THE CONTRIBUTION OF FIBRINOGEN AND RED BLOOD CELLS TO ARTERIAL THROMBOSIS

Bethany Lynne Walton

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Approved by:

Alisa S. Wolberg

William B. Coleman

Brian C. Cooley

Nigel S. Key

Leslie V. Parise

ABSTRACT

Bethany Lynne Walton: The Contribution of Fibrinogen and Red Blood Cells to Arterial Thrombosis
(Under the direction of Alisa S. Wolberg)

Cardiovascular disease is the leading cause of death and disability worldwide. This dissertation explores the role of the clotting factor fibrinogen and red blood cells (RBCs) to arterial thrombosis.

Elevated plasma fibrinogen is associated with arterial thrombosis in humans and directly promotes thrombosis in mice, but the contribution of the γA/γ′ fibrinogen isoform to thrombosis is controversial. To determine if γA/γ′ is prothrombotic, we separated γA/γA and γA/γ′ from human plasma and determined the effects on *in vitro* clot formation and on *in vivo* thrombus formation. Both γA/γA and γA/γ′ were cleaved by murine and human thrombin and were incorporated into murine and human clots. When γA/γA or γA/γ′ was spiked into plasma, γA/γA increased the fibrin formation rate to a greater extent than γA/γ′. In mice, compared to controls, γA/γA infusion shortened the time to carotid artery occlusion, whereas γA/γ′ infusion did not. Additionally, γA/γ′ infusion led to lower levels of plasma thrombin–antithrombin complexes following arterial injury, whereas γA/γA infusion did not. These data suggest that γA/γ′ binds thrombin *in vivo* and decreases prothrombotic activity. Together, these findings indicate that elevated levels of γA/γA promote arterial thrombosis *in vivo*, whereas γA/γ′ does not.

RBCs are the most abundant cell type in blood and increased hematocrit is associated with thrombosis. While it is known that RBCs support thrombin generation and increase platelet activation and aggregation, the specific mechanism by which RBCs influence clotting is unclear. In reconstituted human blood *ex vivo*, RBCs dose-dependently increased thrombin generation in the absence of platelets, although effects were blunted or absent in the presence of platelets. Compared to controls, mice infused with RBCs formed thrombi at a faster rate and had a shortened time to vessel occlusion in a carotid artery injury model. Interestingly, there was no difference in circulating thrombin-antithrombin complexes between RBC^{HIGH} and control mice, and thrombi did not differ in size or fibrin content, suggesting elevated hematocrit promotes arterial thrombosis by a thrombin-independent mechanism. Our data suggest that reducing hematocrit may reduce arterial thrombosis in humans. Future experiments will investigate how the RBC effect on platelets contributes to thrombosis.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
AU	Arbitrary Units
β	Beta
BSA	Bovine Serum Albumin
°C	Degrees Celsius
$CaCl_2$	Calcium Chloride
CAT	Calibrated Automated Thrombography
CGS	Sodium citrate, Glucose, Sodium Chloride
CHD	Coronary Heart Disease
CVD	Cardiovascular disease
DVT	Deep Vein Thrombosis
ELISA	Enzyme-Linked Immunosorbent Assay
$FeCl_3$	Ferric Chloride
FXIII	Factor XIII
γ	Gamma
γ’	Gamma Prime
g	Gram
H&E	Hematoxylin and Eosin
HBS	HEPES Buffered Saline
hNPP	Human Normal Pooled Plasma
Hz	Hertz
IHC	Immunohistochemistry

ICAM	Intercellular Adhesion Molecule
IVC	Inferior Vena Cava
K	Thousand
KCl	Potassium Chloride
L	Liters
µL	Microliter
µM	Micromolar
M	Molar
mg	Milligram
$MgSO_4$	Magnesium Sulfate
Min	Minute
MI	Myocardial infarction
mL	Milliliter
mM	Millimolar
mNPP	Murine Normal Pooled Plasma
NaCl	Sodium Chloride
nm	Nanometer
pA	Polyadenylation
PE	Pulmonary Embolism
PF4	Platelet Factor 4
PGI2	Prostacyclin I_2
PPP	Platelet-poor Plasma
pRBCs	Packed Red Blood Cells

PRP	Platelet-rich Plasma
PS	Phosphatidylserine
PV	Polycythemia Vera
RBCs	Red Blood Cells
RBC^{HIGH}	Mice with Elevated Hematocrit
SCD	Sickle Cell Disease
SD	Standard Deviation
SE	Standard Error
SEM	Standard Error of the Mean
TAT	Thrombin-antithrombin
TF	Tissue Factor
tPA	Tissue-type Plasminogen Activator
TTO	Time to Occlusion
VTE	Venous Thromboembolism
WBC	White Blood Cell
WT	Wild-type

Chapter 1: Introduction: Fibrinogen and Red Blood Cells in Hemostasis and Thrombosis[1]

1.1 Introduction

Arterial thrombosis is a leading cause of death and disability worldwide. Arterial thrombosis is usually initiated following rupture of an atherosclerotic plaque. This causes the formation of thrombi that may become occlusive and cause ischemic damage to the surrounding tissues. Intracardiac thrombosis may also occur due to atrial fibrillation or the presence of a mechanical valve [1]. Arterial thrombi are usually termed "white-thrombi" due to their high platelet count and efforts to understand the pathogenesis of arterial thrombosis have mainly focused on platelets. However growing evidence suggests that the plasma protein fibrin(ogen) and RBCs may also be involved in the development of arterial thrombi.

Venous thrombosis is initiated by endothelial dysfunction and inappropriate expression of plasma and cellular procoagulant activity under low blood flow/stasis (so-called Virchow's Triad). The epidemiology, risk factors, and treatment of venous thrombosis have been recently reviewed in [2]. However, the pathophysiologic mechanisms that contribute to thrombus formation, composition, and stability are still poorly understood. Clues may be found in the distinctive appearance of venous thrombi, which demonstrate regions of high RBC and fibrin content (so-called "red thrombi"). Notably, RBCs can be

[1] This chapter is based on, and reproduced in-part with permission from: Walton BL, Byrnes JR, Wolberg AS. Fibrinogen, red blood cells, and factor XIII in venous thrombosis. *J Thromb Haemost*. 2015, *In Press*

found between layers of fibrin in a "brick and mortar" construction, where they lose their typical discoid shape and acquire a compressed morphology (so-called "polyhedrocytes") [3]. These observations suggest RBCs and fibrin(ogen) interact during venous thrombosis, and that thrombi undergo substantial consolidation during their maturation.

Herein, I will review the contributions of fibrinogen and RBCs to coagulation, and provide evidence supporting their potential roles in both arterial and venous thrombosis.

1.2 Fibrinogen

1.2.1 Fibrinogen structure, fibrin formation, and fibrin mechanical properties.

The fibrinogen molecule consists of 2 sets each of 3 polypeptide chains $(A\alpha B\beta\gamma)_2$. During coagulation, thrombin cleaves N-terminal peptides from the Aα- and Bβ-chains promoting the formation of protofibrils and subsequently, fibrin fibers. Branching results in the characteristic fibrin network seen in micrographs of purified and plasma clots. Fibrinogen circulates at high concentrations (2-4 mg/mL) in plasma, and levels may increase further during inflammation. The concentrations of thrombin and fibrinogen present during clot formation influence fibrin network structure and stability. For example, clots formed in the presence of high thrombin or fibrinogen concentrations have increased fibrin network density and resistance to fibrinolysis compared to clots formed under normal conditions. These processes have been previously reviewed [4, 5].

Crosslinked fibrin is also known for its ability to stabilize clots. This property is determined at both micro- and macro-scales. Individual fibrin fibers have astounding viscoelasticity. Crosslinked fibrin fibers can be stretched to 2.5-times their original length before rupturing, making fibrin as extensible as spider silk [6-8]. Moreover, elastic recovery

of fibers from elongations up to 100% can occur within milliseconds [9]. Branchpoints within the fibrin network are surprisingly strong. When strained, individual fibers fail before branchpoints fail [10]. Thus, it is not surprising that fully-formed fibrin clots have similar extensibility and elasticity as individual fibers.

1.2.2 Fibrinogen and arterial and venous thrombosis.

Elevated total fibrinogen is correlated with increased arterial thrombosis [11-15] and increased venous thrombosis risk [16-20], and risk is concentration-dependent and present in both men and women. Studies using transgenic mice and murine infusion models have associated elevated fibrinogen with increased prothrombotic biomarkers (e.g., D-dimer) [21] and a shorter time to vessel occlusion and increased thrombus fibrin content [22]. Moreover, compared to control mice, thrombi in fibrinogen-infused mice also show increased resistance to fibrinolysis [22]. These findings suggest hyperfibrinogenemia is not merely a biomarker of thrombosis risk, but is causative in arterial and venous thrombosis etiology.

1.2.3 Abnormal fibrin structure and stability in thrombosis.

Several studies have reported abnormal fibrin structure and/or stability in both arterial and venous thrombosis, even when circulating fibrinogen levels are normal. For example, compared to controls, patients with a history of MI produced clots with shorter fibrin fibers with increased stiffness, and an increased resistance to lysis [23, 24]. Additionally, compared to controls, plasma clots from patients with acute ischemic and cryptogenic stroke also displayed reduced permeability and resistance to lysis [25, 26]. In venous thrombosis, plasma clots from patients with a history of idiopathic venous thrombosis show increased

fibrin network density, reduced permeability, and increased lysis times[27]. Interestingly, compared to plasma clots from patients with deep vein thrombosis, clots from patients that experienced pulmonary embolism are less compact and more susceptible to fibrinolysis [27, 28]. In total, these data suggest abnormal fibrin network structure and stability contribute to arterial and venous thrombosis.

1.2.4 Fibrinogen γ'-chain and arterial and venous thrombosis.

The fibrinogen γ-chain can undergo alternative splicing, leading to replacement of 4 C-terminal amino acids with a unique 20 amino acid sequence (γ'; Figure 1.1). The γ'-chain is present in 8-15% of fibrinogen molecules (as γA/γ') in healthy individuals. The genes encoding the fibrinogen chains are co-regulated to maintain the level of fibrinogen in circulation (reviewed in [29]). However, the levels of the γA- and γ'-chains are mediated by independent mechanisms that differentially regulate their expression. Expression of γ'-containing fibrinogen is disproportionally increased by interleukin-6-dependent inflammatory responses [30], suggesting an independent relationship between the γ'-chain, inflammation, and thrombosis. Accordingly, although total fibrinogen levels are positively correlated with thrombosis risk, the fraction of circulating γ'-fibrinogen (γ'/total fibrinogen ratio) modulates risk independently of the total fibrinogen level. Notably, an elevated γ'-to-total fibrinogen ratio is associated with increased risk of arterial thrombosis in numerous epidemiological studies [31-36], suggesting γ' may be driving arterial thrombosis. Interestingly, a reduced γ'-to-total fibrinogen ratio is associated with increased risk of venous thrombosis, suggesting γ' fibrinogen is protective in venous thrombosis [37, 38]. These studies suggest the fibrinogen γ'-chain plays different roles in different vascular beds.

Determining the operant mechanisms has been difficult because γA/γ' fibrinogen has both procoagulant and antithrombotic properties (reviewed in [39]). Briefly, compared to γA/γA clots, clots that contain γ' fibrinogen have a denser network of thin fibrin fibers, reduced permeability, reduced plasminogen binding, and increased resistance to fibrinolysis. The γ'-chain can also bind and sequester thrombin, protecting it from inactivation by antithrombin. These properties are consistent with prothrombotic functions. However, γ' fibrinogen also exhibits impaired polymerization. Recent studies have shown that a γ' carboxyl-terminal peptide reduces plasma thrombin generation even in the presence of anti-factor VIII antibody, suggesting γ'/thrombin interactions reduce factor V activation [40]. By reducing thrombin generation, this peptide also increases the sensitivity of coagulation to activated protein C, thus augmenting endogenous anticoagulant mechanisms [41].

Studies to determine the contribution of the γ'-chain to thrombosis *in vivo* have consistently demonstrated antithrombotic effects. Transgenic expression of the human γ'-chain reduces venous thrombus volume in mice that are heterozygous for the factor V Leiden mutation [42]. A peptide mimicking the γ'-chain C-terminus inhibits fibrin-rich thrombus formation in a baboon model of thrombosis [43]. We recently infused mice with identical levels of either γA/γA or γA/γ' fibrinogen isolated from human plasma [44]. Compared to controls, γA/γA infusion shortens the time to carotid artery occlusion, whereas γA/γ' infusion does not. Additionally, γA/γ' infusion reduces levels of circulating thrombin-antithrombin complexes. These data are consistent with the premise that the γ'-chain reduces thrombin activity. By extension, these data implicate the γA-chain as the prothrombotic mediator in hyperfibrinogenemia-related thrombosis. *These experiments are discussed in detail in*

Chapter 2 of this dissertation. Together, these findings illustrate pleotropic contributions of fibrinogen to arterial and venous thrombosis.

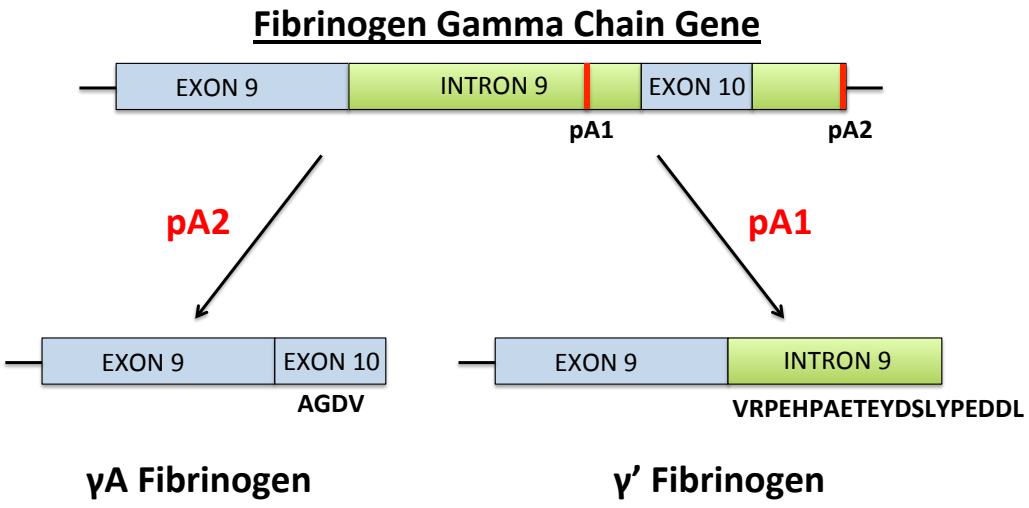

Figure 1.1: The fibrinogen γ chain undergoes alternative processing to form the γA and γ' isoforms. The fibrinogen γ-chain mRNA transcript may undergo splicing at two main polyadenylation sites. The γA-chain forms when polyadenylation (pA) occurs downstream of exon 10 (pA2), leading to translation of exon 10 and the formation of a γ-chain with 10 exons, 9 introns, and 411 amino acids (ending in AGDV). The γ'-chain forms when polyadenylation occurs upstream of exon 10 (pA1), forming a γ-chain which includes intron 9. This results in the translation of an extra 20 amino from intron 9 (VRPEHPAETRYDSLYPEDDL), forming a γ-chain with 427 amino acids.

1.2.5 Fibrin(ogen) interactions with cells and blood proteins.

Most studies of fibrin(ogen) function have used purified systems or plasmas. These studies have identified binding sites on fibrin(ogen) for soluble proteins involved in clot formation, stabilization, and fibrinolysis, including thrombin, FXIII, fibronectin, tissue-type plasminogen activator (tPA), plasminogen, and plasmin [45-49]. Notably, however, fibrin(ogen) also interacts with cells and these interactions may contribute to the incorporation of cells into venous thrombi. For example, fibrin(ogen) contains recognition sequences for integrins including $\alpha M\beta_2$, $\alpha IIb\beta_3$, $\alpha V\beta_3$, which mediate fibrin(ogen) interactions with leukocytes, platelets, and endothelial cells, respectively [50]. These interactions modulate leukocyte function, platelet aggregation and clot retraction, and may anchor thrombi to the endothelium. Fibrin(ogen) also binds to RBCs, which influences both the erythrocyte sedimentation rate and blood viscosity (discussed below) [51-53].

1.3 Red Blood Cells

1.3.1 RBCs in circulation.

RBCs are anucleate cells derived from bone marrow and are the largest cellular component of blood. RBCs have a characteristic biconcave and flexible shape that allows them to traverse the microvasculature and fulfill their primary function of hemoglobin-mediated oxygen transport throughout the body. RBCs circulate at ~4.2-6.1x10^9/mL, although levels are slightly higher in men than women. Clinical observations suggest RBC levels contribute to hemostasis and thrombosis. Bleeding times shorten as hematocrit rises [54-57], and elevated levels of RBCs are associated with increased risk of thrombosis [58-61].

1.3.2 Causes of high hematocrit.

The normal range of RBCs in blood (hematocrit) is 41-46% in men and 36-44% in women, and can be influenced by a number of physiologic and pathologic situations. In high altitude, the bone marrow increases RBC production to compensate for decreased oxygen saturation [62]. RBC levels may also increase in disease states such as polycythemia vera (PV) [63] and as a result of increased erythropoietin, either through exogenous erythropoietin use or abnormal erythropoietin production by certain types of tumors [64]. Conditions associated with hypoxia, such as smoking, lung disease, and heart disease, are also associated with increased RBC production [64].

1.3.3 Hematocrit in arterial and venous thrombosis.

Thrombosis is a common complication in patients with PV, with arterial thrombosis including MI and cerebrovascular events making up the majority of all thrombotic events in these patients [65]. The Cytoreductive Therapy in Polycythemia Vera (CYTO-PV), a large-scale, multicenter, prospective, randomized clinical trial compared maintaining hematocrit <45% or between 45-50% in patients with PV [66]. Compared to maintaining a hematocrit <45%, maintaining hematocrit in the higher range was associated with four times the rate of death from CVD and major thrombosis suggesting hematocrit is a cause for thrombosis in PV. However, this study did not control for patients taking hydroxyurea which complicates the interpretation of these results.

Numerous longitudinal, prospective studies have suggested elevated hematocrit is associated with both arterial and venous thrombosis in patients with an elevated hematocrit not caused by PV. However these studies are more conflicted in their results. Some studies

suggest hematocrit is not independently associated with thrombosis [67, 68] while other studies find an association [15, 58, 60, 69]. Similar conflicted findings are seen in studies on elevated hematocrit and venous thrombosis [70-72]. These studies are summarized in Tables 1.1 and 1.2. Thus it is hard to reconcile the role of elevated hematocrit in thrombosis.

Table 1.1 Epidemiological studies are conflicted on the association between hematocrit and arterial thrombosis

Study	N	Methods	Results
Sorlie (1981) *Puerto Rico Health Program* [58]	-2555 Rural/6151 urban men (45-64 years old)	-Subjects examined 3 times in 8 year follow-up	-High HCT (>49%) associated with an increased risk of MI, coronary insufficiency, and CHD death compared to low HCT (<42%). -Risk more than double in high HCT group
Carter (1983) [68]	-8006 Japanese men	-Subjects completed questionnaires & underwent interviews & medical screening -10 year follow-up	-HCT correlated with predictors or CHD risk, related to non-fatal MI, CHD death, and total CHD -When adjusted for other variables, HCT not independent risk factor
Gagnon (1994) *Framingham Study* [60]	-5209 men & women (30-62 years old) -1073 deaths	-Subjects received biannual examinations -34 year follow-up	-Men and women in the highest HCT quintile had increased risk of CVD death (men ≥49%; women ≥46%) -Correlation strongest in younger men
Brown (2001) *Second National Health & Nutrition Examination Survey (NHANES II) Mortality Study* [67]	-8896 men & women (30-75 years old)	-HCT categorized by sex-specific tertiles -16.8 year follow-up	-Women in the upper tertile were 1.3 times more likely to die from CHD than women with HCT in lowest tertile after multivariate adjustment -Risk was stronger in women <65 years old -HCT not associated with CVD or death in men after multivariate adjustment
Kunnas (2009) *TAMRISK Study* [69]	-670 Finnish Men (55 years old) -188 deaths from CVD	-Health survey data taken on 670 men in 1980 -28 year follow-up -Divided into two groups-HCT ≥50 or <50	-Men with HCT >50% were 2.4 more likes to die from CHD than men with HCT <50% -After adjusting for CHD risk factors, risk was 1.8 fold
Toss (2013) [61]	-417,099 Swedish men (18-19 years old) -9322 1st MI	-Baseline health tests and measures during conscription -36 year follow-up	-HCT ≥49% had a 1.4 fold increased risk of MI compared to men with HCT ≤44%. -Dose dependent relationship and remained constant through follow-up period.

Table 1.2 Epidemiological studies are conflicted on the association between hematocrit and venous thrombosis

Study	N	Methods	Results
Tsai (2002) *The Longitudinal Investigation of Thromboembolism Etiology* [70]	19292 men & women	-Combines data from, the Atherosclerosis Risk In Communities (ARIC) study and Cardiovascular Heath Study (CHS) -8 year follow-up	-No relation between HCT and VTE -**Limitation**: Low HCT cut-off at 43.5%
Vaya (2002) [71]	-109 1st time VTE patients -121 controls	-Subjects had no inherited or acquired risk factors for DVT -DVT documented with ultrasonography or venography and PE with ventilation perfusion scanning or pulmonary angiography -Blood collected from patients 6-36 months after the VTE episode (85% collected within the first year)	-No relation between HCT, RBC aggregation, plasma viscosity and DVT
Braekkan (2010) *Tromsø Study [72]*	-26108 men & women	- 12.5 year follow-up	-Multivariate hazard ratios per 5% increase in HCT were 1.25 for total VTE and 1.37 for unprovoked VTE. -Men with HCT in upper 20th percentile had a 1.5-fold increased risk for total VTE and 2.4-fold risk for unprovoked VTE

1.3.4 RBCs mediate blood rheology.

Hemorheology is the study of how flowing blood influences hemostasis. RBCs are the major determinant of blood rheology because of their prevalence, size, deformability, and ability to undergo reversible aggregation. Under high shear in the arterial circulation, (typically 500-1500 s^{-1}), RBCs promote platelet flux toward the vessel wall (so-called platelet margination), which increases the frequency of platelet-endothelial cell interactions, and platelet-platelet interactions which promotes platelet adhesion, activation, and aggregation (Figure 1.2) [73]. However, under low shear in the venous circulation (typically 10-100 s^{-1}), RBCs increase blood viscosity via their tendency to aggregate (rouleaux formation). Increased blood viscosity is a risk factor for arterial and venous thrombosis [74-76]. Notably, RBC aggregation is mediated by plasma proteins including fibrinogen [51-53]. Consequently, inflammatory processes that increase fibrinogen levels also increase blood viscosity. These effects have been implicated in the association between elevated hematocrit and hyperfibrinogenemia with thrombosis. However, it remains unclear whether this relationship is correlative or causative.

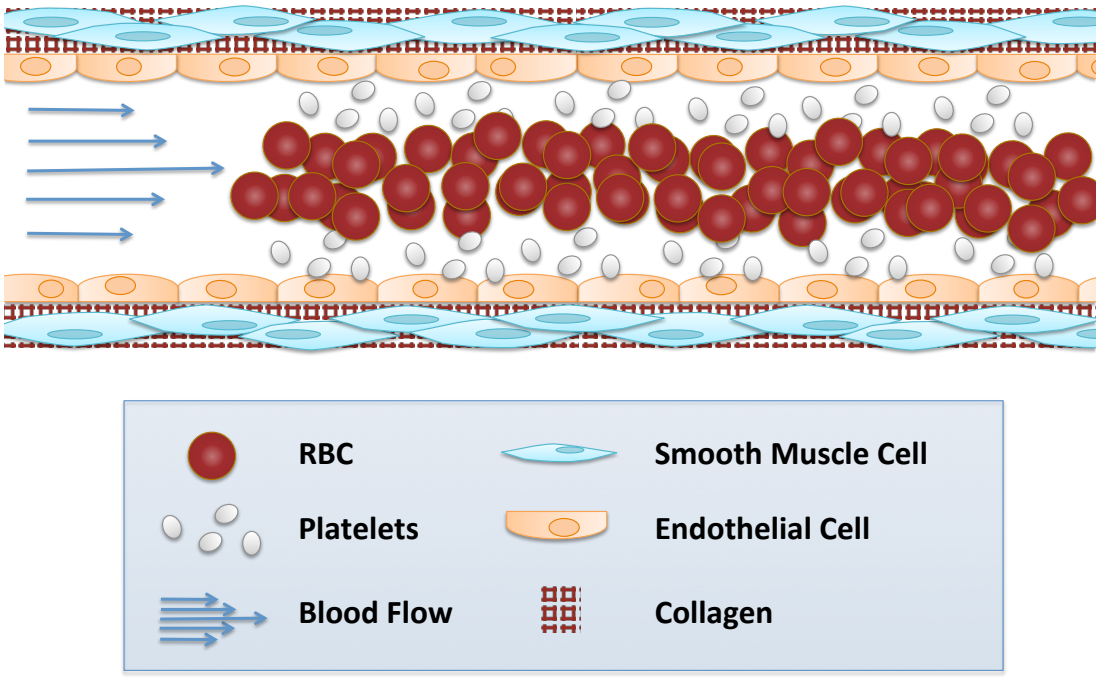

Figure 1.2: RBCs marginate platelets toward the vessel wall under arterial shear. RBCs dominate the rheology of blood due to their high number, large size, and deformability. Under the high shear rates present in arteries, RBCs move toward the center of the blood vessel. This marginates platelets towards the arterial walls, which promotes increased platelet-platelet interactions and platelet-endothelial interactions.

1.3.5 RBCs interact with fibrin(ogen).

RBCs interact specifically with fibrin(ogen) and the fibrinogen motif that mediates RBC interactions involve fibrinogen Aα-chain residues 207-303 [51]. Two potential RBC receptors have been implicated in this interaction. Fibrinogen-RBC interactions can be inhibited by the integrin-blocking molecule eptifibatide and are not supported by RBCs lacking β3 isolated from patients with Glanzmann thrombasthenia [77], implicating β3 or a β3-like molecule on the RBC surface. However, that study did not rule out the possibility that RBC-bound platelets mediate this interaction [77]. Fibrinogen-RBC interactions can also be blocked with an antibody against the integrin-associated protein CD47 [78]. Since CD47 was originally identified for its interaction with αvβ3, αIIbβ3, and α2β1 integrins, it is possible that the RBC binding site comprises a complex with both of these molecules. It is interesting to speculate that that blocked fibrin(ogen)-RBC interactions may reduce whole blood viscosity and thus thrombosis risk.

1.3.6 RBCs interact with cells.

RBCs can interact with leukocytes, platelets, and endothelial cells. For example, RBC ICAM-4 can bind leukocyte β1 and β2 integrins [79, 80] and platelet αIIbβ3 [81]. RBC ICAM-4 also interacts with integrin αv [82]. RBCs are the first cells to adhere to ferric chloride ($FeCl_3$)-treated, intact arterial endothelium, prior to the arrival of platelets [83]. This interaction is not dependent on von Willebrand factor or GPIbα. However, the molecular receptors on RBCs and the endothelium that mediate this interaction have not been identified [83]. Interestingly, RBCs exhibit temporal changes in gene expression during erythropoiesis

[84], suggesting stage-specific receptors may decorate RBCs during differentiation and further refine these interactions.

1.3.7 RBCs influence platelet reactivity.

RBCs have been shown to alter the biochemical responsiveness and functional responsiveness of activated platelets [85]. Silvain et al. [86] showed that RBCs increase ADP-induced platelet activation and aggregation *in vitro* in blood from healthy volunteers. The same group went on to show that patients who received RBC transfusions displayed increased ADP-induced platelet reactivity [87]. Moreover, it was specifically shown that RBCs amplify platelet activation and degranulation by increasing platelet serotonin release, increasing enzymatic ADP removal, and inhibiting proteases [85]. The increase in platelet reactivity by RBCs could not be decreased by aspirin, suggesting that RBC level influences therapeutic effect of aspirin. RBCs have also been shown to enhance the activation of αIIbβ3 and P-selectin on platelets, suggesting RBCs increase platelet activation and aggregation [88]. In sum, these studies suggest that RBCs contribute to thrombus formation by increasing platelet reactivity.

1.3.8 RBCs support thrombin generation.

A small percentage (~0.5%) of RBCs circulate with exposed phosphotidylserine (PS) on their outer membranes [89], suggesting RBCs can assemble prothrombinase complexes and support thrombin generation (Figure 1.3). Interestingly, although levels of both PS-positive RBCs and platelets are elevated in patients with sickle cell disease (SCD) genotypes, only PS-positive RBCs correlate with circulating biomarkers of coagulation activation,

including F1.2 and D-dimer [89]. This finding suggests PS-positive RBCs are the primary cell responsible for thrombophilia in SCD. *In vitro* studies support this premise; when added to platelet-poor plasma, RBCs shorten the lag time and increase the peak of thrombin generation similar to that seen with platelets [90, 91], although in contrast to platelets, thrombin generation on RBCs occurs through the meizothrombin pathway [92]. RBCs can also produce microvesicles that activate procoagulant and complement pathways in vitro [93-95].

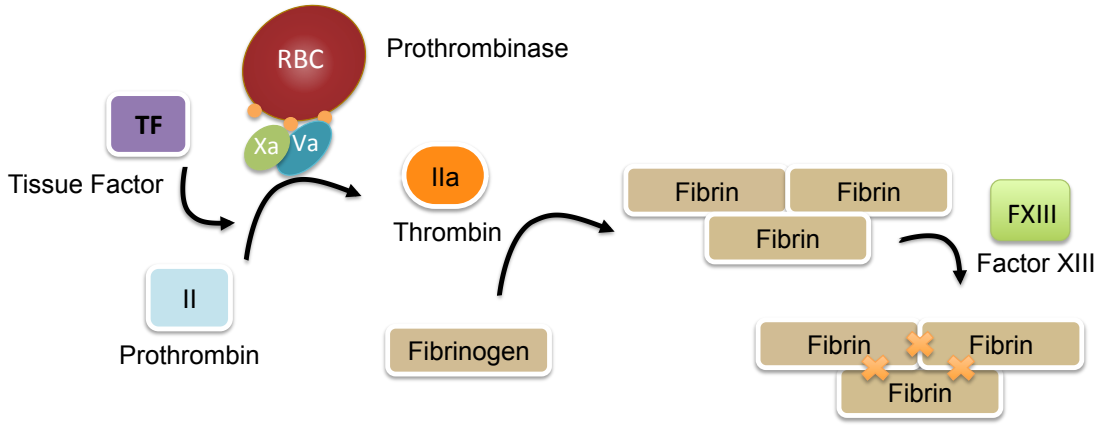

Figure 1.3: A portion of RBCs express PS on their cell membrane. A small percentage (0.5%) of RBCs express PS exposure on their cell surface. This surface allows for prothrombinase complex assembly, prothrombin conversion to thrombin, and fibrin formation. Fibrin can then be crosslinked by the transglutaminase factor XIII, to form the structural support for a clot.

1.3.9 RBCs alter fibrin structure and stability.

RBCs alter fibrin network structure [96, 97] and reduce fibrin network permeability [98]. RBCs also suppress plasmin generation and reduce clot dissolution [97]. In the presence of the substantial contractile forces induced by platelets during clot retraction [99, 100], RBCs are dramatically compressed, which further reduces clot permeability and restricts access of fibrinolytic enzymes to the clot [3, 101]. Importantly, this phenomenon was noted in thrombi harvested from the arterial vasculature [3] but is also likely to have a significant impact on venous thrombosis, since these thrombi contain platelets and large numbers of RBCs. These data suggest reducing RBC content in thrombi may increase clot dissolution, thus reducing thrombosis.

1.4 Conclusions

Both fibrinogen and RBCs are essential components of blood and major players in coagulation. Continued studies are needed to delineate the pathophysiologic mechanisms that mediate the roles of both fibrinogen and RBCs in thrombosis. Specifically, identifying the specific fibrinogen isoform that promotes thrombosis and knowledge of the role of elevated hematocrit in thrombosis will provide new insight into mechanisms that drive abnormal clot formation. Additionally, identification of the RBC-receptor that binds fibrinogen may provide new therapeutic targets to prevent thrombosis.

1.5 Focus of this dissertation

Growing evidence suggests fibrinogen and RBCs play an important role in arterial thrombosis. Previous work on the role of fibrinogen in arterial thrombosis has focused on

total fibrinogen (combined γA/γA and γA/γ') and previous work on RBCs in arterial thrombosis has been performed in murine models with co-morbidities. Therefore, this dissertation will specifically focus on i) the effect of the γA/γA and γA/γ' individually to arterial thrombosis, and ii) the influence of elevated hematocrit to arterial thrombosis in a RBCs transfusion model of elevated hematocrit. Knowledge on how fibrinogen isoforms and RBCs contribute to thrombosis may help elucidate mechanisms involved in the formation of arterial thrombi, reveal biomarkers to predict thrombosis, and provide clues into the best strategies to prevent thrombosis.

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Chapter 2: The fibrinogen γA/γ' isoform does not promote acute arterial thrombosis in mice[2]

2.1 Introduction

Fibrinogen is a 340 kDa glycoprotein that circulates in plasma at 2-4 mg/mL, but during acute inflammation can exceed 7 mg/mL. Fibrinogen is composed of two sets of three polypeptide chains: Aα, Bβ, and γ. Alternative splicing of the main γA chain leads to the γ' chain. Molecules containing the γ' chain circulate as a heterodimer with the γA chain (2Aα, 2Bβ, and γA/ γ') and comprise 8-15% of total fibrinogen in healthy individuals [1, 2]. Elevated fibrinogen levels are associated with increased risk of arterial thrombosis [3-5], and we previously showed that when mice are infused with unfractionated human fibrinogen (~90% γA/γA and 10% γA/γ') and subjected to $FeCl_3$-mediated carotid artery injury, elevated plasma fibrinogen shortens the time to vessel occlusion [6]. These findings suggest elevated fibrinogen is a causative, etiologic agent in arterial thrombosis. However, the specific contributions of γA/γA and γA/γ' fibrinogen isoforms to thrombosis *in vivo* are unknown.

In vitro studies to define the biochemical role of the γ' chain have shown that clots made with purified γA/γ' fibrinogen polymerize at a slower rate than clots made with purified γA/γA fibrinogen [7]. Additionally, the γ' chain supports high affinity binding to thrombin exosite II [8, 9], and studies have shown that thrombin binding to the γ' chain

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competitively inhibits thrombin-mediated platelet activation [10] and reduces thrombin-mediated FpB cleavage [7], and factor VIII [11] and V [12] activation. These properties suggest γA/γ' fibrinogen has anticoagulant activity *in vitro*. Conversely, the γ' chain does not inhibit thrombin-mediated cleavage of FpA [7, 13], and has been reported to support higher affinity binding of FXIII than the γA chain [14], although more recent studies suggest only slightly tighter [14], or even similar [15], binding of FXIII to the γA/γ' isoform compared to the γA/γA isoform. Additional studies in purified systems report contradictory effects of the γ' chain on clot structure and mechanical properties, demonstrating that the γ' chain induces the formation of alternately smaller [7, 13, 16] or larger [17] pores, and stiffer [18] or less stiff [17] clots. These conflicting observations make it difficult to predict the role of γA/γ' fibrinogen under physiologic conditions in thrombosis *in vivo*.

The role of the human γ' chain in thrombosis has previously been tested in two *in vivo* studies. Since the murine γ' chain does not contain the thrombin-binding sequence found on the human γ' chain, Mossesson et al. developed a transgenic mouse that replaced the murine γ' chain with the human γ' chain [19]. Following electrolytic injury to the femoral vein, there was no difference in thrombus volume between mice containing the human γ' chain and wild type (WT) controls, although the presence of the human γ' chain reduced thrombus volume in mice that were also heterozygous for the factor V Leiden mutation [19]. However, interpretation of these findings is complicated by the higher total fibrinogen in WT mice compared to mice expressing the human γ' chain. In a baboon model in which an arteriovenous shunt was placed between the femoral artery and vein, an 18 amino acid peptide mimicking the γ' chain C-terminus (γ' 410-427) inhibited fibrin-rich

thrombus formation [11]. These studies suggest the γ' chain reduces fibrin accumulation and is antithrombotic during venous thrombosis.

Given these findings, it is interesting that retrospective epidemiological studies have correlated elevated γA/γ' fibrinogen levels with *increased* incidence of coronary artery disease [20], myocardial infarction [21], and stroke [22-24]. In particular, the finding that some patients have an increased γ'-to-total fibrinogen ratio [22-25] indicates γA/γ' fibrinogen is not merely a biomarker of increased total fibrinogen, and suggests a specific role for γA/γ' in arterial thrombosis. However, these studies do not and cannot demonstrate causality of γ' chain-containing fibrinogen in thrombosis. The objective of our study was to determine the contribution of γA/γA and γA/γ' fibrinogen to arterial thrombosis.

2.2 Materials and Methods

2.2.1 Proteins and Materials.

Polyclonal rabbit anti-human fibrinogen antibody was from DAKOCytomation (Carpinteria, CA). Monoclonal anti-fibrin(ogen) antibody (59D8) was a generous gift of Drs. Marschall Runge (University of North Carolina), Charles Esmon (Oklahoma College of Medicine), and Rodney Camire (University of Pennsylvania). Mouse anti-human γ' chain-specific antibody (2.G2.H9) was from Millipore (Temecula, CA). Biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA). The AlexaFluor-488 protein labeling kit and 10% pre-cast Tris-glycine gels were from Invitrogen (Carlsbad, CA). Human α-thrombin and murine thrombin were from Enzyme Research Laboratories (South Bend, IN). Lipidated tissue factor (TF, Innovin) was from Siemens (Newark, DE). Phospholipid vesicles (phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine)

were prepared as described [26]. Bovine serum albumin was from Sigma-Aldrich (St. Louis, MO). Peroxidase substrate was from KPL (Gaithersburg, MD).

2.2.2 Plasma preparation.

Contact-inhibited human normal pooled plasma (hNPP) was prepared from 40 healthy subjects (50% female, 68% nonwhite) as described [27], in a protocol approved by the UNC Institutional Review Board. γA/γ' fibrinogen levels in hNPP were measured by ELISA, as described [28]. Murine normal pooled plasma (mNPP) was prepared by collecting blood from 49 female C57Bl/6 mice by inferior vena cava (IVC) venipuncture into 3.2% sodium citrate (1:9 ratio sodium citrate:blood). Pooled whole blood was centrifuged (4000*xg*, 20 minutes), and platelet-poor plasma was aliquoted and frozen at -80^{o}C.

2.2.3 Isolation of γA/γA and γA/γ' fibrinogen.

The γA/γA and γA/γ' fibrinogen variants were separated from human plasminogen-, von Willebrand Factor-, and fibronectin-depleted human fibrinogen (Enzyme Research Laboratories Ltd., Swansea, UK), based on the method described previously [7]. After purification, variants were concentrated using Vivaspin 20 MWCO 100,000 columns (GE Healthcare, Uppsala, Sweden) and dialyzed into 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.4) containing 150 mM NaCl (HBS). Fibrinogen concentration was determined by absorbance at 280 nm using an extinction coefficient of 1.51 mL/(mg/cm). Both variants were functionally active (>95%) in a standard clotability assay.

2.2.4 SDS-PAGE and western blotting.

Fibrinogen preparations were assessed by 10% SDS-PAGE and Coomassie Brilliant Blue staining or western blotting for total fibrinogen or fibrinogen γ' chain. For western blots, membranes were blocked with Tris-buffered saline with 1% Tween containing 5% milk, washed, and probed sequentially with mouse-anti human γ'-specific primary antibody and AlexaFluor-488 conjugated anti-mouse secondary antibody. Fluorescent signal was detected on a Typhoon 900 FLA fluorescent scanner.

2.2.5 Clot formation with purified fibrin(ogen).

Purified fibrinogen, thrombin, and $CaCl_2$ (0.5 mg/mL, 5 nM, and 10 mM, final, respectively) were combined in 96-half-well plates and polymerization was monitored by turbidity at 405 nm using SpectraMax Plus340 plate reader (Molecular Devices, Sunnyvale, CA).

2.2.6 Clot formation in plasma.

hNPP or mNPP was spiked with HBS (Control), or γA/γA or γA/γ' fibrinogen, and clotting was initiated with TF (1:30,000 dilution of Innovin, final), 10 mM $CaCl_2$, and 4 μM phospholipid vesicles in 96-well plates. Clot formation was monitored by turbidity at 405 nm.

2.2.7 Intravital microscopy.

Procedures were approved by the UNC Institutional Animal Care and Use Committee. Laser-induced thrombosis to cremaster muscle venules was performed as described [29].

Briefly, 6-8 week old male C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) were anesthetized and laser injuries were induced with an Ablate! photoablation system equipped with an attenuatable 532 nm pulse laser (Intelligent Imaging Innovations, Denver, CO). Five minutes before injury, mice were injected via the retro-orbital plexus with AlexaFluor 595-labeled anti-glycoprotein IX antibody (0.3 mg/g body weight; Emfret, Eibelstadt, Germany), and AlexaFluor 647-labeled murine anti-fibrin antibody (0.2 mg/g body weight), and trace amounts (5% of total fibrinogen) of AlexaFluor 488-labeled γA/γA or γA/γ' fibrinogen. Five venules maximum were studied per mouse.

2.2.8 $FeCl_3$ thrombosis model.

$FeCl_3$ injury to carotid arteries was performed as described [6]. Briefly, 6-8 week old male C57Bl/6 mice were anesthetized, and human fibrinogen or vehicle (HBS) was administered through the left saphenous vein cannula on a per-weight basis 5 minutes before injury. The right common carotid artery was exposed, dried and treated with $FeCl_3$ (10% on 0.5×1.0-mm filter paper) for 2 minutes. We specifically titrated the conditions to perform these experiments at a threshold at which some mice do not form thrombi, to allow for sensitivity to both increased and decreased procoagulant activity. Blood flow was monitored by Doppler ultrasonic flow probe, and the time to occlusion (TTO) was defined as the time between $FeCl_3$ administration and lack of flow for 60 consecutive seconds, as previously described [6].

2.2.9 Measurement of circulating TAT complexes.

TAT levels were measured by ELISA (Enzygnost TAT micro ELISA, Siemens) using plasma prepared from IVC blood draws from mice subject to $FeCl_3$ carotid artery thrombosis (blood was drawn ~5 minutes following vessel occlusion). Samples showing hemolysis were excluded.

2.2.10 Statistical Methods.

Descriptive statistics (mean, median, standard deviation [SD], standard error of the mean [SEM]) were calculated. Groups were compared using Student's t-tests (normally-distributed data determined by Lilliefors test for normality) or Wilcoxon-Mann-Whitney Rank Sum Tests (non-normally distributed data) in Kaleidagraph v4.1.3. Correlations were performed using SAS 9.2 (SAS Inc., Cary, NC). $P<0.05$ was considered statistically significant.

2.3 Results

2.3.1 γA/γA fibrinogen increases the fibrin polymerization rate to a greater extent than γA/γ' fibrinogen.

Purified γA/γA fibrinogen contained all three fibrinogen chains (Aα, Bβ, and γ) at expected molecular weights (Figures 2.1A-B). No γ' chain was detected in γA/γA fibrinogen (Figure 2.1C), whereas purified γA/γ' fibrinogen showed equal intensities of γA and γ' bands (Figures 2.1A-B). We first clotted purified fibrinogens with purified human thrombin and followed clotting by turbidity. Although fibrinogen γA/γA and γA/γ' isoforms were not explicitly depleted of FXIII, Allen et al. previously showed that the presence or absence of

FXIII does not affect differences in polymerization between γA/γA and γA/γ' fibrinogen [17]. Indeed, consistent with previous reports [7, 13, 17], purified γA/γA exhibited a faster polymerization rate (2.7-fold, $P<0.05$) and higher final turbidity (1.5-fold, $P<0.05$) than purified γA/γ' (Figure 2.1D, Table 2.1). Findings were similar when murine thrombin was used (Figure 2.1D, Table 2.1), showing murine thrombin can convert human fibrinogen to fibrin.

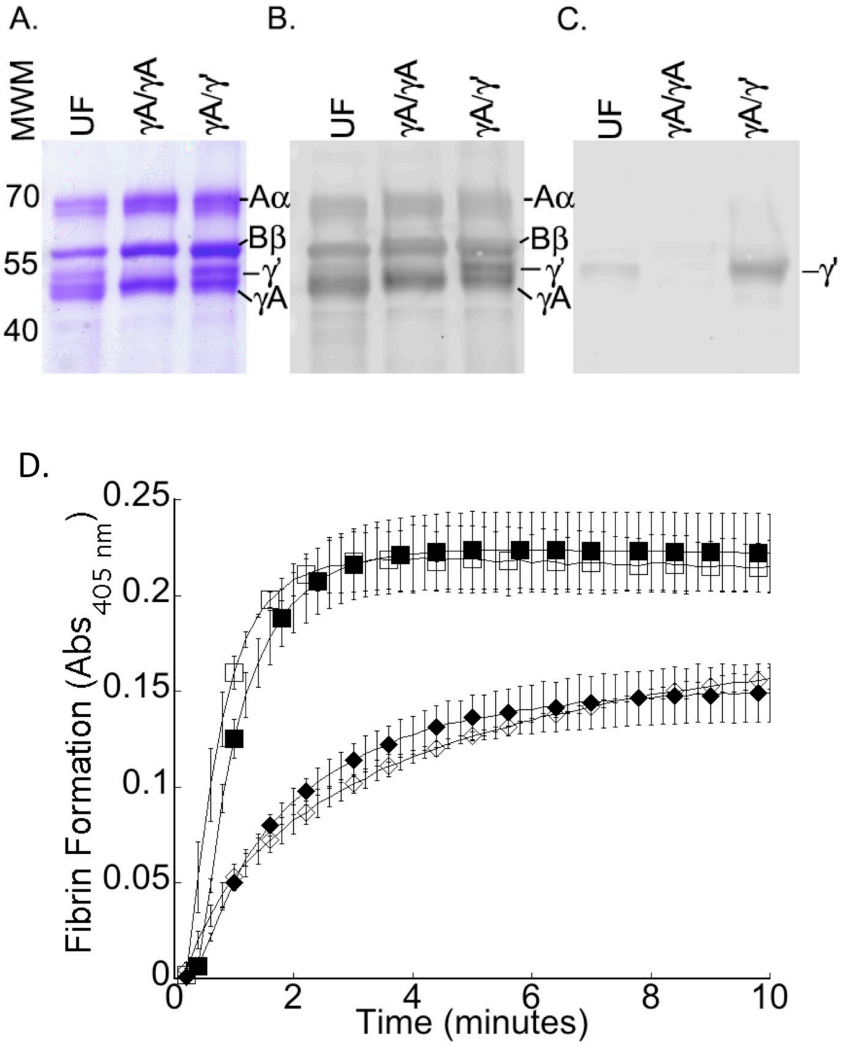

Figure 2.1. Purified fibrinogen contains all three chains (Aα, Bβ, and γA and/or γ') at the expected molecular weights and is equally cleaved by human and mouse thrombin. Unfractionated (UF), or purified γA/γA, or γA/γ' fibrinogen were reduced and separated by 10% SDS-PAGE and detected by: A) Coomassie Brilliant Blue staining, B) polyclonal anti-fibrin(ogen) antibody, or C) 2.G2.H9 antibody against the γ' chain. D**)** Purified human γA/γA (squares) or γA/γ' (diamonds) fibrinogen was clotted in the presence of $CaCl_2$ and human (closed symbols) or murine (open symbols) thrombin. Data show mean±SD, for experiments with human (n=3) and mouse (n=2) thrombin.

Table 2.1. Polymerization of purified fibrinogen isoforms by human and murine thrombin

	Human Thrombin			**Murine Thrombin**		
	Lagtime (seconds)	**Change in Turbidity (OD)**	**Vmax (mOD/min)**	**Lagtime (seconds)**	**Change in Turbidity (OD)**	**Vmax (mOD/min)**
γA/γA	14.2±4.7	0.222±0.022	179.6±16.5	8.25±4.9	0.214±0.024	208.7±38.4
γA/γ'	13.7±5.0	0.149±0.016[#]	66.3±6.5[#]	6.25±6.2	0.164±0.012[#]	61.1±14.7[#]

Mean±SD, [#]P<0.05 versus γA/γA

To determine the effect of elevated γA/γA and γA/γ' fibrinogen on plasma clot formation during *in situ* thrombin generation, we spiked purified γA/γA, γA/γ', or HBS (control) into hNPP. The concentration of fibrinogen in hNPP was 3.1±0.1 mg/mL (100%) and baseline concentration of γA/γ' fibrinogen in hNPP was 0.42 mg/mL (13.5% of total fibrinogen). We increased the total fibrinogen concentration to 3.5 (114%), 3.9 (127%), or 4.4 (143%) mg/mL by spiking in purified γA/γA or γA/γ', so that the γA/γ'-to-total fibrinogen ratios ranged from 9.6-40.1% (Table 2.2). These levels span the range of γA/γ' levels measured in healthy individuals and patients with thrombosis [23-25, 30, 31]. Elevating either γA/γA or γA/γ' fibrinogen increased final clot turbidity compared to plasma spiked with HBS (Figure 2.2B, Table 2.2). When total fibrinogen was raised to 114%, neither γA/γA nor γA/γ' fibrinogen increased the clot formation rate. However, elevating total fibrinogen to 127% or 143% with γA/γA or γA/γ' significantly and dose-dependently increased the clot formation rate versus baseline (HBS). Notably, at each concentration, elevating total fibrinogen with γA/γA increased the clot formation rate to a significantly greater extent than elevating total fibrinogen with γA/γ' (Figure 2.2C, Table 2.2). Linear regression analysis showed that the clot formation rate correlated positively with elevated total fibrinogen ($r=0.667$, $P<0.001$) and negatively with the γ'-to-total fibrinogen ratio ($r=-0.0245$, $P=0.17$), although the relationship between γ'-to-total and clot formation rate did not reach significance. Moreover, the level of γA/γA isoform correlated strongly with the clot formation rate ($r=0.795$, $P<0.001$) whereas the level of γA/γ' did not.

Spiking purified human γA/γA, γA/γ', or HBS (Control) into mNPP produced similar results. For these experiments, the fibrinogen concentration in mNPP was 2.4±0.2 mg/mL (100%), and we spiked mNPP to 3.2 (135%) and 4.1 mg/mL (170%) with γA/γA or γA/γ',

yielding human γ'-to total fibrinogen ratios ranging from 0-41.2%. Consistent with previous observations [6], the final turbidity of murine plasma clots was lower than that of human plasma clots, likely reflecting increased fibrin density of murine fibrin networks versus human networks (unpublished observation). As in human plasma, both γA/γA and γA/γ' increased the clot formation rate, but γA/γA increased the rate to a greater extent than γA/γ' at each concentration tested ($P<0.02$, Figure 2.2F, Table 2.3). These findings suggest that during *in situ* thrombin generation, both elevated γA/γA and γA/γ' fibrinogen promote clot formation, but γA/γA does so to a greater extent.

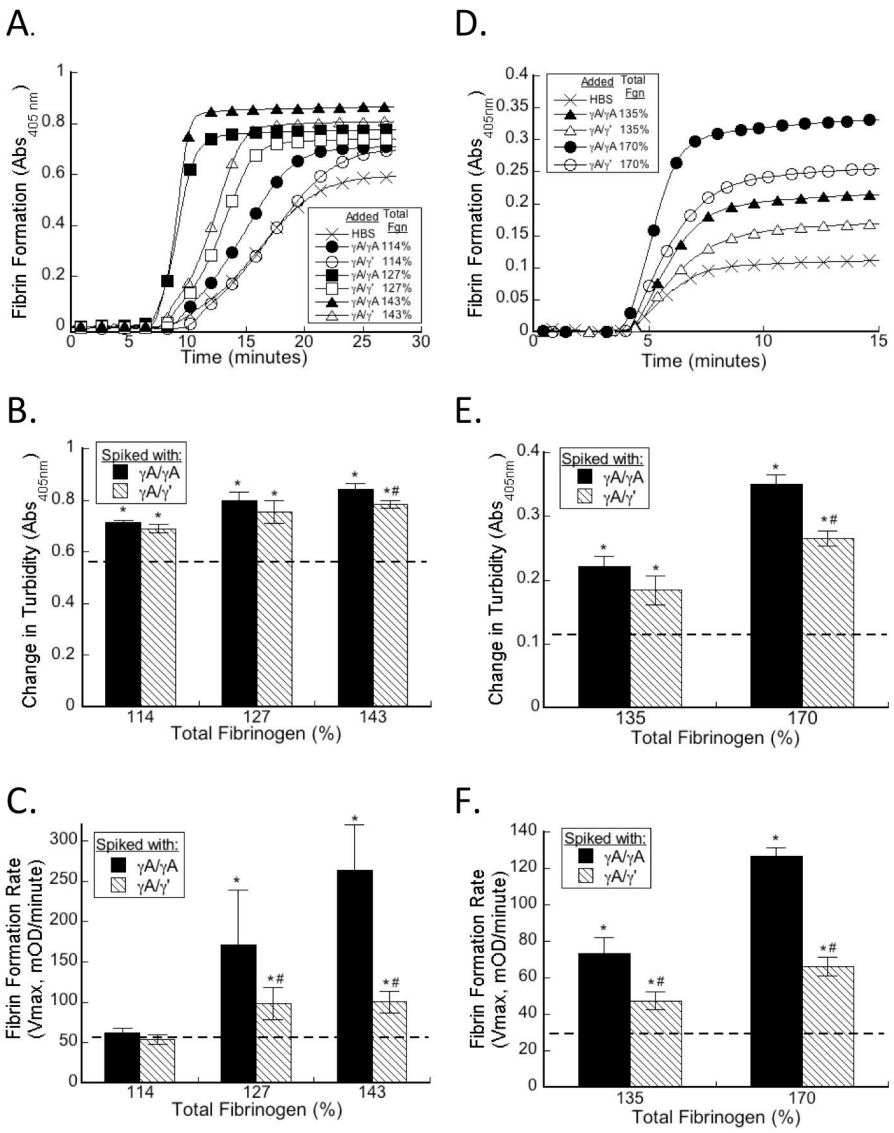

Figure 2.2. Both γA/γA and γA/γ' fibrinogen accelerate clotting in human and mouse plasma. A-C) hNPP was spiked with γA/γA or γA/γ' to increase total fibrinogen to 114%, 127%, or 143% of normal (symbols appear in figure legend), and clot formation was triggered by addition of TF and $CaCl_2$. D-F) mNPP was spiked with human γA/γA or γA/γ' to increase total fibrinogen to 135% or 170% of normal (symbols appear in figure legend) and clot formation was triggered by addition of TF and $CaCl_2$. A, D) Polymerization was monitored by turbidity; for clarity, only a subset of points is shown. B, C, E, F) The contribution of increasing total fibrinogen with γA/γA (solid bars) or γA/γ' (striped bars) on final turbidity (B, E) and fibrin formation rate (C, F) in human (B, C) and mouse (E, F) plasma. Dashed lines represent final turbidity and clot formation rate of HBS controls. Data show means, n=3. *$p<0.05$ versus HBS; $^{\#}p<0.05$ versus γA/γA.

Table 2.2. Effect of fibrinogen isoforms on human plasma clotting

Total Fibrin-ogen (mg/mL [%])	Fibrin-ogen/ Buffer Infused	Human γA/γ' Final (mg/mL)	Human γ'-to-Total Ratio (%)	Lagtime (minutes)	Change in Turbidity (OD)	Vmax (mOD/min)
3.1 (100%)	HBS	0.4	13.5	9.7±3.0	0.587±0.034	54.8±9.3
3.5 (114%)	γA/γA	0.4	11.9	9.7±1.5	0.715±0.007*	62.5±5.7
3.5 (114%)	γA/γ'	0.8	23.9	8.7±0.6	0.690±0.016*	54.3±5.8
3.9 (127%)	γA/γA	0.4	10.7	8.7±1.9	0.789±0.032*	171.7±67.1*
3.9 (127%)	γA/γ'	1.3	32.0	10.0±3.4	0.755±0.043*	98.2±19.9*,#
4.4 (143%)	γA/γA	0.4	9.6	8.5±1.1	0.844±0.022*	263.9±56.6*
4.4 (143%)	γA/γ'	1.8	40.1	10.1±13.5	0.784±0.016*,#	100.4±13.5*,#

Mean±SD, *P<0.05 versus HBS; #P<0.04 versus γA/γA (at same total fibrinogen)

Table 2.3. Effect of fibrinogen isoforms on mouse plasma clotting

Total Fibrin-ogen (mg/mL [%])	Fibrinogen/ Buffer Infused	Human γA/γ' Final (mg/mL)	Human γ'-to-Total Ratio (%)	Lagtime (minutes)	Change in Turbidity (OD)	Vmax (mOD/min)
2.4 (100%)	HBS	0	0	4.0±0.3	0.111±0.003	28.0±2.5
3.2 (135%)	γA/γA	0	0	3.5±0.3	0.222±0.016*	73.3±8.9*
3.2 (135%)	γA/γ'	0.8	25.9	4.0±0.4	0.184±0.023*	47.4±5.0*,#
4.1 (170%)	γA/γA	0	0	4.0±0.3	0.350±0.015*	126.7±4.6*
4.1 (170%)	γA/γ'	1.7	41.2	4.1±0.2	0.265±0.012*,#	66.2±5.2*,#

Mean±SD, *P<0.03 versus HBS; #P<0.02 versus γA/γA (at same total fibrinogen)

2.3.2 Both γA/γA and γA/γ' fibrinogen are incorporated into murine thrombi *in vivo*.

Drouet et al. previously suggested that an increased γ'-to-total fibrinogen ratio is detected in patient plasmas because γA/γA is incorporated into platelet thrombi, whereas γA/γ' is not [25]. Therefore, we determined whether γA/γ' was incorporated into thrombi *in vivo*. We infused mice with AlexaFluor 594-labeled anti-platelet (anti-GPIX) antibody, AlexaFluor 647-labeled antibody against fibrin(ogen) (59D8), and trace amounts (5% of total fibrinogen) of fluorescently-labeled γA/γA or γA/γ' fibrinogen. We then triggered vascular injury to the cremaster vessels and detected γA/γA or γA/γ' fibrinogen within thrombi using intravital microscopy. We initially performed this experiment with arterioles, but observed substantial vessel constriction in response to the injury. However, the venule provided a reasonable alternative that enabled us to avoid the issue of vasoconstriction while observing platelet and fibrin(ogen) accumulation at the injury site *in vivo*. Figure 2.3 shows that both γA/γA and γA/γ' isoforms were incorporated into murine thrombi *in vivo*.

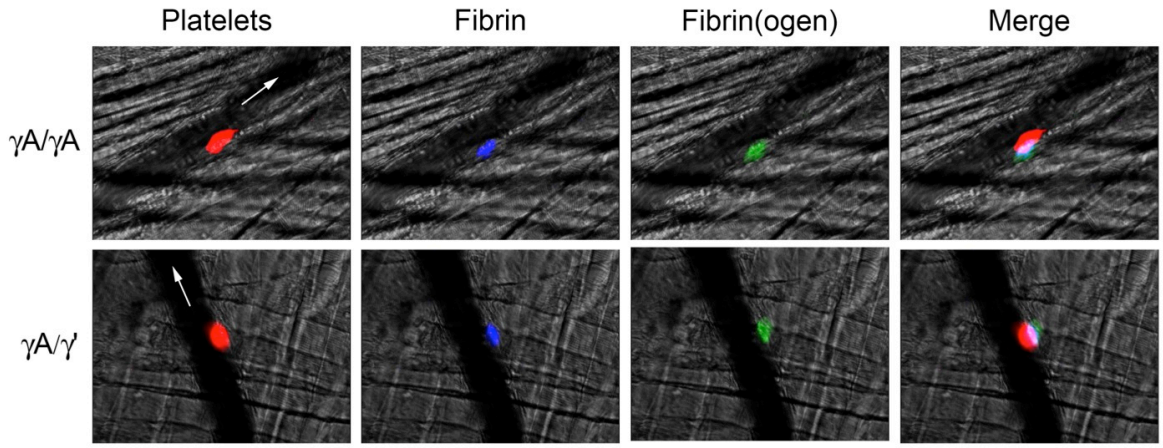

Figure 2.3. Intravital microscopy shows both γA/γA and γA/γ' isoforms are incorporated into murine thrombi. Venules were visualized in the cremaster muscle of mice infused with HBS (control) or AlexaFluor 594-labeled anti-platelet (anti-GPIX) antibody, AlexaFluor 647-labeled anti-fibrin antibody, and purified γA/γA or γA/γ' directly labeled with AlexaFluor 488. Thrombosis was triggered via laser injury. Flow is indicated by white arrows. Colors are: platelets (red), fibrin(ogen) (green), and fibrin (blue). In the merged image, colors are: platelets plus fibrin(ogen) (pink), platelets plus fibrin (purple), and fibrin(ogen) plus fibrin (teal). Images show representative thrombi from 3-4 mice with 14-20 injuries total.

2.3.3 Following $FeCl_3$ injury, γA/γA, but not γA/γ', fibrinogen shortens the time to artery occlusion.

To determine the effect of elevated circulating γA/γA and γA/γ' fibrinogen on arterial thrombosis, we infused mice with HBS or purified human γA/γA, γA/γ', or unfractionated fibrinogen and induced thrombosis via $FeCl_3$ application to the carotid artery. Both human and mouse fibrinogen can be cleaved by human and murine thrombin, cross-linked by factor XIIIa, and digested by plasmin [32]. Additionally, human fibrinogen circulates in mouse plasma, and is incorporated into murine thrombi (Figure 2.3, [6, 33-35]). For these experiments, we obtained total fibrinogen levels of 135% and 170% of normal levels, with human-γ'-to-total fibrinogen ratios of 0%, 25.9%, and 41.2%, consistent with ratios found in normal and pathological conditions [23-25, 30, 31, 36].

Consistent with previous findings, following $FeCl_3$ injury, there was no significant difference in TTO between control mice or mice infused to 135% mg/mL total fibrinogen with either γA/γA or γA/γ' (data not shown) [6]. When total fibrinogen was raised to 170% with γA/γA fibrinogen, the median TTO was faster than that of mice infused with HBS (5.48±0.50 versus 7.25±3.03 minutes [median±SEM], $P<0.05$, Figure 2.4A), similar to that seen in mice infused with unfractionated fibrinogen. However, raising the level of fibrinogen to 170% with γA/γ' fibrinogen did not shorten the median TTO compared to controls (Figure 2.4A). Moreover, 7.25 minutes after $FeCl_3$ injury, 100% and 86% of mice infused with unfractionated or γA/γA fibrinogen, respectively, had an occluded vessel, whereas only 50% of mice infused with γA/γ' fibrinogen developed vessel occlusion (Figure 2.4B). Together, these data indicate that elevated γA/γA fibrinogen promotes arterial thrombosis, whereas elevated γA/γ' does not.

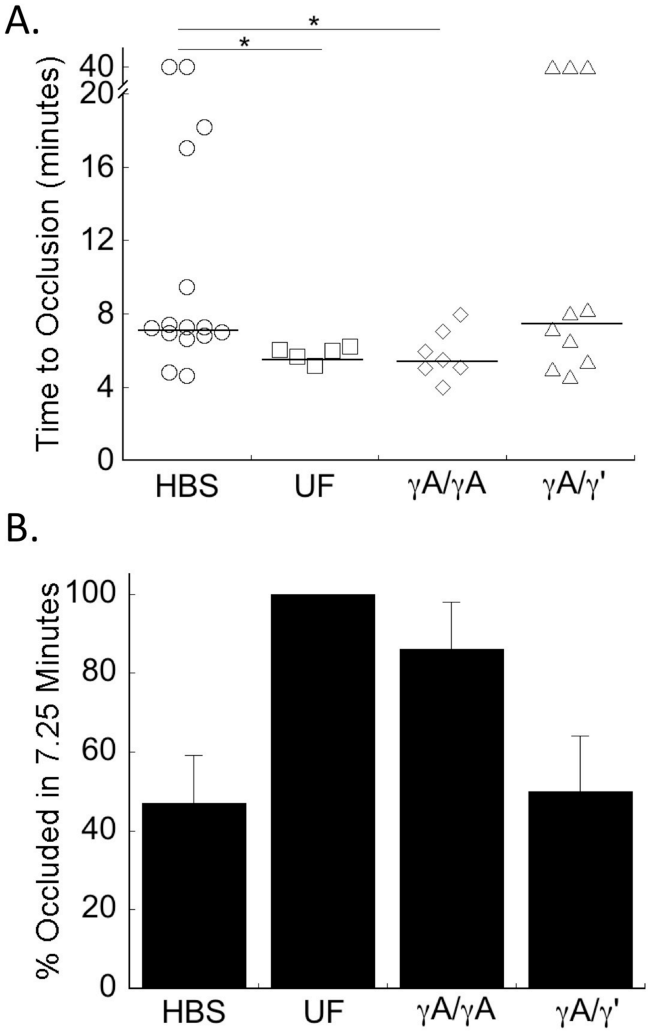

Figure 2.4. γA/ γA fibrinogen shortens the time to vessel occlusion after arterial injury, but γA/γ' does not. Mice were infused with HBS, unfractionated (UF), γA/γA, or γA/γ' fibrinogen to 170%, total fibrinogen. Thrombosis was induced by $FeCl_3$ application to the carotid artery and TTO was determined by Doppler flow probe. In vessels that did not occlude, the TTO was recorded as 40 minutes. A) Each point represents a separate mouse. Lines indicate median values, *$p<0.05$ versus HBS. B) Percent of mice occluded at 7.25 minutes (the median TTO of HBS-infused mice), using the data from (A); 100%, 86%, and 50% of UF-, γA/γA-, and γA/γ'-infused mice, respectively, had occluded vessels at this time.

2.3.4 Following $FeCl_3$ injury, mice infused with γA/γ' fibrinogen have lower circulating TAT complexes than mice infused with γA/γA fibrinogen.

The γ' chain supports high affinity binding to thrombin exosite II [8, 9], and prior studies have shown that γA/γ' fibrinogen has anticoagulant properties (antithrombin I activity) *in vitro* [10-12]. To determine the effect of γA/γ' on procoagulant activity *in vivo*, we measured TAT complexes in murine plasma following $FeCl_3$ injury and stable vessel occlusion. Whereas mice infused with unfractionated or γA/γA fibrinogen had similar circulating TAT complexes as HBS-infused mice, mice infused with γA/γ' had significantly lower circulating TAT complexes (6.2±8.4 versus 18.9±10.9 ng/mL [median±SEM] for γA/γ' and HBS-infused mice, respectively, $P<0.01$, Figure 2.5), consistent with the concept that thrombin binding to γA/γ' fibrinogen sequesters thrombin [10-12, 37] and protects it from inhibition by antithrombin. These findings suggest γA/γ' fibrinogen binds and sequesters thrombin *in vivo* and limits thrombin activity following vascular injury.

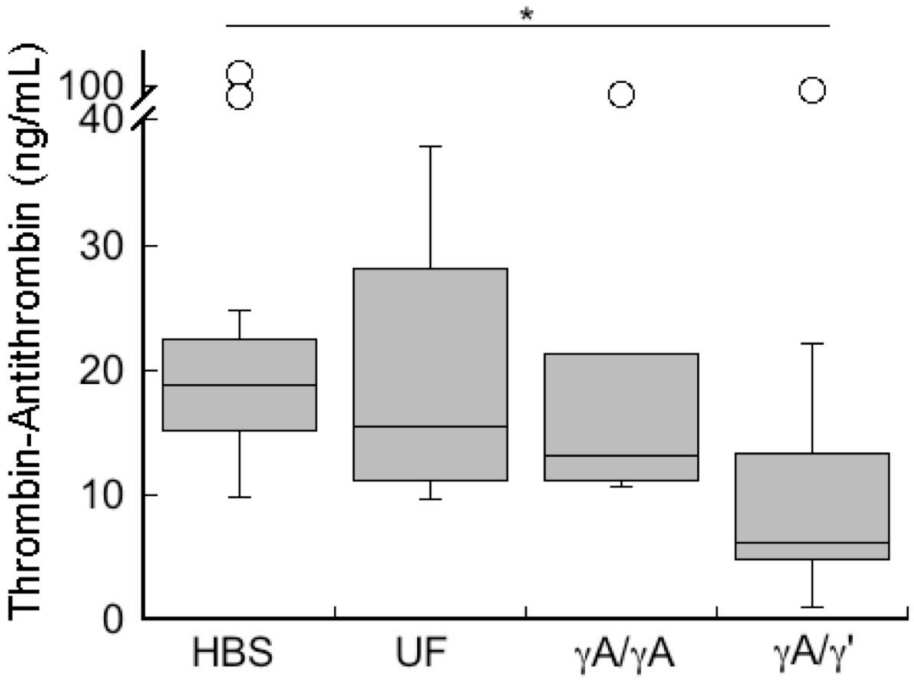

Figure 2.5. Following arterial injury, mice infused with γA/γ' fibrinogen have reduced circulating TAT complexes. TAT levels were measured in plasmas collected from mice subjected to the $FeCl_3$ carotid artery thrombosis. Box plots indicate medians and upper and lower quartiles, *p<0.05 versus HBS**.**

2.4 Discussion

Although epidemiologic studies have associated elevated plasma fibrinogen with arterial thrombosis [3-5], the operant pathogenic mechanisms have been controversial. We previously showed that increased total plasma fibrinogen directly promotes arterial thrombosis in mice [6]. Herein, we separately tested the role of γA/γA and γA/γ' fibrinogen and show that both elevated γA/γA and γA/γ' increased the plasma clot formation rate, but that γA/γA increased the rate to a greater extent than γA/γ'. Although both γA/γA and γA/γ' fibrinogen were incorporated into murine clots, γA/γA fibrinogen shortened the TTO, whereas γA/γ' did not. Interestingly, compared to controls, mice infused with γA/γ' fibrinogen had lower levels of circulating plasma TAT complexes following arterial injury, whereas mice infused with γA/γA did not, suggesting that γA/γ' fibrinogen binds and sequesters thrombin *in vivo*. Together, our data indicate that γA/γ' fibrinogen is not prothrombotic *in vivo* and may even have a protective role in preventing elevated total fibrinogen levels from promoting thrombosis.

Our data support the premise that γA/γ' fibrinogen has both procoagulant and anticoagulant properties and exhibits both of these activities during thrombosis *in vivo*. Similar to γA/γA fibrinogen, γA/γ' increased the fibrin formation rate and final turbidity, though to a lesser extent than γA/γA. Consequently, increased total fibrinogen levels, via either increased γA/γA or γA/γ', would be expected to promote fibrin formation. However, unlike γA/γA, γA/γ' fibrinogen exhibits antithrombin I activity *in vitro* [10-12, 37] and *in vivo* (Figure 2.5). Thus, our finding that elevated γA/γA fibrinogen shortened the TTO, but elevated γA/γ' did not, suggests that the net effect of γA/γ' fibrinogen's opposing procoagulant and anticoagulant activities yielded no change in the TTO (Figure 2.6). These

data suggest that a peptide representing the C-terminus of the γ' chain would have strong anticoagulant effects *in vivo,* since the procoagulant properties of the full length fibrinogen molecule would not be present, whereas the thrombin binding properties of the γ' chain would decrease circulating thrombin. Indeed, this effect was previously demonstrated during *in vivo* thrombosis, in which Lovely et al. saw decreased platelet and fibrin accumulation in the presence of γ' chain peptide [11].

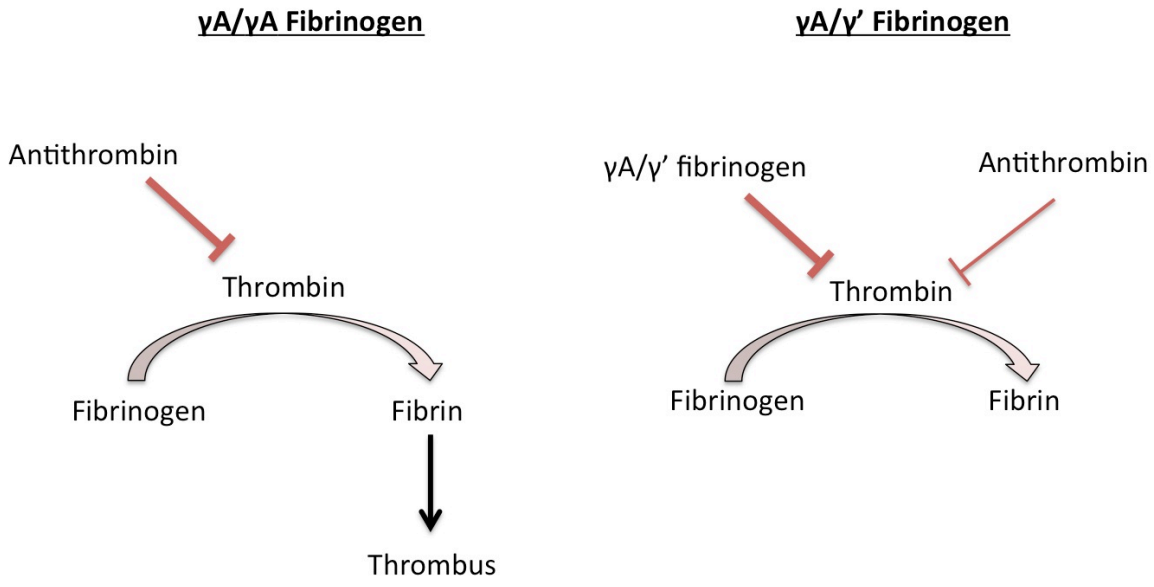

Figure 2.6. γA/γ' fibrinogen binds thrombin, resulting in lower thrombin-antithrombin levels and similar TTO as controls. When mice were infused with γA/γA fibrinogen, fibrinogen was converted to fibrin by thrombin to eventually cause a shortened TTO. In these mice, thrombin was inhibited by antithrombin (left). When mice were infused with γA/γ' fibrinogen, the γ' binds to thrombin, preventing its activity from shortening the TTO, resulting in less thrombin bound to antithrombin (right).

Although previous studies have compared isolated γA/γA and γA/γ' fibrinogens in purified systems, only one has done so during *in situ* thrombin generation in plasma. Using plasmas from apparently healthy Black South Africans, Pieters et al. correlated total fibrinogen levels, γA/γ' fibrinogen levels, and the γ'-to-total fibrinogen ratio with the plasma clot formation rate and turbidity change [38]. Their data suggest that the clot formation rate increases with total fibrinogen, but decreases with elevated γ'-to-total fibrinogen ratio. Our data extend these findings in a system that enabled us to precisely control fibrinogen isoform levels and avoid variability between donor plasmas. Consistent with Pieters et al., we found the clot formation rate correlated positively with elevated total fibrinogen. Importantly, the level of γA/γA isoform correlated strongly with the clot formation rate, whereas the level of γA/γ' did not, suggesting the increase in clot formation rate caused by elevated total fibrinogen is due to γA/γA fibrinogen.

Two prior studies evaluated the effect of the γ' chain on thrombosis *in vivo*. Those studies were limited by differences in the total fibrinogen level expressed by WT and human γ'-expressing mice [19] and use of isolated γ' peptide rather than full length γA/γ' fibrinogen [11]. Moreover, Mosesson et al. [19] evaluated γA/γ' fibrinogen in a venous thrombosis model, and although the arteriovenous shunt model used by Lovely et al. [11] included aspects of arterial thrombosis, it did not recapitulate endothelial denudation and subendothelial exposure associated with plaque rupture and arterial thrombus formation. Consequently, our study supports and extends the prior findings in several important ways. First, our infusion strategy enabled us to tightly-control the level of circulating γA/γA and γA/γ' fibrinogen, allowing us to specifically attribute effects to the levels of isoform and total fibrinogen. Second, our study demonstrated the antithrombin I properties of the full-length

form of the γ' chain. Third, our findings extend previous data from venous thrombosis to arterial pathology. This extension is important since the role of γA/γ' in arterial thrombosis has been controversial. Our findings provide important evidence that γA/γA fibrinogen is causative in the etiology of arterial thrombosis, whereas γA/γ' fibrinogen is not.

Given our findings showing that γA/γ' fibrinogen does not promote arterial thrombosis, it remains unclear why epidemiological studies find a positive association between elevated γA/γ' fibrinogen and arterial thrombosis. Previous studies have suggested that clots formed from γA/γ' fibrinogen are more resistant to lysis, and conflicting studies report abnormal structure and mechanical stability in γ'-chain containing clots [7, 17, 18]. Thus, γA/γ' fibrinogen may produce clots with increased stability that are detected because they persist longer than clots that contain γA/γA. Interestingly, hypofibrinolysis is correlated with increased risk of arterial thrombosis in young (<~50) [39, 40], but not older (>~50) individuals [41, 42], suggesting abnormal clot stability explains some, but not all, of the mechanisms leading to arterial thrombosis. Future studies are warranted to determine the effect of the γA/γ' isoform on arterial clot stability.

Interestingly, Rein-Smith et al. recently showed interleukin-6 preferentially up-regulates hepatocyte production of γA/γ' fibrinogen compared to γA/γA [43]. These data suggest γA/γ' ("antithrombin I") expression is upregulated to limit endogenous procoagulant activity triggered by inflammation. Indeed, C-reactive protein is elevated in patients with a history of arterial thrombosis [23], reflecting the proinflammatory pathology. Increased γA/γ' levels detected in patients after arterial thrombosis are likely a consequence of disease rather than cause, and reflect an innate, antithrombotic response to inflammation. Although our fibrinogen infusion/acute thrombosis model enabled us to isolate and investigate the

immediate, direct effects of elevated γA/γA and γA/γ' on thrombus formation, it did not recapitulate the inflammatory process associated with atherosclerosis. Consequently, long-term exposure to circulating γA/γ' fibrinogen may have additional effects on plaque formation and/or stability. Notably, however, Mosesson et al. did not report evidence of chronic inflammation or atherosclerosis in their model of chronically-elevated fibrinogen γ' levels [19] suggesting even chronic exposure to elevated γA/γ' fibrinogen levels does not cause thrombosis.

In summary, our results show that both γA/γA and γA/γ' fibrinogen increased the fibrin formation rate in plasma, but γA/γA fibrinogen accelerated the rate to a greater extent than γA/γ' fibrinogen. After arterial injury, γA/γA fibrinogen promoted thrombosis, whereas γA/γ' did not. Mice infused with γA/γ' had lower levels of circulating TAT complexes, suggesting that following vascular injury, γA/γ' fibrinogen binds thrombin *in vivo* and limits thrombin activity. Our data establish independent roles of fibrinogen γA/γA and γA/γ' in arterial thrombosis, and suggest γA/γA fibrinogen promotes thrombosis, whereas γA/γ' sequesters thrombin and protects against procoagulant processes induced by inflammation.

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Chapter 3: Elevated Hematocrit Promotes Arterial Thrombosis Independent of Thrombin Generation

3.1 Introduction

Red blood cells (RBCs) are the most abundant cell type in blood. Normal RBC levels range from ~ 4.2 to 6.1×10^9/mL in humans, with numbers being slightly higher in males than in females. RBCs develop in the bone marrow, and circulate for ~120 days. RBCs are primarily known for their role in oxygen transport via their hemoglobin-rich cytoplasm.

Despite being a major component of thrombi, RBCs have been thought to be passive participants in hemostatic and thrombotic processes. However, growing evidence suggests RBCs directly contribute to coagulation. Longitudinal, prospective studies have implicated elevated hematocrit as an independent risk factor for cardiovascular disease (CVD) and CVD-related deaths. In a cohort of 2555 rural and 6151 urban men, incidence of myocardial infarction (MI), coronary insufficiency, or coronary heart disease (CHD) deaths were more than double in the high hematocrit group (hematocrit greater than 49%) compared to the low group (hematocrit less than 42%) [1]. Additionally, in a cohort of 2014 healthy men that were followed over a 16-year period, a high hematocrit was associated with increased risk of dying from CVD [2]. Moreover, health data were examined from 5209 men and women (age range 30-62) participating in the Framingham study and in a 34-year follow-up, it was determined that men and women in the highest hematocrit quintile (men ≥49%; women ≥46%) had an increased risk of death from CVD [3]. More recently, Toss et al. [4] found that younger men (18-19 years old) with an elevated hematocrit (≥49%) had a 1.4-fold

increased risk of MI compared to men with lower hematocrit (≤44%) over the 36-year follow-up. This relationship was linear and remained constant throughout the follow-up period.

RBC transfusion can alleviate bleeding in anemic patients [5], but is also associated with thrombosis in cancer patients [6], following surgery [7], and after subarachnoid hemorrhage [8]. In the latter study, thrombotic risk was dose-dependent with number of RBC units transfused and independent of unit storage time. Moreover, a recent, large meta-analysis of blood transfusion following MI found that transfusion was associated with higher risk of mortality and subsequent MI compared to non-transfused controls [9]. These studies strongly associate elevated hematocrit with thrombosis. However, it remains unclear whether an elevated hematocrit is directly causal in arterial thrombus formation.

Recent studies have documented specific properties of RBCs that may contribute to thrombosis. First, RBCs express phosphotidylserine (PS) on their cell membranes [10] and these PS-positive surfaces support prothrombinase assembly and thrombin generation [11-13]. Patients with prothrombotic disorders such as polycythemia vera (PV), β-thalassemia, and sickle cell disease (SCD), have increased numbers of circulating PS-positive RBCs [14-16], and in patients with SCD, the number of circulating PS-positive RBCs correlates with prothrombin fragment 1.2, a measure of ongoing thrombin generation [17]. These data suggest RBC procoagulant activity supports thrombin generation *in vivo*. Second, RBCs increase platelet reactivity. ADP released by RBCs has been shown to activate platelets and this activation has been shown to be resistant to aspirin [18, 19]. These data suggest hematocrit may influence the therapeutic effect of aspirin. Third, RBCs are the principle determinants of blood viscosity and therefore blood flow. Blood viscosity increases

exponentially with a rise in the hematocrit; thus, a relatively small increase in hematocrit produces a logarithmic increase in viscosity [20]. For example, a rise in hematocrit from 40% to 60% may increase blood viscosity 2.5-fold at high shear and 3.0-fold at low shear [20]. Increased blood viscosity reduces blood flow and is associated with increased risk of thrombosis [21]. Finally, hematocrit mediates the rate of platelet flux toward the vessel walls [22]; in the high shears of the arterial circulation, elevated hematocrit causes increased margination of platelets toward the vessel wall, promoting increased platelet-endothelial, platelet-platelet interactions and platelet activation [23]. However, it is unclear if these RBC properties contribute to thrombosis *in vivo.*

Herein, we determined *in vitro* that RBCs dose-dependently increased thrombin generation in the absence of platelets, but this effect is lost when platelet concentration is increased. We also developed a novel *in vivo* model of elevated hematocrit in mice. Following RBC infusion, RBC^{HIGH} mice maintained normal WBC and platelet numbers but had an elevated hematocrit. When challenged with an arterial injury, RBC^{HIGH} mice had a faster time to occlusion (TTO) compared to controls. However, there was no difference in plasma thrombin-antithrombin (TAT) levels and thrombus fibrin content suggesting RBCs cause thrombosis independent of their procoagulant activity.

3.2 Materials and Methods

3.2.1 Proteins and Materials.

Monoclonal anti-fibrin(ogen) antibody (59D8) was a generous gift of Drs. Marschall Runge (University of North Carolina [UNC]), Charles Esmon (Oklahoma Medical Research Foundation). Lipidated tissue factor (TF, Innovin) was from Siemens (Newark, DE). P2Rho

thrombin-specific substrate was a generous gift from Dr. Bas de Laat (Synapse, Maastricht, Netherlands). Calcium ionophore (A23187) is from Sigma (St. Louis, MO). Anti-TER119 antibody was purchased from Thermo Scientific (Waltham, MA) and anti-annexin V antibody was purchased from BD Pharmingen (San Jose, CA), Prostacyclin I_2 (PGI_2) was purchased from Cayman Chemical (Ann Arbor, MI). Mineral Oil was purchased from Sigma (St. Louis, MO).

3.2.2 Phlebotomy.

Phlebotomy was conducted from healthy, consenting human donors in accordance with the Declaration of Helsinki and the Institutional Review Board at the University of North Carolina. Donors had not taken aspirin for 5 days prior to phlebotomy. Blood was collected via antecubital venipuncture into 0.105 M sodium citrate, pH 5.5 (10% v/v, final concentration).

3.2.3 Reconstituted whole blood model.

Whole blood was centrifuged at 150*xg* for 20 minutes to separate the platelet-rich plasma (PRP), buffy coat, and the RBC fraction. The PRP was then separated into two fractions. For fraction 1, PGI_2 was added to PRP which was then centrifuged to produce a platelet pellet in platelet-poor plasma (PPP). This PPP (containing PGI_2) was discarded. For fraction 2, PRP was centrifuged to make PPP (without PGI_2). The PPP from fraction 2 was used to resuspend the platelet pellet. The RBC fraction was further centrifuged to produce packed RBCs (pRBCs). Cell counts were obtained on a Sysmex pocH-100*i*™ Automated

Hematology Analyzer (Lincolnshire, IL). pRBCs, platelets, and PPP were combined to the hematocrit and platelet concentrations indicated.

3.2.4 Thrombin generation in whole blood.

Thrombin generation was measured in whole blood as described [24]. Briefly, 30 μL of reconstituted blood was mixed with 10 μL thrombin specific substrate (P2Rho; 1.8 mM). Clotting was activated with 20 μL of tissue factor and $CaCl_2$ (1.5 pM and 50 mM, respectively). A 5 μL aliquot of the clotting reactions was immediately transferred to a 96-well plate containing Whatman 589/1 filter paper (St. Louis, MO) and covered with 40 μL of mineral oil. Calibration wells were run for each hematocrit using 20 μL of α2M-thrombin. Fluorescence was recorded every 5 seconds with a Fluoroskan Ascent microplate fluorometer with λex = 485 nm and λem = 538 nm. Reactions were performed at 37 ^{o}C.

3.2.5 Mouse model of elevated hematocrit.

Procedures were approved by the UNC Institutional Animal Care and Use Committee. RBCs were harvested from healthy, 6- to 8-week old male and female C57Bl/6 mice. Briefly, mice were anesthetized and blood was drawn from the inferior vena cava (IVC) into 3.2% sodium citrate (10% v/v, final concentration). Blood was centrifuged at 150xg for 10 minutes to separate RBCs from PRP. RBCs were suspended in sterile 1.29 mM sodium citrate (pH 7.2), 3.33 mM glucose, 124 mM NaCl (CGS) and centrifuged at 250xg for 5 minutes 3 times to wash RBCs. Washed RBCs were suspended in sterile 20 mM N-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (pH 7.4) containing 150 mM NaCl (HBS) and centrifuged at 400xg for 10 minutes to "pack" RBCs. RBCs were counted on a

HV950FS Hemavet cell counter (Drew Scientific, Dallas, TX) and the hematocrit was adjusted to 70%. For RBC transfusions, male, 6- to 8-week old C57Bl/6 mice anesthetized and injected with pRBCs via the retro-orbital plexus. After 24 hours, blood was drawn from the IVC or animals were subjected to thrombosis models.

3.2.6 Blood smears.

Differential staining was performed by fixing smears in methanol, and then incubating in Eosin Y and Azure B for one minute each (Diff-stain kit, IMEB, San Marcos, CA). Stained smears were imaged using an Olympus BX61 microscope (Waltham, MA).

3.2.7 Flow cytometry.

Whole blood and washed RBCs were diluted 1:500 and 1:1000, respectively, in RBC wash buffer (21 mM Tris-base, 140 mM NaCl, 11.1 mM dextrose, 4.7 mM KCl, 1.2 mM $MgSO_4$, and 0.1% PEG 8000 at pH 7.4 in the absence and presence of 2.5 mM $CaCl_2$). Diluted samples (100 µL) were stained in the dark at room temperature for 30 minutes with 10 µL FITC-Annexin V for PS detection and 5 µL PE-TER119 for RBC detection. After staining, samples were further diluted with 1000 µL RBC wash buffer and analyzed on a Stratedigm S1000Ex flow cytometer (San Jose, CA) using appropriate compensation controls. Samples stained in the absence of calcium were used as negative controls. RBCs treated with 10 mM N-ethylmaleimide plus 4 µM calcium ionophore A23187 for 30 minutes prior to staining were used as positive controls. PS-positive RBCs were determined by dual positive staining for TER119 and Annexin V. Percent PS-positive RBCs was calculated by dividing the number of dual positive events by the total number of TER119 events in each sample.

3.2.8 Blood viscosity measurements.

Whole blood viscosity was measured using a stress-controlled cone-and-plate rheometer (AR-G2, TA Instruments, New Castle, DE), where the cone had a 60 mm diameter and a 1/2º cone angle and with the operating temperature set at 37ºC. Startup effects were reduced by subjecting samples to a 30 s^{-1} preshear before beginning the sweep. The viscosity recorded at each shear-rate was the result of the rheometer reaching steady state, where three consecutive viscosity measurements integrated over 10 seconds were required to vary from one another by less than 5%. To ensure the history of high shear rates did not affect the overall rheology of the blood in repeated measurements, we first ran the shear rate sweep with increasing shear rates (from 30 to 2300 s^{-1}) and then repeated the measurement in the opposite direction (from 2300 to 30 s^{-1}).

3.2.9 $FeCl_3$ model of arterial thrombosis.

$FeCl_3$ injury to carotid arteries was performed as described [25-27]. Briefly, 6- to 8-week old male C57Bl/6 mice were anesthetized, and the right common carotid artery was exposed, dried, and treated with $FeCl_3$ (10% on 0.5 X 0.5-mm filter paper) for 2 minutes. Blood flow was monitored by Doppler ultrasonic flow probe, and the time to vessel occlusion was defined as the time between $FeCl_3$ administration and lack of flow for 60 seconds. Vessels were excised and fixed in 10% formalin for 24 hours and then transferred to 70% ethanol.

3.2.10 Measurement of circulating thrombin-antithrombin (TAT) complexes.

TAT complexes were measured with plasma prepared from IVC blood draws at baseline and after $FeCl_3$/carotid artery thrombosis (in separate mice) by ELISA (Enzygnost TAT micro ELISA, Siemens, Munich, Germany).

3.2.11 Histology.

Fixed tissues were dehydrated and paraffin-embedded, and consecutive, 5-μm sections cut and mounted with vectamount (UNC Lineberger Comprehensive Cancer Center Animal Histopathology Core). Slides were stained with hematoxylin and eosin (H&E), and imaged using an Aperio scanner (Leica Biosystems Inc, Buffalo Grove, IL). Immunohistochemistry was performed as described [25]. Briefly, antigen retrieval was performed in a 95°C water bath using Target Retrieval Solution (DakoCytomation, Capinteria, CA). Slides were blocked with mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) and stained with anti-fibrin antibody (59D8) for 1 hour in a humidity-controlled chamber. Slides were developed using an avidin-biotin complex (DakoCytomation, Capinteria, CA). Simultaneously, negative controls were stained in the absence of primary antibody. Staining intensity of thrombi was performed blindly by six individuals on a scale of 0-3.

3.2.12 Measurement of circulating platelet factor 4 (PF4) levels.

PF4 complexes were measured with plasma prepared from IVC blood draws at baseline and after $FeCl_3$/ carotid artery thrombosis (in separate mice) by ELISA (Mouse CXCL4/PF4 Quantikine ELISA, R&D Systems, Minneapolis, MN)

3.2.13 Statistical methods.

Descriptive statistics (mean, median, standard deviation [SD], and standard error of the mean [SEM]) were calculated. Groups were compared using Student's t-tests (normally distributed data determined by Lilliefors test for normality) or Wilcoxon-Mann–Whitney rank sum tests (non-normally distributed data) in Kaleidagraph version 4.1.3 (Synergy Software, Reading, PA). For the viscosity experiments, we logarithmically transformed both viscosity and shear rate and used a linear model to describe the relationship between the variables.

3.3 Results

3.3.1 The RBC increase in thrombin generation in whole blood is dependent on platelet concentration.

Previous studies have shown that RBCs express PS on their cell membrane and that these surfaces can support thrombin generation [10-13]. These prior experiments were limited in that either only one hematocrit was studied, platelets were absent, or only one platelet concentration was tested. Therefore, we separated whole blood from healthy human donors into plasma, concentrated PRP, and pRBCs and reconstituted these components to reach hematocrits of 0, 20, and 45% in the presence or absence of platelets ($200x10^3/\mu L$ or $450x10^3/\mu L$ platelets, final concentration). As expected, in the absence of platelets, increasing hematocrit strongly increased thrombin generation ($P<0.005$, Figure 3.1). When platelet concentration was raised to $200x10^3/\mu L$, increasing hematocrit still increased thrombin generation but to a lesser extent ($P<0.05$) than in the absence of platelets (Figure 3.1). Interestingly, when platelet concentration was raised to $450x10^3/\mu L$, increasing

hematocrit did not increase thrombin generation (Figure 3.1). These data suggest the effect of hematocrit on thrombin generation is dependent on platelet concentration.

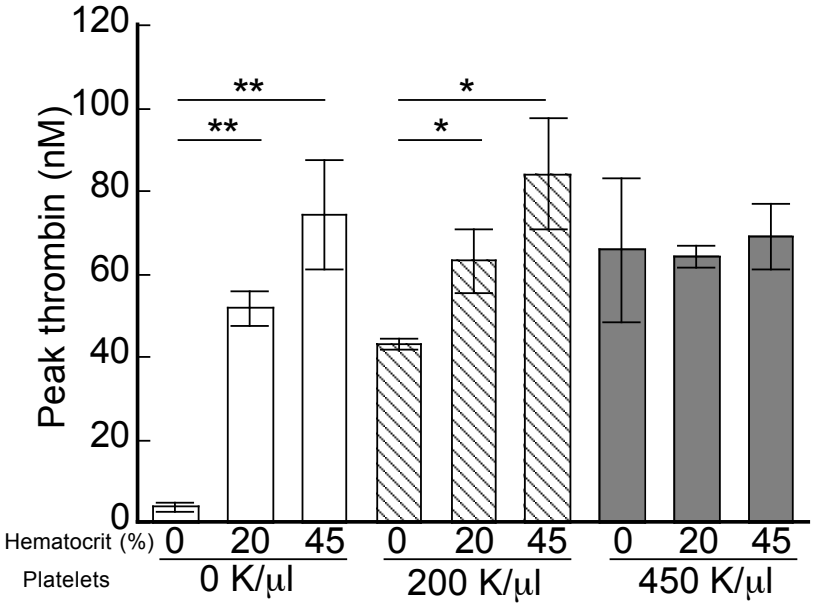

Figure 3.1: The RBC effect on thrombin generation is dependent on platelet concentration. Platelet-poor plasma was reconstituted with 0, 200, or 450 K/μl platelets, and 0, 20, or 45% hematocrit, final. Thrombin generation was measured by calibrated automated thrombography. Data are shown as mean±SEM. N=3-6. **P<0.005, *P<0.05.

3.3.2 RBC infusion raises hematocrit in mice without altering RBC morphology or PS-exposure.

To determine the effects of elevated hematocrit *in vivo*, we developed a mouse model in which we infused healthy mice with washed pRBCs from healthy donor mice. Complete blood counts showed RBC^{HIGH} mice had elevated hematocrit compared to control mice (46.6±0.69 versus 39.32±0.74%, P<0.0001, mean ± SE, Figure 3.2A). Levels of neutrophils and monocytes were the same in controls and RBC^{HIGH} mice (1.05±0.28 versus 1.71±0.35 K/μL, P=0.15, and 0.28±0.06 versus 0.18±0.04 K/μL, P=0.16 respectively, mean ± SE, Figure 3.2B,C). Platelet levels were slightly lower in RBC^{HIGH} mice compared to controls (699.0±33.3 versus 802.2±33.7 K/μL, P=0.04, mean ± SE, Figure 3.2D), likely due to expanded blood volume following RBC infusion. Importantly, platelet counts in both controls and RBC^{HIGH} remained within the normal range. Blood smears demonstrated normal RBC morphology in pRBCs before infusion (Figure 3.2E, left), and whole blood drawn from control mice (Figure 3.2E, middle), and RBC^{HIGH} mice (Figure 3.2E, right). Consistent with previous reports [10, 11, 28, 29], only ~0.5% of pRBCs or circulating RBCs in control and RBC^{HIGH} mice expressed PS (Figure 3.2F). Compared to control mice, blood from RBC^{HIGH} mice displayed increased blood viscosity over a range of venous and arterial shear rates (30-2300 s^{-1}) (Figure 3.2G). Together, these data show pRBCs can be infused in mice without increasing other cell types, changing RBC morphology, or increasing RBC-PS exposure, and establish the murine infusion model as a novel way to increase hematocrit in mice.

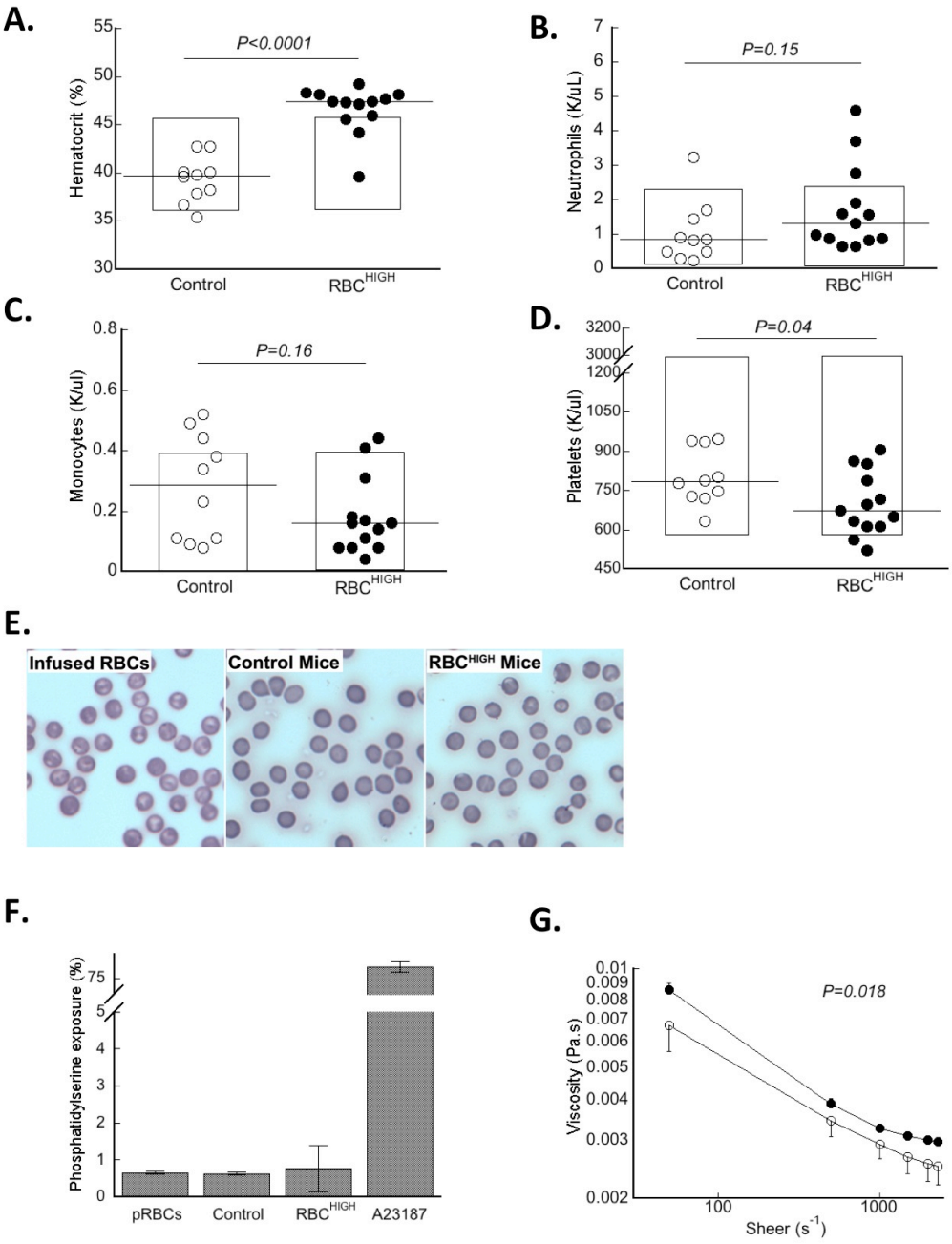

Figure 3.2. RBC transfusion raises hematocrit in recipient mice. RBCs from "donor" mice were transfused into "recipient" mice (RBC^{HIGH}). Blood was drawn from control and RBC^{HIGH} mice after 24 hours. Complete cell count indicates A) elevated hematocrit, but normal B) neutrophil, C) monocyte, and D) platelet numbers. Blood smears on whole blood from control and RBC^{HIGH} mice indicate normal RBC morphology (E) and PS exposure (F). G) Viscosity measurements show RBC^{HIGH} mice (closed circles) have increased viscosity at low and high sheer compared to control mice (open circles). In A-D each dot is a separate mouse, lines indicate median values, and boxes represent the normal range. Error bars represent standard deviation.

3.3.3 Compared to controls, RBC^{HIGH} mice have a faster time to arterial occlusion.

To determine the effect of elevated hematocrit on thrombus formation, we then subjected control and RBC^{HIGH} mice to the $FeCl_3$/carotid artery injury model. Compared to control mice, RBC^{HIGH} mice had a significantly shorter time to occlusion (TTO,12.8±2.2 minutes versus 5.3±0.4, respectively [mean ± SEM], $P<0.001$) Figure 3.3A). Infusion of RBC wash supernatant into mice did not shorten the time to occlusion (Figure 3.3A) indicating the HBS wash the RBCs were packed in did not cause thrombosis. Moreover, 7.7 minutes after $FeCl_3$ injury, 100% of RBC^{HIGH} mice had an occluded vessel, whereas only 50% and 40% of control and HBS-infused mice, respectively, developed vessel occlusion (Figure 3.3B). Inspection of the Doppler curves indicated that compared to control mice, RBC^{HIGH} mice exhibited an earlier onset (3.0±0.5 versus 1.6±0.4 minutes, respectively, $P=0.06$) and faster rate (0.016±0.002 versus 0.009±0.001 Hz/min, respectively, $P<0.009$) of thrombus formation (Figure 3.3C,D).

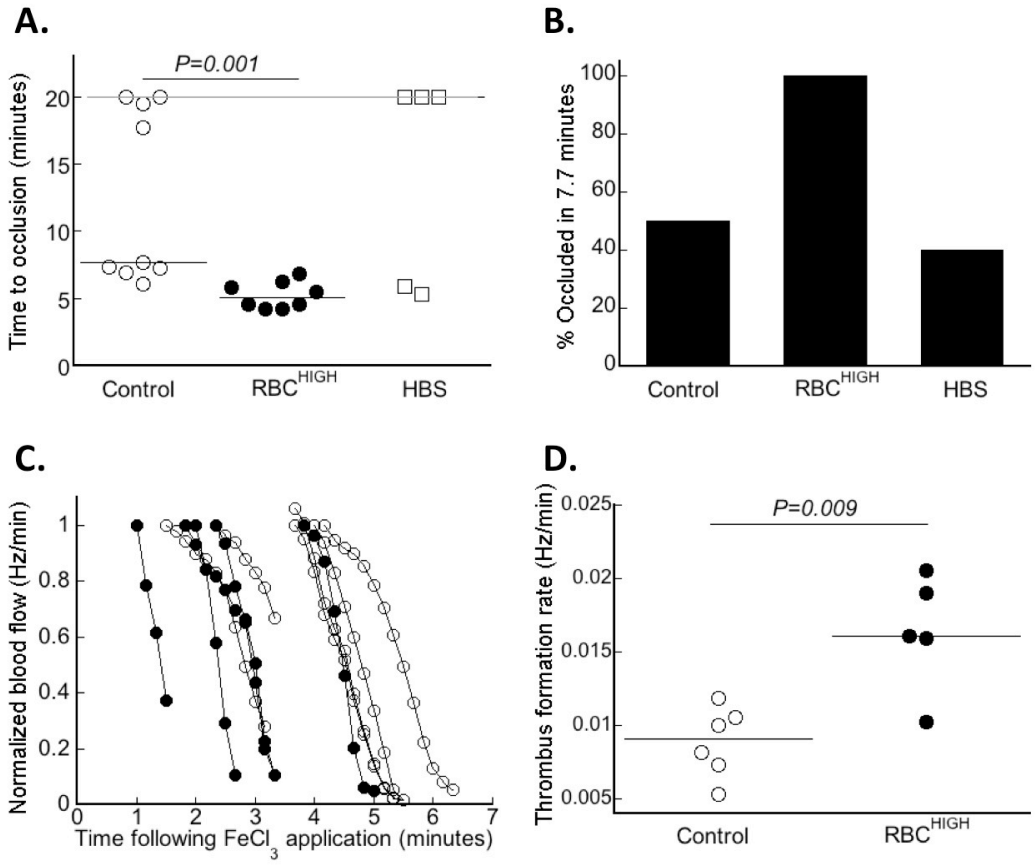

Figure 3.3. RBC^{HIGH} mice have a shortened time to vessel occlusion following $FeCl_3$ injury to the carotid artery. Control and RBC^{HIGH} mice were subjected to $FeCl_3$ injury to the carotid artery. A) Time to vessel occlusion was recorded via Doppler flow probe. In vessels that did not occlude, TTO was recorded as 20 minutes**.** B) Percent of mice occluded in 7.7 minutes (the median TTO of control mice). C) Normalized Doppler curves from control (open circles) and RBC^{HIGH} (closed circles) mice indicate the loss of blood flow during thrombus formation. D) Rate of thrombus formation in control and RBC^{HIGH} mice was determined by fitting a line to the Doppler curves once blood flow decreased.

3.3.4 *In vivo* thrombin generation does not differ between control and RBC^{HIGH} mice.

Figure 3.1 shows that RBCs dose-dependently increase thrombin generation in a static *in vitro* system but not when platelets are present at $450x10^3/\mu L$. To determine if RBCs increase thrombin generation *in vivo*, we drew blood from mice following $FeCl_3$-triggered thrombus formation and measured plasma TAT complexes. We found there was no difference in plasma TAT levels between RBC^{HIGH} and control mice (7.35±1.09 versus 5.78±0.65 ng/mL, respectively, P=0.25, mean±SE, Figure 3.4A). Furthermore, there was also no correlation between hematocrit and TATs (Figure 3.4B) and platelet concentration and TATs (Figure 3.4C). These data suggest RBCs do not shorten the TTO by increasing thrombin generation.

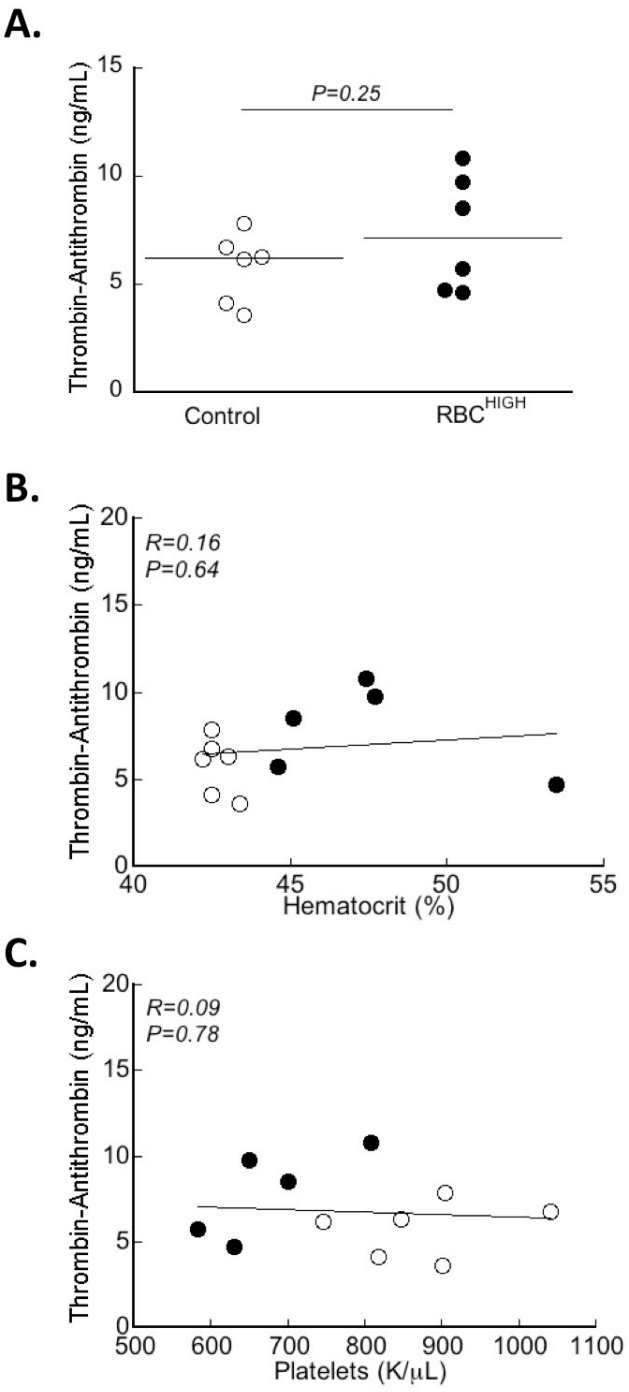

Figure 3.4. Thrombin-antithrombin complexes are similar in control and RBC^{HIGH} mice following $FeCl_3$ injury. A) Plasma TAT complexes were measured by ELISA in control and RBC^{HIGH} mice at baseline and after $FeCl_3$ injury. B) Correlation between hematocrit and TATs. C) Correlation between platelet concentration and TATs.

3.3.5 Thrombi from control and RBC^{HIGH} mice do not differ in size or fibrin content.

To determine the mechanism of faster TTO in RBC^{HIGH} mice, we excised the occluded carotid arteries from mice and stained thrombi with H&E and antibodies against fibrin. Thrombi in control and RBC^{HIGH} mice were occlusive and similar in size (Figure 3.5C), and H&E staining showed thrombi were primarily composed of protein with small islands of RBCs (Figure 3.5A). Thrombi from control and RBC^{HIGH} mice also did not differ in fibrin content (Figure 3.5B). No fibrin staining was detected in the absence of primary antibody, confirming the secondary antibody did not bind to mouse tissue nonspecifically. These data indicate that increasing RBCs produces thrombi of similar size and content, and does not increase fibrin deposition in thrombi.

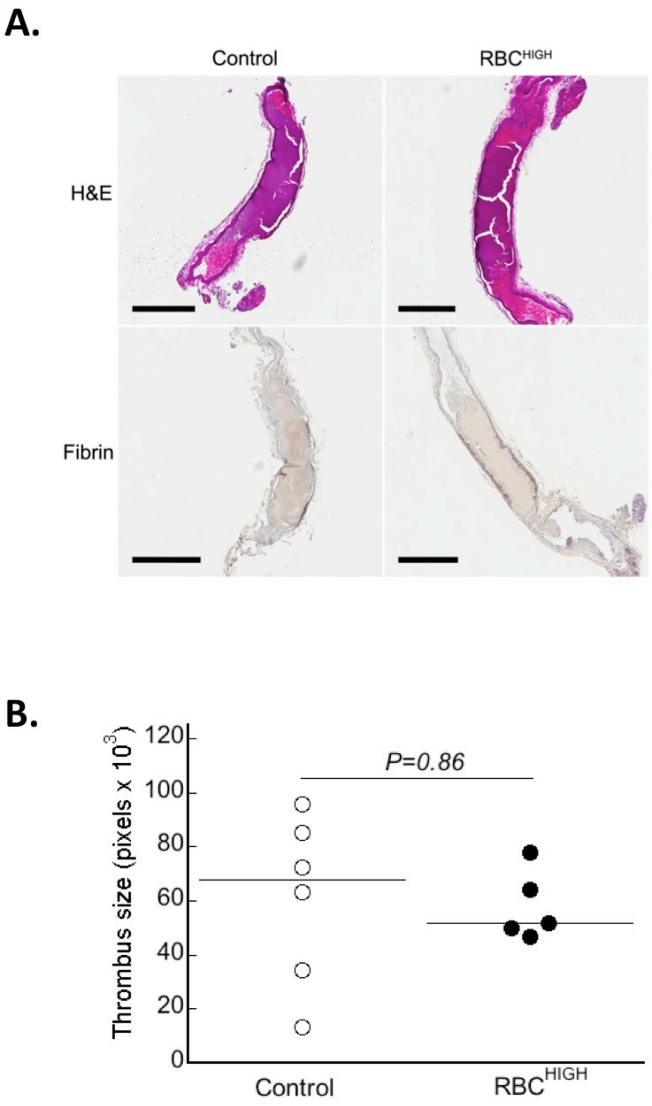

Figure 3.5. Thrombi from control and RBC^{HIGH} mice have similar morphology and fibrin content. Thrombi were excised, fixed, and analyzed by A) H&E staining and immunohistochemistry for B) fibrin (antibody 59D8). C) Thrombus size was determined by measuring pixel area of the thrombus within the vessel. Scale bar represents 500 μm.

3.3.6 PF4 levels did not differ between control and RBC^{HIGH} mice.

To determine if platelet activation was increased in RBC^{HIGH} mice, we used PF4 as a marker of platelet activation in plasma following $FeCl_3$ arterial injury (Figure 3.6). However, arterial thrombus formation did not increase plasma levels of PF4 despite the formation of platelet-rich thrombi in mice (Figure 3.5) suggesting PF4 is a poor marker of platelet activation in murine plasma. Alternatively, since little PF4 was measured in plasmas following platelet-rich thrombus formation, it could be possible that platelets are aggregating, without degranulating, and thus platelet PF4 is maintained in the α-granuoles without being released into the plasma.

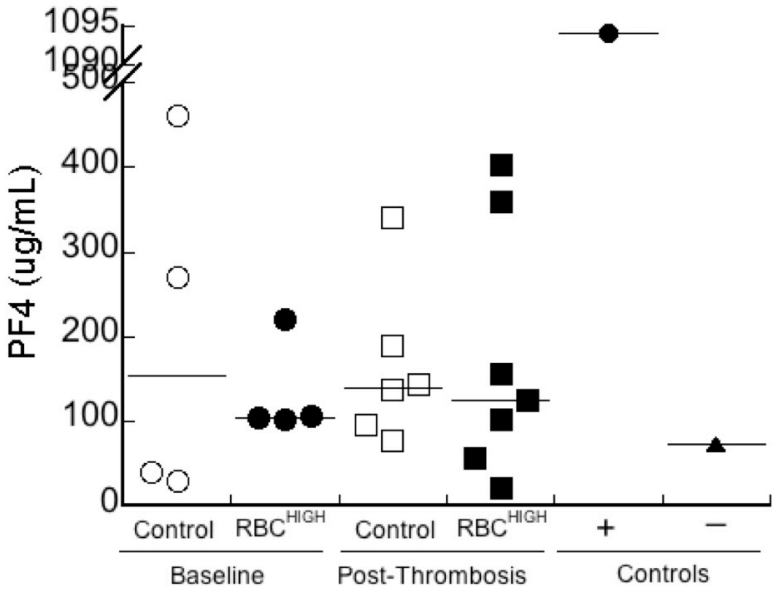

Figure 3.6. PF4 levels did not differ between control and RBC^{HIGH} mice. PF4 was measured by ELISA in plasma from control and RBC^{HIGH} mice at baseline and following $FeCl_3$ injury and clot formation. Uninjured murine whole blood spun to plasma served as a negative control. Murine whole blood activated with thrombin, then centrifuged to PPP served as a positive control.

3.4 Discussion

Although epidemiological studies have associated RBC transfusion and high hematocrit with arterial thrombosis, the causal role of RBCs in thrombosis has been controversial. Additionally, the mechanism by which RBCs promote thrombus formation is unclear. Herein, we measured thrombin generation *in vitro* in reconstituted blood with increasing RBCs and platelets. We found that RBCs increased thrombin generation in the absence of platelets, although effects were blunted and eventually absent as platelet count increased. To determine if RBCs cause arterial thrombosis *in vivo,* we infused RBCs into mice and showed that RBC^{HIGH} mice have a faster TTO than controls. TAT measurements from plasma following arterial injury showed no difference between RBC^{HIGH} and controls. These data suggest thrombosis was not accelerated by RBC-driven thrombin generation.

Similar to previous studies, we found that RBCs increased thrombin generation *in vitro*, but extended these findings by showing that the RBC influence on thrombin generation was dependent on the concentration of another PS-positive surface (platelets). In our *in vivo* model, control and RBC^{HIGH} mice had an average platelet count of 802.2±33.7 and 699.0±33.3 K/μL, respectively. This high platelet count may be a reason why RBC-supported thrombin generation did not drive thrombosis *in vivo*. However, RBC-supported thrombin generation may play a greater role *in vivo* role under thrombocytopenic conditions. Since in our *in vivo* model, RBCs accelerated thrombosis independent of thrombin generation, and our model was performed in an arterial bed in which thrombi are platelet-rich, it is likely that RBCs cause thrombosis via their influence on platelets. Experiments to test this hypothesis and preliminary data are described in Chapter 4.

Since our study shows elevated hematocrit did not accelerate thrombosis via increasing thrombin generation, we suggest that use of anticoagulants would be ineffective in preventing thrombosis in patients with an elevated hematocrit. In fact, a retrospective analysis of patients with PV receiving secondary prophylaxis with oral anticoagulants showed 20% of patients displayed recurrent arterial thrombosis, and 20% of patients developed bleeding [30]. Even though this study was performed in patients with a backdrop of PV, RBCs from patients with PV have been shown to have increased PS-exposure [14] which would suggest RBC-supported thrombin generation may play a greater role in thrombosis in this population compared to a population with elevated RBCs with normal PS exposure. These data further support the idea that RBCs cause thrombosis independent of thrombin generation and that anticoagulation may even cause more harm then good in patients with an elevated hematocrit.

Since RBCs did not accelerate thrombosis via increasing thrombin generation, we hypothesized that RBCs promoted thrombosis via a platelet-mediated mechanism. Antiplatelet therapy has been shown to be successful in preventing thrombosis in patients with elevated hematocrit caused by PV. The efficacy of low-dose aspirin was tested by the European Collaboration on Low-Dose Aspirin in Polycythemia Vera Project (ECLAP) in a double-blind, placebo controlled, randomized clinical trial [31]. Aspirin significantly lowered the risk of cardiovascular death, non-fatal MI, and non-fatal stroke compared to placebo control without causing significant bleeding. These data show platelet inhibition helps prevent thrombosis with patients with PV and suggest elevated RBCs may cause arterial thrombosis via a platelet-mediated mechanism.

Previous studies have used animal models to study the effects of elevated hematocrit on bleeding, thrombosis, and blood flow. These models induced RBC production through overproduction of erythropoietin or by JAK 2^{V617F}-induced PV. In erythropoietin-induced erythrocytosis, studies have found a range of phenotypes. Zebrafish with increased expression of erythropoietin (via mRNA injection) show increased blood viscosity, reduced blood flow, localized vascular stasis, and had a high mortality rate, however thrombosis models were not utilized in this study [32]. Interestingly, mice overexpressing human erythropoietin that reached a hematocrit of 80-85% demonstrated a bleeding phenotype [33]. The authors conclude bleeding is due to reduced coagulation activity due to a lack of plasma volume. However, this hematocrit is not physiologic, making it difficult to translate these results to high hematocrits seen in patients. Hematocrit has also been raised in mice via treatment with human recombinant erythropoietin but no difference in thrombosis was found compared to controls [34]. However, erythropoietin has been implicated in a wide range of activities on different cell types and organ systems including immune cells, endothelial cells, bone marrow stromal cells, and cells of the heart, reproductive system, gastrointestinal tract, muscle, kidney, pancreas, and nervous system [35-40]. Thus, it is hard to draw conclusions about RBCs and thrombosis in models using erythropoietin.

Lamrani et al studied thrombosis in mice that developed JAK 2^{V617F}-induced PV [34]. Interestingly, mice with PV had increased bleeding in a tail bleed model but had a prothrombotic phenotype following $FeCl_3$ injury to mesenteric vessels. However, PV mice also had a platelet GPVI deficiency, and a reduced number of plasma von Willebrand factor multimers [34]. Therefore, studying the effect of high hematocrit in the backdrop of PV makes defining the role of RBC in thrombosis difficult to interpret.

Our novel model of elevated hematocrit using RBC infusion represents a means of studying the effects of RBCs in mice. Although mice had a slight decrease in platelet count this model allowed for elevation of just RBCs without substantially affecting other cell types. This model also showed consistent elevation of RBCs to levels that resembled pathologically high levels in humans. Finally, this model lends itself to further manipulation of RBCs *ex vivo* (i.e. storage or gluteraldehyde treatment to increase stiffness) or use of RBCs from mice strains with RBC abnormalities such as SCD or PV so different RBC properties can be studied.

$FeCl_3$ is a commonly used method to induce thrombosis in animal models. A previous study suggested RBCs play a role in thrombosis following $FeCl_3$-induced vascular injury by being the first cells to adhere to $FeCl_3$-treated endothelial surfaces [41]. This RBC adhesion could serve as a limitation of interpreting our data since in this model increasing hematocrit may increase the number of RBCs binding to the endothelium following $FeCl_3$ injury. Therefore, future experiments will test the role of RBCs in other models of hemostasis and thrombosis.

In summary, our results show that RBCs increased thrombin generation *in vitro* in the absence of platelets, but platelet concentration mediates the RBC effect on thrombin generation. Moreover, we developed a novel model of elevated hematocrit in mice and found elevated hematocrit is causative in arterial thrombus formation *in vivo*. However, thrombosis was not due to increased thrombin generation or fibrin formation. Future studies (discussed in Chapter 4) are warranted to determine the mechanism by which elevated hematocrit causes thrombosis including RBC influences on blood rheology and biochemical

effects on platelet activation and aggregation. Knowledge of the mechanism by which RBCs cause thrombosis will help guide in therapeutic strategies in patients with elevated hematocrit.

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Chapter 4: Summary and Future Directions

4.1 Summary

Arterial thrombosis is a leading cause of death and disability worldwide, but the specific mechanisms leading to thrombus formation are not well-defined. Numerous epidemiological studies have made associations between blood hypercoaguability and risk of arterial thrombosis. These associations have primarily been studied in *in vitro* systems where purified components of blood are studied in isolation or in the presence of a limited number of additional blood components. Therefore, this dissertation utilized plasma-based *in vitro* systems and novel *in vivo* murine models to study how a form of plasma hypercoaguability (elevated γ'-fibrinogen) and cellular hypercoagability (elevated RBCs) influence arterial thrombus formation. The goal of this research was to determine if elevated levels of γ'-fibrinogen and/or RBCs are directly causative in arterial thrombosis or just biomarkers of a different pathology.

4.2 Future Directions

We have shown using a murine model that γA/γA, not γA/γ', is the fibrinogen isoform responsible for causing arterial thrombosis [1]. This was an interesting finding since epidemiological studies have associated elevated γA/γ' fibrinogen with arterial thrombosis [2-7]. To date, studies measuring γA/γ' fibrinogen levels in patient plasma have sampled blood following arterial thrombosis. We hypothesize that the elevated γA/γ' fibrinogen levels measured in these plasmas is due to an inflammatory response following thrombus

formation. Data to support this hypothesis came from a study on the effects of the proinflammatory cytokines (e.g. IL-6) on fibrinogen expression by HEPG2 cells [8]. Using an *in vitro* system of cultured cells, the authors found that IL-6 upregulates γ' production to a greater extent than γA production [8]. Therefore we propose γ' may be increased in response to the thrombus and not causative in the development of the thrombus as was previously thought. Prospective studies are needed to determine if γA/γ' levels are actually elevated prior to thrombus formation. This will give further insight into the role of γA/γ fibrinogen in arterial thrombosis in humans. Even though our model showed γA/γ' is not causative in arterial thrombosis, it may serve as a good biomarker of an ongoing inflammatory process.

There are several mechanisms by which RBCs can promote thrombosis including increasing platelet margination toward the vessel wall, increasing platelet aggregation and activation, and increasing plasma thrombin generation. These mechanisms present several different therapeutic strategies to prevent thrombus formation including phlebotomy, platelet antagonism, and anticoagulation (Figure 4.1). However, our data suggest RBCs do not increase thrombus fibrin content or thrombin generation in a mouse model of arterial thrombosis suggesting anticoagulation would not be an appropriate therapeutic strategy in patients with high hematocrit.

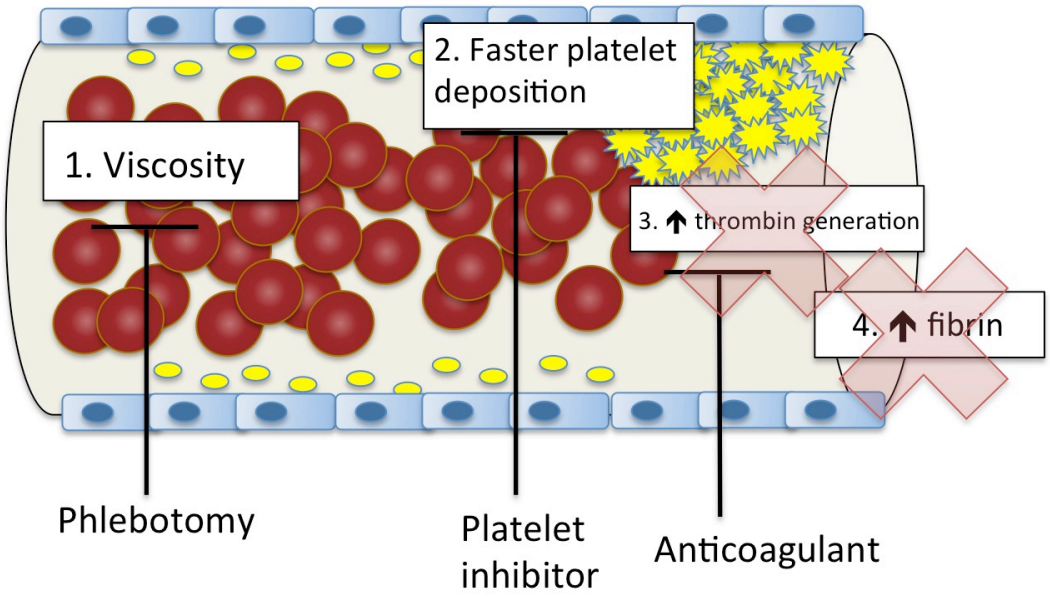

Figure 4.1. Conceptual model showing influence of RBCs on thrombus formation and therapeutic targets. RBCs increase viscosity, promote platelet interactions with the vessel wall, and increase thrombin generation. Phlebotomy, platelet antagonism, and anticoagulants are candidates for reducing thrombotic risk with elevated hematocrit. Our data suggest anticoagulants would not be a good therapeutic strategy since elevated hematocrit promotes thrombosis independent of thrombin generation and fibrin formation.

Future experiments on RBCs should focus on determining the mechanism by which RBCs promote thrombosis *in vivo*. To test the hypothesis that RBCs accelerate thrombosis via a platelet dependent mechanism, we will use *ex vivo* microfluidic chambers to test the effect of elevated hematocrit on the rate and total amount of platelet and fibrin accumulation on collagen. We have an active collaboration with Drs. Keith Neeves and Adam Wufsus at Colorado School of Mines; they are currently performing these experiments and have preliminarily found increasing hematocrit dose-dependently increases platelet accumulation on collagen (Figure 4.2A). However, increasing hematocrit did not increase fibrin(ogen) accumulation between 45% and 60% hematocrit with only a slight increase from 30%-45%. (Figure 4.2B). These data are consistent with our murine model described in Chapter 3 in that RBCs do not increase fibrin deposition in clots. Furthermore, this data suggest RBCs contribute to thrombosis via a platelet-mediated mechanism.

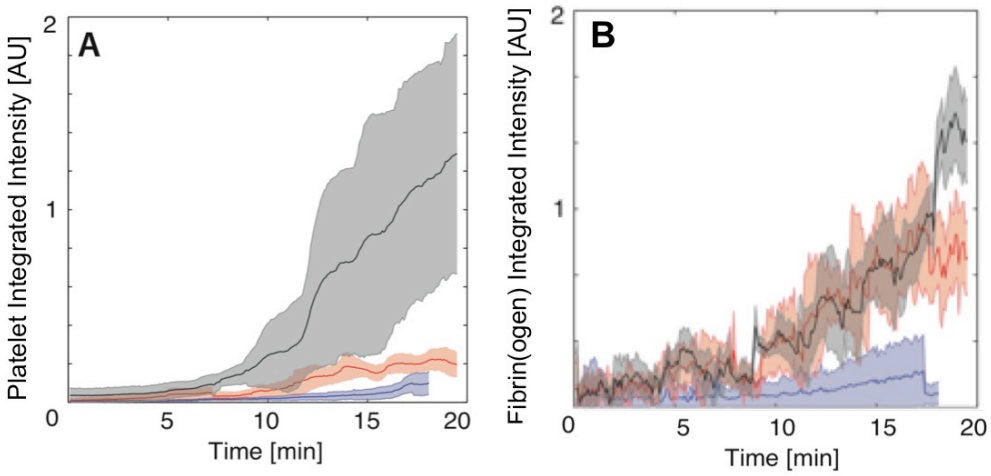

Figure 4.2. Platelet and fibrin(ogen) fluorescent intensity. A) Integrated fluorescent intensity of Pacific-Blue anti-CD41 labeled platelets and B) Alexa Fluor-488 labeled fibrinogen as a function of time for the 30 (blue), 45 (red), and 60 (black) hematocrit blood clots. Lines are the mean and the shaded region is the standard deviation of 6-10 collagen spots from three separate assays. Experiments performed in collaboration with, and printed with permission from Drs. Keith Neeves and Adam Wufsus at the Colorado School of Mines.

Next, we plan to add a platelet antagonist to the reconstituted blood used in the microfluidic model to determine if RBCs promote thrombosis via a platelet mediated mechanism. Moreover, we will utilize a murine model where RBC^{HIGH} and control mice are treated with a platelet antagonist and subjected to $FeCl_3$ injury to the carotid artery. We expect inhibiting platelets will eliminate the effect of elevated hematocrit on thrombosis by reducing RBC-induced platelet activation and aggregation. In sum, these experiments will determine if RBCs promote thrombosis via increasing platelet activation and aggregation in response to a procoagulant stimulus.

Our novel mouse model of elevated hematocrit may be useful for studying other RBC-related pathologies. For example, in transfusion medicine it is controversial whether RBC storage increases the procoagulant nature of RBCs [9, 10]. Similarly, sickled RBCs have been shown to have abnormal PS expression, stiffness, and adhesive properties but it is unclear if these properties contribute to thrombus formation [11]. It would be interesting to combine the RBC infusion model using RBCs that have been stored, treated with gluteraldehyde, or that have been isolated from mice with sickle cell disease to determine if RBCs are specifically responsible for thrombosis in these pathologies. Additionally, it would be interesting to perform an RBC transfusion and subject mice to models of venous thrombosis to determine how RBCs influence clot formation under different vascular conditions. Knowledge of the influence of both normal and abnormal RBCs to arterial and venous thrombosis may help provide insight in the mechanisms by which RBCs cause thrombosis, and thus prevent thrombosis in patients with elevated hematocrit, and patients with RBC abnormalities. Additionally, knowledge of the mechanism by which RBCs cause

thrombosis may also guide therapeutic strategies in patients with elevated hematocrit and RBC disorders.

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