GENETIC REGULATION OF THE EXPRESSION OF TWO FIBRINOGEN GAMMA CHAIN SPLICE VARIANTS BY FIBRIN DEGRADATION PRODUCTS AND INFLAMMATORY CYTOKINES

by

Chantelle Rein

A DISSERTATION

Presented to the Department of Molecular and Medical Genetics
and Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
March 2010

Department of Medical and Molecular Genetics
School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Chantelle Rein

has been approved

Dr. David Farrell
Thesis Advisor, Professor

Dr. Monica Hinds
Assistant Professor

Dr. Owen McCarty
Assistant Professor

Dr. Cheryl Maslen
Professor

Dr. Kent Thornburg
Professor

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aPTT- Activated Partial Thromboplastin Time

AP-1- Activator Protein-1

BCA- Bicinchoninic Acid Assay

BSA- Bovine Serum Albumin

CAD- Coronary Artery Disease

CRP- C-Reactive Protein

CVD- Cardiovascular Disease

DAPI- 4',6-diamidino-2-phenylindole

DTT- Dithiothreitol

EGCG- Epigallocatechin Gallate

ELISA- Enzyme-Linked Immunosorbent Assay

EMSA- Electromobility Shift Assay

FDP- Fibrin Degradation Product

GAPDH- Glyceraldehyde 3-Phosphate Dehydrogenase

GAS- Interferon-γ Activated Sequence

GP1b/V/X- Glycoprotein1b/V/IX Complex

GPIIbIIIa- Glycoprotein IIbIIIa

HRP- Horseradish Peroxidase

IFN-γ- Interferon-γ

IFNGR- Interferon-γ Receptor

IL-1β- Interleukin-1β

IL-6- Interleukin-6

JAK- Janus Kinase

LRP- Low-density lipoprotein Receptor Related Protein

MEM- Minimal Essential Medium

MI- Myocardial Infarction

PAI-1- Plasminogen Activator Inhibitor-1

PCR- Polymerase Chain Reaction

PMSF- Phenylmethylsulfonyl Fluoride

PT- Prothrombin Time

qRT-PCR- Quantitative Real-Time Polymerase Chain Reaction

RAP- Receptor Associated Protein

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SNP- Single Nucleotide Polymorphism

STAT- Signal Transducer and Activator of Transcription

TF- Tissue Factor

TGF-β- Transforming Growth Factor-β

TMB- 3,3', 5,5''-tetramethylbenzidine

TNF-α- Tumor Necrosis Factor-α

tPA- Tissue Plasminogen Activator

TT- Thrombin Time

uPA- Urokinase Plasminogen Activator

vWF- von Willebrand Factor

Acknowledgements

First and foremost I would like to thank my mentor, Dr. David Farrell, for all of his patience, wisdom, and guidance, and also for teaching me that you can be a good scientist and still have a life outside of the lab. He has helped me to learn and understand techniques, both new and ancient, and I am truly thankful for his mentorship. I would also like to thank the members of my thesis advisory committee for guiding me through this entire process and for all of their constructive criticisms and ideas along the way. You have been a wonderful source of support for me. To all of my wonderful friends, I thank each and every one of you that I have ever borrowed a reagent from, asked for technical advice from, complained over coffee with, done retail therapy with, got some aggression out on the soccer field with and anyone else who doesn't fall into any of the above categories. A special shout out goes to Dr. KV for helping me with the fluorescence microscopy. Next I have to thank Matt, who has somehow managed to stand by me and stay with me for the last insane four and a half years. Thank you for being there to listen, putting me in my place when I need it, encouraging me, supporting me, cooking me dinner after long days in the lab, listening to presentations when you clearly had no idea what I was talking about, and attending numerous nerdy school functions because I asked you to. I would not be half as sane as I am after this journey if it wasn't

for you. And finally, I would like to thank my family, especially my Mom, Dad and sister Stephanie, for always believing in me. You bought me my first chemistry set when I decided that science was cool and you've always encouraged me to follow my dreams, even when they changed on a weekly basis. I would not be the person and science geek that I am today if it was not for your unending love and support. I love you and am truly thankful for all of the things you have given me in life.

Abstract

Fibrinogen is the zymogen precursor of fibrin, the protein that makes up blood clots. Fibrinogen is synthesized in the liver and circulates in plasma at a concentration of approximately 3 mg/mL. Fibrinogen is comprised of two sets of three polypeptide chains, which are encoded by the fibrinogen α (*FGA*), β (*FGB*) and γ (*FGG)* genes. Plasma fibrinogen concentration is an independent predictor of cardiovascular disease (CVD) including myocardial infarction (MI), coronary artery disease (CAD) and stroke. Recently, a gamma chain variant of fibrinogen, γ' fibrinogen, was discovered as an additional independent risk factor for the development of MI and CAD. However, the mechanisms that regulate the production of this variant isoform have not been elucidated. The aim of this dissertation was to examine novel mechanisms that regulate the production of total and γ' fibrinogen. Additionally, we sought to examine the molecular defect in a patient that presented with hemorrhage and a diagnosed dysfibrinogenemia.

Interferon-γ (IFN-γ) was identified as a novel regulator of total fibrinogen synthesis. We identified an IFN-γ activated sequence (GAS) within the promoter of *FGG*. We have shown that IFN-γ signals to activate signal transducer and activator of transcription-1 (STAT1), which dimerizes and binds to the novel GAS element within the *FGG* promoter to decrease transcription. We also show that IFN-γ is able to inhibit interleukin-6 (IL-6)

induced fibrinogen synthesis by interfering with the ability of IL-6 induced STAT3 to bind to known response elements within the *FGG* promoter.

We have also identified IL-6, IFN-γ and tumor necrosis factor-α (TNF-α) as molecules that differentially regulate the production of the fibrinogen γ chain isoforms. IL-6 acts by increasing total fibrinogen while decreasing γ' fibrinogen production, thereby lowering the ratio of γ'/total fibrinogen. TNF-α decreases γ' fibrinogen while not affecting the levels of total fibrinogen produced, again decreasing the γ'/total fibrinogen ratio. IFN-γ decreases total fibrinogen synthesis, but not γ' synthesis, to increase the γ'/total fibrinogen ratio.

Additionally, we have discovered that the fibrin degradation product D-dimer is able to enter HepG2 cells and travel to the perinuclear space. Subsequently, we see a decrease in the amount of γ' fibrinogen produced, suggesting that D-dimer is a negative regulator of γ' fibrinogen synthesis. While we were not able to identify a receptor for D-dimer on the cell surface, we found ample evidence that D-dimers are taken up via endocytosis.

Finally, we identified an R275C mutation in the γ chain of fibrinogen in a 54-year-old female. Most patients with this mutation are asymptomatic, and those that do exhibit symptoms usually present with thrombosis. Our patient was rather unique in that their presentation was hemorrhagic, and this work shows that the γR275C mutation is enough to sustain a hemorrhagic phenotype.

Chapter 1: Introduction

1.1 **Blood Coagulation and Fibrinolysis**

1.1.1 Primary Hemostasis

In healthy humans, the blood circulates in liquid form. However, upon injury or trauma to a vessel, it is necessary for the blood to solidify and form a clot in order to prevent massive blood loss. The formation and breakdown of a clot are quickly initiated and very highly regulated events. Coagulation begins when the endothelium of a vessel becomes damaged, exposing the subendothelial matrix to flowing blood and initiating primary hemostasis. Damage of the endothelial layer leads to exposure of subendothelial collagen to flowing blood containing von Willebrand Factor, a protein that is synthesized and stored within endothelial cells and is secreted into the plasma. Exposed collagen binds to circulating von Willebrand Factor (vWF) through the vWF A3 domain, which in turns binds to the glycoprotein Ib-V-IX complex on the surface of platelets via its A1 domain, effectively tethering platelets to the site of vessel injury. Additionally, free exposed collagen is able to bind to the surface of platelets through glycoprotein VI. By utilizing many binding sites on their surface, the platelets are able to spread themselves over the exposed subendothelium, forming a monolayer of platelets (Figure 1.1).

Adhesion of platelets to exposed collagen through glycoprotein VI and integrin $\alpha_2\beta_1$ also leads to a series of changes on and within the platelets, known as platelet activation. When bound to exposed collagen, glycoprotein VI interacts with the platelet FcRI gamma chain, resulting in phosphorylation of FcRI and subsequent intracellular signaling. Platelet activation culminates with a change in the shape of the platelets, release of

certain contents of the platelet granules, exposure of phosphatidylserine onto the platelet surface and, most importantly for this discussion, activation of glycoprotein IIb/IIIa on the platelet surface. Glycoprotein IIb/IIIa binds to circulating fibrinogen, an important plasma protein zymogen, in the presence of calcium. Each fibrinogen molecule is divalent, so that one fibrinogen molecule is able to bind to two platelets. Free platelets near the growing platelet plug become activated and these free platelets are recruited to the forming plug by binding to fibrinogen (Figure 1.2). By bridging adjacent activated platelets, fibrinogen is able to aggregate the platelets at the site of injury to form a platelet plug and slow the loss of blood at this site.

1.1.2 Secondary Hemostasis

The newly formed platelet plug is relatively unstable and may be easily dislodged from the site of injury. Therefore it is necessary to stabilize the plug and secure it to the vessel wall to form a stable physical barrier to trap escaping blood. This process occurs when coagulation factors interact with one another in a series of enzymatic reactions commonly known as the coagulation cascade (Figure 1.3). In addition to the exposure of collagen mentioned above, damage to endothelial cells exposes tissue factor (TF), an integral membrane protein, to circulating factor VII (fVII), forming the TF/fVIIa complex and initiating the extrinsic pathway of the coagulation cascade. The TF/fVIIa complex then activates factor X (fX) to factor Xa (fXa) directly, but also activates factor IX (fIX) from the intrinsic pathway to fIXa which then complexes with fVIII on the surface of platelets to produce additional fXa. fXa, in the presence of its cofactor factor Va and the platelet phospholipid surface, cleaves prothrombin (fII) into thrombin (fIIa), which in turn

cleaves fibrinogen into fibrin, forming a fibrin clot at the site of the platelet plug. The clot is then stabilized by fXIIIa, a transglutaminase that forms covalent bonds between the glutamine and lysine residues of neighboring fibrin fibers. The final two steps of the cascade will be discussed in more detail in the following section. However, the end product of the coagulation cascade is a stable fibrin meshwork that is able to trap red blood cells, white blood cells and platelets, effectively stemming the flow of blood from a wounded vessel.

As mentioned earlier, the formation of a functional blood clot is a highly regulated process. The formation of a clot needs to be restricted to the area of vascular injury and must only occur for a time period long enough to form the clot. In order to ensure that this happens, there are several mechanisms in place to regulate coagulation. The coagulation cascade itself is its own form of regulation, in that each protein is sequentially activated so that only small amounts of active enzymes are present at any given time. Additionally, the enzymatic reactions that give rise to active coagulation factors can only occur on the negatively charged surface of activated cells, such as activated platelets with exposed phosphatidylserine molecules. Similarly, TF is only exposed upon injury to the vessel and is not always exposed to flowing blood. Finally, there are numerous anticoagulant proteins and cofactors present during coagulation, which serve to keep the activity of active proteases localized both temporally and spatially. Tissue factor pathway inhibitor is able to reversibly inhibit fXa as well as the fVIIa/TF complex in a fXa dependent manner, aiding in the control of the extrinsic pathway of coagulation. Antithrombin III and heparin cofactor II are glycoproteins

synthesized in the liver that inhibit the common pathway of coagulation by inhibiting thrombin in the presence of heparin by trapping thrombin in a conformation in which its active site is not accessible to its substrates. Antithrombin III is also able to inhibit fIXa and fXa in the presence of heparin. Additionally, the activated protein C pathway is able to inhibit coagulation by degradation of fV and fVII, the cofactors necessary for formation of the prothrombinase and tenase complexes, respectively. Protein C is cleaved by thrombin to activated protein C, and in the presence of its cofactor, protein S, and a negatively charged surface, cleaves fV and fVIII at specific residues. By cleaving fV and fVIII, activated protein C inhibits the formation of the complexes required for fX activation and the conversion of prothrombin to thrombin, both of which are part of the common coagulation pathway.

1.1.3 Fibrinolysis

Once the fibrin clot is formed and performs its hemostatic functions, it must also be broken down in order to restore normal blood flow in the vessel and to prevent embolization and subsequent lodging of the clot in other vessels. The process of fibrinolysis, or the breaking down of the clot, must also occur only at the site of the clot and in the correct time frame to prevent permanent damage due to blockage of the vessel or embolization of the clot leading to blockage in the microvasculature. This process commences in response to the initiation of the coagulation cascade. The fibrinolytic system is made up of inactive proenzymes, namely plasminogen, and its activators and inhibitors, which provide regulation of proper physiological clot dissolution. The zymogen plasminogen circulates in the plasma and is activated by tissue plasminogen

activator (tPA) and/or urokinase plasminogen activator (uPA) into plasmin in the presence of fibrin. Plasmin binds to lysine residues on fibrin molecules at distinct proteolytic sites and cleaves fibrin into several fibrin degradation products (Figure 1.4). The presence of fibrin is required for the activation and function of plasminogen as well as its inhibitors (1) and also serves to direct and restrict fibrinolysis to the location of the blood clot. Plasminogen and tPA form a ternary complex with fibrin (2) via interactions between Kringle domains on the plasminogen and tPA molecules and cryptic Lysine resides exposed following conformational changes in the fibrin molecule after fibrinopeptide cleavage (reviewed in 3). Once activated, plasminogen cleaves fibrin at specific Lysine resides, exposing more C-terminal Lysine residues at the sites of cleavage, further enhancing the activation and activity of plasmin(ogen).

Much like coagulation, fibrinolysis is a tightly regulated event. Activators of plasminogen are only secreted under certain conditions, and several physiological inhibitors of fibrinolysis serve to keep the breakdown of a fibrin clot in check. Most importantly, the activities of α_2-antiplasmin and plasminogen activator inhibitor-1 (PAI-1) serve to directly inhibit the activity of plasmin and prevent the activation of plasmin from plasminogen, respectively. Additionally, the requirement of fibrin as a scaffold for enhanced plasmin activation and activity serves to localize both the activators and inhibitors of fibrinolysis to the site of the blood clot and provides its own level of regulation.

1.2 Fibrinogen Structure and Function

1.2.1 Fibrinogen Gene Expression and Assembly

The α, β and γ chains of fibrinogen are encoded by three separate single copy genes which reside on human chromosome 4q23-q32 (4). The genes are arranged in the order γ, α and β, with the β chain transcribed in the opposite direction of the α and γ genes (Figure 1.5).

The expression of fibrinogen is limited to hepatocytes, although small amounts of fibrinogen mRNA have been found in the lung (5). Due to its expression as a zymogen, fibrinogen is able to be expressed at high basal levels and circulates at approximately 3 mg/ml. Additionally, the expression of the three chains is highly coordinated such that an increase in the expression of one gene leads to an up regulation of expression of the other two genes (6). Examination of the promoter regions of the three fibrinogen genes has shown that there are regulatory elements common to all three gene promoters as well as unique motifs present in each gene (Figure 1.6).

All three fibrinogen chain promoters contain Type I (CAAT-enhancing binding element) and Type II (STAT3 binding element) IL-6 response elements as well as glucocorticoid response elements, allowing fibrinogen to be up regulated during the acute phase response. The α and β chains each contain a binding site for hepatic nuclear factor 1 (HNF-1), a tissue specific sequence required for expression in hepatocytes (7). HNF-1 has been shown to be the main protein involved in the basal expression of the α and β genes, while the γ gene possesses an adenovirus major late promoter element (MLP) that

is required for its basal expression (8). Although transcription of the three genes is highly coordinated, disruption of the transcription of the fibrinogen α chain in mice did not affect the transcription of the β and γ chains (9). It has been hypothesized that since the γ gene of fibrinogen is encountered by the transcriptional machinery first, that its expression may exert influence on the downstream α and β genes, further validating the results of Suh et al (12).

Once transcribed and translated, the polypeptide chains are assembled in the endoplasmic reticulum of hepatocytes. The first step in assembly is the formation of Aα-γ complexes, which is dependent upon the levels of each chain. When fibrinogen is expressed at basal levels, there is a steady state surplus of Aα and γ chains (10). This first assembly step is followed by addition of the third chain, Bβ, to form Aα-Bβ-γ molecule (11). Finally two Aα-Bβ-γ molecules dimerize to form intact fibrinogen. Glycosylation of the full fibrinogen molecule begins in the endoplasmic reticulum and is finished in the Golgi apparatus, followed by phosphorylation and sulfation of certain residues. Functional fibrinogen is then trafficked to the cell membrane where it is released into the circulation. Any unassembled chains are retained within the endoplasmic reticulum and targeted to the lysosome or the proteasome for degradation (12).

1.2.2 Biochemistry of Fibrinogen and Fibrin Clot Formation

Fibrinogen is a 340 kD disulfide linked dimer of three polypeptide chains: Aα, Bβ and γ. The amino termini of all six chains come together to form a globular domain in the center of the molecule, known as the disulfide knot, within the E domain of the molecule. This

region is important in the formation of a fibrin clot in that the amino termini of the Aα and Bβ chains include fibrinopeptides A and B, cleavage of which, as described in more detail below, is required for formation of a clot. The carboxyl termini of each chain also end in globular domains, and the region in between these domains form α helical coiled-coils. These C-terminal regions of the molecule are known as the D-domains (Figure 1.7).

The process of fibrin clot formation is initiated when the protease thrombin is activated during the coagulation cascade as described earlier. Thrombin cleaves fibrinopeptides A and B at the amino termini of the Aα and Bβ chains. The cleavage of these small peptides exposes cryptic polymerization sites on each chain, known as knob A and knob B, within the fibrin monomer. The exposure of these sites allows adjacent fibrin monomers to associate with one another in a half staggered conformation to form protofibrils (Figure 1.8). This association is mediated by 'knob-hole interactions' in which knob A interacts with a pocket, hole A, formed by the γ chain C-termini of two neighboring fibrin molecules. Additionally, knob B is able to interact with 'hole B' within the C-terminus of the Bβ chain on a fibrin protofibril on the opposite side, allowing the fiber to grow in thickness. The D-domains of neighboring fibrin monomers also interact with one another, allowing for elongation of the growing fibrin protofibril. These two sites of interaction allow the fibrin clot to grow in both length and thickness (Figure 1.9).

1.3 γA/γ' Fibrinogen

1.3.1 Fibrinogen γ Chain Isoform Production

The γ chain of fibrinogen is known to have two splice isoforms, γA and γ'. The γA chain arises from what is considered canonical mRNA processing and splicing, where intron 9 is spliced out and exon 9 is ligated to exon 10 (Figure 1.10). The ribosome then translates exon 9 and exon 10 until it encounters a stop codon found after the first four residues coded by exon 10. This isoform constitutes approximately 90-95% of all γ chains and plays an important role in hemostasis, as the last 4 residues encoded by exon 10, AGDV, constitute the binding site for the platelet receptor for fibrinogen, GPIIbIIIa, which is responsible for platelet bridging and aggregation during primary hemostasis, as discussed earlier.

Approximately 5% percent of the γ chain that is incorporated into fibrinogen molecules is the γ' isoform. This isoform arises from an alternative splicing event within intron 9. It is believed that a polyadenylation sequence within intron 9 is recognized and cleaved by a multiprotein complex known as cleavage and polyadenyaltion specificity factor (CPSF) before intron 9 can be spliced out, resulting in the loss of exon 10 and the retention of a portion of intron 9 in the mRNA. Translation of this mRNA isoform reads through the end of exon 9 into intron 9 (also known as exon 9a), leading to a 20 amino acid C-terminal extension, VRPEHPAETEYDSLYPEDDL, that is not found in the γA chain (Figure 1.10) (13,14).

1.3.2 Pathophysiology of the γ' Chain of Fibrinogen

The γ' chain carboxyl terminus is very highly negatively charged as it contains seven glutamate and aspartate residues within this region as well as two tyrosine residues that are O-sulfated (15). As previously mentioned, the functional fibrinogen molecule is a dimer of three strands with the stoichiometry (αβγ)(αβγ). Due to the relative abundance of each of the γA chains, approximately 93% of all functional fibrinogen contains two γA chains, and is termed γA/γA fibrinogen. The remaining 7% of functional fibrinogen is made up predominantly of fibrinogen heterodimers containing one of each γ chain isoform, and is called γA/γ' fibrinogen. γ'/γ' homodimers do exist, although their relative abundance in humans is thought to be less than 1% of the total fibrinogen in circulation due to the scarcity of the γ' chain.

Several studies have shown that clots formed with γA/γ' fibrinogen have unique physical properties and differ significantly from clots made from γA/γA fibrinogen. These unique properties of the γ' chain itself and clots formed by incorporation of γA/γ' or γ'/γ' fibrinogen are summarized in Table 1.1. The γ' chain binds directly to the proenzyme form of fXIII (16), suggesting that the γ' chain serves as a cofactor for fXIII activation (17). This may lead to a higher local concentration of fXIIIa at the growing clot, thus increasing the amount of cross-linking between the fibrin monomers and creating a more stable fibrin clot. This would account for observations made by our lab and others that clots made with γ' fibrinogen incorporated lyse more slowly than those made with γA/γA fibrinogen (18). Additionally, previous work in our lab has shown that not only does γA/γ' fibrinogen serve as a carrier for fXIII, but also increases the rate of fXIII activation,

leading to a high local concentration of active fXIIIa (17). However, the γ' chain lacks the binding site for platelet adhesion and aggregation that is present in the γA chain (19-22), but this has been thought to have no functional consequence due to the excess of bivalent γA/γA fibrinogen. However, the γ' chain has been shown to inhibit thrombin induced platelet aggregation (28).

γA/γ' fibrinogen is known to contain two thrombin-binding sites, a high affinity site and a low affinity site. The low affinity thrombin-binding site has been localized to the E domain of fibrinogen and requires cleavage of fibrinopeptide B for efficient binding (23). This low affinity binding site is present in both γA/γA fibrinogen and γA/γ' fibrinogen. The high affinity binding site has been localized to the γ' chain (24,25). The highly negatively charged γ' chain binds to thrombin's exosite II, a highly positively charged region, through electrostatic interactions (25). This binding creates an additional site of clot bound thrombin, and is clinically significant in that this it is resistant to heparin-mediated inhibition (26,27). Importantly, the binding of thrombin's exosite II to the γ' chain of fibrinogen has also been shown to alter the specificity and activity of thrombin towards certain substrates including protease activated receptor 1 (PAR1) on the surface of platelets (28) and fVIII (29).

Finally, work in our lab and others has shown that clots formed with γA/γ' fibrinogen are more resistant to clot breakdown and have altered clot architecture (18). Clots formed with only recombinant γ'/γ' fibrinogen were less compact and contained thicker fibrin fibrils than clots formed with γA/γA fibrinogen. Additionally, the γ'/γ' clots were 20%

more permeable than the γA/γA clots. More dramatically, the viscoelastic measurements of the clots formed with γ'/γ' fibrinogen were three times stiffer than γA/γA clots, and the rates of lysis were 8 times slower (18).

1.4 Fibrinogen and Disease

Elevated plasma fibrinogen is a well-documented risk factor for cardiovascular disease. High plasma fibrinogen levels are an independent risk factor for myocardial infarction (29), stroke (30) and other cardiovascular diseases. The magnitude of the increase in fibrinogen levels above baseline correlates with the presence and severity of disease. Surprisingly, elevated fibrinogen levels have also been associated with nonvascular mortality. This may be due, in part, to the role of fibrinogen as an acute phase reactant that is substantially up regulated during injury or infection. Fibrinogen levels have also been correlated with several established cardiovascular risk factors such as blood pressure and serum cholesterol levels (30).

The biochemical basis for the association of high plasma fibrinogen with cardiovascular disease has recently begun to be elucidated. In a study using clots formed from whole blood of stroke patients with high fibrinogen levels, patient clots lysed slower than those formed from the blood of patients with low fibrinogen levels (31). However, definitive evidence that high fibrinogen levels directly cause cardiovascular disease has yet to be discovered. High fibrinogen levels may simply be a marker for inflammation, which is usually present in the vasculature in cardiovascular disease. Alternatively, high fibrinogen levels could change the hemodynamic properties of blood by increasing viscosity or altering the interactions between any number of the enzymes in the coagulation cascade. A recent study using a mouse line genetically altered to produce high plasma fibrinogen levels has shown that high fibrinogen levels lead to increased fibrin deposition in certain organs and exacerbated neointimal hyperplasia. However,

high fibrinogen levels did not affect the extent or incidence of arterial thrombosis (32). Moreover, high fibrinogen levels showed an inverse correlation with the amount of thrombin generated, suggesting that high fibrinogen levels may indeed alter the interactions between enzymes involved in coagulation.

While total fibrinogen is a known independent risk factor for cardiovascular disease, the observations that γA/γ' fibrinogen forms stiffer and more highly cross-linked clots that are more resistant to clot breakdown have led to the hypothesis that the γ' chain of fibrinogen may play an important role in the development of cardiovascular disease as well. There have been several studies looking at the role of total fibrinogen on the risk of heart attacks, stroke and other thrombotic events. These studies have shown that total fibrinogen is a risk factor for stroke, myocardial infarction, ischemic heart disease and atherosclerosis (29,30,33,34). However, there have been significantly fewer studies looking at the role of γA/γ' fibrinogen levels in cardiovascular disease. Studies from our laboratory have shown an association between γA/γ' fibrinogen levels and coronary artery disease (35) and myocardial infarction (36). Others have shown an inverse correlation between γA/γ' fibrinogen levels and deep vein thrombosis (94). Although polymorphisms located within the γ gene of fibrinogen have been shown to influence the γ':γA ratio (37), factors that regulate the levels of γA/γ' fibrinogen are generally uncharacterized. Therefore, a focus of this research is to examine novel mechanisms involved in the regulation of the γ chain splice isoforms of fibrinogen that may serve as future therapeutic targets in the fight against cardiovascular disease.

Fig. 1.1

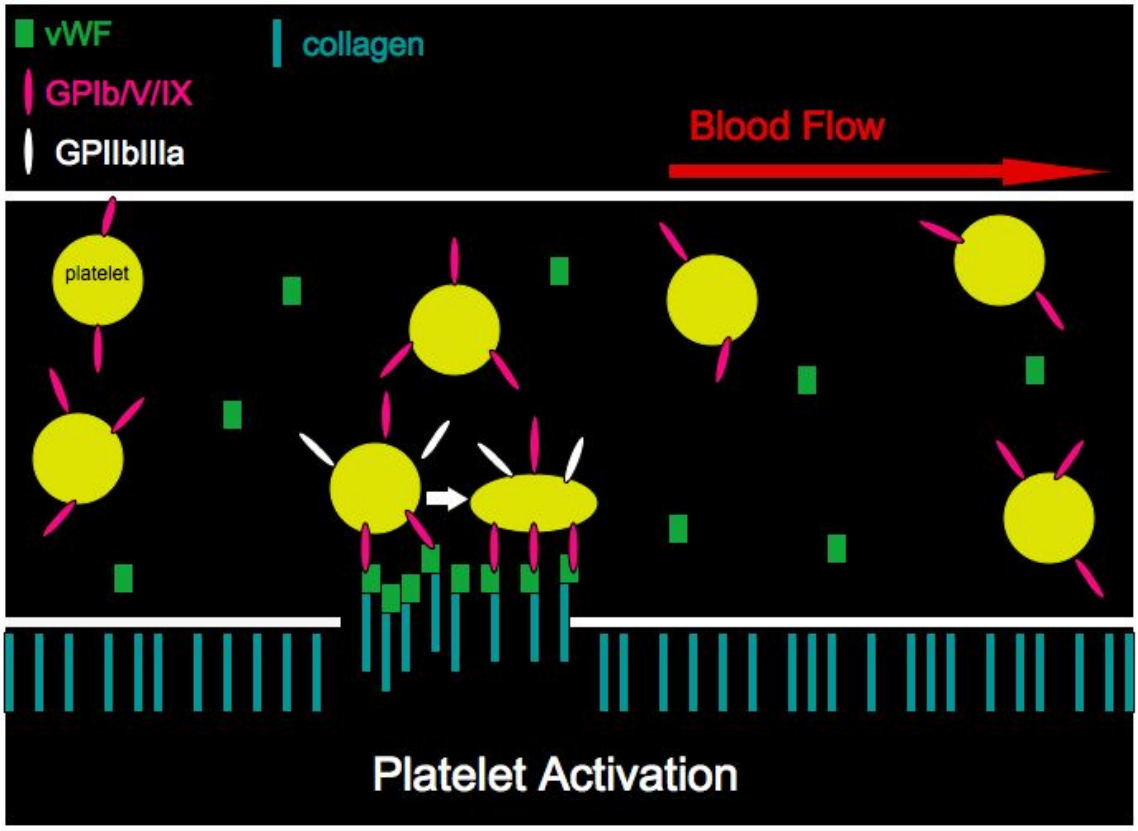


Figure 1.1: Platelet Adhesion and Activation- Adhesion of platelets (yellow circles) to the site of vascular injury. The endothelium of the blood vessel is shown in white lines. The subendothelial matrix, including collagen (cyan), is exposed following injury to the endothelium. Exposed collagen binds with circulating vWF (green squares), which in turn binds to the GPIb/V/IX complex (pink) on the platelet surface, tethering the platelets to the vessel. Platelets become activated (yellow oval) and GPIIbIIIa (white ovals) is subsequently exposed on the platelet surface.

Fig. 1.2

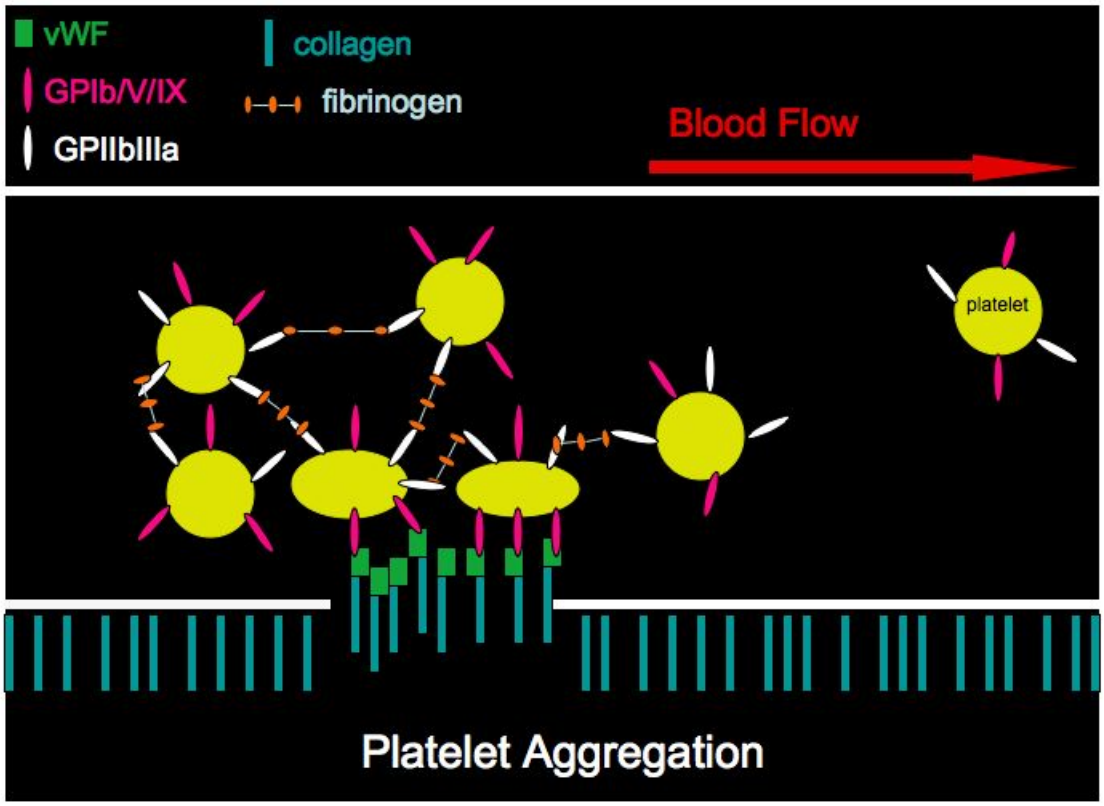


Figure 1.2: Platelet Aggregation- Adhered activated platelets (yellow ovals) are able to recruit adjacent activated platelets to the site of injury to form a platelet plug. Exposure of GPIIbIIIa (white ovals) on activated platelets leads to binding of circulating fibrinogen (orange) to the platelet. The dimeric nature of fibrinogen allows it to bind a second nearby activated platelet, bridging the platelets and retaining them at the site of injury.

Fig. 1.3

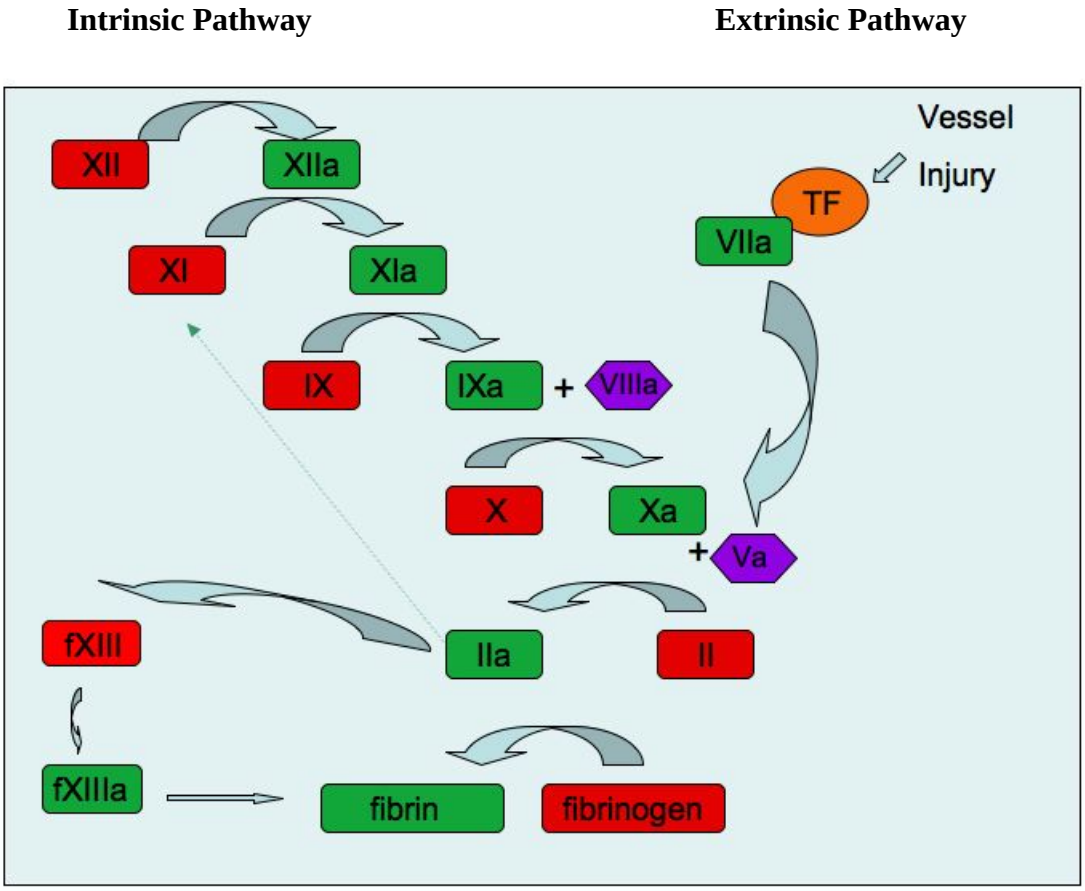


Figure 1.3: The Coagulation Cascade- Injury to the blood vessel exposes TF on the endothelium, initiating the extrinsic pathway of the cascade. A series of proteolytic activations occur in the presence of cofactors and the negatively charged surface of the platelet. The cascade leads to the activation of thrombin (IIa), which is able to feedback to positively regulate the cascade. Thrombin also cleaves fibrinogen into fibrin, forming a clot at the site of the platelet plug. The clot is then stabilized by the transglutaminase fXIIIa, which is also activated by thrombin, which crosslinks neighboring fibrin fibers.

Fig 1.4

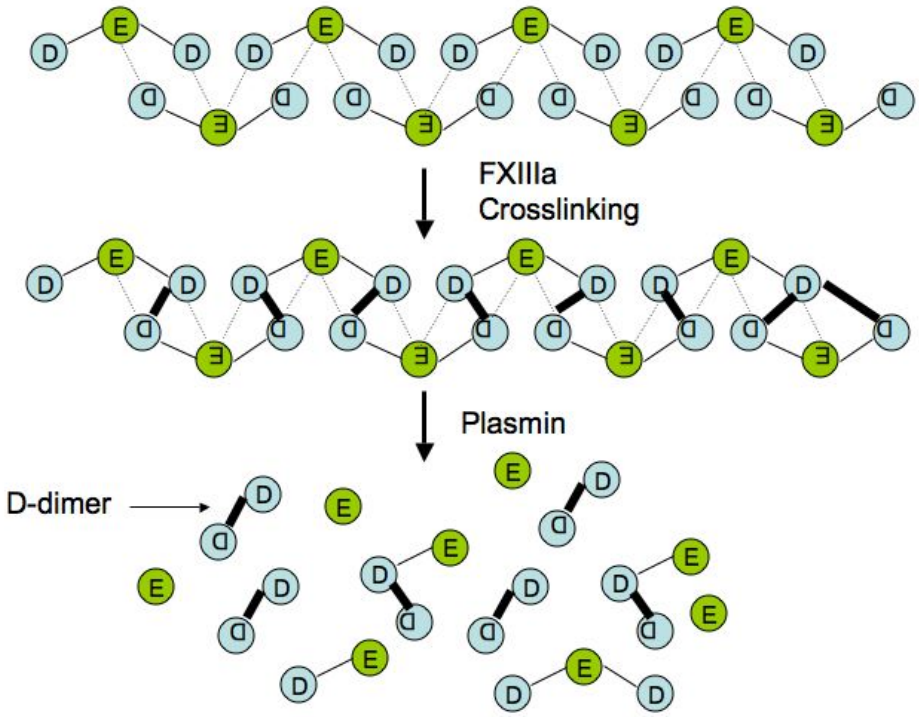


Figure 1.4: Fibrin Clot Dissolution- The zymogen plasminogen is activated to plasmin in the presence of fibrin. Plasmin then cleaves fibrin at distinct proteolytic sites, forming fibrin degradation products, including D-fragments, E-fragments, and D-dimers.

Fig. 1.5

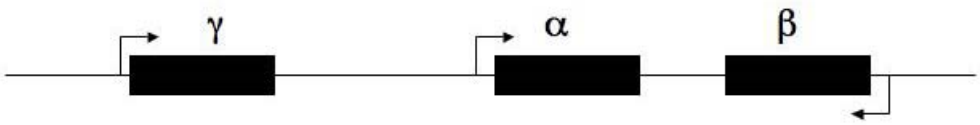


Figure 1.5: Arrangement of Fibrinogen Gene Loci on Human Chromosome 4- The human fibrinogen gene cluster on chromosome 4q23-q32 (Adapted from (38)).

Fig 1.6

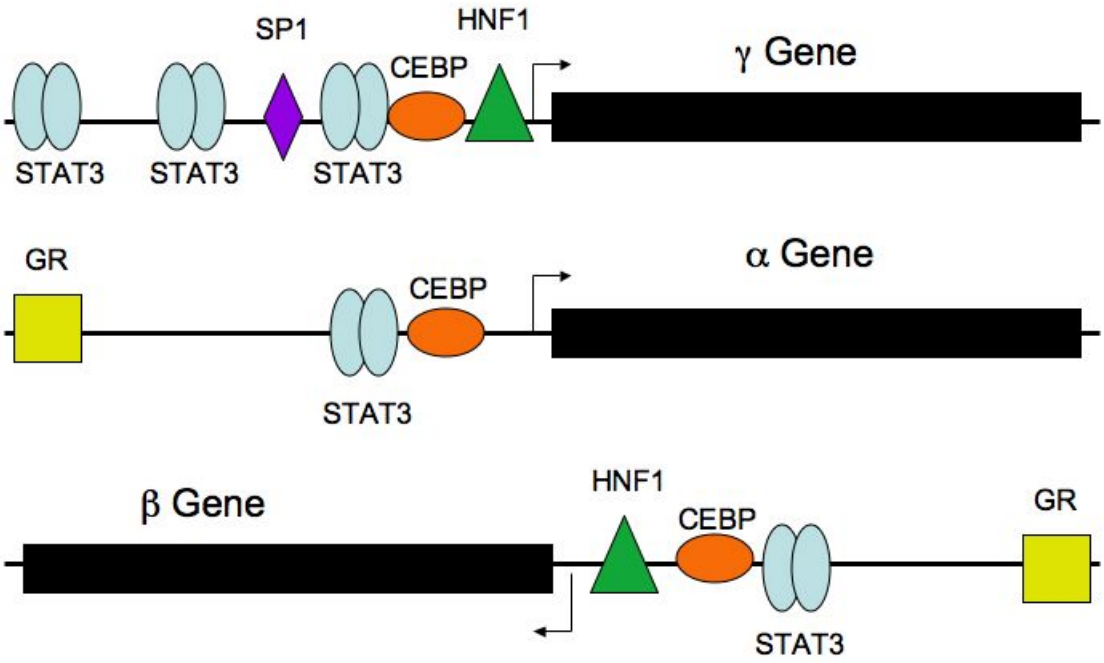


Figure 1.6: Known Transcriptional Response Elements Within the Promoters of the Three Fibrinogen Genes- The promoter region of each of the fibrinogen genes is shown with some of the known transcription factors that bind to this region. The transcriptional start site is depicted by an arrow, and the coding regions of the genes are shown as black boxes.

Fig. 1.7

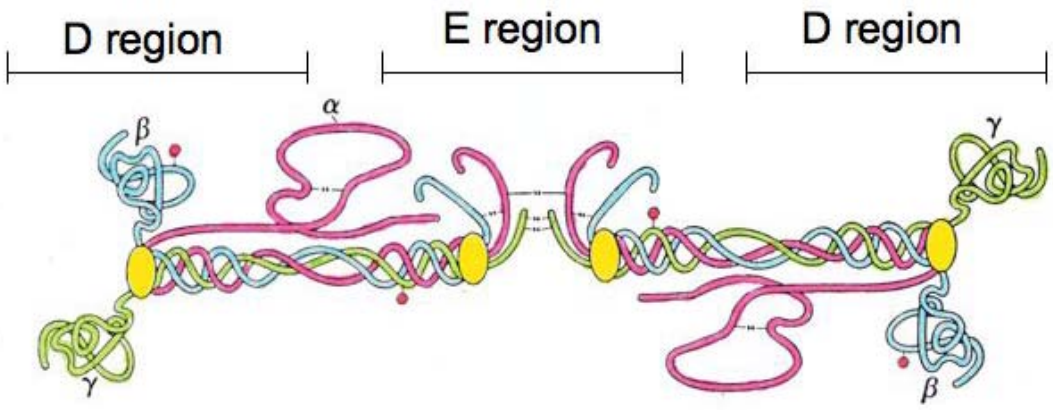


Figure 1.7: Representative Structure of Fibrinogen- The crystal structure of bovine fibrinogen is shown. Each of the three fibrinogen chains is shown in a different color: α in red, β in blue and γ in green. The amino termini of the α and β chains form the disulfide knot that contains fibrinopeptides A and B. This central region is known as the E-domain, while the two C-terminal regions of the molecule form the D-domains.

Fig. 1.8

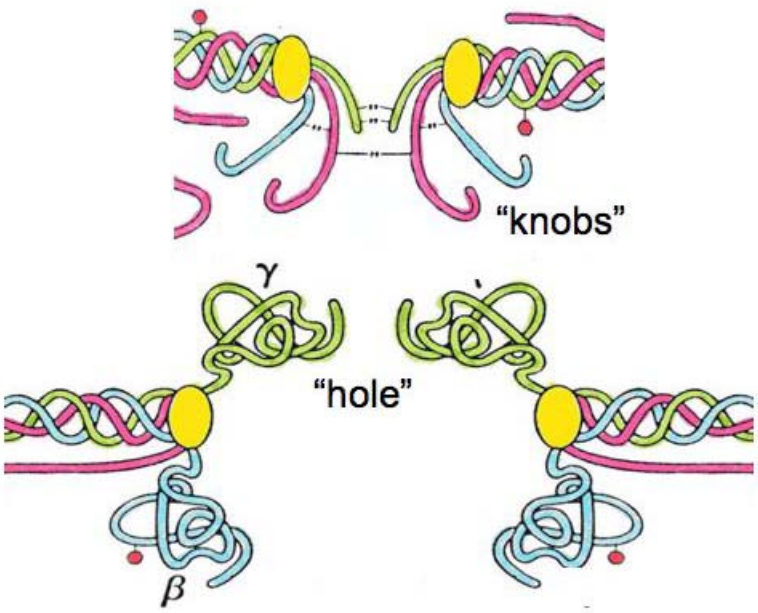


Figure 1.8: Half-staggered Interactions of Adjacent Fibrin Monomers- Following cleavage of Fibrinopeptides A and B, cryptic polymerization sites are uncovered, allowing adjacent fibrin monomers to associate with one another through knob-hole interactions. Knobs A and B are within in the E –domain of one fibril, while the γ hole, is formed by two D-domains on adjacent fibrin fibrils.

Fig. 1.9

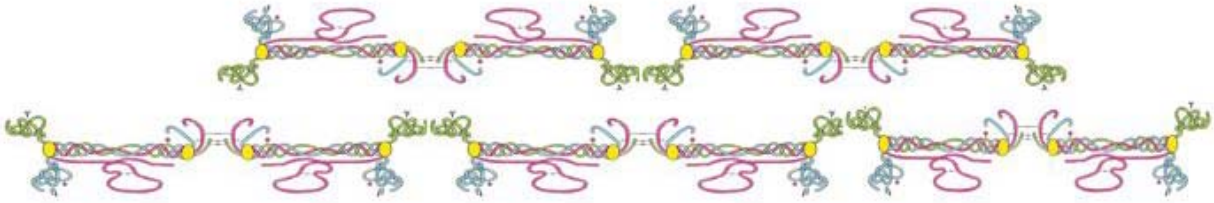


Figure 1.9: Lateral and End-to-End Association of Fibrin Protofibrils- The D-domains of neighboring protofibrils (shown in green) also interact with one another, allowing for elongation of the growing protofibril.

Fig. 1.10

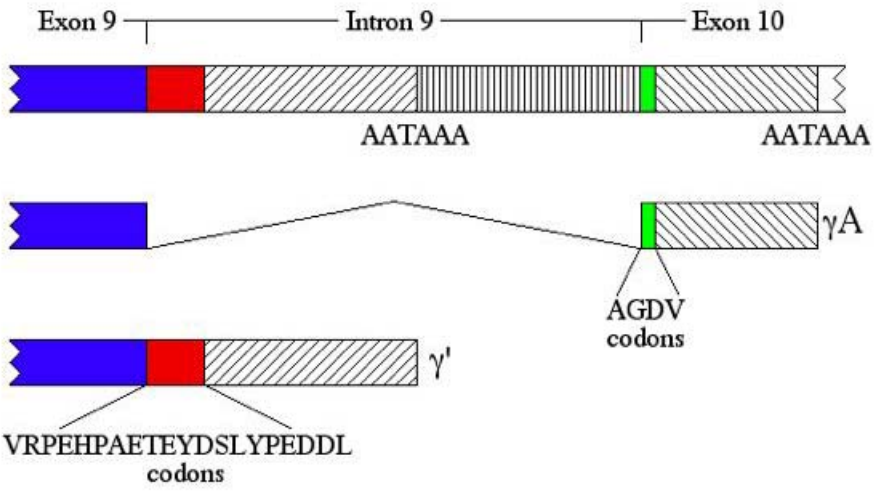


Figure 1.10: Alternative Splicing of the Fibrinogen γ Chain pre-mRNA- Alternative splicing of the fibrinogen γ chain pre-mRNA results in two γ chain isoforms: γA and γ' fibrinogen. The γA form is formed by canonical splicing, while the γ' isoform undergoes polyadenylation and cleavage within intron 9, leading to the formation of a polypeptide with an extended C-terminus.

Table 1.1

	γA	γ'
Site Produced	Liver (mRNA also found in other organs)	ONLY in liver
Crosslinking	Slower	More Rapid
Platelet Binding Site	Yes, AGDV integrin binding	No
Polymerization	Faster	Slower and altered
Fibrinolysis	Faster	Slower
Thrombin Binding	Low affinity binding site	Low and high affinity sites
fXIII Binding Site	No	Yes

Table 1.1: Comparison of the γA and γ' chains of fibrinogen and clots formed with each isoform incorporated

Chapter 2: General Methods

Cell Culture–These studies employ the HepG2 human hepatocellular carcinoma cell line as an *in vitro* model system. HepG2 cells, like native hepatocytes, produce the majority of the circulating coagulation factors. Most importantly for our work, HepG2 cells have been shown to produce fibrinogen (40). Moreover, HepG2 cells have been shown to produce both γ chain isoforms of fibrinogen, the γA and γ' forms (41). HepG2 cells have been utilized to examine the hepatic acute phase response in many studies and provide a useful tool to examine the acute phase response of the two fibrinogen γ chain isoforms in the studies presented here.

HepG2 cells were grown to 90% confluence in 24-well plates in minimal essential medium (MEM) containing 10% fetal calf serum. Cells were then serum-starved for 24 hours and treated with recombinant cytokines (Chapters 3 and 5) or fibrin degradation products (Chapter 4) for 24 hours. In inhibitor experiments (Chapter 3), cells were pretreated with 5 μM epigallocatechin gallate (EGCG), a specific STAT1 inhibitor, for 30 minutes then treated with recombinant IFN-γ for 24 hours. Conditioned media were harvested from the wells, and cells were washed with PBS and lysed in RIPA buffer (150mM NaCl/20mM Tris, pH7.4/0.2% SDS/5mM EDTA/1% NP-40/5mM EACA/1μM leupeptin/1μM pepstatin/0.1mM N-ethylmaleimide/0.1mM phenylmethylsulfonyl fluoride (PMSF)). Total cellular protein concentration was measured by bicinchonic assay (BCA) (Pierce) and was used to normalize ELISA results.

Total Fibrinogen ELISAs–To measure total fibrinogen produced by HepG2 cells, 96 well plates were coated with 1.5 μg/ml AXL203 rabbit anti-human polyclonal antibody (Accurate Chemical) and incubated overnight at 4ºC. Wells were washed three times with PBS/0.1% Triton and blocked in PBS containing 1% BSA/0.1% Triton at 37ºC for 2 hours. Wells were washed and 50 μl of conditioned medium was added to each well and incubated at 37ºC for one hour. Wells were washed again with PBS/0.1% Triton and a 1:2500 dilution of sheep anti-human fibrinogen-horseradish peroxidase (HRP) conjugate was added to the wells and incubated for 1 hour at 37ºC. Wells were washed and incubated with 3,3', 5,5''-tetramethylbenzidine (TMB) substrate for 30 minutes at room temperature. Stop solution was added to each reaction and absorbance was measured at 450 nm.

γ' Fibrinogen ELISAs- Previous studies evaluating γ' fibrinogen in patient samples lacked a standardized and well-characterized assay. ELISAs for γ' fibrinogen were developed by our laboratory so as to be able to easily determine γ' fibrinogen concentrations in plasma samples (42). This assay was modified slightly for measuring γ' levels in cell culture supernatant. Briefly, 96 well plates were coated overnight at 4^{o}C with the monoclonal 2.G2.H9 antibody which is directed against the unique C-terminus of the γ' chain and does not bind to the γA chain (42). Wells were washed three times with PBS/0.1% Triton and blocked in PBS containing 1% BSA/0.1% Triton at 37ºC for 2 hours. Wells were washed and 50 μl of conditioned medium was added to each well and incubated at 37ºC for one hour. Wells were washed again with PBS/0.1% Triton and a 1:2500 dilution of sheep anti-human fibrinogen-HRP conjugate was added to the wells

and incubated for 1 hour at 37ºC. Wells were washed and incubated with TMB substrate for 30 minutes at room temperature. Stop solution was added to each reaction and absorbance was measured at 450 nm.

Western Blotting–HepG2 cells were prepared and treated as described above. Following 24 hours of cytokine treatment, cells were washed with PBS and lysed in RIPA buffer. Total cellular protein concentration was determined using a BCA assay and equivalent amounts of protein from the cell lysates were separated by 10% resolving/4% stacking sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred to a nitrocellulose membrane and the membrane was blocked overnight in Western A buffer containing 50mM Tris, pH 7.4/5 mM EDTA/0.05% NP-40/150 mM NaCl/0.25% gelatin . The membrane was probed using rabbit anti-human STAT1 or STAT3 and GAPDH antibodies (Cell Signaling) in Chapter 3, or polyclonal rabbit anti-fibrinogen antibody in Chapter 4. The membrane was stripped and reprobed for phospho-STAT1 or phospho-STAT3 using a rabbit anti-human pSTAT1 or rabbit anti-human pSTAT3 antibody (Cell Signaling) in Chapter 3. Goat anti-rabbit Alexafluor 680 secondary antibodies were used for detection and membranes were imaged using the LiCor Odyssey Imaging System.

qRT-PCR–HepG2 cells were prepared and treated with increasing doses of fibrin degradation products or inflammatory cytokines for 24 hours as described. Cells were then washed with PBS and mRNA was harvested using the RNeasy kit (Qiagen). 2 μg of total mRNA was reverse transcribed with Superscript III Reverse Transcriptase (Qiagen)

and a random hexamer primer set. 50 μg cDNA was then used in a quantitative real-time PCR reaction with the following primer and probe sets: fibrinogen γ chain forward primer 5'-CCACTATGAAGATAATCCCATTC-3', reverse primer 5'-CGGTCTTTTAAACGTCTCCAGC-3', and internal FAM labeled probe 5'-CCCAGGTGGTGTTGCTGTCCTTCTC-3' (ABI). The human 18S primer probe control set (ABI) was used as an endogenous control, and all results were normalized to 18S using the ΔΔCT method. Each experiment was performed in triplicate on each plate, and each plate was replicated three times.

Chapter 3: Regulation of Fibrinogen Synthesis by IFN-γ

3.1 Introduction

Inflammation can lead to a vast number of changes both locally and distantly from the site of trauma or infection. The changes that arise are known as an acute phase response and can occur in both acute and chronic inflammatory states. These changes involve alterations in behavioral, biochemical, and physiological systems. Importantly, the acute phase response involves changes in the concentrations of many plasma proteins, including those involved in complement activation, protein transport, inflammation, coagulation and fibrinolysis. An acute phase protein has been defined as one whose plasma concentration increases or decreases by at least 25% during an inflammatory response. The changes in these protein levels are due mainly to a change in their expression by hepatocytes, which are the main producers of plasma proteins. While different acute phase proteins may respond in a coordinated fashion during an acute phase response, not all proteins will increase or decrease in a uniform fashion and there is a high level of variability in the protein concentrations seen from individual to individual. These findings suggest that many of the acute phase proteins are regulated individually, and may be controlled by many different modulators of the acute phase response that are activated under inflammatory conditions.

The major players that regulate the acute phase response are a group of proteins known as cytokines. Cytokines are signaling proteins that are produced by different types of activated immune cells, mainly monocytes and macrophages, at the site of inflammation. The cytokines produced during the acute phase response are commonly referred to as

inflammatory cytokines and include interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon γ (IFN-γ) and transforming growth factor β (TGF-β). These cytokines are often present in different combinations under different inflammatory conditions, and while they are capable of acting alone to affect the production of acute phase proteins, they often work in combination with each other to produce a specific effect dependent on the inflammatory condition. Several studies in primary hepatocytes and hepatocyte cell lines have shown the involvement of IL-6 as a major modulator of acute phase response (43), and have shown roles for IL-1, TNF-α and TGF-β in modulating the IL-6 response (44,45).

Interferon-γ, a type II interferon, is most notably known for its role as an immunomodulatory cytokine produced in response to bacterial and viral infection. While only immune cells are known to produce IFN-γ, all cells of the body with the exception of erythrocytes express the IFN-γ receptor (IFNGR) and are able to respond to IFN-γ (46). Signaling through IFNGR is distinct from the signaling pathways elicited in response to the type I interferons IFN-α and IFN-β. Binding of IFN-γ to its receptor IFNGR induces dimerization of the receptor, leading to phosphorylation of Janus Kinases 1 and 2 (JAK1/2), which are constitutively associated with the receptor. Cytoplasmic Signal Transducer and Activator of Transcription-1 (STAT1) then binds to the phosphorylated JAKs and becomes phosphorylated. Two phosphorylated STAT1 molecules then dimerize and are translocated to the nucleus where the dimer binds to IFN-γ Activated Sequences (GAS) containing the sequence TTNCNNNAA (where N

represents any base), in the promoter regions of IFN-γ responsive genes, thus exerting an effect on target genes (Figure 3.1).

IFN-γ administered systemically has been shown to impair wound healing in mouse models, possibly by impairing collagen cross-linking (47). More recently, it has been shown that IFN-γ suppresses infection-mediated fibrin deposition via a STAT1 mediated mechanism in response to *Toxoplasma gondii* (48). While these effects have been partially attributed to a reduction in fibrinolytic enzyme production during infection stimulated IFN-γ signaling (49), the question of whether or not IFN-γ may reduce fibrin deposition by directly interfering with the production of fibrinogen has not been answered. Moreover, a recent genome wide association study has shown that a locus located at or near the gene encoding interferon regulatory factor-1 (IRF1) regulates levels of circulating fibrinogen (50). IRF1 is a known transcriptional activator of genes induced by both type I and type II interferons, and its expression has been shown to be significantly induced by IFN-γ (51). Therefore this chapter will focus on determining the effects of IFN-γ on the production of total fibrinogen.

3.2 Materials and Methods

Reagents–HepG2 cells were purchased from ATCC and grown in MEM containing 0.2 mM L-glutamine/100 units/ml penicillin/100 μg/ml streptomycin/10% fetal calf serum (Invitrogen). Recombinant human IFN-γ and IL-6 were purchased from Leinco Technologies. Rabbit anti-human pSTAT1 (Y701), rabbit anti-human STAT1, rabbit anti-human STAT3, rabbit anti-human pSTAT3 (Y705) and rabbit anti-human GAPDH polyclonal antibodies were purchased from Cell Signaling Technologies. IR-700 labeled oligonucleotides were purchased from IDT Technologies. AXL203 rabbit anti-fibrinogen antibody and HRP-labeled sheep anti-human fibrinogen antibody were purchased from Accurate Chemical Corp. TMB HRP substrate and 450nm Stop Solution were purchased from BioFX. Epigallocatechin gallate (EGCG), a specific STAT1 inhibitor from green tea extract, was purchased from Sigma.

qRT-PCR– qRT-PCR was performed as described in the general methods section using cells treated with increasing concentrations of IFN-γ.

Electrophoretic Mobility Shift Assays–HepG2 nuclear extracts were prepared following 4 hours of incubation with IFN-γ (25 ng/ml), IL-6 (100 ng/ml) or a combination of the two. Cells were washed in ice cold PBS and lysed in 0.1M HEPES, pH 7.5/0.6M KCl/1 mM EDTA/1mM dithiothreitol (DTT)/0.1mM PMSF/2% NP-40 and centrifuged for 5 minutes at 4ºC at 3000 rpm. Cell pellets were resuspended in buffer containing 0.1M HEPES, pH 7.5/0.6M KCl/1 mM EDTA/1mM DTT/0.1mM PMSF and incubated on ice for 5 minutes. Following centrifugation for 5 min at 4ºC and 3000 rpm, cell pellets were

resuspended in 0.67 M Tris, pH 7.8/60 mM KCl/1 mM DTT/ 1mM PMSF. The mixture was frozen in liquid nitrogen and stored at -70ºC until use.

Single stranded probes labeled at the 5' end with IRDye-700 were designed to contain the putative promoter GAS sequences from each of the fibrinogen gene promoters or one of the three IL-6 response elements (IL6REs) found in the fibrinogen γ chain promoter. The sequences of the probes can be found in Table 3.1. A rat α-macroglobulin promoter oligonucleotide that is known to bind to STAT1 and STAT3 was used as a positive control (48). Single stranded forward and reverse probes were annealed at 95ºC for 10 minutes and allowed to cool to room temperature to form double stranded molecules for use in binding reactions. DNA-protein binding reactions were performed using the Odyssey Infrared EMSA Kit (LiCor Biosciences) according to the manufacturer's protocol. Briefly, 200 fmole labeled oligonucleotides were incubated with 10 μg nuclear extract at room temperature for 20 minutes in a buffer containing 100 mM Tris pH 7.5/500 mM KCl/10 mM DTT/0.25% Tween and 50 ng poly dI-dC. For antibody binding, the cell lysate was incubated with 1 μl of antibody for 10 minutes prior to addition to the labeled oligonucleotide mix, then incubated an additional 10 minutes upon addition to the oligos. DNA-protein complexes were resolved on a 5% TBE native acrylamide gel at 4ºC in 1X TBE buffer (89 mM Tris, pH 8.3/ 89mM borate/ 2mM EDTA) , avoiding light exposure. The gels were imaged at 700 nm using the LiCor Odyssey Imaging System.

Co-Immunoprecipitation (Co-IP)-HepG2 nuclear extracts were prepared as above following 4 hours of treatment with both IL-6 (100 ng/ml) and IFN-γ (25 ng/ml). Rabbit anti-human STAT1 or STAT3 antibody was crosslinked to protein A/G beads using disuccinimidylsuberate. The rabbit anti-human fibrinogen antibody AXL203 was crosslinked to protein A/G beads as a negative pulldown control. Antibody-bead conjugates were incubated with 500 μg cell lysate overnight at 4^{o}C on a rotating platform. Beads were centrifuged to the bottom of the tube and washed three times in phosphate buffered saline. Protein complexes were eluted from the beads using IgG Elution Buffer, pH 2.8 (Pierce) and the eluates were added to SDS-PAGE loading buffer and boiled. Eluates were run on a 10% resolving, 4% stacking SDS-PAGE gel and proteins were transferred to nitrocellulose membrane. The membrane was blocked overnight in 1% gelatin. The membrane was then probed with a rabbit anti-human STAT3 antibody overnight at 4^{o}C. Secondary goat anti-rabbit 680 Alexafluor was incubated with each membrane and bands were visualized using the LiCor Odyssey System. Western blots were also performed on the same IP samples using a rabbit anti-human STAT1 antibody and secondary goat anti-rabbit 680 Alexafluor antibody.

Statistical Analysis- Data were analyzed using the SPSS version 17.0 Software package. All experiments were performed three separate times, with three replicates for each experiment. Calculation and comparison of the means between treated and untreated groups was performed using a Mann-Whitney test to determine statistical significance with a cutoff of $p<0.05$ and data are presented as mean $\pm$ SEM. For qRT-PCR

experiments, statistics were performed on ΔCt values after normalization to 18S using a paired t-test with a significance cutoff of $p<0.05$.

Table 3.1

Oligo Name	Sequence (5'-3')
α GAS sense	ACCCTGTTCCTGGAATGTGAGA
α GAS antisense	TCTCACATTCCAGGAACAGGGT
β GAS sense	AAGATGTTGCTTAAATGATAAA
β GAS antisense	TTTATCATTTAAGCAACATCTT
γ GAS sense	AGGAGCTTACATAAAGGGACAA
γ GAS antisense	TTGTCCCTTTATGTAAGCTCCT
IL6RE1 sense	GCAAAATCTGGGAACCTG
IL6RE1 antisense	CAGGTTCCCAGATTTTGC
IL6RE2 sense	ATGTACTGGAAGAAGTTG
IL6RE2 antisense	CAACTTCTTCCAGTACAT
IL6RE3 sense	CATTCCTGGGAATAGTG
IL6RE3 antisense	CACTATTCCCAGGAATG
α2-macroglobulin sense	AATCCTTCTGGGAATTC
α2-macroglobulin antisense	GAATTCCCAGAAGGATT

Table 3.1: Sequences of Single Stranded IR-700 Labeled Oligonucleotides Used in EMSAs.

3.3 Results

Characterization of the Effects of IFN-γ on Basal Fibrinogen Production in HepG2 Cells–Previous studies have shown that the presence of IFN-γ leads to a decrease in fibrin deposition during *Toxoplasma gondii* infection in mice (49) and delayed wound healing (47). Additionally, the association between a locus located near the gene encoding IRF-1 and circulating fibrinogen levels (50) led us to investigate the role of IFN-γ in regulating fibrinogen production. As shown by ELISA, treatment of HepG2 cells with increasing concentrations of IFN-γ within the physiological range caused a dose dependent, although not statistically significant, decrease in the amount of total fibrinogen secreted into the media after 24 hours (Figure 3.2A). At the highest concentration, production of fibrinogen from HepG2 cells was decreased by approximately 25%. The production of total fibrinogen mRNA was also decreased following treatment of HepG2 cells with IFN-γ, as shown by quantitative real-time PCR (Figure 3.2B) but again, this data was not statistically significant. These data therefore show that at physiological concentrations, IFN-γ may be able to inhibit production of fibrinogen at the protein and mRNA level in HepG2 cells, although the data are inconclusive.

Effect of IFN-γ on IL-6 Mediated Fibrinogen Synthesis- IL-6 is a potent inducer of fibrinogen synthesis through the activation of STAT3, which in turn binds to three Type II IL-6 response elements within the γ promoter of fibrinogen (53). It is also one of the first and most significant enhancers of the acute phase response. Therefore, in order to test the effects of IFN-γ on a known inducer of fibrinogen synthesis, HepG2 cells were treated with 100 ng/ml IL-6 alone or in combination with increasing doses of IFN-γ. As

seen in Figure 3.3A, IFN-γ is able to inhibit the IL-6 mediated induction of fibrinogen synthesis in HepG2 cells. These findings were statistically significant, with $p<0.01$ for the induction of fibrinogen synthesis by IL-6 and $p<0.05$ for the inhibition of IL-6 by IFN-γ at IFN-γ concentrations of 3.13 ng/ml, 6.25 ng/ml and 12.5 ng/ml, respectively, as analyzed by Mann-Whitney tests. Treatment with IFN-γ in combination with IL-6 leads to levels of secreted fibrinogen comparable to baseline levels. Additionally, induction of total fibrinogen mRNA in HepG2 cells by IL-6 was also inhibited by treatment with IFN-γ, as shown by qRT-PCR (Figure 3.3B), however these results did not yet reach statistical significance. These results suggest that IFN-γ is a powerful negative regulator of IL-6 induced fibrinogen production.

The Fibrinogen α, β and γ Chain Promoters Contain IFN-γ Regulatory Sequences– IFN-γ signals through a specific pathway that differs from the signaling pathway utilized by IFN-α and -β. IFN-γ binds to the IFNGR, which has been shown to be present on HepG2 cells (54). IFNGR signals through a JAK/STAT-mediated mechanism to induce phosphorylation and dimerization of STAT1 molecules that can then translocate to the nucleus where they exert their effects on target genes (Figure 3.1). Therefore, we postulated that there may be STAT1 binding sites located in the promoter regions of the fibrinogen gene promoters that are capable of affecting the production of fibrinogen following IFN-γ treatment. Using the PROMO transcription factor binding site predictor program (55), we identified a STAT1 consensus motif (GAS element) at base pairs -244 to -236 of the α fibrinogen promoter, -124 to -116 of the β fibrinogen promoter and -20 to -28 of the γ fibrinogen promoter (Figure 3.4A). The presence of these GAS elements

within the fibrinogen promoters suggests that activated STAT1 may be able to bind to the promoter regions to regulate fibrinogen synthesis. Due to the proximity of the γ promoter GAS to both the transcriptional start site and the three known IL-6 response elements within the γ promoter (Figure 3.4B), we chose to focus this work on characterizing the γ chain GAS and its role in the regulation of both basal and IL-6 induced fibrinogen synthesis.

Examination of STAT1 Phosphorylation at Tyr 701 in HepG2 Cells in Response to IFN-γ treatment- STAT1 is the major signaling molecule involved in the signaling of IFN-γ and requires phosphorylation at tyrosine 701 in order to dimerize and translocate to the nucleus (56). Phosphorylated STAT1 is able to homodimerize with other phosphorylated STAT1 monomers, as well as form heterodimers with phosphorylated STAT2 and STAT3 (56,58). In order to determine whether IFN-γ is affecting basal and IL-6 induced fibrinogen γ gene promoter function via a STAT1 mechanism, we confirmed that STAT1 was phosphorylated at tyrosine 701 and activated in HepG2 cells in response to IFN-γ. Western blotting of HepG2 nuclear lysates treated with increasing doses of IFN-γ showed a dose-dependent increase in STAT1 phosphorylation after 24 hours, while total STAT1 levels showed in initial increase in response to IFN-γ, but no subsequent increase in protein levels (Figure 3.5A and B). Therefore we conclude that treatment of HepG2 cells with IFN-γ leads to an increase in the levels of phosphorylated and activated STAT1.

STAT1 Forms a Complex with the Fibrinogen γ Promoter GAS Site–To determine whether activated STAT1 is able to bind to the fibrinogen γ gene promoter GAS site, we

performed electromobility shift assays (EMSAs) using IFN-γ treated HepG2 cell nuclear extracts. HepG2 cells were treated with 25 ng/ml IFN-γ for 4 hours. Nuclear extracts from these cells were incubated with labeled probes containing the γ promoter GAS site or the α_2-macroglobulin GAS as a positive control. IFN-γ treated extracts formed a complex with the γ GAS probe (Fig. 3.6, lane 3), which could be competed off with unlabeled competitor, indicating site specificity (Figure 3.6, lane 4). Furthermore, the addition of a STAT1 antibody to the nuclear lysates prior to incubation with the labeled γ promoter GAS probe inhibited the formation of this complex (Figure 3.6, lane 5), indicating that STAT1 is present in the complex which binds to this region of the promoter. The addition of an isotype matched control antibody against GAPDH did not lead to a shift of the STAT1-DNA complex (data not shown). Taken together, these results show that STAT1 is able to form a complex at the fibrinogen γ gene promoter, specifically at the GAS site.

Effects of a specific STAT1 inhibitor on fibrinogen synthesis after treatment with IFN-γ- In order to confirm that the functional responsiveness of the fibrinogen promoter to IFN-γ is mediated by STAT1, HepG2 cells were pretreated with 5 μM epigallocatechin gallate (EGCG), a specific inhibitor of STAT1, for 30 minutes prior to the addition of IFN-γ. ELISAs were then performed to quantitate the amount of fibrinogen secreted from the HepG2 cells. As seen in Figure 3.7, pretreatment with EGCG did not significantly alter fibrinogen production after IFN-γ treatment, suggesting that EGCG did not suppress the IFN-γ mediated decrease in fibrinogen levels.

Investigation of STAT1 and STAT3 Heterodimer Formation in HepG2 Cells Treated with IFN-γ and IL-6-In order to determine the mechanism by which IFN-γ is able to inhibit IL-6 stimulated fibrinogen synthesis, we tested the hypothesis that STAT1 and STAT3 form heterodimers, which may prevent full STAT3 activity at IL6REs when cells were treated with both IL-6 and IFN-γ. HepG2 cells were co-treated with 100 ng/ml IL-6 and 25 ng/ml IFN-γ and lysed in RIPA. As seen in Figure 3.8, co-immunoprecipitation experiments did not show any evidence of STAT1/STAT3 heterodimer formation when proteins were immunoprecipitated with an antibody against STAT1 and probed using an antibody against STAT3. A similar experiment was performed by immunoprecipitating with an antibody against STAT3 and probing with an antibody against STAT1 with the same result (data not shown). We therefore conclude that IFN-γ does not inhibit IL-6 mediated fibrinogen synthesis by binding of STAT1 to STAT3.

Characterization of STAT3 Phosphorylation at Tyr 705 in Response to IL-6 Alone and IL-6/ IFN-γ Co-treatment- Unlike STAT1, STAT3 does not require phosphorylation for translocation to the nucleus, but phosphorylation at tyrosine 705 downstream of IL-6 receptor signaling is required for binding of STAT3 to its target DNA sequences (59). Therefore, we hypothesized that treatment of HepG2 cells with IFN-γ may inhibit IL-6 induced phosphorylation of STAT3. To test this, HepG2 cells were treated with increasing doses of IL-6 alone, or IL-6 and IFN-γ, and the presence of STAT3 phosphorylated at tyrosine 705 was assayed by Western blot using an antibody specific for STAT3 phosphorylated at tyrosine 705. Figure 3.9 depicts an increase in the amount of pSTAT3 following IL-6 treatment up to 100 ng/ml IL-6 (3.9A top and 3.9B). Addition

of IFN-γ did not seem to have an effect on the phosphorylation of STAT3 at tyrosine 705 up to 6.25 ng/ml IFN-γ (3.9A bottom and 3.9C), although inhibition of phosphorylation of STAT3 at Tyr 705 was observed at the two highest concentrations of IFN-γ examined. This suggests that in our experiments, IFN-γ does not inhibit IL-6 mediated fibrinogen synthesis by inhibiting phosphorylation of STAT3 at tyrosine 705 at low concentrations, but may play a role at higher concentrations.

Investigation of STAT3 Binding to IL-6 Response Elements Within the Fibriongen γ Chain Promoter in the Presence of IL-6 and IFN-γ- In order to further investigate the mechanism leading to the decrease in IL-6 mediated fibrinogen synthesis following IFN-γ and IL-6 co-treatment, EMSAs were performed using three labeled oligonucleotides, each containing one of the IL-6 response elements found in the γ gene promoter (52) (Figure 3.4). We tested the hypothesis that IFN-γ treatment reduces pSTAT3 binding to IL-6 response elements within the γ promoter. Lysates from cells treated with IL-6 (100 ng/ml), IFN-γ (25 ng/ml) or a combination of the two were incubated with the labeled oligonucleotides. Electromobility shift assays were performed to determine complex formation at each of the three IL6REs within the fibrinogen γ chain promoter. Figure 3.10A depicts the results from an experiment examining complex formation at IL6RE1, the IL6RE furthest from the transcriptional start site (Figure 3.4B). Lysates from cells treated with IL-6 (lane 4)showed complex formation (arrow) that was not seen in untreated cells (lane 2). This represented a 1.16 fold increase of complex formation at this site. Interestingly, pretreatment of the lysate with an antibody against pSTAT3 showed an increase in intensity of this band (lane 3), corresponding to a 2.15 fold increase over

untreated lysates. IFN-γ is also known to induce STAT3 activation, although not to the same extent as STAT1 activation. As seen in Figure 3.10A (lane 6), lysates from cells treated with IFN-γ also showed formation of a complex with the same migration pattern as that of IL-6 treated cell lysates (a 1.4 fold increase over untreated lysates), and this complex was not competed off by pretreatment of the lysate with an antibody against pSTAT3 suggesting that this complex does not contain STAT3. Finally, lysates from cells co-treated with IL-6 and IFN-γ showed a similar migration pattern to those of IL-6 and IFN-γ treated lysates (corresponding to a 1.37 fold increase over untreated lysates) and this band was also not competed off with an antibody against pSTAT3 (lanes 8 and 7 respectively). These results suggest that a protein complex is formed at IL6RE1 after treatment with IL-6 and IFN-γ, but IFN-γ does not interfere with the formation of this complex and STAT3 does not seem to be a major component of this complex.

The binding of protein complexes was also examined at the site of IL6RE2 (Figure 3.10B). Similarly to IL6RE1, complex formation at IL6RE2 occurred in the presence of lysate from cells treated with IL-6 (lane 4), which was not seen in lysates from untreated control cells (lane 2). This represented a 3.07 fold increase in complex formation at this site compared to untreated lysates. Unlike the complex formed at IL6RE1, formation of one of the complexes (arrow) was inhibited by pretreatment of the lysate with an antibody against pSTAT3, suggesting that STAT3 is a major component of this complex (lane 3). Lysates from cells treated with IFN-γ did not induce formation of this complex (lane 6) (a 1.18 fold change compared to untreated lysates), although addition of an antibody against pSTAT3 did lead to formation of this complex (lane 5). Additionally,

there is an apparent shift of the second complex (star) to a slower migrating complex when IL-6 treated lysates were pretreated with an antibody against pSTAT3, suggesting that this complex may also contain pSTAT3. This shift was also seen in lysates treated with IFN-γ. Finally, lysates from cells co-treated with IL-6 and IFN-γ showed complex formation that resembled both migration patterns of lysates treated with IL-6 and IFN-γ individually (lane 8) and this represented a 2.36 fold increase as compared to untreated lysates. These complexes were partially competed off by pretreatment with an antibody against pSTAT3 (lane 7), suggesting that STAT3 may be a component of these complexes.

The most interesting finding from this set of EMSA experiments were those of complex formation at IL6RE3, the IL-6 response element closest to the transcriptional start site of the γ gene and closest to the putative GAS (Figure 3.4). As seen in Figure 3.10C, lysates from cells treated with IL-6 formed two complexes (lane 3), one of which was not seen in untreated control cells (arrow) and one which was enhanced slightly (1.14 fold) over untreated control cells (star). Both of these complexes were competed off by pretreatment of the lysates with an antibody against pSTAT3 (lane 2), suggesting that STAT3 is a major component of these complexes. Neither of these complexes were formed at IL6RE3 when IFN-γ treated lysates were used (lane 5). Most importantly though, these complexes were formed to a lesser extent when using lysates from cells co-treated with IL-6 and IFN-γ (lane 7), which corresponded to a 2 fold decrease in formation of this complex. These results suggest that IFN-γ treatment is interfering with formation of the IL-6 induced complexes which contain pSTAT3.

Binding of Phosphorylated STAT1 to IL-6 Response Elements Within the Fibriongen γ Promoter- In order to determine whether pSTAT1, induced by IFN-γ, may be interfering with STAT3 function by binding to the IL6REs within the γ chain promoter, especially IL6RE3 as described above, EMSA was performed using labeled oligonucleotides containing each of the three IL6REs along with an antibody against pSTAT1. As seen in Figure 3.11, complex formation at all three IL6REs was seen when lysates from cells co-treated with IL-6 and IFN-γ were used (lanes 2, 4 and 6). However, only the complex formation at IL6RE3 and, to a lesser extent, IL6RE1 was inhibited by pretreatment of the lysates with an antibody against pSTAT1 (lanes 3, 5 and 7). These results imply that pSTAT1 is able to bind to IL6RE1,and more importantly, IL6RE3 where it can inhibit binding of pSTAT3 to the site, thus inhibiting pSTAT3 function.

Fig 3.1

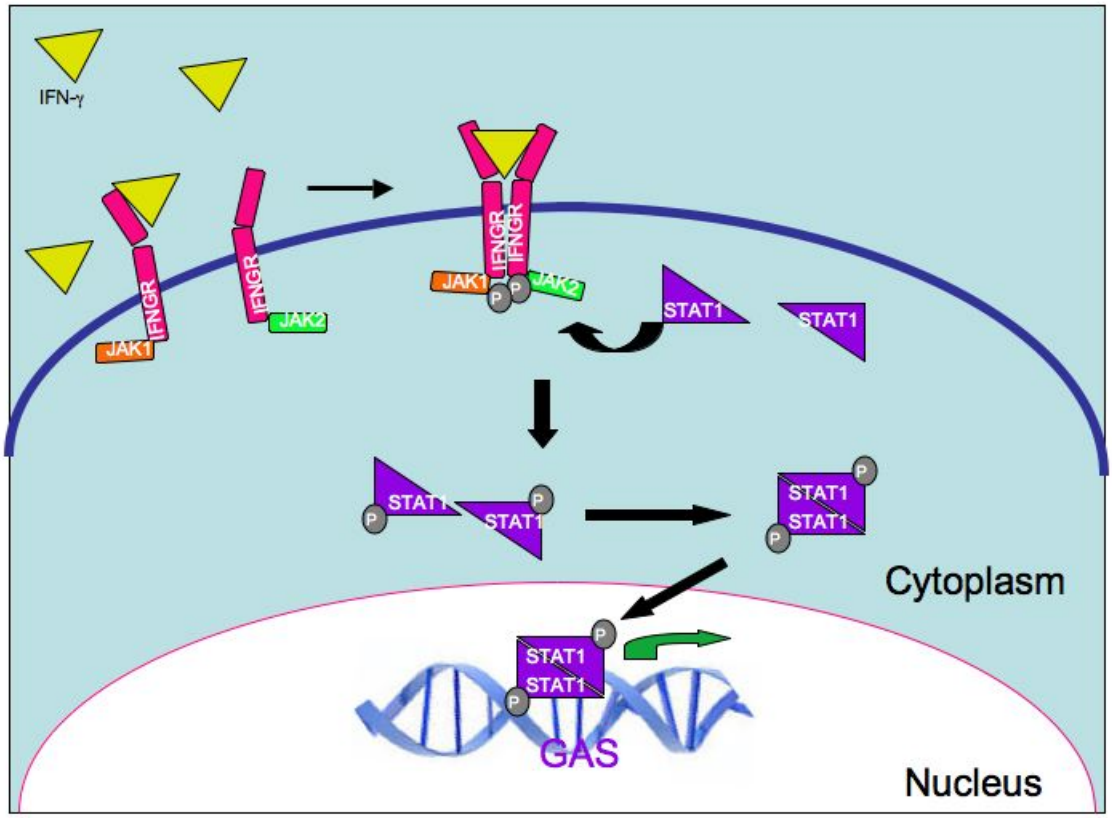


Figure 3.1: Interferon-γ Signaling Pathway IFN-γ binds to its cell surface receptor, IFNGR, and causes dimerization of the receptor. JAK1/2, which are constitutively associated with the receptor, phosphorylate the IFNGR and autophosphorylate each other. Phosphorylation of IFNGR leads to the recruitment of STAT1 to the receptor, where it is phosphorylated by JAK1/2. Phosphorylated STAT1 then homodimerizes and translocates to the nucleus where it binds to GAS elements within the promoters of IFN-γ responsive genes.

Fig. 3.2A

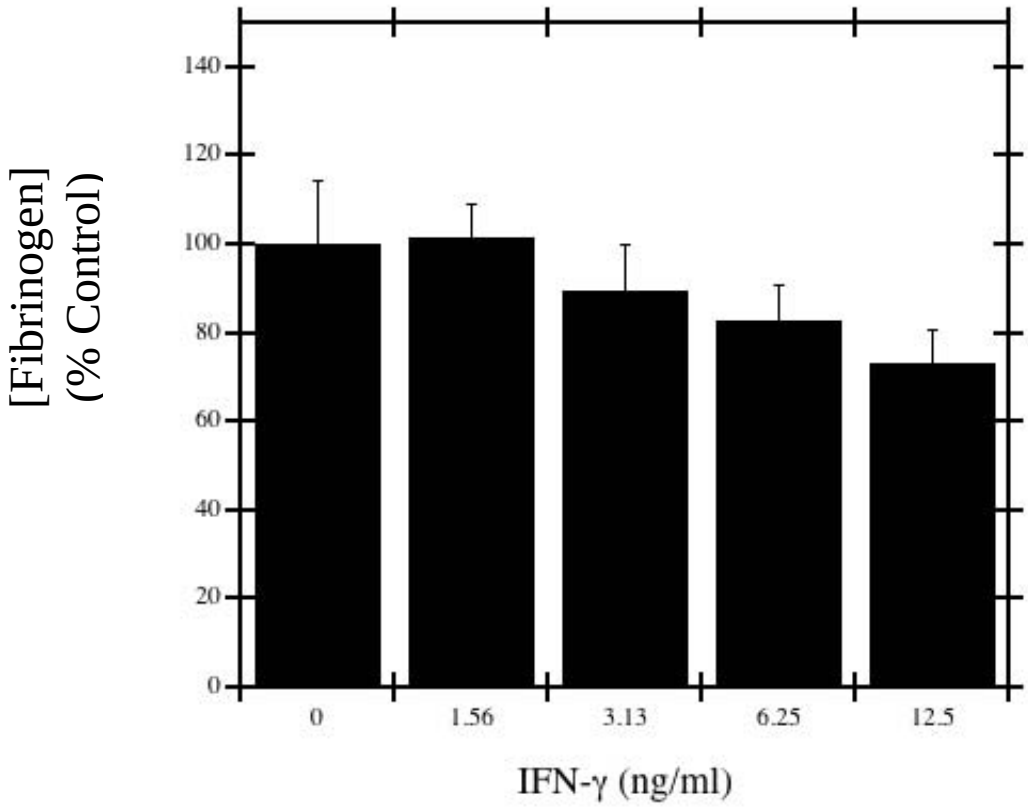

[Fibrinogen]
(% Control)
140
120
100
80
60
40
20
0
0
1.56
3.13
6.25
12.5
IFN-γ (ng/ml)

Fig. 3.2B

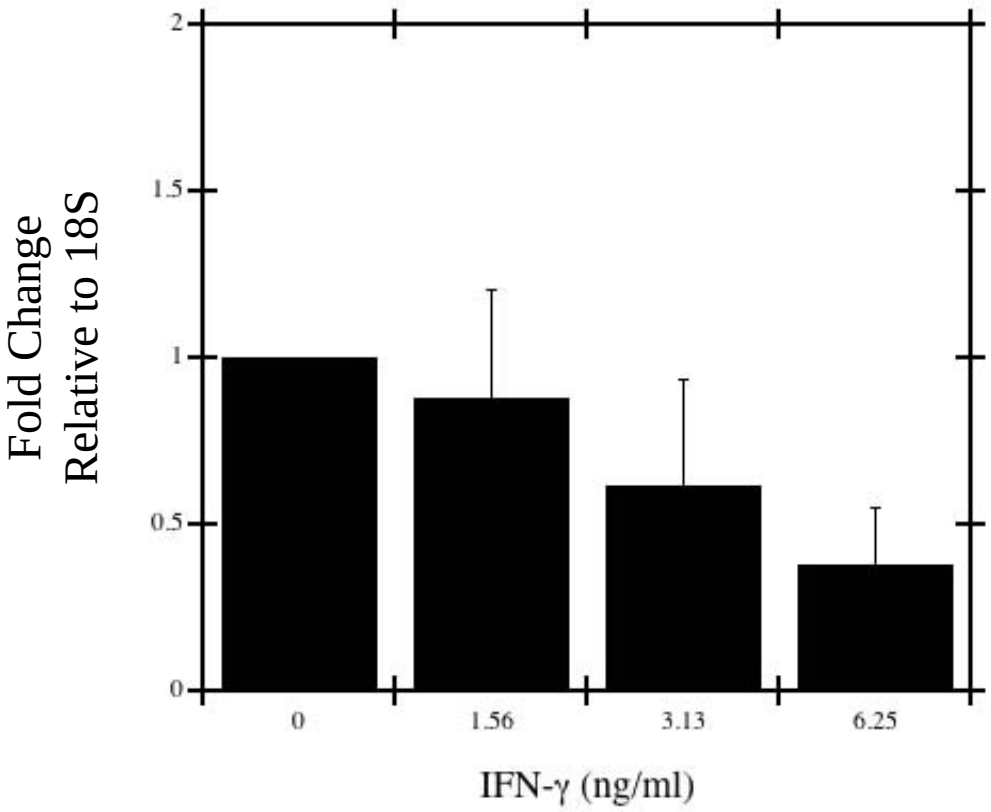


Figure 3.2: Characterization of the Effects of IFN-γ on Basal Fibrinogen Production in HepG2 Cells. HepG2 cells were grown to confluence and incubated with medium containing increasing concentrations of IFN-γ for 24 hours. A) Fibrinogen levels were assayed by ELISA and the results normalized to the amount of total protein from whole cell lysates. B) Fibrinogen mRNA harvested from IFN-γ treated cells was quantitated using qRT-PCR and normalized to 18S mRNA. There was no statistically significant difference between untreated samples and any IFN-γ treated samples at the protein or mRNA level as analyzed by Mann-Whitney tests.

Fig. 3.3A

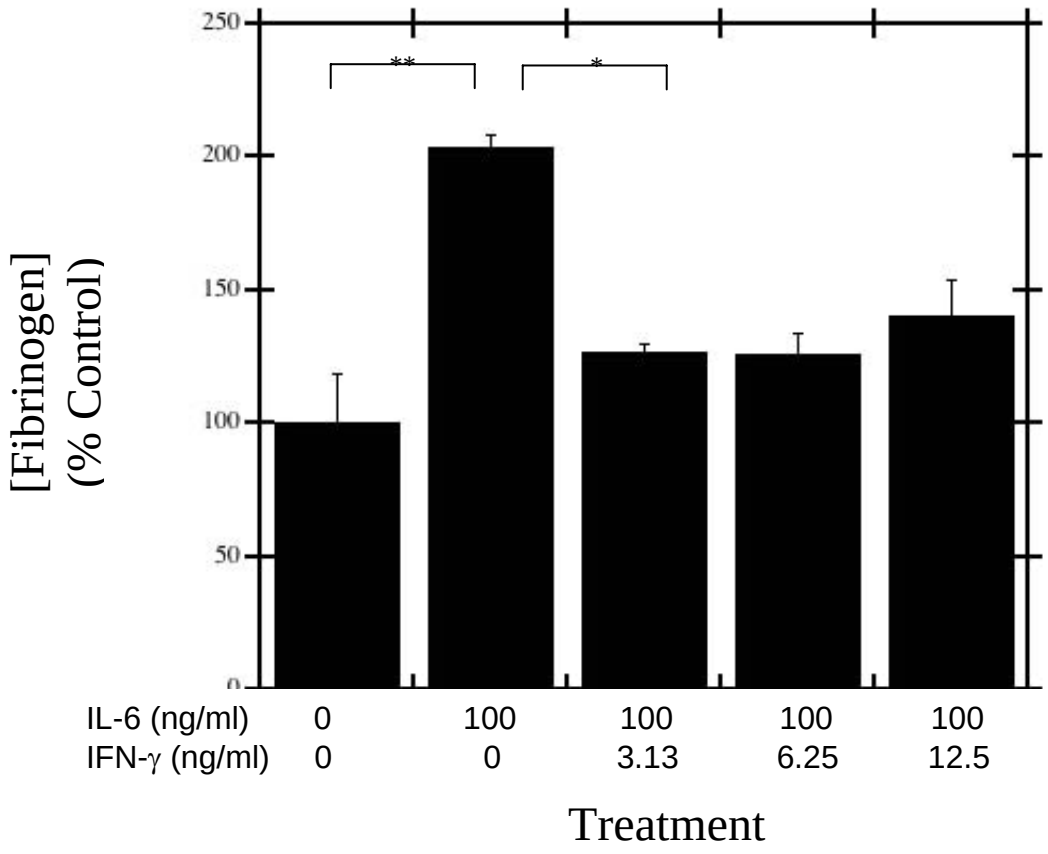

[Fibrinogen]
(% Control)
250
200
150
100
50
0
**
*
IL-6 (ng/ml) 0 100 100 100 100
IFN-γ (ng/ml) 0 0 3.13 6.25 12.5
Treatment

Fig. 3.3B

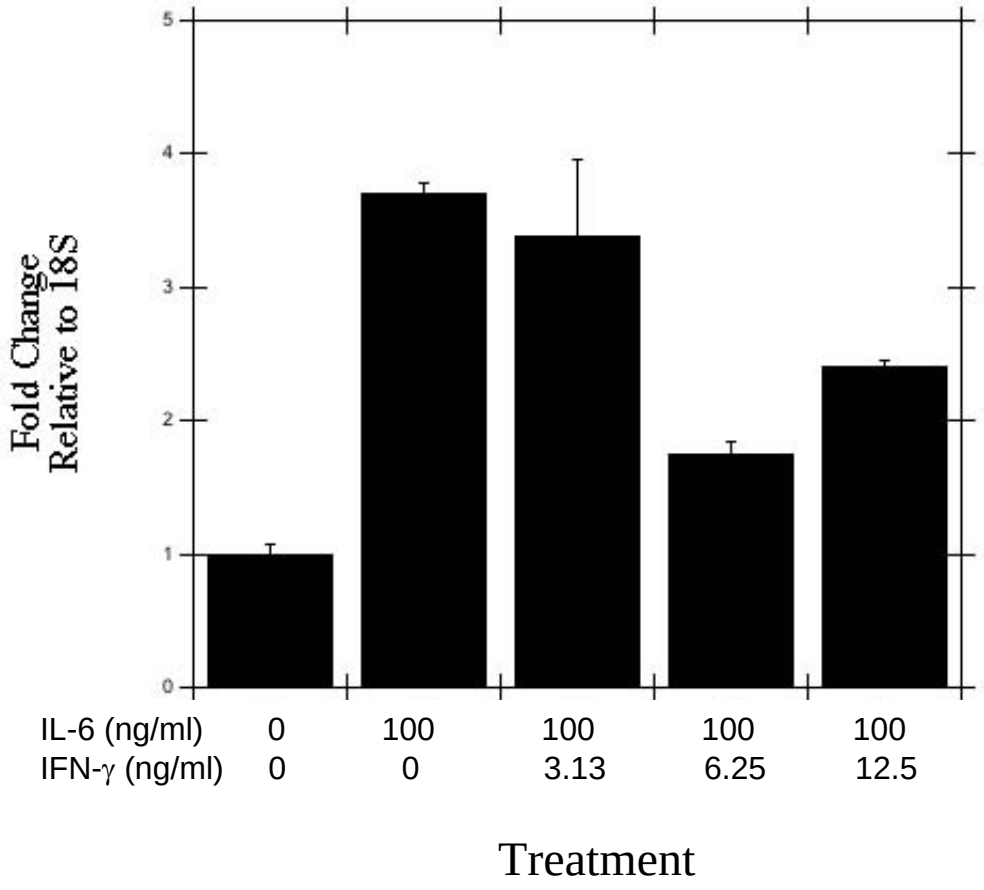


Figure 3.3: The effects of IFN-γ on IL-6 induced fibrinogen synthesis.

HepG2 cells were treated with 100 ng/ml recombinant IL-6 in the presence or absence of increasing concentrations of IFN-γ. A) Fibrinogen levels were quantitated using ELISA and normalized to total cellular protein. **$p<0.01$ when comparing IL-6 to untreated, and *$p<0.05$ when comparing IFN-γ treated to IL-6 treated, by Mann-Whitney test. B) Total fibrinogen mRNA was quantified by qRT-PCR. Fibrinogen mRNA was normalized to 18S mRNA.

Fig. 3.4A

Consensus IFN-γ Activated Sequence (GAS):

TTNCNNNAA

Fbg Gene Promoters:

α: -250 accctgTTCCTGGAAtgtgaga -222

β: -130 aagatgTTGCTTAAAtgataaaat-105

γ: -35 aggagcTTACATAAAgggacaa -9

Fig 3.4B

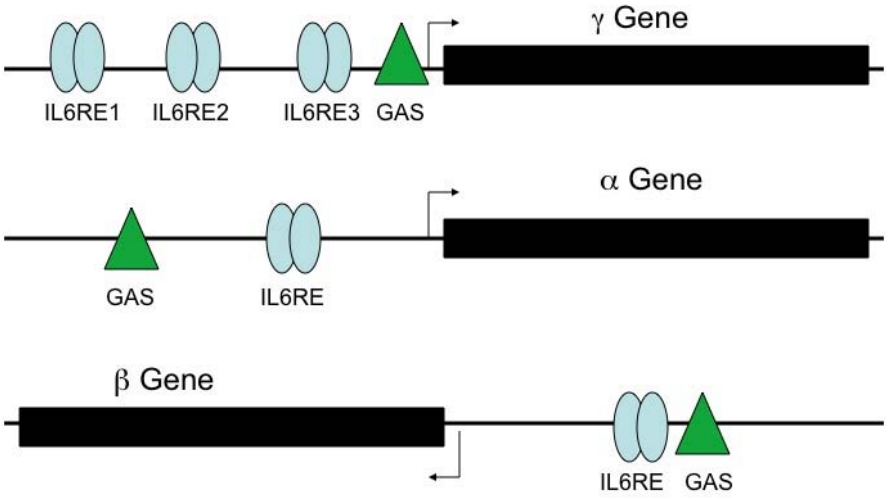


Figure 3.4: All three fibrinogen gene promoters contain IFN-γ regulatory sequences. A) The consensus GAS sequence is shown alongside the putative GAS sequences located within the fibrinogen gene promoters. B) Diagram of the 600 bp upstream of the fibrinogen promoter transcriptional start sites showing the positions of the putative GAS sequence (triangle) in relation to known IL-6 response elements (STAT3 binding sites-ovals).

Fig. 3.5A

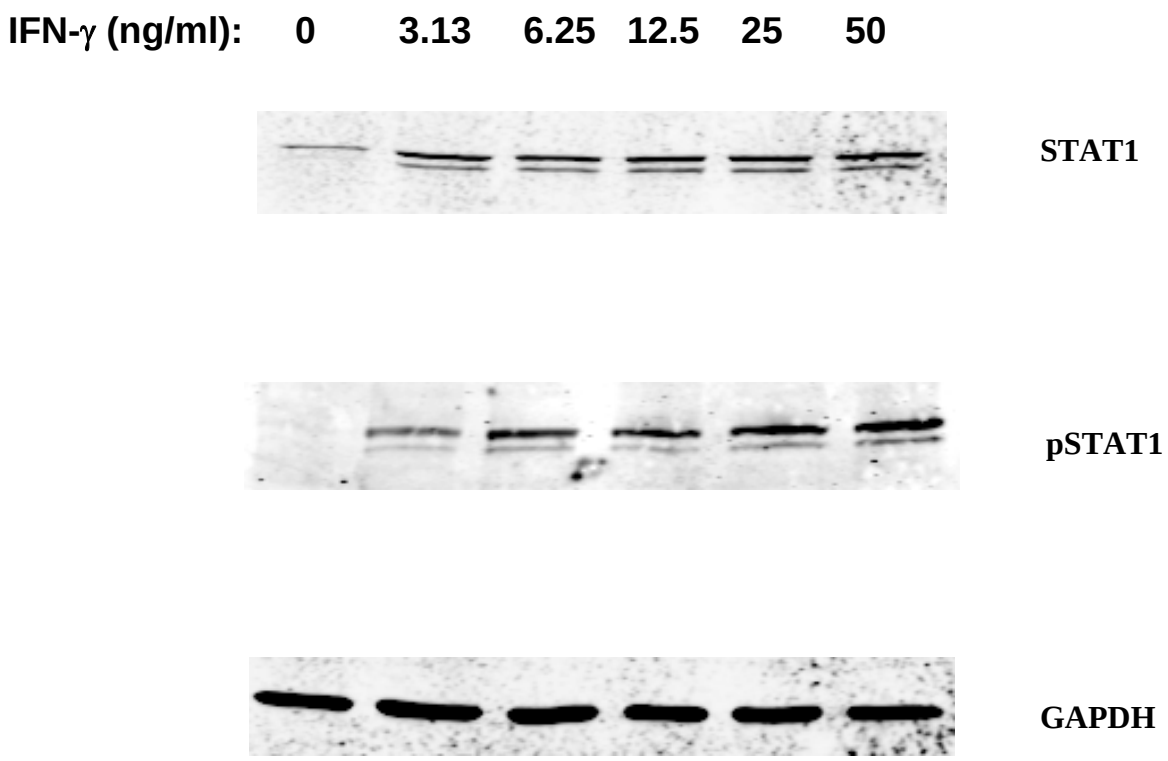


Fig. 3.5B

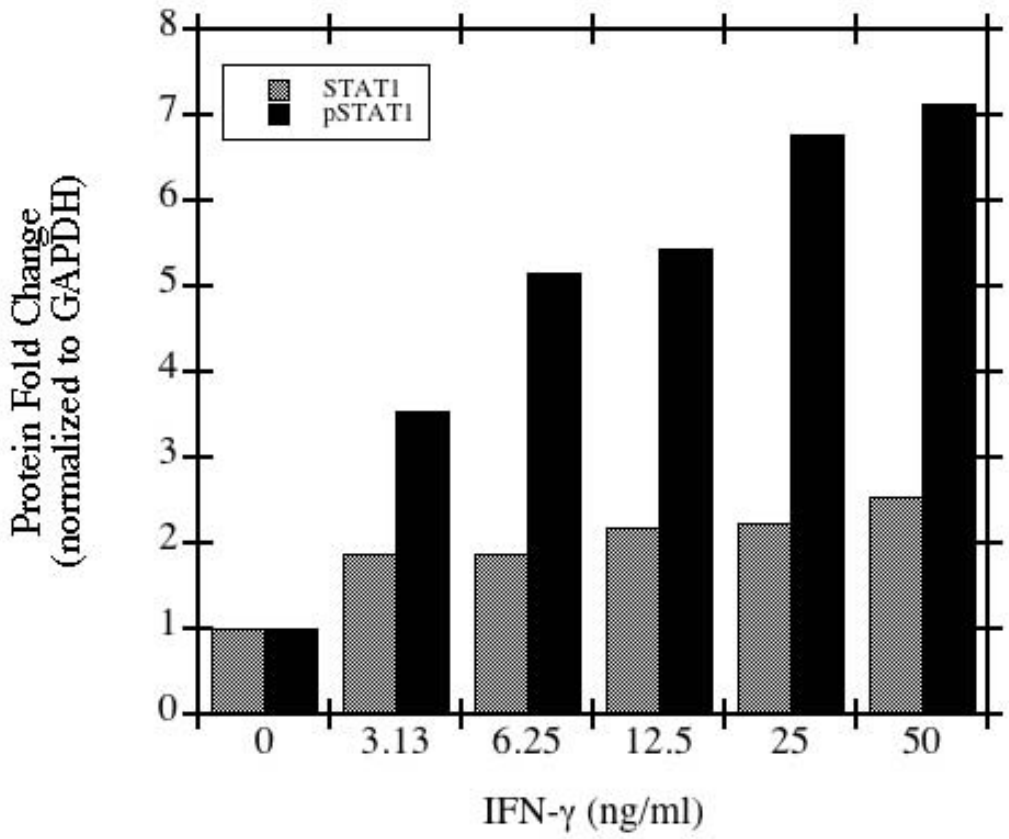


Figure 3.5: Effect of treatment with IFN-γ on phosphorylation of STAT1 at Tyr 701 in HepG2 cells. HepG2 cells were treated with increasing concentrations of IFN-γ and incubated for 24 hours. A) Cells were lysed and equivalent amounts of protein were resolved on a 10% SDS-PAGE gel. Proteins were transferred onto nitrocellulose membranes and probed using antibodies against STAT1 and GAPDH or pSTAT1 (Tyr 701). B) Quantification of changes in protein level by band desitometry. STAT1 and pSTAT1 (Tyr701) levels were normalized to GAPDH and compared to untreated cells.

Fig. 3.6

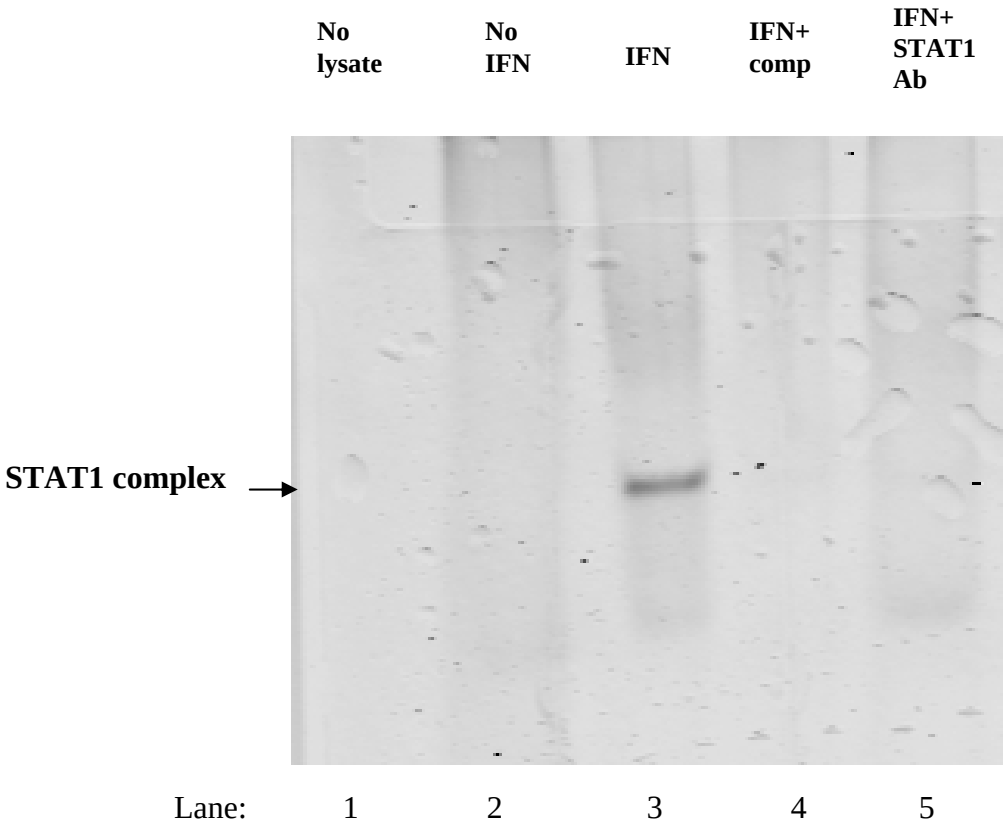


Figure 3.6: Treatment with IFN-γ results in binding of STAT1 to the human fibrinogen γ-chain promoter. HepG2 cells were treated with 25 ng/ml IFN-γ for 4 hours. Nuclear extracts prepared from these cells were used in a binding reaction with IR700 labeled oligos containing the γ-chain GAS sequence. Specificity of STAT1 binding was tested by including 200X nonlabeled oligo (lane 4) and by antibody binding assays with antibodies against STAT1 (lane 5).

Fig. 3.7

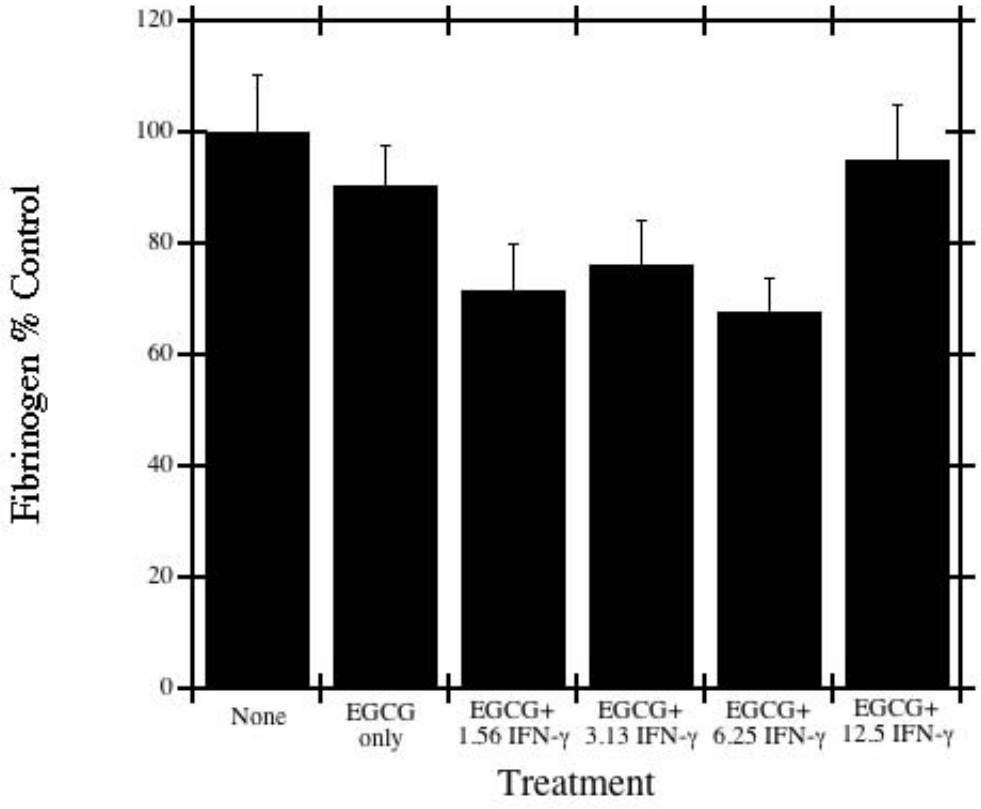


Figure 3.7: Effects of the STAT1 inhibitor EGCG on fibrinogen production in the presence of IFN-γ. HepG2 cells were pretreated with 5 μM EGCG, a specific STAT1 inhibitor, for 30 minutes prior to IFN-γ treatment. Fibrinogen levels were quantitated using ELISA and normalized to total cellular protein. Significance measurements were tested using Mann-Whitney tests.

Fig. 3.8

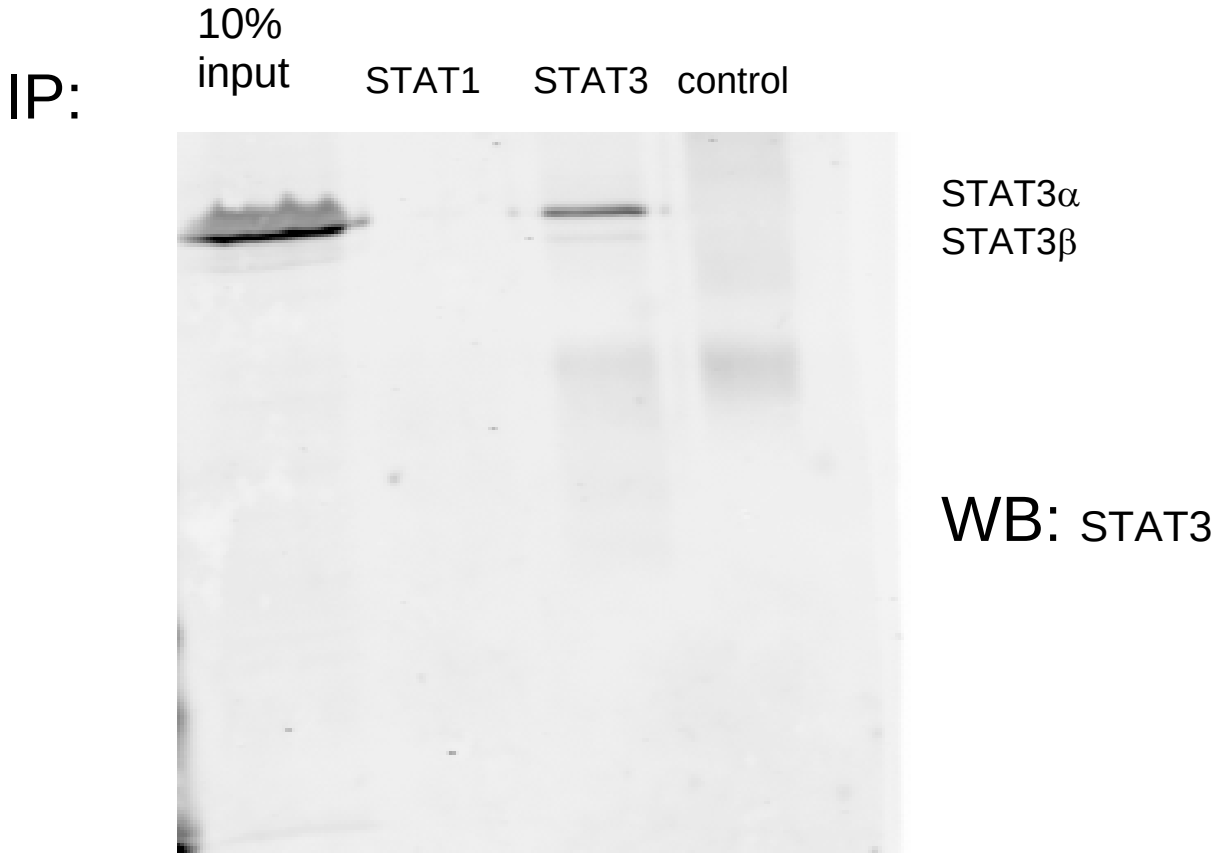
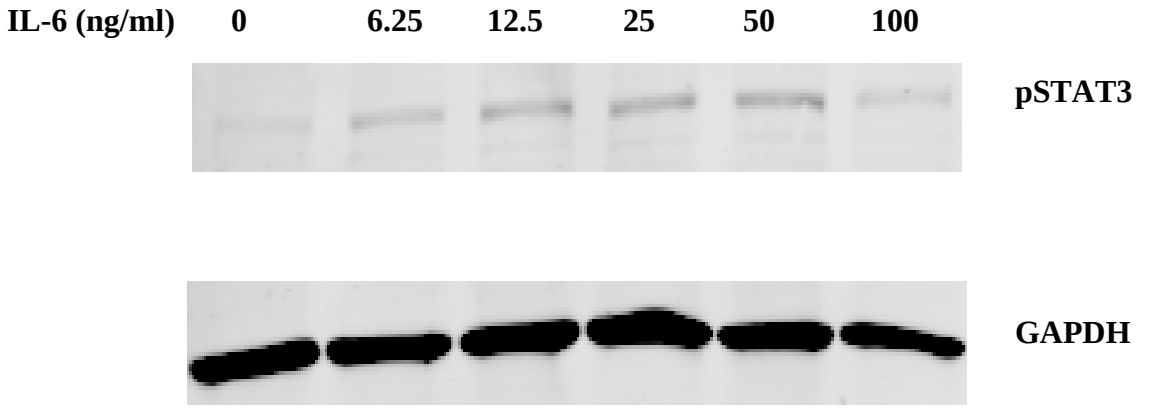


Figure 3.8: Investigation of STAT1 and STAT3 heterodimer formation in HepG2 cells treated with IFN-γ and IL-6. STAT1 and STAT3 were immunoprecipitated from HepG2 cells treated with 100 ng/ml IL-6 and 25 ng/ml IFN-γ and Western blotted using an antibody against STAT3.

Fig 3.9A


IL-6 (ng/ml) 0 6.25 12.5 25 50 100
pSTAT3
GAPDH

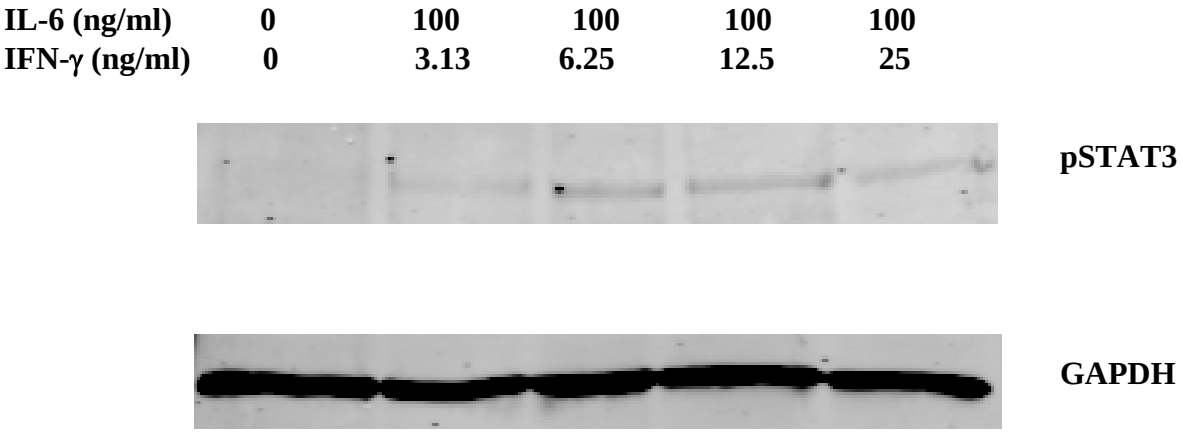

IL-6 (ng/ml) 0 100 100 100 100
IFN-γ (ng/ml) 0 3.13 6.25 12.5 25
pSTAT3
GAPDH

Fig. 3.9B

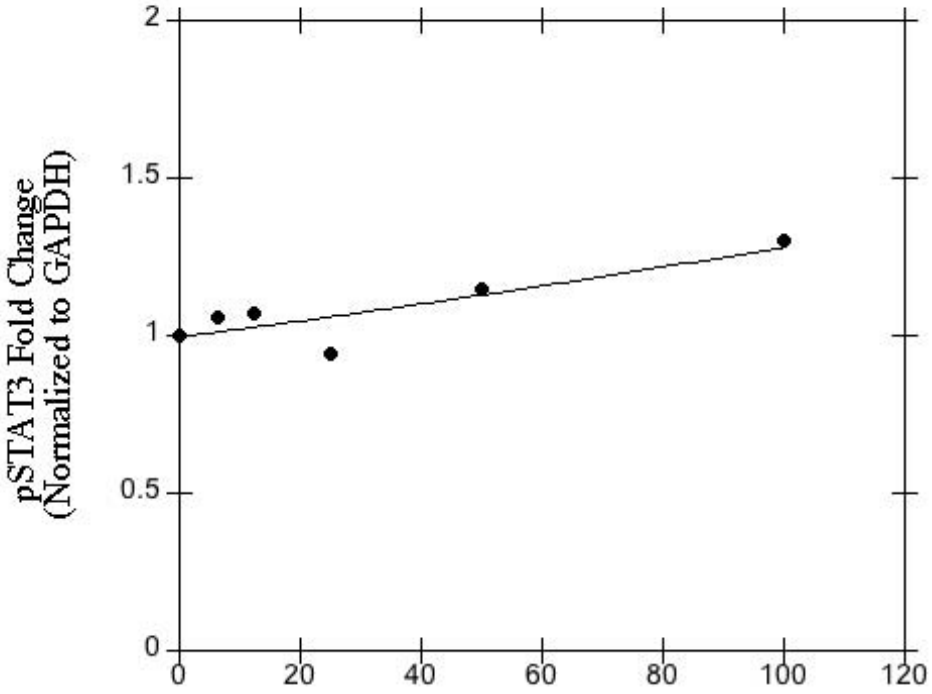

pSTAT3 Fold Change
(Normalized to GAPDH)
2
1.5
1
0.5
0
0
20
40
60
80
100
120
IL-6
(ng/ml)

Fig 3.9C

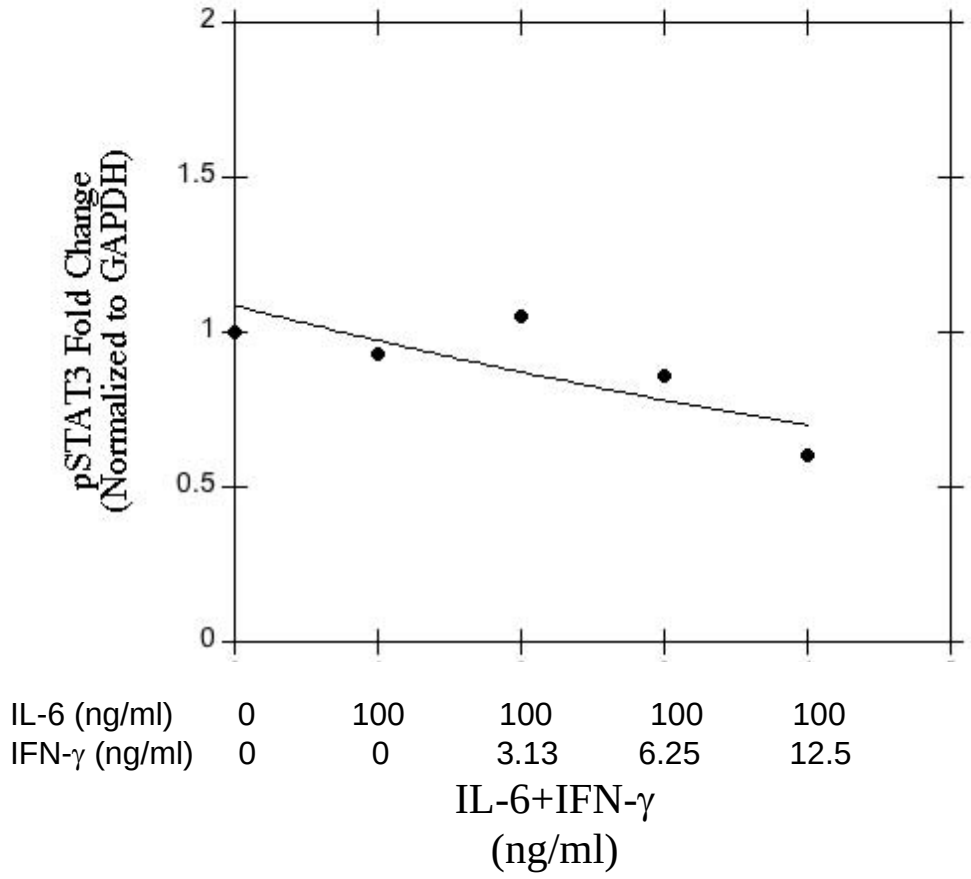


Figure 3.9: Characterization of STAT3 phosphorylation at Tyr 705 in response to IL-6 alone and IL-6/ IFN-γ co-treatment. HepG2 cells were treated with 0-100 ng/ml IL-6 (top) or 100 ng/ml IL-6 and 0-25 ng/ml IFN-γ (bottom). A) Cells were lysed and equivalent amounts of protein were resolved on a 10% SDS-PAGE gel. Proteins were transferred onto nitrocellulose membranes and probed using antibodies against pSTAT3 (Tyr705) and GAPDH. B) Quantification of changes in protein level by band desitometry. pSTAT3 (Tyr705) levels were normalized to GAPDH and compared to untreated cells.

Fig. 3.10A

Lane:	1	2	3	4	5	6	7	8
Lysate:	-	no treatment	IL6	IL6	IFN-γ	IFN-γ	IL6+ IFN-γ	IL6+ IFN-γ
IL6RE1:	+	+	+	+	+	+	+	+
pSTAT3 Ab:	-	-	+	-	+	-	+	-

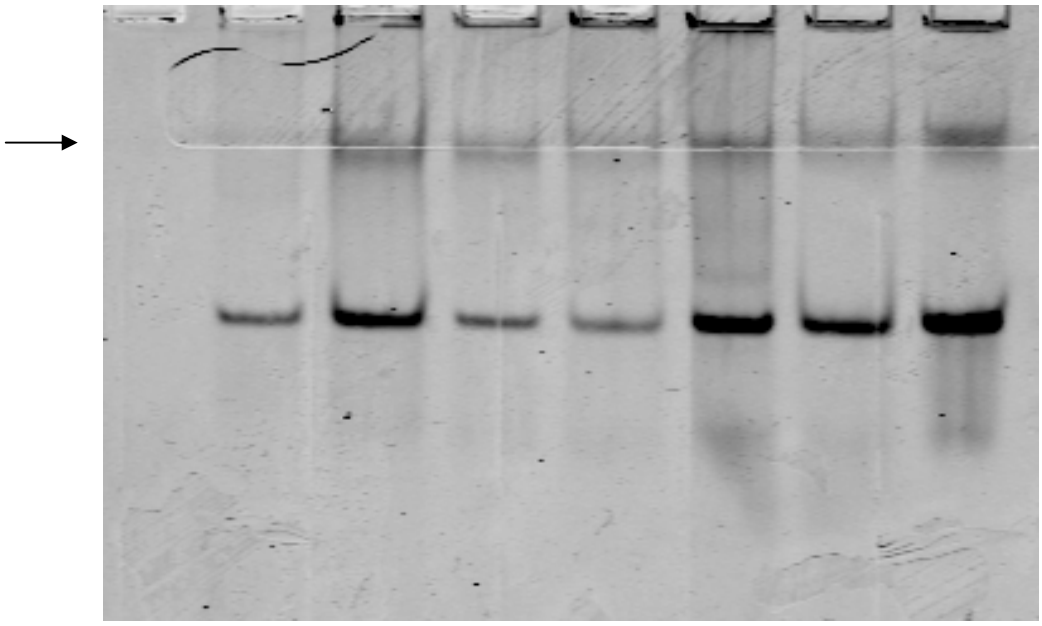


Fig 3.10B

Lane:	1	2	3	4	5	6	7	8
Lysate:	-	no treatment	IL6	IL6	IFN-γ	IFN-γ	IL6+ IFN-γ	IL6+ IFN-γ
IL6RE2:	+	+	+	+	+	+	+	+
pSTAT3 Ab:	-	-	+	-	+	-	+	-

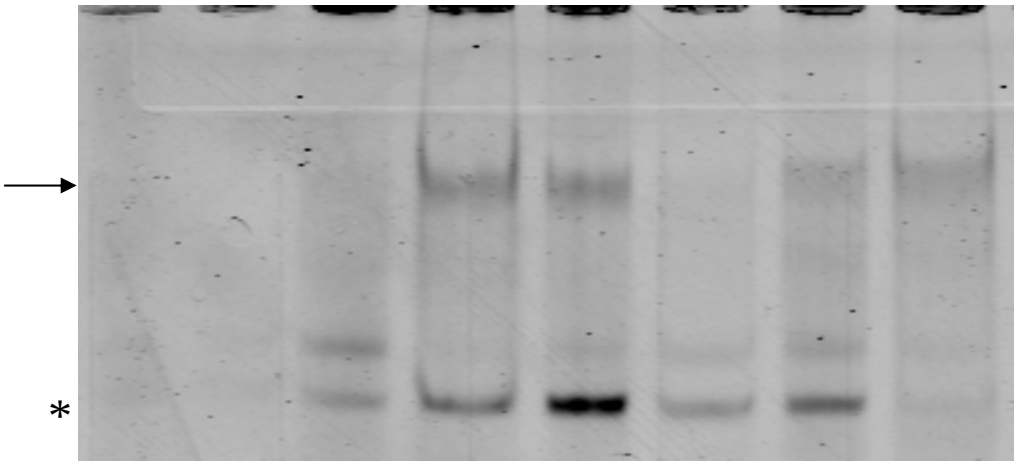


Fig. 3.10C

Lane:	1	2	3	4	5	6	7
Lysate:	no treatment	IL6	IL6	IFN-γ	IFN-γ	IL6+ IFN-γ	IL6+ IFN-γ
IL6RE3:	+	+	+	+	+	+	+
pSTAT3 Ab:	-	+	-	+	-	+	-

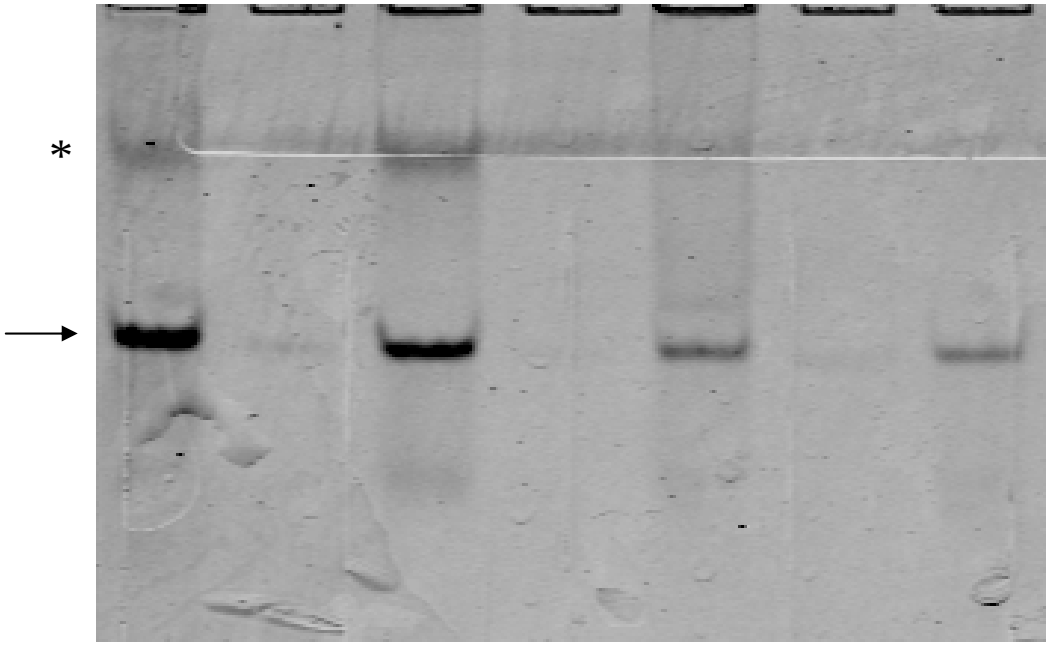


Figure 3.10: Investigation of STAT3 binding to IL-6 response elements within the fibrinogen γ chain promoter in the presence of IFN-γ and IL-6. HepG2 cells were treated with IL-6 (100 ng/ml), IFN-γ (25 ng/ml) or a combination of the two. Cell lysates were harvested and used in EMSAs with labeled oligonucleotides containing each of the IL-6 response elements of the fibrinogen γ gene promoter. An antibody against pSTAT3 was used for antibody binding. Treatment with IFN-γ and IL-6 does not significantly alter the binding of STAT3 to IL6RE1 (A) or IL6RE2 (B) as compared to treatment with IL-6 alone . Treatment with IFN-γ and IL-6 decreases the formation of two STAT3 protein complexes at IL6RE3.

Fig. 3.11

Lane:	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Lysate:	**no treatment**	**IL6+ IFN-γ**	**IL6+ IFN-γ**	**IL6+ IFN-γ**	**IL6+ IFN-γ**	**IL6+ IFN-γ**	**IL6+ IFN-γ**
IL6RE:	**1**	**1**	**1**	**2**	**2**	**3**	**3**
pSTAT1Ab:	**-**	**-**	**+**	**-**	**+**	**-**	**+**

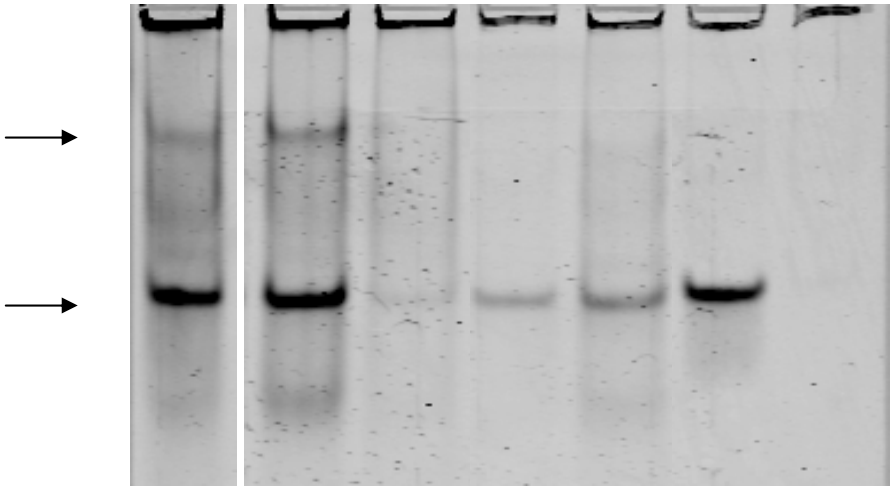


Figure 3.11: Binding of phosphorylated STAT1 to IL-6 response elements within the fibrinogen γ gene promoter. HepG2 cells were treated with a combination of IL-6 and IFN-γ (25 ng/ml). Cell lysates were harvested and used in EMSAs with labeled oligonucleotides containing each of the IL-6 response elements of the fibrinogen γ gene promoter. An antibody against pSTAT1 was used for antibody binding.

3.4 Discussion

IFN-γ is a key player in the regulation of immune and inflammatory responses and is one of several important cytokines that regulate the induction and duration of the acute phase response. Being that fibrinogen is an acute phase reactant, this study sought to determine the effects of IFN-γ on fibrinogen production in HepG2 hepatocellular carcinoma cells. We have shown that IFN-γ slightly downregulates the production of fibrinogen in HepG2 cells in response to IL-6 (Figure 3.3A). These results may explain an *in vivo* study in which cancer patients treated with IFN-γ prior to limb perfusion had lower fibrinogen levels before and during perfusion as compared to those who did not receive IFN-γ treatment (60). In contrast, a study looking at the effects of cytokines on fibrinogen production in the context of alveolar inflammation in A549 human alveolar epithelial cells did not show a statistically significant difference in the fibrinogen produced by these cells following IFN-γ treatment, although there did seem to be a decrease in fibrinogen production (61). Since the lung is not a significant source of circulating fibrinogen, the relevance of these data on the levels of circulating fibrinogen is questionable. IFN-γ is known to downregulate the expression of several other proteins, including the stem cell factor (SCF) receptor (62), NHE 2 and 3 which are involved in inflammation in inflammatory bowel disease (63) and type I collagen (64). The latter was shown to occur through an IFN-γ mediated increase in pSTAT1 at the Col1A2 promoter, similar to the model proposed here.

IFN-γ signaling is a well-defined pathway that is distinct from the IFN-α and IFN-β signaling pathways. IFN-γ activation of the IFNGR leads to phosphorylation of the

receptor by the constitutively associated Janus Kinase (JAK) 1 and 2. This phosphorylation leads to the recruitment of STAT1 molecules to the receptor where they are phosphorylated on Tyr 701. This phosphorylation event allows for homodimerization of STAT1 molecules followed by their translocation to the nucleus where they bind to GAS within IFN-γ responsive genes. We have identified a novel GAS sequence within the fibrinogen γ promoter at base pairs -29 to -21, very close the transcriptional start site (Figure 3.4). We have shown that treatment of HepG2 cells with IFN-γ leads to a dose responsive increase in the amount of phosphorylated STAT1 within the cells (Figure 3.5). We also show that the novel GAS sequence is functionally active as nuclear phosphorylated STAT1 is able to bind to the GAS sequence within the fibrinogen γ promoter (Figure 3.6). This binding is sequence specific in that competition with excess unlabeled oligonucleotides containing the novel GAS sequence is able to compete for STAT1 binding. Additionally, pretreatment of IFN-γ stimulated nuclear lysates with an antibody against STAT1 abrogates the interaction between STAT1 and the GAS sequence, showing that STAT1 is the molecule binding to the novel GAS sequence (Figure 3.6). Pharmacological inhibition of STAT1 using the specific STAT1 inhibitor EGCG did not show complete inhibition of IFN-γ responsiveness (Fig. 3.7), which can most likely be attributed to the fact that higher doses are required for complete inhibition. However, at higher EGCG concentrations we see a decrease in fibrinogen levels when cells are treated with EGCG alone, which would confound our results. Hence we were forced to use lower concentrations that do not fully inhibit the phosphorylation of STAT1 (data not shown), which most likely accounts for the incomplete inhibition of IFN-γ responsiveness in HepG2 cells.

We also saw a significant inhibitory effect of IFN-γ on the IL-6 induced increase in fibrinogen production in our cells (Figure 3.3). IL-6 is a known upregulator of fibrinogen synthesis and the fibrinogen γ promoter contains three IL-6 responsive STAT3 binding sites (53). Inhibition of IL-6 induced fibrinogen synthesis by other inflammatory cytokines has been shown, particularly in the case of IL-1β. IL-1β mediated NF-κB activation was shown to block the binding of STAT3 to the IL-6 responsive STAT3 binding sites within the rat fibrinogen γ promoter (65). Therefore, it is possible that activation of STAT1 may lead to inhibition of STAT3 binding to the IL-6 response elements within the human fibrinogen γ chain promoter by binding to and sequestering STAT3 away from the promoter. However, we do not see an association of STAT1 with STAT3 in HepG2 cells by immunoprecipitation when co-stimulated with IL-6 and IFN-γ (Figure 3.8). Alternatively, there is the possibility that IFN-γ induced STAT1 activation may lead to an inhibition of STAT3 phosphorylation and consequent activation and nuclear localization by activating Supressor of Cytokine Signaling (SOCS) family members. SOCS1 and SOCS3 are known to be activated by IFN-γ in macrophages and lead to subsequent inhibition of STAT3 phosphorylation and activation (66). Phosphorylation of STAT3 at Tyr705 is known to be required for the binding of STAT3 to IL-6 response elements within target gene promoters, whereas phosphorylation at Ser727 is required for full STAT3 mediated transcriptional regulation. Upon stimulation with IL-6, we see an increase in STAT3 phosphorylation at Tyr705, which is not affected by the addition of IFN-γ (Figure 3.9), suggesting that the addition of IFN-γ is not interfering with phosphorylation of STAT3. However, we do see a change in formation

of a protein complex at IL6RE3, the γ chain IL-6 response element closest to the putative GAS, by EMSA (Figure 3.10). IL6RE3 showed a specific band following IL-6 treatment that was not seen in either IFN-γ treatment or IFN-γ and IL-6 co-treatment. Additionally, the IL-6 induced formation of this complex was inhibited by pre-incubation of the lysate with an antibody against pSTAT3, suggesting that STAT3 is a major component of the complex forming at IL6RE3 following IL-6 treatment. Furthermore, a complex that is formed at IL6RE3 in the presence of IL-6 and IFN-γ (Figure 3.11) is fully inhibited by pretreatment of the lysate with an antibody against pSTAT1. These results provide a mechanism by which pSTAT1 is able to bind to IL6RE3, inhibiting the binding of pSTAT3 to this region, thus inhibiting the full function of pSTAT3 to induce transcription at the fibrinogen γ promoter in the presence of pSTAT1. This would explain the inhibition of complex formation seen in lysates treated with IL-6 and IFN-γ that was seen upon treatment with IL-6 alone (Figure 3.10). This finding is particularly interesting as previous studies have shown that it is the second-furthest IL-6 response element, IL6RE2, that is the major IL-6 responsive site, and that the other flanking IL-6 response elements are less responsive, but still contribute to full γ gene promoter activity in response to IL-6 (53).

Overall, these studies identify IFN-γ as a novel regulator of IL-6 induced fibrinogen production in the liver. Here we have provided a model for this regulation, in which STAT1 is phosphorylated and activated in HepG2 cells following treatment with IFN-γ. pSTAT1 is able to bind to a novel GAS element within in the fibrinogen γ gene promoter and inhibits basal levels of fibrinogen expression via downregulation of transcription of

the γ chain mRNA. Additionally, pSTAT1 is also able to bind to IL-6 response elements within the γ gene promoter, namely IL6RE3 that resides only 115 base pairs upstream from the novel GAS element identified here. Binding of pSTAT1 to IL6RE3 may inhibit the binding of pSTAT3 to this site, which leads to a decrease in the ability of IL-6 mediated signaling to fully induce transcription at the fibrinogen γ gene promoter. This mechanism would clarify why we saw an incomplete inhibition of IL-6 mediated fibrinogen induction by IFN-γ . IFN-γ was not able to fully inhibit IL-6 mediated fibrinogen expression probably due to the fact that the other two IL-6 response elements, IL6RE1 and, to a greater extent, IL6RE2, did not show significant STAT1 binding and were still responsive to STAT3.

Investigation into the mechanism of IFN-γ mediated inhibition of IL-6 induced fibrinogen production may lead to the discovery of novel interactions between IFN-γ and IL-6 signaling pathways and will further our knowledge of how fibrinogen is regulated. Additionally, the identification of IFN-γ as a downregulator of basal fibrinogen synthesis may lead to the future development of a potential therapeutic molecule for patients with high fibrinogen levels to lower their risk of cardiovascular disease.

Chapter 4: Fibrin Degradation Products D-dimer and D-fragment Regulate Fibrinogen Expression

4.1 Introduction

Persistence of cross-linked fibrin is frequently seen during the development of atherosclerosis and thrombotic disease. Much like the formation of a fibrin clot, a complex cascade of events leads to the activation of enzymes capable of breaking down the cross-linked fibrin. The major enzyme responsible for fibrinolysis is plasmin, which is cleaved from its inactive zymogen plasminogen by tPA or uPA. The interaction of plasmin with cross linked fibrin at sites located within the C-terminal D-domains of the fibrin molecules leads to the formation of several major fibrin degradation products (FDPs), as described in Chapter 1. In this study we focus on the fibrin degradation product D-dimer, a 190 kDa molecule consisting of two cross linked D-domains, which can sometimes be non-covalently linked to an E domain (Figure 4.1).

D-dimer is considered to be an independent marker for thrombotic disease and is also correlated with an increased risk of severe atherosclerosis and vascular complications (67). A recent study using microarray technology to look at the effects of D-dimer on macrophage function showed that 27 macrophage genes were up regulated greater than two-fold over controls following D-dimer treatment, suggesting that D-dimers are capable of affecting the transcription of responsive genes (68). Additionally, a separate study showed that D-dimers are capable of inducing transcription of plasminogen activator inhibitor I (PAI-I) by increasing the binding activity of the transcription factor

AP-1 components c-fos/junD to an AP-1 like element in the promoter region (69). PAI-I modulates fibrinolysis by inactivating plasminogen, the zymogen precursor of the major clot breakdown enzyme plasmin. D-dimers are able to induce the transcription of PAI-I and therefore decrease the production of active plasmin, leading to a decrease in fibrinolytic activity and subsequent persistence of fibrin clots.

Studies of plasmin derived fibrin breakdown products in mice have shown that clearance of D-dimers and D-fragments from the circulation is performed mainly by the liver, in particular the hepatocytes of the liver. In a study examining I^{125}-labeled fibrin degradation products including D fragment, E fragment and D-dimer, Pizzo and Pasqua (70) showed that D-dimer and D fragment are cleared from the circulation almost identically, and that this uptake may be receptor-mediated. Investigation into whether the carbohydrate side chains of fibrinogen lead to receptor-mediated endocytosis via the galactose or mannose receptor showed that neither of these were responsible for the uptake of D-dimer or D fragment by hepatocytes. However, uptake of the D-dimers into the liver is suggested to occur via a receptor-mediated event *in vivo* as well, although the receptor has yet to be identified (70). We hypothesized that the fibrin degradation product D-dimer is capable of binding to the surface of hepatocytes and becomes endocytosed through a receptor specific for D-dimer. Once inside the cell, the D-dimers may be capable of feeding back to regulate synthesis of total and γ' fibrinogen, which may alter one's susceptibility to thrombosis.

The interaction of D-dimers with membrane associated proteins or intracellular proteins and possible signaling mechanisms in the cell will not only provide insight into factors and mechanisms governing the clearance of fibrin degradation products, but may also serve to elucidate novel therapeutic targets in the fight against cardiovascular disease.

4.2 Materials and Methods

Reagents- Human fibrinogen was purchased from George King Biomedical. Human thrombin, factor XIII and plasmin were purchased from Haematologic Technologies. DEAE-cellulose was purchased from Sigma and Sephacryl 200-HR resin was purchased from GE Healthcare. Rabbit anti-human TLR4 was purchased from Anaspec, Inc., mouse anti-human gC1qR was purchased from Abcam, Inc., and mouse anti-human LRP antibody was purchased from Meridian Life Science, Inc. Mouse anti-human D-dimer antibody was purchased from AbD Serotec. Vybrant Cell Labeling Solution was purchased from Molecular Probes Inc. Vectashield with DAPI fixation medium was purchased from Vector Technologies, Inc. Affigel-15 affinity column resin was purchased from BioRad Technologies Inc.

Clot formation and degradation- 200 mg of human fibrinogen was clotted in the presence of 50 U human thrombin and 20 U FXIIIa overnight at 37^{o}C in 20 mL buffer consisting of 5 mM $CaCl_2$/0.15 M Nacl/0.1 M Tris, pH 7.4. The resulting clot was washed in the above buffer and digested with 20 U plasmin in 20 ml of the above buffer overnight at 37^{o}C. The resulting clot lysis products were dialyzed against 1 mM $CaCl_2$/0.01M Tris, pH 8.6 over a course of 3 days with fresh buffer changes every 6-8 hours. The products were then subjected to chromatography as described below.

DEAE Anion Exchange Chromatography- A DEAE anion exchange column was poured and equilibrated in buffer consisting of 0.1 M Tris, pH 7.4/ 1mM $CaCl_2$ for 5 hours at 4^{o}C. Sample was added to the column and the column was washed overnight in

equilibration buffer at 4°C at a flow rate of 20 ml/hr. Sample was eluted off the column by a 400 ml buffer gradient with the equilibration buffer as starting buffer and 0.1 M Tris, pH 7.4/1 mM $CaCl_2$/0.17 M NaCl as the end buffer. Fractions were collected in 5 ml volumes and absorbance at 280 nm was monitored using a UV monitor. Eluates with A280 readings above the baseline were analyzed by gel electrophoresis on 10% resolving/4% stacking SDS-PAGE gels and stained with Coomassie Blue stain. This chromatography step served to separate out fractions of D-dimers/D-fragment from fractions containing E fragment. Those fractions free of E fragment were pooled and concentrated using aquacide, and dialyzed into 0.1 M Tris, pH 7.4 for further separation on a sizing column.

Sephacryl 200-HR Size Exclusion Chromatography- A Sephacryl 200-HR size exclusion column of 350 ml was poured, packed by gravity flow and equilibrated in 0.1 M Tris, pH 7.4 at 4°C. The column was washed overnight at 4°C in 700 ml of this buffer, and a concentrated 8 ml sample of D-dimer/D fragment (2.2% of the total sizing column volume) was loaded onto the column. The proteins were eluted, based on their stokes radii, in 0.1 M Tris, pH 7.4. The A280 of the samples was measured by spectrophotometry and fractions with A280 above baseline were analyzed on 10% resolving/4% stacking SDS-PAGE gels and stained with Coomassie Blue. Fractions containing mostly or only D-dimers were pooled and run over the sizing column a second time, and again separated on an SDS-PAGE gel and stained with Coomassie Blue. Fractions of pure D-dimers were then pooled and concentrated in aquacide. Fractions

containing pure D-fragments were also pooled, concentrated and run on an SDS-PAGE gel to be used as a control in our experiments.

ELISAs- Cells were treated with increasing concentrations of purified D-dimer for 24 hours and conditioned media were collected. ELISAs for total and γ' fibrinogen were performed as described in the General Methods section in Chapter 2.

qRT-PCR–HepG2 cells were prepared as described above and treated with increasing concentrations of purified D-dimers for 24 hours. qRT-PCR was performed as described in the General Methods section in Chapter 2.

Fluorescein Labeling of Fibrinolytic Fragments- D-dimers and D fragments were labeled with fluorescein using the EZ Label Fluorescein Protein Labeling Kit (Pierce) according to the manufacturers instructions. Concentrated D-dimer and D fragment samples from the sizing column were dialyzed into 50 mM borate, pH 8.5 and were conjugated to N-hydroxysuccinimide fluorescein by addition of a 24 molar-excess of fluorescein. The mixtures were mixed and incubated at room temperature for 1 hour. The samples were then dialyzed into PBS and absorbance at 280nm and 518 nm were used to determine the concentration of labeled protein and the extend to which the protein was labeled, respectively.

Microscopy- Real-time microscopy was performed by the OHSU Microscopy Core within the Department of Molecular Microbiology and Immunology. For real-time

imaging, HepG2 cells were plated onto chambered cover glass slides. Cells were serum starved for 24 hours and treated with 5 μl/ml Vybrant Cell Labeling Solution for 20 minutes at 37^{o}C according to the manufacturer's instructions to label cell membranes. Cells were then treated with 370 nM fluorescein labeled D-dimer or 500 nM fluorescein-labeled BSA for 5 minutes at 37^{o}C, then washed with serum-free MEM to remove remaining labeled proteins. Cells were imaged continuously for 30 minutes using the Applied Precision DeltaVision image restoration system.

For fluorescence microscopy, HepG2 cells were plated onto chambered cover glass slides, serum-starved for 24 hours, and treated with either 10 nM fluoresceinated D-dimer or BSA for up to 60 minutes in a time course experiment. For inhibition studies, cells were pretreated with either a polyclonal antibody against human gC1qR (10 μg/ml), a monoclonal blocking antibody against human TLR4 (10 μg/ml) or receptor associated protein (RAP) (40 μg/ml), an inhibitor of the low-density lipoprotein-related receptor (LRP). Cells were fixed every 10 minutes using Vectashield fixation medium containing 4',6-diamidino-2-phenylindole (DAPI). Cells were then imaged, using an epifluorescence microscope equipped with appropriate filters to visualize fluorescein, in the laboratory of Dr. Allison Fryer. Exposure times were fixed to allow comparison between treatment groups. Exposure limits were chosen to be short enough not to collect any background fluorescence in samples not treated with fluoresceinated protein, and were then kept constant throughout the collection of data.

D-dimer Affinity Column Chromatography and Mass Spectrometry- Purified D-dimers were dialyzed into 0.1M HEPES, pH 7.5 overnight at 4^{o}C. A 5ml Affi-gel 15 affinity column was poured in the above buffer and dialyzed D-dimers were added to the resin solution to create a D-dimer affinity column. Protein was coupled to the resin in a buffer consisting of 100 mM HEPES, pH 7.4. All remaining unoccupied Affi-gel sites were blocked by incubation of the resin solution with 1mM ethanolamine-HCl, pH 8.0 for one hour at room temperature with shaking. The column was then washed with 0.1 M HEPES, pH 7.5, followed by sequential 20 ml washes in each elution buffer: 0.15 M NaCl/10 mM HEPES, pH 7.4/10 mM EDTA/10 mM CHAPS; 1M NaCl/10 mM HEPES, pH 7.4/ 10 mM CHAPS; or 8 M urea/10 mM HEPES, pH 7.4/10 mM CHAPS, all containing protease inhibitors. The column was then equilibrated in 0.15 M NaCl/10 mM HEPES, pH 7.5/10 mM CHAPS overnight. HepG2 cells were harvested in 0.15 M NaCl/10 mM HEPES, pH 7.5/10 mM CHAPS and incubated for 30 minutes at room temperature to solubilize the membrane. Cells were spun at 15,000 rpm for 30 minutes to separate the cytoplasm and soluble membrane fraction, contained in the supernatant, from the particulate fraction, which is pelleted. This supernatant membrane fraction was run over the D-dimer affinity column and eluted sequentially with the above mentioned elution buffers in 1 ml fractions. Eluates were monitored by measuring the absorbance at 280 nm. Eluted proteins were concentrated by adding 60% TCA to a final concentration of 15% TCA in each fraction. Solutions were incubated on ice for 10 min and centrifuged at 14,000xg for 10 minutes. Pellets were resuspended in 1 ml ice cold acetone and spun again. The pellet was washed again in acetone, spun and air dried. Pellets were resuspended in SDS-PAGE loading buffer and separated on SDS-PAGE gels. Gels were

stained with Coomassie Blue and bands were extracted and sent to the OHSU Proteomics Core for mass spectrometry.

Co-Immunoprecipitation- Cell membrane fractions were prepared as described above. Protein G beads were incubated with a mouse anti-human D-dimer polyclonal antibody. Bead-bound antibodies were then crosslinked using the disuccinimidyl suberate method (Pierce). Antibody-bead complexes were incubated with 300 μg of cell membrane fractions overnight at 4^{o}C on a rotating platform. Beads were washed three times in phosphate buffered saline, and bound protein was eluted using IgG elution buffer (Pierce). Eluted proteins were separated on a 10% resolving/4% stacking SDS-PAGE gel. Proteins were then transferred to a nitrocellulose membrane and the membrane was blocked for 2 hours at room temperature in Western A buffer containing 50mM Tris, pH 7.4/5 mM EDTA/0.05% NP-40/150 mM NaCl/0.25% gelatin. Membranes were washed three times in Western A and incubated with monoclonal antibodies against each of three potential D-dimer receptors: gC1qR, TLR4 and LRP. Membranes were washed three times in Western A and incubated with a 1:25,000 dilution of Alexafluor 680 rabbit anti-mouse secondary antibody for one hour at room temperature. Membranes were then visualized using the LiCor Odyssey Imaging System.

Binding Studies- HepG2 cells were plated on black tissue culture-treated 96-well plates and allowed to grow to approximately 80% confluence in MEM containing 10% fetal bovine serum. Cells were then incubated in serum free and bicarbonate-free minimum essential medium containing 10mM HEPES, pH 7.4 and 1 mg/ml BSA. Cells were

incubated with serial dilutions of fluoresceinated D-dimer, D-fragment or BSA for 1 hour. Unbound labeled proteins were removed by washing three times in serum- and bicarbonate- free MEM, and bound proteins were solubilized and quantitated by emission at 518 nm after excitation at 491 nm on a BioTek 96-well plate reader. Non-specific D-dimer binding was defined empirically as the amount of binding observed in the presence of a 20-fold molar excess of unlabeled D-dimers.

Statistical Analysis- Data were analyzed using the SPSS version 17.0 Software package. All experiments were performed at least three separate times, with three replicates for each experiment. Calculation and comparison of the means was performed using ANOVA with Tukeys post-hoc correction to determine statistical significance with a cutoff of $p<0.05$ and data are presented as mean $\pm$ SEM. For qRT-PCR experiments, statistics were performed on ΔCt values after normalization to 18S using a paired t-test.

4.3 Results

Purification of D-dimers From Fibrin Clots- D-dimers were purified from intact fibrin clots by plasmin digestion of the clot followed by sequential ion-exchange and size exclusion chromatography on DEAE and Sephacryl-200 HR columns, respectively. DEAE chromatography was able to separate out D-dimers, D-dimers bound to E-fragments, and D-fragments from the remainder of the clot degradation products as shown in representative A280 tracings and SDS-PAGE analysis of eluates (Figures 4.2 and 4.3). Fractions with D-domain containing fragments were then run over a size exchange column in order to separate pure D-dimers from D-fragments and D-dimers bound to E-fragments (Figures 4.4 and 4.5). Thus, we were able to isolate several fibrin degradation products from plasmin digested fibrin clots and purify them to ensure no contamination from other fibrin degradation products.

Investigation of the binding of D-dimers to the surface of HepG2 cells- Previous studies have shown that D-dimers and D-fragments are cleared from the circulation mainly by the liver, and the majority of this clearance is performed by hepatocytes. Additionally, these studies have suggested that this clearance may be mediated by a receptor on the surface of hepatocytes (70). Therefore, we investigated the uptake of fluorescently labeled D-dimers and D-fragments by HepG2 cells using fluorescent binding studies. Labeled D-dimers were taken up by HepG2 cells, but this uptake was not saturable even up to 1 μM, one hundred times the physiological concentration of D-dimers. Surprisingly, D-fragment showed minimal uptake by HepG2 cells when compared to D-dimers (Figure 4.6). This suggests that D-dimers, but not D-fragment, have an active uptake mechanism

in HepG2 cells. However, this uptake does not seem to indicate the presence of a saturable D-dimer receptor on the surface of these cells.

D-dimers are able to enter HepG2 cells- Previous reports using labeled fibrinogen and fibrin degradation products in mice have suggested that D-dimers are taken up in the liver by hepatocytes as a clearance mechanism (70). In order to confirm these results, D-dimers were fluorescently labeled and incubated with HepG2 cells and monitored over the course of one hour using fluorescence microscopy. As seen in Figure 4.7, fluorescein labeled D-dimers (green) are able to enter the HepG2 cells before the 20 minute time point and are mainly cytoplasmically localized for up to 20 minutes. However, after 30 minutes of incubation, the fluorescent signal is lost, suggesting that the D-dimers are either degraded intracellularly or exported out of the cell. Additionally, the remaining fluorescence seen is concentrated around the nucleus (blue) at 30 minutes. D-dimer fluorescence is further decreased by 40 minutes, with the remaining fluorescence still centered around the nucleus. Real-time cell imaging was also performed by incubating HepG2 cells with either fluorescently labeled D-dimers or fluorescently labeled BSA as a control. As seen in Figure 4.8, both fluorescently labeled proteins were taken up by HepG2 cells within 5 minutes. However, only the labeled D-dimer seemed to migrate to the space around the nucleus, while the labeled BSA seemed to remain cytoplasmic. These results suggest that D-dimers are able to enter hepatocytes and travel to the perinuclear space.

Investigation of Membrane Proteins that Interact with D-dimers- In order to identify

possible membrane-bound binding partners of D-dimers, a D-dimer affinity column was created by covalently linking purified D-dimers to N-hydroxysuccinimide esters of a derivatized crosslinked agarose gel bead support (Affi-gel). HepG2 membrane fractions were run over the affinity column and eluted under 3 different elution conditions (low salt, high salt, high pH) (Figure 4.9). Eluted proteins were sent for proteomic analysis and several potential binding partners for D-dimers were identified, although these did not include any cell surface receptors. Table 4.1 details some of the more interesting among the identified proteins that bound to D-dimers. Most notably for this set of experiments is the inclusion of several proteins, such as Rab10 and Sorting nexin-9 which are involved in endocytosis at the plasma membrane. Additionally, Sec 23B, a protein involved in vesicle trafficking and fibronectin, a protein that binds to integrins, were also identified as binding partners for D-dimers. Together with the results of surface binding experiments, these results imply that D-dimers are taken up by hepatocytes via endocytosis, although whether or not this event is receptor-mediated remains to be seen.

Characterization of potential receptors for D-dimers- Although we did not see a saturable binding curve when fluorescently labeled D-dimers were incubated with HepG2 cells, nor did proteomic analysis identify any membrane receptors that interact with D-dimers, we tested three candidate receptor proteins that have been shown previously to bind to either fibrinogen, fibrin, a fibrin degradation product or other coagulation proteins. Low-density lipoprotein receptor-related protein-1 (LRP-1) is an endocytic receptor belonging to a superfamily of proteins relating to the low-density lipoprotein (LDL) receptor. LRP-1 is known to bind and endocytose several coagulation proteins and

complexes including fVIIa/Tissue Factor complex, fVIIIa, fIXa, and FXIa (71). Knowing that this receptor is capable of binding many coagulation proteins, we investigated whether it was capable of binding to D-dimers using co-immunoprecipitation. As shown in Figure 4.10A, membrane fractions immunoprecipitated with an anti-D-dimer antibody did not show the presence of LRP-1, suggesting that D-dimers do not bind to LRP-1. Additionally, pre-treating HepG2 cells with receptor associated protein (RAP), an inhibitor of LRP, did not significantly inhibit the uptake of fluorescently labeled D-dimers from the media when compared to control cells(Figure 4.11A and B). Similarly, gC1qR and Toll-like Receptor-4 (TLR4) did not show binding D-dimers by immunoprecipitation (Figure 4.10B and C). gC1qR is the globular C1q complement binding protein receptor and has been shown to bind to fibrinogen and fibrin via the D-domain (72), therefore it was identified as a candidate receptor in that D-dimers contain two crosslinked D-domains. Toll-like Receptor-4 is known to be activated by lipopolysaccharide (LPS) treatment and has shown to be involved in the development of atherosclerosis by upregulating chemokine expression (73). Interestingly, TLR4 signaling is also induced by fibrinogen (73), leading us to include it as a potential candidate receptor for D-dimer. As mentioned previously, none of these three receptor molecules showed binding to D-dimer by co-immunoprecipitation, suggesting that none are the receptor for D-dimer. Confirming these findings, pretreatment of HepG2 cells with blocking antibodies to either gC1qR or TLR4 did not inhibit the uptake of fluorescently labeled D-dimer by HepG2 cells compared to controls (Figure 4.11B).

Effects of D-dimers on the production of γ' and total fibrinogen- Previous studies have shown that D-dimers are able to induce the transcription of target genes via the transcription factor AP-1 (69), however the effect of D-dimers on fibrinogen γ chain isoform production has not been well studied. In order to determine the effects of D-dimers on the production of fibrinogen, HepG2 cells were treated with increasing doses of purified D-dimers for 24 hours. As shown by ELISA, treatment with increasing doses of D-dimer led to a slight, although not significant, decrease in the amount of γ' fibrinogen produced by the cells while having no effect on the amount of total fibrinogen produced (Figure 4.12). This suggests that D-dimers may be affecting the relative production of the two fibrinogen splice isoforms, γA and γ'. Additionally, the addition of D-dimers significantly inhibited the production of γ' mRNA in HepG2 cells (Figure 4.13), suggesting that D-dimers may be able to interfere with the alternative splicing of the γ chain pre-mRNA.

Fig. 4.1

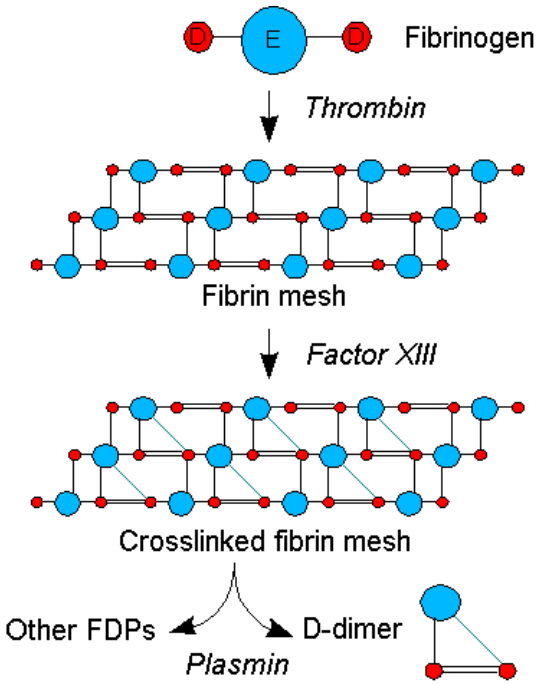


Figure 4.1: Formation and Degradation of a Fibrin Clot. The fibrin clot is formed and cross-linked by fXIIIa, as described in Chapter 1. Activation of plasminogen to plasmin leads to the cleavage of specific arginine and lysine bonds within fibrin fibrils and the production of fibrin degradation products, including D-dimer.

Fig. 4.2

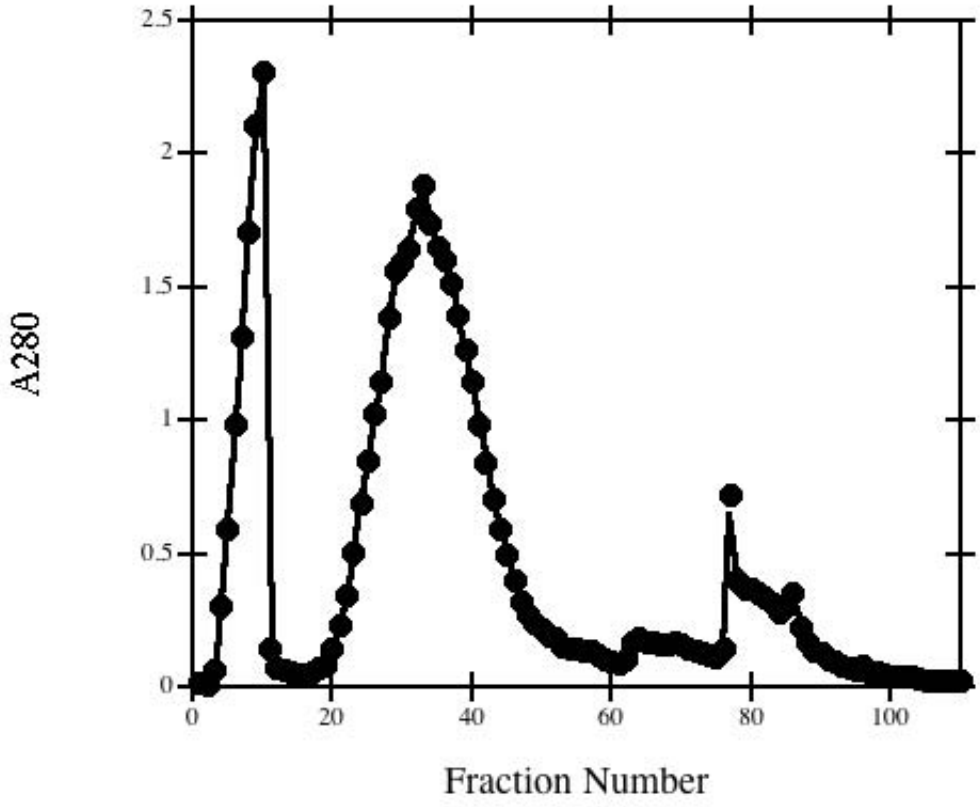


Figure 4.2: Separation of Fibrin Degradation Products By Ion Exchange Chromatography. Fibrin clots were prepared and lysed with plasmin. Lysed clots were run over a DEAE column and fibrin degradation products were eluted with a 400 ml gradient using of 0.1 M Tris, pH 7.4/ 1mM $CaCl_2$ as the starting buffer and 0.1 M Tris, pH 7.4/1 mM $CaCl_2$/0.17 M NaCl as the final buffer. Absorbance was monitored at 280 nm.

Fig. 4.3

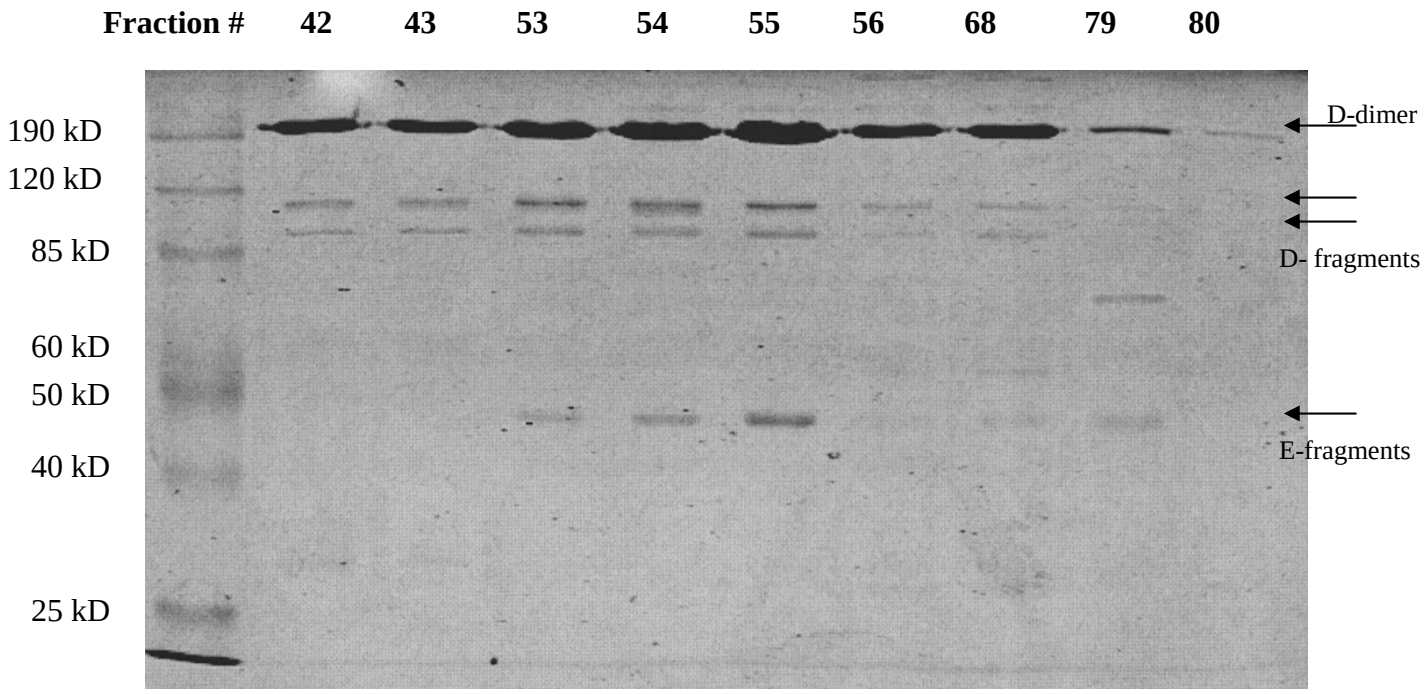


Figure 4.3: Representative SDS-PAGE of Fibrin Degradation Products Eluted From DEAE Column. Peak fractions of fibrin degradation products eluted from a DEAE column were loaded onto a 10% SDS PAGE gel and resolved. Protein bands were stained with Coomassie Blue stain.

Fig 4.4

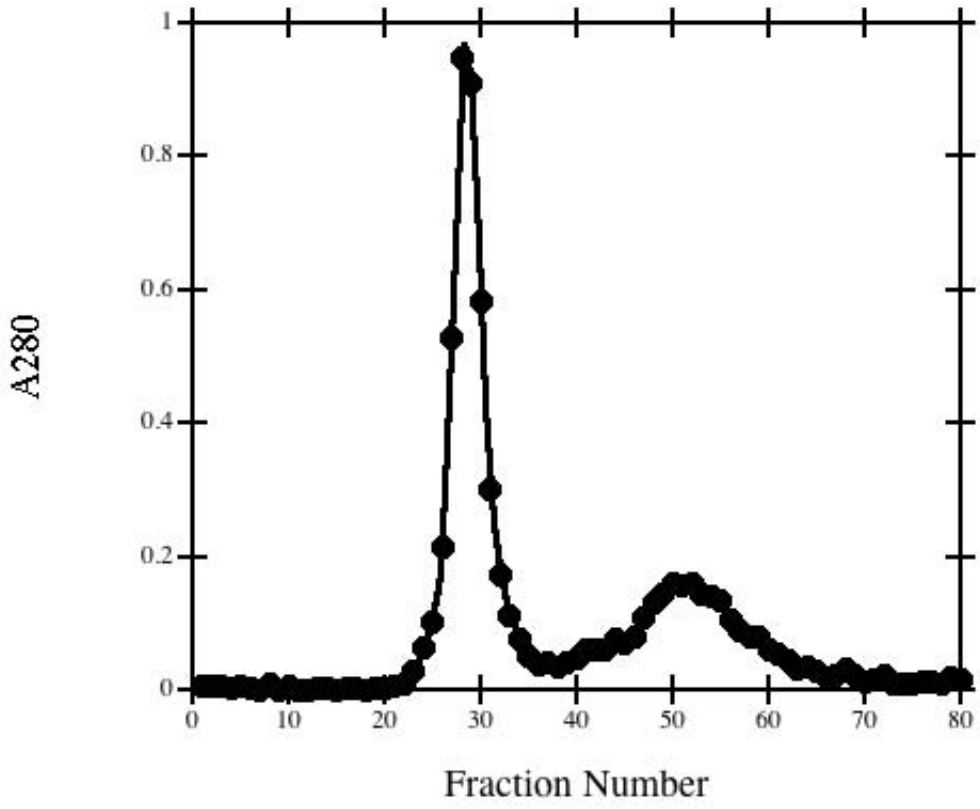


Figure 4.4: Separation of Fibrin Degradation Products By Size Exclusion Chromatography. Peak fractions containing D-dimers were pooled and run over a Sephacryl-200HR size exclusion column. Separated proteins were eluted in 0.1 M Tris, pH 7.4/ and absorbance was monitored at 280 nm.

Fig 4.5

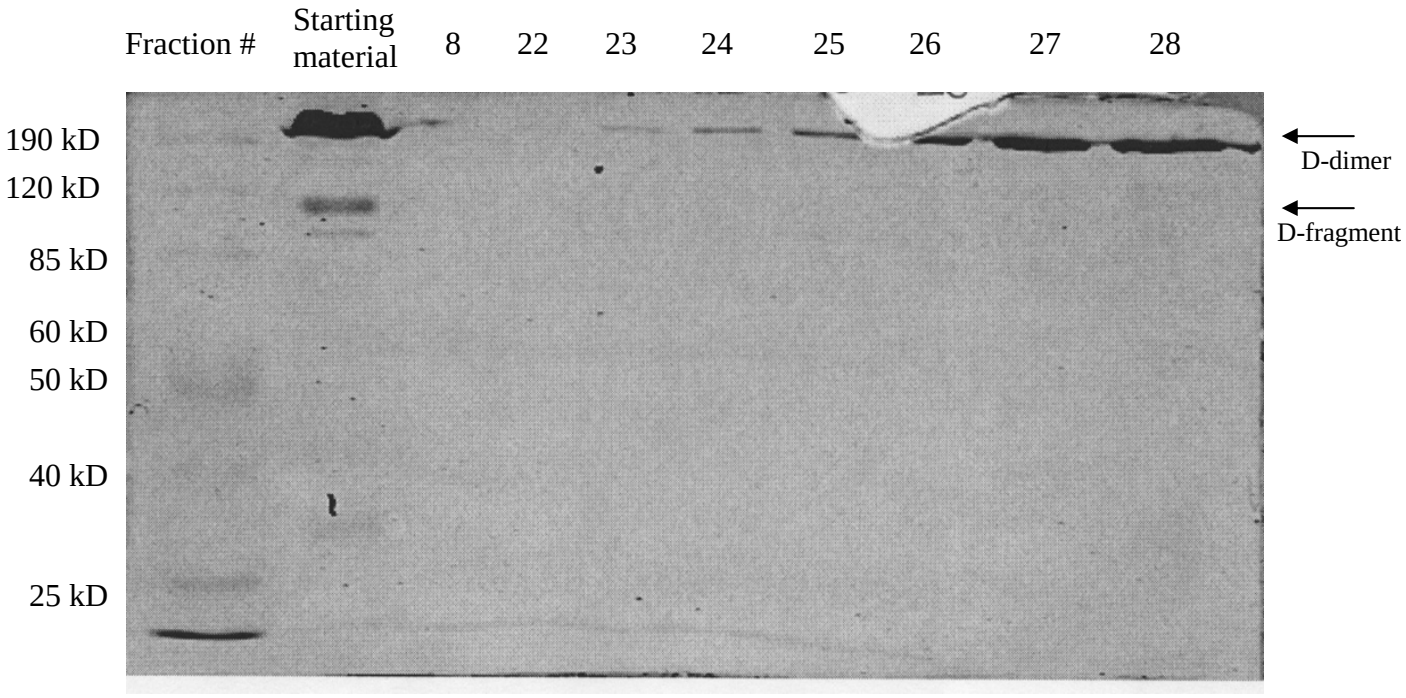


Figure 4.5: Representative SDS-PAGE Illustrating Separation of D-dimers by Size Exclusion Chromatography . Fractions containing D-dimers were pooled and separated using size exchange chromatography. Proteins eluted from the sizing column were loaded onto a 10% SDS PAGE gel and resolved. Protein bands were stained with Coomassie Blue stain.

Fig. 4.6

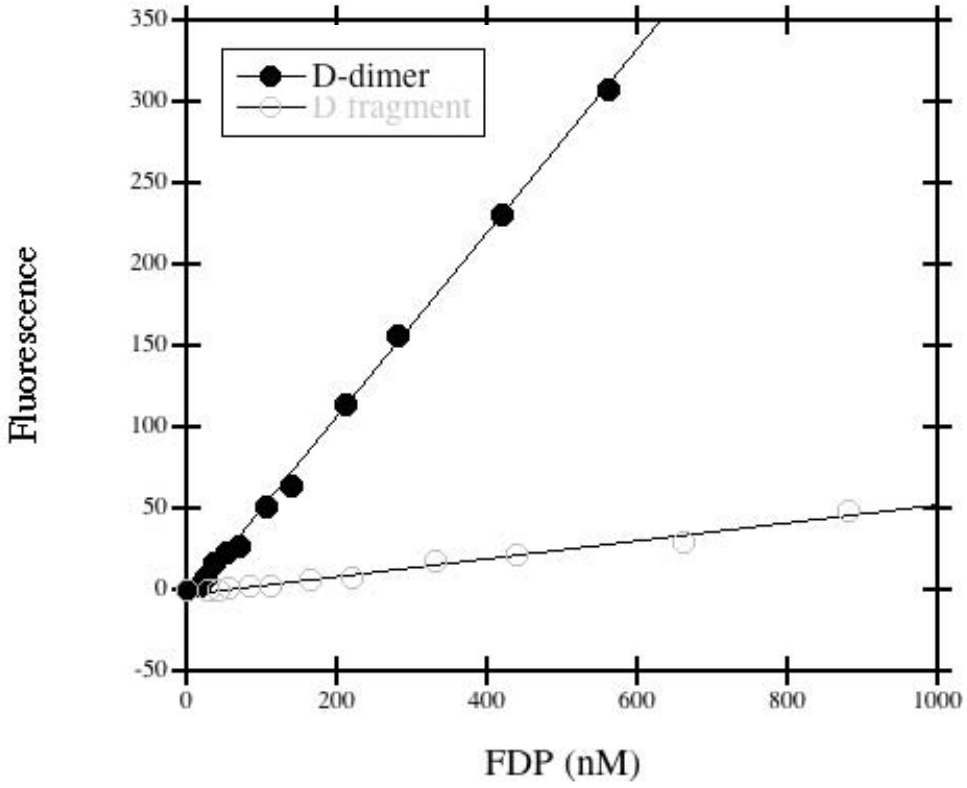


Figure 4.6: Uptake of D-dimers, but not D Fragments, by HepG2 Cells. Fluorescent uptake studies were performed on HepG2 cells using fluorescein-labeled D-dimers or D-fragments. Increasing concentrations of fluorescently labeled proteins were added to HepG2 cells and incubated for 1 hour. Cells were washed and bound protein was quantitated by analyzing emission at 518 nm following excitation at 491 nm.

Fig. 4.7

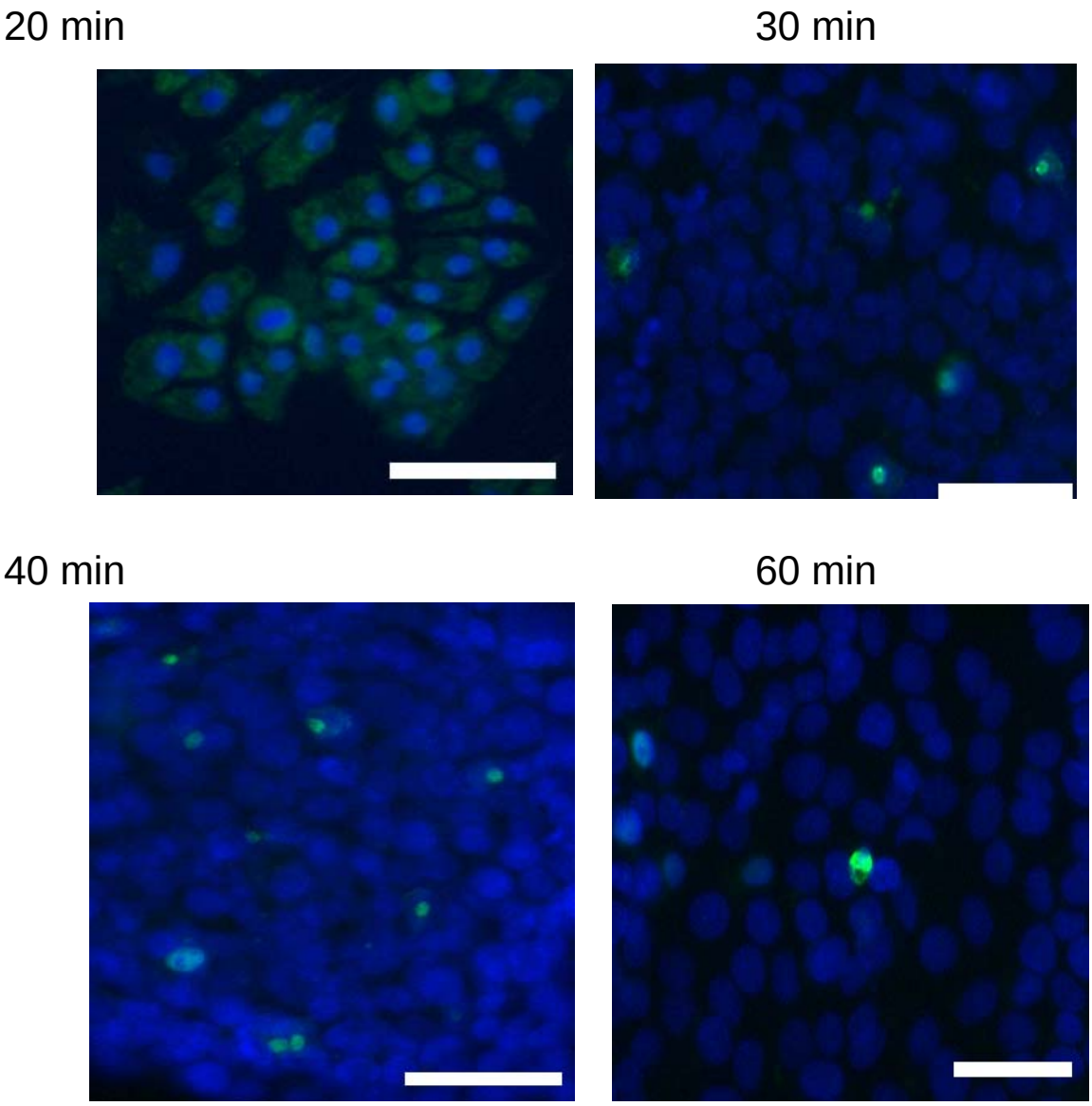
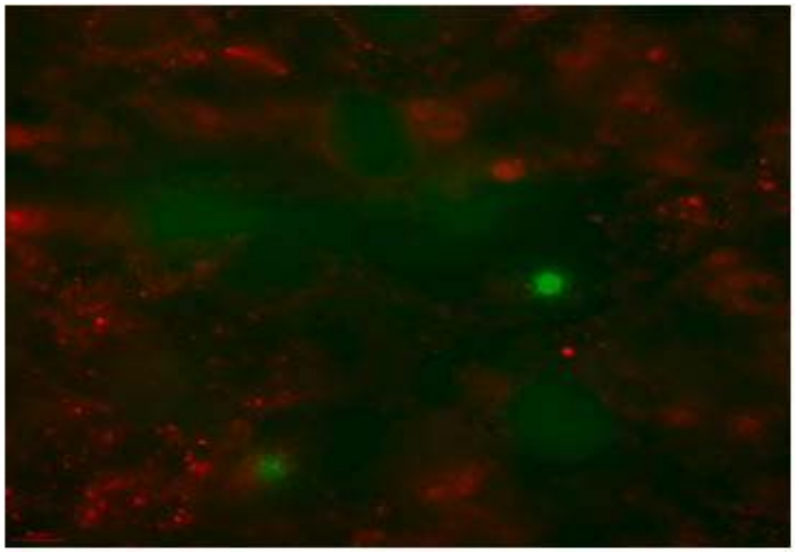
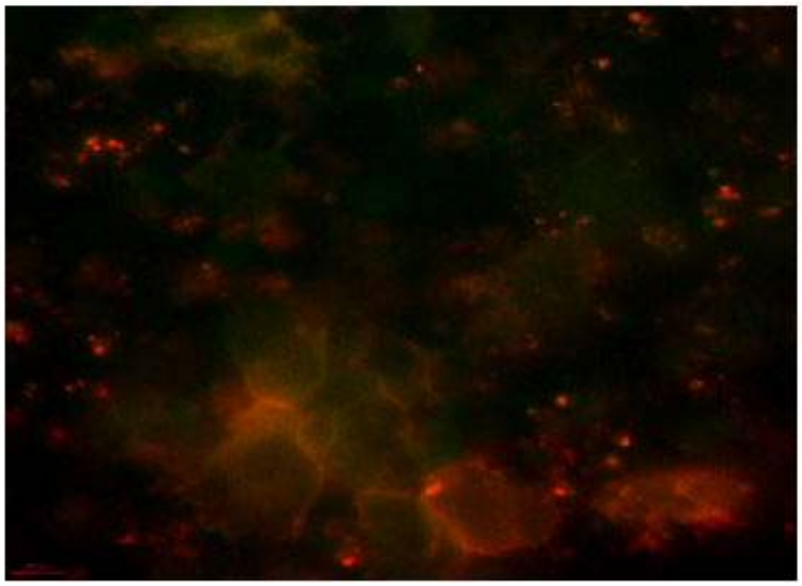


Figure 4.7: Investigation of D-dimer uptake by HepG2 Cells. HepG2 cells were incubated with fluorescein labeled D-dimers in a time course experiment. Cells were washed and fixed every 10 minutes. Uptake was visualized at 40x. Nuclei are stained blue with DAPI stain and D-dimers are shown in green. Scale bars are 45 μm length and 10 μm in height.

Fig. 4.8A

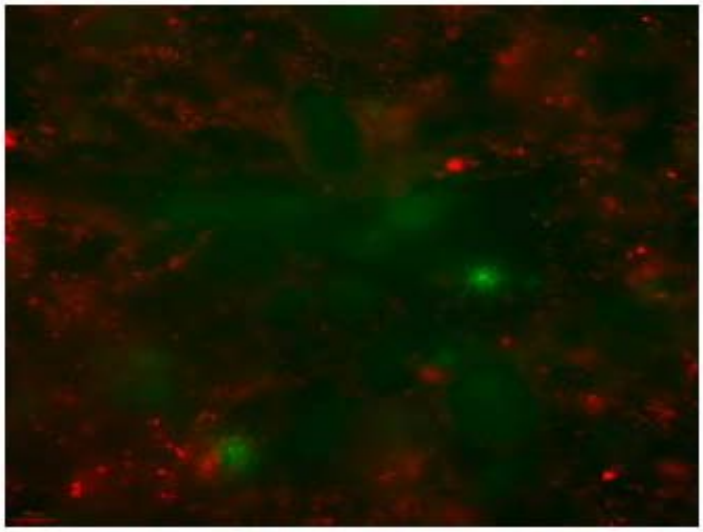


D-dimer
0 minutes

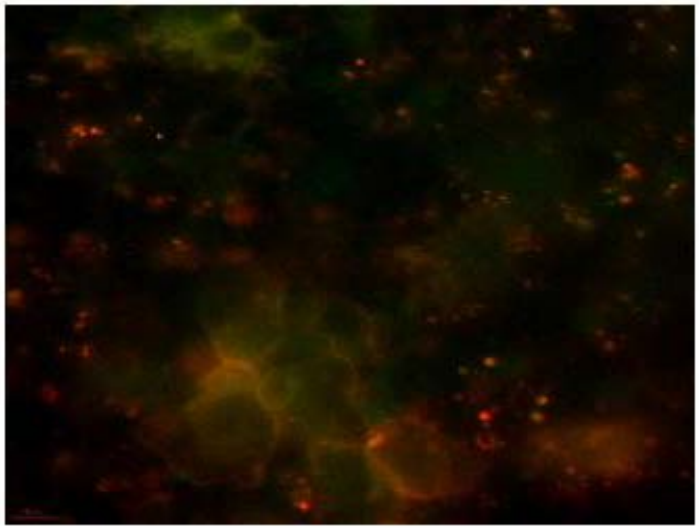


Albumin
0 minutes

Fig. 4.8B

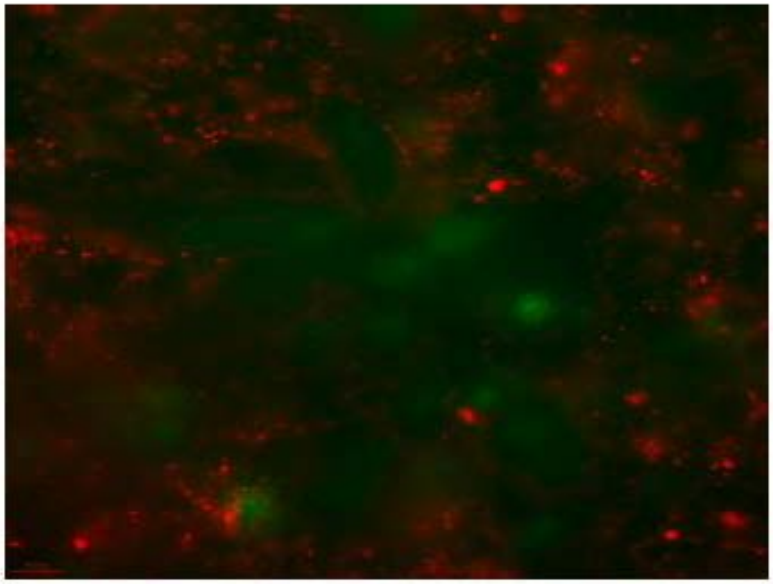


D-dimer
3 minutes

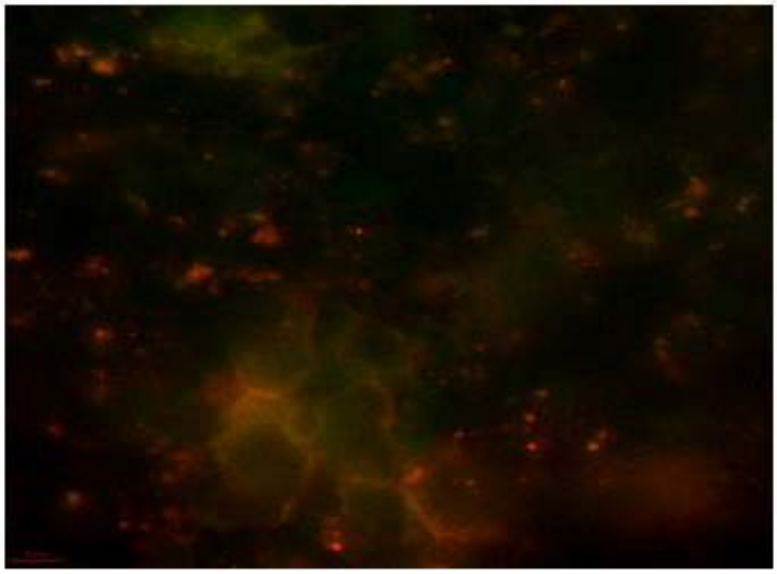


Albumin
3 minutes

Fig. 4.8C

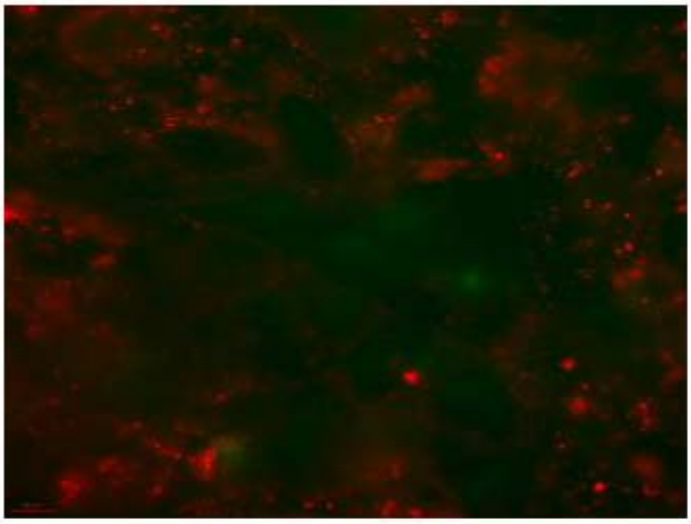


D-dimer
5 minutes

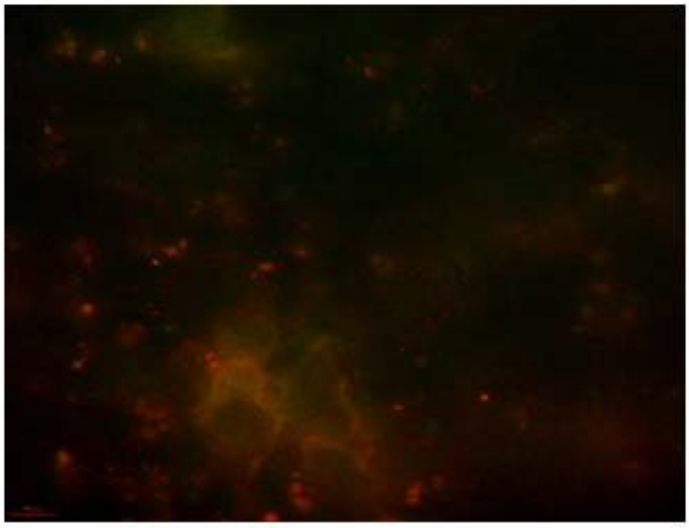


Albumin
5 minutes

Fig. 4.8D



D-dimer
10 minutes



Albumin
10 minutes

Figure 4.8: Localization of D-dimers within HepG2 cells. Fluorescently labeled D-dimers or BSA controls were incubated with HepG2 cells for 5 minutes. Cells were washed to remove excess labeled protein and the movement of labeled protein was visualized using real time cell imaging. The above panels are still frames taken from live cell movies. Cell membranes are labeled in red by staining with Vybrant Cell Labeling solution. Scale bars shown in the lower left hand corner of each picture represent 10 μm.

Fig. 4.9

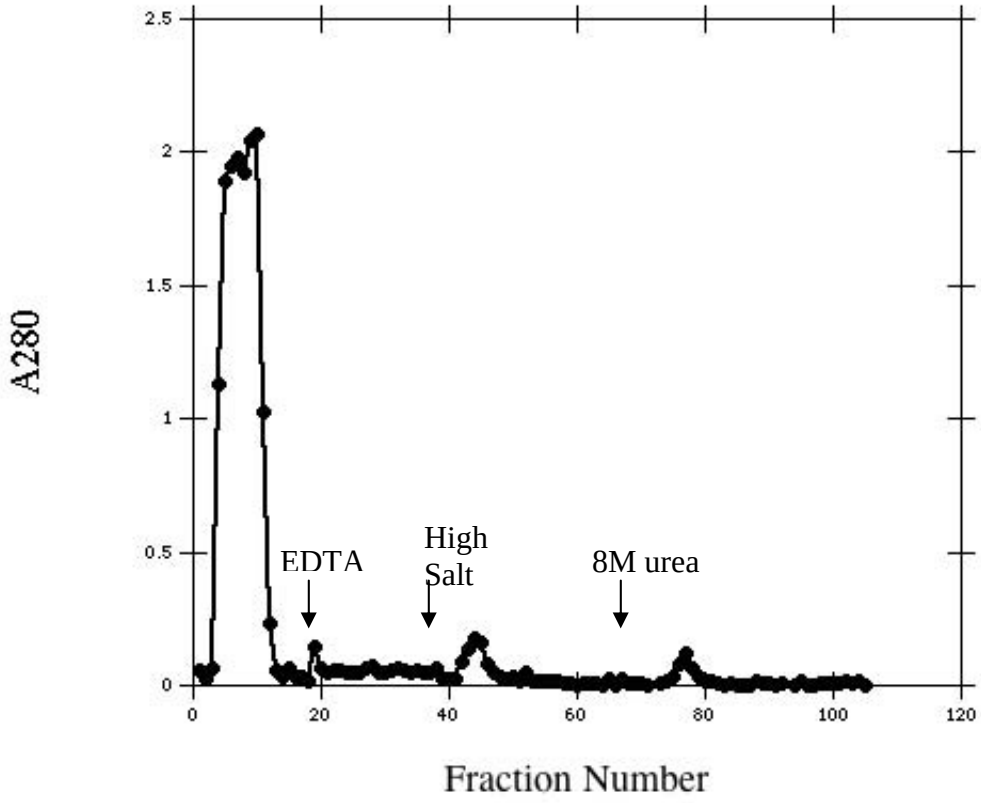


Figure 4.9: Elution of Proteins that Bind to D-dimers on a D-dimer Affinity Column. Purified D-dimers were coupled to Affy-Gel 15 resin. HepG2 membrane fractions were run over the column in 0.1 M HEPES, pH 7.5. Proteins were eluted sequentially in buffers containing 10 mM EDTA, 1M NaCl, and 8M urea in 10 mM HEPES, pH 7.5/10 mM CHAPS. Eluted proteins were sent for proteomic analysis by mass spectrometry.

Fig. 4.10

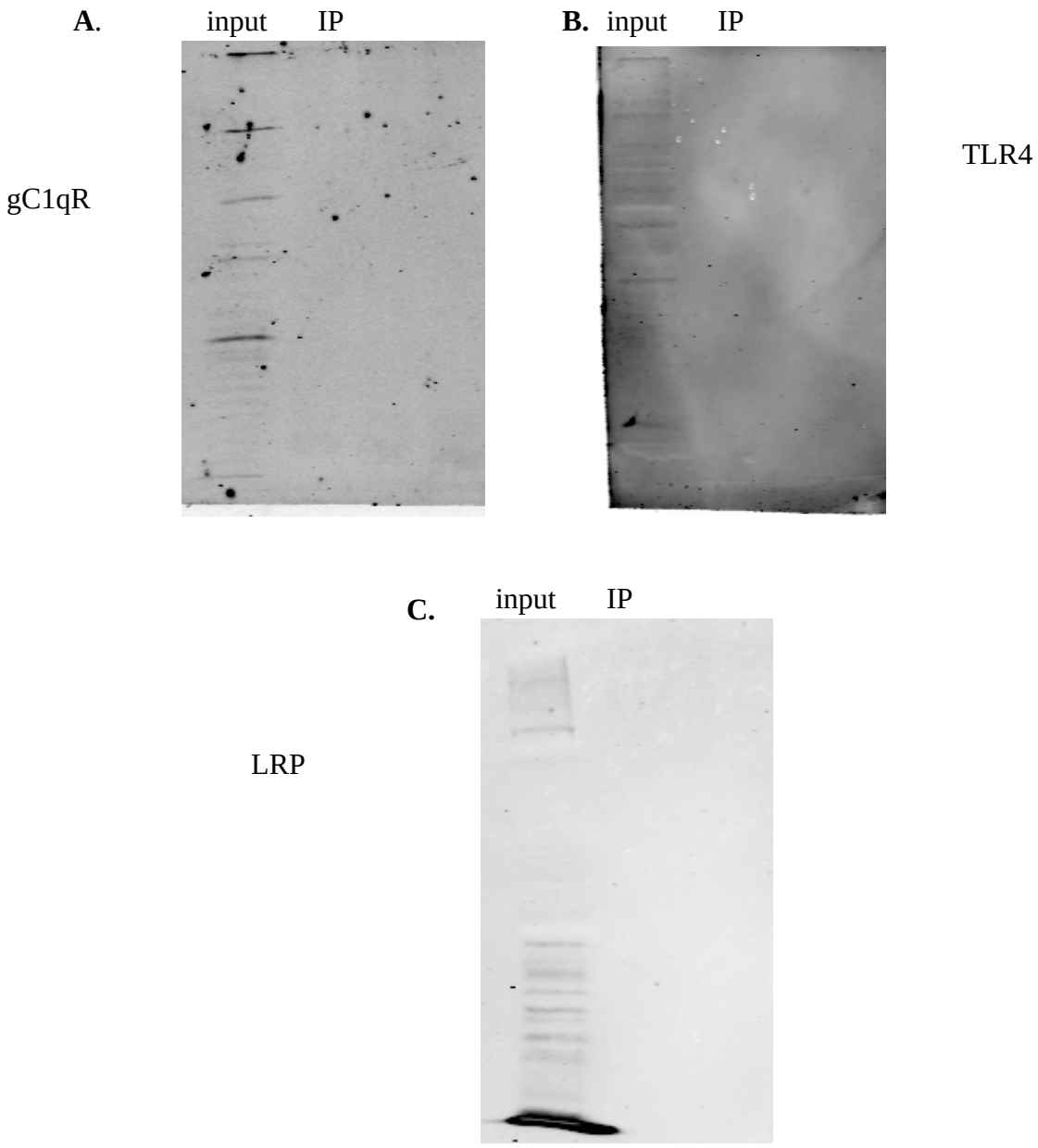


Figure 4.10: Characterization of potential D-dimer receptors on HepG2 cells. HepG2 membrane fractions were immunoprecipitated with a monocloncal antibody against D-dimers and separated on 10% SDS PAGE. Proteins were blotted onto a nitrocellulose membrane and probed for either gC1qR (Panel A), TLR4 (Panel B) or LRP (Panel C).

Figure 4.11A

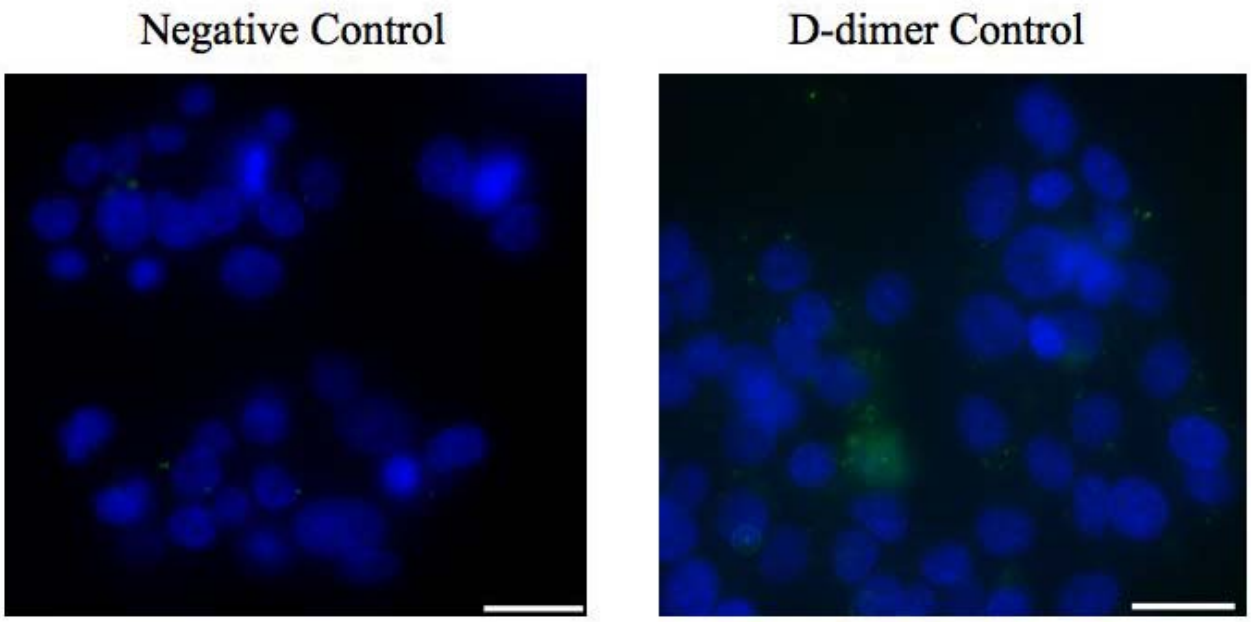


Fig. 4.11B

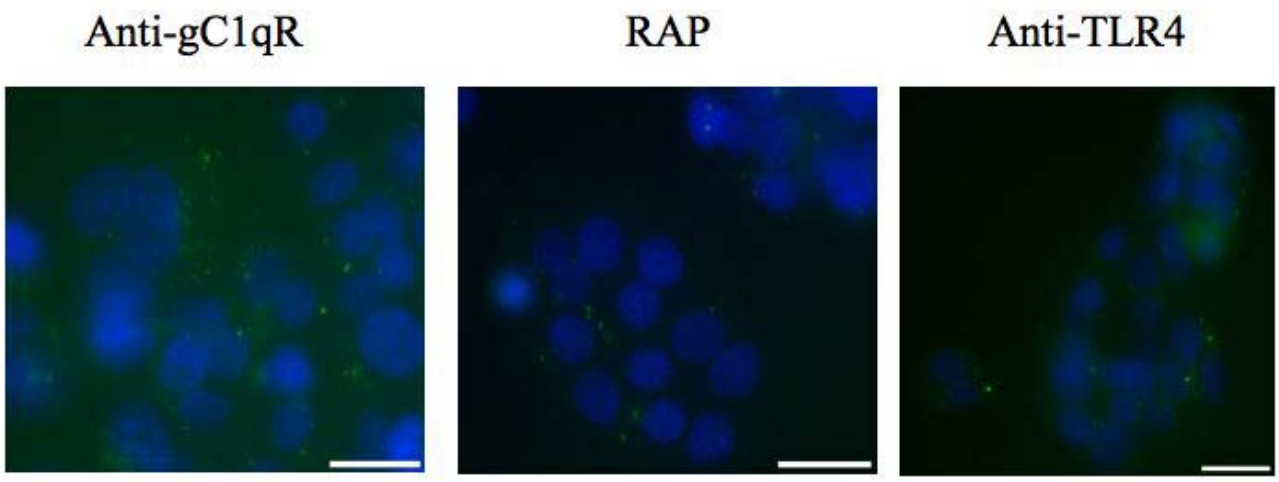


Figure 4.11: Characterization of potential receptors for D-dimer on the surface of HepG2 cells. Fluorescently labeled D-dimers were incubated with HepG2 cells for 20 minutes with or without pretreatment with an antibody against gC1qR (10 μg/ml), RAP (40 μg/ml) or an antibody against TLR4 (10 μg/ml). Cells were fixed and visualized at 60x. Nuclei are stained blue with DAPI and fluorescently labeled D-dimer is shown in green. Scale bars are 25 μm in length and 10 μm in height.

Figure 4.12

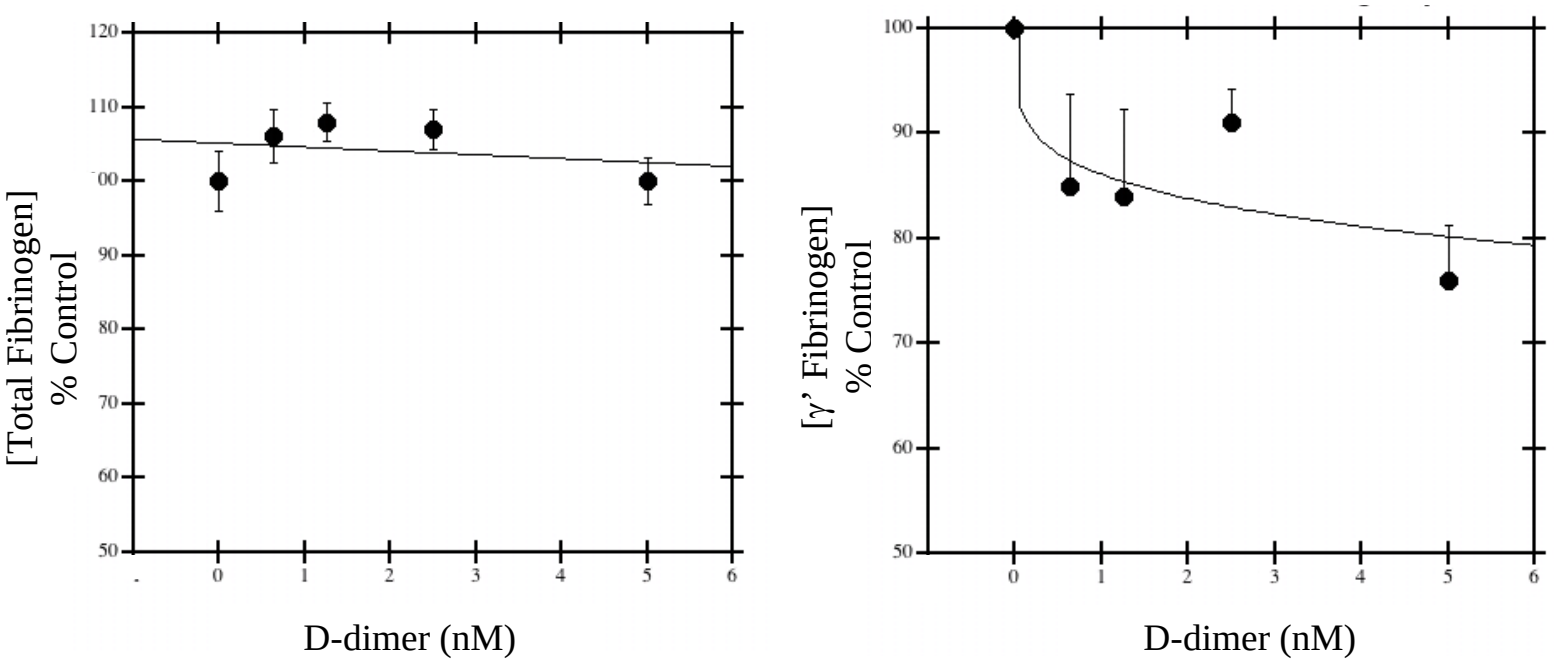


Figure 4.12: Investigation of the effects of D-dimer treatment on fibrinogen production in HepG2 cells. HepG2 cells were incubated with increasing concentrations of purified D-dimers for 24 hours. γ' and total fibrinogen levels in the supernatant were measured by ELISA and results were normalized to protein levels in each well. Western blots for γ' and total fibrinogen were also performed.

Fig. 4.13

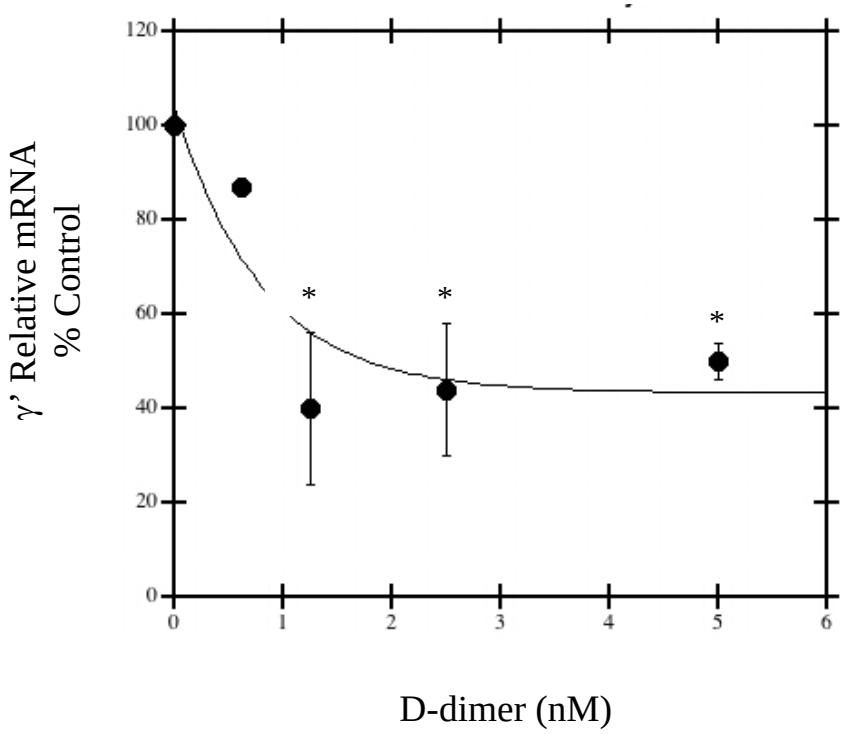


Figure 4.13: Investigation of D-dimer treatment on fibrinogen mRNA levels in HepG2 cells. HepG2 cells were treated with increasing concentrations of purified D-dimers for 24 hours. Cytoplasmic mRNA was harvested and qRT-PCR was performed using primers specific for the γ' isoform. *$p<0.01$ as compared to untreated cells using a student's t-test.

Table 4.1

Protein Name	Protein Function
Rab-10	Interacts with AP1S1 Endocytic Protein
Sorting Nexin-9	Interacts with Clathrin
Sec23B	Involved in Vesicle Trafficking
Fibronectin	Binds to Cell Surface Integrins

Table 4.1. HepG2 Proteins Identified as Potential Binding Partners of D-dimers. HepG2 membrane fractions were run over a D-dimer affinity column and eluted under three different conditions (EDTA, high salt, 8M urea). Eluted proteins were separated on a 10% SDS-PAGE gel and stained with Coomassie Blue stain. Bands were extracted and subjected to mass spectrometry to ascertain their identity.

4.4 Discussion

This study demonstrates for the first time that the fibrin degradation product D-dimer is endocytosed at the plasma membrane and negatively regulates the production of γ' fibrinogen in HepG2 cells. There has been a substantial amount of controversy in the literature concerning the regulatory effects of FDPs on fibrinogen production. Most importantly in this debate is the question of which cell type is affected by FDPs and how these cells in turn regulate fibrinogen synthesis. For example, in cultured rat hepatocytes, D-fragments have been shown to stimulate fibrinogen synthesis directly while E-fragments had no effect on fibrinogen synthesis (74). However a separate study suggested that E-fragments affected the synthesis of fibrinogen while D-fragments had no effect (75). Additionally, the addition of FDPs to monocytes or leukocytes was shown to increase the production of several cytokines including IL-6 and IL-1β, which in turn can affect the production of fibrinogen from nearby hepatocytes (76). This finding is especially interesting in that levels of D-dimer correlate with the levels of the proinflammatory cytokines IL-6, IL-8 and TNF-α and disease severity and death in critically ill patients (77).

D-dimers contain two crosslinked D-fragments and can also contain a bound E-fragment. Several reports have shown differential regulatory events when cells are treated with D-fragment as opposed to D-dimer. D-dimer was a more potent stimulator of IL-1α and IL-1β in peripheral blood monocytes as compared to D-fragment (78). Moreover, D-dimer induced the transcription and synthesis of plasminogen activator inhibitor-I (PAI-I) in rat lung fibroblasts while intact fibrinogen had no effect (69). These effects may be due to

conformationally dependent epitopes found in D-dimers but not in D-fragments. These conformationally dependent epitopes may lead to specific binding of D-dimer, but not D-fragment, to surface receptors. D-dimer has been shown to specifically bind to monocytes by the integrin CD11b/CD18 (MAC-I) (79). We hypothesized a similar epitope specific binding for D-dimer on the surface of HepG2 cells. Uptake studies examining the uptake of labeled D-dimer and D-fragment byHepG2 cells provides support for this hypothesis, as we see D-dimer taken up while very little D-fragment is taken up (Fig 4.6). This suggests that the molecular mechanism by which D-dimer is taken into the cells is specific. The speckled pattern of fluorescence that is seen once the D-dimers are taken into the cells (Figs 4.7 and 4.11) is suggestive of the presence of D-dimers in endosomes, which in turn would imply that the protein had been taken up via receptor-mediated endocytosis. We did not see saturable binding of D-dimers to HepG2 cells even at 1 μM, 100 times physiological concentration, which could suggest one of two scenarios. First, there may not be a receptor for D-dimer present on HepG2 cell surfaces and D-dimer may be passively taken up by the cells via fluid-phase endocytosis. Fluid-phase endocytosis of the labeled protein would still be consistent with the presence of fluorescent speckles suggestive of endosomes, although it would not explain the lower uptake of D fragments. Second, the potential receptor at the HepG2 cell surface may have such a low affinity that even higher doses of D-dimer are required to attain saturation. LRP in particular is known to bind to many different ligands, including several coagulation factors, and the affinity of this receptor for its ligands ranges from the low nM scale up to 4 μM. If LRP were indeed the receptor for D-dimer on the surface of HepG2 cells, it is possible that we were not able to saturate the binding at 1 μM. The

scenario of an abundant low-affinity receptor led us to examine whether any known fibrin(ogen) binding receptors may play a role in this uptake. We chose three candidate receptor proteins, LRP, gC1qR and TLR4, based on the existing literature that shows that each of these three receptors is known to bind to fibrin, fibrinogen or a fibrin degradation product (71-73). Inhibition of any of the three receptors using either blocking antibodies, as with gC1qR and TLR4, or a known receptor associated protein that interferes with receptor function for LRP, did not show significant inhibition of uptake in HepG2 cells (Fig 4.11). Treatment with RAP may have slightly inhibited uptake of fluorescently labeled D-dimer, as less total fluorescence is seen and the diffuse fluorescence seen in control cells is absent, but further investigation with higher concentrations of RAP and other LRP inhibitors is necessary to confirm this result.

Interestingly, elution of HepG2 membrane fractions from a D-dimer affinity column also did not identify any likely membrane receptors for D-dimers, although it did identify several membrane-bound proteins involved in endocytosis as potential binding partners for D-dimers (Table 4.1). Notably, Rab10 is a protein related to Ras that is known to bind to the protein AP1S1. AP1S1 is a protein responsible for linking clathrin to receptors within coated vesicles at the plasma membrane (80). Sorting Nexin-9 is also known to interact with clathrin as well as dynamin2 during the process of endocytosis (81). Sec23B is also a vesicle-associated protein, although its exact function has not yet been identified. The identification of these three proteins as potential D-dimer binding partners strongly suggests that D-dimers are taken up via endocytosis. The presence of AP1S1, a clathrin

binding protein, would suggest that this event is receptor-mediated, however further studies are needed to confirm this finding and to identify the receptor.

Once hepatocytes have taken up circulating D-dimer, we see a regulatory effect on the production of γ' fibrinogen, but not total fibrinogen. We did not see this effect when cells were treated with D-fragment, E-fragment or the C-terminal γ' peptide (May-Lynn Chu, unpublished data). Treatment with D-dimer was effective at reducing γ' fibrinogen protein levels by approximately 20%, whereas total fibrinogen levels remained constant over all concentrations of D-dimer treatment (Fig 4.12). We also saw a corresponding decrease in the production of γ' mRNA, although the levels of mRNA were reduced by approximately 50% as measured by qRT-PCR (Fig 4.13). While several studies mentioned above have looked at the effect of FDPs on total fibrinogen production, this is the first study suggesting that FDPs may differentially regulate the production of γA and γ' fibrinogen. This regulation is important due to the association of γ' fibrinogen levels with several types of cardiovascular disease.

We originally hypothesized that FDPs may act as a sensor in the bloodstream that could feed back to the liver, where the FDPs are taken up, to signal that more or less fibrinogen of either isoform should be produced. In this scenario, we would have expected that increased concentrations of D-dimers, stemming from increased clot formation and breakdown, would lead to an increase in total fibrinogen since the fibrinogen is being used to actively create a clot. However, we do not see any change in the amount of total fibrinogen produced by HepG2 cells following D-dimer treatment, showing that D-

dimers do not directly affect fibrinogen production in HepG2 cells, but rather may act by binding to monocytes and increasing IL-6, a known stimulator of fibrinogen synthesis. Moreover, we saw a decrease in the amount of γ' fibrinogen produced following D-dimer treatment suggesting that an increase in plasma D-dimer, which may contain the γ' chain, may feed back to inhibit further γ' fibrinogen production. This scenario is supported by the existing knowledge of γ' fibrinogen biochemistry. Due to the thrombotic nature of γ' fibrinogen, it is plausible that as a clot is being degraded, a mechanism would exist that communicates to the liver that γ' fibrinogen, which forms clots that are not readily broken down, should not be synthesized.

In summary, this study has shown that D-dimer is a newly identified regulator of fibrinogen γ chain isoform production, leading to a decrease in the amount of γ' fibrinogen produced while not affecting the level of total fibrinogen. We show that HepG2 cells are able to take up D-dimers, as suggested by previous work, but that this uptake is possibly the result of fluid-phase endocytosis rather than a receptor-mediated event. The identification of a novel regulator of γ' fibrinogen production is an important step towards understanding the regulation of fibrinogen γ chain isoform production and its emerging role as a risk factor in cardiovascular disease.

Chapter 5: Regulation of Fibrinogen γ Chain Isoform Expression by IL-6, IFN-γ TNF-α, TGF-β and IL-1β

The work presented in this chapter has been submitted for publication to Cytokine.

5.1 Introduction

Inflammation is known to play an important role in both the development of cardiovascular disease and increasing the risk of a cardiovascular event in the setting of cardiovascular disease. The presence of a chronic inflammatory state can lead to cardiovascular events by contributing to the buildup and rupture of atherosclerotic plaques and thrombus formation. Additionally, surgery or trauma may give rise to an acute inflammatory response that may increase the risk of a thrombotic event. Studies have shown that up to 5% of all patients who undergo coronary artery bypass graft (CABG) surgery will suffer from a subsequent stroke (82). The systemic inflammatory response to surgery or trauma may lead to an increase in circulating coagulation factors. Studies from our laboratory have shown that both total fibrinogen and γ' fibrinogen increase two-fold following CABG surgery (Figure 5.1, Rehana Lovely unpublished data). This is an important finding since increased levels of fibrinogen have been implicated in stroke, and may play a role in the high incidence of stroke related deaths following CABG surgery.

As mentioned previously, fibrinogen is a known Type II acute phase reactant that is induced mainly by IL-6 and glucocorticoids. Specific combinations of inflammatory cytokines have been shown to have differing effects on the production of fibrinogen in

the HepG2 human hepatocellular carcinoma cell line (83). These studies showed that IL-1α, TNF-α and TGF-β were able to decrease the production of fibrinogen individually, while the addition of IL-6 more than doubled the production of fibrinogen. However, when treated with IL-6 in combination with IL-1α, TNF-α and TGF-β, each of these cytokines inhibited the IL-6 mediated production of fibrinogen. Interestingly, *in vivo* studies on the effects of certain cytokines on acute phase protein production show paradoxical results, with IL-1 inhibiting acute phase protein production *in vitro*, while enhancing it *in vivo* (84, 85). Therefore it is important to examine the effects of inflammatory cytokines in both models in order to discern whether the cytokines act directly on liver cells or act indirectly through other mediators.

Several molecular mechanisms by which cytokines affect the production of total fibrinogen have been studied extensively in recent years (53, 86). IL-6 exerts its effects through a two-component cell surface receptor, resulting in phosphorylation and activation of STAT3 in the cytoplasm. Phosphorylation of STAT3 in the active state leads to STAT3 dimerization and translocation of the complex to the nucleus where it exerts its effects on transcription (Figure 5.2). All three human fibrinogen genes contain a consensus type II IL-6 response element of the sequence CTGGGAA within their promoters, with the promoter of the γ gene of fibrinogen containing three of these elements (53). Binding of dimerized STAT3 to each gene promoter leads to an increase in the transcription of all three fibrinogen genes, ultimately leading to an increase in fibrinogen protein production.

In addition to regulating the transcriptional activity of target genes, cytokines also play a role in post-transcriptional regulation of splicing of several genes. McKay et al. (87) have shown that IL-1β and TGF-β are able to regulate alternative splicing of the fibronectin gene in cell culture. Additionally, Li et al. (88) have shown that IL-6 is able to alter the splicing pattern of the Bcl-XL gene, switching from the canonically spliced short form to the alternatively spliced long form over time when treated with IL-6. These are relevant observations to the present investigations, as the fibrinogen γ gene is also alternatively spliced, producing both the γA and γ' fibrinogen chains.

An ongoing study in our laboratory, the Periodontitis And Vascular Events (PAVE) study, is investigating the levels of total and γ' fibrinogen in patients with MI and periodontitis, a chronic inflammatory condition. This study indicates that patients with both periodontitis and MI have much higher levels of total and γ' fibrinogen as compared to patients with only MI (Kristine Alexander, data not published). This increase in the amount of total and γ' fibrinogen also correlated with the well-known inflammatory marker C-Reactive Protein (CRP). An alteration in the ratio of γA/γA fibrinogen to γA/γ' fibrinogen could have significant consequences since, as mentioned previously, increases in γA/γ' fibrinogen concentration have been shown to be associated with cardiovascular disease. Therefore, the study of factors and signals that regulate the production of γ' fibrinogen is an important step in understanding the physiological and pathophysiological functions of γ' fibrinogen. Here we examine the role of the acute phase reaction in the regulation of γ' fibrinogen and find that inflammatory cytokines play a role in the regulation of both total and γ' fibrinogen levels. Specifically, IL-6 and TNF-α decrease

the ratio of γ'/total fibrinogen in HepG2 cells. IFN-γ increases the ratio of γ' to total fibrinogen and TGF-β and IL-1β do not affect the ratio of γ'/total fibrinogen, but affect both fibrinogen isoforms in tandem.

5.2 Materials and Methods

Reagents- Recombinant human IL-6, TNF-α, TGF-β, IL-1β and IFN-γ were purchased from Leinco Technologies.

Cell Culture- HepG2 cells were cultured as described in the General Methods Section in Chapter 2. Cells were treating with increasing doses of individual cytokines as well as combinations of cytokines for 24 hours.

ELISAs- ELISAs for total and γ' fibrinogen were performed as described in the General Methods section in Chapter 2. Calculations of the ratio of γ'/total fibrinogen were performed by dividing the mean γ' fibrinogen concentration by the mean total fibrinogen concentration and these values were plotted as a function of cytokine concentration.

Statistical Analysis- Dose-response curves were generated for γ' and total fibrinogen production for each cytokine. Data is shown as mean $\pm$ SEM. Data was analyzed using the SPSS Version 17.0 Statistics Software Package. One-way ANOVA with Tukey's post hoc test was used to compare differences between treated and untreated cells for each fibrinogen isoform. Paired student t-tests were used to compare total fibrinogen to γ' fibrinogen at each cytokine concentration. Additionally differences between the two dose response curves were investigated using ANOVA with Tukey's pot-hoc correction. Significance cutoffs for all analyses were set at $p \leq 0.05$.

5.3 Results

Analysis of the Effects of TNF-α on Fibrinogen γ Chain Isoform Production in HepG2 Cells- TNF-α is a known modulator of the acute phase response and is involved in systemic inflammation. The role of TNF-α in the modulation of the type II acute phase proteins, especially fibrinogen, has been controversial in the literature. In an *in vivo* study examining the role of TNF-α in *Candida albicans* infection, Riipi et al demonstrated that administration of recombinant murine TNF-α to mice leads to a significant increase in the concentration of circulating fibrinogen (89). However, a study performed in HepG2 cells showed an inhibition of fibrinogen synthesis by TNF-α (83). Therefore we sought to determine the effects of TNF-α on the production of both total and γ' fibrinogen in HepG2 cells. As shown in Figure 5.3, addition of recombinant TNF-α did not alter the levels of total or γ' fibrinogen significantly as compared to untreated cells, which is in contrast to both of the above findings. Additionally, TNF-α had a slightly inhibitory effect on the production of γ' fibrinogen, reducing γ' fibrinogen up to 30% at the highest doses tested. There was a significant difference between the γ' and total fibrinogen dose response curves by one-way ANOVA ($p=0.031$). The ratio of γ'/total fibrinogen also decreased in a dose dependent manner (Figure 5.4). These findings show that TNF-α has differential effects on the production of total fibrinogen and γ' fibrinogen production in HepG2 cells.

Effects of IL-1β and TGF-β on Fibrinogen γ Chain Isoform Production- IL-1β is a member of the proinflammatory IL-1 superfamily produced by macrophages, monocytes

and dendritic cells in response to infection. IL-1β has several effects on the coagulation system, including increasing the release of von Willebrand Factor and plasminogen activator and inhibiting the anticoagulant protein C pathway (90). Interestingly, IL-1β is also known to bind to fibrinogen and fibrin, and this binding leads to increased activity of IL-1β (91). Previous studies have shown that IL-1β has an inhibitory effect on fibrinogen production in HepG2 cells (83), however the effects of IL-1B on γ' fibrinogen have not previously been examined. As shown in Figure 5.5, the addition of recombinant IL-1β to HepG2 cells caused an initial decrease in the production of both total and γ' fibrinogen, which was significant at 0.125 ng/ml and 0.25 ng/ml IL-1β ($p< 0.05$). There was no significant difference between the dose response curves by one-way ANOVA ($p=0.35$). Since both total and γ' fibrinogen decreased to a similar extent, this suggests that the inhibition may be occurring at the promoter level. The ratio of γ'/total fibrinogen remained unchanged upon treatment with IL-1β (Figure 5.4).

TGF-β is a cytokine known to have both pro- and anti-inflammatory functions and is produced in several different cell types. Its specific function depends on the extracellular environment and cues received from surrounding cells. TGF-β signaling has been implicated in numerous pathological processes, including atherogenesis (92). Hence, we wanted to examine the effects of TGF-β on the production of total and γ' fibrinogen in HepG2 cells. Several previous studies have shown that TGF-β inhibits the production of total fibrinogen in both HepG2 and Hep3B hepatocellular carcinoma cell lines (93, 94). As seen in Figure 5.6, our data is consistent with these prior studies in that addition of TGF-β to HepG2 cells led to a dose-dependent decrease in total fibrinogen produced

($p<0.05$ when compared to untreated cells). Additionally, a dose-dependent decrease in the production of γ' fibrinogen was also observed. However, there was no significant difference between the dose response curves by one-way ANOVA ($p=0.86$). Moreover, the ratio of γ'/total fibrinogen remained relatively unchanged upon treatment, except at the highest concentration tested (Figure 5.4) suggesting that the action of TGF-β on fibrinogen production, like IL-1β, may occur at the promoter level. These results suggest that both IL-1β and TGF-β lead to decreased expression of both total and γ' fibrinogen in tandem.

Effects of IL-1β and TGF-β on IL-6 Mediated Fibrinogen Induction- The initial pro-inflammatory cytokines produced during the acute phase response include TGF-β, IL-1β and IL-6. TGF-β and IL-1β are also capable of increasing the production of IL-6, the major regulator of hepatic gene response during the acute phase reaction. Therefore, we sought to examine the effects of TGF-β and IL-1β on the IL-6 induced production of total and γ' fibrinogen. In agreement with a previous study performed by Zhang and Fuller (65), we found that IL-1β inhibited IL-6 mediated fibrinogen expression. The initial addition of IL-6 led to a significant increase in both total and γ' fibrinogen production ($p<0.001$), and co-treatment with IL-1β and IL-6 led to a significant decrease in the production of both isoforms as compared to IL-6 alone ($p<0.001$ for total fibrinogen, $p<0.01$ for γ' fibrinogen). This inhibition did not show statistical significance when comparing the dose response curves for production of the two isoforms by ANOVA ($p=0.65$) and was dose dependent for γ' fibrinogen, but did not exert any dose-dependent

effects on total fibrinogen (Figure 5.7). Additionally, TGF-β inhibited the IL-6 mediated increase in both total and γ' fibrinogen in a dose-dependent manner (Figure 5.8). In this particular experiment γ' fibrinogen was not as responsive to IL-6 or to TGF-β mediated inhibition of IL-6, leading to a significant difference between the two dose response curves as analyzed by ANOVA ($p=0.01$). IL-6 treatment alone led to an expected significant increase in both total and γ' fibrinogen ($p<0.001$). Co-treatment with IL-6 and TGF-β led to a significant decrease in total fibrinogen at 1.25, 2.5 and 5 ng/ml TGF-β ($p<0.05$ as compared to untreated cells) and a significant decrease in γ' fibrinogen at all concentrations tested ($p<0.05$ as compared to untreated cells). Together, these results demonstrate that IL-1β and TGF-β inhibit the IL-6 mediated induction of total and γ' fibrinogen. Since each of the γ chain isoforms was affected similarly in each case, the simplest hypothesis is that inhibition of IL-6 mediated fibrinogen induction occurs at the promoter level.

Analysis of the Effects of IFN-γ on Fibrinogen γ Chain Isoform Production- As described in earlier chapters, IFN-γ is a newly discovered regulator of fibrinogen synthesis. Upon treatment with IFN-γ, total fibrinogen expression is inhibited, although not significantly, via a STAT1 mediated mechanism. We also sought to measure the effects of IFN-γ on γ' fibrinogen production. As expected for a mechanism that affects transcription at the γ chain promoter, IFN-γ also slightly decreased the expression of γ' fibrinogen (Figure 5.9). However, the extent of inhibition of γ' fibrinogen was not as great as that of total fibrinogen and neither was statistically significant as compared to untreated cells.

Additionally, there was no significant difference between the two using one-way ANOVA (p=0.4). Additionally, the ratio of γ'/total fibrinogen increased upon the addition of IFN-γ (Fig 5.4), as would be expected when total fibrinogen is decreased to a greater extent than that of γ' fibrinogen.

Analysis of the effects of IL-6 on fibrinogen γ chain isoform production- IL-6 is a known inducer of fibrinogen synthesis in HepG2 cells, and in agreement with several previous reports of its induction potential, we saw an approximately 2-fold increase in the production of total fibrinogen when HepG2 cells were treated with recombinant IL-6 (Figure 5.10), which was statistically significant ($p<0.001$). Unexpectedly, we did not see a corresponding increase in the production of γ' fibrinogen. γ' fibrinogen production increased only at supraphysiological levels of IL-6, in the range of 100 ng/ml. There was no statistically significant difference in γ' fibrinogen production achieved in any of the concentrations tested. There was a significant difference between the dose response curves by one-way ANOVA (p=0.034). This increase in only total fibrinogen led to a decrease in the ratio of γ'/total fibrinogen (Figure 5.4). Together these results show that IL-6 is able to differentially regulate the production of total and γ' fibrinogen in HepG2 cells.

Fig. 5.1

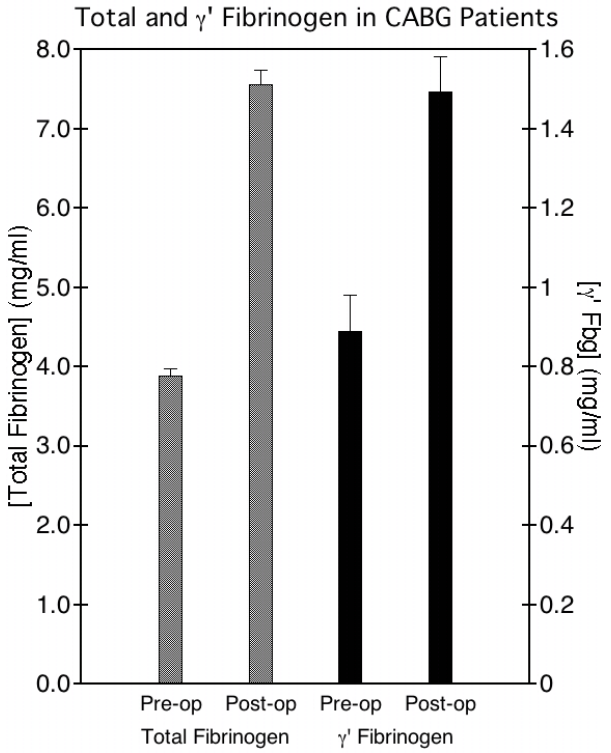


Figure 5.1: Total and γ' fibrinogen levels increase following coronary artery bypass graft surgery Pre- and post- operative levels of total and γ' fibrinogen were measured by ELISA in 76 patients scheduled to undergo CABG surgery. Results are shown +/- standard deviation.

Fig 5.2

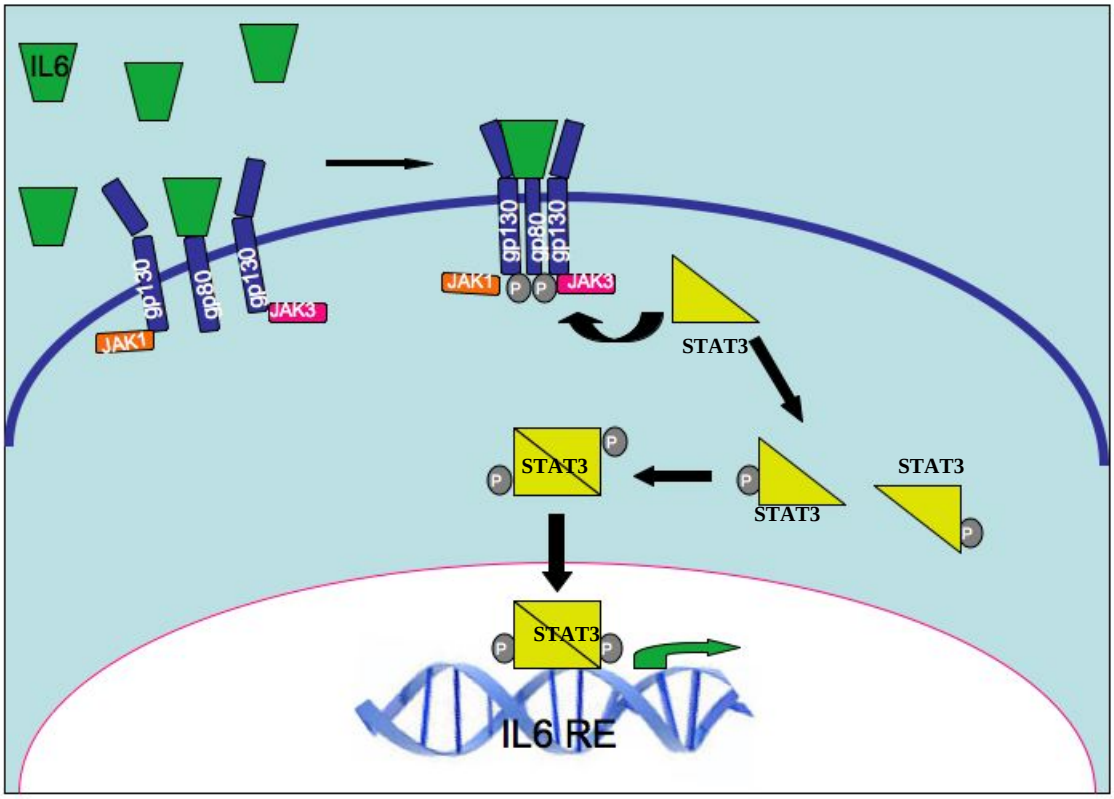


Figure 5.2 The IL-6 Signaling Pathway. Signaling through IL-6 is mediated by the cell surface receptors gp130 and gp80. Binding of IL-6 to gp80 leads to dimerization of the receptor complex and activation of JAK1/3, which phosphorylates the receptor. Subsequently, STAT3 is recruited to the complex where it is phosphorylated. Phosphorylated STAT3 dimerizes and travels to the nucleus where it binds to IL-6 Response Elements (IL6 RE's) to affect target gene transcription.

Fig 5.3

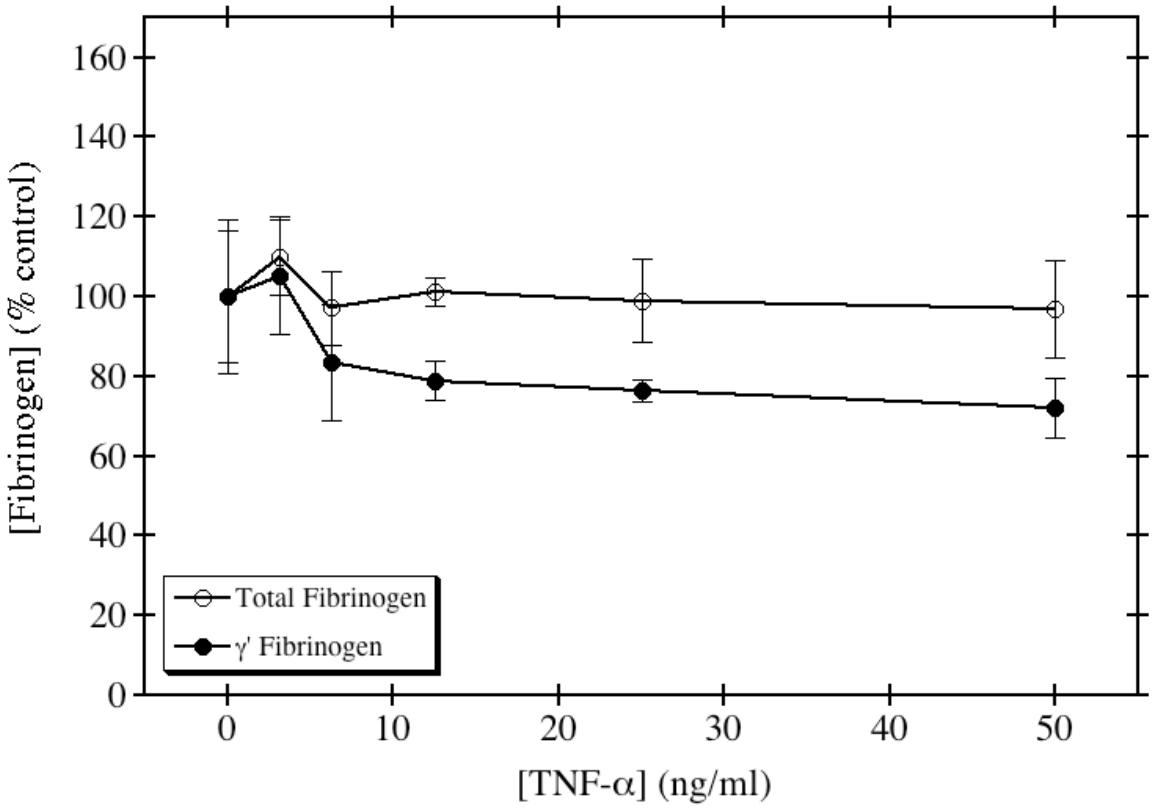


Figure 5.3 Analysis of the effects of TNF-α on fibrinogen γ chain isoform production in HepG2 cells. HepG2 cells were grown to confluence and incubated with medium containing increasing concentrations of TNF-α for 24 hours. Total and γ' fibrinogen levels were assayed by ELISA and the results normalized to the amount of total protein from whole cell lysates.

Fig. 5.4

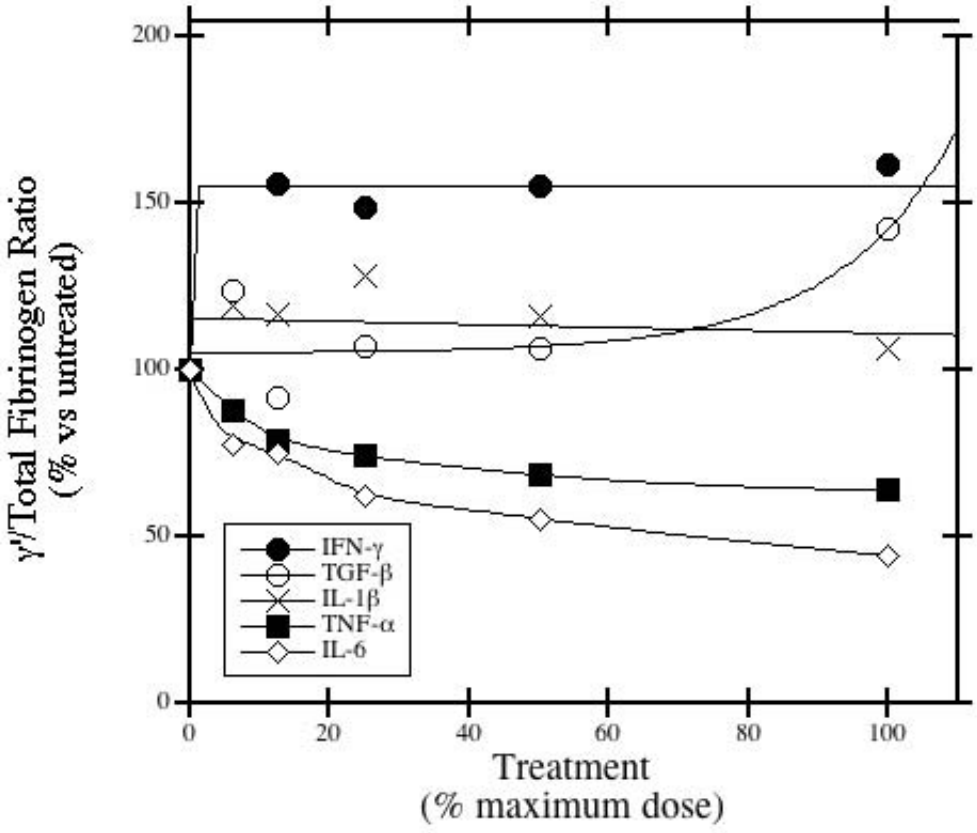


Figure 5.4 Effect of Cytokine Treatment on the Ratio of γ' Fibrinogen to Total Fibrinogen. ELISA measurements of γ' fibrinogen were divided by those of total fibrinogen for each of the above experiments. Ratios are plotted as percent change compared to untreated cells on the y-axis and as % of maximum cytokine dose on the x-axis. The maximum doses of each cytokines were 12.5 ng/ml IFN-γ, 5 ng/ml TGF-β, 1 ng/ml IL-1β, 50 ng/ml TNF-α and 100 ng/ml IL-6.

Fig. 5.5

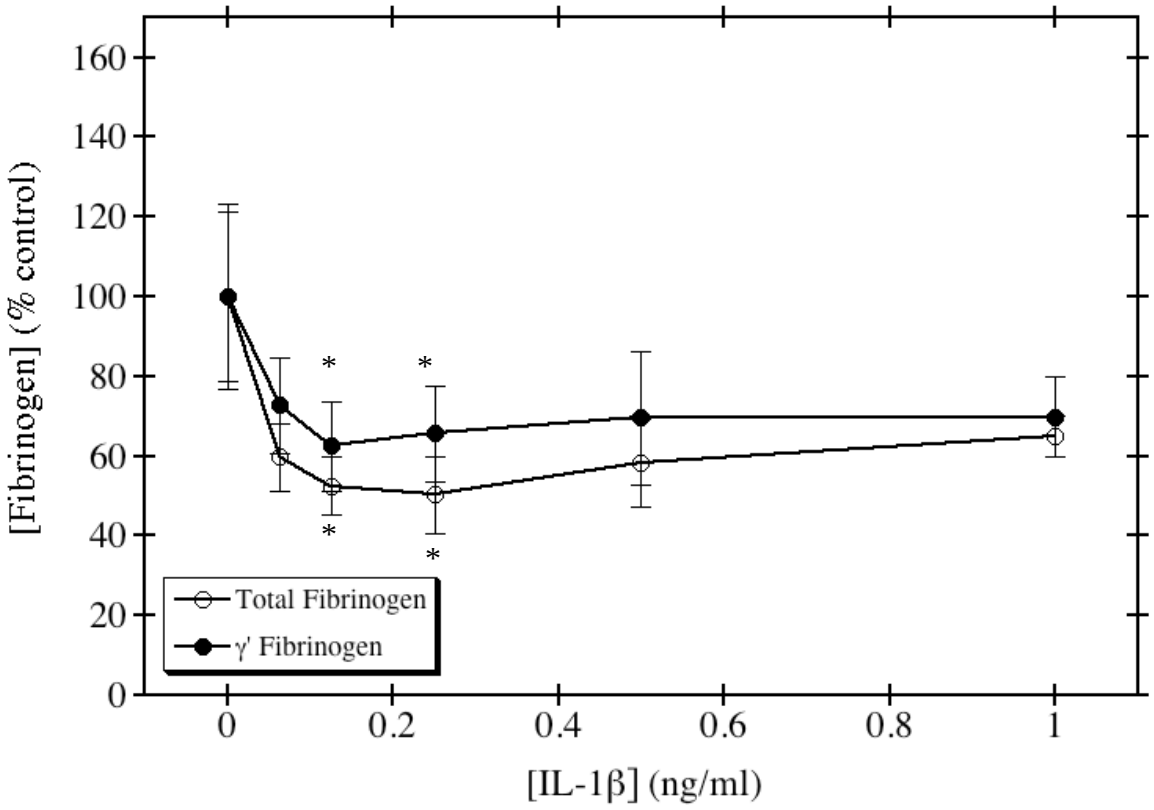


Figure 5.5 Effects of IL-1β treatment on fibrinogen γ chain isoform production.

HepG2 cells were grown to confluence and incubated with medium containing increasing concentrations of IL-1β for 24 hours. Total and γ' fibrinogen levels were assayed by ELISA and the results normalized to the amount of total protein from whole cell lysates. * $p<0.05$ compared to untreated cells as analyzed by ANOVA.

Fig. 5.6

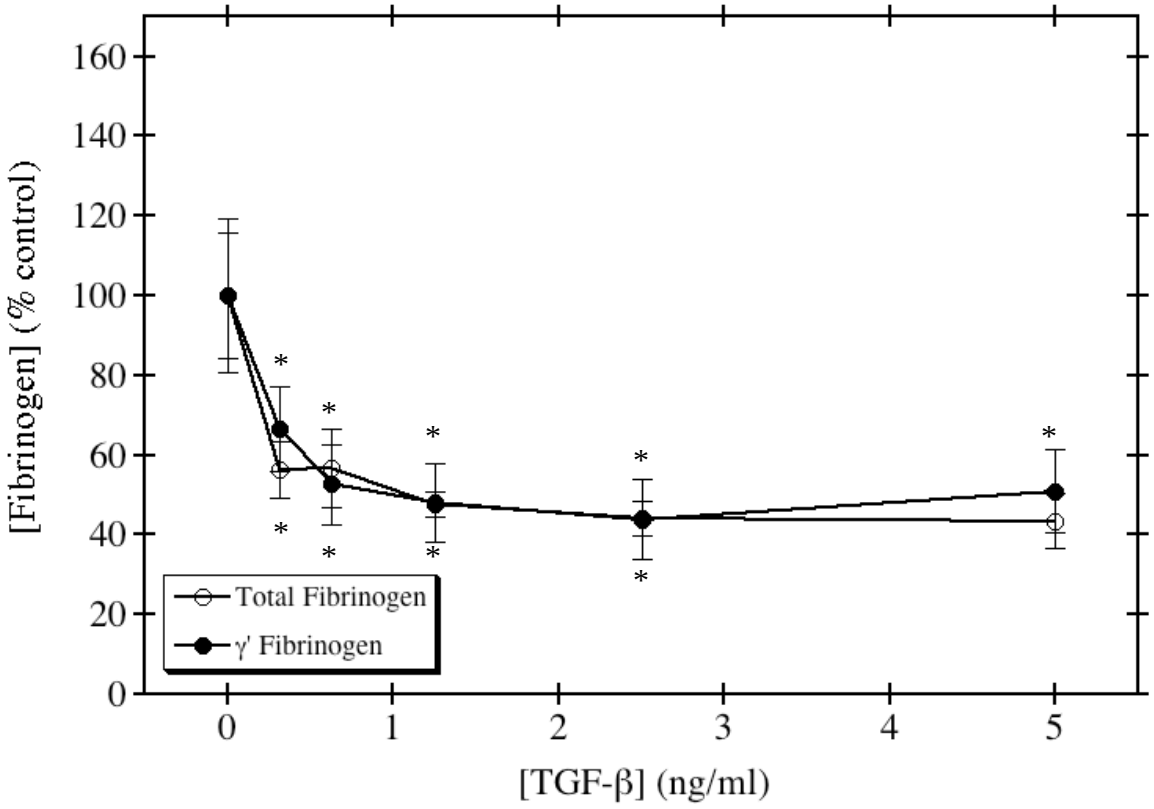


Figure 5.6 Effects of TGF-β on fibrinogen γ chain isoform production. HepG2 cells were grown to confluence and incubated with medium containing increasing concentrations of TGF-β for 24 hours. Total and γ' fibrinogen levels were assayed by ELISA and the results normalized to the amount of total protein from whole cell lysates. * $p<0.05$ compared to untreated cells as analyzed by ANOVA.

Fig. 5.7

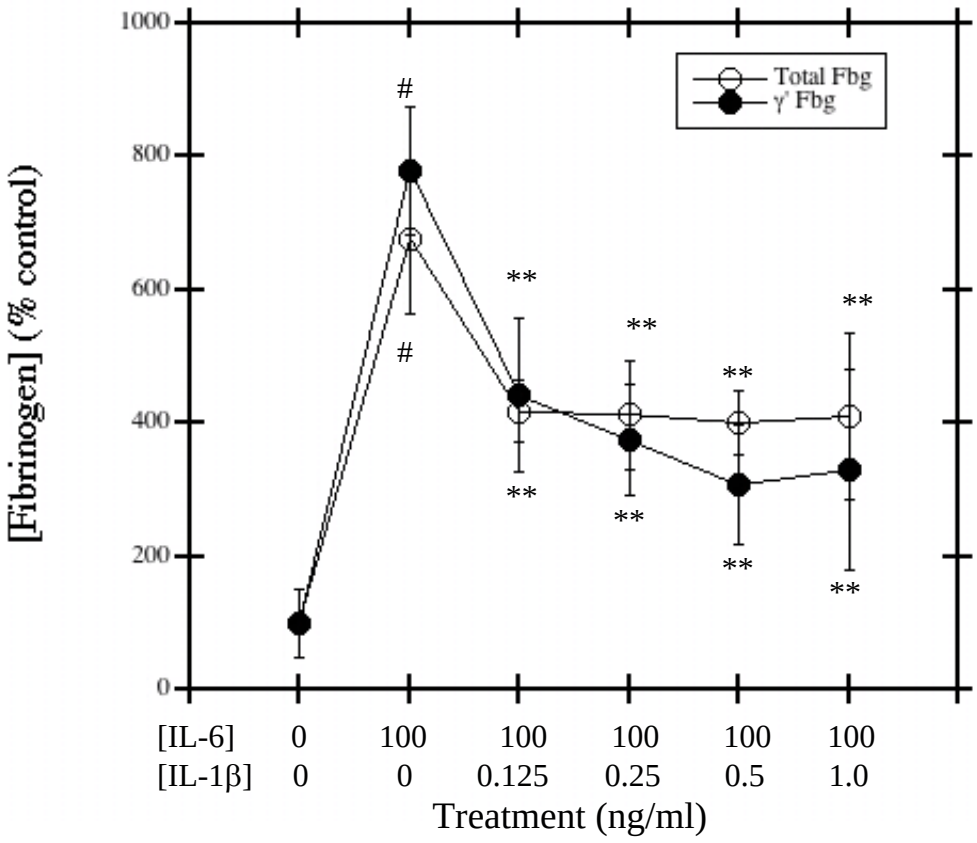


Figure 5.7 Effects of IL-1β on IL-6 mediated fibrinogen induction. HepG2 cells were treated with 100 ng/ml IL6 alone or in combination with increasing concentrations of IL-1β. Cells were harvested and γ' and total fibrinogen was measured by ELISA. # $p<0.001$ when compared to untreated cells by ANOVA. ** $p<0.001$ when compared to cells treated with IL-6 alone by ANOVA.

Fig 5.8

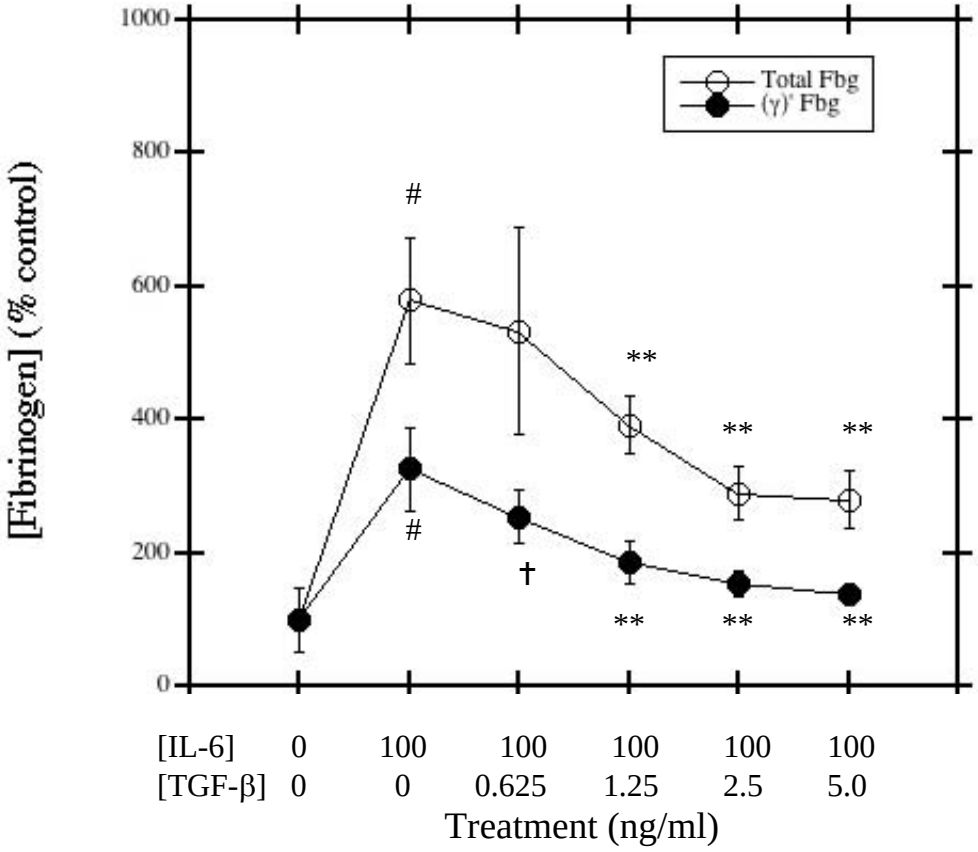


Figure 5.8 Effects of TGF-β on IL-6 mediated fibrinogen induction. HepG2 cells were treated with 100 ng/ml IL6 alone or in combination with increasing concentrations of TGF-β. Cells were harvested and γ' and total fibrinogen was measured by ELISA. #p<0.001 when compared to untreated cells by ANOVA. †p<0.01 when compared to cells treated with IL-6 alone by ANOVA. **p<0.001 when compared to cells treated with IL-6 alone by ANOVA.

Fig. 5.9

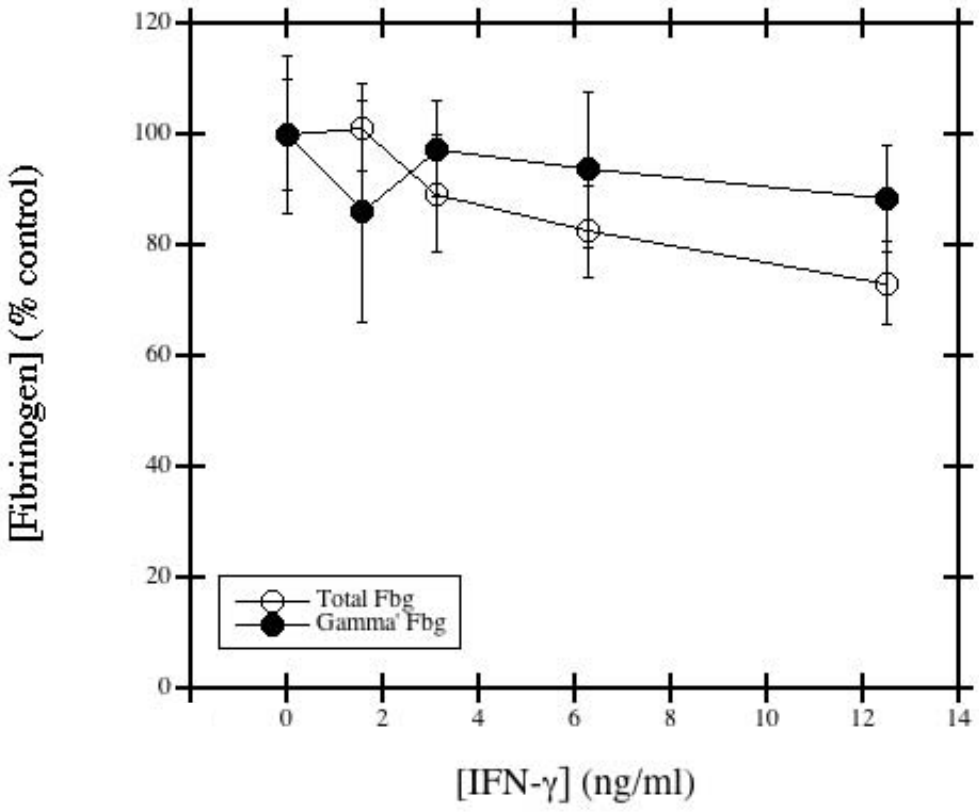


Figure 5.9 Effects of IFN-γ treatment on fibrinogen γ chain isoform production.

HepG2 cells were grown to confluence and treated with increasing concentrations of IFN-γ for 24 hours. Total and γ' fibrinogen levels were assayed by ELISA and the results were normalized to total protein from whole cell lysates.

Fig. 5.10

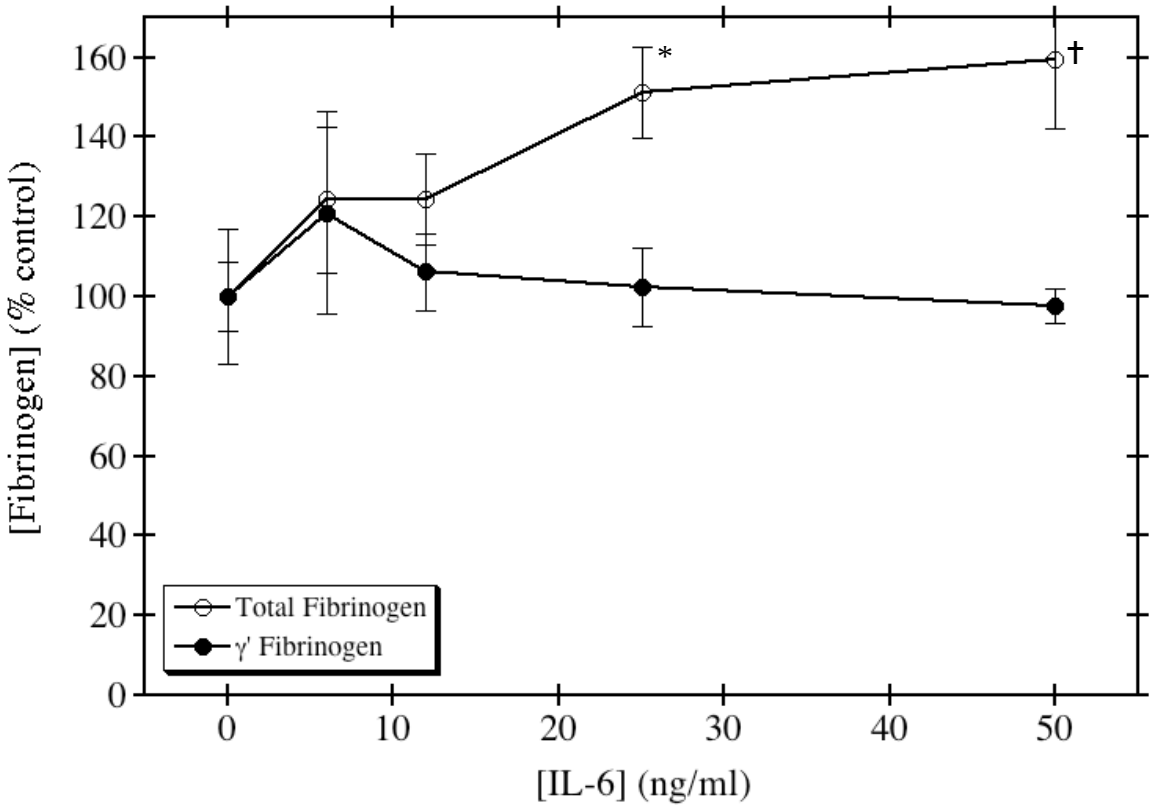


Figure 5.10 Effects of IL-6 treatment on fibrinogen γ chain isoform production.

HepG2 cells were treated with increasing concentrations of IL-6 for 24 hours. Total and γ' fibrinogen were assayed by ELISA and results were normalized to total protein from whole cell lysates. * $p<0.05$ when compared to untreated cells. † $p<0.01$ when compared to untreated cells.

5.4 Discussion

Total fibrinogen is a known acute phase reactant, responding to the actions of several cytokines generated during an acute phase response to injury or infection, but the role of γ' fibrinogen as an acute phase reactant was previously unknown. This study is the first to show that three inflammatory cytokines, IL-6, TNF-α and IFN-γ, are able to differentially regulate the production of γ' fibrinogen, thereby altering the γ'/total fibrinogen ratio. The γ' isoform of fibrinogen is a newly-emerging risk factor for cardiovascular disease (95,96) that arises from alternative processing of fibrinogen γ chain pre-mRNA (13,14). However, the events that regulate the alternative splicing event remain unknown. A recent report demonstrated that concentrations of γ' fibrinogen are higher in patients with ischemic stroke during the acute phase, as compared to the convalescent phase (97), suggesting that there is an increase in the alternative splicing event that produces γ' fibrinogen during this period.

While both total and γ' fibrinogen levels are individually associated with cardiovascular disease, the ratio of γ'/total fibrinogen has also been implicated in thrombotic disease. Previous studies have linked an increased γ'/total fibrinogen ratio to arterial thrombosis (98), while decreased γ'/total fibrinogen ratios have been associated with deep vein thrombosis (99). Therefore the ratio of γ'/total fibrinogen may be a useful predictive tool for thrombotic risk, and the factors that modulate this ratio may play an important role in the development of cardiovascular disease.

Although IL-1β and TGF-β inhibited the production of both total and γ' fibrinogen in HepG2 cells (Figs 5.5 and 5.6), the fact that both fibrinogen isoforms decreased by similar amounts and the ratio of γ'/total fibrinogen did not change suggests that IL-1β and TGF-β exert their effects at the promoter level, decreasing the total amount of γ chain mRNA. TGF-β has previously been shown to inhibit the production of total fibrinogen in a hepatocyte cell line (93), presumably mediated by Smads 3 and 4, downstream effector molecules of the TGF-β signaling pathway. TGF-β is also known to inhibit the IL-6 mediated increase in fibrinogen synthesis via Smad3 and Smad4 (94), which inhibit STAT3 activity at the promoters of IL-6 responsive genes (100). Our finding that TGF-β inhibits IL-6 mediated total fibrinogen and γ' fibrinogen synthesis (Fig 5.8) supports this previous finding. Similarly, IL-1β is also known to inhibit IL-6 mediated fibrinogen synthesis, although this inhibition is caused by activation and nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which directly inhibits the binding of STAT3 to the IL-6 response elements within the fibrinogen promoter (65). Once again, we see a similar trend when we examine the effects of IL-1β on IL-6 mediated total and γ' fibrinogen production (Fig 5.7).

The effects of TNF-α on the production of total and γ' fibrinogen were less robust than those of the other cytokines we examined. We did not see any change in the amount of total fibrinogen produced by HepG2 cells following treatment with increasing concentrations of TNF-α (Figure 5.3). This is in contrast to *in vivo* studies in mice, which showed an increase in circulating fibrinogen levels after they were treated with *Candida albicans*, an inducer of TNF-α, or with recombinant TNF-α (89). However, treatment

with *C. albicans* may be inducing the expression of several other cytokines as well as TNF-α in this study, which may play a role in increasing fibrinogen levels. As previously mentioned, there is seldom a scenario in which only one cytokine is present and affecting expression of acute phase proteins, and TNF-α may itself be inducing or repressing other cytokines that may affect the production of fibrinogen. More interesting to us was the significant decrease of γ' fibrinogen production following TNF-α treatment. TNF-α is not known to alter the splicing or polyadenylation of mRNA of other genes directly, and its role in signaling has generally been to induce cell death. However, signaling through TNF-α is known to activate the MAP kinase pathway, which has been shown to play a role in alternative pre-mRNA splicing as well as polyadenylation (101,102).

As described in Chapter 3, we identified IFN-γ as a novel regulator of total fibrinogen production in HepG2 cells via a STAT1 mediated mechanism. As we saw that STAT1 was affecting fibrinogen transcription at the promoter level, we were surprised to see a differential regulation in the amounts of total and γ' fibrinogen produced. While we again observed a decrease in the amount of total fibrinogen produced by HepG2 cells following IFN-γ treatment, we did not see any significant change in the amount of γ' fibrinogen produced. Similar to TNF-α, IFN-γ is not known to directly alter splicing or polyadenylation of genes, but usually acts at the transcriptional level to alter the amount of total mRNA of target genes. However, it is possible that activation of the IFN-γ signaling pathway may induce expression or activation of other molecules that may affect splice site selection or polyadenylation.

IL-6 is a well-studied inducer of fibrinogen synthesis, due to the presence of several IL-6 response elements within the promoters of the three fibrinogen genes. Most notably, the fibrinogen γ chain promoter contains three such IL-6 response elements (53), making it very responsive to IL-6 stimulation. This promoter level regulation would lead us to expect similar increases in the levels of total fibrinogen and γ' fibrinogen. Surprisingly, we saw an increase in the amount of total fibrinogen produced but no increase in γ' fibrinogen production (Fig 5.10), leading to a decrease in the ratio of γ'/total fibrinogen (Figure 5.4). This surprising decrease in the amount of γ' fibrinogen produced does not directly contradict previous work (97) due to the fact that IL-6, and all of the individual cytokines mentioned in this chapter, do not work alone, but instead they interact with each other leading to cross-talk between signaling pathways. The temporal regulation of the expression of these cytokines will determine their interactions with one another during the acute phase, whether they are synergistic or antagonistic. These interactions may differentially regulate the response of acute phase reactants, such as fibrinogen, depending on which cytokines are present at the time and site of inflammation.

Extracellular signals have been known to alter the splicing of several genes by changing the phosphorylation patterns of proteins of the spliceosome and the polyadenylation pathway (103). Specifically, IL-6 has been shown to alter the splicing pattern of Bcl-XL in a leukemia cell line, and this alteration in splicing requires different intronic sequences be present in the downstream intron (88). As mentioned earlier, TNF-α, as well as IL-6 and IFN-γ, are known to activate different members of the MAP kinase family, some of which are involved in splicing and polyadenylation reactions. These alterations in

splicing or polyadenylation may provide a mechanism for the change in γ' fibrinogen following treatment with IL-6, IFN-γ and TNF-α that we see in this study. Alternatively, addition of these cytokines may alter the protein or mRNA stability of the γ chain isoforms, leading to preferential degradation of one isoform over the other. Investigation into each of these mechanisms will provide us with a much greater understanding of how splicing and polyadenylation of the fibrinogen γ chain pre-mRNA is regulated, and may provide us with ways of altering the production of the two isoforms, as well as the γ'/total fibrinogen ratio, thus altering the risk for cardiovascular disease.

Chapter 6: Identification and Characterization of a γ Chain R275C Mutation in a Patient with Dysfibrinogenemia

The work presented in this chapter has been accepted for publication in Blood Coagulation and Fibrinolysis

6.1 Introduction

Congenital abnormalities of fibrinogen are a set of rare disorders that include afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia. Afibrinogenemia and hypofibrinogenemia are quantitative defects resulting in the complete absence of circulating fibrinogen or low circulating fibrinogen levels, respectively. Afibrinogenemia is inherited in an autosomal recessive fashion and affects both males and females equally. Patients with afibrinogenemia have a severe bleeding phenotype during infancy and childhood, but symptoms usually decrease with age (104). Hypofibrinogenemia has several modes of inheritance and patients usually suffer from bleeding episodes early in life that become less severe as the patient ages.

Dysfibrinogenemias are qualitative defects in the fibrinogen molecule that lead to a decrease in the function, but usually not the quantity, of fibrinogen. Interestingly, most patients with a dysfibrinogenemia are asymptomatic and are only identified by chance through routine coagulation tests. In those that are symptomatic, some have hemorrhagic phenotype, some a thrombotic phenotype and others show both hemorrhagic and thrombotic tendencies. To date, there have been over 450 dysfibrinogenemias identified

in all three fibrinogen genes, the majority of which have a defect at the site of fibrinopeptide release, leading to abnormal fibrin polymerization.

The γ chain mutation γR275C has been identified in 17 named dysfibrinogens (105). All of these reported dysfibrinogens have the classic phenotype of a mutant fibrinogen, with a prolonged clotting time and a high antigen to activity ratio (106). Yet the phenotype of these dysfibrinogens varies considerably, with the majority of dysfibrinogens containing the γR275C mutation being phenotypically silent. However, Fibrinogen Hannover IV and Hershey IV are associated with hemorrhage, while Fibrinogens Bellingham, Bologna, Cedar Rapids, and Villajoyosa are associated with thrombosis, although all of these dysfibrinogens contain the γR275C mutation. A mechanistic explanation for the hemorrhagic phenotype associated with Fibrinogen Hershey IV is also complicated by the fact that the Hershey IV proband is a compound heterozygote with a novel γV411I mutation in the platelet integrin αIIbβ3 binding site (107). The present chapter provides evidence that the γR275C mutation alone is sufficient to cause a hemorrhagic phenotype.

6.2 Materials and Methods

Reagents- AXL203 polyclonal rabbit anti-human fibrinogen antibody was purchased from Zymed, Inc. Immunoprecipitation kits were purchased from Pierce. Micro protein trap cartridges were purchased from Michrom Bioresources, Inc.

Clinical testing- All experiments were conducted with the understanding and the consent of the proband, and the experiments were approved by the OHSU Institutional Review Board, IRB #2792. Standard clinical coagulation tests were performed on the proband, including the activated partial thromboplastin time (aPTT), prothrombin time (PT), thrombin time (TT), and fibrinogen level. Fibrinogen antigen testing was performed using radial immunodiffusion by ARUP Laboratories (Salt Lake City, UT, USA). Standard liver function tests were also performed on the proband.

DNA sequencing- Genomic DNA was purified from whole blood using the QIAamp DNA blood kit. DNA sequencing of the coding regions of each of the three fibrinogen genes as well as the intron-exon boundaries was performed as described previously (108). The DNA sequence for the coding regions was obtained for the Aα, Bβ and γ genes (*FGA*, *FGB*, and *FGG*, respectively) from both strands.

Fibrinogen purification- Citrate-anticoagulated plasma was collected from the proband and from an anonymous control donor. Fibrinogen was purified from each sample using glycine precipitation (107). Purified fibrinogens were analyzed on a 10% sodium

dodecyl sulfate polyacrylamide gel under reducing conditions followed by Coomassie blue staining.

Immunoprecipitation and Western blotting- Fibrinogen was immunoprecipitated following purification using a Co-Immunoprecipitation Kit (Pierce) according to the manufacturer's instructions. Briefly, AXL203 polyclonal rabbit anti-human fibrinogen antibody was covalently linked to agarose resin. Antibody bound-beads were washed and incubated with 250 μg of purified control and proband fibrinogen overnight at 4˚C. Bound proteins were eluted in a low pH buffer and were separated on a 10% sodium dodecyl sulfate polyacrylamide gel under reducing conditions. Protein was transferred to a nitrocellulose membrane and the membrane was blocked in 1% gelatin for two hours. The membrane was then probed using a rabbit anti-human albumin antibody at 4˚C overnight. The membrane was then incubated with a goat anti-rabbit Alexafluor 680 secondary antibody for one hour at room temperature and the membrane was imaged using a LiCor Odyssey Imaging System.

Protein sequencing- Protein sequencing was performed at the OHSU Protein Core. Five to 10 μg portions of purified control and mutant fibrinogen were dried by vacuum centrifugation and dissolved in 10 μl of 8M urea/1.0M Tris/0.2M methylamine, pH 8.5. One μl of 0.2M dithioerythritol was then added, and the samples were incubated at 50˚C for 15 minutes, followed by the addition of 2 μl of 0.5M iodoacetamide and incubation at room temperature for 15 minutes. An additional 2 μl of 0.2M dithioerythritol was then added and samples were diluted to a final 40 μl volume with 5% formic acid. One μg of

each reduced and alkylated sample was injected onto a micro protein trap cartridge and fibrinogen subunits were separated by reverse phase chromatography using a 1.0 x 250 mm C4 column (214 MS C4, Vydac, Hesperia, CA, USA). The column used a 2-60% acetonitrile gradient over 70 minutes in a mobile phase containing 0.1% formic acid and a 19 μl/minute flow rate. Spectra were collected in profile mode over a 600-2000 m/z range while averaging 20 μscans. Data collected during the elution of the fibrinogen γ chain were averaged and deconvoluted using BioMass Calculation and Deconvolution Software (Bioworks 3.2, ThermoFinnigan, San Jose, CA, USA). Mass measurements with an error of less than 0.01% were confirmed using horse myoglobin as a standard.

5.4 Results

Case Report- The proband is a 54 year old female with Hepatitis C who presented in 2001 with heavy menstrual bleeding that became progressively worse, required a hysterectomy in 2002, and was diagnosed at that time with dysfibrinogenemia. She has a history of excessive menstrual blood flow since menarche at age nine, but delivered three children with no noted bleeding abnormalities. Minor cuts and abrasions generally stopped bleeding spontaneously in 1–2 minutes. She has had rare epistaxis, the last one in her late teens, and has had dental extractions without excessive bleeding. Prior to hysterectomy, she had not been challenged with major surgical procedures, but did have a D&C with polyp removal. She was bleeding at the time of the D&C and continued to have some vaginal bleeding following the procedure. She had not required transfusion therapy prior to hysterectomy for her bleeding episodes. Her hysterectomy was managed with the use of cryoprecipitate prior to surgery, and she did well without excessive bleeding. No fibrinolytic inhibitors were used prior to, during, or after surgery.

The family history was unremarkable; the proband's mother (age 76) had a history of breast cancer, and her father had died at age 72 of what was believed to be a ruptured abdominal aortic aneurysm. The most recent laboratory coagulation tests on the proband revealed a prolonged thrombin time of 47.9 seconds (Table 6.1) suggestive of a fibrinogen abnormality. Fibrinogen antigen levels were normal at 308 mg/dL, but the fibrinogen activity level was low at 132 mg/dL (Table 6.1), suggesting that the patient has a dysfibrinogenemia. Liver function tests showed only mild abnormalities. Alkaline phosphatase, aspartate aminotransferase, and alanine aminotrasnferase were slightly

elevated, but total bilirubin and albumin were within the normal range. These laboratory results suggested little impairment of liver function that might have accounted for a bleeding diathesis.

Characterization of the Mutation- All three fibrinogen genes were sequenced on both DNA strands. Sequencing revealed heterozygosity for a mutation in the fibrinogen γ chain within exon 8 (Figure 6.1). This led to an amino acid substitution of Cys for Arg at residue 275 (γR275C). This mutation has been reported previously to cause dysfibrinogenemia (105). The mutation is located at the interface between D-domains (Fig. 6.2) in polymerized fibrin (110).

Purified Fibrinogen Portland I showed only a very slight change in mobility in the γ chain, consistent with a point mutation (Figure 6.3).

Albumin Binding Studies- Introduction of a new unpaired cysteine residue has been reported to result in disulfide-linked albumin conjugates in Fibrinogen Milano VII containing a γ Ser358Cys mutation (111). To determine whether the proband's fibrinogen contained bound albumin, both proband and normal fibrinogen were immunoprecipitated and western blotted with an antibody against human albumin. There was no detectable binding of albumin to either the proband's or the control fibrinogen (Figure 6.4). In addition, mass spectrometry did not detect any peptide sequences in the purified fibrinogen that corresponded to albumin fragments.

Table 6.1

Parameter (normal range)	July 2002	August 2008
Fibrinogen Antigen (149-353 mg/dl)	326 mg/dl	308 mg/dl
Fibrinogen Activity (250-450 mg/dl)	48-52 mg/dl	132 mg/dl
Prothrombin Time (11-13s)	17-19 s	19.6 s
Thrombin Time (15-17s)	-	47.9 s
CBC	Normal	Normal
APTT (28-30s)	-	36.8 s
Alkaline Phosphatase (35-105 U/ml)	-	120 U/ml
Aspartate Aminotrans. (5-32 U/ml)	-	44 U/ml
Alanine Aminotrans. (5-35 U/ml)	-	43 U/ml
Total Bilirubin (0.1-1.0 mg/dl)	-	0.4 mg/dl
Albumin (3.5-5.2 g/dl)	-	4.4 g/dl

Table 6.1: Clinical Laboratory Results from the Patient with Fibrinogen Portland I.

Standard clinical coagulation and liver function tests were performed on the patient with Fibrinogen Portland I. The normal range of each assay is noted in parentheses.

Fig 6.1

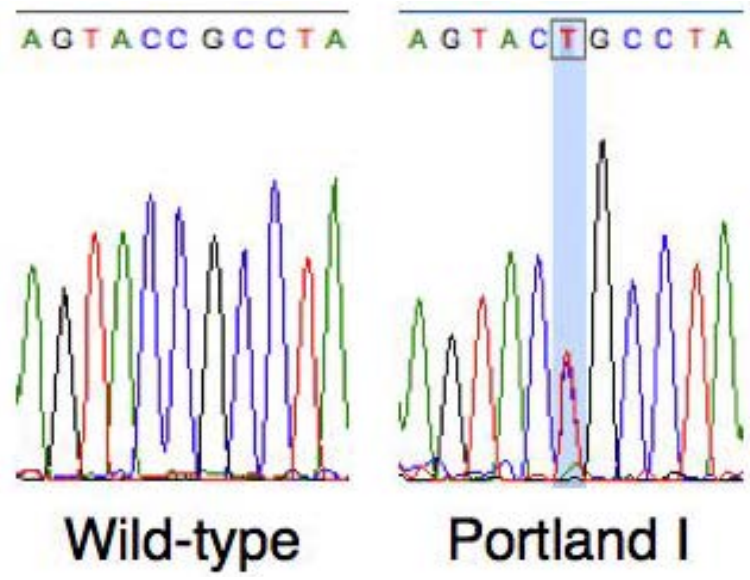


Figure 6.1 Sequencing of Fibrinogen Portland I γ Gene A heterozygous C to T transition is shown at nucleotide position 981 that changes a CGC codon to TGC, resulting in a γ Arg275 to γ Cys275 mutation

Fig. 6.2

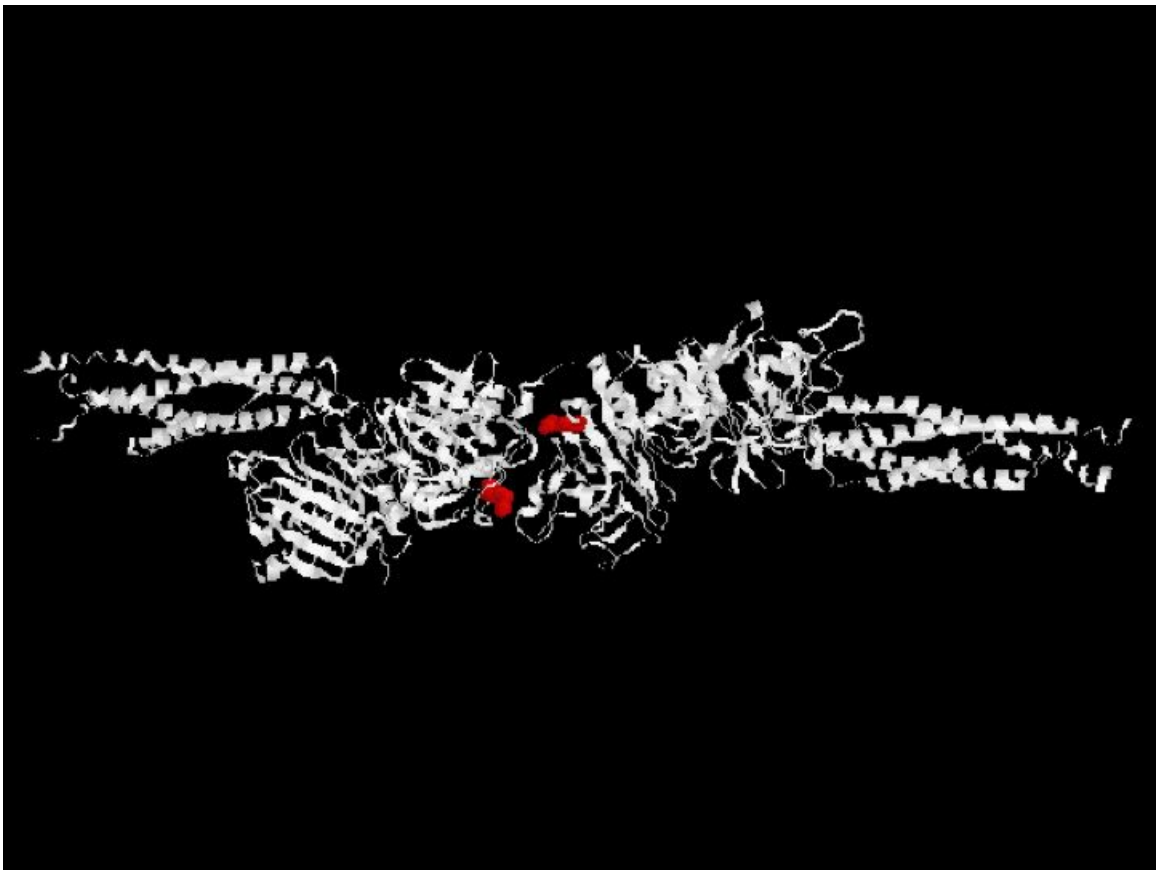


Figure 6.2 Location of the γ Arg275Cys Mutation at the Interface of Fibrin D-domains The γ Arg275 residue that is mutated to a Cys in Fibrinogen Portland I is shown in red. This figure shows the critical location of this amino acid at the interface between polymerized D-domains.

Fig. 6.3

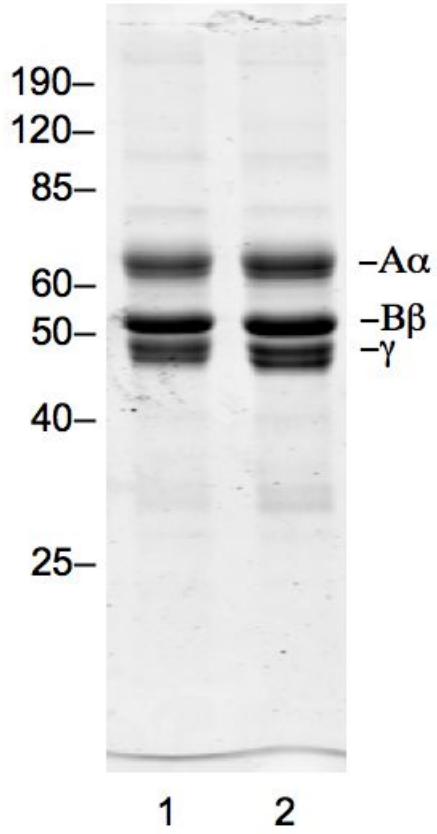


Figure 6.3 Purified Fibrinogen Portland I and Control fibrinogen The fibrinogens were purified using glycine precipitation and analyzed by 10% SDS-PAGE under reducing conditions. Lane 1, Fibrinogen Portland I; lane 2, control fibrinogen. The molecular weights in kDa are shown at left.

Fig. 6.4

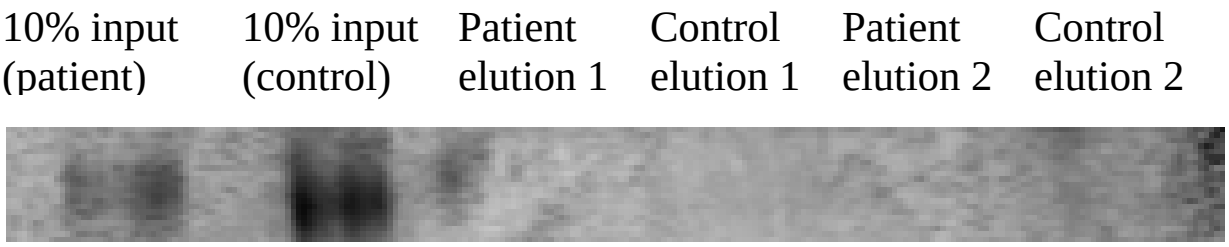


Figure 6.4 Co-Immunoprecipitation of Human Albumin and Fibrinogen from Patient and Control Fibrinogen Samples Fibrinogen was precipitated from patient and control plasma, pulled down using an anti-human fibrinogen antibody, and western blotted for human albumin. Two sequential elutions of fibrinogen off of the antibody-bead complex were performed in order to maximize protein concentration for western blotting.

5.5 Discussion

The γR275C mutation most often results in a lack of clinical symptoms, as seen in Fibrinogens Tokyo II (112), Milano V (113), and Villajoyosa (114). Extensive biochemical studies have revealed that this mutation does not lead to abnormal t-PA or plasminogen binding (114) and does not affect factor XIIIa mediated crosslinking (112). However, this mutation does lead to formation of abnormal fibers within the clot network, suggesting that the γR275C mutation leads to an abnormal D:D association, resulting in impaired fibrin formation (112). These results are in agreement with the location of the mutation at the critical D:D interface within the fibrinogen molecule (Fig. 6.2). In contrast to the asymptomatic patients with the molecular defects described above, the clinical symptoms of this dysfibrinogen usually present as thrombosis (115, 116) and not hemorrhage as seen in the patient described here.

Excessive bleeding was also reported in another patient with the γR275C mutation whose dysfibrinogen, Fibrinogen Hershey IV, was characterized previously by our laboratory (107). In this case, the proband was a 52-year-old woman who gave a history of frequent, sometimes prolonged epistaxis as a child. In addition, she had menorrhagia, most likely secondary to uterine fibroids. Like the proband for fibrinogen Portland I, this patient also underwent a hysterectomy, and a work-up prior to surgery showed a prolonged thrombin time and low functional plasma fibrinogen. However, this patient was a compound heterozygote for another mutation, γV411I, which interrupted the

platelet binding site for integrin GPIIbIIIa, resulting in lower affinity fibrinogen binding. The contribution of each mutation to the bleeding diathesis was therefore unclear. The present case report of fibrinogen Portland I suggests that the γR275C mutation by itself is sufficient to account for the hemorrhagic phenotype.

An additional confounding factor in the etiology of this patient's hemorrhagic phenotype is the presence of Hepatitis C. A recent analysis of intracerebral hemorrhage indicated that the incidence of Hepatitis C infection was greater in patients with spontaneous intracerebral hemorrhage as compared to controls, suggesting that Hepatitis C infection is a risk factor for intracerebral hemorrhage (117). Additionally, patients with Hepatitis C infection also had prolonged PT values, lower platelet counts and lower fibrinogen levels than controls (117), suggesting that Hepatitis C infection may be playing a role in hemorrhage. However, Hepatitis C infection is not traditionally considered to be a cause of spontaneous systemic hemorrhage. The patient's liver function tests were also normal to mildly abnormal, leading us to believe that the primary cause of hemorrhage in this patient is a dysfibrinogenemia, with the possibility that Hepatitis C infection led to exacerbated bleeding.

Chapter 7: Summary and Conclusions

Total fibrinogen and γ' fibrinogen are two independent risk factors that have been shown to increase the risk for myocardial infarction, coronary artery disease and stroke. Determining novel mechanisms that regulate the production of both γ chain isoforms of fibrinogen is an important step in understanding of the etiology of cardiovascular disease.

In addition to being an important hemostatic protein, fibrinogen is also a known acute phase reactant. Fibrinogen levels increase in response to certain inflammatory- and infection-mediated stimuli due to increased hepatic synthesis. Most importantly for this work, IL-6 is known to increase the production of fibrinogen up to 6-fold in HepG2 cells (53). Previously, little was known about the ability of IFN-γ to regulate fibrinogen levels. Following a recent study identifying a member of the interferon signaling pathway as a mediator of circulating fibrinogen levels (50), we examined the role of IFN-γ in fibrinogen regulation. As discussed in Chapter 3, we have shown that IFN-γ is a novel regulator of basal and IL-6 mediated fibrinogen production and that this regulation occurs at the promoter level. We saw an approximate 25% decrease in the production of fibrinogen from HepG2 cells treated with IFN-γ for 24 hours. We also saw a concomitant decrease in the amount of fibrinogen mRNA produced. During this time period, the downstream mediator of IFN-γ, STAT1, was phosphorylated and activated. We also identified a novel GAS within the promoter of the fibrinogen gamma gene, which we have shown is functional and binds to activated STAT1 within the nucleus. We have also shown that pSTAT1 is able to bind to an identified IL-6 response element, blocking the

ability of STAT3 to bind to this element downstream of IL-6 signaling. This led to a decrease in the induction of fibrinogen synthesis by IL-6 when co-treated with IFN-γ. These findings support observations by others that the loss of IFN-γ leads to increased fibrin deposition during *T. gondii* infection (48). While some of these effects have been attributed to an increase in fibrinolysis following IFN-γ administration (49), our results suggest that a decrease in the production of fibrinogen may also play a role. While our results point to IFN-γ as a regulator of fibrinogen synthesis *in vitro*, the role of IFN-γ must also be assessed *in vivo*. In the future, administration of IFN-γ to normal and hyperfibrinogenemic mice, in the presence and absence of infection, will allow us to more definitively assign a role for IFN-γ in fibrinogen synthesis. There are also two additional putative GAS sequences that we have identified, one each within the α and β fibrinogen gene promoters. While our preliminary results suggest that pSTAT1 is not able to bind to the β promoter GAS, the α GAS is able to bind to pSTAT1. Future studies will allow us to determine whether this GAS site is functional and whether binding of STAT1 to this GAS positively or negatively influences the production of fibrinogen.

Because little is known about the mechanisms regulating γ' fibrinogen synthesis, identifying potential modulators of γ' fibrinogen levels will allow us to expand our understanding of its physiological and pathophysiological functions. At this point in time, our understanding of how the γ' isoform of fibrinogen is produced is, for the most part, an unproven theory that is nonetheless accepted by the field. This theory states that γ' fibrinogen mRNA is produced when intron 9 of the fibrinogen gene is polyadenylated and cleaved before it can be removed (13,14). This suggests that spatiotemporal

regulation of both the spliceosome and enzymes involved in polyadenylation may play a significant role in generating more of one isoform over the other. Moreover, any modifications to these enzymes may alter their affinity for the target pre-mRNA, in turn altering the splicing or polyadenylation of intron 9. In Chapter 4, we discuss the novel role of fibrin degradation products, namely D-dimer, in the regulation of γ' fibrinogen synthesis. Upon treatment with D-dimer, HepG2 cells produce less γ' fibrinogen protein and mRNA while the amount of total fibrinogen protein and mRNA produced remains stable. This suggests that D-dimer is somehow altering the splicing or polyadenylation of the fibrinogen γ chain. This data partially agrees with previous work that has shown the addition of D-dimer and D-fragment to cultured cells does not lead to a change in the amount of total fibrinogen produced (75). Previous reports have shown that D-dimers are able to affect the transcription of target genes, such as PAI-1, by activating the transcription factor AP-1 components c-fos/junD (69). While we see an decrease in the amount of γ' fibrinogen produced by HepG2 cells treated with D-dimer, we do not see a concomitant decrease in the amount of total fibrinogen that would suggest a promoter level effect of D-dimers, similar to the effect seen on PAI-1. A limitation of the work described here is that we are using an immortalized cell line, which has been altered in order to survive multiple passages. In order to truly determine the role of D-dimer signaling as it occurs naturally, future studies include the administration of murine D-dimer to mice in order to determine its effects on circulating plasma fibrinogen levels. Although we do not expect to see a promoter effect following treatment of our cells with D-dimer, EMSAs may be performed in order to assess the binding of transcription factors to the fibrinogen gene promoters following treatment with D-dimers. Chapter 4 also

discusses the search for a receptor for D-dimer on the surface of HepG2 cells. Previous work suggested that fibrin degradation products, especially D-dimer, were taken up by murine hepatocytes as a clearance mechanism (70). The macrophage receptor for murine FDPs containing the D domain was later characterized (118), but the identity of a receptor on the surface of liver cells was not examined. We were unable to detect the presence of a receptor for human D-dimer on the surface of our HepG2 cells, as our binding studies did not suggest saturable binding. In fact, the results of our binding studies coupled with the finding of several endocytic plasma membrane proteins eluted from a D-dimer affinity column strongly suggests that D-dimers are taken up by HepG2 cells via endocytosis. However, these studies remain novel in that we have shown that once HepG2 cells have taken up D-dimers, the D-dimers travel to the perinuclear space where they presumably signal to the nucleus to exert their effects on γ' fibrinogen synthesis. Future studies looking at the exact location of internalized D-dimers will be very informative and will help to determine how D-dimers are exerting their effects intracellularly. These studies would involve staining of the individual cellular compartments with several different fluorescent markers and determining by microscopy which of these cellular markers co-localizes with D-dimers.

In addition to determining the effects of D-dimer on the production of the fibrinogen γ chain isoforms, this work also examined the effects of several inflammatory cytokines on total and γ' chain production, as described in Chapter 5. We have identified IL-6, TNF-α and IFN-γ as molecules that are able to differentially regulate the production of total and γ' fibrinogen. Each of these cytokines seems to alter the ratio of γ'/total fibrinogen in a

unique way, as IL-6 increases total fibrinogen but not γ' fibrinogen, TNF-α decreases γ' fibrinogen but does not affect total fibrinogen levels, and IFN-γ decreases total fibrinogen but does not alter the levels of γ' fibrinogen. This is an important finding in that all three of these mediators are involved in the acute phase response, and are most likely working either synergistically or antagonistically during the acute phase. The identification of known signaling molecules as modulators of total and γ' fibrinogen levels allows us to better understand the origins of cardiovascular disease and may also point to possible future therapeutic targets. While these findings present novel regulators of γ' fibrinogen synthesis, the molecular mechanisms behind these observations must be elucidated in order to fully comprehend the spatial and temporal regulation of fibrinogen γ gene splicing and polyadenylation. An *in vitro* splicing assay coupled with qRT-PCR would be an incredibly useful tool to help determine whether the addition of these cytokines to a splicing reaction truly alters splicing. Should these assays show differential splicing patterns upon the addition of cytokines, western blots and qRT-PCR could be performed in order to detect differences in the expression, localization or phosphorylation of known splicing factors such as the SR family of splicing proteins. This same set of experiments could also be performed using antibodies directed against enzymes known to function in polyadenylation in order to detect differences between treated and untreated cells as well as differences between different cytokine treatments. Finally, the role of two recently identified polymorphisms within intron 9 of the γ chain of fibrinogen in alternative splicing should be investigated. Previous studies have proven that IL-6 was independently associated with total fibrinogen levels and, conversely, a single nucleotide polymorphism (SNP) within the fibrinogen gamma gene, FGG 9340 T/C, influenced

serum IL-6 levels (119). Furthermore, two additional gamma gene SNPs, FGG 9615 C/T and FGG 10034 C/T have been identified as belonging to a haplotype with an increased γ' fibrinogen level in a case-control study of venous thrombosis (99). The authors speculate that the FGG 10034 C/T SNP, located within a region downstream of the polyadenylation site which binds the Cleavage Stimulatory Factor (CstF), may be associated with increased γ' fibrinogen levels as its presence may increase polyadenylation and cleavage (99). The interaction of this set of SNPs with circulating IL-6 levels as well as SNPs within the IL-6 gene could be examined using a genome wide association study and may provide a mechanistic rationale as to why IL-6 may be differentially regulating γ' fibrinogen levels in our experiments.

While regulation of the levels of total and γ' fibrinogen are critical in understanding their role in cardiovascular disease, a dysfunctional fibrinogen presents a different set of physiological challenges. In Chapter 6 we discuss the identification of a fibrinogen γ chain mutation, which was previously shown to be predominantly thrombotic (115), in a patient presenting with bleeding. This patient had a diagnosed dysfibrinogenemia that we identified as a mutation in the fibrinogen γ gene at residue 275. This mutation caused a C>T transition, leading to a missense mutation in which a free cysteine reside was incorporated at γ chain residue 275 in place of an arginine. We show that this free cysteine does not bind to circulating albumin, nor does it drastically change the separation pattern of the γ chain. These results suggest that the R275C alone is capable of producing a bleeding phenotype as well as thrombotic and asymptomatic phenotypes.

In conclusion, this work has shown that IFN-γ is a novel regulator of total fibrinogen synthesis via a STAT1 dependent mechanism, while D-dimers, IL-6, TNF-α and IFN-γ are differential regulators of total and γ' fibrinogen production, both of which have been identified as independent risk factors for cardiovascular disease. These findings may lead to the use of agonists or inhibitors of these cytokines in a therapeutic manner to decrease total or γ' fibrinogen, thus decreasing the risk for cardiovascular disease. Finally, this work has shown that the fibrinogen R275C gamma chain mutation is sufficient to cause a hemorrhagic phenotype. The phenotype of this mutation is known to be predominantly asymptomatic, and symptomatic patients have been overwhelmingly thrombotic. Therefore the finding of a patient with this mutation presenting with a hemorrhagic phenotype is novel and increases our knowledge of the molecular mechanisms of dysfibrinogenemias.

Chapter 8: References

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