB increases as polymerization proceeds. FpB can be cleaved at a faster rate than FpA, but this is conformation dependent (Weisel and Litvinov, 2013). After, monomeric fibrin will self-assemble through knob-hole

interactions to form half-staggered fibrin oligomers. Next, these fibrin oligomers will aggregate laterally which is promoted by the C-terminal portion of fibrinogen's Aα chains (αC)-αC homophilic interactions within and between the fibrin oligomers. αC polymers are formed via cross-linking by FXIIIa. The elongation and thickening of fibrin fibers are accompanied by branching which is important to produce a 3-dimensional network. Also, calcium plays an essential role in fibrin polymerization as fibrinogen has many binding sites for calcium that are important for the stability and promotion of polymerization. In addition, cross-linking occurs between fibrinogen γ chains and can also occur between the α and γ chains. Cross-linking within and between fibrin oligomers makes polymerization irreversible and stabilizes fibrin polymers, rendering them resistant to clot lysis (Weisel and Litvinov, 2013).

1.2.4 Natural anticoagulants

Many physiological inhibitors exist to modulate the extent of coagulation in hemostasis and in the event of a breach to the vascular system, will prevent the propagation of a thrombus (Hoffman, 2003, Mann and Ziedins, 2004, Sagripanti and Carpi, 1998). These inhibitors include antithrombin (AT), heparin cofactor II, the protein C-protein S system, and tissue factor pathway inhibitor (TFPI). AT binds and neutralizes activated clotting factors including thrombin, FXa and the rest of the procoagulant serine proteases. These reactions are enhanced by medicinal heparin, a sulfated polysaccharide. Physiologically, heparin can be found in human mast cell granules. However, mast cell granule heparin is not the physiological counterpart of medicinal heparin. Instead, heparan sulfate, which is found on the surface of most eukaryotic cells, possesses antithrombotic activity (Weitz, 2003). Similar to AT, heparin cofactor II inhibits thrombin selectively through the formation of an equimolar complex and this reaction is enhanced by dermatan sulfate. Dermatan sulfate is produced mainly in the skin or blood vessels and is

considered a selective inhibitor of thrombin because the exclusive target of heparin cofactor II is thrombin (Sagripanti and Carpi, 1998, Liaw *et al*., 2001). The protein C-protein S system also contributes to attenuation of thrombin formation. Once thrombin has diffused away from the site of injury, it can bind to thrombomodulin (TM) on the endothelial surface. This thrombin/TM complex can activate endothelial protein C receptor (EPCR)-bound protein C to activated protein C (APC). APC with its cofactor protein S, can subsequently degrade FVa and FVIIIa after a stable clot has been formed (Mann and Ziedins, 2004, Sagripanti and Carpi, 1998).

TFPI possesses an amino terminal region followed by three tandem Kunitz-type inhibitory domains and a carboxy-terminal region (Broze, 1995). The presence of more than one Kunitz-type domain allows TFPI to inhibit both FXa as well as the TF/FVIIa complex. TFPI has also been shown to bind FVa (Ndonwi *et al*., 2012). TFPI has the ability to directly inhibit FXa by binding at or near the active site of the clotting factor. The second Kunitz domain is mainly responsible for FXa inhibition, although other parts of TFPI also play a role in FXa inhibition. TFPI binds FXa most efficiently when it is a part of the prothrombinase complex. The inhibition of the TF/FVIIa complex is FXa-dependent. A quaternary complex is formed between FXa, TFPI, and the TF/FVIIa complex where the first Kunitz domain binds FVIIa. TFPI could bind FXa first and then subsequently bind the TF/FVIIa complex or could bind the FXa-TF/FVIIa complex with the end goal of attenuating FX activation (Monroe and Hoffman, 2001, Sagripanti and Carpi, 1998, Broze, 1995). These four inhibitor systems function together to balance out procoagulant forces. In the event of a deficiency of any of these inhibitor systems, thrombosis may result.

1.3 Overview of Thrombosis

Thrombosis is a major cause of mortality and morbidity and is a result of the disruption of the hemostatic balance (Fredenburgh and Weitz, 2018). Arterial thrombosis usually develops upon atherosclerotic plaques as a result of some plaques possessing a thin fibrous cap and upon rupture of this cap, prothrombotic materials from the necrotic core are exposed which results in platelet aggregation and fibrin formation. Hyperlipidemia is a major driver of atherosclerosis (Previtali *et al*., 2011). A thrombus formed in arterial circulation may lead to myocardial infarction or ischemic stroke. Venous thrombosis develops as a result of genetic or acquired hypercoagulable state. Venous thrombosis is associated with age, obesity, cancer, immobility, and some surgeries. All risk factors contributing to venous thrombosis usually involve at least one component of Virchow's triad, which are broad categories thought to contribute to thrombosis. These categories include the hypercoagulability of blood, blood stasis, and endothelial dysfunction or damage (Mackman, *et al*., 2015). Venous thrombosis usually develops in valve cusps of deeper veins within the leg and has the potential to dislodge which could result in pulmonary embolism (PE) if the clot embolizes to the lungs (Rosendaal, 1999, Fredenburgh and Weitz, 2018). Uncontrolled thrombin generation as a result of a lack of the previously mentioned natural anticoagulants or a pathological amount of clotting factors may result in thrombosis (Crowther and Kelton, 2003). For example, thrombosis can develop as a result of even a mild deficiency of AT. Likewise, deficiencies of protein C and protein S, may also lead to thrombosis. Patients with elevated levels of clotting factors VIII, IX and XI are at increased risk for thrombosis compared to individuals without elevation. There is also evidence to suggest that a defective fibrinolytic system is a risk factor for thrombosis. (Crowther and Kelton, 2003, Faioni et al., 1998). Specifically, one study found that if the thrombin activatable fibrinolysis

inhibitor (TAFI) levels were above the 90th percentile, the individual had a two-fold risk increase for thrombosis compared with TAFI levels below the 90th percentile (Van Tilburg *et al.*, 2000).

1.4 Fibrinolysis

The main purpose of fibrinolysis (Figure 3) is to degrade fibrin clots and maintain the fluid state of blood within hemostasis. Thrombosis will result if there is deficient fibrinolysis and bleeding may result with excessive activation of fibrinolysis. Neutrophils release neutrophil elastase and cathepsin G which serve as profibrinolytic enzymes by directly digesting fibrin (Okafor and Gorog, 2015). Neutrophils can become trapped within blood clots and release these fibrinolytic enzymes through secretion or cell lysis. Once exposed to fibrin, these enzymes can facilitate fibrinolysis.

1.4.1 t-PA and u-PA

The vascular endothelium synthesizes and releases tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), both of which convert plasminogen to plasmin (Okafor and Gorog, 2015, Fredenburgh and Weitz, 2018). t-PA is synthesized and released constitutively and is primarily responsible for intravascular fibrin degradation. t-PA is a serine protease that has five distinct domains: a fibronectin like finger domain, an epidermal growth factor domain, two kringle domains, and a protease domain. t-PA is synthesized and released as a single chain polypeptide but is cleaved into a two-chain form by plasmin (Fredenburgh and Weitz, 2018). Both forms of t-PA have the ability to convert plasminogen into plasmin. t-PA is able to bind through its finger and second kringle domain to fibrin C-terminal lysine (Lys) residues on the surface of fibrin that are exposed in partially degraded fibrin, but can also bind intact fibrin and fibrinogen. u-PA is synthesized and released as a single chain

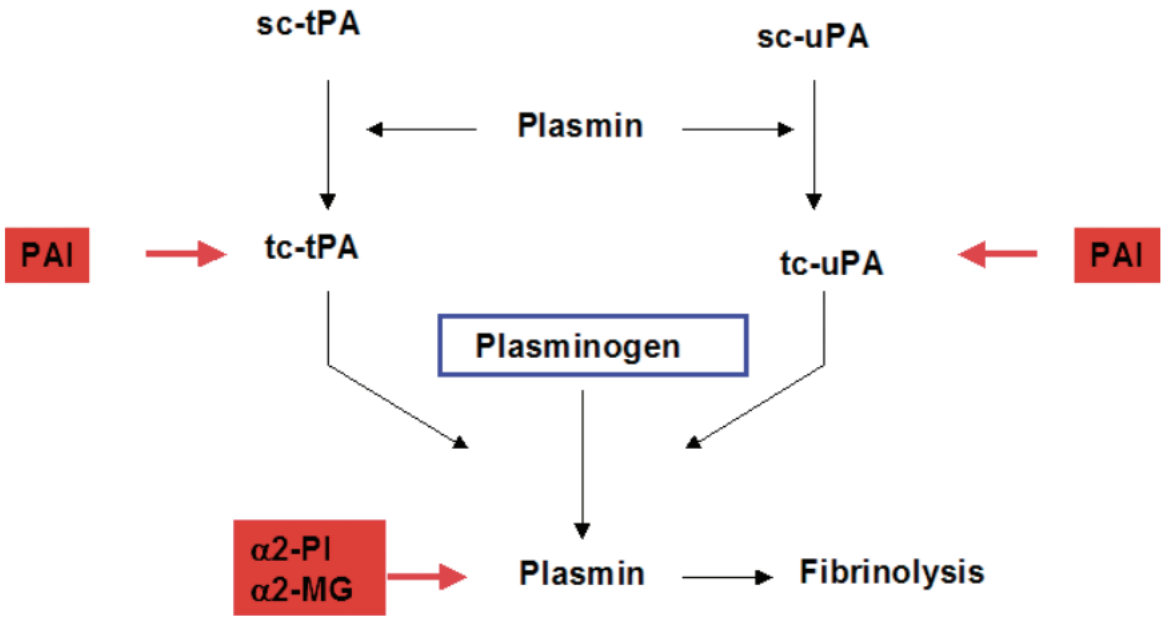


Figure 3: The fibrinolytic cascade.

Shown is the mechanism by which clots are dissolved to maintain hemostasis. Plasminogen is activated to plasmin primarily through the action of two-chain tissue plasminogen activator (tc-tPA), but also two-chain urokinase (tc-uPA). Both tc-tPA and tc-uPA are initially secreted in single chain format (sc-tPA and sc-uPA) from endothelial cells. Plasmin can then cleave single chain t-PA and u-PA to their two chain forms and also will bind to fibrin to facilitate fibrinolysis. Both plasminogen activators can be inhibited by plasminogen activator inhibitor (PAI)-1 and plasmin is inhibited by alpha2-antiplasmin (a2-PI), and to a lesser extent by alpha2-macroglobulin (a2-MG) (Cesarman-Maus and Hajjar, 2005).

polypeptide that possesses little enzymatic activity (Vassalli *et al*., 1991). Plasmin cleaves the inactive single chain u-PA into an active two-chain form that can bind u-PA receptors on the surface of cells. In a non-fibrin dependent manner, the two-chain form of u-PA converts plasminogen to plasmin. u-PA is released in the context of inflammation and is responsible for pericellular proteolysis and tissue remodeling and repair (Gonias and Hu, 2015, Fredenburgh and Weitz, 2018).

1.4.2 Plasmin(ogen)

Plasminogen is secreted primarily by the liver as an 810-amino acid polypeptide. The mature form of this enzyme consists of 791 amino acids. Plasminogen contains a heavy chain which consists of five kringle domains and a light chain which contains the active site. Plasminogen in its native form has a glutamic acid (Glu) at its amino terminus (Glu-plasminogen) and plasmin can cleave this form of plasminogen at Lys (position 77) to generate plasminogen with a Lys residue at its amino terminus (Lys-plasminogen) (Fredenburgh and Weitz, 2018, Gong *et al*., 2001). t-PA cleaves one peptide bond in either Glu-plasminogen or Lys-plasminogen into plasmin at the arginine (Arg) (position 561) and valine (Val) (position 562) bond. t-PA cleavage of Lys-plasminogen occurs more readily because the open conformation of this zymogen allows for exposure of the cleavage site. Glu-plasminogen possess a circular closed conformation and hence the bond to be cleaved is less accessible (Gong *et al*., 2001). The t-PA and plasminogen interaction is accelerated 3 orders of magnitude in the presence of fibrin as it provides a surface for the reaction to take place. Similar to t-PA, plasminogen is able to bind fibrin as a result of the Lys residues on fibrin and the corresponding Lys binding sites in the kringle domains of plasminogen. Localization of both enzymes on fibrin

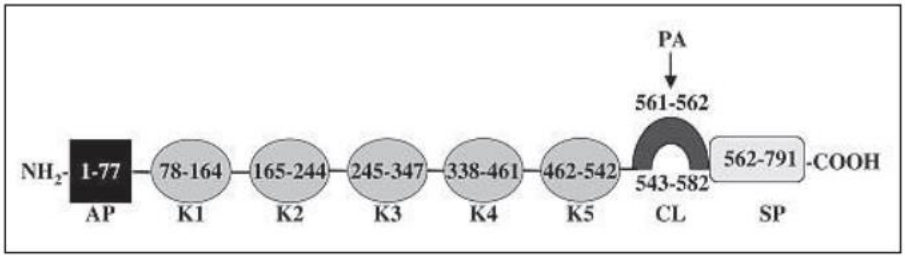


Figure 4: The structure of human plasminogen.

Shown are the structural and functional domains of plasminogen. The amino-terminus activation peptide (AP) is followed by five kringle domains and the plasminogen activator (PA) cleavage site at position 561-562 and a light chain with the catalytic serine protease (SP) domain (Ploplis and Castellino, 2005).

accelerates their interaction and the production of the fibrinolytic enzyme, plasmin (Fredenburgh and Weitz, 2018).

Plasmin is a serine protease and has broad specificity for several proteins including gelatin, casein, fibrinogen, and fibrin (Sherry *et al*., 1959, Ploplis and Castellino, 2005). Once plasminogen has been cleaved to plasmin, plasmin cleaves fibrin which exposes C-terminal Lys residues on the surface of fibrin. This allows for plasminogen and t-PA to continue to bind to fibrin, which enhances plasmin generation and thus fibrin removal (Cesarman-Maus and Hajjar, 2005). Plasmin(ogen) possesses 5 kringle domains (Figure 4) and a light chain with the catalytic domain (Ploplis and Castellino, 2005). Plasmin consists of two polypeptide chains linked by a disulfide bond. Plasmin also has the ability to degrade FV and FVIII which reduces thrombin generation downstream and eventual fibrin clot formation (Vassalli *et al*., 1991). Fibrinolysis also has an inherent self-regulating process.

1.5 Inhibitors of Fibrinolysis

1.5.1 PAI-1

Plasminogen activator inhibitor (PAI)-1 is a serine protease inhibitor and is considered to be the major inhibitor of t-PA in plasma. PAI-1 is secreted by endothelial cells in its active form but is quickly converted to its latent form (Vassalli *et al*., 1991). To be reactivated, it must be exposed to chaotropic agents or PL. PAI-1 binds both the single and two-chain polypeptide versions of t-PA and u-PA, thus inhibiting their activity. PAI-1 is stabilized through association with vitronectin from plasma and the ECM vitronectin. PAI-2 which is derived from monocytes, inhibits u-PA and t-PA, but not as efficiently which suggests that PAI-2 may play additional, yet unidentified roles with other enzymes (Vassalli *et al*., 1991).

1.5.2 TAFI

TAFI is a procarboxypeptidase synthesized in the liver and is activated by thrombin alone and when thrombin binds to thrombomodulin on endothelial cells. TAFI can also be activated by plasmin. Activated TAFI (TAFIa) cleaves the Lys residues located at the C-termini of fibrin which removes the binding site for plasminogen, plasmin and t-PA. Removing the binding site for these profibrinolytic proteins attenuates fibrinolysis. Typically, TAFIa has a short half-life and has no known inhibitors (Fredenburgh and Weitz, 2018, Urano *et al*., 2018).

1.6 Alpha2-Antiplasmin (α2AP)

α2AP is a single chain glycoprotein with a molecular mass of around 70,000 that circulates in human plasma at a concentration of around 70 μg/ml (Holmes *et al*., 1987, Houng *et al*., 2016). In the circulation, the half-life of α2AP is 2.6 days and it is primarily synthesized in the liver. However, low levels of α2AP transcripts have been found elsewhere such as the brain (Houng *et al*., 2016).

1.6.1 α2AP *protein structure*

Mature α2AP consists of 491 amino acids. α2AP (Figure 5) is initially secreted with a methionine (Met) amino acid at its N-terminus (Houng *et al*., 2016, Abdul *et al*., 2015). 70% of circulating α2AP is then cleaved at proline (Pro) residue 12 by α2AP cleaving enzyme, a soluble fibroblast activating protein, resulting in an asparagine (Asn) residue at the N-terminus. This form of α2AP has an N-terminal sequence of Asn-Gln-Glu-Glu-Val (Wiman and Collen, 1979). The reactive site of human α2AP is Ser-Arg-Met-Ser-Leu-Ser where the plasmin complex formation and attack happens on the Arg-Met (position 376 and 377) bond in the reactive center loop of α2AP (Abdul *et al*., 2015, Holmes *et al*., 1987).

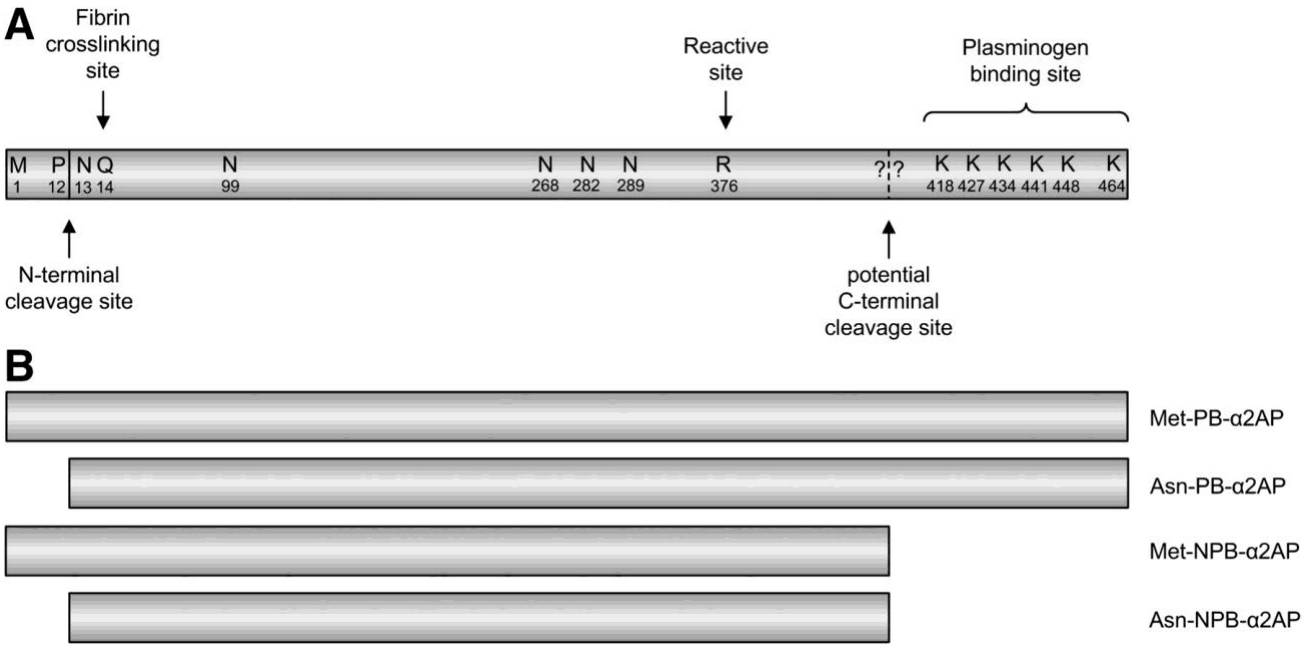


Figure 5: Variants of mature α2AP.

(A) α2AP prior to cleavage with the N-terminus Met residue still present. Shown is the N-terminus cleavage site for the α2AP cleaving enzyme which results in the production of the potent N-terminal Asn residue. After cleavage, this form of Asn-α2AP now has a glutamine (Gln) residue at position 2 which allows for faster cross-linking to fibrin compared with the Met-α2AP form due to less hindrance. Also shown is the C-terminus site responsible for binding to plasminogen with the currently unknown C-terminal cleavage site. (B) Shows various forms of α2AP including two forms that are able to bind plasminogen (PB) due to the presence of the C-terminus which contains Lys residues necessary to interact with the kringle domains of plasmin. Also shown are two forms of α2AP that are not able to bind plasminogen (NPB) due to the absence of the C-terminus, and thus the absence of necessary Lys residues (Abdul *et al.*, 2015).

1.6.2 Binding of α2AP

α2AP is a serine protease inhibitor (serpin) that rapidly inhibits plasmin by binding to its second kringle domain to form an enzymatically inactive and stable 1:1 complex in either the circulation or on the surface of fibrin. If binding to fibrin-bound plasmin, α2AP competes with fibrin (Wiman and Collen, 1979, Miles *et al*., 1982, Abdul *et al*., 2015). The reaction between α2AP and plasmin is one of the fastest protein interactions known to occur *in vivo*. The association rate constant for the reaction between α2AP and plasmin is $2x10^{7}$ M^{-1} s^{-1} (Hall *et al*., 1991). As with other serpins, this stable complex is unable to be dissociated by dodecyl sulfate which suggests a covalent bond is formed between plasmin and α2AP (Wiman and Collen, 1979). Initially, the C-terminal end of α2AP containing six Lys residues binds the Lys binding sites located at the kringle domains on plasmin (Figure 6). Kringle 2 on plasmin is mainly responsible for this interaction with the Lys residues, however kringle 1, 4, and 5 also play a role. Kringle 3 is the only non-Lys binding kringle on plasmin and is responsible for interacting with the sulfotyrosine (position 457) on the C-terminus of α2AP. The Arg residue (position 376) on α2AP forms a covalent bond with the serine (Ser) residue on plasmin (position 741) which results in formation of the plasmin-α2AP (PAP) complex and a subsequent loss of plasmin activity (Abdul et al., 2015). During formation of the PAP complex, a peptide of around 8,000 Da is cleaved as part of the catalytic reaction from the C-terminus of α2AP. This results in the production of a modified form of α2AP at 62,000 Da (Holmes *et al*., 1987). It was recently discovered that the inhibition of plasmin by α2AP causes the half-life of plasmin to be less than 100 milliseconds in vivo (Edy and Collen, 1977, Houng *et al*., 2016).

FXIIIa can cross link α2AP to fibrin. This is a result of a Gln residue at position 2 of the N-terminus of α2AP, which serves as the substrate. Cross linking of α2AP to fibrin protects it

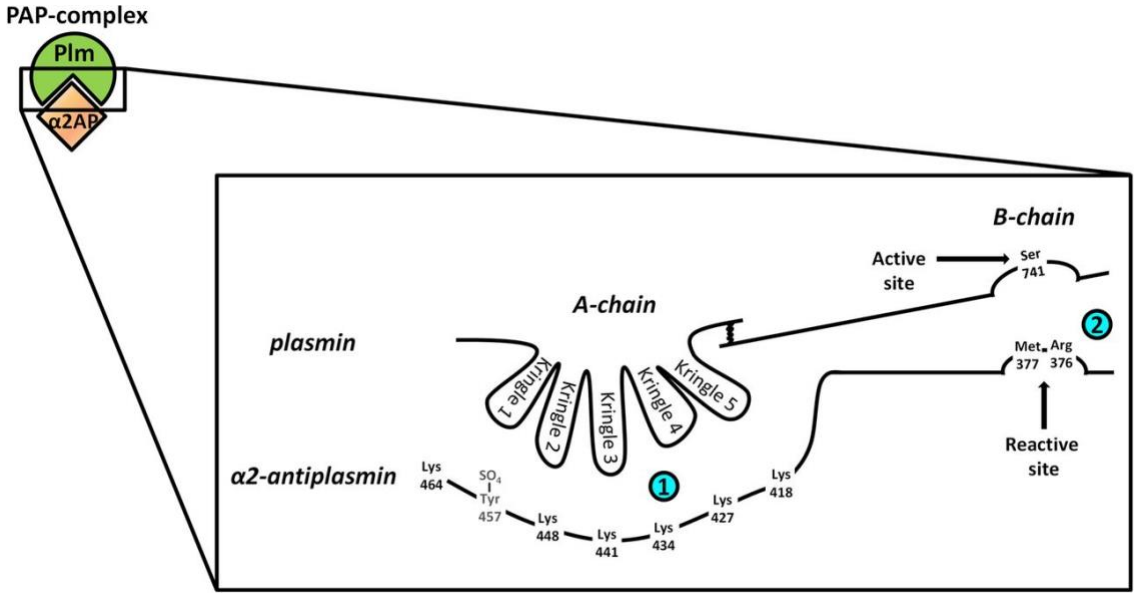


Figure 6: The interaction between plasmin and α2AP.

Shown are the 5 kringle domains of plasmin which interact with the C-terminal Lys residues in α2AP. Kringle 2 on plasmin is mainly responsible for this interaction, however kringle 1, 4, and 5 also play a role. The reactive site of α2AP consisting of Met-Arg residues at position 376, form a covalent bond with the active site Ser at position 741 of plasmin. This results in the formation of the PAP which results in a loss of function of plasmin (Abdul *et al.*, 2015).

from premature fibrinolysis by binding to and inactivating free and fibrin bound plasmin (Houng *et al*., 2016, Abdul *et al*., 2018). The importance of α2AP cross-linking to fibrin is seen in α2AP deficient patients who develop secondary hemorrhage after trauma or surgery (Aoki, 2004). The Asn-α2AP variant of α2AP is cross-linked to fibrin at a faster rate than Met-α2AP due to the Gln residue (position 14) on the Met-α2AP form no longer being hindered by the 12 additional amino acids in the Asn-α2AP form. This suggests that higher levels of Met-α2AP may allow for fibrinolysis due to Met-α2AP being cross linked to fibrin at a slower rate and thus inhibiting the antiplasmin-cleaving enzyme has been a target of potential therapeutics. The N-terminus of α2AP is primarily responsible for its incorporation into a fibrin clot whereas the C-terminus is what allows α2AP to initially interact with plasmin (Abdul *et al*., 2018, Uitte de Willige *et al*., 2018). Around 65% of circulating α2AP has its C-terminus intact and thus is able to bind plasmin, compared with the other 35% which is not able to bind plasmin due to an absent C-terminus. The mechanism by which the C-terminus is cleaved *in vivo* is not well understood. However, according to Abdul *et al*., 2020, cleavage of the C-terminus occurs preferentially at the Gln-aspartic acid (Asp) residue at positions 421 and 422. Cleavage can also occur after leucine (Leu), Glu, Gln or Asp residues at positions 417, 419, 420, or 422, respectively. Individuals with α2AP deficiencies typically suffer from mild or severe bleeding tendencies. Functional clot lysis assays performed using plasma from these patients demonstrates accelerated clot lysis compared with normal plasma. In these experiments, the rate of clot lysis was inversely proportional to the concentration of exogenous α2AP added back to the plasma (Abdul *et al*., 2015).

1.7 Causes and treatment of thrombosis

Thrombotic diseases can present in a variety of forms such as myocardial infarction (MI), pulmonary embolism (PE), deep vein thrombosis (DVT) and acute ischemic stroke (AIS). As

discussed previously, thrombotic diseases are a major cause of morbidity and mortality (Perler, 2005). Many therapies have been developed and subsequently improved to treat these disorders, but several issues still remain which justifies the need for new anticoagulant and thrombolytic therapies. In terms of thrombolytic therapy, streptokinase (SK) was the first drug to be approved by the FDA but was associated with an antigenic response. Urokinase (UK) is a thrombolytic drug that was used to treat PE. Both however, lead to hemorrhagic complications due to their non-fibrin specific effects which has limited therapeutic applications. The current treatment of MI, PE and AIS involves using fibrin specific agents such as altepase, a recombinant tissue plasminogen activator (rt-PA) (Perler, 2005). The goal of fibrinolytic therapy is to have rapid and successful restoration of blood flow to ischemic tissue. For example, the FDA approved standard of care for AIS is intravenous administration of rt-PA and/or thrombectomy. IV fibrinolytic therapy is recommended for patients presenting with acute stroke symptoms within the first 3 to 4.5 hours after onset of symptoms (Lansberg, 2009, Vivien, 2017, Nelson *et al*., 2019). However, in the context of ischemic stroke, only 5-10% of patients receive thrombolysis by rt-PA after weighing the benefits versus risks of treatment which includes considering the symptoms, defining the time of onset and the presence or absence of contraindications to thrombolysis. In addition, 6% of patients receiving rt-PA for ischemic stroke experience bleeding complications which can lead to complications such as intracerebral hemorrhage (ICH). Hemorrhagic transformation results due to the fibronectin like finger domain of rt-PA mediating interactions allowing it to cross the blood-brain barrier (Vivien, 2017, Nelson *et al*., 2019). Thus, the use of rt-PA by clinicians is debated even under ideal conditions (Dewar and Shamy, 2019). As a result, other fibrinolytic agents have been developed to improve the efficacy of fibrinolytic therapy while reducing negative complications. For example, tenecteplase (TNK) is a modified tissue

plasminogen activator with a more specific pharmacodynamic profile than t-PA. TNK contains three amino acid mutations that make it distinct from t-PA. First, a glycosylation site was inserted in kringle 1 through substitution of threonine (Thr) to Asn at position 103. In addition, the high mannose structure at position 117 was removed by substituting an Asn residue with a Gln residue through a deglycosylation mutation. Moving the glycosylation site from position 117 to 103 in kringle 1, increased the affinity for fibrin. Finally, a four-peptide sequence beginning with a Lys residue at position 296 was replaced with four alanine (Ala) residues which increased resistance to PAI-1 (Keyt *et al*., 1994). These changes also resulted in a longer half-life (24 minutes) compared with t-PA (5 minutes). TNK is typically given at lower doses and by bolus (0.1-0.4 mg/kg) instead of bolus (0.9 mg/kg) followed by an infusion as seen with rt-PA (Nelson *et al*., 2019, Thiebaut *et al*., 2018). TNK has demonstrated decreased rates of bleeding in the context of MI treatment and is approved by the FDA. Current American Heart Association guidelines are now recommending TNK as alternative to t-PA for AIS patients. However, further studies are required to evaluate the efficacy of TNK treatment in AIS and PE.

In terms of anticoagulant therapy for thrombotic disorders, heparin (accelerates AT inhibition of FXa, thrombin, and other serine proteases) and warfarin (vitamin K antagonist) are used but sometimes result in bleeding, ineffective thrombi dissolution and post-thrombotic syndrome which is associated with several long-term complications (Perler, 2005, Weitz and Chan, 2020). As a result, direct oral anticoagulants (DOACs) have been developed and are now approved for treatment of VTE. These include dabigatran, which inhibits thrombin, and rivaroxaban, apixaban and edoxaban, which inhibit FXa. Approval was based on phase 3 trials demonstrating that DOACs were as effective as vitamin K antagonists and associated with less bleeding (Van Es *et al*., 2014). Thus, DOACs are now recommended due to their similar

efficacy, less bleeding events, and greater convenience compared with vitamin K antagonists for patients with VTE (Weitz and Chan, 2020). However, in the context of a massive PE, immediate thrombus dissolution is necessary that cannot be provided by DOACs. Thus, there is a need for improved thrombolytic and anticoagulant agents that are not associated with potentially fatal side effects.

One approach to thrombolytic therapy has been to investigate the ability of an inhibitor of the fibrinolytic system, α2AP, to modulate fibrinolysis. Previous *in vitro* studies have demonstrated that a monoclonal antibody (MAb) against α2AP that was added to plasma clots, was able to bind to plasma α2AP and α2AP cross-linked to fibrin to facilitate clot lysis induced by the endogenous fibrinolytic system without exogenous plasminogen activator (Reed *et al.*, 1990). When the MAb was added in conjunction with exogenous plasminogen activator, there was a synergistic effect and clot lysis was accelerated by 20- to 80-fold. In a rabbit jugular vein model, delivery of the same MAb with and without simultaneous delivery of t-PA, enhanced thrombolysis. The combination of the MAb and t-PA did not increase the non-specific fibrinogen consumption, suggesting that it may not lead to bleeding complications seen with other thrombolytics (Reed *et al.*, 1990). Thus, this thesis project will seek to further understand the usefulness of inhibiting α2AP with the use of a MAb alone or in combination with plasminogen activators such as t-PA and TNK. Modulation of α2AP in animal experiments with a MAb will provide insight as to whether this approach may be used for thrombolytic therapy.

1.8 Overview of the thesis and rationale

1.8.1 Objectives

Several thrombolytic therapies to treat thrombosis currently exist but pose risks to patients receiving treatment, with hemorrhagic complications being the primary concern. Many

of these therapies function by delivering exogenous plasminogen activators to generate more of the fibrinolytic enzyme, plasmin. Modulation of α2AP levels to reduce the required amount of plasminogen activator necessary to dissolve thrombi, could reduce the frequency of hemorrhagic complications associated with thrombolytic therapy. We first aimed to evaluate the approach of modulating an inhibitor in the fibrinolytic cascade with the use of an antisense oligonucleotide (ASO) against human α2AP mRNA by observing the effect on fibrinolysis in plasma and purified systems. Next, we aimed to fully characterize a monoclonal IgG antibody against mature human α2AP. This included assessing binding capabilities to various proteins, the effect on plasmin inhibition by α2AP, the effect on fibrinolysis in purified and plasma systems, and finally the *in vivo* efficacy as a thrombolytic agent. Targeting an inhibitor in the fibrinolytic cascade and the main inhibitor of plasmin, α2AP, alone and in adjunct with current thrombolytic therapy and the effect this has on fibrinolysis will be explored. This approach could prove to be a safer and more effective alternative compared with current thrombolytic strategies.

1.8.2 Hypothesis

The proposed function of the A2AP IgG is to inhibit α2AP. As α2AP is the main inhibitor of plasmin, we hypothesize that knockdown of α2AP mRNA or inhibition of α2AP function will enhance fibrinolysis which will be assessed *in vitro* and *in vivo*.

Chapter 2: Materials and Methods

2.1 Materials

The chromogenic plasmin substrate (S-2251) was obtained from Molecular Innovations (Novi, Michigan, USA). Human serum albumin (HSA), purified polyclonal anti-goat IgG horseradish peroxidase (HRP) antibody, purified polyclonal anti-rabbit HRP antibody, and bovine serum albumin (BSA) were from Sigma-Aldrich Inc (St. Louis, Missouri, USA). Affinity purified goat anti-human a2-antiplasmin polyclonal antibody was obtained from Affinity Biologicals Inc (Ancaster, ON, Canada). Polyclonal rabbit anti-human fibrinogen antibody was obtained from Dako (Produktionsvej 42, Denmark). Human α-thrombin, plasminogen, and fibrinogen were obtained from Enzyme Research Laboratories (South Bend, Indiana, USA). Human plasmin, alpha2-antiplasmin, and FXIIIa were obtained from Haematologic Technologies Inc. (Essex Junction, Vermont, USA). Tissue-type plasminogen activator was obtained from Genentech (San Francisco, California). Blood was collected with consent from 20-30 healthy donors and platelet poor plasma (PPP) was removed and stored at -80$_{o}$C. A P4 non-immune IgG was isolated from PPP using an affinity column as follows. Protein A from bacteria was attached to the sepharose column purchased from Bio-Rad (Hercules, California) to isolate the P4 non-immune IgG. Fragment X was prepared by plasmin mediated digest of fibrinogen. Lightning-Link HRP labeling kit was obtained from Expedeon (San Diego, California). TNK-tPA (tenecteplase) was obtained from Roche (Basel, Switzerland). 10X lysing buffer was obtained from BD Scientific (Mississauga, Ontario). Fluorescein (FITC), o-phenylenediamine dihydrochloride (OPD), and gentle elution buffer was obtained from Thermofisher scientific (Mississauga, Ontario). α2AP (SRMSLSS) and fibrinogen (SWNSGSS) peptides were obtained from Biomatik (Cambridge, Ontario).

Male New Zealand white rabbits were obtained from Charles River (Senneville, Quebec). The three ASOs (Table 1) against human α2AP were provided by Ionis (Carlsbad, California, USA). In the ASO and A2AP IgG studies, rabbit plasma was prepared by collecting 1.8 mL of blood and adding it to a 3 mL syringe prefilled with 200 μL of 3.8% trisodium citrate. Next, sedimentation of the red cells by centrifugation was performed at 5,000 g for 10 minutes. The plasma was removed and centrifugated again at 5,000 g for 10 minutes. The plasma was again removed and stored at -80$_{o}$C.

The A2AP IgG1 against human α2AP was developed by Bayer AG (Cologne, Germany) and provided as part of research collaboration. It was selected using a phage display and a synthetic human antibody gene library (BioInvent n-CoDeR) with human and rabbit α2AP as the antigen (Parmely and Smith, 1988). Binding to rabbit and human α2AP was assessed by Bayer AG using surface plasmon resonance (SPR) and enzyme linked immunosorbent assays (ELISA). For rabbit α2AP, the Kd determined was 21 nM and 6 nM in SPR and ELISA, respectively. For human α2AP, the Kd determined was 100 nM and 120 nM in SPR and ELISA, respectively. The IC50 values in functional assays assessing plasmin activity were determined by plotting the curve of best fit and were 15 nM for rabbit α2AP and 170 nM for human α2AP. Affinity maturation was performed by Bayer AG by altering amino acid residues in the complement-determining region of the Fab portion of the A2AP IgG1. The Fc portion of the A2AP IgG1 was subsequently changed to an IgG4 isotype to reduce the immunogenicity of the antibody which yielded A2AP IgG4. The IC50 values in functional assays reading out plasmin activity were 20 nM for rabbit α2AP and 0.5 nM for human α2AP for the A2AP IgG4. Bayer AG also provided human and rabbit alpha2-antiplasmin.

ASO ID	Length (nucleotides)	ASO Sequence	Position in α2AP mRNA	Species of rabbit
ASO2	20	CGGCATACAAATCACTACAA	2136 to 2117	Oryctolagus cuniculus
ASO3	20	CCGGCGGCATACAAATCACT	2140 to 2121	Oryctolagus cuniculus
ASO4	20	TTCATTCTTTATTCCGGCGG	2153 to 2134	Oryctolagus cuniculus

Table 1: Antisense Oligonucleotides (ASO) against mature human α2AP mRNA.

Shown are the ID, length, sequence, position, and species of three different ASOs against mature human α2AP mRNA used in animal experiments to observe the effect of reducing α2AP protein on fibrinolysis. ASOs were provided by Ionis. (Carlsbad, California, USA).

2.2 Methods (Effect of the α2AP ASO)

2.2.1 SDS-PAGE and western blot analysis of plasma from ASO treated rabbits

Western blotting was performed to determine the levels of remaining α2AP in ASO-treated rabbits. Three different ASO's were administered to rabbits. Each ASO group contained six rabbits that were then divided into two groups of three and received either a low or high dosage, 40 mg/kg or 80 mg/kg, respectively. Rabbits received respective doses via injections subcutaneously twice weekly in the intrascapular area for four weeks. Also, two rabbits received a low dose of saline and another two rabbits received a high dose of saline. Blood was collected from the marginal ear artery from all rabbits prior (wk0), three weeks (wk3), and four weeks (wk4) after initiation of ASO administration. Plasma was prepared by collecting 1.8 mL of blood and adding it to a 3 mL syringe prefilled with 200 μL of 3.8% trisodium citrate. Next, sedimentation of the red cells by centrifugation was performed at 5,000 g for 10 minutes. The plasma was removed and centrifugated again at 5,000 g for 10 minutes. The plasma was again removed and stored at -80$_{o}$C. Human plasma and plasma from rabbits that were treated with each ASO and at each time point was quantified using a spectrophotometer at 280 nm and diluted with 20 mM Hepes and 150 mM NaCl, pH 7.4 buffer (HBS) to achieve an A280=1. All samples were then diluted 1:1 (10 uL each) with non-reducing sample buffer containing 0.004% bromophenol blue, 4% SDS, and 20% glycerol. Samples were boiled for five minutes and loaded onto a 4-10% polyacrylamide gradient Mini-PROTEAN TGX pre-cast gel (BIO-RAD Laboratories, Inc.). Gel electrophoresis was then performed in the presence of 0.1% SDS at 70 volts for 10 minutes followed by 185 volts for 40 minutes. The gel and a PVDF membrane were then both equilibrated with 1X transfer buffer solution containing 10% of a 248 mM Tris, 1.92 M glycine solution, 20% methanol, and deionized distilled water. The gel and membrane were then

assembled in the apparatus and placed in 4°C 1X transfer buffer. The transfer was performed over one hour at 100 volts in a cold room. Five percent milk was then used to block proteins on the membrane for one hour on a shaker at room temperature. Next, 0.5 μg of polyclonal affinity purified goat anti-human alpha2-antiplasmin antibody was added to five percent milk and placed on the shaker with the membrane for one hour. After this, 0.5 μg of polyclonal anti-goat IgG horse radish peroxidase (HRP) antibody was added in five percent milk and placed on the shaker with the membrane for 3 hours. Five washes were then performed with 20 mm Tris-HCl and 150 mM NaCl, pH 7.4 (TBS) containing 0.005% Tween 20 for five minutes each to eliminate non-specific binding of proteins. To visualize proteins, Clarity western peroxide reagent and luminol/enhancer reagent (BIO-RAD Laboratories, Inc.) were added at a 1:1 ratio (2.5 mL of each) and the membrane was immediately imaged using Image Lab Software (version 5.2.1.) on the Gel/ChemiDoc MP Imager System (BIO-RAD Laboratories, Inc.). Densitometry was performed using the same software.

2.2.2 Comparing the influence of t-PA versus ASO in rabbit plasma clot lysis times

First, a t-PA titration (0-10 nM) was performed using plasma from rabbits that were treated with ASO2 and ASO4, both at wk4 time point to evaluate the optimal concentration of t-PA to use in the rabbit plasma clot lysis assays. Absorbance of all rabbit plasma samples was determined using a spectrophotometer at 280 nm. Plasma samples were diluted with HBS to A280=1 to normalize protein concentrations. Next, 20 nM of thrombin was used to initiate clotting and 0-10 nM of t-PA was used to initiate clot lysis. Thrombin and t-PA were diluted with HBS and added separately to opposite sides of wells of a multi-well plate. Before dispensing into wells with a multi-channel pipette, rabbit plasma samples were supplemented with 30 mM $CaCl_2$. Absorbance was read at 405 nm every two seconds for 2 hours at 37°C on a

kinetic plate reader (SpectraMax 340PC384 Microplate Reader, Molecular Devices). Clot lysis time was defined as the time to reach half of the maximal decrease in absorbance after clotting was complete. Clotting was determined to occur when the maximal absorbance was reached. The clot lysis times were plotted against the concentration of t-PA and were shown as a dose response.

2.2.3 Effect of reconstituting human α2AP *back to rabbit plasma depleted of* α2AP *by ASO*

Human α2AP was added back to rabbit plasma depleted of α2AP by ASO2 from the wk4 time-point, at increasing concentrations to determine if clot lysis times could be restored. Human α2AP was added to individual wells to achieve final concentrations of 0-1 μM. Clotting and lysis were initiated with 20 nM thrombin and 5 nM t-PA, respectively, and monitored and evaluated as described in 2.2.2. The clot lysis times were plotted against the concentration of human α2AP.

2.2.4 Effect of three different ASO's against α2AP *in rabbit plasma clot lysis assays*

Plasma clot lysis assays were used to evaluate the effect of reduced α2AP levels in ASO-treated rabbits. Plasma clot lysis times were measured using plasma from rabbits that received only the high dosage at each time point for each ASO in addition to the saline treated rabbits. Clotting and lysis were initiated with 20 nM thrombin and 5 nM t-PA, respectively, and monitored and evaluated as described in 2.2.2. The three ASO treatments were individually grouped, where each group consisted of all three time points and was plotted against the clot lysis time. A plot was also made that compared the data from each of the time points and was also plotted against the clot lysis time data.

2.3 Characterization of the A2AP IgG

2.3.1 SDS-PAGE and antibody comparison in western blot detection of proteins of interest

To begin to characterize the binding of the A2AP IgG, western blotting was performed. Human plasma, rabbit plasma, and ASO-treated rabbit plasma were measured using a spectrophotometer at 280 nm and diluted with HBS to achieve an A280 of 1. 5 μg of human fibrinogen, fragment X, human α2AP, the PAP complex, and human and rabbit α2AP provided by Bayer were used to investigate the binding of the A2AP IgG1 and IgG4 to various proteins. Fibrinogen was used because α2AP is cross-linked to the αC-domain of fibrinogen and fragment X was used because the structure of fragment X is the same as fibrinogen except the αC domain is omitted. Bayer reagents were used as controls and to ensure any binding or lack thereof was not due to a difference in source of α2AP. All samples were diluted 1:1 (10 μL each) with reducing sample buffer containing a 0.004% bromophenol blue, 4% SDS, and 20% glycerol. Samples were boiled for five minutes and loaded onto a 4-10% polyacrylamide gradient Mini-PROTEAN TGX pre-cast gel (BIO-RAD Laboratories, Inc.). Gel electrophoresis was then performed in the presence of 0.1% SDS at 50 volts for 10 minutes followed by 175 volts for 40 minutes. The gel and a membrane were then both equilibrated prior to the western blot with 1X transfer buffer containing 10% of 248 mM Tris, 1.92 M glycine, 20% methanol, and deionized distilled water. The western blot was then assembled, placed in refrigerated 1X transfer buffer, and subsequently placed in a 4°C room. The transfer was performed over one hour at 100 volts. Five percent milk was then used to block proteins on the membrane for one hour on a shaker. Next, 1 μg of the A2AP IgG1 (HRP conjugated), A2AP IgG4 (HRP conjugated), or the affinity purified goat anti-human α2AP polyclonal antibody was added to five percent milk and placed on the shaker with the membrane overnight at 4°C. To the membrane that received the goat anti-

human α2AP polyclonal antibody, 0.5 μg of polyclonal anti-goat IgG HRP antibody was added in five percent milk and placed on the shaker with the membrane for 3 hours. Five washes were then performed with TBS containing 0.005% Tween 20 for five minutes each to eliminate non-specific binding of proteins. Visualization of proteins was performed as in 2.2.1. In parallel, gels with the same samples were stained with Coomassie staining solution (BIO-RAD Laboratories, Inc.) for one hour and de-stained with deionized distilled water for three hours. Protein bands were visualized using Image Lab Software (version 5.2.1.) on the Gel/ChemiDoc MP Imager System.

2.3.2 ELISA detection of human α2AP and the PAP complex

To determine if the A2AP IgG1 and IgG4 could bind and their affinities for α2AP, ELISA was performed. The affinity purified goat anti-human alpha2-antiplasmin polyclonal antibody was diluted in 50 mM carbonate buffer to a final concentration of 1 μg/mL and coated in the wells of a 96 well high binding plate (Thermofisher scientific) overnight at 4°C. Next, 100 μL of 1% BSA in 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (PBS) was incubated in each well for two hours at room temperature. After three washes with PBS containing 0.05% Tween 20, human α2AP was titrated from 0 to 1000 ng/mL. In separate experiments, equimolar amounts of human α2AP and plasmin to generate the PAP complex were titrated up to 20 nM. In both titrations, the reagents were diluted in 1% BSA in PBS and left to sit at room temperature for two hours. After three washes with PBS containing 0.05% Tween 20, A2AP IgG1 and IgG4 (both HRP conjugated as directed by Innova Biosciences) were added to a final concentration of 2 μg/mL in 1% BSA in PBS for two hours. After three washes with PBS containing 0.05% Tween 20, 100 uL of 2.3 mM OPD containing 0.03% H_2O_2 were added to 12 mL of PBS after which 100 μL was added to each well. After the plate sat in the dark for 30

minutes, 50 μL of 2.5 M H_2SO_4 was added to stop the reaction. The absorbance was then read using a plate reader at 490 nm. The OD values were plotted against the concentration of human α2AP and the PAP complex and IC50 values were determined where applicable by determining a curve of best fit.

2.3.3 ELISA detection of human fibrinogen

To further characterize the binding of the A2AP IgG to fibrinogen, ELISA was performed. the A2AP IgG The polyclonal rabbit anti-human fibrinogen antibody was diluted in 50 mM carbonate buffer pH 9.6 to a final concentration of 1 μg/mL and coated in the wells of a 96 well high binding plate overnight at 4°C. Blocking and washing were performed as in 2.3.2. Human fibrinogen diluted in 1% BSA in PBS was then added to the wells at concentrations of 0 to 1000 ng/mL. After washing as in 2.3.2, HRP-conjugated A2AP IgG1 at a concentration of 2 μg/mL was prepared in 1% BSA in PBS was added and incubated for two hours. Next, washing and the detection was performed as in 2.3.2. The OD values were plotted against the concentration of fibrinogen and IC50 values were determined where applicable.

2.3.4 Detection of the fibrinogen-α2AP complex in western blotting

To determine whether the A2AP IgG could detect fibrinogen bound α2AP, human fibrinogen-α2AP complexes were made. This was performed by incubating 1 μM of human fibrinogen with 1.5 μM of human α2AP in the presence of 200 nM FXIIIa and 2.5 mM $CaCl_2$ for 30 minutes at 37°C. The complexes were prepared for analysis by gel electrophoresis and western blotting. Controls were performed where each reagent was omitted. Samples were diluted 1:1 (10 μL each) with reducing sample buffer as in 2.3.1. Samples were then loaded onto a 4-10% polyacrylamide gradient Mini-PROTEAN TGX pre-cast gel and electrophoresis was then performed under the same conditions as in 2.3.1. This gel was stained with Coomassie

staining solution and the bands were visualized as in 2.3.1. A parallel experiment was performed, and the gel was prepared for western blotting as in 2.3.1. Next, 1 μg of the A2AP IgG1 (HRP conjugated), A2AP IgG4 (HRP conjugated), affinity purified goat anti-human α2AP polyclonal antibody, or polyclonal rabbit anti-human fibrinogen was added to five percent milk and placed individually on the shaker with four identical membranes overnight at 4°C. To the membrane that received the goat anti-human α2AP polyclonal antibody, 0.5 μg of polyclonal anti-goat IgG HRP antibody was added in five percent milk. To the membrane that received the rabbit anti-human fibrinogen polyclonal antibody, 0.5 μg polyclonal anti-rabbit HRP antibody was added in five percent milk. Both were placed on the shaker with the membrane for 3 hours. Washes and visualization of protein bands were performed as in 2.3.1.

2.3.5 A2AP IgG detection of human and rabbit fibrinogen degradation products

To identify the A2AP IgG binding site on fibrinogen, human and rabbit fibrinogen plasmin-mediated digests were performed. First, human or rabbit fibrinogen at 11.8 μM was incubated with 200 nM or 150 nM of plasmin, respectively. This was performed in the presence of 2 mM $CaCl_2$. Next 10 μL aliquots were removed from the reaction at different time points (prior to addition of plasmin, 1, 5, 10, 20, 30, and 60 minutes after addition of plasmin). Each aliquot was placed in reducing sample buffer as in 2.3.1 to obtain final concentrations of 5.9 μM of fibrinogen. The degradation products were analyzed by gel electrophoresis and we examined A2AP IgG binding to fibrinogen degradation products in western blotting. Samples were loaded onto a 4-10% polyacrylamide gradient Mini-PROTEAN TGX pre-cast gel and electrophoresis was then performed under the same conditions as in 2.3.1. This gel was stained with Coomassie staining solution and the bands were visualized as in 2.3.1. A parallel experiment was performed, and the gel was prepared for western blotting as in 2.3.1. 1 μg of the A2AP IgG1 (HRP

conjugated) was used to probe the membrane. Washes and visualization of protein bands was performed as in 2.3.1.

2.3.6 Characterization of A2AP IgG4 binding to plasma proteins by affinity chromatography

Affinity chromatography was performed to determine whether the A2AP IgG4 binds α2AP and fibrinogen in PPP. First, Sepharose beads were swollen and washed with 1 mM HCl for 20 minutes in a sintered glass filter using a vacuum pump. Next, the beads were washed with deionized distilled water after which they were equilibrated with 0.1 M $NaHCO_3$ pH 8.3 (coupling buffer). Next, the A2AP IgG4 was dialyzed into TBS pH 7.4. After, 25 mg of the A2AP IgG4 was incubated with the beads overnight at 4°C. The beads with the A2AP IgG4 immobilized were then incubated for 2 hours at 4°C in 0.1 M Tri-HCl pH 8.0 to block unbound sites. 30 mL of PPP was passed over the 10 mL microscale column twice at a flow rate of 1 mL/min. Next, elution was performed using gentle elution buffer (Thermofisher scientific) and samples were collected in 2 mL fractions. Protein containing fractions were identified by monitoring absorbance at 280 nm. Next, in addition to 5 μg of both human α2AP and fibrinogen, the peak protein containing eluted samples (10 μL) were loaded onto a 4-10% polyacrylamide gradient Mini-PROTEAN TGX pre-cast gel and electrophoresis was then performed under the same conditions as in 2.3.1. This gel was stained with Coomassie staining solution and the bands were visualized as in 2.3.1. A parallel experiment was performed, and the gel was prepared for western blotting as in 2.3.1. Next, 1 μg of the affinity purified goat anti-human α2AP polyclonal antibody or the polyclonal rabbit anti-human fibrinogen was added to five percent milk and placed on the shaker with the membrane overnight at 4°C. To the membrane that received the goat anti-human α2AP polyclonal antibody, 0.5 μg of polyclonal anti-goat IgG HRP antibody was added in five percent milk. To the membrane that received the rabbit anti-human fibrinogen

polyclonal antibody, 0.5 μg polyclonal anti-rabbit HRP antibody was added in five percent milk. Both were placed on the shaker with the membrane for 3 hours. Washes and visualization of protein bands was performed as in 2.3.1.

2.3.7 Determination of binding affinities of A2AP IgG1 and IgG4 for human α2AP, fibrinogen and fragment X using surface plasmon resonance (SPR)

SPR experiments were performed on a BIAcore T200 (GE Health Sciences). A2AP IgG1 and IgG4 were immobilized onto separate flow cells on a CM5 chip to response units (RU) values of 2198 and 2172, respectively using an amine coupling kit (GE Healthcare). A blank flow cell was used as a control. Human α2AP was titrated from 0 to 250 nM, and both fibrinogen and fragment X were titrated from 0 to 10 μM. All proteins were diluted in HBS containing 0.01% Tween 20. The contact time was 180 s, the dissociation time was 1800 s, and the flow rate was 60 uL/min. The regeneration buffer was glycine pH 2.5. The sensograms were analyzed using BIAcore T200 evaluation software. Affinity was measured by determining the equilibrium dissociation constant (KD) through both steady state affinity and kinetic (1:1) binding analysis. The RU values were plotted against the concentration of human α2AP, fibrinogen and fragment X used in the reactions for both the A2AP IgG1 and IgG4.

2.3.8 Effect of A2AP IgG1 on α2AP inhibition of plasmin

The effect of A2AP IgG1 on plasmin inhibition by α2AP was determined by monitoring hydrolysis of a plasmin substrate. First, 1 μM of the A2AP IgG1 or 1 μM of control IgG were mixed with human α2AP (0-100 nM) for 15 minutes at room temperature. Next, 25 nM of plasmin was incubated with these reactions at room temperature for 15 minutes. After, S-2251 was then added to a final concentration of 400 μM. Also, to determine if the A2AP IgG1 could dose-dependently inhibit the α2AP mediated neutralizing effect on plasmin activity, the A2AP

IgG1 or the P4 non-immune IgG were titrated from 0-1 μM and incubated with 30 nM of human α2AP for 15 minutes. After which, 25 nM of plasmin was added was incubated for 15 minutes, followed by the addition of 400 μM of S-2251. This experiment was performed in the absence or presence of fibrinogen. All enzymes and substrates were diluted with HBS containing 0.005% Tween 20. Upon the addition of S-2251, absorbance was immediately read at 405 nm at 2 second intervals for one hour on a kinetic plate reader at room temperature. Linear portions of the absorbance-time plots were analyzed by determining the slopes to obtain initial rates of pNA formation and were plotted against the concentration of α2AP or antibody, respectively.

2.3.9 Effect of the A2AP IgG1 on plasmin- or t-PA-mediated clot lysis

2.3.9.1 Clot lysis mediated by plasmin

A2AP IgG1 was titrated in a purified system to confirm its specificity. A master mix was added to each well to achieve final concentrations of 60 nM human α2AP, 3 μM fibrinogen, and 2 mM $CaCl_2$. Clotting was initiated by 20 nM thrombin and clot lysis was mediated by 50 nM plasmin. The A2AP IgG1 was titrated from 0-5 μM in the reaction wells. The reaction was performed in a 96-well plate at 37°C and all reagents were in HBS-Tw buffer. Thrombin, plasmin, and A2AP IgG1 were dispensed individually in wells. As a control, α2AP was omitted in one sample. Absorbance was read and clot lysis times were defined as in 2.2.2. The clot lysis times were plotted against the concentration of A2AP IgG antibody.

2.3.9.2 Clot lysis mediated by t-PA and plasminogen

Additional experiments were performed where conditions were as above except that lysis was mediated by t-PA and plasminogen. 5 nM t-PA replaced plasmin in the wells. The master mix had 1 μM human α2AP due to the larger amount of plasmin that would be generated and contained 0.5 μM plasminogen. Here, two additional sets of experiments were performed as

negative controls. The first was using a non-immune IgG P4 antibody in place of A2AP IgG1. The second negative control was the omission of α2AP in one sample. Absorbance was read, and clot lysis times were measured using the same conditions as in 2.2.2. The clot lysis times were plotted against the concentration of A2AP IgG1 antibody.

2.3.10 Effect of the A2AP IgG1 and IgG4 on t-PA-mediated clot lysis in human or rabbit plasma systems

The A2AP IgG1 and IgG4 antibodies were examined for their effect on clot lysis in human and rabbit plasma. Thrombin and t-PA were dispensed into wells as in 2.2.2. The A2AP IgG1 and IgG4 antibodies from 0-5 μM were aliquoted in the wells. Human pooled plasma recalcified with 30 mM $CaCl_2$ was to initiate the reaction. Absorbance was read, and clot lysis times were measured using the same conditions as in 2.2.2 at 37°C. The clot lysis times were plotted against the concentration of A2AP IgG1 and IgG4.

2.3.11 The effect of α2AP and fibrinogen peptides on A2AP IgG1-, IgG4-, and t-PA-mediated clot lysis in human plasma

Peptide analogs of the α2AP reactive center loop (SRMSLSS) and fibrinogen α2AP-like (SWNSGSS) peptides were incubated (0-20 μM) with 2 μM of the A2AP IgG1 or A2AP IgG4 for 15 minutes and subsequently loaded into wells of a 96-well plate. Thrombin at 20 nM and t-PA at 5 nM were also loaded in the wells in separate aliquots. Human pooled plasma recalcified with 30 mM $CaCl_2$ was added to initiate the reaction. Absorbance was read, and clot lysis times were measured using the same conditions as in 2.2.2. The clot lysis times were plotted against the concentration of the α2AP and fibrinogen peptides present in the reaction.

2.3.12 Effect of the A2AP IgG1 and IgG4 on t-PA- and TNK-mediated lysis of preformed human plasma clots

To make preformed clots, 400 μL of human pooled plasma containing 100 nM of in house FITC-fibrinogen and 30 mM $CaCl_2$ was clotted in a 5-mL tube with 100 nM of thrombin and incubated for 30 minutes at room temperature. A bacterial inoculation loop was inserted immediately after addition of plasma (Chan *et al*., 2012). The clot was removed using the loop and then washed in a tube containing 5 mL TBS-0.01% Tween 20 for one minute and then immediately placed in a 1 mL bathing solution of human plasma containing 1 or 10 nM of t-PA or TNK and 1 μM of the A2AP IgG1 or IgG4 alone, or a combination of the A2AP IgG1 or IgG4 with 1 nM TNK or t-PA. At intervals up to 3 h, a 250 μL aliquot was taken from the bathing plasma and read in a fluorescent plate reader (SpectraMax M3 Microplate Reader, Molecular Devices) by taking an endpoint read. After the read, the aliquots were then placed back into the bathing solution of plasma. The excitation and emission wavelengths were 485 and 538 nm, respectively. The change in fluorescence overtime which indicates FITC-fibrin degradation products being released from the plasma clot was plotted against the time. For multiple comparisons, analysis of variance (ANOVA) Holm-Sidak method was used.

2.3.13 Efficacy of A2AP IgG4 in a rabbit thrombosis model

To assess the *in vivo* thrombolytic potential of A2AP IgG4, its effect on the lysis of preformed jugular vein clots was assessed in rabbits (Weitz *et al*., 1993).

2.3.13.1 Jugular vein thrombosis model

All animal utilization protocols were approved by the Animal Research Ethics Board at McMaster University, and all studies were performed in accordance with the Canadian Council on Animal Care guidelines. Male New Zealand white rabbits weighing between 2-4 kg were

acclimatized for a minimum of 5 days prior to the procedure. Anesthesia was induced by the intramuscular injection (GEN 578) of ketamine (50 mg/kg) and xylazine (2 mg/kg) (GEN 581) and was maintained throughout the duration of the procedure with a mixture of oxygen (1 litre/min) and 2.5% isoflurane delivered by face mask. Tracheostomy was performed for intubation. The left femoral artery and vein were surgically isolated and then cannulated for blood sampling and drug delivery, respectively. After exposing the right jugular vein through a ventral skin incision, a 2 cm venous segment was identified. Using a 4-Fr Fogarty catheter introduced through the facial vein, the isolated segment of the jugular vein was denuded of endothelium by 15 passages of the inflated balloon. Blood flow through the venous segment was then occluded by tourniquets placed proximally, distally, and around the side branches. The Fogarty catheter was withdrawn and replaced with a cannula through which the venous segment was injected with a mixture containing 8 nM human α-thrombin, 10 μM of FITC-fibrinogen, and autologous rabbit plasma that was prepared ex vivo in a 1 mL syringe. The total volume injected was 100 μL/kg. These maneuvers resulted in rapid clot formation within the isolated vein segment. After allowing the thrombus to mature for 30 min, blood flow through the jugular vein was restored for 10 minutes by removing the tourniquets, and the rabbits were given 15 mg/kg of A2AP IgG4, 0.5 mg/kg (therapeutic dose) of TNK, 0.1 mg/kg TNK or 2 mL of saline IV. Combinations of 15 mg/kg A2AP IgG4 with 0.1 mg/kg TNK were also given. At the end of the experiment the rabbits were anticoagulated with unfractionated heparin 1000 U/rabbit IV and then euthanized using sodium pentobarbital (240 μg/mL) also administered intravenously.

2.3.13.2 Extent of thrombolysis

The extent of thrombolysis was determined by measuring plasma fluorescence over time. At each time point, a 2 mL blood sample was taken, subjected to centrifugation at 5,000 g, after

which the plasma was collected. After a second centrifugation step, 250 μL aliquots were read in a fluorescent plate reader by taking an endpoint read. The excitation and emission wavelengths were 485 and 538 nm, respectively. The change in fluorescence overtime which indicates FITC-fibrin degradation products (FDP) being released from the plasma clot inserted into the jugular vein will be plotted against the time after deliver of the treatment. For multiple comparisons, analysis of variance (ANOVA) Holm-Sidak method was used.

2.3.13.3 Extent of bleeding

Bleeding was assessed by ear incision. At 60 minutes, following blood sampling, three 9-mm full-thickness incisions were made in the left ear using a no. 11 Bard-Parker scalpel blade. The pierced ear was then immediately immersed in a beaker containing 1 L of sterile saline which was maintained at 37°C and constantly stirred using a heated stir plate and a magnetic stir bar. One hour after immersing the ear, three 10 mL aliquots of saline were removed, and the red cells will be sedimented by centrifugation at 2,500 g for 7 min at 23°C. After aspirating the supernatant, the red cell pellet was resuspended in 2 mL of 1X lysis buffer. After 10 minutes, the samples were then spun again for 20 minutes. The absorbance of the samples was then measured at 550 nm to quantify the hemoglobin content, and by comparing these values with those of known quantities of rabbit blood treated in the same fashion, the volume of blood lost at each time point was estimated. For multiple comparisons, analysis of variance (ANOVA) Holm-Sidak method was used.

2.3.13.4 Extent of fibrinogen degradation

To determine the integrity of systemic fibrinogen and assess whether degradation occurred after delivery of various treatments, SDS-PAGE and western blots were performed on plasma samples taken at different time points after administration of various treatments from

rabbits as done in 2.2.1 with the exception of the primary antibody being a polyclonal rabbit anti-human fibrinogen antibody and the secondary antibody being a purified polyclonal anti-rabbit HRP antibody.

2.3.14 Statistical analysis

Data are presented as mean ± standard error. The significance of differences in the means was determined using t tests. For paired comparisons, the Mann-Whitney rank sum test was used. For multiple comparisons, ANOVA (Holm-Sidak) method was used. For all analyses, p-values < 0.05 were considered statistically significant. Graphs and Statistical analysis were completed using SigmaPlot (v.11, San Jose, CA).

Chapter 3: ASO Study Results

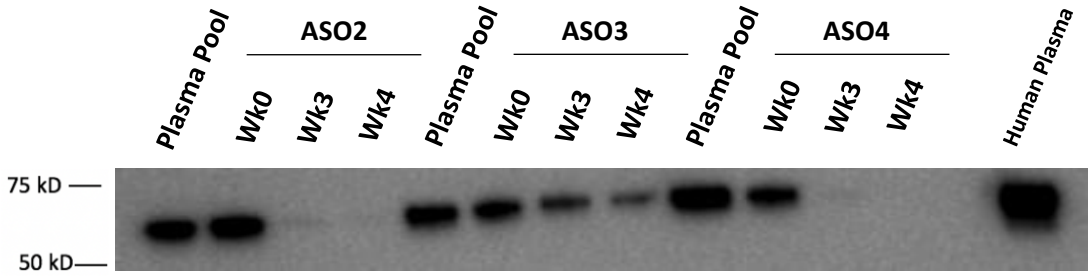
3.1 SDS-PAGE and western blot analysis of plasma from ASO treated rabbits

Initially, three ASOs were examined for the purpose of selecting one for further study. To evaluate the efficacy of the ASOs and to quantify the amount of α2AP present in the plasma from rabbits treated with 80 mg/kg of each ASO, samples were subjected to SDS-PAGE and western blot analysis. Samples from weeks 1 and 2 were not analyzed because it takes 3-4 weeks for ASO to achieve full effect. Samples from different ASOs and time-points were run and the bands for α2AP stain less intensely towards the wk4 time-point (Figure 7A). Densitometry (Figure 7B) was performed on the bands from the western blot corresponding to the wk4 time-point for each ASO. It was determined that the percentage of α2AP remaining relative to saline treated plasma controls were 1.3%, 20.83%, and 0.37% in the bands for ASO2-wk4, ASO3-wk4, and ASO4-wk4, respectively. In conclusion, administration of ASO2 and ASO4 resulted in the greatest reduction in plasma α2AP. From here it was determined to use the wk4 samples for remaining experiments.

3.2 The influence of t-PA versus lack of α2AP on clot lysis in rabbit plasma

To determine the optimal concentration of t-PA to use to initiate clot lysis, a dose-response of t-PA was performed using normal rabbit plasma and rabbit plasma treated with ASO2 and ASO4, both at the wk4 time-point. This would allow us to determine optimal t-PA concentration that is influenced by α2AP. t-PA was titrated from 0-10 nM in each type of plasma and clot lysis times were compared with the rabbit plasma pool. A dose-dependent shortening of lysis time was observed with increasing concentrations of t-PA (Figure 8). At 2 nM t-PA, there appears to be differences in lysis time between the control samples and plasma samples from ASO treated rabbits. The lysis times converge at t-PA concentrations above 4 nM for control and

A


Plasma Pool
ASO2
Wk0
Wk3
Wk4
Plasma Pool
ASO3
Wk0
Wk3
Wk4
Plasma Pool
ASO4
Wk0
Wk3
Wk4
Human Plasma
75 kD
50 kD

B

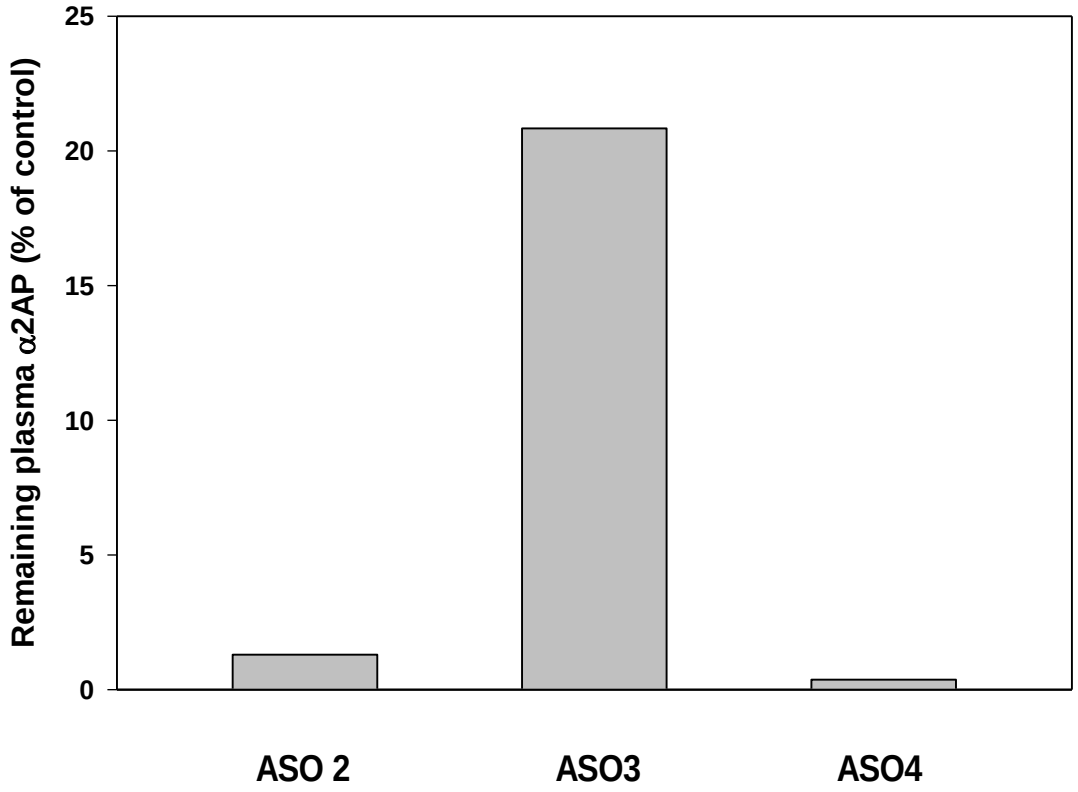

Remaining plasma α2AP (% of control)
25
20
15
10
5
0
ASO 2
ASO3
ASO4

Figure 7: Western blot analysis to detect rabbit α2AP in plasma from rabbits treated with three different ASOs.

A) Plasma samples from each time-point and each ASO were diluted 1:1 (10 μL each) with non-reducing sample buffer and electrophoresed onto a 4-10% polyacrylamide gradient gel in the presence of SDS. Samples were then transferred to membrane and probed with affinity purified goat anti-human a2-antiplasmin polyclonal antibody as the primary antibody and purified polyclonal anti-goat IgG horseradish peroxidase antibody as the detecting antibody. The membrane was imaged using Image Lab Software. (B) Densitometry was performed on the bands from the wk4 time-point in each condition to detect the amount of α2AP remaining in the bands relative to the amount of α2AP in the plasma pool. Representative data shown. n=2.

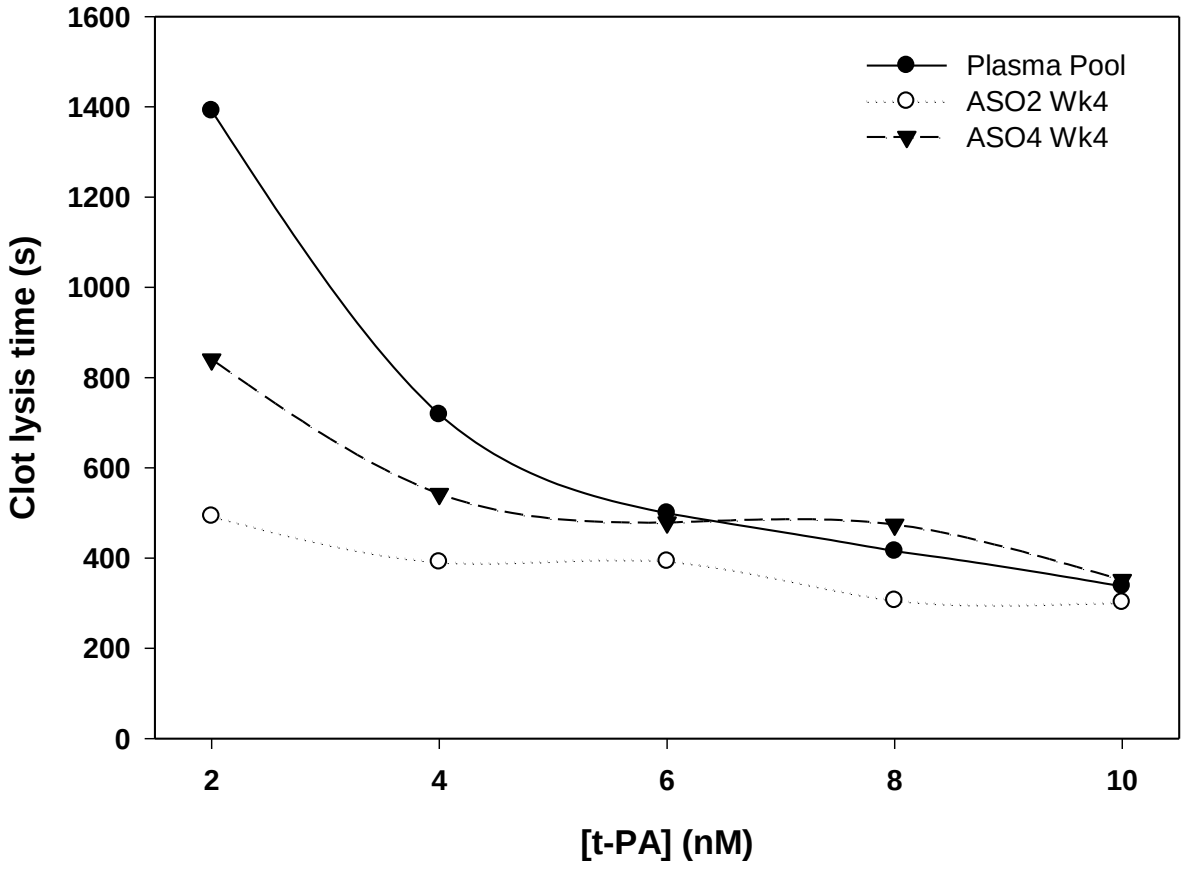

Plasma Pool
ASO2 Wk4
ASO4 Wk4
Clot lysis time (s)
1600
1400
1200
1000
800
600
400
200
0
2
4
6
8
10
[t-PA] (nM)

Figure 8: Effect of t-PA concentration on clot lysis of normal and α2AP deficient plasma. Recalcified plasma from rabbits treated with saline, ASO2, or ASO4 at the wk4 time-point were clotted with 20 nM of thrombin in the presence of 0-10 nM of t-PA. The plate was read at 405 nm at 2 second intervals for two hours at 37°C. Clot lysis time was defined as the time to reach half of the maximal decrease in absorbance after clotting was complete. n=1.

treated rabbit plasmas. This indicates that t-PA at 6 nM can overcome the inhibitory effect of α2AP and thus we demonstrate that a t-PA concentration of 4 nM or less is most suitable for evaluating the function of α2AP.

3.3 Effect of three different ASOs against α2AP in rabbit plasma clot lysis assays

To determine the effectiveness of the ASOs, plasma clot lysis times from rabbits that received ASO2, ASO3, and ASO4 were compared. The rabbits used here received the high dosage (80 mg/kg). Blood was collected from the rabbits at three time-points (wk0, wk3, wk4) and the plasma was used from each ASO and time point in clot lysis assays. 20 nM thrombin was used to initiate clotting and 5 nM t-PA was used to initiate clot lysis. Figure 9 depicts the clot lysis profile in plasma from a rabbit given ASO2. Comparisons were made across all three time-points (wk0, wk3, wk4) for each ASO. For each ASO, clot lysis times were shorter as the wk4 time-point was approached compared with saline treated rabbits suggesting there is a progressive acceleration in clot lysis with increasing time of exposure to the ASO. More importantly, a progressive decrease in α2AP levels correlates with shorter clot lysis times. Lysis times in the saline treated rabbits were around 700-800 s. Data for all rabbits are shown in Figure 10. All wk0 plasma clot lysis times were about 700 s and for each ASO there was a dose-dependent shortening. In plasma from rabbits treated with ASO3, wk0, wk3, and wk4 clot lysis times were around 700 s, 500 s and 450 s, respectively. The clot lysis times with plasma from rabbits treated with ASO2 and ASO4 were faster at every time point compared with plasma from rabbits treated with ASO3. However, in the plasma from rabbits treated with ASO2 and ASO4, there was a slight difference in clot lysis times at wk0 (prior to rabbit ASO treatment) compared with the plasma from saline treated rabbits. These numbers should have ideally been the same, but their difference could be due to a number of factors such as inter-assay variation, pipetting error or

between rabbit differences in α2AP levels. Due to wk4 having the shortest lysis times for each ASO, it appears as though the longer the rabbit α2AP mRNA is exposed to ASO, the greater effect the ASO has. This is due to progressively reduced α2AP levels. In addition, Figure 9B demonstrates that the clot lysis times are 300-500 s at the wk4 time-point. This suggests that the ASOs have achieved the greatest effect relative to the other time-points as once a2AP levels are reduced below 5% of normal, it is unlikely that clot lysis times will get shorter. The results in Figure 7 demonstrate that the ASOs are reducing α2AP levels and thus allowing plasmin to facilitate quicker clot lysis. Thus, the plasma from rabbits that received 80 mg/kg of ASO2 was chosen for future experiments.

3.4 Effect of adding human α2AP back to rabbit plasma depleted of α2AP by ASO

To confirm that a decrease in α2AP in rabbit plasma due to treatment with the ASOs resulted in reduced clot lysis times, human α2AP was added back to plasma from a rabbit that received ASO2. As shown in Figure 11, with 5 nM t-PA, the lysis time of control plasma was around 4500 s and in plasma from an ASO2-treated rabbit was 900 s. With increasing concentrations of α2AP, clot lysis times increased in a dose-dependent manner and were restored to times similar to the plasma from rabbits treated with saline at an α2AP concentration of 500 nM. This profile may not be accurate as 650 nM α2AP resulted in a quicker lysis time. Beyond 500 nM, the clot lysis times continued to increase and at a concentration of 1 μM of α2AP, no lysis occurred within the two-hour assay (not shown). The lack of α2AP resulted in accelerated clot lysis times and confirms the effect of reduced α2AP on clot lysis.

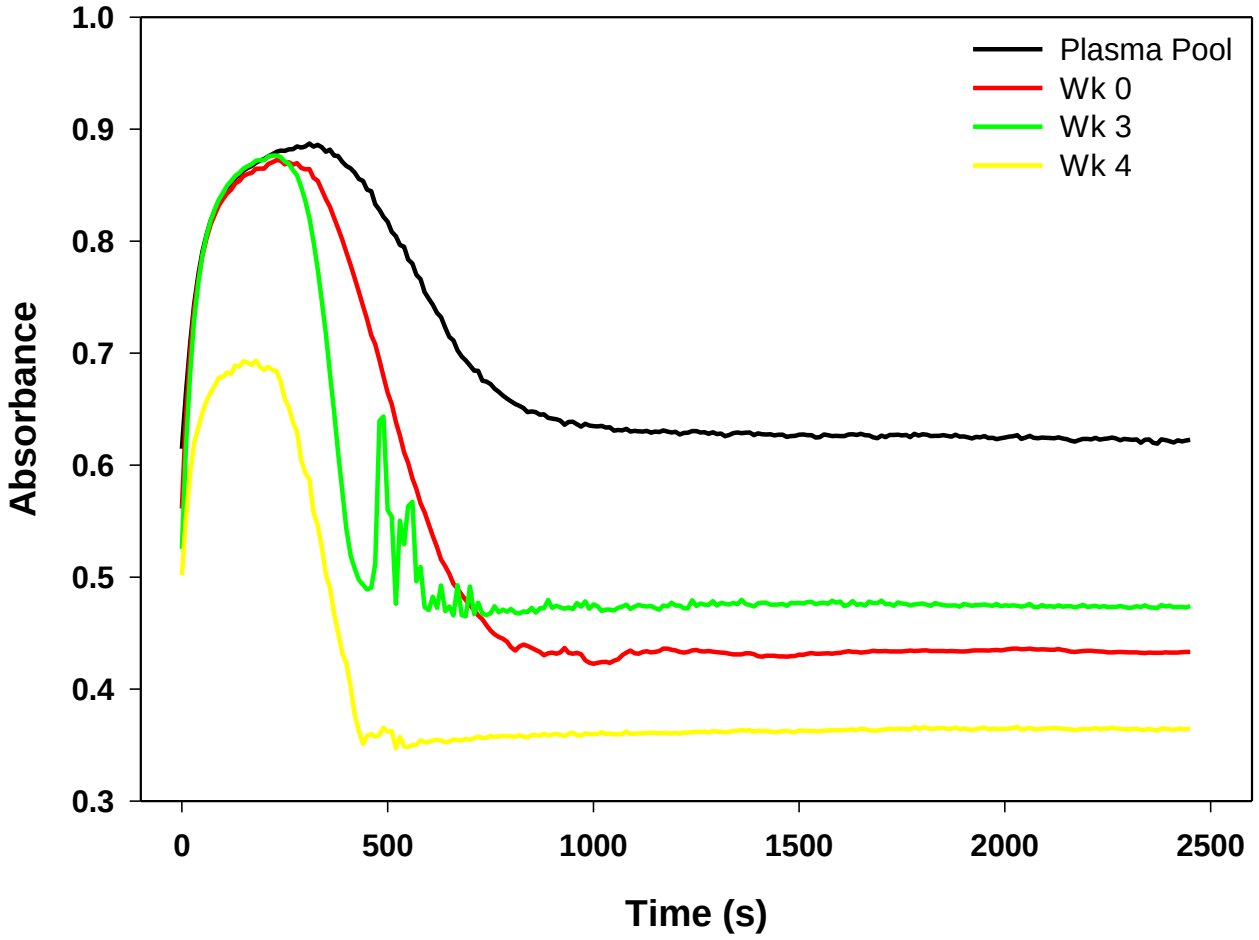
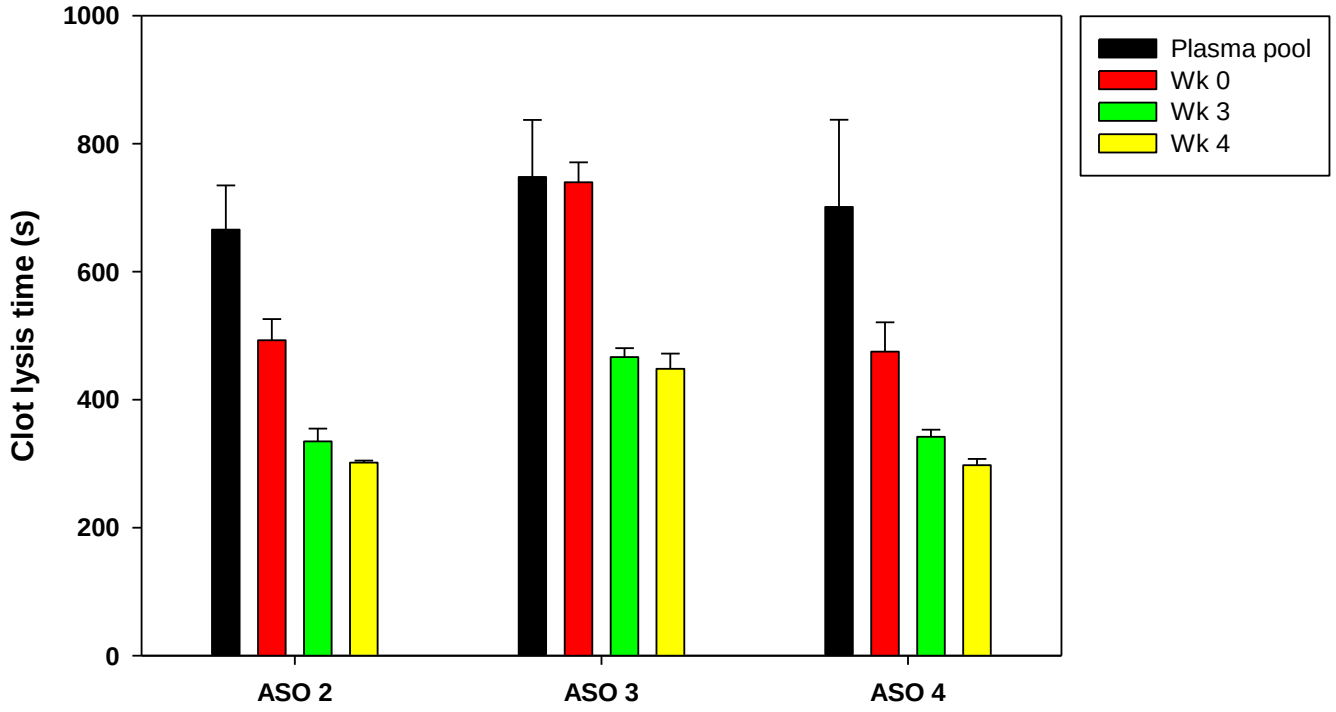

Plasma Pool
Wk 0
Wk 3
Wk 4
Absorbance
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
500
1000
1500
2000
2500
Time (s)

Figure 9: t-PA-mediated rabbit plasma clot lysis after treatment with ASO2.

Clot lysis times were measured using plasma from rabbits that received saline or a high dosage treatment of ASO2 (80 mg/kg). Plasma samples supplemented with 30 mM $CaCl_2$ were clotted with 20 nM thrombin in the presence of 5 nM of t-PA. Absorbance was read at 405 nm at 2 second intervals for two hours at 37°C. n=2.

A


1000
800
600
400
200
0
Clot lysis time (s)
ASO 2
ASO 3
ASO 4
Plasma pool
Wk 0
Wk 3
Wk 4

B

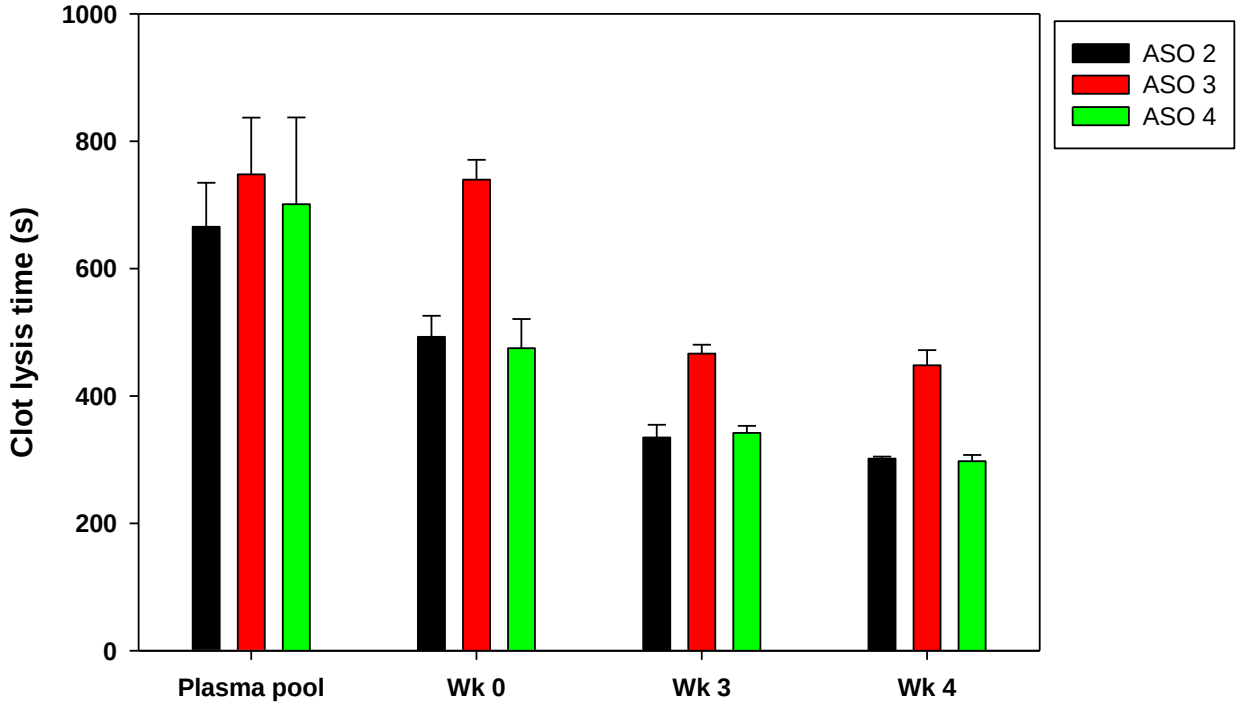

1000
800
600
400
200
0
Clot lysis time (s)
Plasma pool
Wk 0
Wk 3
Wk 4
ASO 2
ASO 3
ASO 4

Figure 10: Effect of three different ASOs against human α2AP on t-PA-mediated rabbit plasma clot lysis.

Recalcified plasma from rabbits treated with saline and the three ASO's against α2AP at different time points after administration of the ASO. 20 nM of thrombin was used to initiate clotting and 5 nM of t-PA was used to mediate clot lysis. The rabbit plasma was supplemented with 30 mM of $CaCl_2$. The plate was read at 405 nm at 2 second intervals for two hours at 37°C. Clot lysis time was defined as the time to reach half of the maximal decrease in absorbance after clotting was complete. Comparisons of clot lysis times made between each ASO (A) and between each time-point (B). Mean ± SE. n=2.

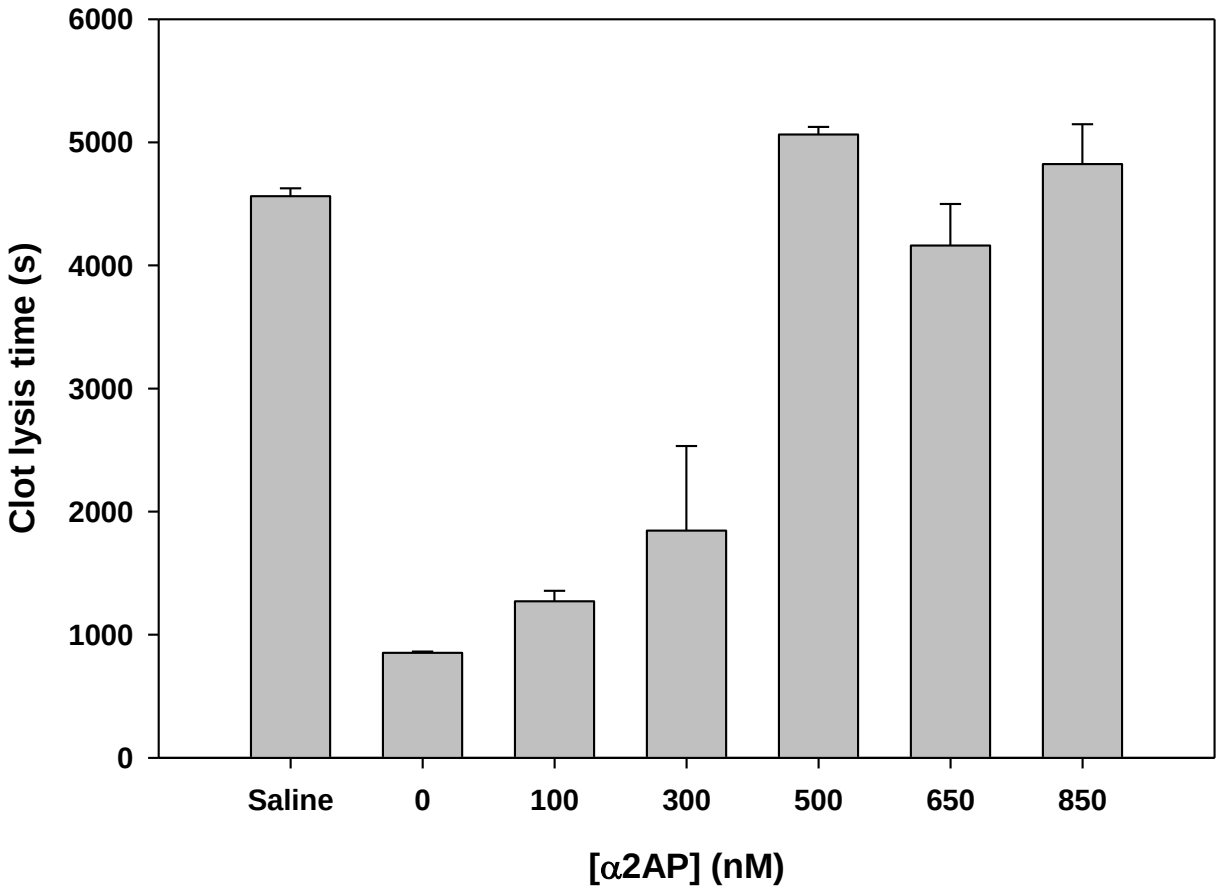

6000
5000
4000
3000
2000
1000
0
Clot lysis time (s)
Saline
0
100
300
500
650
850
[α2AP] (nM)

Figure 11: α2AP add back to plasma from the wk4 time-point treated with ASO2.

α2AP was titrated 0-1 μM in recalcified plasma from rabbits treated with saline and 80 mg/kg of ASO2. 20 nM of thrombin was used to initiate clotting 5 nM of t-PA was used to mediate clot lysis. The plate was read at 405 nm at 2 second intervals for two hours at 37°C. Clot lysis time was defined as the time to reach half of the maximal decrease in absorbance after clotting was complete. Lysis times were plotted against the concentration of α2AP added back to the reaction. Mean ± SE. n=2.

Chapter 4: A2AP IgG

4.1 Antibody characterization

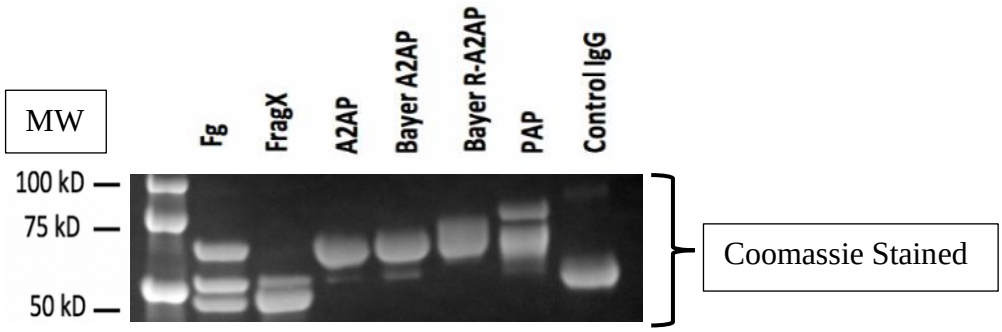
The A2AP IgG1 was the initial candidate selected using a phage display and an antibody gene library with human and rabbit α2AP as the antigen (Parmely and Smith, 1988). Affinity maturation was performed by Bayer AG by altering amino acid residues in the complement-determining region of the Fab portion of the A2AP IgG1. The Fc portion of the A2AP IgG1 was subsequently changed to an IgG4 which yielded A2AP IgG4. Various plasma samples and proteins of interest (Figure 12A) were probed in a western blot to assesses antibody specificity of the A2AP IgG1 and A2AP IgG4 antibodies in comparison with polyclonal human α2AP and fibrinogen antibodies. Studies were done under reducing conditions. A2AP IgG1 and IgG4 demonstrate similar binding (Figure 12B). Both isotypes of the antibody recognize similar bands in human and rabbit plasma that could be α2AP or α-chain of fibrinogen based on their molecular weights of around 70 kDa. Neither antibody appears to bind to purified α2AP from various sources (Haematologic Technologies Inc and Bayer AG). Both forms of the antibody bind the α- and β-chain of human fibrinogen and the β-chain of fragment X. Both are also able to bind the PAP complex, suggesting a conformational requirement for binding α2AP in this type of experiment. Next, polyclonal antibodies were used for comparison. The polyclonal human α2AP antibody does not bind human fibrinogen or fragment X but does bind α2AP from various sources in addition to the PAP complex. This confirms the detection of the PAP complex by the A2AP IgG1 and IgG4. Finally, the fibrinogen polyclonal antibody was tested and as expected binds fibrinogen and fragment X and also binds fibrinogen in human plasma. Fragment X was tested because it lacks the αC-domain which is the site where α2AP is cross-linked. Due to the bands for α-chain of fibrinogen and α2AP running at approximately the same position and the

fact that both the α2AP and fibrinogen polyclonal antibodies detect a band in human plasma, it is unclear what the A2AP IgG4 is detecting in human plasma. Also, it is unclear what the A2AP IgG1 and IgG4 antibodies are detecting in the rabbit plasma samples at around 80 kD. In conclusion, the A2AP IgG1 and IgG4 fail to bind α2AP in a western blot but do bind fibrinogen and fragment X. Due to the polyclonal human α2AP antibody failing to bind fibrinogen, A2AP IgG1 or IgG4 binding is unlikely to be as a result of α2AP cross-linked to fibrinogen. Also, due to the fact that A2AP IgG1 and IgG4 bound to fragment X, the binding to fibrinogen is likely non-specific.

4.2 ELISA detection of human α2AP and the PAP complex by A2AP IgG1 and IgG4

A sandwich ELISA was next used to validate the results of western blotting experiments. The polyclonal anti-human α2AP antibody was immobilized to the bottom of the wells of a 96 well high binding plate prior to blocking. Then human α2AP was titrated from 0-14.3 nM and the PAP complex titrated up to 20 nM. A2AP IgG1 and IgG4 (both HRP conjugated) were added to a final concentration of 2 μg/mL, and detection occurred with 2.3 mM OPD containing 0.03% H_2O_2. As seen in Figure 13A, both isotypes exhibit dose-dependent binding to human α2AP, with higher absorbance values seen for higher concentrations of human α2AP. Curves of best fit were determined and show that the A2AP IgG4 and IgG1 appear to bind with similar affinity as evidenced by their Kd values of 2.3 ± 0.1 nM and 4 ± 0.7 nM, respectively. These affinities were not significantly different (P=0.085). Also, as seen in Figure 13B, both antibodies bind the PAP complex dose dependently with a linear response, suggesting saturation was not achieved. As a result, the sensitivity of the A2AP IgG1 and IgG4 for α2AP appears to be greater compared with the PAP complex. Thus, similar to western blotting, both isotypes were able to detect the PAP

A


MW
Fg
FragX
A2AP
Bayer A2AP
Bayer R-A2AP
PAP
Control IgG
100 kD
75 kD
50 kD
Coomassie Stained

B

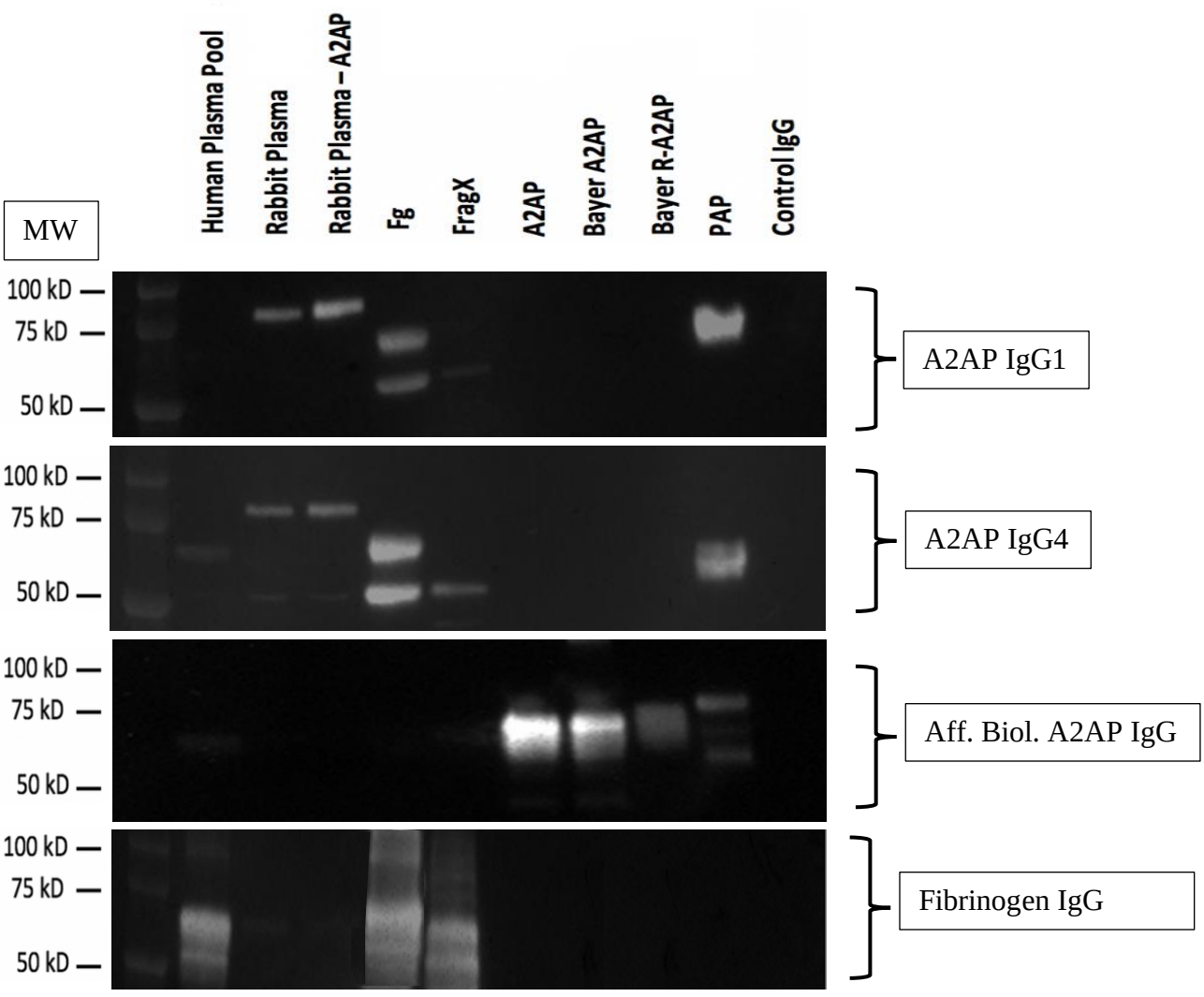
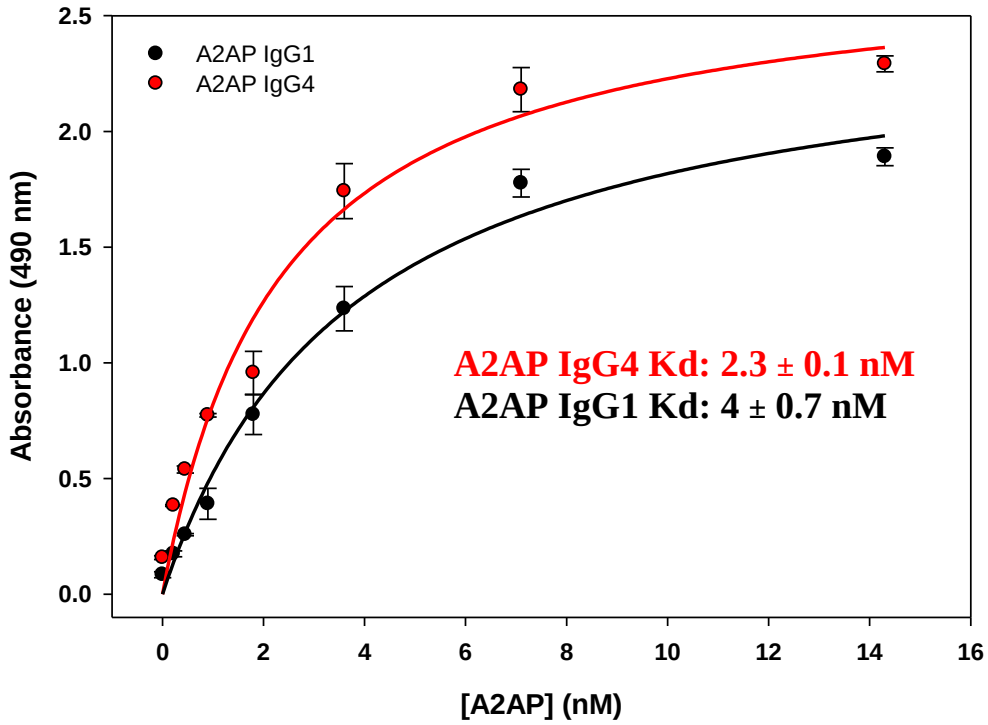

MW
Human Plasma Pool
Rabbit Plasma
Rabbit Plasma – A2AP
Fg
FragX
A2AP
Bayer A2AP
Bayer R-A2AP
PAP
Control IgG
100 kD
75 kD
50 kD
A2AP IgG1
A2AP IgG4
Aff. Biol. A2AP IgG
Fibrinogen IgG

Figure 12: Antibody specificity determined by western blot.

Gel electrophoresis various proteins was performed in the presence of SDS under reducing conditions (A). Western blots (B) were then performed and samples were then transferred to the membrane and probed with the A2AP IgG1 (HRP conjugated), A2AP IgG4 (HRP conjugated), an affinity purified goat anti-human α2AP polyclonal antibody (Aff. Biol. A2AP IgG), and a rabbit anti-human fibrinogen polyclonal antibody (Fibrinogen IgG). For the Aff. Biol. A2AP IgG, a purified polyclonal anti-goat IgG HRP conjugated antibody was used as the secondary antibody. For the Fibrinogen IgG, a purified polyclonal anti-rabbit HRP antibody was used as the secondary antibody. The membrane was imaged using Image Lab Software. Representative data shown. n=2.

A


A2AP IgG1
A2AP IgG4
A2AP IgG4 Kd: 2.3 ± 0.1 nM
A2AP IgG1 Kd: 4 ± 0.7 nM
Absorbance (490 nm)
[A2AP] (nM)
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8
10
12
14
16

B

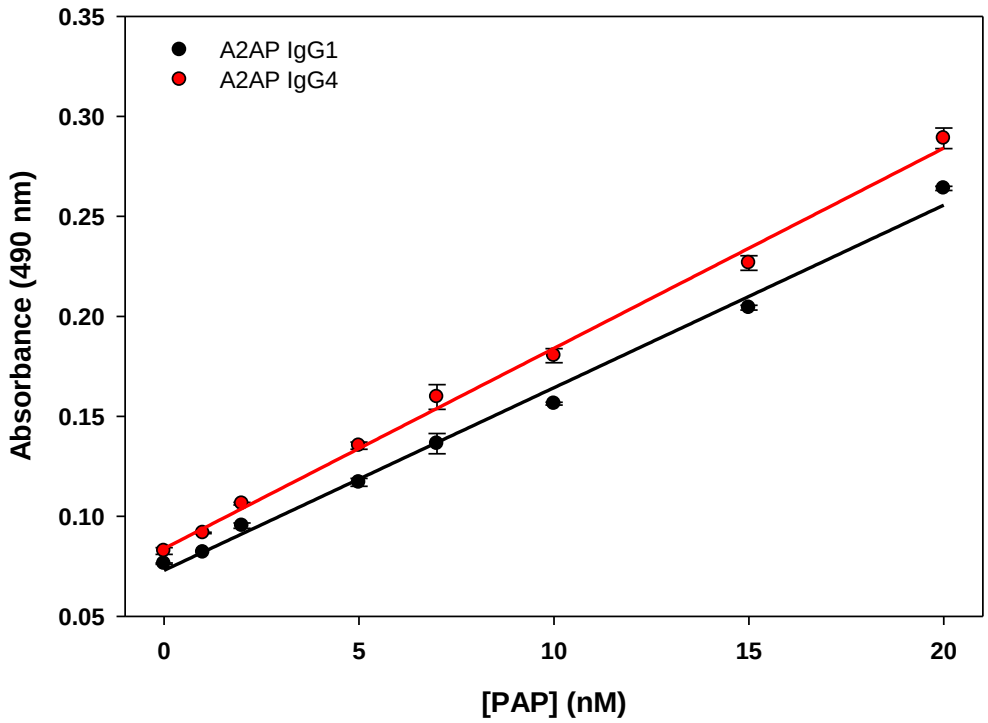

A2AP IgG1
A2AP IgG4
Absorbance (490 nm)
[PAP] (nM)
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0
5
10
15
20

Figure 13: Comparing antibody detection in α2AP and PAP complex ELISA

An affinity purified goat anti-human a2-antiplasmin polyclonal antibody was coated to a 96 well high binding plate, prior to blocking, to a final concentration of 1 μg/mL. After three washes with PBS containing 0.05% Tween, (A) human α2AP was titrated at 0 to 14.3 nM and (B) equimolar amounts of human α2AP and plasmin up to 20 nM. For detection, the A2AP IgG1 and IgG4 (both HRP conjugated) were added to a final concentration of 2 μg/mL for two hours after which detection occurred with 2.3 mM OPD containing 0.03% H_2O_2. The absorbance was then read using a kinetic plate reader by taking an endpoint read at 490 nm. The OD values were plotted against the concentration of human α2AP and the PAP complex. Curves of best fit were determined. Mean ± SE. n=3.

complex. However, unlike the results with western blotting, both isotypes were able to bind α2AP suggesting a conformational requirement for binding. Cleavage of α2AP by plasmin upon complex formation may expose the reactive center loop of α2AP and enable the binding of A2AP IgG1 and IgG4.

4.3 ELISA detection of human fibrinogen by A2AP IgG1

Using a sandwich ELISA approach, the binding of the A2AP IgG1 antibody to human fibrinogen was explored to validate the results from the western blotting experiments. A polyclonal rabbit anti-human fibrinogen antibody was immobilized to the surface of a 96 well high binding plate. Then human fibrinogen was titrated from 0-14.3 nM. Bayer A2AP IgG1 (HRP conjugated) was added to a final concentration of 2 μg/mL. As seen in Figure 14, A2AP IgG1 exhibits dose-dependent and saturable binding to human fibrinogen. In this experiment, the affinity of the A2AP IgG1 for fibrinogen was not significantly different than that for α2AP as evidenced by its Kd value of 3 ± 0.2 nM in comparison to 4 ± 0.7 nM for α2AP (P=0.258). Thus, similar to data from western blotting, the A2AP IgG1 is able to bind human fibrinogen.

4.4 Antibody comparison in western blot detection of fibrinogen-α2AP complex

To further understand if the A2AP IgG1 and IgG4 antibodies were binding non-specifically to fibrinogen or to the αC domain of fibrinogen, human fibrinogen-α2AP complexes were generated by incubating human fibrinogen with human α2AP in the presence of FXIIIa and $CaCl_2$ for 30 minutes at 37°C after which the bands were analyzed by gel electrophoresis and probed with both A2AP IgG1 and A2AP IgG4 and compared with the polyclonal human α2AP and fibrinogen antibodies. As seen in Figure 15A, the human fibrinogen-α2AP complex is successfully formed in the presence of FXIIIa and runs at around 140 kDa on SDS-PAGE (Ritchie *et al*., 2000). In the absence of FXIIIa, due to the bands for α-chain of fibrinogen and

α2AP co-migrating, fibrinogen and α2AP are indistinguishable. Blots of similar gels were probed with both A2AP IgG1 and A2AP IgG4 (Figure 15B). Both detect human fibrinogen and fail to detect α2AP. Both antibodies are unable to detect the fibrinogen-α2AP complex. In comparison as seen in Figure 15C, the polyclonal human α2AP and fibrinogen antibodies are able to detect the fibrinogen-α2AP complex as evidenced by a band around 140 kD. This suggests that the interaction between A2AP IgG1 or IgG4 and fibrinogen may be non-specific and not as a result of cross-linked α2AP.

4.5 Detection of human and rabbit fibrinogen degradation products by A2AP IgG1

To further characterize where on fibrinogen the A2AP IgG antibodies were binding, human and rabbit fibrinogen were incubated with plasmin and the degradation products were then subjected to SDS-PAGE and western blot analysis. Human or rabbit fibrinogen was incubated with 200 nM or 150 nM of plasmin, respectively in the presence of $CaCl_2$. Aliquots were removed from the reaction at different time points (prior to addition of plasmin, 1, 5, 10, 20, 30, and 60 minutes after addition of plasmin) and were analyzed by SDS-PAGE and western blotting. As seen in Figure 16A depicting the stained gel for human fibrinogen, the α-chain of fibrinogen shows degradation starting five minutes after the addition of plasmin and by 20 minutes after plasmin addition, the α-chain is completely degraded. The β-chain of fibrinogen shows degradation starting 10 minutes after the addition of plasmin and by 20 minutes after plasmin addition, the β-chain is completely degraded. The γ-chain is partially degraded 60 minutes after plasmin addition (completion of experiment). The corresponding western blot (Figure 16B) shows that the A2AP IgG1 initially binds the α- and β-chains of fibrinogen, and shows minimal binding to γ-chain, consistent with Figures 12 and 15. The A2AP IgG1 is able to

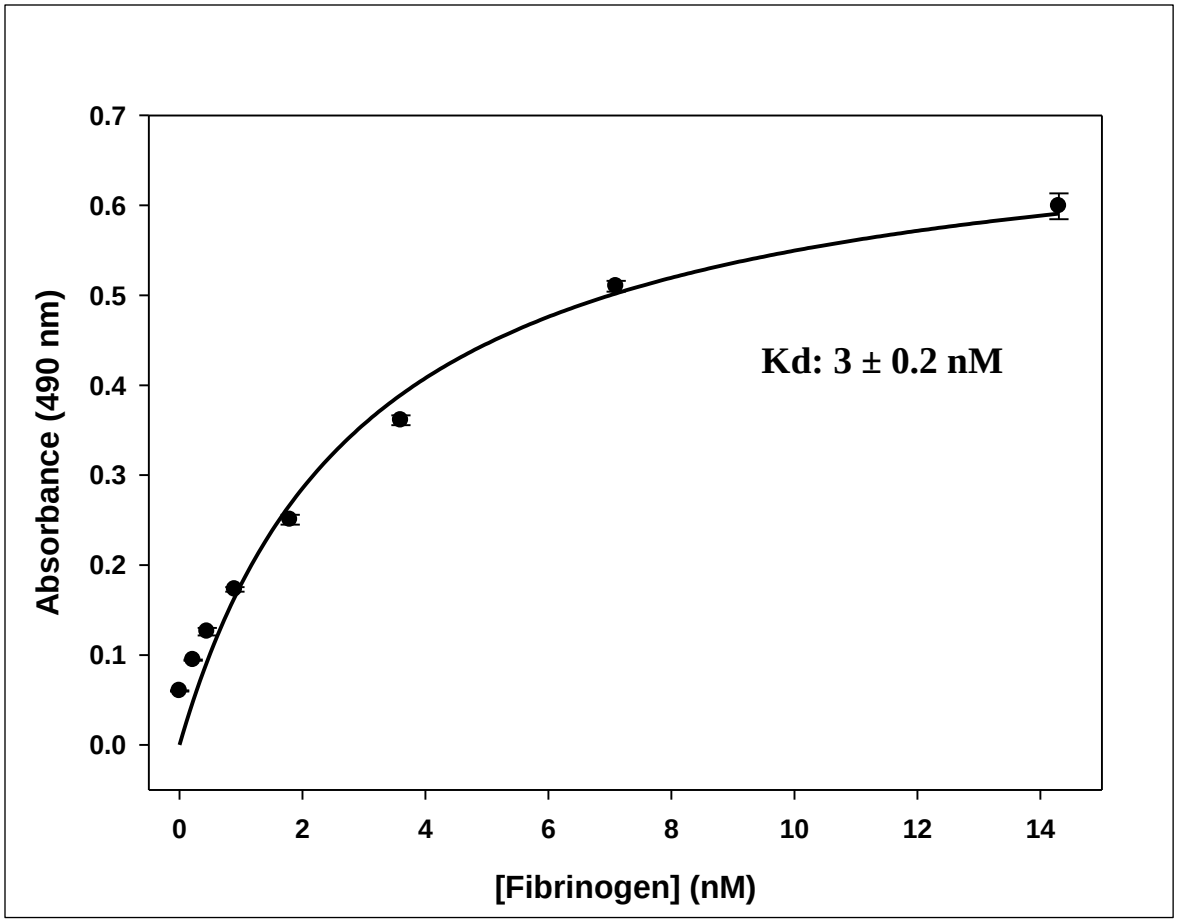
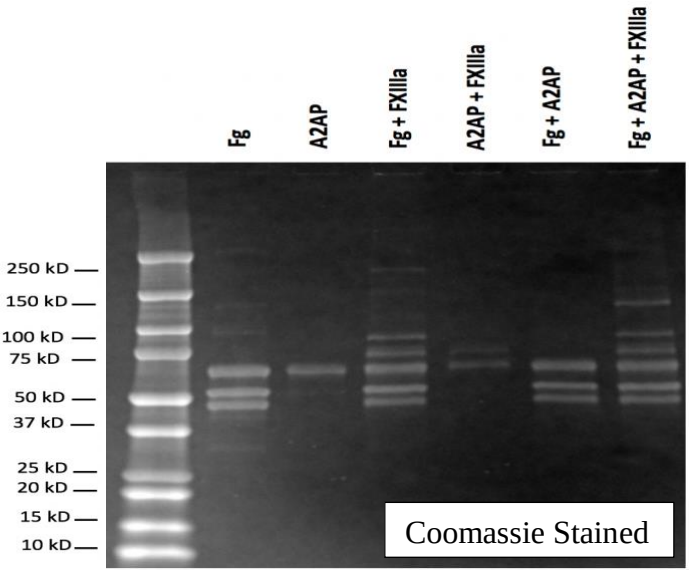

Absorbance (490 nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
2
4
6
8
10
12
14
[Fibrinogen] (nM)
Kd: 3 ± 0.2 nM

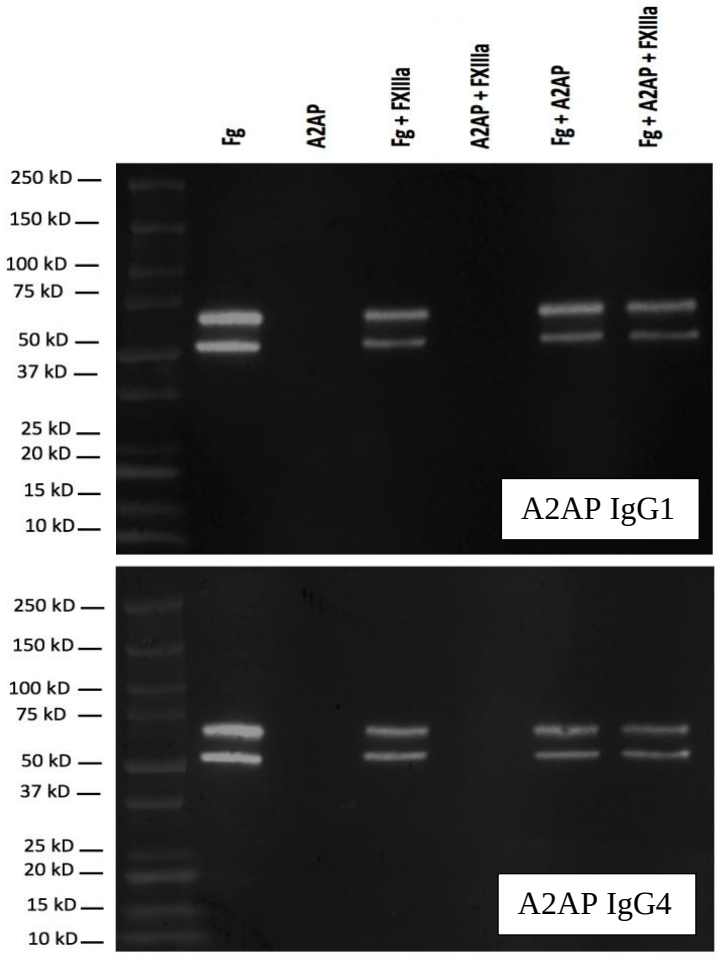
Figure 14: Antibody binding in ELISA detection of human fibrinogen.

A polyclonal rabbit anti-human fibrinogen antibody was coated to a 96 well high binding plate, prior to blocking, to a final concentration of 1 μg/mL. After three washes human fibrinogen was titrated at 0-14.3 nM. For detection, Bayer A2AP IgG1 (HRP conjugated) was added to a final concentration of 2 μg/mL after which 2.3 mM OPD containing 0.03% H_2O_2 was added. The absorbance was then read using a kinetic plate reader by taking an endpoint read at 490 nm. The OD values were plotted against the concentration of human fibrinogen. Curve of best fit was determined. Mean ± SE. n=3.

A


Fg
A2AP
Fg + FXIIIa
A2AP + FXIIIa
Fg + A2AP
Fg + A2AP + FXIIIa
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD
Coomassie Stained

B


Fg
A2AP
Fg + FXIIIa
A2AP + FXIIIa
Fg + A2AP
Fg + A2AP + FXIIIa
250 kD
150 kD
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75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD
A2AP IgG1
A2AP IgG4

C

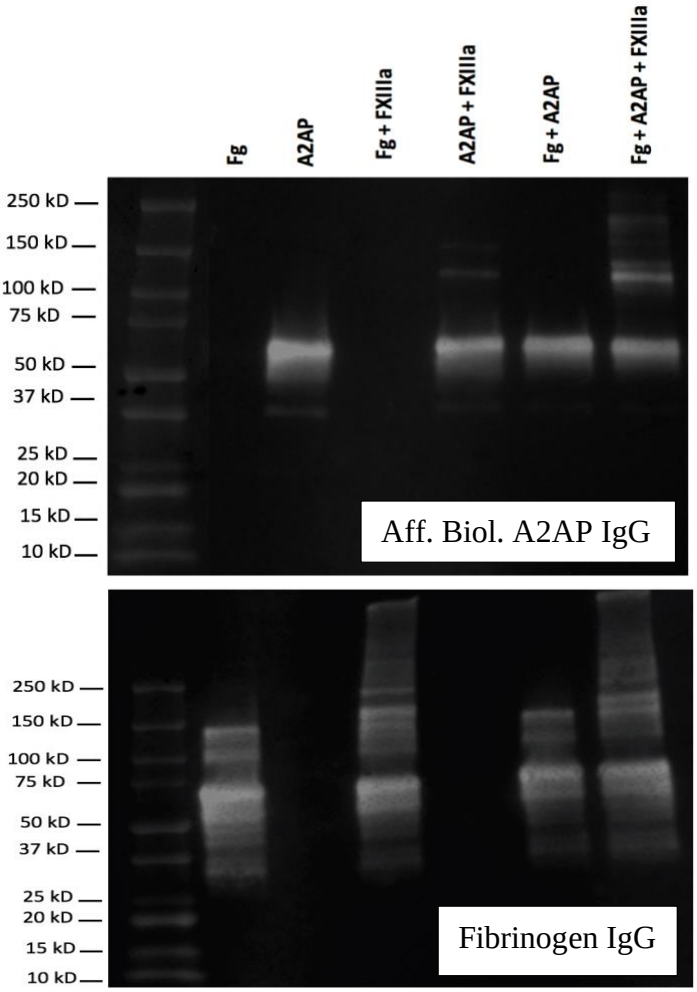

Fg
A2AP
Fg + FXIIIa
A2AP + FXIIIa
Fg + A2AP
Fg + A2AP + FXIIIa
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD
Aff. Biol. A2AP IgG
Fibrinogen IgG

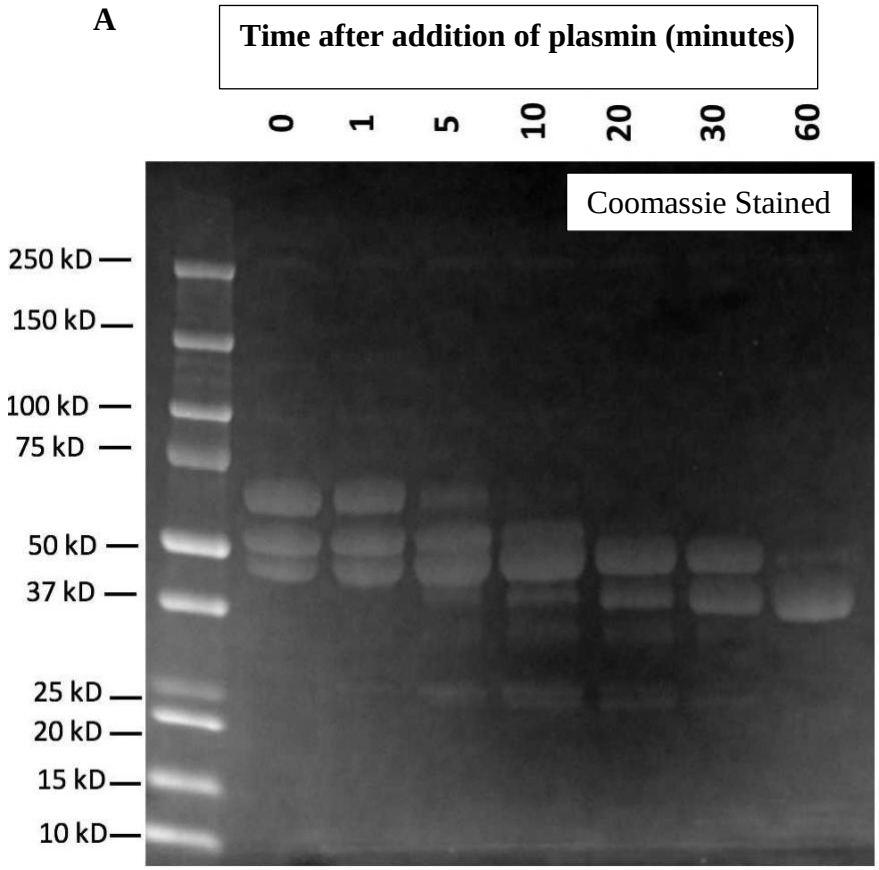
Figure 15: Antibody comparison in western blot detection of fibrinogen-α2AP complex. Reduced gel electrophoresis in the presence of SDS of fibrinogen-α2AP complexes was performed (A). Western blots (B and C) were then performed and samples were transferred to the membrane and probed with A2AP IgG1 (HRP conjugated), A2AP IgG4 (HRP conjugated), an affinity purified goat anti-human α2AP polyclonal antibody (Aff. Biol. A2AP IgG), and a rabbit anti-human fibrinogen polyclonal antibody (Fibrinogen IgG) as primary antibodies. For the Aff. Biol. A2AP IgG, a purified polyclonal anti-goat IgG HRP conjugated antibody was used as the secondary antibody. For the Fibrinogen IgG, a purified polyclonal anti-rabbit HRP antibody was used as the secondary antibody. The membrane was imaged using Image Lab Software. Representative data shown n=2.

detect the α-chain degradation products and potentially the β-chain degradation products. Thus, it appears that the non-specific interactions between the A2AP IgG1 and human fibrinogen are mediated through the α- and β-chains of fibrinogen and not the γ-chain. Figure 17A depicts the SDS-PAGE gel for rabbit fibrinogen and a similar order of degradation seen with human fibrinogen. The α-chain is degraded first starting five minutes after plasmin addition and is completely degraded 10 minutes after plasmin addition. The β-chain shows degradation five minutes after plasmin addition and is completely degraded 20 minutes after plasmin addition. In the corresponding western blot (Figure 17B) the A2AP IgG1 only appears to detect the α-chain, β-chain, and α-chain degradation products. Thus, the α- and β-chains of both human and rabbit fibrinogen are recognized by the A2AP IgG1 and appear to be the sites of non-specific interactions.

4.6 Characterization of A2AP IgG4 binding to plasma proteins in affinity chromatography

After concluding that A2AP IgG4 binds α2AP in ELISA and fibrinogen in western blotting and ELISA, we next determined whether A2AP IgG4 could recognize α2AP, fibrinogen or fibrinogen-α2AP in plasma. Affinity chromatography was performed using A2AP IgG4 immobilized to Sepharose. PPP was passed over the column twice. Next, the column-bound proteins were eluted with gentle elution buffer and analyzed by gel electrophoresis and western blotting. Polyclonal α2AP and fibrinogen antibodies were used to probe the eluted sample. The SDS-PAGE gel (Figure 18A) shows multiple bands, some which run at the same molecular weight as α2AP and fibrinogen. On western blotting, both the polyclonal α2AP (Figure 18B) and fibrinogen (Figure 18C) antibodies detect the eluted proteins indicating that both α2AP and fibrinogen were bound to the A2AP IgG4 column. Both the fibrinogen and α2AP antibodies

A


Time after addition of plasmin (minutes)
0
1
5
10
20
30
60
Coomassie Stained
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD

B

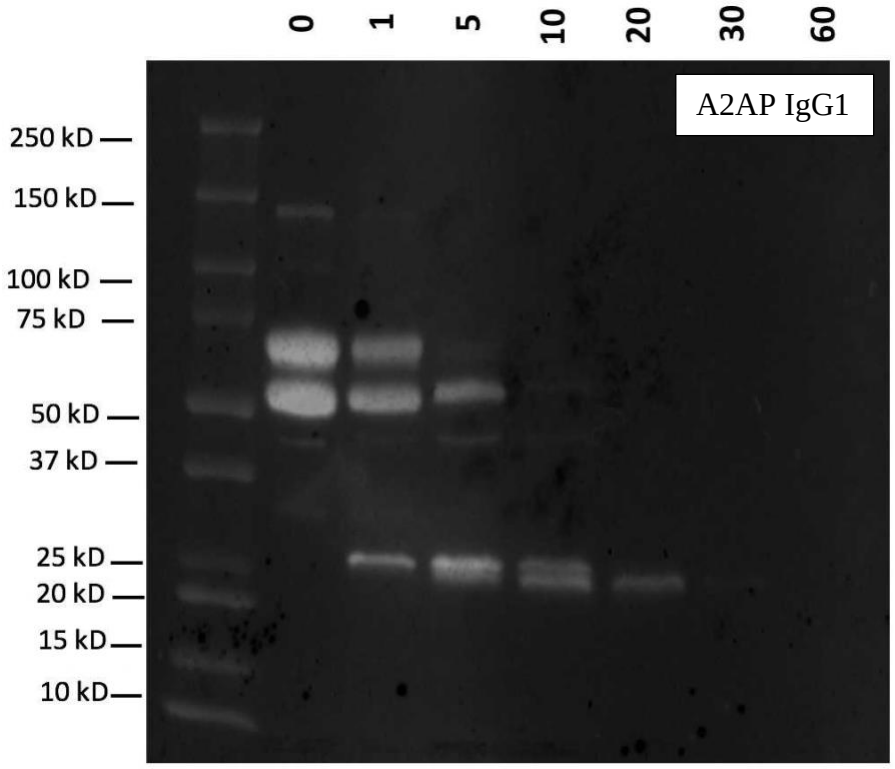
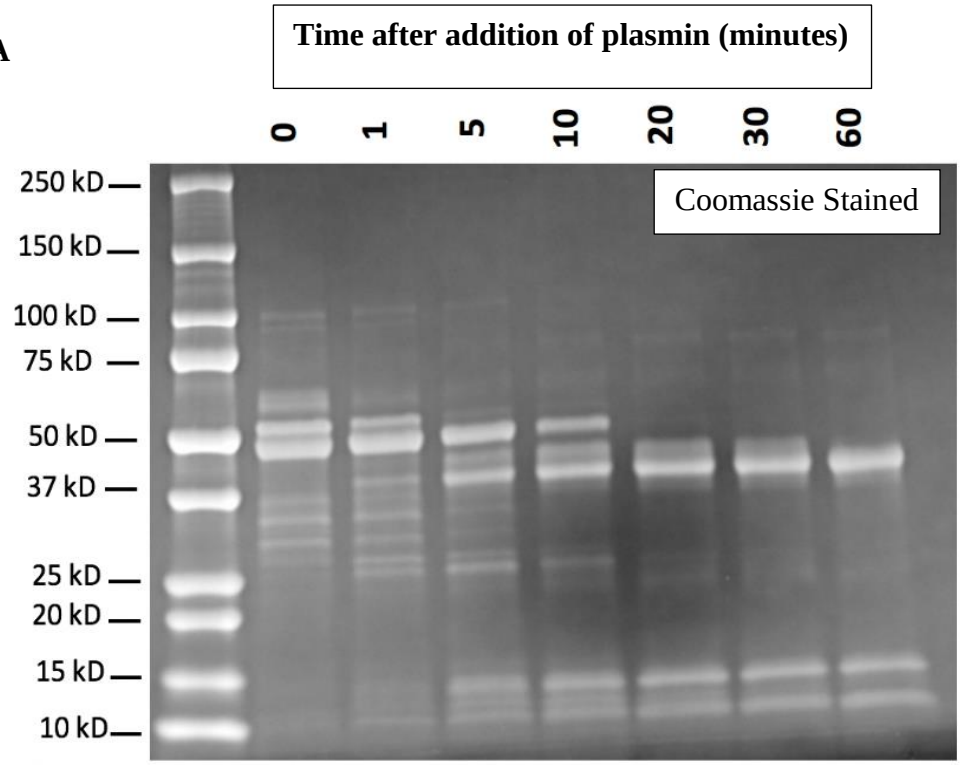

0
1
5
10
20
30
60
A2AP IgG1
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD

Figure 16: Detection of plasmin-mediated human fibrinogen degradation products by A2AP IgG1

SDS-PAGE analysis of fibrinogen degradation products under reducing conditions of plasmin-mediated digest of human fibrinogen over time was performed (A). Next, western blots (B) were then performed and samples were transferred to the membrane and probed with A2AP IgG1 (HRP conjugated. The membrane was imaged using Image Lab Software. Representative data shown. n=2.

A


Time after addition of plasmin (minutes)
0
1
5
10
20
30
60
Coomassie Stained
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD

B

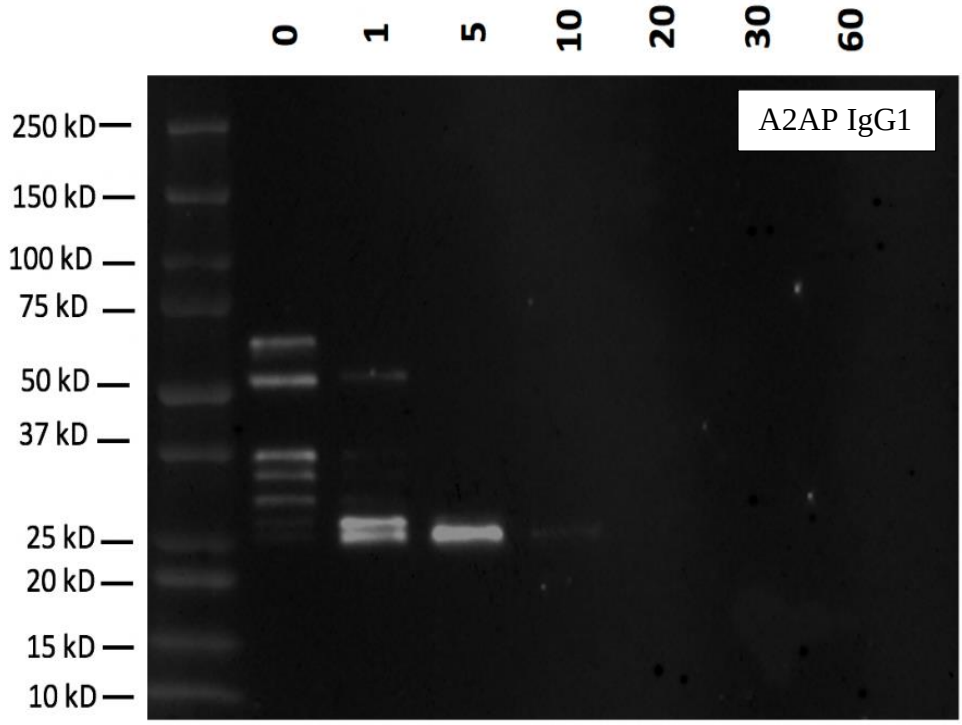
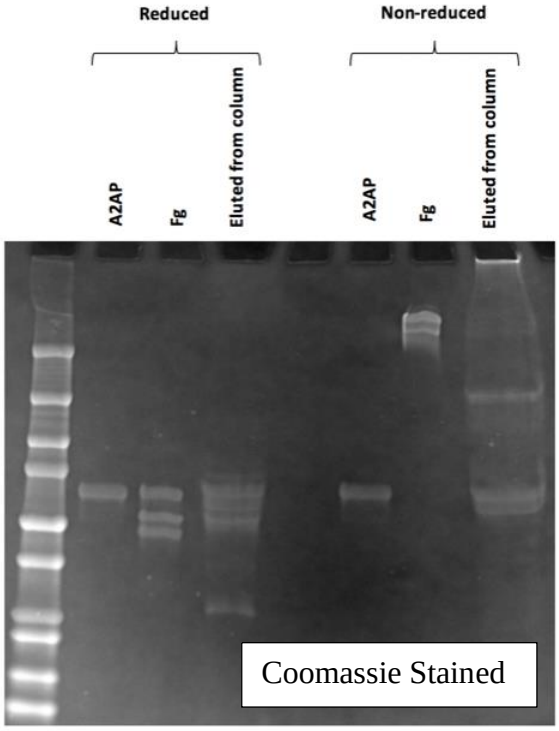

0
1
5
10
20
30
60
A2AP IgG1
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
15 kD
10 kD

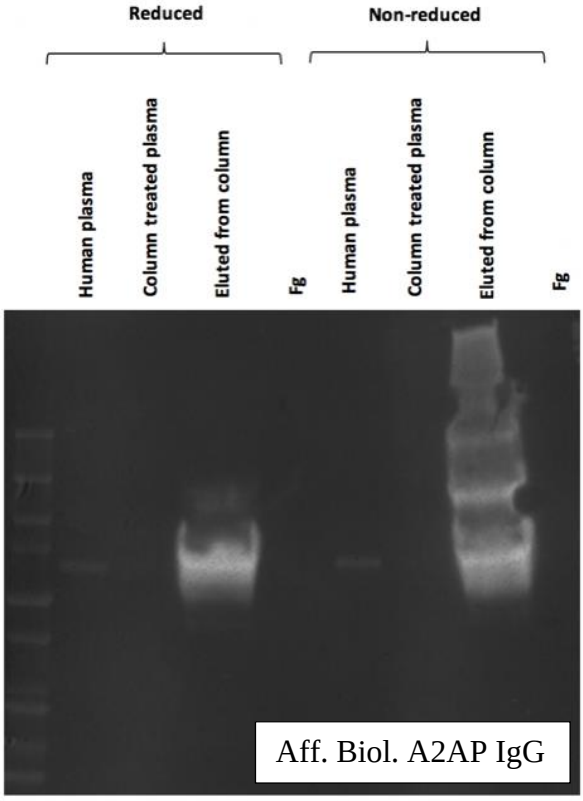
Figure 17: Detection of plasmin-mediated rabbit fibrinogen degradation products by A2AP IgG1

SDS-PAGE analysis of fibrinogen degradation products under reducing conditions of plasmin-mediated digest over time of rabbit fibrinogen was performed (A). Next, western blots (B) were then performed and samples were transferred to the membrane and probed with A2AP IgG1 (HRP conjugated. The membrane was imaged using Image Lab Software. Representative data shown. n=2.

A


Reduced
Non-reduced
A2AP
Fg
Eluted from column
A2AP
Fg
Eluted from column
Coomassie Stained

B


Reduced
Non-reduced
Human plasma
Column treated plasma
Eluted from column
Fg
Human plasma
Column treated plasma
Eluted from column
Fg
Aff. Biol. A2AP IgG

C

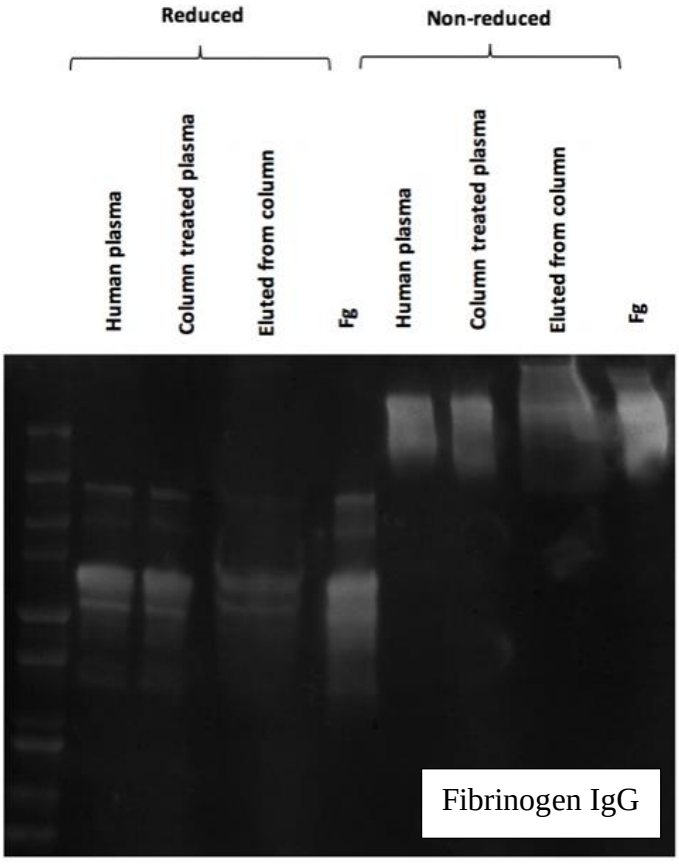

Reduced
Non-reduced
Human plasma
Column treated plasma
Eluted from column
Fg
Human plasma
Column treated plasma
Eluted from column
Fg
Fibrinogen IgG

Figure 18: Characterization of A2AP IgG4 binding to plasma proteins in affinity chromatography

The A2AP IgG4 was immobilized to a Sepharose column and human plasma was passed over twice. The bound proteins were eluted with gentle elution buffer and the sample was subjected to SDS-PAGE analysis (A). Western blotting (B and C) was then performed where an affinity purified goat anti-human α2AP polyclonal antibody (Aff. Biol. A2AP IgG), and a rabbit anti-human fibrinogen polyclonal antibody (Fibrinogen IgG) were used as primary antibodies. For the Aff. Biol. A2AP IgG, a purified polyclonal anti-goat IgG HRP conjugated antibody was used as the secondary antibody. For the Fibrinogen IgG, a purified polyclonal anti-rabbit HRP antibody was used as the secondary antibody. The membrane was imaged using Image Lab Software. Representative data shown. n=2.

detect bands at higher molecular weights around 140 kDa which could be cross-linked chains of fibrinogen or the α2AP-fibrinogen complex, although the latter is unlikely due to the polyclonal α2AP failing to detect α2AP at higher molecular weights. Thus, the A2AP IgG4 binds both plasma α2AP and fibrinogen, and potentially the α2AP-fibrinogen complex.

4.7 Determination of binding affinities of the A2AP IgG1 and IgG4 for human α2AP, fibrinogen and fragment X using SPR

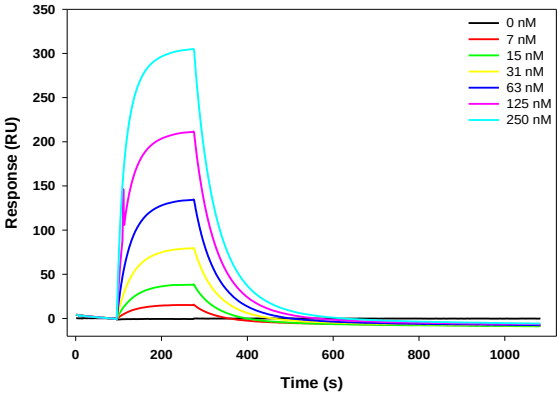
A2AP IgG1 and IgG4 antibodies were immobilized to flow cells on a CM5 chip to explore the binding of various proteins using SPR. Human α2AP was titrated from 0 to 250 nM, and fibrinogen and fragment X were titrated from 0 to 10 μM. Antigens were passed over flow cells with a contact time of 180 s, a dissociation time of 1800 s, and flow rate of 60 uL/min. As seen in Figure 19A and 19D, human α2AP bound to both the A2AP IgG1 and IgG4 in a dose-dependent manner. The α2AP binding to A2AP IgG1 achieved a maximum response of roughly 300 RU, whereas the α2AP binding to A2AP IgG4 achieved a maximum response of roughly 450 RU. However, equilibrium occurred for each titration of A2AP IgG1 but did not occur for the A2AP IgG4, suggesting a higher affinity of the A2AP IgG4 for α2AP. The affinity was determined using both steady state affinity and kinetic (1:1) binding analyses. Binding of α2AP to A2AP IgG4 appears to be higher affinity than to A2AP IgG1 as evidenced by the Kd values in Table 2. A Kd value of 120.8 ± 11.7 nM was determined for the A2AP IgG1 and 1.9 ± 0.2 nM for the A2AP IgG4 for binding to human α2AP using the kinetic (1:1) binding analysis. These Kd values are significantly different (P=0.029). Fibrinogen was tested due to binding seen in ELISA and western blotting. As seen in Figure 19B and 19E, fibrinogen bound both antibodies in a dose-dependent manner, and both achieved a maximal response around 120 RU with similar profiles. The A2AP IgG1 bound to fibrinogen with a Kd value of 99.3 ± 5.8 nM and the A2AP

IgG4 had a Kd value of 122.5 ± 18.9 nM. These Kd values were not significantly different (P=0.629). Fragment X was tested due to binding seen in western blotting. As seen in Figure 19C and 19F, for fragment X, the maximal response was around 40 RU for both antibodies and the Kd value was 65.4 ± 16.6 nM for A2AP IgG1 and 79.7 ± 22.7 nM for A2AP IgG4, although the shapes of the curves limit the accuracy of these results due to inability to perform a kinetic (1:1) binding analysis. Also, these Kd values were not significantly different (P=0.708). In conclusion, using SPR, the A2AP IgG1 and IgG4 both bind human α2AP, fibrinogen, and fragment X, consistent with data seen in ELISA and western blotting. Also, the A2AP IgG4 binds α2AP with significantly higher affinity than A2AP IgG1.

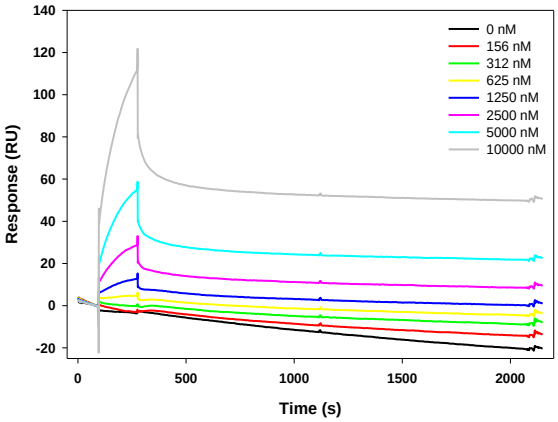
4.8 Effect of the A2AP IgG1 on α2AP inhibition of plasmin

The functional specificity of the A2AP IgG was examined by determining their effect on α2AP inhibition of plasmin. The effect that the A2AP IgG1 had on plasmin inhibition by α2AP was assessed by measuring plasmin cleavage of a chromogenic substrate as α2AP was titrated. A fixed amount of plasmin was used at 25 nM and human α2AP was titrated up to 100 nM in the presence of 1 μM of the A2AP IgG1 or control IgG and incubated for 15 minutes. The chromogenic substrate S-2251 was added to detect plasmin activity. As seen in Figure 20A, at approximately equimolar amounts of plasmin and α2AP, complete inhibition of plasmin occurred in the presence of control IgG. A curve of best fit was determined and the IC50 in this experiment was 4.7 ± 0.7 nM. However, in the presence of the A2AP IgG1, at least four times molar excess of α2AP was necessary to abolish plasmin activity. The IC50 in the presence of the A2AP IgG1 was 26 ± 4.5 nM which was significantly different than the IC50 in the absence of the A2AP IgG1 (P=0.029). This suggests that the presence of the A2AP IgG1 significantly reduces the potency of α2AP. Next, to determine if the A2AP IgG1 could inhibit the α2AP

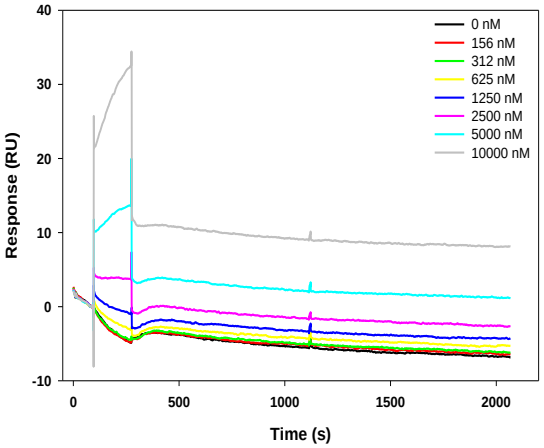
A


Response (RU)
Time (s)
0 nM
7 nM
15 nM
31 nM
63 nM
125 nM
250 nM

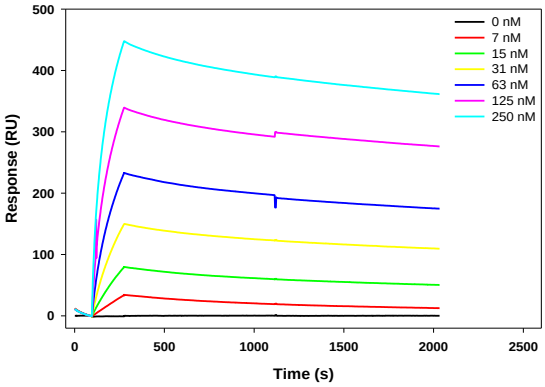
B


Response (RU)
Time (s)
0 nM
156 nM
312 nM
625 nM
1250 nM
2500 nM
5000 nM
10000 nM

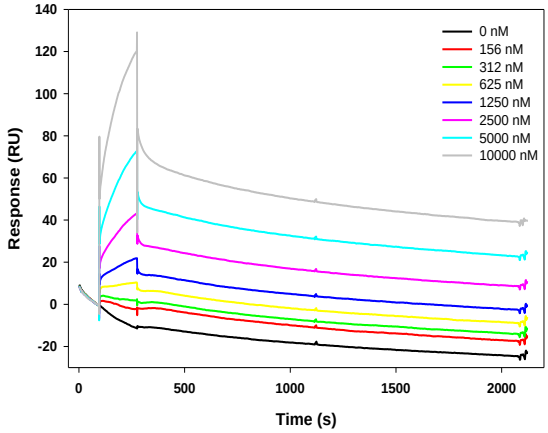
C


Response (RU)
Time (s)
0 nM
156 nM
312 nM
625 nM
1250 nM
2500 nM
5000 nM
10000 nM

D


Response (RU)
Time (s)
0 nM
7 nM
15 nM
31 nM
63 nM
125 nM
250 nM

E


Response (RU)
Time (s)
0 nM
156 nM
312 nM
625 nM
1250 nM
2500 nM
5000 nM
10000 nM

F

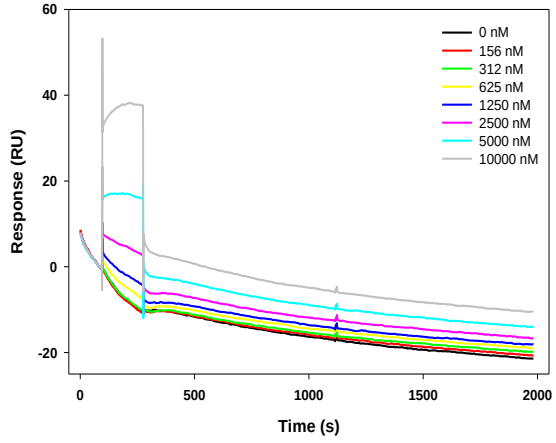

Response (RU)
Time (s)
0 nM
156 nM
312 nM
625 nM
1250 nM
2500 nM
5000 nM
10000 nM

Figure 19: Determination of binding affinities of the A2AP IgG1 and IgG4 for human α2AP, fibrinogen and fragment X using SPR

A2AP IgG1 was immobilized to a CM5 chip to 2198 RU and A2AP IgG4 was immobilized to a separate flow cell at RU of 2172. A blank flow cell served as the control. Human α2AP from 0 to 250 nM (A and D), and 0-10 uM fibrinogen (B and E) or fragment X (C and F) were injected over the flow cells for 200 s in separate experiments. The dissociation time was 1800s and the flow rate was 60 uL/min. Binding affinity was measured by determining the equilibrium dissociation constant (Kd) through both steady state affinity and kinetic (1:1) binding analysis. Representative runs from 2-4 experiments are shown.

Ligand	Analyte	Kd (nM)	
		Steady State Affinity	**Kinetic (1:1 Binding)**
A2AP IgG1	α2AP	177 ± 2.7	120.8 ± 11.7
A2AP IgG1	Fibrinogen	N/A	99.3 ± 5.8
A2AP IgG1	Fragment X	N/A	65.4 ± 16.6
A2AP IgG4	α2AP	N/A	1.9 ± 0.2
A2AP IgG4	Fibrinogen	N/A	122.5 ± 18.9
A2AP IgG4	Fragment X	N/A	79.7 ± 22.7

*

Table 2: Binding affinities of the A2AP IgG1 and IgG4 for human α2AP, fibrinogen and fragment X using SPR.

Kd values reported in nM. Mean ± SE. n=2-4. *P=0.029.

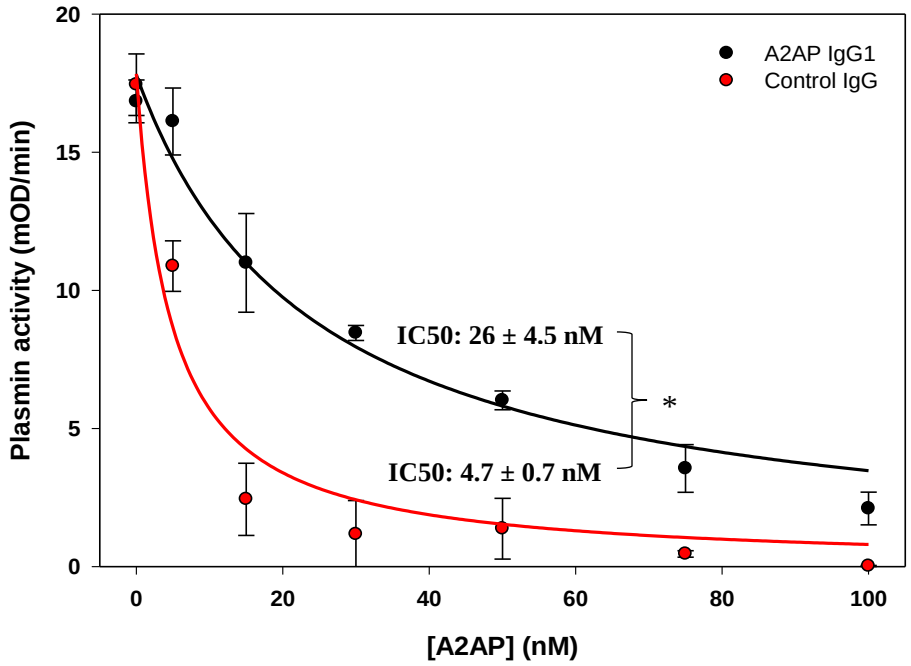
mediated neutralizing effect on plasmin activity in the presence of an equimolar amount of α2AP, the A2AP IgG1 was titrated up to 500 nM. As seen in Figure 20B, the A2AP IgG1 was able to partially neutralize α2AP and thereby allow for residual plasmin activity back to similar levels as it was in the absence of any α2AP. In conclusion, the A2AP IgG1 has a protective effect on plasmin activity in the presence of α2AP by partially neutralizing α2AP-mediated inhibition of plasmin.

Due to the binding of the A2AP IgG1 and IgG4 to fibrinogen, we next determined if fibrinogen influenced the inhibitory effect of A2AP IgG1 on α2AP. A2AP IgG1 was titrated from 0-1 μM in the presence of 3 μM fibrinogen. Plasmin activity was assessed in the presence of α2AP ± fibrinogen or in the presence of fibrinogen without α2AP. As seen in Figure 21, plasmin activity in the presence of fibrinogen, but in the absence of α2AP is around 25 mOD/min regardless of the concentration of the A2AP IgG1 and was determined to compare plasmin activity in the presence of α2AP, fibrinogen and the A2AP IgG1. In the presence of α2AP, no plasmin activity is observed as expected. In the presence of increasing concentrations of A2AP IgG1, there is less α2AP-mediated inhibition of plasmin but not a complete removal of plasmin inhibition. However, in the presence of fibrinogen, the neutralizing effect of the A2AP IgG1 on α2AP appears to be enhanced with plasmin activity around 22 mOD/min. In conclusion, the potency of the A2AP IgG1 is enhanced in the presence of fibrinogen.

4.9 Effect of the A2AP IgG1 on plasmin- and t-PA-mediated clot lysis

Next it was determined whether the A2AP IgG1 antibody could accelerate clot lysis in a purified system by dose-dependently inactivating α2AP. Thrombin at 20 nM and plasmin at 50 nM were used to initiate clotting and clot lysis, respectively. Reactions were performed in the

A


A2AP IgG1
Control IgG
IC50: 26 ± 4.5 nM
IC50: 4.7 ± 0.7 nM
*
Plasmin activity (mOD/min)
[A2AP] (nM)
0
5
10
15
20
0
20
40
60
80
100

B

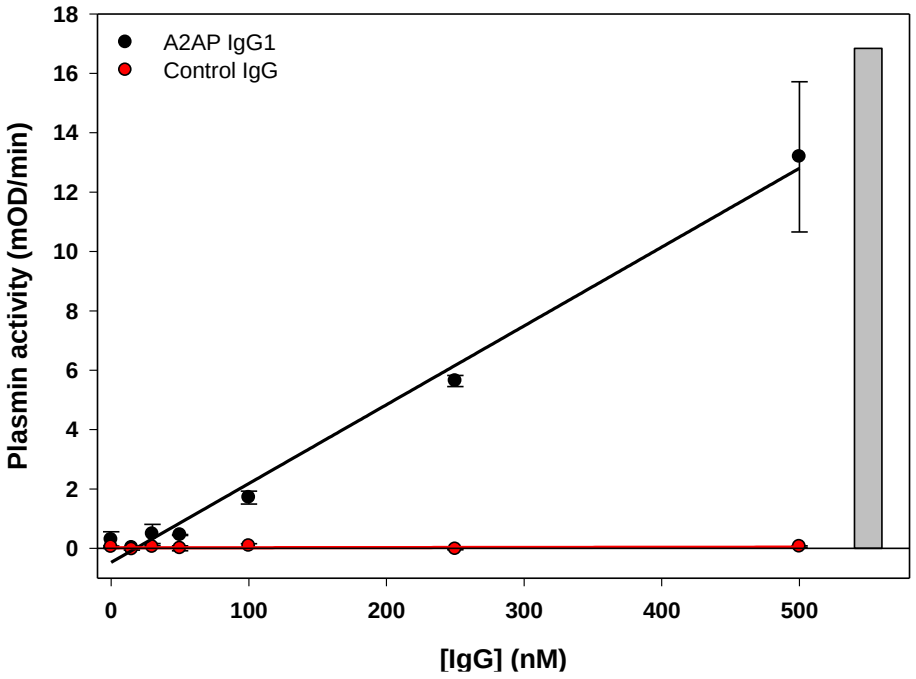

A2AP IgG1
Control IgG
Plasmin activity (mOD/min)
[IgG] (nM)
0
2
4
6
8
10
12
14
16
18
0
100
200
300
400
500

Figure 20: Effect of the A2AP IgG1 on plasmin inhibition by α2AP

(A) α2AP (0-100 nM) was titrated in the presence of 1 μM A2AP IgG1 or 1 μM Control IgG for 15 minutes at room temperature. Next, 25 nM of plasmin was incubated with these reactions for 15 minutes. After, chromogenic substrate S-2251 was added at a final concentration of 400 μM and plasmin cleavage was measured. (B) The A2AP IgG1 or the P4 IgG were titrated from 0-500 nM and incubated with 30 nM of human α2AP for 15 minutes. After which, 25 nM of plasmin was added and was incubated for 15 minutes, followed by the addition of 400 μM of S-2251. Upon the addition of S-2251 (A and B), absorbance was immediately read at 405 nm for one hour at 2 second intervals on a kinetic plate reader at room temperature. Shown are rates of pNA formation. Mean ± SE. n=3-4. *P=0.029.

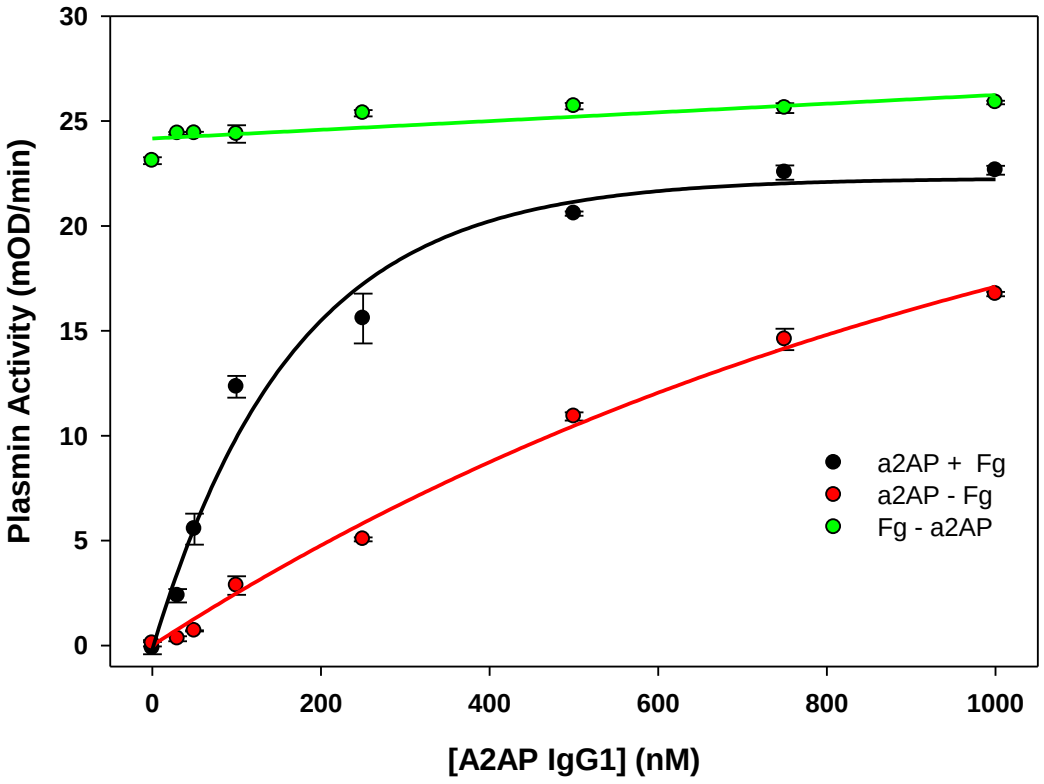
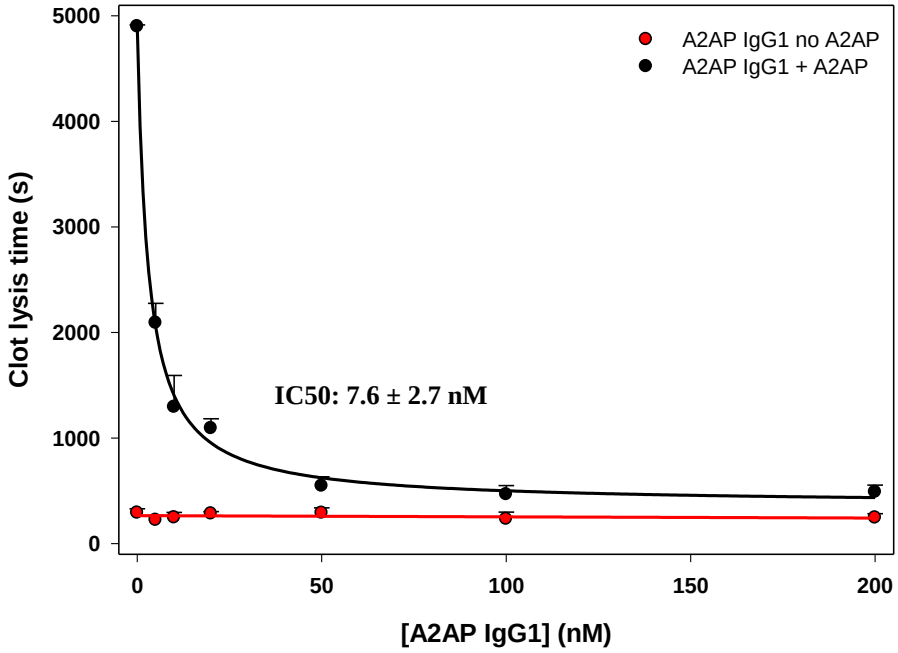

Plasmin Activity (mOD/min)
30
25
20
15
10
5
0
0
200
400
600
800
1000
[A2AP IgG1] (nM)
a2AP + Fg
a2AP - Fg
Fg - a2AP

Figure 21: Effect of the A2AP IgG1 on α2AP inhibition of plasmin in the presence of human fibrinogen

The A2AP IgG1 was titrated from 0-1000 nM in the presence of 3 μM fibrinogen. Plasmin activity was assessed in the presence of α2AP ± fibrinogen and in the presence of fibrinogen without α2AP. After mixing the relevant reagents, 25 nM of plasmin was added and was incubated for 15 minutes, followed by the addition of 400 μM of S-2251. Upon the addition of S-2251, absorbance was immediately read at 405 nm for one hour at 2 second intervals on a kinetic plate reader at room temperature. Shown are rates of pNA formation. Mean ± SE. n=3.

A


A2AP IgG1 no A2AP
A2AP IgG1 + A2AP
IC50: 7.6 ± 2.7 nM
Clot lysis time (s)
[A2AP IgG1] (nM)
0
1000
2000
3000
4000
5000
0
50
100
150
200

B

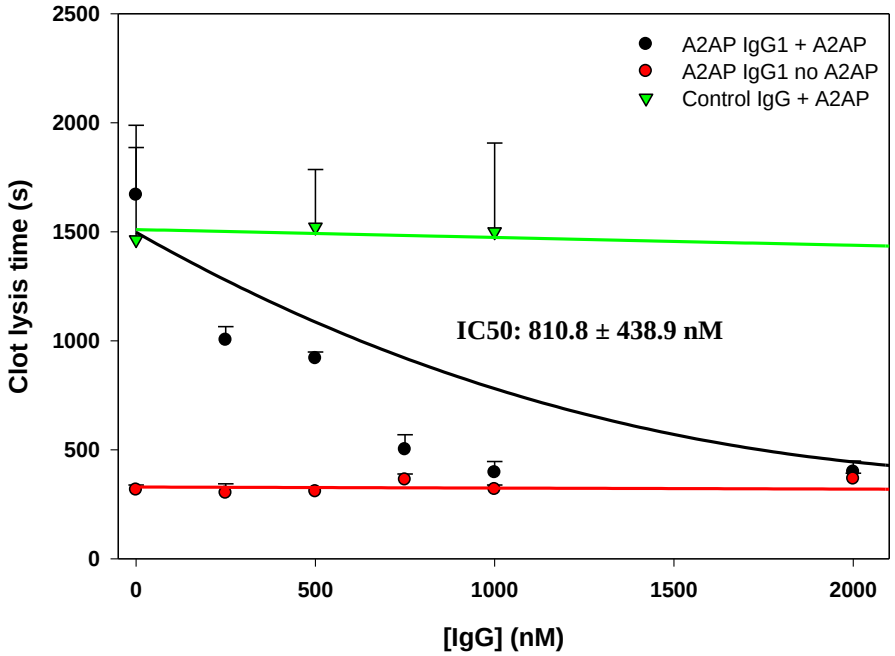

A2AP IgG1 + A2AP
A2AP IgG1 no A2AP
Control IgG + A2AP
IC50: 810.8 ± 438.9 nM
Clot lysis time (s)
[IgG] (nM)
0
500
1000
1500
2000
2500
0
500
1000
1500
2000

Figure 22: Effect of the A2AP IgG1 on plasmin- and t-PA-mediated human purified clot lysis.

(A) The A2AP IgG1 antibody was titrated in a human purified system to confirm its specificity and ability to accelerate clot lysis. The clot lysis was mediated by plasmin at 50 nM, thrombin at 20 nM and the A2AP IgG1, which was titrated from 0-5 μM. The reaction was initiated by addition of a solution containing final concentrations of 60 nM human α2AP, 3 μM fibrinogen, and 2 mM $CaCl_2$. As a control, the same conditions as the A2AP IgG1 antibody titration experiment was performed, but with no human α2AP. (B) The same experiments performed as in (A) with the clot lysis mediated by t-PA and plasminogen. 5 nM t-PA was used to facilitate clot lysis and the samples contained α2AP of 1 μM and contained 0.5 μM plasminogen. As a control, a non-specific IgG P4 antibody at the same concentrations as the A2AP IgG1 antibody was used. Also, the same control (no human α2AP) in (A) was also used. Absorbance was read at 405 nm at 37°C and clot lysis times were determined using instrument software and were defined as the time to reach half of the maximal absorbance in the reaction. The clot lysis times were plotted against the concentration of A2AP IgG antibody present in the reaction. Curves of best fit were determined. Mean ± SE. n=4.

presence or absence of 60 nM α2AP and the corresponding lysis times for these conditions were 5000 s and 700 s, respectively. A2AP IgG1 was titrated from 0-5 μM. As seen in Figure 22A, A2AP IgG1 in the presence of α2AP, was able to shorten clot lysis times in a dose-dependent manner. The clot lysis times in the absence of the A2AP IgG1 antibody were around 5000 s. With the A2AP IgG1, the clot lysis times maximally shortened at around 50 nM of antibody and at 700 s with an IC50 value of 7.6 ± 2.7 nM. In this experiment, a negative control was used to confirm the specificity of the A2AP IgG1 antibody. This control involved omitting α2AP, which resulted in clot lysis times that were around 300-400 s as the A2AP IgG1 had no effect on the clot lysis. In Figure 22B, the same experiment was performed with the lysis being mediated by t-PA instead of plasmin. Here, two controls were performed. First, the control IgG antibody at the same concentrations as the A2AP IgG1 antibody and second, omitting α2AP. These were run to confirm that any effect seen was because of the specificity of the A2AP IgG1.

The presence of α2AP increased the clot lysis time from 300 s to 1400 s. The lysis times were dose-dependently shortened with maximal response at ~ 1 μM and at roughly 500 s with an IC50 value of approximately 810.8 ± 438.9 nM. The control IgG resulted in lysis times that were unchanged in the presence of α2AP. The second negative control in this experiment, omitting α2AP, resulted in clot lysis times that were around 300-400 s. The A2AP IgG1 was significantly more potent in the plasmin-mediated clot lysis (P=0.029). In conclusion, these results demonstrate the specificity of the A2AP IgG1 and its ability to shorten clot lysis in a dose-dependent manner.

4.10 Effect of the A2AP IgG1 and A2AP IgG4 on t-PA-mediated clot lysis in human or rabbit plasma

A2AP IgG1 and IgG4 were examined for their effect on lysis of human or rabbit plasma clots. 20 nM thrombin was used to initiate clotting and 5 nM t-PA was used to initiate clot lysis. The raw data plot for the A2AP IgG4 titration in rabbit plasma is shown in Figure 23. Clotting occurs between 600-1000 s for all groups, followed by a decrease in absorbance consistent with lysis. Lysis times were determined and are plotted against the corresponding concentration of the A2AP IgG4. As seen in Figure 24A, A2AP IgG1 and IgG4 reduced lysis times in human plasma in a dose-dependent manner. The clot lysis times were maximally shortened at an antibody concentration of around 1 μM and at roughly 1000 s with IC50 values of 359.6 ± 34.1 nM and 161.9 ± 47.2 nM, respectively. These values are significantly different (P=0.027). In Figure 24B, the same effect is observed in rabbit plasma with both antibodies shortening clot lysis in a dose-dependent manner with IC50 values of 335 ± 24.9 nM for the A2AP IgG1 and 237 ± 19 nM for the A2AP IgG4. These values are also significantly different (P=0.037). In other experiments that are not shown, the P4 IgG had no effect, confirming the specificity of the A2AP IgG1 and IgG4 antibodies for human α2AP. Also, the A2AP IgG1 had non-significant differences in potencies between human and rabbit plasma (P=0.650). The same was true for the A2AP IgG4 (P=0.214). In conclusion, the potency of the A2AP IgG4 appears to be greater in human and rabbit plasma compared with the A2AP IgG1.

4.11 The effect of α2AP and fibrinogen peptides on A2AP IgG1-, IgG4-, and t-PA-mediated lysis in human plasma

α2AP reactive center loop (SRMSLSS) and fibrinogen α2AP-like (SWNSGSS) peptides were used to determine if they could compete with the A2AP IgG1 and IgG4 and reduce their

potencies. The fibrinogen peptide was chosen due to serine residues being located in the same positions as they are in the α2AP reactive center loop. Both peptides were titrated (0-20 μM) in the presence of 2 μM of the A2AP IgG1 or A2AP IgG4 in a plasma clot lysis assay. Thrombin at 20 nM and t-PA at 5 nM were used to initiate clotting and lysis, respectively. Human pooled plasma recalcified with 30 mM $CaCl_2$ was added simultaneously to all reaction wells to initiate the reaction. As seen in Figure 25, both the α2AP and fibrinogen α2AP-like peptides had no effect on the influence of the A2AP IgG1 on t-PA-mediated plasma clot lysis as all lysis times were around 1000 s similar to what was seen in Figure 24 at similar concentrations of antibody. However, with A2AP IgG4, clot lysis was reduced in the presence of only the α2AP peptide and not the fibrinogen peptide. The clot lysis times in the absence of α2AP peptide were around 1000s. However, in the presence of increasing concentrations of the α2AP peptide, the lysis times were eventually prolonged to around 2500 s. This demonstrates the specificity of the antibody for the reactive center loop of α2AP and also is consistent with the binding data from SPR in Figure 19 and Table 2 where the affinity of the A2AP IgG4 for α2AP was significantly greater compared with the A2AP IgG1.

4.12 Effect of the A2AP IgG1 and IgG4 in t-PA- and TNK-mediated lysis of preformed clots

Preformed clot lysis assays were performed to give an assessment of how the A2AP IgG1 and IgG4 affect lysis of preformed plasma clots as this is a more accurate representation of what would happen *in vivo*. Lysis was initiated with t-PA or TNK. Clots were formed on plastic inoculation loops with 400 μL of human pooled plasma containing 100 nM of FITC-fibrinogen and 30 mM $CaCl_2$.100 nM of thrombin was used to initiate clotting and the clot was allowed to mature for 30 minutes at room temperature. After washing the clots, they were immersed in a 1

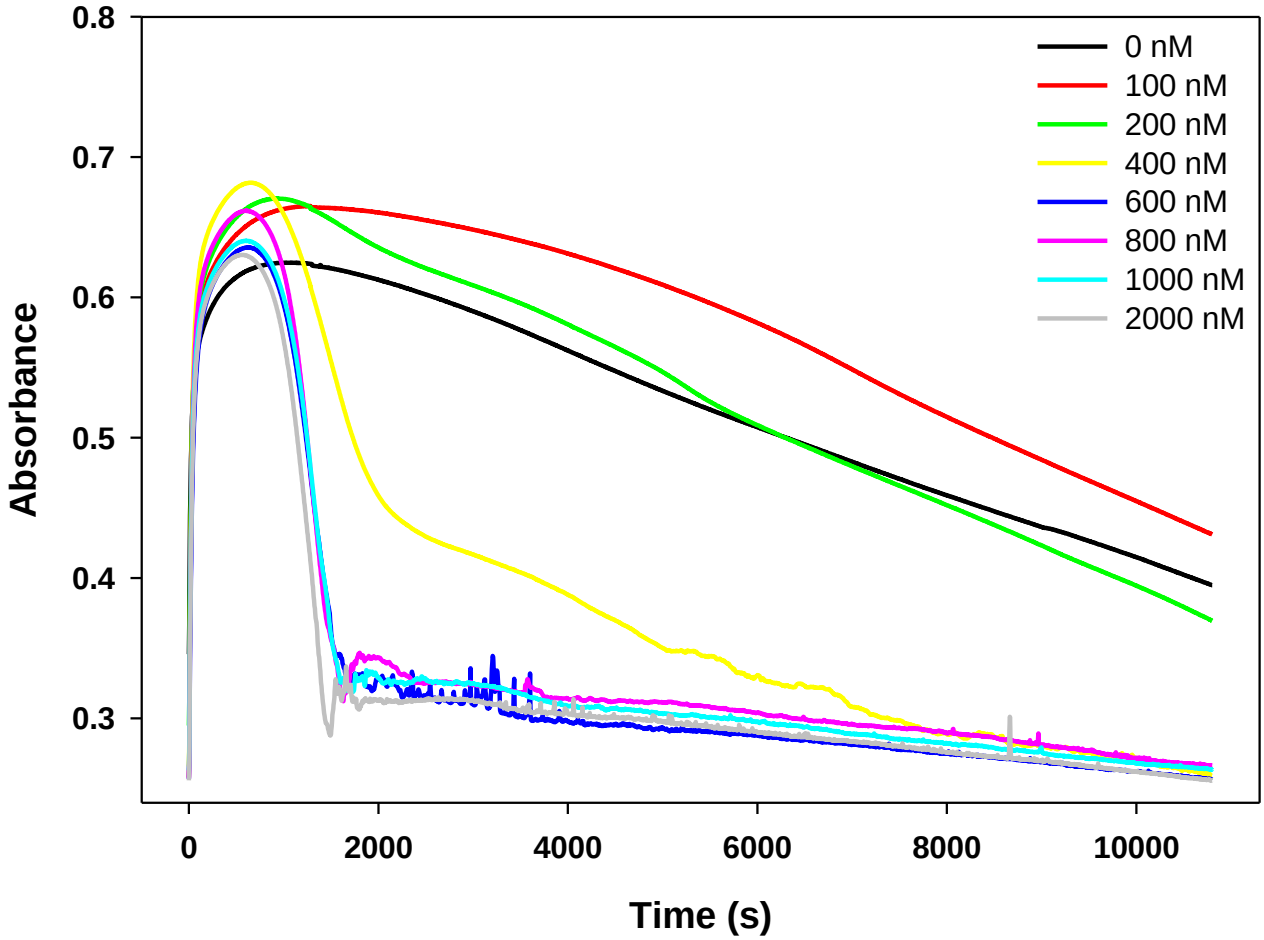
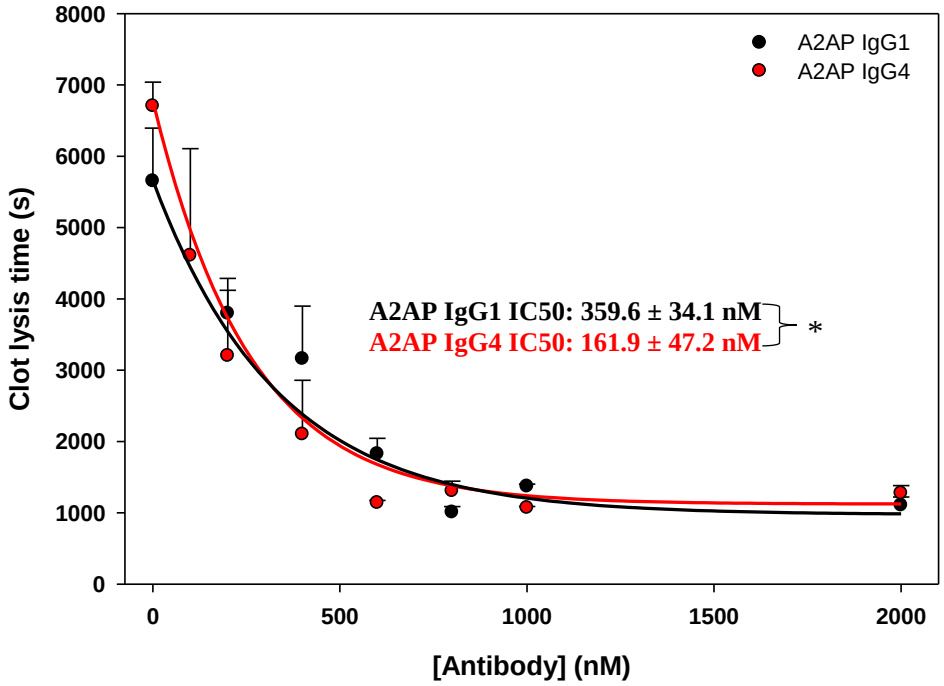

0.8
0.7
0.6
0.5
0.4
0.3
Absorbance
0
2000
4000
6000
8000
10000
Time (s)
0 nM
100 nM
200 nM
400 nM
600 nM
800 nM
1000 nM
2000 nM

Figure 23: Effect of the A2AP IgG4 on t-PA-mediated rabbit plasma clot lysis.

Clot lysis was evaluated in plasma from rabbits that received saline. Plasma samples were supplemented with 30 mM $CaCl_2$ were clotted with 20 nM thrombin in the presence of 5 nM of t-PA. The plate was read at 405 nm for three hours at 37°C. The A2AP IgG4 antibody was titrated to explore the effect it has on clot lysis times in a rabbit system. Absolute values were plotted against time after the addition of plasma. Representative plot is shown.

A


A2AP IgG1
A2AP IgG4
A2AP IgG1 IC50: 359.6 ± 34.1 nM
A2AP IgG4 IC50: 161.9 ± 47.2 nM
*
Clot lysis time (s)
[Antibody] (nM)
0
1000
2000
3000
4000
5000
6000
7000
8000
0
500
1000
1500
2000

B

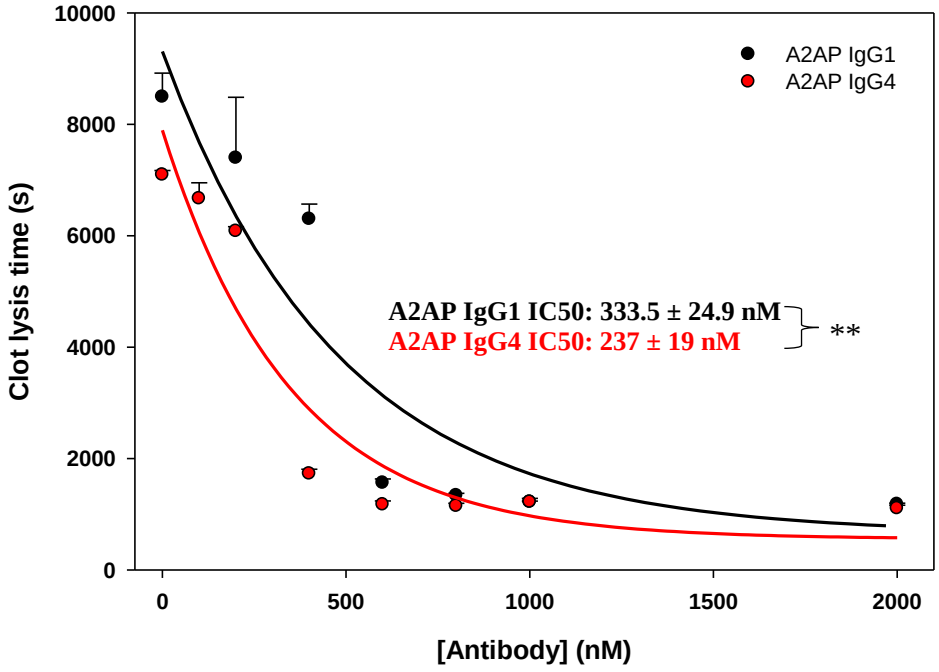

A2AP IgG1
A2AP IgG4
A2AP IgG1 IC50: 333.5 ± 24.9 nM
A2AP IgG4 IC50: 237 ± 19 nM
**
Clot lysis time (s)
[Antibody] (nM)
0
2000
4000
6000
8000
10000
0
500
1000
1500
2000

Figure 24: Effect of the A2AP IgG1 and IgG4 on t-PA mediated human and rabbit plasma clot lysis.

The A2AP IgG1 and IgG4 antibodies were titrated in human pooled plasma and untreated rabbit plasma to explore the effect it has on clot lysis times in a human and rabbit system. Thrombin at 20 nM and t-PA at 5 nM were used to stimulate clotting and initiate clot lysis, respectively. The A2AP IgG1 and IgG4 antibodies were titrated from 0-5 μM at various concentrations and loaded into the reaction wells. Human and rabbit pooled plasma contained 30 mM $CaCl_2$ and was added simultaneously to all reaction wells to initiate the reaction. Absorbance was read at 405 nm at 37°C and clot lysis times were determined using instrument software. The clot lysis times were plotted against the concentration of A2AP IgG antibody present in the reaction. Curves of best fit were determined. Mean ± SE. n=3. *P=0.027. **P=0.037.

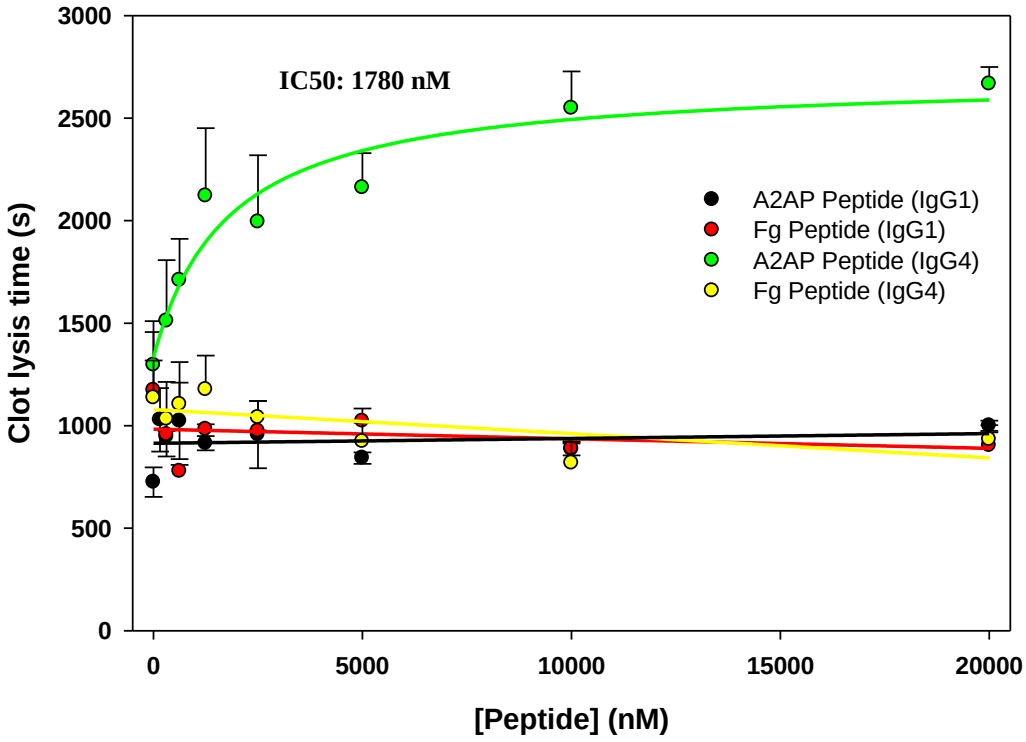
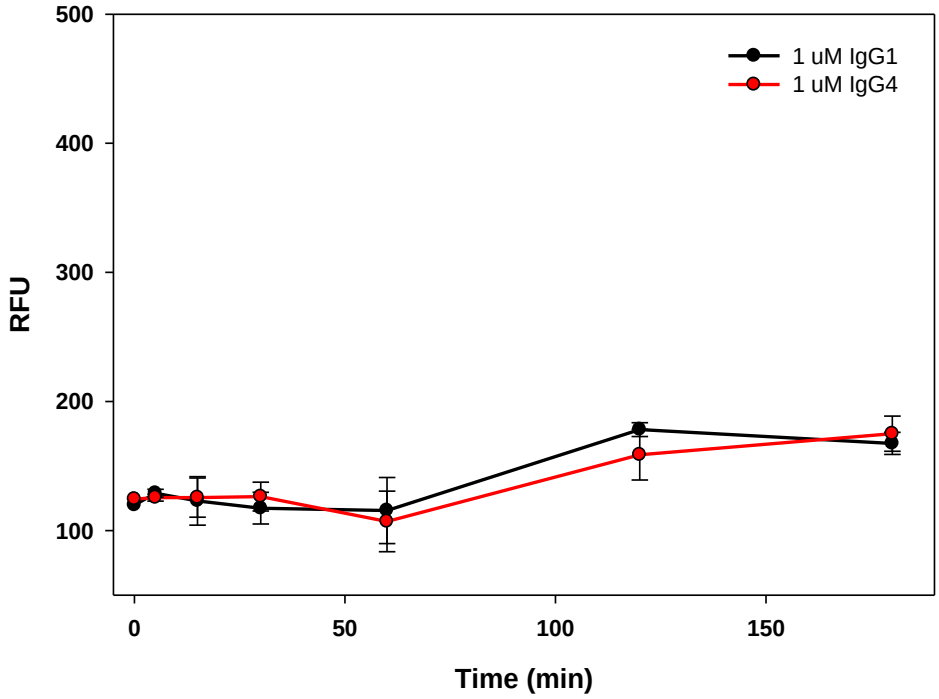

IC50: 1780 nM
A2AP Peptide (IgG1)
Fg Peptide (IgG1)
A2AP Peptide (IgG4)
Fg Peptide (IgG4)
Clot lysis time (s)
[Peptide] (nM)
0
500
1000
1500
2000
2500
3000
0
5000
10000
15000
20000

Figure 25: The effect of α2AP and fibrinogen peptides on A2AP IgG1-, IgG4-, and t-PA-mediated human plasma clot lysis.

α2AP reactive center loop (SRMSLSS) and fibrinogen α2AP-like (SWNSGSS) peptides were titrated (0-20 μM) in the presence of 2 μM of the A2AP IgG1 or A2AP IgG4. Thrombin at 20 nM and t-PA at 5 nM were used to initiate clotting and facilitate clot lysis, respectively. Human pooled plasma recalcified with 30 mM $CaCl_2$ was added simultaneously to all reaction wells to initiate the reaction. Absorbance was read at 405 nm at 37°C. The clot lysis times were plotted against the concentration of A2AP IgG antibody present in the reaction. Curves of best fit were determined. Mean ± SE. n=3-5.

mL bathing solution of human plasma containing 1 μM of the A2AP IgG1 or IgG4 and in combination with 1 or 10 nM of t-PA or TNK. At fixed intervals, 250 μL aliquots were taken from the bathing solution of human plasma to measure the FITC-FDPs. As seen in Figure 26A, neither antibody had an effect on plasma clot lysis in the absence of a plasminogen activator. Relative to the control, both antibodies resulted in less than 0.5% clot lysis and they were not statistically significant from one another (Figure 26B). In Figure 27A, profiles show linear increase in RFU consistent with lysis and in Figure 27B, percent lysis values were calculated based on total fluorescent units present. In Figure 27A and B, 1 nM of t-PA alone achieved 27% clot lysis after three hours. The combination of 1 nM t-PA with 1 μM of the A2AP IgG1 or IgG4 both achieved around 55% clot lysis. The combination groups were both statistically significant from the 1 nM t-PA alone group (P=0.013 and P=0.01 for the A2AP IgG1, and IgG4 respectively), but not statistically significant from each other. 10 nM t-PA alone achieved 47% clot lysis after three hours compared to 27% with 1 nM t-PA. The combination of 10 nM t-PA with 1 μM of the A2AP IgG1 or IgG4 achieved around 86% and 91% clot lysis, respectively. These combination groups were both statistically significant from the 10 nM t-PA alone group (P=<0.001 for both the A2AP IgG1 and IgG4), but not statistically significant from each other. Also, the combination of 1 nM t-PA with 1 μM of the A2AP IgG4 was significantly greater than 10 nM t-PA alone (P=0.037). This suggests that a greater thrombolytic result can be achieved with a lower dose of t-PA when combined with an inhibitor to α2AP. TNK was used in separate experiments to confirm its thrombolytic effect as it was selected as the plasminogen activator for the *in vivo* thrombolysis study. In Figure 28A and B, 1 nM TNK alone achieved 14% clot lysis after three hours, which was less than 1 nM t-PA. The combination of 1 nM TNK with 1 μM of the A2AP IgG1 or IgG4 both achieved around 70% clot lysis, which was greater than the t-PA

A


500
400
300
200
100
RFU
0
50
100
150
Time (min)
1 uM IgG1
1 uM IgG4

B

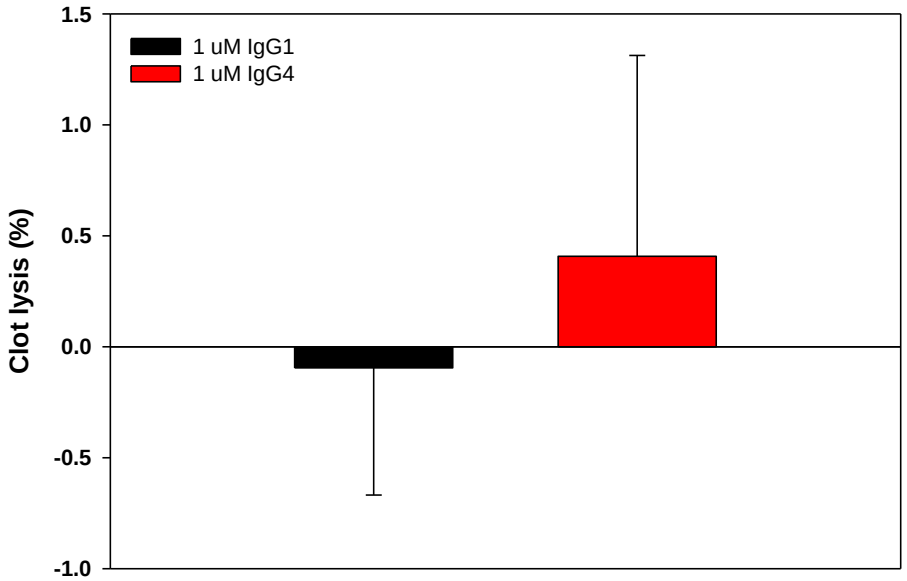
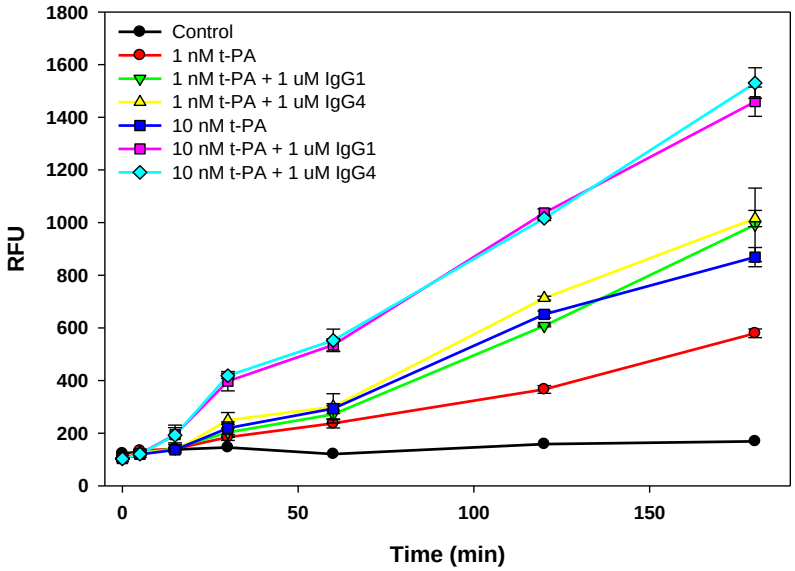

1.5
1.0
0.5
0.0
-0.5
-1.0
Clot lysis (%)
1 uM IgG1
1 uM IgG4

Figure 26: Effect of A2AP IgG1 and IgG4 on lysis of preformed human plasma clots.

The A2AP IgG1 and IgG4 antibodies were tested in the absence of any plasminogen activator in preformed clot lysis assays. In each experiment, 400 μL of human pooled plasma containing 100 nM of FITC-fibrinogen and 30 mM $CaCl_2$ was clotted with 100 nM of thrombin for 30 minutes at room temperature on an inoculation loop. The clot was washed in TBS-0.01% Tween then immediately placed in a 1 mL bathing solution of human plasma containing 1 μM of the A2AP IgG1 or the A2AP IgG4., 250 μL aliquots were taken from the bathing solution of human plasma before addition of the plasma clot, and at 5, 15, 30, 60, 120, and 180 minutes after the addition of the plasma clot and read using a fluorescent plate. The excitation and emission wavelengths were 485 and 538 nm, respectively. The fluorescence was plotted against the time after addition of the plasma clot to the bathing solution. Mean ± SE. n=3.

A


Control
1 nM t-PA
1 nM t-PA + 1 uM IgG1
1 nM t-PA + 1 uM IgG4
10 nM t-PA
10 nM t-PA + 1 uM IgG1
10 nM t-PA + 1 uM IgG4
RFU
Time (min)
0
200
400
600
800
1000
1200
1400
1600
1800
0
50
100
150

B

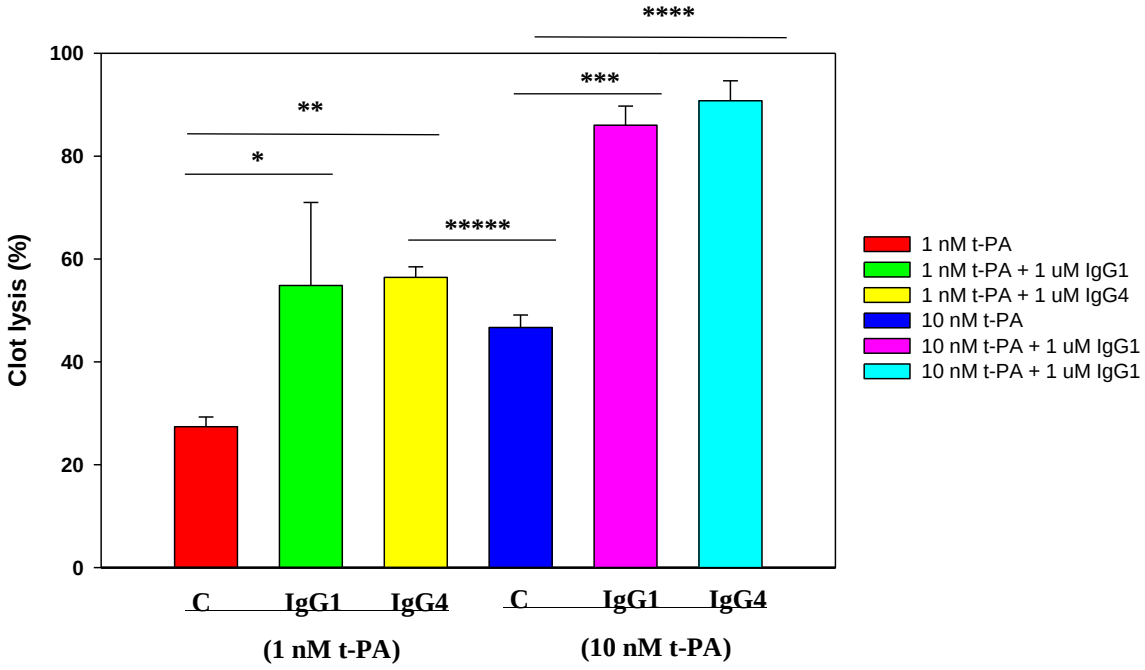
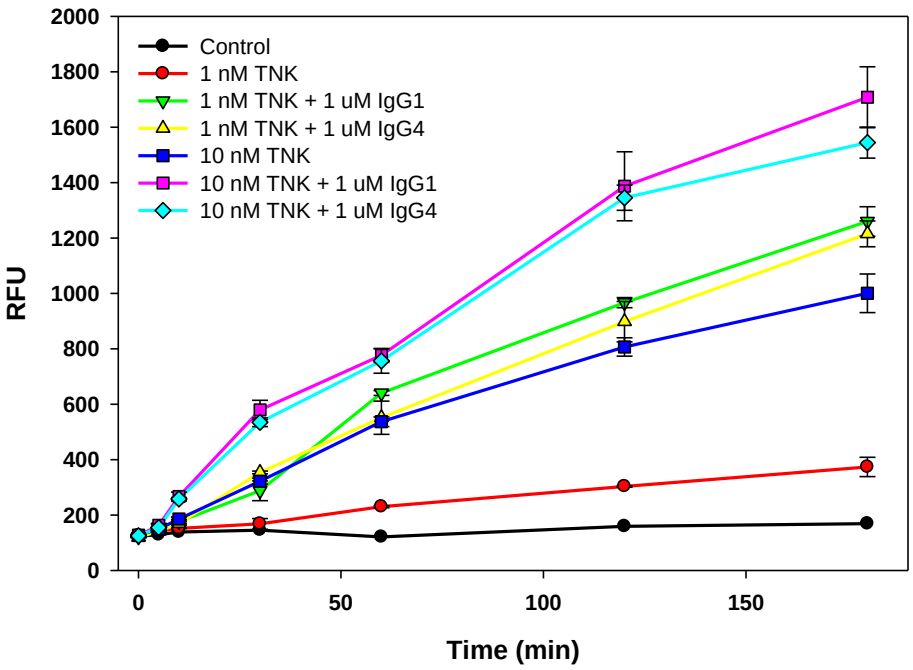

Clot lysis (%)
0
20
40
60
80
100
*
**
***
****
*****
C
IgG1
IgG4
C
IgG1
IgG4
(1 nM t-PA)
(10 nM t-PA)
1 nM t-PA
1 nM t-PA + 1 uM IgG1
1 nM t-PA + 1 uM IgG4
10 nM t-PA
10 nM t-PA + 1 uM IgG1
10 nM t-PA + 1 uM IgG1

Figure 27: The effect of the A2AP IgG1 and IgG4 on t-PA-mediated preformed plasma clot lysis.

The effect of A2AP IgG1 and IgG4 antibodies on lysis of preformed clot lysis was assessed in the presence of t-PA. 400 μL of human pooled plasma containing 100 nM of FITC-fibrinogen and 30 mM $CaCl_2$ was clotted with 100 nM of thrombin for 30 minutes at room temperature on an inoculation loop. The clot was then washed in TBS-0.01% Tween then immediately placed in a 1 mL bathing solution of human plasma containing 1 μM of the A2AP IgG1 and IgG4 in the presence of 1 or 10 nM of t-PA. After, 250 μL aliquots were taken from the bathing solution of human plasma before addition of the plasma clot, 5, 15, 30, 60, 120, and 180 minutes after the addition of the plasma clot and read using a fluorescent plate reader. The excitation and emission wavelengths were 485 and 538 nm, respectively. The fluorescence was plotted against the time after addition of the plasma clot to the bathing solution. Calculations are plotted in B. Mean ± SE. n=3. *P=0.013. **P=0.01. ***, ****P=<0.001.*****P=0.037.

A


Control
1 nM TNK
1 nM TNK + 1 uM IgG1
1 nM TNK + 1 uM IgG4
10 nM TNK
10 nM TNK + 1 uM IgG1
10 nM TNK + 1 uM IgG4
RFU
Time (min)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
0
50
100
150

B

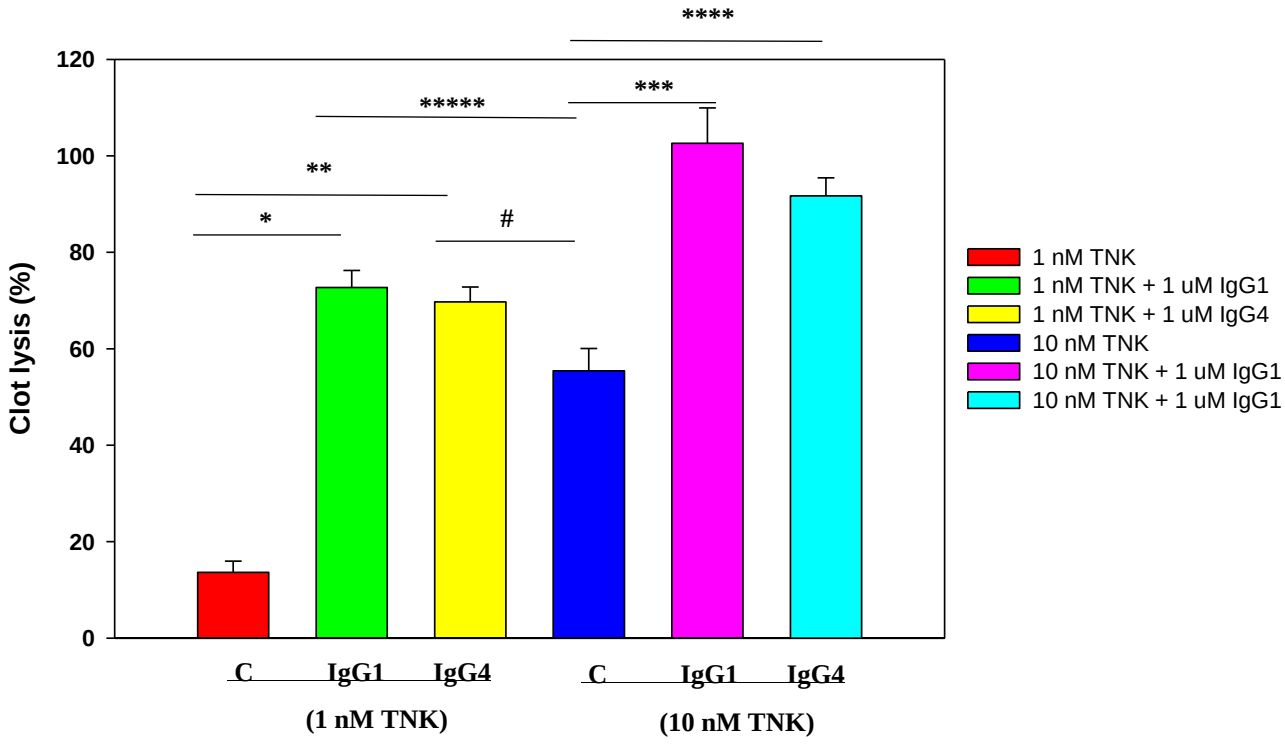

Clot lysis (%)
0
20
40
60
80
100
120
*
**
*****
***
****
#
C
IgG1
IgG4
(1 nM TNK)
C
IgG1
IgG4
(10 nM TNK)
1 nM TNK
1 nM TNK + 1 uM IgG1
1 nM TNK + 1 uM IgG4
10 nM TNK
10 nM TNK + 1 uM IgG1
10 nM TNK + 1 uM IgG1

Figure 28: The effect of the A2AP IgG1 and IgG4 on TNK-mediated preformed plasma clot lysis.

The effect of A2AP IgG1 and IgG4 antibodies on lysis of preformed clot lysis was assessed in the presence of TNK. 400 μL of human pooled plasma containing 100 nM of FITC-fibrinogen and 30 mM $CaCl_2$ was clotted with 100 nM of thrombin for 30 minutes at room temperature on an inoculation loop. The clot was then washed in TBS-0.01% Tween then immediately placed in a 1 mL bathing solution of human plasma containing 1 μM of the A2AP IgG1 and IgG4 in the presence of 1 or 10 nM of TNK. After, 250 μL aliquots were taken from the bathing solution of human plasma before addition of the plasma clot, 5, 15, 30, 60, 120, and 180 minutes after the addition of the plasma clot and read using a fluorescent plate reader. The excitation and emission wavelengths were 485 and 538 nm, respectively. The fluorescence was plotted against the time after addition of the plasma clot to the bathing solution. Mean ± SE. n=3. *, **P=<0.001. ***P=<0.001. **** P=0.003. *****P=0.018. #P=0.038.

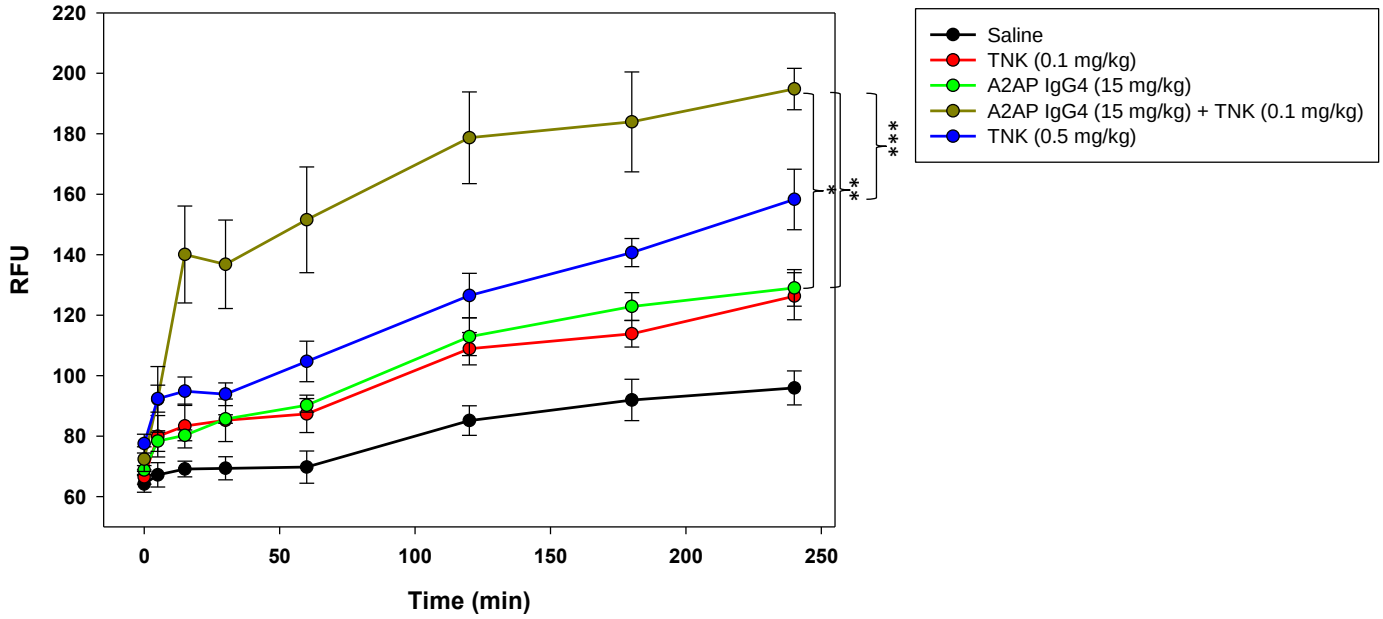
combinations. The combination groups were both statistically significant from the 1 nM TNK alone group (P=<0.001 for both the A2AP IgG1 and IgG4), but not statistically significant from each other. 10 nM TNK alone achieved 55% clot lysis after four hours, compared to 14% of 1 nM TNK. The combination of 10 nM TNK with 1 μM of the A2AP IgG1 or IgG4 achieved around 100% and 91% clot lysis, respectively. These combination groups were both statistically significant from the 10 nM TNK alone group (P=<0.001 and P=0.003 for the A2AP IgG1 and IgG4, respectively), but not statistically significant from each other. Also, the combination of 1 nM TNK with 1 μM of the A2AP IgG1 and IgG4 were statistically significantly greater than 10 nM TNK alone (P=0.018 and 0.038, respectively). In conclusion, neither antibody alone has an effect on clot lysis, however a synergistic response is observed when either antibody is combined with t-PA or TNK. A lower dose of t-PA or TNK combined with either antibody achieves equal or greater clot lysis compared to a higher dose of t-PA or TNK alone.

4.13 *In vivo* efficacy of the A2AP IgG4 in a rabbit thrombosis model

To assess the in vivo efficacy and thrombolytic potential of the A2AP IgG4, rabbit thrombolysis studies were performed (Weitz et al., 1993). The A2AP IgG4 was chosen due to its greater affinity for α2AP and its reduced immunogenicity compared with the A2AP IgG1. As seen in Figure 29A, all groups with the exception of the saline controls display a rapid increase in fluorescence indicative of initiation of clot lysis within the first 15 minutes, with slower lysis over the duration of the experiment. By analyzing the area under the curve, it was determined these data were significantly different (P=<0.001). The combination group was significantly different than 15 mg/kg of A2AP IgG4, 0.1 mg/kg of TNK, and 0.5 mg/kg TNK alone. The percent lysis after 4 hours was then determined and plotted in Figure 29B. A correction was made to omit the background signal of the plasma. At 4 hours, the saline control exhibited

around 12% lysis which could likely be attributed to endogenous lysis mechanisms or embolization of the clot fragments. Next, 0.1 mg/kg TNK dose showed approximately 20%, the 0.5 mg/kg TNK dose showed approximately 30% and the A2AP IgG4 alone showed approximately 23% (Figure 29B). The combination of 20% therapeutic TNK and A2AP IgG4 achieved 41% lysis which was significantly greater than, the 20% therapeutic TNK dose alone or the A2AP IgG4 alone ($P=0<0.001$). Also, the combination group achieved significantly greater lysis than the therapeutic TNK dose alone ($P=0.007$). Bleeding was assessed in standardized ear incisions (Weitz *et al.*, 1993). The saline control resulted in the least volume of blood loss, around 300 μL. Amounts for the therapeutic dose of TNK were 624 μL, the 20% therapeutic dose 248 μL, and the A2AP IgG4 alone 233 μL. The combination group showed roughly 640 μL (Figure 30). None of these groups were statistically significant from one another due to the high degree of variation ($P=0.219$). As another test of specificity, the integrity of circulating fibrinogen was assessed by western blot using an anti-rabbit Fg antibody. Four hours post-treatment, no treatment showed consumption of systemic fibrinogen which was similar to saline controls (Figure 31).

A


RFU
Time (min)
Saline
TNK (0.1 mg/kg)
A2AP IgG4 (15 mg/kg)
A2AP IgG4 (15 mg/kg) + TNK (0.1 mg/kg)
TNK (0.5 mg/kg)
***
***
***

B

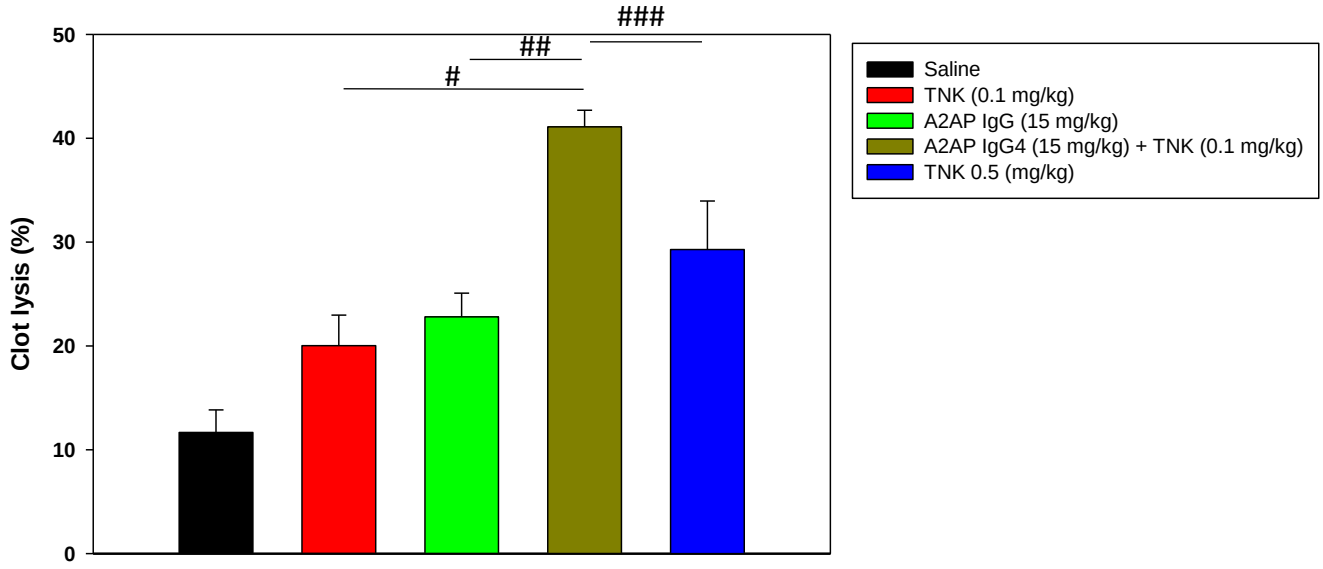

Clot lysis (%)
###
##
#
Saline
TNK (0.1 mg/kg)
A2AP IgG (15 mg/kg)
A2AP IgG4 (15 mg/kg) + TNK (0.1 mg/kg)
TNK 0.5 (mg/kg)

Figure 29: Extent of clot lysis in a rabbit thrombosis model.

To assess the in vivo efficacy and thrombolytic potential of the A2AP IgG4, rabbit thrombosis studies were performed. Male New Zealand white rabbits were given a mixture containing 8 nM human α-thrombin, 10 μM of FITC-fibrinogen, and homologous rabbit plasma in their jugular vein. After clotting was allowed for 30 minutes, blood flow through the jugular vein was restored and the rabbits were given differing doses of the A2AP IgG4 and TNK alone and also a combination of the two. Blood samples were collected to measure the plasma fluorescence (A) over time (before treatment, 5, 15, 30, 60, 120, 180 and 240 min after treatment), with increasing fluorescence suggesting more thrombolysis. (B) Percent clot lysis calculations are plotted. Mean ± SE. n=5-6. *,**,***P=<0.001. #, ##P=<0.001. ###P=0.007.

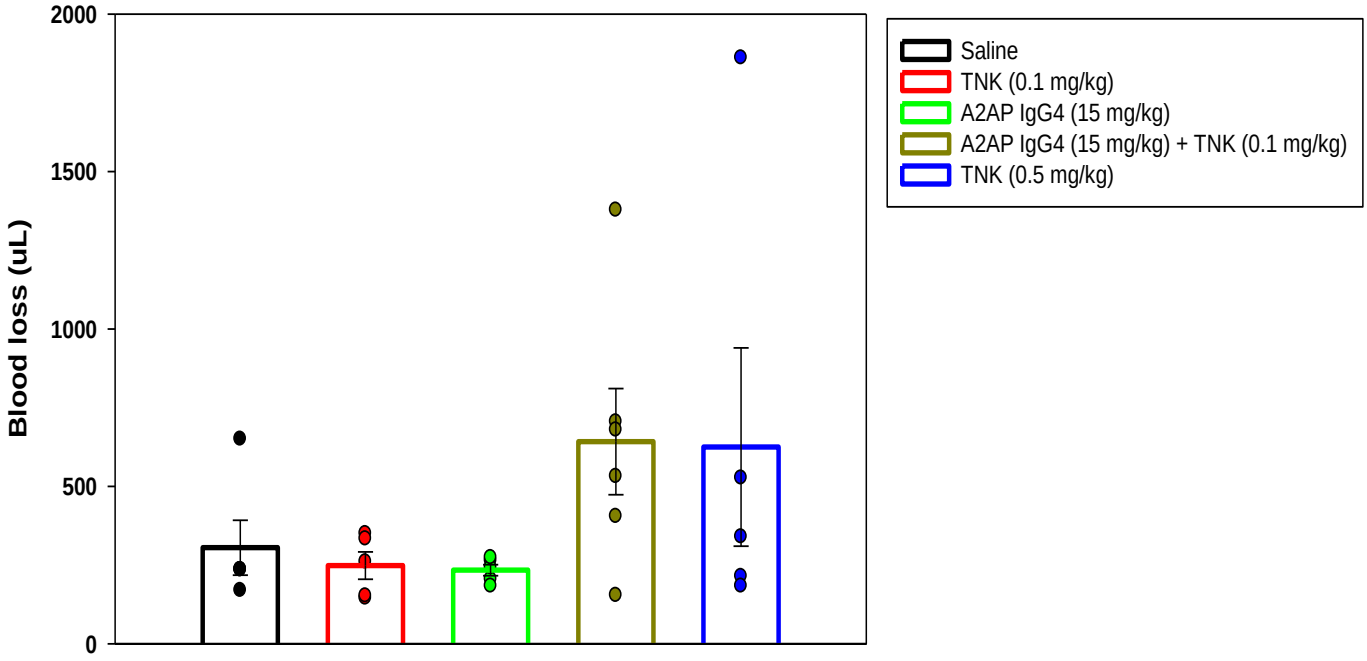

2000
1500
1000
500
0
Blood loss (uL)
Saline
TNK (0.1 mg/kg)
A2AP IgG4 (15 mg/kg)
A2AP IgG4 (15 mg/kg) + TNK (0.1 mg/kg)
TNK (0.5 mg/kg)

Figure 30: Bleeding in a rabbit thrombosis model.

In rabbits undergoing thrombolysis experiments, ear bleeding was quantified. At 60 minutes following collection of a blood sample, three 9-mm full-thickness incisions were made in the left ear which was then immediately immersed in a beaker containing 1 L of sterile saline maintained at 37°C. After one hour, three 10 mL aliquots of saline were be removed. Hemoglobin was assessed by BD lysis buffer assay and quantified by comparison with a standard curve prepared with known quantities of blood. Mean ± SE. n=5-6.

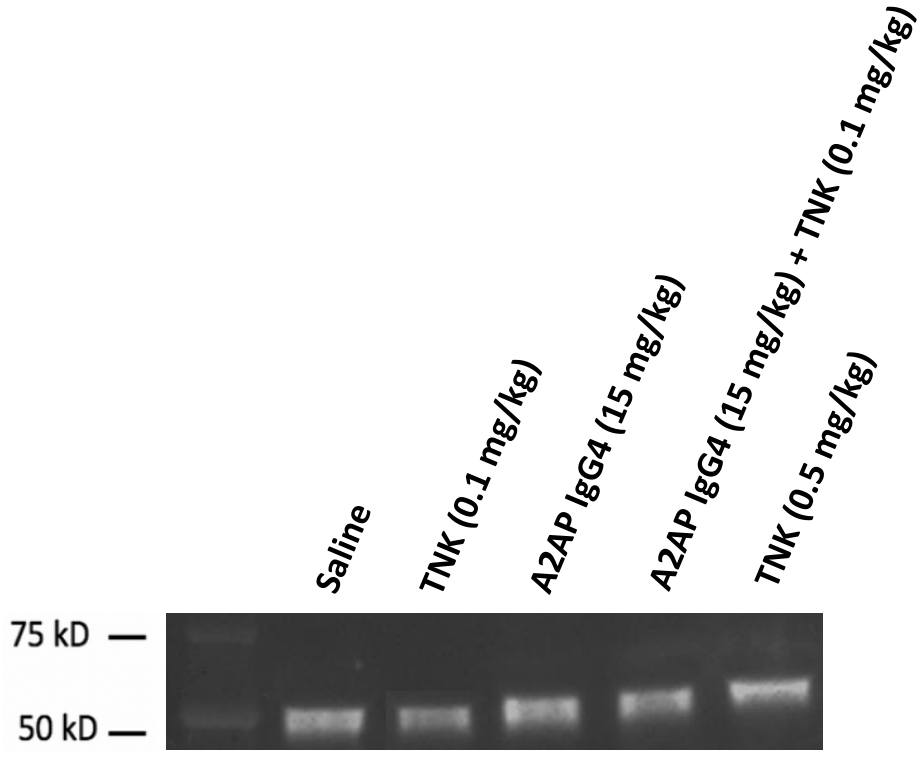

Saline
TNK (0.1 mg/kg)
A2AP IgG4 (15 mg/kg)
A2AP IgG4 (15 mg/kg) + TNK (0.1 mg/kg)
TNK (0.5 mg/kg)
75 kD
50 kD

Figure 31: Fibrinogen degradation in a rabbit thrombosis model.

In rabbits undergoing thrombolysis experiments, the integrity of systemic fibrinogen was analyzed by SDS-Page and western blots. Experiments were performed on plasma samples from the 240-minute time-point from rabbits. The primary antibody was a polyclonal rabbit anti-human fibrinogen antibody and the secondary antibody was a purified polyclonal anti-rabbit peroxidase antibody. Representative image shown. n=5-6.

Chapter 5: Discussion

5.1 Overview

Thrombosis is a leading cause of mortality and morbidity worldwide and can present in several forms including MI, AIS and VTE in both arterial and venous circulation (Perler, 2005, Fredenburgh and Weitz, 2018). Many therapies have been developed to treat these disorders, but several issues still remain which justifies the need for new anticoagulant and thrombolytic therapies. Antithrombotic strategies typically involve anticoagulants such as heparin and warfarin, or fibrinolytics such as rt-PA and TNK. Despite the availability of these treatments, they are limited in use due to fatal or disabling bleeding complications (Perler, 2005).

In terms of thrombolytic therapy, the focus has been to activate the fibrinolytic system with the goal of rapid restoration of blood flow in ischemic tissue. Typically, t-PA and plasminogen bind to fibrin clots which leads to activation of plasminogen to plasmin and subsequent degradation of fibrin. SK, which is a plasminogen activator, was the first drug to be approved by the FDA for PE and MI but was associated with an immune response (Hirsh *et al*., 1968, Perler, 2005). UK, another plasminogen activator, is a thrombolytic drug that was used to treat PE. Both however, lead to hemorrhagic complications due to their non-fibrin specific effects which limited therapeutic applications. This led to the desire to develop new thrombolytic agents that would theoretically only act on pathological thrombi to reduce bleeding complications. In the 1980s, genetic sequencing of t-PA and recombinant DNA technology made the mass production of fibrin specific agents such as rt-PA possible. The current treatment of MI, AIS, and some PE involves using the fibrin specific agent alteplase, a rt-PA (Perler, 2005). Despite this, clinical data demonstrates that rt-PA is still associated with the risk of bleeding (The GUSTO Investigators, 1993, Kohn, 1992). Also, plasminogen activation by rt-PA is more

efficient in the presence of the D-dimer-E complex, a crosslinked fibrin degradation product. Systemic plasmin can then degrade fibrinogen which releases fragment X, a clottable fibrinogen degradation product. Fragment X can be incorporated into hemostatic plugs elsewhere and contribute to unexpected bleeding (Weitz, 1995). As a result of these unintended effects, roughly 6% of patients receiving rt-PA develop ICH (Nelson *et al*., 2019). TNK, another plasminogen activator which is more specific to fibrin, more resistant to PAI-1, and has a longer half-life, was developed to address these negative side effects (Nelson *et al*., 2019, Thiebaut *et al*., 2018). TNK has demonstrated improved efficacy relative to rt-PA in the context of MI treatment and is recommended in the context of AIS. As a result, TNK was chosen to be evaluated alone and in combination with the A2AP IgG4 in the rabbit thrombosis model. Despite the availability of TNK, only 5-10% of patients requiring thrombolytic care receive thrombolysis (Nelson *et al*., 2019). Thus, reducing the amount of exogenous plasminogen activator administered to patients while combining it with other thrombolytic agents presents as an attractive alternative to thrombolytic therapy.

α2AP is a potent inhibitor of plasmin and thus a key regulator of fibrinolysis. Deficiencies of α2AP are inherited in an autosomal recessive fashion and may result in a bleeding disorder. Homozygotic patients possess less than 2% of normal α2AP and display a severe bleeding tendency including joint hemorrhages. Heterozygotic patients possess 40-60% of normal α2AP levels and this may result in a mild bleeding tendency (Abildgaard *et al*., 1993, Owen *et al*., 2005, Griffin *et al*., 1982). Thus, the effect that modulating α2AP levels has on fibrinolysis is clear. Due to these findings and the relatively high concentration (~ 1 μM) of α2AP in plasma, α2AP has been identified as a potential target for thrombolytic therapy to enhance fibrinolysis with the goal of dissolving thrombi present in thrombotic diseases (Lijnen

and Collen, 1995, Lijnen, 2001, Houng *et al*., 2016). The advantage would be to reduce the amount of PA necessary and thus bleeding, to facilitate clot lysis by simultaneously inhibiting the plasmin inhibitor, α2AP.

In this study, we aimed to characterize the A2AP IgG1 and A2AP IgG4 antibodies in experimental development by Bayer AG (unpublished) to determine if these antibodies and this method of targeting α2AP could serve as a viable thrombolytic therapy. The A2AP IgG1 against human α2AP was selected using a phage display of a library (BioInvent n-CoDeR) of syntethic human antibody genes with human and rabbit α2AP as the antigen. Affinity maturation was performed by altering amino acid residues in the complement-determining region of the Fab portion of the A2AP IgG1. The Fc portion of the A2AP IgG1 was subsequently changed to an IgG4 isotype to reduce the potential immunogenicity of the antibody, which yielded A2AP IgG4. The antibodies were tested *in vitro* using various binding, activity, and clot lysis assays as well as *in vivo* using a rabbit thrombosis model.

5.2 ASO Study

Administering ASOs against α2AP to rabbits was performed to validate the approach of inhibiting α2AP to accelerate clot lysis by attenuating plasmin inhibition. Initially, three ASOs were examined for the purpose of selecting one for further experimentation. Western blotting and densitometry analysis confirmed that the amount of α2AP protein remaining at the wk4 time-points was the lowest for all three ASOs compared with the other two time-points. Specifically, relative to plasma from saline treated rabbits, plasma from ASO2 and ASO4 treated rabbits showed the greatest reduction in α2AP levels with 1.3% and 0.37% α2AP remaining, respectively. This level of protein reduction is consistent with ASOs for other targets in the coagulation system (Yau *et al*., 2014, Dias and Stein, 2002). These results correlated with the *in*

vitro clot lysis assays using plasma from rabbits treated with each ASO, that demonstrated progressively shortened clot lysis times toward the wk4 time-point. This suggests that α2AP deficiencies resulting from ASO administration led to quicker clot lysis and supports the idea of inhibiting α2AP to accelerate lysis of clots. α2AP-deficient rabbit plasma from rabbits that received ASOs was used in later experiments to characterize the A2AP IgG. It was determined that concentrations of t-PA below 4 nM were required to differentiate normal and α2AP-deficient plasma from ASO treated rabbits. This likely results from rapid activation of plasminogen at t-PA concentrations above 5 nM that overwhelms the ability of α2AP to regulate clot lysis. However, t-PA concentrations below 4 nM displayed inconsistent lysis times in normal rabbit plasma. To address this, 5 nM t-PA generated consistent lysis times and was thus chosen for further experimentation. Although clot lysis began prior to full clot formation in some cases, the effect of α2AP deficiency was clear. Our results in the rabbit plasma are also supported by the fact that reintroducing human α2AP back into α2AP-deficient rabbit plasma, restored clot lysis times in a dose-dependent manner similar to clot lysis times seen using plasma from rabbits treated with saline. In fact, when adding back α2AP to a concentration of 1 μM, clot lysis was completely inhibited within the two-hour experiment, demonstrating the potential effect that modulating α2AP levels might have. The fact that normal rabbit plasma still lysed under these conditions demonstrates the potent effect that altering α2AP levels can have on clot lysis. However, the observed effect could have been due to an over estimation of the amount of α2AP added back or the fact that human α2AP was added to a rabbit system. Despite this, these experiments support the idea of reducing α2AP function to increase plasmin activity and thereby accelerate fibrinolysis.

5.3 Specificity of A2AP IgG1 and A2AP IgG4

The A2AP IgG was selected using a phage display of a synthetic human antibody gene library (BioInvent n-CoDeR) with human and rabbit α2AP as the antigen. After a series of Fab purification steps assessing plasmin activity, 14 IgGs were produced. Affinity for α2AP was measured using SPR and ELISA and the lead candidate, A2AP IgG was selected with the reactive center loop being the epitope. Initially, the specificity of the antibodies was examined by western blotting to characterize binding to various proteins. The A2AP IgG1 and IgG4 antibodies failed to bind human and rabbit α2AP but did bind the PAP. Inability to bind human α2AP in western blotting is likely because the reactive center loop was unexposed due to how α2AP was transferred to the membrane. As a result of these antibodies being monoclonal, no binding would occur if the reactive center loop was inaccessible. Binding to PAP likely occurred because cleavage by plasmin changed the conformation of α2AP, exposing its epitope. Binding to fibrinogen was unexpectedly observed and could have been mediated through the αC domain, where α2AP is known to be cross-linked (Sakata and Aoki, 1980, Mosesson *et al*., 2008, Fraser *et al*., 2011). However, both antibodies also bound the β chain of fibrinogen and fragment X, which lacks the αC domains. The A2AP IgG1 and IgG4 antibodies also detected a faint band in human plasma. However, due to α2AP and the α-chain of fibrinogen running at approximately the same molecular weight, it was difficult to distinguish between the two. In rabbit plasma, the antibodies detected similar bands that migrated at molecular weights different than the bands detected by the affinity purified polyclonal α2AP IgG and fibrinogen IgG antibodies, making it difficult to determine their identity.

Sandwich ELISA was performed to validate the results from western blotting. The ELISA showed that the antibodies can bind α2AP, unlike in western blotting. It is important to

note that a direct ELISA approach where α2AP was coated to the well and detected with the A2AP IgG1 and IgG4 was unsuccessful. Sandwich ELISA was also performed where the A2AP IgG1 was used as the capture and detector antibody and was unsuccessful. Also, the ELISA confirmed the ability of both antibodies to bind the PAP complex, which was dose-dependent. In addition, dose-dependent binding of fibrinogen using a direct and sandwich approach was observed. Binding to fragment X was also observed in ELISA. Together, these results support the requirement for exposure of the reactive center loop to enable binding to the A2AP IgG1 and IgG4. Also, these results suggest non-specific interactions with fibrinogen. In SPR, we determined that the A2AP IgG4 had a significantly greater affinity for α2AP compared with the A2AP IgG1. We also found that in SPR, the A2AP IgG1 and IgG4 both bound to fibrinogen and fragment X.

Fibrinogen-α2AP complexes were made to determine whether A2AP IgG1 and IgG4 binding to fibrinogen seen in western blotting, ELISA and SPR was mediated through the αC domain of fibrinogen or non-specific interactions. The αC domain is where α2AP would be cross-linked. In western blotting, a polyclonal α2AP antibody detected the fibrinogen-α2AP complex but the A2AP IgG1 and IgG4 antibodies did not. This supported our initial suspicions from western blot and ELISA results that the A2AP IgG1 and IgG4 were binding to fibrinogen non-specifically. To determine where the A2AP IgG1 and IgG4 antibodies were binding to on fibrinogen, plasmin-mediated digests of human and rabbit fibrinogen were performed. The A2AP IgG1 and IgG4 appear to interact the most with the α- and β- chains of both human and rabbit fibrinogen. The signal achieved for the fibrinogen α-chain seemed uncharacteristically high for the amount of α2AP that would be present. This combined with the binding to the β-chain of fibrinogen further supported the idea of non-specific binding. Next, we were interested

to determine whether the A2AP IgG4 could immunodeplete the fibrinogen fraction in plasma with α2AP crosslinked to it. Affinity chromatography was thus performed where the A2AP IgG4 was immobilized on a Sepharose column and human plasma was passed over. The eluted sample depicted several bands, one of which ran at the same molecular weight (70 kDa) as both purified α2AP and human fibrinogen and showed both α2AP and fibrinogen bands when probed in a western blot with the polyclonal α2AP and fibrinogen antibodies, respectfully. Thus, together these data suggest the A2AP IgG1 and IgG4 bind human α2AP, but also bind fibrinogen non-specifically. The cause of cross reactivity could be due to similar amino acid sequences to the reactive center loop of α2AP in the α or β chains of fibrinogen. It could also be due to interference from the generic Fc portion of the IgG4 antibody as opposed to the unique Fab region. Bayer AG did not find non-specific binding of A2AP IgG4 to recombinant α, β or γ chains of fibrinogen. However, they did corroborate our findings of non-specific binding of the A2AP IgG4 to human fibrinogen in ELISA. The non-specific interactions with fibrinogen may limit its therapeutic potential as the A2AP IgG4 could preferentially bind fibrinogen over α2AP or the fibrinogen interaction could interfere with α2AP binding. Also, binding to fibrinogen could enhance the effect of the A2AP IgG4 by aiding localization of the antibody to fibrin clot. Thus, the potential effect of this interaction should be considered prior to moving into clinical trials. Also, α2AP being a serpin, shares conserved residues with other serpins that facilitate folding into a conformation comprising of three β-sheets, eight to nine α-helices, and a reactive center loop (Whisstock *et al.*, 2010). Thus, this similarity between α2AP and other serpins could impact the efficacy of the antibody.

5.4 Functional evaluation of A2AP IgG1 and A2AP IgG4

Next, we evaluated the ability of the A2AP IgG to neutralize α2AP in enzymatic assays and in clot lysis. In chromogenic experiments evaluating plasmin inhibition by α2AP, we demonstrated that A2AP IgG1 protects plasmin activity and significantly reduces the potency of α2AP. Four times as much α2AP to plasmin was required to abolish plasmin activity in the presence of the A2AP IgG1. Also, with equimolar amounts of plasmin and α2AP, the A2AP IgG1 neutralizes α2AP and recovers plasmin activity. This corroborates findings from another study testing the ability of a MAb to neutralize α2AP. Singh *et al*., 2017 found that a MAb against α2AP had the ability to protect plasmin activity in the presence of α2AP. However, they found that equimolar amounts of their MAb and α2AP was enough to neutralize inhibition on plasmin (Singh *et al*., 2017). In our case, over ten-fold excess of A2AP IgG1 was required to neutralize α2AP in the chromogenic assay. Given the affinities determined in ELISA and SPR of A2AP IgG1 for α2AP which had Kd values of 4 ± 0.7 nM and 120.8 ± 11.7 nM, respectively, this result was surprising. This suggests a reduced potency of the A2AP IgG1 compared to the MAb in the Sing *et al*., 2017 study and that binding of the A2AP IgG1 may not translate directly into neutralization. However, these results still suggest that the A2AP IgG1 antibody preserves the activity of plasmin by inactivating α2AP. Based on this experiment it appears that excess A2AP IgG over α2AP is necessary to restore plasmin activity which is an important consideration for potential *in vivo* use. With the concentration of α2AP in human plasma being ~ 1 μM, it appears the A2AP IgG would have to be given at a dose to maintain a plasma concentration of several fold higher than α2AP. Also, the effect of the A2AP IgG1 on neutralizing α2AP was enhanced in the presence of fibrinogen and thus, binding to fibrinogen is unlikely to be important.

Clot lysis assays were used to evaluate the A2AP IgG1 and IgG4 in the presence of a clot. Purified assays were initially performed to allow for a defined system for comparison with plasma. Purified assays involved a titration of the A2AP IgG1 with lysis being initiated by plasmin or t-PA and plasminogen. In both plasmin- and t-PA/plasminogen-mediated lysis, the A2AP IgG1 demonstrated a dose-dependent shortening of clot lysis. However, the A2AP IgG1 was significantly more potent in plasmin-mediated lysis compared with t-PA-mediated lysis. IC50 values of 7.6 ± 2.7 nM and 810.8 ± 438.9 nM were reported for plasmin- and t-PA/plasminogen-mediated lysis, respectively. In addition, a non-specific IgG and the omission of α2AP were used as controls. Both supported the specificity to α2AP. The results from the plasma clot lysis correlated with the affinities determined in SPR. The A2AP IgG4 was determined to have a significantly higher affinity (63-fold) for α2AP compared with the A2AP IgG1. Our findings corroborated those from Bayer AG. They determined 300- and 10-fold greater affinity of the A2AP IgG4 for α2AP compared with the A2AP IgG1 as determined in ELISA and SPR, respectively. In line with this, the A2AP IgG4 was significantly more potent in human plasma compared with the A2AP IgG1 when comparing IC50 values. Although significance was determined, approximately 1 μM of each antibody was required to neutralize endogenous α2AP. This suggests similar potencies. In rabbit plasma, A2AP IgG4 was significantly more potent compared with the A2AP IgG1. Also, approximately 1 μM of the A2AP IgG4 was required to neutralize endogenous rabbit α2AP compared with approximately 1.5 μM of A2AP IgG1. Bayer AG determined that the A2AP IgG4 displayed 20-fold higher affinity by ELISA and 2.5-fold higher affinity by SPR compared with A2AP IgG1 for rabbit α2AP. Thus, our result in the clot lysis assays is consistent with their affinity measurements. In conclusion, although the affinity of the A2AP IgG4 for α2AP is greater, the potencies in human plasma appear to be similar. Also,

these results suggest that *in vivo,* a minimum plasma concentration of 1 μM of the A2AP IgG4 will need to be maintained to effectively neutralize α2AP.

The α2AP peptide was used to examine specificity by exploring its ability to antagonize A2AP IgG1 and IgG4. A2AP IgG1 was not affected, but the A2AP IgG4 activity was attenuated by the α2AP peptide as clot lysis times were dose-dependently delayed by the peptide. This influence is likely due to the 63-fold greater affinity of the A2AP IgG4 for α2AP (as determined by SPR) relative to the A2AP IgG1. Together these results confirm the specificity of both antibodies to α2AP, their ability to modulate fibrinolysis, and the increased potency of the A2AP IgG4 due to its greater affinity for α2AP. The fibrinogen peptide had no effect on A2AP IgG1 or A2AP IgG4 activity.

Experiments examining the lysis of pre-formed clots were performed to better model clinical thrombolysis. In the routine clot lysis assay, the antibody was mixed with the plasma before clotting, but this is not representative of what would happen clinically where the antibody would be given after thrombus formation. In the preformed clot lysis experiments, clots formed by clotting plasma containing fluorescent fibrinogen around plastic inoculation loops were washed and then immersed in plasma containing t-PA or TNK without or with A2AP-IgG1/IgG4. The antibodies alone had no effect on clot lysis in the absence of t-PA. t-PA or TNK alone achieved 47% and 55% clot lysis, respectively. However, in combination with a low dose of t-PA or TNK, both antibodies elicited almost 100% clot lysis and achieved significantly greater clot lysis than higher doses of t-PA or TNK alone. This demonstrates the potential synergy between the antibodies and plasminogen activators. It suggests that less t-PA or TNK is required in the presence of an α2AP inhibitor to achieve the same or greater effect than a higher dose of t-PA or TNK alone. Also, our results show that the A2AP IgG1 and IgG4 are effective

with outside-in lysis and not just internal. The A2AP IgG4 was tested *in vivo* due to a lower likelihood of immunogenicity.

5.5 In vivo efficacy of A2AP IgG4

Finally, the in vivo thrombolytic potential was assessed in a rabbit thrombosis model to investigate whether the A2AP IgG4 can augment the action of TNK. A mixture containing autologous rabbit plasma, human α-thrombin, and fluorescent fibrinogen was prepared ex vivo and injected into an isolated jugular vein segment. After the clot matured, rabbits were administered various treatments. Three doses of the A2AP IgG4 were tested to determine responsiveness of the model, and 15 mg/kg was selected based on its effect on thrombolysis and extent of bleeding. The dose of 7.5 mg/kg provided a response similar to the saline control and 30 mg/kg was not superior to 15 mg/kg based on the response in the plasma. From western blotting and ELISA experiments, we recognized the importance of exposure of the reactive center loop for antibody binding. This requirement questioned the effectiveness of the A2AP IgG4 *in vivo*. However, these results suggest that despite the requirement, thrombolysis can occur. Lower doses of TNK than the therapeutic dose (0.5 mg/kg) were tested alone and in combination with the A2AP IgG4. We first found that when administered alone, the A2AP IgG4 resulted in thrombolysis *in vivo* despite no effect in the pre-formed plasma clot lysis experiments. This is possibly due to plasminogen activator being released from the vascular endothelium or the absence or presence of flow. We found that the combination of the A2AP IgG4 with low dose TNK resulted in significantly greater thrombolysis compared with a higher dose of TNK alone, the A2AP IgG4 alone, and the lower dose of TNK alone. These results were obtained with 20% of the therapeutic dose of TNK. A dose of 10% of therapeutic TNK was initially tested. However, thrombolysis was comparable with the A2AP IgG4 alone and thus was

considered to be equivalent to background lysis. This was inconsistent with what was seen in a similar study that explored an α2AP MAb in PE. Singh *et al.*, 2017 found synergy when 10% of therapeutic rt-PA dose (0.1 mg/kg) was combined with the MAb, whereas we needed 20% of therapeutic TNK in the combination. They observed significantly greater thrombolysis compared with a clinical dose of rt-PA (1.2 mg/kg) alone and the same dose of the MAb used alone. However, it was a different antibody, different PA, and different model. Despite this, our results still corroborated the findings from this study that showed a synergy and a greater thrombolytic effect can be achieved by delivering lower doses of PAs while simultaneously inhibiting α2AP.

However, the combination of 20% therapeutic dose of TNK with the A2AP IgG4 displayed a non-significant difference in terms of volume of blood loss compared with the therapeutic dose of TNK. Also, similar to this dose of TNK, the combination resulted in excessive bleeding from the surgical sites, although this was not quantified. The bleeding could be attributed to variation between rabbits or experimenter error, which could be corrected with a higher sample size. Despite this, the combination of 20% therapeutic dose of TNK with the A2AP IgG4 did not result in degradation of fibrinogen non-specifically as evidenced by western blotting. Also, reperfusion studies were performed on a few rabbits receiving the combination treatment as well as one saline treated rabbit. In two of three rabbits that were administered the combination treatment, flow was completely restored as evidenced by measuring the venous flow before clotting, immediately after clotting, and four hours after treatment administration. The saline treated rabbit did not exhibit restoration of blood flow. Our model is limited to acute applications and does not assess the thrombolytic potential of the A2AP IgG4 in a chronic setting. Also, using whole blood as opposed to plasma to form the venous clot could have led to a more stable clot that could be removed and measured at the end of the experiment. Also,

administering the A2AP IgG4 to rabbits prior to inducing thrombosis and measuring the thrombotic load could determine whether the antibody could serve as a prophylactic therapy.

Our findings corroborate several studies where the effect of α2AP inhibition with a MAb on t-PA activity was examined. Reed *et al*., 1990 demonstrated *in vitro* that inhibition of α2AP with a MAb enhances clot lysis in the presence of PAs including t-PA. They next showed that when administering the same MAb with t-PA in a rabbit jugular vein thrombosis model, the combination causes significantly greater clot lysis than the equivalent doses of t-PA alone (Reed *et al*., 1990). Also, the combination did not consume fibrinogen non-specifically. Further, Butte *et al*., 1997 found that when administering a MAb with rt-PA to ferrets with PE, there was enhanced clot lysis compared with the same or higher doses of rt-PA alone. Like our findings, the combination did not result in fibrinogen consumption. Another study found that α2AP contributes to the persistence of venous thrombosis, justifying it as a target for thrombolytic therapy. Thus, thrombus weight and the fibrin content of thrombi formed after inferior vena cava (IVC) ligation were lower in α2AP deficient mice than in wild type mice. Furthermore, in mice with preformed venous thrombi, a MAb against α2AP decreased the size and weight of the thrombi compared with treatment with pharmacological doses of t-PA alone (Singh *et al*., 2017). These findings are consistent with our preliminary reperfusion measurements that revealed reperfusion of the jugular vein four hours after administration of A2AP IgG4 plus TNK which was not seen in saline controls. Thus, attenuating α2AP while administering a PA appears to have a synergistic effect on thrombolysis.

Regulation of the fibrinolytic system depends not only on α2AP, but also on PAI-1 and TAFIa. Gene deletion studies in mice demonstrate that the absence of TAFI significantly increases lysis in a pulmonary clot lysis model compared with wildtype mice (Swaisgood *et al*.,

2002). Also, studies in mice deficient in both PAI-1 and α2AP showed significantly prolonged bleeding times after tail amputation compared with either deficiency alone, demonstrating the contribution of PAI-1 to fibrinolysis regulation (Dewerchin *et al*., 2001). However, in this study, mice with α2AP deficiency with or without PAI-I deficiency displayed greater spontaneous lysis of intravenously injected 125I-fibrin labeled plasma clots than wildtype mice. This effect was not observed in mice deficient in PAI-1 with or without α2AP deficiency. Also, there was much less kidney fibrin deposition in mice deficient in α2AP with or without PAI-1 deficiency compared to control mice. This effect was not observed in mice deficient in PAI-1 with or without α2AP deficiency. Also, therapies targeting PAI-1 are considered antithrombotic as opposed to thrombolytic because PAI-1 is also important for enhancing growth of a thrombus under shear stress (Urano and Suzuki, 2017). These findings show the important regulatory role of α2AP on thrombosis and fibrinolysis (Dewerchin *et al*., 2001). As a result, targeting α2AP appears to provide unique benefit, but targeting other fibrinolytic inhibitors such as TAFIa or PAI-1 presents as potential thrombolytic therapy. The role of α2AP in regulating fibrinolysis is apparent in many other studies. Mice deficient in α2AP had significantly reduced mortality due to acute PE compared with controls in a jugular vein venous thrombosis model. Plasminogen deficient mice in this study did not survive due to acute PE. Lungs of α2AP deficient mice had only a few thrombi compared to plasminogen deficient mice which had extensive thrombosis. When α2AP was administered to α2AP deficient mice, mortality was not different than wild type mice. t-PA administration in α2AP deficient mice further reduced mortality compared to α2AP deficient mice and tail bleeding was not prolonged (Matsuno *et al*., 2003). Our findings corroborate this study and show the potential synergy of simultaneously inhibiting α2AP while administering a PA. Other studies show that mice deficient in α2AP have enhanced fibrinolytic

potential due to spontaneous lysis 4 hours after intravenous injection of plasma clots and that inhibition of FXIIIa-mediated cross linking of α2AP enhances lysis of PE in a ferret model (Lijnen *et al*., 1999, Reed and Houng, 1999).

Thrombolytic therapy must overcome regulation by α2AP, but some regulation must be maintained to ensure hemostasis and avoid a bleeding scenario (Leebeek et al., 1988). Modulation of α2AP provides unique strategy to enhance fibrinolysis while simultaneously administering an exogenous PA. Reducing the doses of PA administered while inhibiting α2AP may not lead to consumption of other coagulation factors (Aoki, 2004, Urano and Suzuki, 2017). Thus, the key role that α2AP plays to regulate fibrinolysis is clear and this identifies α2AP as a novel target for thrombolytic therapy that can potentially reduce the risk of bleeding while enhancing clot lysis.

Chapter 6: Future directions

There are several areas that require further investigation to determine the utility of A2AP IgG4 for thrombolytic therapy. We found substantial evidence that A2AP IgG1 and IgG4 bind to fibrinogen. It will therefore be important to determine the potential consequences of this *in vivo*. Preliminary chromogenic experiments suggest that the presence of fibrinogen does not interfere with the potency of the antibody. In our rabbit studies, fibrinogen does not appear to prevent enhancement of clot lysis. Bayer AG could perform further rounds of affinity maturation by altering amino acid residues in the complement-determining region of the Fab portion of A2AP IgG4. Also, by assessing amino acid sequences and removing similarities found in fibrinogen, sites of non-specific binding could be removed.

In the rabbit thrombosis model, the efficacy for the combination of the 20% therapeutic dose of TNK with A2AP IgG4 was promising. The combination provided a significantly greater response in the plasma and total percent clot lysis than all other groups. However, differences in ear bleeding as a result of treatment administration were non-significant between groups. A larger sample size is needed to be sure of the effect of the combination on bleeding. The side effect of bleeding in thrombolytic therapy is an important consideration so it will be necessary to learn how to manage this while still providing improved efficacy. In addition to the ear bleed model, future studies could also weigh the gauze sponges used to manage bleeding during procedures. Also, the chosen rabbit thrombosis model made it difficult to determine the change in fluorescence in the plasma clot itself. Being able to measure the fluorescence change in the clot will help us accurately assess the response to treatment. Using a fluorescent marker also made it difficult to accurately quantify the response due to some of the marker being attached to the isolated segment of the vessel wall after the experiment was finished.

Further studies will also need to be performed to assess the usefulness of the A2AP IgG4 in thromboembolism, arterial thrombosis and in combination with known anticoagulants used in DVT. In terms of anticoagulant therapy, heparin, warfarin or DOACs are typically used on patients with DVT and for DVT prophylaxis. Heparin functions by accelerating AT-mediated inhibition of FXa, thrombin and other serine proteases. Warfarin inhibits the vitamin K-dependent synthesis of prothrombin, FVII, FIX, and FX (Sagripanti and Carpi, 1998, Perler, 2005). DOACs such as dabigatran (thrombin inhibitor) and rivaroxaban, apixaban, or edoxaban (factor Xa inhibitors) have proven to be an effective and safe alternative in the context of acute VTE (Van Es *et al*., 2014). Despite the use of these anticoagulants, post-thrombotic syndrome develops within one to two years in 20-50% of patients with symptomatic DVT (Kahn and Ginsberg, 2004). This condition may result due to ineffective thrombolysis and is associated with several long-term complications (Perler, 2005). Thus, in a chronic setting, there is a need for improved anticoagulant agents that are not associated with potentially negative side effects. Exploring the efficacy of the A2AP IgG4 in adjunct to the previously mentioned anticoagulant therapy could improve outcomes for patients with venous thrombosis. If results in these subsequent *in vitro* and *in vivo* studies are promising, clinical trials involving patients with thrombotic disorders will need to be carried out in patients with emergent arterial thrombi such as heart attacks or ischemic strokes and/or DVT and patients with PTS. Studies have tried to improve thrombolysis for over 30 years and some have succeeded as new fibrinolytic agents have been developed. The present study confirms the thrombolytic efficacy of inhibiting α2AP, but further tests and evaluations will need to be performed to assess the therapeutic potential of this antibody specifically.

Chapter 7: References

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