FIBRINOGEN AND PLASMINOGEN: THEIR ROLES BEYOND HEMOSTASIS

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Hemostasis is maintained by a well-coordinated series of vascular and chemical reactions that regulate the balance between coagulation and fibrinolysis. Under certain pathological conditions an exaggerated or insufficiently haemostatic response may lead to a situation in which coagulation/fibrinolysis contribute to disease. In the first part of this work, we undertook a study of the function of fibrinogen (Fg) in a murine model of acute inflammation, viz., lipopolysaccharide (LPS)-induced lethal endotoxemia. This study demonstrated that mice with a total deficiency of fibrinogen (Fg<sup>-/-</sup>) presented with reduced mortality, coagulation, and inflammatory responses when compared to their wild-type (WT) counterpart. The attenuated inflammatory responses in Fg<sup>-/-</sup> mice correlated with a lack of fibrin deposition in organs. Inflammatory cells appeared early in the tissues of challenged WT mice, but occurred at later times in Fg<sup>-/-</sup> mice. This delayed response in Fg<sup>-/-</sup> mice was confirmed by studies that showed a strong dependence on Fg for binding of neutrophils to endothelial cells in the presence of LPS. Inflammatory cytokines were elevated in both genotypes, however their levels were generally lower at early times in Fg<sup>-/-</sup>. Therefore, a Fg deficiency enhances survival from lethal endotoxemia through attenuation of inflammatory responses that result from reduced leukocyte
infiltration to the inflammatory foci, and, from downregulation of chemokine/cytokine expression. Our results suggest that fibrin(ogen) plays an important role as an early mediator in the cross-talk between coagulation and inflammation.

In the second part of this dissertation, we utilized a biochemical approach to study the role of plasminogen (Pg) in the pathogenic mechanisms of Group A streptococcus (GAS). GAS is the etiologic agent responsible for a number of human diseases that range from common pharyngitis to severe infections. Streptokinase (SK) is a 414 amino acid protein secreted by several streptococcal species, and an efficient activator of Pg. Interestingly, SK is not an enzyme; it activates Pg indirectly by the formation of a 1:1 complex with Pg. Furthermore, Pg activation by SK is highly species specific with activity towards human Pg (hPg), but exhibiting no activity against mouse plasminogen (mPg). In an attempt to define which amino acid regions within Pg may account for the species specificity of SK, several mutants, and chimeric mouse-human Pg constructs were generated. The hPg light chain (hL) was identified as the region responsible for SK sensitivity, specifically the amino acid sequences encoded by exons 16 and 18. In addition, surface plasmon resonance (SPR) experiments demonstrated high affinity binding between all Pg variants and SK, including mPg, indicating that SK has the ability to form “catalytic complexes” in a non-species specific manner. However, no active site is formed within the moiety of the activator complex. In summary, we identified the loci within hPg that productively interacts with SK. The data generated herein presents novel insights for the understanding of the activation mechanism of hPg by SK.
To Anna Regina

“Always bear in mind that your resolution to succeed is more important than any one thing” Abraham Lincoln (1809-1865)
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ABBREVIATIONS

AP…………………………………………………………………………Activation Peptide
BM…………………………………………………………………………Bone marrow
BMM……………………………………………………………………Bone marrow derived-macrophages
BALF……………………………………………………………………Bronchoalveolar lavage fluid
CD14……………………………………………………………………Cluster of differentiation-14
COX-2……………………………………………………………………Cyclooxygenase-2
ECs………………………………………………………………………Endothelial cells
Fg………………………………………………………………………Fibrinogen
GAS……………………………………………………………………Group A streptococcus
GM-CSF…………………………………………………………Granulocyte Monocyte Colony-Stimulating Factor
H&E…………………………………………………………………Hematoxylin II and eosin Y stain
HMWK……………………………………………………………High molecular weight kininogen
IL-1……………………………………………………………………Interleukin-1
IL-1R…………………………………………………………………Interleukin-1 receptor
IL-3……………………………………………………………………Interleukin-3
IL-2……………………………………………………………………Interleukin-2
IL-4……………………………………………………………………Interleukin-4
IL-5……………………………………………………………………Interleukin-5
IL-6……………………………………………………………………Interleukin-6
IL-7……………………………………………………………………………Interleukin-7
IFN-γ……………………………………………………………………..Interferon gamma
iNOS……………………………………………………………………. Inducible nitric oxide synthase
IRAK…………………………………………………………………... Interleukin-1 receptor-associated kinase
K……………………………………………………………………………………Kringle
LBT4…………………………………………………………………….. Leukotrine B4
LPS……………………………………………………………………... Lipopolysaccharide
MAP3K…………………………………………………………………… Mitogen-activated protein kinase
MCP-1…………………………………………………………………… Monocyte Chemoattractant Protein-1
MHC……………………………………………………………………… Major-histocompatibility-complex
MIP-1…………………………………………………………………… Macrophage Inflammatory Protein-1
MIP-2…………………………………………………………………… Macrophage Inflammatory Protein-2
MPO………………………………………………………………………. Myeloperoxidase
MyD88…………………………………………………………………… Myeloid differentiation primary response gene-88
NEO……………………………………………………………………… Neomycin phosphotransferase gene
NF-κB…………………………………………………………………….. Nuclear factor-kappa B
NO………………………………………………………………………… Nitric Oxide
NPGB………………………………………………………………….. \( \rho \)-Nitrophenyl-\( \rho \)'-Guanidino-Benzoate
PAR-2…………………………………………………………………… Protease activated receptor-2
PBS……………………………………………………………………….. Phosphate buffer solution
PC………………………………………………………………………… Protein C
PCR……………………………………………………………………… Polymerase chain reaction
Pg………………………………………………………………………… Plasminogen
PK……………………………………………………………………………..Prekallikrein
PLP………………………………………….Periodate-Lysine-Paraformaldehyde fixative
Pm…………………………………………………………………………..Plasmin
PMN…………………………………………………………...Polymorphonuclear cells
Pol B………………………………………………………..Polymyxin B
sE-Sel……………………………………………….Soluble Endothelial selectin
SAK……………………………………………………………………..….Staphylokinase
SK…………………………………………………………………………….Streptokinase
S2251…………………………………………………………………..H-D-Val-L-leu-L-lys-ρ-nitroanilide
TF……………………………………………………………………………..Tissue factor
Th………………………………………………………………………………...Thrombin
TMB……………………………………………………………….Tetra Methyl Benzidine
TLRs…………………………………………………………………Toll-like receptors
TLR-4………………………………………………………….Toll-like receptor 4
TNF-α…………………………………………………………………..Tumor necrosis factor alpha
TRAF-6…………………………………………………………..Tumor necrosis factor associated factor-6
PAI-1…………………………………………………………..Plasminogen activator inhibitor-1
uPA…………………………………………………………..Urokinase-type plasminogen activator
tPA…………………………………………………………..Tissue-type plasminogen activator
WBC………………………………………………………………………White blood cell
WT……………………………………………………………………..……..….Wild-type
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CHAPTER 1:
BACKGROUND

The activation of the innate immune response is the first mechanism of protection against external pathogens. An effective immunity depends not only on the proper activation of the immune cells, but also on the regulation of their molecular interactions with other components of the host defense system. Upon exposure to an antigen, pathogen-associated molecular patterns are recognized through “pattern recognition receptors” (1). These interactions trigger signaling pathways resulting in an acute inflammatory response, characterized by increased vascular permeability, leukocyte chemotaxis, and enhanced procoagulant activity.

Under normal circumstances inflammation-induced coagulation is a physiologically efficient process; however, inflammation as a result of severe infection or trauma induces signaling pathways that contribute to disease, as illustrated by the coagulopathy that is associated with sepsis (2, 3). Recently, several links among the inflammation and coagulation pathways have been established. Inflammation not only leads to activation of coagulation, but coagulation strongly affects the inflammatory response. Coagulation factors participate in the inflammatory response, either as chemoattractants or as inflammatory mediators, such as cytokines. Inflammatory mediators also regulate coagulation processes via induction of tissue factor or thrombin expression, through signal transduction processes (4).
Fibrinogen (Fg), the precursor of fibrin has a central role in hemostasis as the main component of blood clots. Studies have suggested that Fg has an additional function in other pathophysiological processes, such as atherosclerosis, tumor formation, and infection (5, 6). Further, *in vitro* and *in vivo* studies have demonstrated that Fg is an important regulator of the inflammatory response. Fg can stimulate leukocyte function through induction of various chemotactic factors and surface receptors (7). Additionally, Fg can stimulate neutrophil aggregation and macrophage expression of chemokines (MIP-1, MIP-2 and MCP-1) attracting inflammatory cells to the site of injury. Interestingly, it has also been suggested that Fg and Lipopolysacharide (LPS) may share a similar signal pathway through toll like receptor-4 (8, 9).

Due to these important functions of fibrinogen in inflammation, we undertook a study of the function of Fg in a model of continuous administration of endotoxin (LPS) to induce acute inflammation. In addition, *in vitro* studies were performed in order to gain better understanding of the molecular mechanisms by which Fg contributes to the crosstalk between the hemostatic and inflammatory systems. A detailed background section is presented with the objective of elucidating the rationale and motivations of this work.

1.1. Hemostasis

Hemostasis is defined as a balance between physiological processes that regulate thrombus formation and dissolution. Normally, when a vessel is injured, a cascade of reactions occurs to limit blood loss. Blood vessels constrict to reduce blood flow at the site of injury. Platelets aggregate and bind to exposed collagen to form a plug that can
stop minor bleeding. Finally, the aggregating platelets and the damaged tissue lead to the activation of the coagulation cascade, whereby the blood clot or thrombus is formed by the action of proteolytic reactions. Once the bleeding is arrested and the injured vessel is repaired, regulatory processes limit the growth of the thrombus. The clot is dissolved by action of the fibrinolytic system. The equilibrium between coagulation and fibrinolysis is highlighted by the severe phenotypes observed in patients with deficiencies in components of these pathways. Moreover, mutations and inappropriate expression of hemostatic factors can lead to thrombotic and hemorrhagic stroke, heart attack, cancer, atherosclerosis and sepsis. (10, 11)

1.1.1. The coagulation pathway

Endothelial cells (ECs) are lined on the inner wall of blood vessels, serving as a barrier between blood and the tissues. Under normal physiological conditions, the endothelium is maintained in an antithrombotic environment. Upon injury, this environment is switched to a prothrombotic state promoting thrombus formation (12). Two pathways can lead to clot formation: the intrinsic and extrinsic pathway. The extrinsic pathway triggers the initial phase of blood coagulation, whereas amplification requires the intrinsic pathway.

The activation of the intrinsic pathway is triggered when blood comes into contact with negatively charged surfaces in the presence of Ca$^{2+}$. This pathway is initiated by the activation of FXII to $\alpha$-FXIIa, conversion of prekallikrein (PK) to kallikrein, and the cleavage of high molecular weight kininogen (HMWK). FXII activation leads to a series of enzymatic reactions involving the serine proteases FXI, FIX, and FX. This sequence of
reactions comprises the mechanism for initiating thrombin generation and subsequent clot formation. While the intrinsic pathway is a key mechanism for initiating coagulation in vitro, the absence of important hemostatic abnormalities associated with FXII and FXI deficiencies indicates that the intrinsic pathway is not important for normal blood coagulation in vivo, however its activation is required for the sustained propagation of the thrombus initiated by the extrinsic pathway (13, 14).

The extrinsic pathway is activated in response to vascular injury that results in exposure of the subendothelial layers of the vessel wall to blood components. Upon injury, the transmembrane receptor tissue factor (TF) is exposed on injured cells and monocyte membranes, triggering blood coagulation by its binding to plasma factor VII/VIIa (FVII/FVIIa). The TF/FVIIa complex is the primary activator of the coagulation protease cascade in vivo. This complex catalyzes the conversion of FIX to FIXa, and FX to FXa in the presence of Ca\(^{+2}\) and phospholipids. Subsequently, FXa catalyzes the activation of prothrombin to thrombin. Thrombin formation then leads to the proteolytic cleavage of fibrinogen to fibrin, which rapidly assembles into a fibrin polymer, the main component of the blood clot. Sustained generation of thrombin to consolidate the blood clot requires FIXa and FVIIIa, however during some types of significant injury, the FIXa generated by FVIIa/TF appears insufficient to sustain coagulation, and additional FIXa generated through the intrinsic pathway is then required.

A diagram of the coagulation cascade is presented in Figure 1.1.
1.2. The immune response

The mammalian immune system consists in two major forms of host defense, the innate and adaptive immune responses. Innate immunity is the immediate host defense against invading micro-organisms, and is based on the recognition of micro-organism-associated molecular patterns. It is not antigen specific and reacts equally well to a variety of organisms. In contrast, the adaptive immune response is specific, and depends on the clonal expansion of two classes of specialized lymphocytes, T and B cells. These cells express receptors that are somatically generated in response to a specific antigen (15). This response is based on “immunological memory”; it reacts more efficiently on subsequent exposure to the same organism.

Although the innate and adaptive immune systems both function to protect against invading organisms, they differ in a number of ways. The activation of the adaptive immune response does not occur immediately after a novel pathogen enters into the host system, long-term humoral and cell-mediated immune memory need to be developed before a specialized response is mounted, whereas the innate immune system includes defenses that are ready to be mobilized upon infection. Therefore, the innate and adaptive immune responses cannot be seen as independent processes, both responses need to be coordinated in order to control the replication of the infecting pathogen.

1.2.1. The innate immune response

The innate immune system operates by the recognition of a broad range of pathogen-associated molecular patterns, which are produced only by microbial pathogens, and not by their hosts. Among the best-characterized pathogens-associated
molecular patterns are bacterial lipopolysaccharide, peptidoglycans, lipoteichoic acids, mannans, bacterial DNA, double stranded RNA, and glucans. The recognition of these pathogen-associated molecular patterns depends on the expression of patterns recognition receptors by the host effector cells, most importantly on macrophages, dendritic cells, and B cells. Structurally, pattern-recognition receptors belong to several families of proteins: Leucine-rich repeat domains, calcium-dependent lectin domains, and scavenger-receptor protein domains. Based on their function, pattern-recognition receptors can be divided into three classes: secreted, endocytic, and signal receptors. Secreted pattern-recognition receptors function as opsonins. They bind to microbial cell walls and mark them for recognition by the complement system and phagocytes. Endocytic pattern-recognition receptors are expressed on the surface of phagocytes. These receptors mediate the uptake and delivery of pathogens into lysosomes. Once the pathogen is destroyed, pathogen-derived proteins are processed, and the resulting peptide is then expressed on the cell surface in order to be presented to other cells by the major-histocompatibility-complex (MHC) molecules. Signaling receptors recognize pathogen-associated molecular patterns and activate signal-transduction pathways that induce the expression of a variety of immune response genes. The toll-like receptors (TLRs) are well-characterized members of this class of pattern-recognition receptors. They are similar in structure and function to the *Drosophila* Toll protein (16-18). Structurally, TLRs cytoplasmic domain is similar to that of the mammalian interleukin-1 receptor (IL-1R) and an extracellular domain containing leucine-rich repeats (19-21). To date, there have been 12 TLRs identified in mice and 10 TLRs in humans (22-24). Toll-like receptor 4 (TLR-4) was the first human toll-like receptor to be characterized; TLR-4 was established as the LPS signaling
receptor (25). LPS, also know as endotoxin, is a major cell wall constituent of gram-negative bacteria. It is composed of three elements: a core of oligosaccharide, an O-specific chain made of repeating sequences of polysaccharides and a lipid A component, which is responsible for the proinflammatory properties of LPS (26). It was originally believed that LPS activated immune cells through a non-specific mechanism that involved the spontaneous intercalation of lipid A into the mammalian lipid bilayer. However, early studies suggested that the biological actions of LPS were facilitated by its binding to endogenous proteins (27). In vivo studies demonstrated that TLR-4 is essential for LPS recognition. Mice with either a spontaneous mutation of the tlr4 gene or a targeted disruption of the gene have no response to LPS (28, 29). LPS recognition is mediated by CD14, a receptor expressed on macrophages and B cells, and a complex formed by MD-2 and TLR-4. The binding of LPS to CD14 leads to the association of CD14 with TLR-4/MD-2 complex, inducing the dimerization of TLR-4. Once, this receptor becomes activated, it recruits the adapter protein MyD88, which is associated with the serine-threonine protein kinase interleukin-1 receptor-associated kinase (IRAK). IRAK is then phosphorylated and bound with the tumor necrosis factor associated factor 6 (TRAF-6) adapter protein. Further oligomerization of TRAF-6 activates the MAP3K pathway (Mitogen-activated protein kinase kinase kinase), leading to the activation of IκB kinase 1 (IKK1) and IκB kinase 2 (IKK2). These kinases phosphorylate IκB on serine residues, leading to the activation of NF-κB, which moves into the cell nucleus and induces the transcription of a wide variety of inflammatory-and immune-response genes (Figure 1.2) (30).
1.2.2. The inflammatory response

The activation of the innate immune system is crucial for initiating defensive action against pathogens. The detection of molecular patterns by innate immunity receptors lead to the activation of a complex network of molecular and cellular interactions directed to induce the expression of genes encoding proteins, such as cytokines, with regulatory functions that facilitate cell to cell signaling. Cytokines mediate the activation and extravasation of neutrophils and mononuclear cells to the sites of infection or injury leading to the local and systemic activation of the inflammatory response.

Inflammation is a protective mechanism of the immune system triggered in response to cellular and vascular injury due to exogenous or endogenous stimuli (31). Inflammation has been recognized by centuries as an indicator of disease. More than 2,000 years ago, the Roman physician Celsus recognized “the cardinal signs of inflammation” (warmth, redness, swelling, and pain). These signs described by Celsus are caused by cellular and tissue responses to mechanical injury or an infectious agent. The inflammatory process is activated to destroy and to prevent the pathogen spread, as well as to aid in homeostasis restoration.

Inflammation can be divided into different phases: The acute vascular response, the acute cellular response, chronic cellular response, and resolution.

1. Acute vascular response. During this phase, alterations of the vascular endothelium occur. These alterations are characterized by increased blood flow (vasodilation) and the transudation of fluids into the surrounding tissues
(increased permeability). These events lead to edema, accompanied by the exudation of proteins and immune cells into the injured tissue.

2. Acute cellular response. This phase is characterized by leukocyte movement from the capillaries to the tissues through a process known as extravasation. This process consists of the attachment of inflammatory cells to endothelial cells (margination and adhesion) and the subsequent migration towards the surrounding tissues (transmigration). Cell transmigration occurs via chemotaxis. Chemokine gradients stimulate the movement of leukocytes between endothelial cells and basement membranes into inflamed tissues. This process is known as diapedesis. The hallmark of this phase is the appearance of inflammatory cells, particularly neutrophils, in the injured tissues. Neutrophils, also known as polymorphonuclear cells (PMN), are the first cells to be recruited to sites of infection or injury.

3. Chronic cellular response. Under normal circumstances, a healing process follows the acute cellular response. However, if there is ineffective elimination of the infectious agents, a chronic cellular response may follow. This phase of inflammation is characterized by the appearance of a mononuclear cell infiltrate composed of macrophages and lymphocytes, angiogenesis, and fibrosis.

4. Resolution. Once the infectious agent has been eliminated, under normal circumstances, a healing process follows. Homeostasis will be restored by the removal of blood clots and other tissue debris, the formation of scar tissue by fibroblasts, collagen, and endothelial cells (fibrosis), and by the development of new vessels (angiogenesis) to restore the normal blood flow to the injured tissues.
1.2.3. Acute and chronic inflammation

**Acute Inflammation**

An acute inflammatory response will follow after an injury occurs by the action of physical, chemical, or biologic agents. The events involve the release of vasoactive molecules, lipid mediators (prostaglandins and leukotrienes), the activation of plasma enzyme systems (complement system, the coagulation system, the fibrinolytic system and the kinin-kallikrein system), the recruitment of white blood cells (WBC), and the expression and secretion of inflammatory cytokines and chemokines.

Acute inflammation unfolds a series of physiological events within minutes after an “injurious” agent enters into the host system:

- Increased Blood Vessel Dilation. Small blood vessels (capillaries and venules) dilate. This allows more blood to flow into the affected area. The increase in blood flow leads to increased numbers of blood cells entering the affected area.

- Increased Blood Vessel Permeability. The secretion of chemical mediators such as histamine and bradykinin, causes endothelial cell contraction and increases in local blood pressure. The combination of these events lead to changes in the blood vessel lining (endothelial) cells causing the opening of intercellular junctions. These junctions allow a filtrate of plasma out of the capillaries by a process known as “transudation.” The rapid exodus of protein-rich plasma is known as “exudation.” As plasma proteins move out of the capillaries, tissue swelling, pain, and loss of function will occur due to the fluid accumulation and increased pressure within the damaged area.

- Blood Flow Stagnation. Plasma leakage from the blood stream increases the red blood cell concentration. Blood viscosity elevates, and the flow rate decreases. These
effects allow blood leukocytes to contact the endothelial lining cells.

- **Cell rolling and adhesion.** Endothelial cells express surface receptors and ligands that mediate WBC adhesion to the vascular endothelium. Leukocytes adhere to the endothelium by rolling (mediated by selectins) followed by adhesion (mediated by integrins). Increasing number of leukocytes (mainly neutrophils) line up along the endothelium, an appearance called “margination”. Adherent neutrophils and monocytes then insert pseudopods into the open junctions between endothelial cell, in order to squeeze through the intercellular gap (diapedesis) and migrate into the interstitial tissue (extravasation).

- **Leukocytes activation.** Neutrophils and monocytes are the first cells to be attracted to the injured area via a process termed chemotaxis. Chemotaxis is defined as locomotion oriented along a chemical gradient. Neutrophils and monocytes will migrate along the chemotactic gradient into the injured area. Several chemotactic agents have been identified, among them: C3a and C5a (components of the complement system), leukotrienes (products of arachidonic acid), and cytokines (mainly, those from the chemokine family). In addition to chemotaxis, these chemical mediators induce leukocyte activation. The activation of neutrophils and the monocyte/macrophage system lead to a variety of responses such as cell locomotion, degranulation, secretion of lysosomal enzymes, the activation of oxidative burst, expression of adhesion molecules, and the release of antibacterial enzymes such as alkaline phosphatases, or myeloperoxidase (MPO) by neutrophils and macrophages. Neutrophils are capable of engulfing bacteria that have been tagged by opsonins (immunoglobins, IgG and IgA, or components of the complement system, C3b, C4b). Phagocytosis plays a major role in the inflammatory
process. Coated microorganisms are brought into the cell into a body called phagosome. Neutrophil lysosomes fuse with phagosomes releasing enzymes, such as hydrogen peroxide, hypochlorite, and other substances lethal to bacteria. However, in this process, many neutrophils are killed. When this happens, monocytes/macrophages appear in larger numbers and replace neutrophils. Macrophages are monocytes that entered an area of tissue injury. They have a long life span and can survive in acidic environments. These cells are a key component of the cell-based immunity. Macrophages (macro- = large; phago = eater) are phagocytes, their function is to engulf and process antigenic substances, in order to present “altered antigens” to more specialized cells for destruction. Macrophages also secrete chemokines (to recruit additional cells to the site of inflammation), and a number of enzymes such as collagenases and elastases, which aid in antigen destruction.

**Chronic Inflammation**

Chronic inflammation is characterized by the proliferation of lymphocytes (T cells) and plasma cells (B cells) as a consequence of the inability to eliminate and/or the continual reacquisition of an antigen. Chronic inflammation is long lasting, however the signs and symptoms are not as drastic as those associated with acute inflammation. If an inflammatory reaction starts as acute but persists, it will enter a chronic phase. Once the infection has been controlled, collagen will build up to scar tissue proportions leading to the development of deformed tissue in a process known as fibrosis. Examples of a chronic inflammatory response are the symptoms that develop in allergy and asthmatic reactions.

In chronic inflammation, monocytes/macrophages, lymphocytes and plasma cells are the predominant cells. Phagocytosis is the main function of macrophages. They
engulf and process antigens to aid in the recognition by other cells. The most predominant cells in chronic inflammation are lymphocytes. These cells are divided into two different groups: T and B. T-lymphocytes arise from the thymus gland and participate in the cell-based immunity. B-lymphocytes are generated from bone marrow and mainly mediate humoral immunity. Chemical mediators, such as cytokines produced by macrophages, activate T-Lymphocytes. Activated lymphocytes can react with certain antigens to eliminate them. They can also induce monocyte activation by the production of lymphokines. Under normal circumstances, B-Lymphocytes are not present in circulation, they are converted to plasma cells in lymphoid organs or at the site of chronic inflammation. These plasma cells produce and secrete antibodies against specific antigens. Eosinophils and multinucleated giant cells may also appear in chronic inflammatory processes, such as hypersensitivity reactions, asthma, and autoimmune disease. Eosinophils are polymorphonuclear leukocytes, characterized by the presence of bright red granules within their cytoplasm. When eosinophils degranulate, they release a series of chemical mediators, such as histamine, peroxidases, RNases, DNases, lipases, and major basic protein, that are toxic to both antigen and host tissues. Multinucleated giant cells are formed by the fusion of macrophages, in response to certain microorganisms, such as mycobacterium.

Resolution

Fibroblasts play an important role in the healing phase of the infection. They will be recruited to the areas surrounding the injured tissues, once there, they will produce collagen to repair destroyed tissue leading to the formation of scars and the resolution of the infection. (32,46,47,48)
1.2.4. Chemical inflammatory mediators

Chemical mediators modulate the inflammatory response by inducing vascular changes, endothelium activation, leukocyte adhesion and transmigration, chemotaxis, etc. Amongst the cellular sources of these mediators are platelets, neutrophils, monocytes/macrophages and mast cells, endothelial cells, and fibroblasts. Activity of chemical mediators is accomplished through binding to respective receptors on target cells or by inducing expression or release of other mediators by target cells. This mechanism serves to amplify the inflammatory response. As discussed earlier, a variety of factors are involved in the inflammatory response, vasoactive amines (histamine and serotonin), plasma proteases (complement system and the clotting system), arachidonic acid metabolites (prostaglandins, leukotrienes, and lipoxins), nitric oxide, lysosomal enzymes from leukocytes, and oxygen-derived free radicals from leukocytes, etc. Of all the factors, cytokines constitute the largest and most pleitropic group of such mediators. Cytokines are small proteins, which mediate and regulate immunity, inflammation, and hematopoiesis (15, 33). They are produced *de novo* in response to an immune stimulus. The initiation, maintenance, and resolution of inflammatory responses depend upon the cytokine signaling through receptor-ligand interactions on target cell populations. Once a population of cells responds to a specific stimulus, they may express and produce cytokines that exert distinct effects upon another population of cells. Inflammatory cells, such as neutrophils and monocytes are recruited and activated in response to specific chemotactic signals, resulting in further amplification of a cytokine cascade. Cytokines can be classified based on their origin as monokines (expressed by mononuclear phagocytes), and lymphokines (expressed by lymphocytes). Cytokine denomination is
also related to their function. The largest group of cytokines stimulates immune cell proliferation and differentiation. This group includes Interleukin 1 (IL-1), which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, and IL-6, which stimulate proliferation and differentiation of B cells; Tumor necrosis factor alpha (TNF-α) which stimulates cell proliferation, differentiation, and apoptosis; Interferon gamma (IFN-γ), which activates macrophages; and IL-3, IL-7, and Granulocyte Monocyte Colony-Stimulating Factor (GM-CSF), which stimulate hematopoiesis. Regulation of natural immunity is crucial in the very early steps of inflammation and involves TNF-α and IL-1β, IFN-γ, and IL-6. Chemokines belong to a family of small proteins, secreted by inflammatory cells. Structurally, chemokines are characterized by the presence of four cysteine residues in conserved locations. Functionally, they have the ability to direct the movement of immune cells to the inflamed sites by establishing chemical gradients. Chemokines are produced by an array of cells including monocytes/macrophages, neutrophils, platelets, eosinophils, T and B lymphocytes, and endothelial cells. These cells can produce chemokines in response to a variety of factors that trigger an immune response, including pattern recognition ligands, IL-1, TNF-α, C5a, Leukotriene B4 (LBT4), and IFNs (34).

1.3. Coagulation and inflammation

Inflammation-induced coagulation activation is characterized by enhanced fibrin formation and impaired fibrin degradation (35). The vascular endothelium represents one of the main links between coagulation and inflammation. Endothelial cells express adhesion molecules, growth factors, and cytokines that promote inflammation and
coagulation activation (36). Studies have revealed that both endothelial cells and monocytes can be stimulated by inflammatory markers such as IL-1, IL-6, and TNF-α to produce TF which then triggers coagulation activation (37, 38). Early experiments showed that LPS-induced coagulation activation increased the production of inflammatory cytokines in peripheral blood monocytes (39). Further studies have shown that several coagulation proteases induced the expression of proinflammatory mediators in response to LPS (40). There is evidence that binding of TF-FVIIa to protease activated receptor-2 (PAR-2) effects the production of reactive oxygen species and cell adhesion molecules in macrophages. It has also been shown to affect neutrophil infiltration and proinflammatory cytokine (TNF-α, IL-1β) expression. Additionally, other hemostatic proteins such as, FXa, thrombin, and Fg increase the synthesis of IL-6 and IL-8 in EC and mononuclear cells in vitro (41-44).

Another important link between inflammation and coagulation is the Protein C (PC) system. Studies employing low-PC expressing mice demonstrated that lethality was exacerbated in these mice following LPS challenge. Low-PC expressing mice present a more severe inflammatory response. This response is characterized by disseminated intravascular coagulation, thrombocytopenia, hypotension, and multiple organ dysfunction (45).

Taken together, these results suggest a close link between hemostasis and inflammation. Further studies of these “linking points” will provide important information to understand the full relevance of these interactions.
Figure 1.1 Coagulation pathway. The solid arrows indicate procoagulant reactions. Red dots show inhibitory pathways. Green dotted arrows indicate some of the feedback activation loops. Black lettering shows the anticoagulant pathway, and brown lettering depicts the fibrinolytic pathway. This illustration is courtesy of Dr. Francis J. Castellino.
Figure 1.2 The Signaling Pathway of LPS. Three different gene products mediate the recognition of Lipopolysaccharide (LPS): CD-14, toll-like receptor 4 (TLR-4), and MD-2. Once TLR-4 is activated, MyD88-IRAK-TRAF-6 complexes activate the mitogen-activated protein kinase (MAP3K) family, leading to a series of phosphorylations and the releasing of NF-κB, which induces the expression of pro-inflammatory cytokines such as Tumor Necrosis alpha (TNF-α), Interleukins-1, 6, 8 (IL-1,6,8), and antibacterial metabolites such as Nitric Oxide (NO) and Cyclooxygenase-2 (COX-2).
1.4. References


CHAPTER 2:
DELAYED INFLAMMATORY RESPONSES TO ENDOTOXIN IN FIBRINOGEN-
DEFICIENT MICE

2.1. Introduction

Fibrinogen (Fg) is a 340,000-Dalton (Da) protein with a primary structure consisting of three pairs of disulfide bonded polypeptide chains named Aα, Bβ, and γ, composed of 610, 461, and 411 amino acids, respectively. Fg is the precursor of fibrin, which is the main component of the blood clot formed during the hemostatic response to tissue injury. Each polypeptide chain, of Fg, Aα, Bβ, and γ, is encoded by three separate genes, FGA, FGB, and FGG, respectively, located on the 50-kb region of chromosome 4q28-q31. The three genes are translated in a coordinated fashion to code for each individual chain (1-3). Each arm of the Fg molecule contains a single Aα, Bβ, and γ from each of the three pairs of polypeptide chains and a dimeric central domain containing the amino terminals of each individual arm. The amino terminal ends of the Aα and Bβ chains represent fibrinopeptides A and B (Figure 2.1). The cleavage of fibrinopeptides A and B is the first step toward Fg polymerization by the formation of fibrin. Fibrinopeptides A and B are cleavaged by thrombin. The removal of the fibrinopeptides leads to the polymerization of the monomers into fibrin dimers. These fibrin dimers can grow laterally by addition of monomers forming a protofibril. FXIIIa then catalyzes the formation of cross-links between the protofibrils increasing the blood clot stability (4,5).
Interestingly, besides fibrin(ogen)’s importance as the main component of the blood clot, studies have suggested that fibrin(ogen) possesses functions outside hemostasis. Fibrin(ogen) deposition is a universal feature within injured tissues and inflammatory foci. There is clear evidence that Fg has an additional function as regulator of the inflammatory response. Fg and fibrin influence the production of proinflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, and MIP-2) by mononuclear cells and endothelial cells (6). Fg deficient mice indeed have shown inhibition of macrophage adhesion and less thrombin-mediated cytokine production (7). In addition, fibrin(ogen) can stimulate leukocyte function (8,9), platelets aggregation (10), and NF-κB activation (11). Moreover, studies have suggested that Fg and LPS may share a similar signalling pathway, requiring the expression of functional toll like receptor-4 (12).

Because of these important functions of Fg in inflammation, an interest arose to analyze the role of Fg in an in vivo model of endotoxemia. A model of continuous LPS administration was employed to induce acute inflammation. $Fg^{-/-}$ mice were exposed to continuous administration of LPS. The hemostatic and inflammatory responses were analyzed to further understand the role of Fg as a mediator in the cross-talk between coagulation and inflammation. The results presented herein provide experimental evidence of the importance of Fg as a central regulator of the inflammatory response.
2.2. Materials and Methods

2.2.1. Animal handling

The mice were housed in micro-isolation cages on a constant 12-hour light/dark cycle with controlled temperature and humidity and given access to food and water ad libitum. The breeding and housing was performed according to the Institutional Animal Care Comitee (IACUC) and Association for Assessment and Accreditation of Laboratory Animal Care (AALAC ) regulations.

2.2.2. Generation of Fg\(^{-/-}\) Mice

The generation and characterization of mice with a total targeted deletion of Fg (Fg\(^{-/-}\)) have been described previously (13). Briefly, mice were generated by the targeted deletion of the gamma-chain of Fg, which resulted in the absence of detectable circulating Fg. The entire coding sequence of the FGG chain was replaced with a neomycin phosphotransferase gene (NEO) by targeted homologous recombination. Mice were back-crossed for more than seven generations into the BALB/c strain. Male WT and Fg\(^{-/-}\) littermates at 7-8 weeks of age were used in this study.

2.2.3. LPS challenge: Model of systemic endotoxemia

LPS (Serotype 0111:B4; Sigma, St Louis, MO) was dissolved at 5 µg/µl in isotonic saline. A volume of 100 µl of this solution was placed in osmotic pumps (Model 1003 D; Duret Cooperation, Cupertino, CA), which were implanted intraperitoneally into mice. Male, WT and Fg\(^{-/-}\) mice at 7-8 weeks of age were sedated with isofluorane using a
ventilator and the surgery was performed using a surgical microscope (Leica Nustock, Germany) under sterile conditions. The abdomen of the mice was shaved and disinfected with 70% ethanol. Mice were laid on their backs and their extremities taped to a surgical board. A longitudinal 1 cm incision was made in the skin and into the peritoneum with sterilized forceps and scissors. The pumps were inserted. The pumps released LPS or saline at a rate of 1 µl/h. The dose of LPS used was determined from preliminary experiments with WT BALB/c mice such that 50-60% of the mice expired within 72 h. At 0, 3, 6, 12, 24, and 48 hours after pump implantation, the mice were sacrificed and subjected to necropsy. For survival statistics, mice were monitored for 96 h after implantation of the pumps.

2.2.4. Necropsy

Mice were sacrificed at various time points, as indicated above. Animals were anesthetized with isoflurane at the proposed sacrifice time points. Blood was collected by vena cava puncture into the appropriate anticoagulant solution at a volume ratio of 9:1 for plasma assays of cytokines, chemokines and, other inflammatory markers. The mice were then perfused through the iliac artery with saline until no additional blood emerged from the vena cava. Lungs were removed and immersed either in RLT buffer (Qiagen, Valencia, CA), Periodate-Lysine-Paraformaldehyde fixative (PLP), or saline solution for quantitative RT-PCR, histological analysis, and Myeloperoxidase (MPO) activity determination.
2.2.5. Histological Analysis

Mice were sacrificed at various times after pump implantation. Tissues were collected and fixed in PLP overnight followed by 70% ethanol before processing. After fixation, samples were dehydrated and embedded in paraffin utilizing a Renaissance tissue processor (Ventana Medical Systems, Inc., Tucson, AZ). Sections (3-4 µm) were collected on microscope slides (Fisher Scientific, Fair Lawn, NJ) and stained with hematoxylin II and eosin Y (H&E) or specific immunostains for fibrin(ogen) and neutrophils.

**H&E stains**

The slides were deparaffinized by three five-minute washes in xylene (Fisher, Fair Lawn, NJ) and rehydrated by consecutive two-minute washes in a series of ethanol solutions (from 100% to 70%) and then in distilled water. Slides were then incubated in a hematoxylin solution (Richard-Allan Scientific, Kalamazoo, MI) for 1.5 minutes and washed in distilled water (three changes), clarifier 2 (2% acetic acid in 95% ethanol) (Richard-Allan Scientific, Kalamazoo, MI) 20 dips or 30 seconds, tap water (two minutes), Scott’s water substitute (0.36% NaCO₃, 2% MgSO₄ in H₂O (Sigma, St. Louis, MO) 20 dips or 30 seconds and distilled water (two minutes). Slides were incubated in EosinY (1.5 minutes) washed in 95% ethanol for 1 minute and dehydrated by three two-minute washes in FLEX100 and cleared by three two-minute washes in xylene. Finally, coverslips were placed on the slides with mounting media (Cytoseal XYL, Richard Allan Scientific, Kalamazoo, MI). For immunostaining of fibrin(ogen), and neutrophils, tissue sections were deparaffinized and placed in avidin and biotin blocking solutions (Zymed Laboratories, San Francisco, CA) to block endogenous avidin, followed by Peroxo-block
(Zymed Laboratories, San Francisco, CA) to inhibit endogenous peroxidase activity. After the blocking steps, a pre-immune rabbit serum was applied.

**Fibrinogen immunostaining**

The sections were incubated in a goat anti–mouse fibrin(ogen) antibody (Nordic Immunology, Tillburg, The Netherlands) solution, followed by rabbit anti–goat IgG (DAKO, Carpinteria, CA) in 10% normal mouse serum (DAKO, Carpinteria, CA). A complex of horseradish peroxidase (HRP), conjugated to a goat anti–HRP IgG (DAKO, Carpinteria, CA) was then added. The slides were developed with 3-amino-9-ethylcarbazole (AEC) and followed with a hematoxylin QS counterstain (Vector Laboratories, Burlingame, CA).

**Neutrophil immunostaining**

The primary antibody used for neutrophil immunostaining was rat anti–mouse neutrophil NIMP-R14 (Abcam, Cambridge, MA). The secondary antibody was HRP-conjugated goat anti–rat IgG (STAR 72; Serotec, Raleigh, NC). The slides were then developed with Nova Red and followed by hematoxylin QS counterstain.

2.2.6. Tissue Assays

**Myeloperoxidase activity (MPO)**

The assay to determine MPO activity was performed as previously described (14). Briefly, 0.2 g to 0.4 g of lung tissue from saline-perfused animals was homogenized in a 2 ml screw-cap tube containing 0.1 g of zirconia beads and 1 ml of 0.5% Hexadecyl Tri-Methyl Ammonium Bromide (CTAB) in 50 mM PBS, pH 6.0 in an orbital tissue-homogenizer (Fast Prep Bio 101, Thermo Savant, Holbrook, NY) and shaken for 30
seconds at the maximum speed. The homogenates were then transferred to new tubes and the volumes were adjusted with 0.5% CTAB in 50mM PBS, pH 6.0, to make a final concentration of 0.19 g tissue/ml. The samples were then incubated in a 60° C water bath for two hours. The cells were lysed by repeated freeze/thawing in liquid nitrogen and a water bath. The tubes were then spun by centrifugation for ten minutes at 13,000 g and the supernatants containing MPO were transferred to clean tubes. In a 96-well plate, 50 µl of 0.75mM H₂O₂, 80mM PBS, pH 5.4, 50µl of sample or standard, and 25 µl 8mM TMB (Tetra Methyl Benzidine) in DMSO was added in triplicate. The plates were incubated in the dark for two minutes and the reaction was stopped with 50 µl of 1M HCl. The absorbance at 540 nm was measured in a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA) and the values were converted to units of peroxidase activity using a standard curve. The standards were prepared using solutions of concentrations ranging between 0 and 0.5 units of horseradish peroxidase (POD, Roche, Indianapolis, IN) in 0.5% CTAB, 50mM Phosphate buffer, pH 6.0.

**RNA purification and quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) assays**

Total RNA was extracted from tissues harvested at different time points after LPS administration. All the instruments, such as blades and spatulas employed during the isolation were wiped with RNAzap (Ambion, Austin, TX) and carefully handled. A 15- to-20 mg piece of lung tissue was submerged in a 2 ml DNAse / RNAsase free screw cap tube containing 1 g of 1 mm diameter zirconia beads (Biospec Products, INC. Bartlesville, OK) and 600 µl RLT buffer (Qiagen, Valencia, CA). The samples were homogenized in a Fast-Prep FP120 shaker (Thermo Savant, Holbrook, NY). The purification procedure was conducted using the Qiagen RNeasy Mini-extraction kit (Qiagen Ltd, Valencia, CA),
following the manufacturer specification. Once isolated, the total RNA concentration was determined by measuring the absorption at 260 nm using the Nanodrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at -80 °C for future use.

Primers and probes for quantitative QRT-PCR of inducible nitric oxide (iNOS) along with the housekeeping gene ribosomal protein (RPL-19) were designed using the software Primer Express and were synthesized by either MWG-Biotech Inc. (Highpoint, NC) or Applied Biosystems (ABI) (Foster City, CA). The primer sets used for the reactions are listed in table 2.1. QRT-PCR was carried out using the ABI Prism 7700 Sequence Detector. The 30 µl reactions were assayed in duplicate using a final concentration of 1.2 mM dNTPs, 4 mM MgCl₂, 50 nM of each primer, 20 nM of the FAM-TAMRA probe, 1 unit of TaqGold (Roche, Indianapolis, IN), Prime RNAse Inhibitor (Eppendorf, Westbury, NY), and Multiscribe reverse transcriptase (ABI, Foster City, CA). For each reaction, 100 ng of total RNA was used. The thermocycling conditions were: 48° C for 30 min and 95° C for 10 min, followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min, with fluorescent readings at the end of each cycle. Serial dilutions of standard RNA from pCYTO/CHEM or pPC/HYPO (calculated copy numbers: 1.0x10⁴ to 1.0x10⁹) were used to construct standard curves for determination of copy numbers of the relevant genes. mRNA copy number of each individual gene was determined as previously described (15).

2.2.7. Plasma assays

**Cytokine levels**

Plasma levels of soluble TNF-α, IL-6, and MIP-2 were determined using
Quantikine-M murine kits (R&D Systems, Minneapolis, MN). Briefly, the assay was based on the classic quantitative sandwich enzyme linked immunosorbent assay technique. A monoclonal antibody or an affinity purified polyclonal antibody specific for the desired analyte was pre-coated onto a microplate, then 100 µl of standards and samples were dispensed on each well in duplicate, and incubated for two hours. After emptying the microplates, washing, and removing the unbound material, an HRP-labeled secondary antibody against the same analyte (usually an affinity purified goat-anti-mouse polyclonal antibody), was dispensed onto the wells and incubated for two hours. After repeated washes, 50 µl of the substrate solution (TMB and H₂O₂) was added to the wells and the reaction stopped with 50 µl of 1 M H₂SO₄ acid after 30 minutes. The absorbance at 540 nm was subtracted from the absorbance at 450 nm, and measured in a plate reader (Spectra Max Plus) (Molecular Devices, Sunnyvale, CA).

**Soluble E-Selectin levels**

E-selectin is expressed on activated endothelium as an early mediator of leukocyte-endothelial adhesion. Soluble E-selectin is present in the supernatant of cytokine-activated endothelial cells and elevated serum levels are found in a variety of inflammatory conditions (16). In order to determine the contribution of fibrin(ogen) to endothelial cell damage, plasma levels of sE-selectin were measured using Quantikine-M murine kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s directions. The assay is based on the classic quantitative sandwich enzyme linked immunosorbent assay technique, as previously described.

**Plasma NOₓ (NO₂ and NO₃) levels**

Plasma NOₓ (NO₂ and NO₃) levels were determined with a total NOₓ assay kit
(Pierce, Rockford, IL) following the manufacturer’s directions. Nitric oxide is detected by the measurement of two stable breakdown products, nitrate (NO\textsuperscript{3-}) and nitrite (NO\textsuperscript{2-}). This assay relies on the complete conversion of nitrate to nitrite by the action of the enzyme nitrate reductase, and the chemical detection of total nitrite by the Griess reaction, which uses sulfanilamide and N-1-naphthylenediamine dihydrochloride (NED) under acidic conditions. This system is based on nitrite detection as a colored azo dye product that absorbs visible light at 540 nm. Briefly, following conversion of nitrate to nitrite, 50 µl of each individual sample was transferred to a 96-well plate. Next, 50 µl of a sulfanilamide solution was added to each individual well. Following sulfanilamide addition, samples were incubated for 10 min at room temperature and protected from light. After incubation, 50 µl of NED solution was dispensed to all wells. After a 10 min incubation, a purple color began to form as an indicator of the formation of an azo compound. Absorbance was measured at 540 in a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA). Serial dilutions of nitrite standard were used to generate a reference curve for nitrite quantification.

**Thrombin-Antithrombin (TAT) complex levels in plasma**

A two antibody-sandwich ELISA was used to detect TAT complex levels in plasma according to the manufacturer’s instructions. Briefly, 96-well microtiter plates (immuno break apart modules, Nunc maxisorp) were coated with an affinity purified Sheep monoclonal antibody generated against human thrombin (Enzyme Research Laboratories, South Bend, IN, USA); the capture antibody was diluted 1:100 in 50 mM carbonate buffer pH 9.6, and incubated overnight a 4°C. After incubation, the microtiter plates were blocked with 100 µl of PBS-BSA (2% w/v), pH 7.4, at room temperature and washed four times in
PBS/Tween. At this point, the coated plates were ready to be treated with the sample and standards. An aliquot of 100 µl of plasma sample (1:4 dilution in HBS-BSA/0.01% Tween 20 pH 7.2) or standard (0 to 60 ng/ml) TAT complex (Enzyme Research Laboratories, South Bend, IN, USA) in HBS-BSA/0.01% Tween 20 was added to the 96-well plates and incubated for two hours at room temperature. After four washes with PBS/Tween, 100 µl of 1:100 peroxidase conjugate affinity purified Sheep antibody to Antithrombin III (Enzyme Research Laboratories, South Bend, IN, USA) in HBS-BSA/0.01% Tween 20 was added to the wells and incubated for two hours at room temperature. The plates were then washed and 100 µl of the colorimetric substrate TMB and H₂O₂ (R&D Systems, Minneapolis, MN) was added and incubated for 30 minutes at room temperature. The reaction was stopped with 50 µl of 1 M H₂SO₄ acid and the absorbance was measured at 450 nm with a wavelength correction set at 540 nm in a plate reader (Spectra Max Plus, Molecular Devices, Sunnyvale, CA).

2.2.8. Bronchoalveolar lavage fluid collection

After systemic LPS administration, the animals were euthanized at various time points. Their thoraxes were opened, the lungs were dissected, and the tracheas were cannulated in order to collect bronchoalveolar lavage fluid (BALF). Approximately, 0.5 ml of BALF was recovered by gentle suction in phosphate buffer solution (PBS), pH 7.4, and placed on ice for immediate processing. The lavage fluid was spun at 200g for 30 min, and the supernatant was removed aseptically and stored in individual aliquots at 80°C. Albumin in bronchoalveolar lavage fluid (BALF) was determined by a capture ELISA kit following the manufacturer’s protocol (Bethyl Laboratories, Montgomery, TX).
standard curve was generated by serial dilution of purified albumin (7.8-10,000 ng/ml) and used to quantitate the extent of albumin permeabilized in the lungs.

2.2.9. Isolation and fluorescence labeling of human peripheral blood neutrophils

Neutrophils were separated from fresh EDTA-treated whole human blood using Polymorphprep (Axis-Shield, Oslo, Norway). Briefly, 5 ml of anticoagulated whole blood was layered over an equal amount of Polymorphprep in a 15 ml centrifuge tube. The samples were spun by centrifugation at 500 g for 35 min in a swing-out rotor at 18°C. After centrifugation, two leukocyte bands were visible. The lower band consisted of polymorphonuclear cells (PMN) while the erythrocytes were pelleted. The PMN band was harvested using a 200 µl pipette. The cells were then resuspended in PBS, and spun down at 400 g for 10 min at 18°C. The cell pellet was resuspended in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA). The number of neutrophils was quantified using a hemocytometer, and the concentration was adjusted to 1.0×10⁶ cells/ml. The neutrophils (1.0×10⁶ cells) were fluorescence-labeled with 2 mM 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR) in 1 ml of DMEM for 30 min at 37°C. After labeling, the cells were resuspended in 1 ml of DMEM, containing 10 ng of LPS, and incubated for 30 min at 37°C.

2.2.10. Isolation and culture of bone marrow derived macrophages

Bone marrow cells were extracted from the femurs of male WT mice, using a sterile technique. Both femurs were dissected from each mouse. Skin and muscle was removed completely from the bones using a razor blade. The bones were then gently
cleaned with a paper towel, and cut off with sterile scissors. Each individual bone was flushed using a pre-chilled syringe containing 5 ml bone marrow media. The samples were filtered through a 100 µm cell strainer, and spun down at 57 g for 1 min at 4 °C. The supernatant was discarded, and the cell pellet was resuspended with 5 ml of media. The cells were counted, and seeded at 7 x 10⁶ cells in 150 mm petri dishes with 25 ml of BM media. Cells were ready for subculture after 8 days.

2.2.11. Cell cultures

**EA.hy926 cells**

The human cell line EA.hy926, which is a hybrid of human umbilical vein endothelial cells (ECs) and A549 cells, derived from a human lung carcinoma, were provided by Dr CJ Edgell (University of North Carolina, Chapel Hill, NC, USA) and used as the source of ECs. The cells were grown in DMEM/10% FBS/10 U/ml penicillin/10 mg/ml streptomycin. Monolayers of these cells were prepared in each well of 96-well culture plates.

**L-cells**

L-Cell conditioned medium (LCM) is used as a source of monocyte colony stimulating factor (M-CSF) to induce BM cells to differentiate into adherent macrophages. The medium was prepared as follows; L-cells were grown in 10ml of RPMI1640/10%FBS/10 U/ml penicillin-10 mg/ml streptomycin/1%L-glutamine. Once the cells reached 80% confluency, they were expanded into 75 cm² flasks with 25 ml of media and incubated for 5 days. The supernatants were collected and stored at –80° C.
2.2.12. Endotoxin (LPS) detection test

Endotoxin levels in the culture medium and proteins used in this study, were determined by the Limulus Amebocyte Lysate (LAL) test kit (Cambrex, Walkerville, MD) according to the manufacture’s instructions. Briefly, the samples were mixed with the LAL supplied in the test kit and incubated at 37°C for 10 minutes. A substrate solution (Limulus Amebocyte Lysate in water) was then added to the LAL-samples mixture and incubated at 37°C for an additional 6 minutes. The reaction was then stopped with stop reagent (25% v/v glacial acetic acid in water). Yellow color formation was the indicator of the presence of endotoxin in the samples. Absorbance was then determined spectrophotometrically at 405-410 nm. The concentration of endotoxin in each sample was calculated from a standard curve of known endotoxin concentrations.

2.2.13. Neutrophil binding assays

EA.hy926 monolayer wells were washed with DMEM/1% FBS, after which 200 µl of human Fg (0-4000 µg/ml) (Enzyme Research, South Bend, IN), or the same concentrations of bovine serum albumin (BSA), was added to each well. The plate was incubated at 37°C in a CO₂ incubator for 120 min. Fluorescence-labeled, LPS-activated human neutrophils (8.0×10⁴ cells), resuspended in 200 µl of DMEM (Dulbecco’s Modified Eagle’s Medium), were added to each EC-containing well at 37°C in a CO₂ incubator for 30 min. Next, the supernatant and the non-adherent neutrophils were removed by inverting the plate and centrifugation at 500 g for 5 min. ECs and adherent neutrophils were lysed with 100 µl of 50 mM Tris-HCl/0.1% SDS, pH 8.4. The
fluorescence intensity from each well was measured using a FLUOstar galaxy microplate reader (BMG Labtechnologies, Durham, NC).

2.2.14. Bone marrow macrophage assays

BM-derived macrophages (BMM) were subcultured in 4 ml of serum free media (RPMI1640/10 U/ml penicillin/10 mg/ml streptomycin/1%L-glutamine) and seeded at 1x10^6 cells/well in six-well plates. The cells were then incubated with LPS (10 nM), Fg (0 and 2.5 mg/ml), thrombin (0.5 U/ml), hirudin (10U/mL), and Polymyxin B (10µg/ml) for a period of 24 hrs. Culture supernatants were then collected at 0, 6, 12, and 24 h after stimulation for ELISA assays. Soluble MIP-2, IL-6 and TNF-α production were determined using Quantikine-M murine kits (R&D Systems, Minneapolis, MN).

Human Fg and thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Hirudin was purchased from Berlex (Montville, NJ). Polymyxin B was purchased from Sigma (St. Louis, MO).

2.2.15. Measurements of leukocytes

An aliquot (50 µl) of EDTA-treated blood from individual mice was applied to an automated CBC analyzer (Hemavet HV950FS, Drew Scientific, Oxford) in order to determine the number of WBCs, including a three-part WBC differential count (lymphocytes, neutrophils, monocytes).
2.2.16. Statistical Analysis

Data was represented as a mean ± SEM. For survival analysis, the data was classified according to the Kaplan-Meyer analysis and the comparison of survivals between different genotypes was performed using the log-rank and Wilcoxon tests with the NCSS software (NCSS, Kaysville, UT). For data analysis and paired comparison, the t-test was used. All analyses were performed using the computer-assisted Statview program (Abacus Concepts, CA), and p < 0.05 was considered significant.

2.3. Results

2.3.1. Survival Rates

Survival rates were evaluated up to 96 h after LPS administration. Statistical analysis showed that Fg^-/- mice had a lower mortality rate (44.4% survival at 96 h) than WT mice (34.8% survival at 96 h). These survival differences were statistically significant at early stages during challenge, but at later times mortality rates equalized (Figure 2.2). No deaths were observed in control experiments employing saline-containing pumps.

2.3.2. Pathological Analysis

**Fibrin(ogen) in lungs**

Fibrin deposition appeared 6 h post-LPS administration in the capillaries of the lung alveolar wall (Figures 2.3A and 2.3B) and increased up to 24 h (Figures 2.3C, 2.3D,
and 2.3I) in WT mice. No fibrin formation was observed in $Fg^{-/-}$ mice at any time (Figures 2.3E-H and 2.3J).

**Neutrophil infiltration in lungs**

An increase in cellular infiltration was observed at early time points (from 6-12 h) in lungs of WT mice (Figures 2.4A-C). In contrast, $Fg^{-/-}$ mice in which a delay in cellular infiltration was observed (Figures 2.4G-I). As expected, these cells were primarily neutrophils (Figures 2.4J-L).

2.3.3. Tissue and Cell assays

**Myeloperoxidase (MPO) levels in lungs**

An increase in myeloperoxidase activity is an indicator of neutrophil activation. Lung tissues from WT and $Fg^{-/-}$ mice were assayed to determine MPO levels. We found that MPO activity increased as a function of time after LPS administration in WT mice up to 24 h (Figure 2.5A) and decreased at 48 h. In contrast, only a slight increase in MPO was observed in $Fg^{-/-}$ mice during 24-48 h.

**Albumin as a measure of lung permeability**

Bronchoalveolar lavage fluid (BALF) was collected from WT and $Fg^{-/-}$ mice from 0 to 36 h post-LPS administration. Maximal albumin levels were reached 12 h post-LPS administration in BALF of WT mice (149 ± 33 µg/ml for WT mice). In contrast albumin levels in BALF from $Fg^{-/-}$ mice were still rising at 36 h and at this time point were 177 ± 15 µg/ml in $Fg^{-/-}$ mice as opposed to 83.4 ± 22.0 µg/ml in WT mice (Figure 2.5B)
**In vitro assays**

**Effect of Fg on LPS-activated neutrophil binding to ECs in vitro**

Fluorescence-labelled human neutrophils were activated by 1 h incubation with LPS. Following activation, neutrophil binding characteristics to EA.hy926 monolayers were assessed at increasing concentrations of Fg. The results showed that Fg enhances LPS-activated neutrophil binding to ECs (Figure 2.5C). Maximal binding capacity was reached at 2000 µg/ml of added Fg, which is close to its physiological concentration in WT mice.

**Macrophage assays**

We examined the functional consequences of macrophage exposure to Fg, thrombin, and fibrin. Supernatants were harvested from BMM cells cultured in the presence and the absence of Fg or fibrin. Time points of 6-24 h post-Fg administration showed no significant increase in cytokine production above the baseline. In contrast, when fibrin was formed by the addition of 0.5 U/ml of α-thrombin, a significant elevation in MIP-2, TNF-α, and IL-6 production was observed. No significant increase in cytokine production occurred by the addition of thrombin alone (Figure 2.6). To further establish the thrombin independence of fibrin-induced cytokine secretion, cultures were pre-incubated with thrombin for one hour. The cells were then supplemented with hirudin to suppress thrombin activity, followed by Fg addition. No significant contribution of thrombin was observed in the production of inflammatory cytokines, not even after the addition of Fg (Figure 2.7), which indicates that Fg polymerization to fibrin is necessary to trigger cell signaling pathways that lead to the secretion of inflammatory mediators. In order to validate this data, we evaluated the possibility that fibrin-induced cytokine
secretion could be explained by LPS contamination. Cultures were supplemented with polymyxin B (10 µg/ml), and LPS antagonist. Polymyxyn B did affect control experiments supplemented with LPS (10 ng/ml), but no effects were observed in samples incubated with Fg and thrombin (Figure 2.8).

2.3.4. Endotoxin detection assay

In the experiments reported here, culture medium and plasma proteins contained less than 1 U/ml endotoxin. Endotoxin (LPS) contamination did not affect the results obtained in our experiments.

2.3.5. Plasma assays

**Thrombin-Antithrombin (TAT) complex levels in plasma**

TAT levels were measured in plasma samples from WT and Fg⁻/⁻ mice. Interestingly, TAT levels were elevated in resting Fg⁻/⁻ as compared to those in WT mice. These results correlated with the elevated thrombin generation observed in human patients with Fg deficiencies (17). At 3 h post-LPS administration, TAT levels were greatly elevated in WT mice, while the TAT levels in Fg⁻/⁻ mice were unaltered from their resting values. At 6 h post-LPS, a slight elevation of TAT was observed in Fg⁻/⁻ mice and these levels in both genotypes remained similar at later times (Figure 2.9A).

**Soluble Endothelial selectin (sE-Sel) levels in plasma**

The release of E-selectin into circulation is an indicator of ECs damage. sE-selectin levels were assessed in plasma samples from WT and Fg⁻/⁻ mice. Soluble E-selectin levels were dramatically increased 6 h post-challenge in WT mice and remained
elevated through 24 h. In contrast, the E-selectin levels in Fg$^{-/}$ mice were stable up to 12 h with increases noted 24 h post-LPS administration (Figure 2.9B).

**Nitric oxide levels in plasma**

No significant differences in plasma concentration of NOx were found between WT and Fg$^{-/}$ mice in resting conditions. At 3 h post-challenge, NOx was elevated in WT mice and then rapidly decreased to baseline at 6 h, and remained stable up to 24 h. In contrast, the levels of NOx in Fg$^{-/}$ mice at 3 h post-LPS were significantly lower than those observed in WT mice at this same time. NOx levels in plasma from Fg$^{-/}$ mice increased at 12 h post-LPS and were significantly higher than those in WT mice. These values then decreased through 24 h (Figure 2.9C).

**iNOS mRNA levels in lung tissue**

iNOS expression was measured in lung tissue from WT and Fg$^{-/}$ mice. No significant differences in iNOS mRNA levels were detected at resting conditions between genotypes. At 6 h post-LPS administration, iNOS expression was significantly increased in WT mice, then decreased at 12 h, and remained stable through 24 h. In contrast, the iNOS mRNA levels in Fg$^{-/}$ mice were elevated at 12 h post-LPS (Figure 2.9D). Similar trends were observed when NOx was measure in plasma (Figure 2.9C).

**Inflammatory cytokines in plasma**

Plasma levels of inflammatory cytokines were evaluated in WT and Fg$^{-/}$ mice. No differences in cytokine levels were found at resting conditions between genotypes. TNF-α, IL-6, and MIP-2 levels were measured in plasma of WT and Fg$^{-/}$ mice after LPS administration at different time points (0-24h). We observed that TNF-α levels were
elevated in both genotypes 6 h post-LPS, however these levels were significantly higher in WT mice than in Fg⁻/⁻ mice. TNF-α levels in WT mice were decreased at 12 h post-LPS administration and remained low at 24 h. The opposite was observed in Fg⁻/⁻ mice, as TNF-α levels were significantly higher than those in WT mice at 12-24 h post-challenge (Figure 2.10A). Similarly, at 6 h post-LPS administration, the IL-6 plasma concentrations were elevated in both groups, but these levels tended to be higher in WT mice than in Fg⁻/⁻ mice, although the differences were not statistically significant. In both genotypes the plasma IL-6 levels were decreased at 24 h (Figure 2.10B). Increased MIP-2 levels were detected 6 h post-LPS administration in both genotypes, but those in WT mice were significantly higher than those observed in Fg⁻/⁻ mice. Interestingly, plasma levels of MIP-2 in WT mice decreased from 6 to 24 h, while in Fg⁻/⁻ mice increases occurred from 6 to 24 h post-LPS administration. At 6 and 24 h, MIP-2 levels were significantly higher in Fg⁻/⁻ mice than in WT mice (Figure 2.10C).

2.3.6. Measurements of Leukocytes (WBcs)

The number of total WBCs, neutrophils, lymphocytes, and monocytes in both genotypes were very similar in resting mice. Total WBC counts decreased at 3 h in both genotypes (Figure 2.11A). This was mainly reflected by the decrease of lymphocytes, which remained low up to 24 h and were almost identical at all time points, except at 12 h (Figure 2.11B). On the other hand, neutrophil counts increased from 3 to 24 h and were significantly higher in Fg⁻/⁻ mice at later times (Figure 2.11C). The monocyte counts were constant throughout the challenge and not different between the groups (Figure 2.11D).
2.4. Discussion

The hemostatic and inflammatory systems are highly integrated with considerable cross-talk. Inflammatory events trigger the activation of the coagulation and fibrinolytic systems, and vice-versa. The key coagulation factor Fg, is one of the hemostatic factors that “bridge” the coagulation and inflammatory pathways. Fibrin(ogen) deposition on vascular ECs and organs is an important hallmark feature of acute inflammation. The focus of the present study was to evaluate the importance of fibrin(ogen) on lung pathologies, since acute lung injury and malfunction is characteristic in uncontrolled widespread inflammation (18). Additionally, pulmonary inflammation rapidly triggers lethality due to increased alveolar permeability, which leads to defective gas exchange and hypoxia (19).

Fibrin deposition normally occurs very quickly after mechanical trauma, thermal trauma, or infection. LPS-induced endotoxemia is characterized by the formation of fibrin(ogen) matrices within challenged organs, serving as a signal of “local damage”. The formation of fibrin deposits is important in leukocyte recruitment, and the subsequent expression of cytokines and chemokines. In this study, following the LPS challenge, fibrin(ogen) deposits appeared early in lung tissue of WT mice and remained high through later time points. As expected, in correlation with increased fibrin deposition, neutrophil diapedesis occurred at early time points in WT mice tissues; however neutrophil infiltration was delayed in Fg−/− mice, due to the lack of fibrin deposits. These observations were further supported by in vitro assays employing ECs, primary human neutrophils and Fg. A positive correlation between Fg levels and the binding of LPS-activated neutrophils to activated ECs occurred. Endothelial cells serve as
a barrier for the exchange of macromolecules and fluids between the vascular compartment and the tissues. Increased vascular permeability allows inflammatory cells to contact the endothelial lining (20). Lung microvascular permeability was measured as increases in albumin concentration levels in BALF following LPS administration. Increased permeability of lung vasculature occurred at early times post-LPS treatment in WT mice, but at later times in Fg−/− mice as indicated by the albumin concentration levels in BALF. These results indicated that increased fibrin formation correlates with early EC dysfunction, which contributes to the organ failure characteristic of systemic inflammatory disorders. Furthermore, the endothelium expresses selectin molecules, which are involved in the initial recruitment of the leukocytes to the endothelial surface. LPS increases the expression and release to circulation of soluble E-selectin. In septic patients, a high level of endothelial selectins in plasma correlates with poor prognosis (21). In this study, elevation of soluble E-selectin levels in plasma was observed at 6 h in WT mice and at 24 h in Fg−/− mice, indicating a delay in EC activation, which also contributes to attenuated neutrophil migration into the tissues in a Fg−/− state. Together these results indicated that Fg accelerates the inflammatory process. Fibrin within the damaged tissues label the site of any challenge and triggers the recruitment and activation of leukocytes by its interaction with specific receptors on ECs, macrophages, neutrophils and other cells.

Thrombin is an important coagulation factor, which cleaves Fg to fibrin promoting the arrest of bleeding by the formation of the blood clot. Furthermore, thrombin is involved in inflammatory processes via its interactions with multiple proteolytic targets and pathways, e.g., through interactions with G-protein-coupled
receptors (PARs), and through the stimulation of ECs, platelets, and other cells leading to the production of cytokines and chemokines (22,23). Afibrinogenemia patients present with several hemostatic alterations, such as high levels of TAT. In correlation with their human counterpart, Fg−/− mice show elevated levels of TAT. Interestingly, this high TAT condition did not measurably up-regulate the resting levels of inflammatory mediators such as, TNF-α, IL-6, or MIP-2. After LPS treatment, TAT levels in WT mice were elevated early and remained high through 24 h post-LPS administration. However, Fg−/− plasma TAT levels were similar to resting levels through the entire challenge period. These findings indicate that a high thrombin level, in itself, is inadequate to enhance inflammatory responses. This observation was further supported by in vitro assays employing bone marrow derived macrophages. When cells were supplemented with thrombin, no significant increases in the levels of TNF-α, IL-6, or MIP-2 occurred. Interestingly, addition of Fg had no effects on cytokine production. In contrast, when cells were cultured with fibrin, formed by thrombin incubation with Fg, significant upregulation in the secretion of inflammatory cytokines occurred. These results suggest that Fg conversion to fibrin is necessary to transmit signals to prompt macrophage cytokine expression and secretion. Our results indicate that inflammatory cell activation is rapidly triggered by Fg conversion to fibrin. Once fibrin deposits are formed, tissue macrophages express and secrete a wide variety of inflammatory mediators as a response to the stimuli.

The similarity of the differential expression patterns of TNF-α, iNOS, and MIP-2 during the LPS challenge in both groups of mice support the proposed role of fibrin(ogen) in the regulation of leukocyte function. Neutrophils are known to specifically
engage immobilized fibrin(ogen) through the leukocyte integrin receptor $\alpha_M\beta_2$. The engagement of increased number of neutrophils on the inflamed endothelium leads to the up-regulation of the early response mediator TNF-\(\alpha\), which is known to induce transcription factor NF-\(\kappa\)B (24), which then stimulates cell migration, production of cytokines and chemokines, e.g., IL-6, and MIP-2, and the expression of a number of inflammatory mediators, e.g., iNOS. After LPS administration, MIP-2 and iNOS levels peaked early in WT mice and decreased through 24 h. In contrast, the levels of these inflammatory markers were increased in Fg\(^{-/}\) mice up to 24 h. These results confirm that fibrin(ogen) systematically accelerated the production of MIP-2 and iNOS, likely via activation of the transcription factor NF-\(\kappa\)B by increased expression of TNF-\(\alpha\).

Nitric oxide (NO) is synthesized from the amino acid L-arginine by the action of NO synthase (NOS). NO is a highly reactive radical important for the regulation of cellular functions in a normal physiological state. In pathological conditions, high output of NO contributes to tissue damage and hypotension. LPS induces the production and release to circulation of large amounts of NO by alveolar macrophages and lung endothelial cells (25). In the present study, plasma levels of NO were measured. Plasma NO was elevated early in WT mice. This trend correlated with the expression partner of iNOS, and the increased albumin concentration in BALF, which were also delayed in Fg\(^{-/}\) mice. A possible mechanism to explain these findings is that once activated neutrophils accumulate on the injured tissue, these cells release a number of chemical mediators that affect EC permeability, a process delayed in a Fg deficient state (26). Early neutrophil recruitment into the lungs in WT mice correlates with the increase in cellular permeability indicated by the albumin levels observed in BALF. This phenomenon does
not occur as early in Fg−/− mice as in the WT counterparts. Thus, the albumin levels were low at early time points but increased at later time points, in accord with the delayed neutrophil recruitment.

The up-regulation of the inflammatory cytokines IL-6 and MIP-2, observed post-LPS administration in both genotypes, is also relevant. Fibrin(ogen) can stimulate expression of IL-6 and MIP-2 by ECs and mononuclear cells (12,26). Nevertheless, differences in Fg levels do not have a major affect on IL-6 production in this model. IL-6 appears to have an effect on the expression of Fg. Fg up-regulation leads to enhanced MIP-2 production by alveolar macrophages and activated neutrophils. MIP-2 contributes to lung injury by regulating neutrophil infiltration, NO generation, and pulmonary edema. Elevated plasma levels of MIP-2 correlated closely with the degree of neutrophil infiltration in lung reflected by the increased MPO activity and the pathological findings shown by immunohistochemistry. Furthermore, MIP-2 generation leads to the activation of neutrophil elastases (27), which contributes to tissue damage by LPS-activated neutrophils. Overall these results indicate that a Fg deficiency attenuates EC damage by delaying neutrophil recruitment to the site of injury, and subsequent processes, such as cytokine expression, increase in permeability, and further activation of the resident alveolar macrophages. In figure 2.12 a schematic representation of a possible mechanism by which Fg regulates lung injury in this model is proposed.

In conclusion, the present work highlighted the role of fibrin(ogen) in bridging the coagulation system and the inflammatory response by regulating macrophage chemokine secretion, and neutrophil binding to EC, promoting the development of a pro-inflammatory endothelium.
Figure 2.1 Representation of the fibrinogen molecule. Fg structure consists of three pairs of polypeptide chains: $\alpha$, $\beta$, and $\gamma$. Fibrinopeptides A and B (FPA and FPB). *Carbohydrate clusters. † Disulfide rings.
Figure 2.2 Survival rates of WT and Fg\textsuperscript{-/-} mice after implantation of the LPS-containing osmotic pumps. The solid line indicates the survival curve of WT mice (N=23 mice) and the dashed line depicts the survival curve of Fg\textsuperscript{-/-} mice (N=27). The difference in the survival rates was compared using the log-rank and the generalized Wilcoxon tests. The p values were 0.1332 and 0.0373, respectively.
Figure 2.3 Fibrinogen in lung tissue of LPS-challenged WT and $Fg^{-/-}$ mice. Fibrinogen immunohistochemical analysis of lung tissue from (A-D) WT and (E-H) $Fg^{-/-}$ mice at 0, 6 h, 12 h, and 24 h after administration of LPS. Original magnification $\times$ 400. (I, J) Higher magnified images corresponding to the black square in panels D and H, respectively. Original magnification $\times$ 1000.
Figure 2.4 Histochemical and immunohistochemical analysis of murine lung tissue after LPS administration via osmotic pumps. (A-C) H&E staining of WT and (G-I) Fg<sup>-/-</sup> mouse lung sections 0, 6 h, and 12 h post-LPS administration. Anti-neutrophil immunostaining of lung sections of WT mice at (D) 0, (E) 6 h, and (F) 12 h post-LPS treatment. Anti-neutrophil immunostaining of lung sections of Fg<sup>-/-</sup> mice at (J) 0, (K) 6 h, and (L) 12 h post-LPS treatment. Original magnification × 400.
Figure 2.5 Neutrophil infiltration into lung endothelium post-LPS. (A) MPO activity in lung tissue from WT (black bars, $N = 5$-7 mice at each time point) and Fg^{-/-} (grey bars, $N = 5$-9 mice at each time point) mice at various times after initiation of LPS challenge. (B) Albumin concentrations in BALF from WT (black bars, $N = 5$-7 mice at each time point) and Fg^{-/-} (grey bars, $N = 5$-9 at each time point) mice. (C) LPS-activated neutrophil binding to endothelium in the presence of increasing concentrations of Fg (black bars, $N = 6$ mice) and BSA as a control (grey bars, $N = 5$ mice). *$p < 0.05$
Figure 2.6 Fibrinogen conversion to fibrin stimulates cytokine secretion by bone marrow-derived macrophages (BMM). Secretion of TNF-α (A), IL-6 (B), and MIP-2 (C) by BMM. BMM (1 x 10^6) were seeded in 6-well plates and cultured in the presence of fibrinogen (Fg) (2.5 mg/ml), thrombin (0.5 U/ml), and fibrinogen supplemented with thrombin to promote fibrin formation. Cytokines levels were significantly elevated by the formation of fibrin. No increases in cytokine production were observed by incubation with fibrinogen or thrombin. The experiments were performed in triplicate.
Figure 2.7 Thrombin is inadequate in inducing cytokine secretion by BBM. BBM cells were assayed for TNF-α (A), IL-6 (B), and MIP-2 (C) production in response to fibrinogen (2.5 mg/ml), thrombin (0.5 U/ml), thrombin supplemented with fibrinogen, and thrombin supplemented with 10 U/ml of hirudin, followed by fibrinogen (Fg) addition. After 1 h incubation with thrombin, no effects in inflammatory cytokine production were observed. Cytokine secretion was upregulated upon supplementation with fibrinogen. No elevation in TNF-α, IL-6, and MIP-2 levels occurred in the presence of fibrinogen after thrombin activity was suppressed by the addition of hirudin.
Figure 2.8 LPS does not contribute to fibrin-stimulated cytokine secretion. Cultures were stimulated with fibrinogen (2.5 mg/ml), thrombin (0.5 U/ml), LPS (10 ng/ml) in the presence of the LPS antagonist polymyxin B sulfate (10 µg/ml). Supernatants were collected at different time points and assayed for TNF-α, IL-6, and MIP-2. Polymyxin B suppressed cytokine secretion in the cultures supplemented with LPS, but no affects were observed on fibrin-stimulated cells.
Figure 2.9 Markers for thrombin generation and EC damage after LPS. (A) Plasma TAT levels in WT (black bars, N=6-9) and Fg<sup>−/−</sup> (grey bars, N=5-10) mice at 0, 3 hr, 6 hr, 12 hr, and 24 hr after initiation of LPS administration. (B) Plasma soluble E-selectin levels in WT (black bars, N=5-7) and Fg<sup>−/−</sup> (grey bars, N=5-7) mice at 0, 3 hr, 6 hr, 12 hr, and 24 hr after initiation of LPS administration. (C) Plasma NOx levels in WT (black bars, N=6-8) and Fg<sup>−/−</sup> (grey bars, N=6-10) mice at 0, 3 hr, 6 hr, 12 hr, and 24 hr post-LPS administration. (D) The relative expression levels of mRNA of iNOS versus RPL19. WT (black bars, N=8-18 mice at each time point) and Fg<sup>−/−</sup> (grey bars, N=6-13 mice at each time point). * p<0.05.
Figure 2.10 Plasma markers of the inflammatory response to LPS challenge. (A) Plasma levels of TNF-α in WT (black bars) and Fg⁻/⁻ (grey bars) mice at various times after initiation of LPS administration. N = 5-1 mice at each time point. (B) Plasma levels of IL-6 in WT (black bars, N = 7-13 mice at each time point) and Fg⁻/⁻ (grey bars, N = 8-11 mice at each time point) mice. (C) Plasma levels of MIP-2 in WT (black bars, N = 5-13 mice at each time point) and Fg⁻/⁻ (grey bars, N = 5-15 mice at each time point) mice. *p < 0.05
Figure 2.11 WBC counts during the time course. Total WBC (A), lymphocytes (B), neutrophils (C), and monocytes (D) in WT (black bars, N=7-9) and Fg−/− (grey bars, N=8-9) mice at 0, 3 hr, 6 hr, 12 hr, and 24 hr after initiation of LPS administration. * P<0.05.
Figure 2.12 Schematic representation of the role of fibrin(ogen) in acute lung injury. LPS binding to its receptor (TLR-4 in complex with CD14 and MID-2) results in monocyte and endothelial activation. LPS recognition leads to NFκB signaling pathway activation in monocytes and endothelial cells. LPS-induced NFκB activation promotes the expression of inflammatory cytokines on monocytes and injured endothelial cells. Cytokine signaling up-regulates the activation of additional inflammatory cells, mainly neutrophils. The formation of fibrin(ogen) deposits on lung endothelial cells affects permeability, nitric oxide production, and accelerates neutrophil binding and recruitment to the site of injury by the expression of selectin and adhesion molecules, leading to impaired vasoconstriction and edema. Fibrin(ogen) mediates neutrophil adhesion to endothelial cells by its interactions with the integrin receptors expressed on the leukocyte surface. The engaged neutrophils then transmigrate from the vascular compartment into the tissue. Once neutrophils accumulate on the injured endothelium, they release a number of chemical mediators, e.g., neutrophil elastases, that affects endothelial cell permeability by promoting further damage. In addition, increased fibrin(ogen) deposition enhances the production of inflammatory cytokines by alveolar macrophages and endothelial cells.
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2.5. References


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CHAPTER 3:
BACKGROUND

Group A streptococcus (GAS) is the etiologic agent responsible for a wide variety of human diseases which range from common pharyngitis to invasive manifestations. The incidence of diseases caused by GAS has increased considerably in recent years, especially in the invasive form, as necrotizing fasciitis, characterized by skin wounds. During the past several years, the pathogenic mechanisms of streptococci have been an area of intense investigation. Recent studies have found that streptococci have the ability to invade nonphagocytic cells by breaking the host cellular and tissue barriers through interaction with host proteins that facilitate access into the vascular system (1, 2). Interactions between the plasminogen (Pg) system and streptokinase (SK), a protein secreted by GAS, have been hypothesized to promote the bacterial invasion into tissues (3). Interestingly, unlike other Pg activators which activate Pg by limited proteolysis, SK lacks hydrolytic activity. Therefore, the mechanism by which this protein displays its activity remains unclear (4). Streptokinase is a 440 amino acid protein, with a 26-amino acid N-terminal signal peptide that is cleaved during secretion to yield a 414-amino acid mature protein. Streptokinase contains three domains denoted α (residues 1-150), β (residues 151-287), and γ (residues 288-414). Experiments have demonstrated that each individual domain can bind to Pg. However, they cannot activate Pg independently. Until now, investigations have focused mainly on the identification and characterization of
domain interactions between human Pg (hPg) and SK. Mutagenesis studies have shown that the α, β, and γ domains cooperatively induce the formation of an active site within the hPg activator complex moiety, providing a means for the substrate Pg to be recognized by the activator complex, mainly through interactions mediated by the SK α-domain (5, 6). Although the results obtained in these studies have helped to establish the basic principles of Pg activation by SK, the nature and mechanism of species-restricted action of SK are virtually unknown. SK is a specific activator of hPg, and exhibits no activity towards other species, including mouse Pg (mPg). Studies of streptococcal pathogenicity have been hampered by this limitation. Recently, “humanized” transgenic mice expressing hPg were generated, and enhanced virulence and systemic spread of GAS infection were observed (7), supporting the idea that recruitment and activation of Pg by invasive bacterial pathogens enhances its virulence.

In order to gain a more complete understanding of how bacterial pathogens take advantage of the plasminogen/plasmin system, molecular and in vivo experiments needed to be performed. For this study, a systematic interchange of exons between hPg and mPg was performed. This approach allowed for the identification of specific regions of hPg that determine SK sensitivity.

A detailed background section is presented with the objective of elucidating the rationale and motivations of this work.

3.1. The Fibrinolytic System

After tissue injury, coagulation activation is triggered promoting thrombus formation and propagation. Once hemostasis has been restored, the formation of fibrin
clots at the site of vascular injury must be regulated in order to prevent thrombotic complications. One of the processes by which thrombus propagation is restricted beyond the site of injury is known as fibrinolysis. The main active enzyme involved in the fibrinolytic process is plasmin (Pm), which is formed from its precursor, Plasminogen (Pg). Pm cleaves the fibrin mesh at various sites, leading to the formation of fibrin degradation products that are cleared by other proteinases. The fibrinolytic system is activated when Pg is converted to the serine protease Pm, by the physiological activators urokinase-type Pg activator (uPA) or tissue-type plasminogen activator (tPA). Pm then cleaves fibrin at a number of different sites, producing soluble fibrin degradation products without hemostatic activity. The fibrinolytic system is regulated by plasminogen activator inhibitor (PAI-1) and plasmin inhibitors. PAI-1 inactivates tPA and uPA and is released from vascular endothelial cells and activated platelets. The primary plasmin inhibitor is α2-antiplasmin, which inactivates free plasmin escaping from clots. Some α2-antiplasmin is also cross-linked by factor XIIIa to fibrin during clotting in order to prevent excessive plasmin activity within clots. tPA and uPA are also cleared by the liver, which is another mechanism of preventing excessive fibrinolysis (Figure 3.1).

3.2. Human Plasminogen: Gene organization and Primary structure

Human Plasminogen (hPg) is synthesized in the liver as an 810-amino acid polypeptide-chain. During secretion a 19-amino acid leader peptide is cleaved, generating the mature form of this protein, which comprises 791 amino acids (8-10). Extrahepatic organs have also been identified as possible sources of Pg, e.g., adrenal, kidney, brain, testis, heart, lung, uterus, spleen, thymus, and gut tissue (11). The gene for hPg has been
mapped to chromosome 6q26-6q27. The hPg coding sequence comprises a 57 bp signal sequence and a total of 2373 nucleotides for the mature protein. The hPg gene contains nineteen exons, which range in size from 75-387 bp, and eighteen introns of type I, II and O. The primary structure of hPg is presented in Figure 3.2. The Pg molecule contains a total of six structural domains, each with different properties. The N-terminal portion of the molecule consists of an activation peptide (AP). Cleavage of the peptide bond between residues 77 and 78 is required for the release of the activation peptide resulting in the transformation of Glu-Pg to Lys-Pg. The cleavage of the Arg^{561}-Val^{562} peptide bond in hPg leads to the formation of Pm, which contains a heavy chain of 561 amino acid residues, disulfide linked to a light chain of 230 amino acid residues. The heavy chain comprises the N-terminal portion of the Pg molecule, which consists of an activation peptide (AP), and a series of repeating homologous triple-disulfide-linked peptide regions, about 80 amino acids in length, termed kringles. The function of kringles in Pg is primarily to mediate protein-protein interactions, such as those between Fg and hPg, and hPg to mammalian and bacterial cell surfaces. The Pm light chain contains the carboxyl-terminus of hPg, which comprises the catalytic domain that resembles that of the serine protease family. The catalytic triad of amino acids that define serine proteases is present in Pm, and involves His^{603}, Asp^{646}, and Ser^{741}(4).

3.3. Plasminogen activation

The fibrinolytic system is activated when Pg is converted to the serine protease Pm, by the physiological activators uPA or tPA usual activators of the fibrinolytic system. The conversion of Pg to Pm is a consequence of proteolytic cleavage of the
Arg$^{561}$-Val$^{562}$ peptide bond. This results in the generation of Glu$^1$-Pm, which consists of a N-terminal-derived heavy chain (561 amino acids), covalently linked by two disulfide bonds to a COOH-terminal-derived light chain (230 amino acids). The overall scheme for conversion of circulating Glu$^1$-Pg to the final product, Lys$^{78}$-Pm is shown in Figure 3.3 (12, 13). Glu$^1$-Pg and Lys$^{78}$-Pg can adopt two different conformations, T and R, which are highly influential to their activation capability. The T-state (Tight-conformation) is a compact state seen in full-length hPg. In this conformational state, Pg is poorly activated (14-16). Investigations of recombinant (r) Glu$^1$-Pg variants have shown that both negative and positive effector molecules have an influence on the structure and activation of plasminogen (17, 18). For example, it has been suggested that in the presence of Cl$^-$, Lys side chains, and/or pseudo-Lys arrangements in the 77-amino acid NH$_2$-terminal AP, interact with ω-amino acid binding sites of K1$_{Pg}$, K4$_{Pg}$, and to a lesser extent, K5$_{Pg}$. In addition, studies have shown that a number of activation peptide residues interact with these kringle sites, and participate in the stabilization of the T-conformation (19). On the other hand, the conformation of Glu$^1$-Pg changes to a more flexible Relaxed-state (R-state), that is readily activatable when the activation peptide (AP) is displaced from its kringle binding sites upon addition of ω-amino acids, e.g., ε-aminocaproic acid (EACA). Once Glu$^1$-Pm is formed, it acts as a catalyst for cleavage of the AP from Glu$^1$-Pg and Glu$^1$-Pm, thus catalyzing conversion of Glu$^1$-Pg to Lys$^{78}$-Pg or Glu$^1$-Pm to Lys$^{78}$-Pm. Release of the AP renders Lys$^{78}$-Plg a more activatable substrate due to its transformation to the R-state and transition to a protein that is now insensitive to negative and positive effector molecules (20, 21).
3.4. Bacterial Pathogenesis and the fibrinolytic system

The ability to degrade tissue barriers formed by extracellular matrices, and basement membranes is one of the most important factors in the pathogenesis of bacterial infection. Massive tissue and structural damage by bacterial proteases enhances bacterial invasiveness into the host body. However, a number of invasive bacteria are extracellular pathogens and produce very low levels of proteinases. Consequently, degradation and penetration through a network of membranes composed of collagen fibrins, elastin, fibronectin, and proteoglycans, required the use of different mechanisms for invasiveness. Some of these mechanisms rely on the interaction with proteinase-dependent cascade systems of their host. The mammalian fibrinolytic system offers a potential proteolytic system that could be utilized by pathogenic bacteria.

3.4.1. The fibrinolytic system in bacterial infection

The activation of the fibrinolytic system has been suggested as a critical component in bacterial invasiveness. Bacteria interact with the fibrinolytic system in various ways. Once Pg is activated to Pm, it offers a potential proteolytic system that could degrade various extracellular matrices and membranes. Plasmin can activate procollagenase as well as elastases, aiding in the breaking down of extracellular matrices (22). Several pathogenic bacteria, such as Streptococcus and Staphylococcus, produce plasminogen activators (PAs) that are either secreted or surface-bound proteins. Streptokinase (SK) and staphylokinase (SAK) are not enzymes themselves but form 1:1 complexes with Pg and Pm, leading to changes in conformation and specificity of Pg. The mechanism of Pg activation by SK and SAK is similar but differs in some aspects.
While SK-Pg is enzymatically active, SAK-Pg is inactive and requires the conversion of Pg to Pm. Another important difference is that SAK primarily activates fibrin-bound Pg. This requirement has encouraged studies with SAK regarding its ability to function as an *in vivo* thrombolytic agent (23).

### 3.4.2. Activation of hPg by SAK

Staphylokinase (SAK) is a 136-amino acid protein produced by strains of *S. aureus* carrying a prophage, which contain the sak gene. SAK does not possess protease activity by itself. hPg activation by SAK depends on the formation of a stoichiometric complex between SAK and hPm. The SAK-hPm is formed from SAK-hPg in the presence of other PA e.g., tPA (24). The SAK-Pm complex modifies SAK within the complex by cleavage of the Lys$^{10}$-Lys$^{11}$ peptide bond and subsequently converts SAK-Pg to SAK-Pm, which in turn converts free Pg to Pm. There is evidence that the Arg$^{719}$ in Pg and the Met$^{26}$ in SAK are important for the binding between SAK and Pg. Moreover the NH$_2$-terminal region of SAK is important for the active site formation in the Pm molecule in the binary complex (25-28).

### 3.4.3. Activation of human plasminogen by Streptokinase

Invasive bacterial infections caused by streptococci represent one of the most extensively study models for interactions between pathogens and the Pg system (29). SK is a single-chain 414-amino acid protein secreted by β-hemolytic group A, C and G streptococci. SK contains three domains, denoted α (residues 1-150), β (residues 151-287), and γ (residues 288-414) (30). SK is an efficient PA, and plays an important role in
the streptococcal virulence by generating proteolytic activity at the bacterial cell surface, facilitating invasion of host tissues. Interestingly, SK does not exhibit hydrolytic activity. Thus, in order to activate Pg, SK has to bind to Pg and to induce within it an active site by nonproteolytic mechanisms. The activation of Pg by SK involves two major mechanistic steps: (1) formation of a plasminogen activator complex, and (2) the activation of substrate Pg. The formation of the activator complex involves interactions between the COOH-terminal domain of SK and the catalytic domain of Pg, as well as another site in SK and the kringle domains of Pg. In the SK-Pg complex, the active site of Pg is exposed and functional without hydrolysis of the Arg560-Val561 peptide bond (31, 32). Stability of the SK-Pg complex is attributed largely to high affinity binding between components of the β-domain of SK with Pg. Conversely, crystallographic studies determined the surface area of the respective SK domains available to interact with Pm and found that the β-domain is significantly less involved than the α or γ-domains (33).

In the second step of the activation, SK-Pg complexes become catalytic activators of the remaining Pg. Recent equilibrium binding and kinetic studies of the SK-initiated pathway of Pm formation support a mechanism in which rapidly formed SK·Pg* (streptokinase-plasminogen) binds Pg as a specific substrate, followed by intermolecular cleavage to form Pm. SK has approximately 800+ higher affinity with [Lys]Pm as compared to native [Lys]Pg, and 11,000+ higher affinity as compared with [Glu]Pg, which results in displacement of Pg from SK·Pg* and formation of SK·Pm (streptokinase-plasmin), that then catalyzes complete conversion of free Pg to Pm. The Pg activation mechanism is modulated by lysine-binding site (LBS) interactions between SK and the kringle domains
of Pg and Pm (34). The overall activation process of hPg by SK is described in Figure 3.4.

3.5. Species selectivity of streptokinase in activation of different mammalian plasminogens

Through years of intensive investigations, important structural data have been obtained regarding critical interactions within the SK-hPg complex that might contribute to the mechanism of the activation of hPg by this complex. The roles of domains of SK that function in various manners in the activator complex have been revealed (35, 36). However, the basis for the species selectivity of SK in activation of mammalian plasminogens is yet unclear (4, 13). Human and non-human-derived SKs differ from each other and activate Pg in a species-specific manner. Early studies with rabbit Pg (rPg) showed weak activation when incubated with SK produced by a strain of human origin. It was found that although a complex was formed between SK and rPg, SK was rapidly degraded to inactive forms, indicating that the complex was unstable (37). Investigations with SKs isolated from streptococcal strains from equine or porcine origin have extended knowledge further on this subject. Although equine/porcine-derived SKs interact with hPg, no activation was achieved. A similar situation was observed when either equine or porcine Pg was incubated with human raised SK. Comparisons of amino acid sequences of different mammalian-derived SKs have shown that there are few similarities between them. These data indicated that the complex activator formed between SK and Pg could vary in primary structure and conformational properties, affecting Pg activation (38).
Further studies are needed to fully understand the molecular mechanisms of this species-restricted activation to help to elucidate the basis for species-restricted infection.

3.6. Group A streptococci infections

Plasminogen activation is a critical component in establishing invasive bacterial infections. Group A streptococci (GAS) infection incidence has increased considerably in recent years, especially in the invasive form, as necrotizing fasciitis (characterized by skin wounds). Necrotizing fasciitis is a rare condition in which bacteria destroy tissues underlying the skin. Tissue death, necrosis or gangrene, spreads rapidly. This disease can be fatal in as little as 12 to 24 hours. During the past several years the pathogenic mechanisms of streptococci have been an area of intense investigation. Recent studies have found that streptoccoci have the ability to invade non-phagocytic cells by breaking the host cellular and tissue barriers through the interaction with host proteins, such as Pg. Plasminogen interactions with streptococcus contribute to the invasive potential of the microorganism by direct action in degrading host tissue barriers, and facilitating access into the vascular system. Certainly, animal models have provided valuable information toward understanding bacteria-Pg interactions, however they also have limitations in this respect, due to the high species-specificity of pathogenic factors such as SK. Early investigations with mPg suggest very low sensitivity to streptokinase as compared to human and other mammalian species (39). In vivo studies demonstrated that mice generally are highly resistant to skin infection by most human pathogenic GAS (40). Thus, hPg plays a critical role in the pathogenesis of these human GAS by interacting with specific pathogenicity factors (streptokinase, staphylokinase) a function that is not
provided by mPg. Binding studies using surface plasmon resonance indicated that in order to promote the formation and activation of the hPg-SK complex, SK β domain interacts with Pg kringle 5 and all three domains of SK interact with the hPg catalytic domain (6, 41, 42). Animal infection models have been developed using PG−/− mice (43), and more recently Tg+ PG−/− mice (7) (transgenic mice expressing human plasminogen in a PG−/− background). This latter study raised the possibility that the degree of polymorphism between the different plasminogen species determines the susceptibility for specific bacterial infections. However, more specific questions concerning Pg-SK interactions remain to be answered. In order to shed more light on the molecular mechanisms of the species specificity of SK for host Pg, biochemical studies have been performed. Recombinant chimeric proteins sharing human and mouse Pg sequences have been produced to investigate which specific areas of Pg are important for SK sensitivity. Biochemical techniques has provided important information regarding the nature of Pg activation by SK. In the next chapter these novel findings will be discussed.
Figure 3.1 The fibrinolytic system. The main active enzyme of the fibrinolytic pathway is Plasmin (Pm), which is formed from its precursor, Plasminogen (Pg). The conversion of Pg to Pm is a consequence of proteolytic cleavage of the Arg$^{561}$-Val$^{562}$ peptide bond by the action of the physiological plasminogen activators Urokinase Plasminogen activator (uPA) and/or Tissue Plasminogen activator (tPA). Pm cleaves fibrin leading to the formation of fibrin degradation products (FDP).
Figure 3.2 Primary structure of human Plasminogen. http://www.chem.cmu.edu/groups/Llinas/res
Figure 3.3 The overall scheme for conversion of circulating Glu\textsuperscript{1}-Pg to the final product Lys\textsuperscript{78}-Pm. Circulating Glu\textsuperscript{1}-Pg is activated to Glu\textsuperscript{1}-Pm by activator A-catalyzed cleavage of the Arg-Val peptide bond in Glu\textsuperscript{1}-Pg. This step most likely provides the first molecules of plasmin (Pm). The initial Glu\textsuperscript{1}-Pm formed catalyzes cleavage of a 77-amino acid peptide from the amino-terminus of Glu\textsuperscript{1}-Pg, yielding Lys\textsuperscript{78}-Pg. The activation rate of Lys\textsuperscript{78}-Pg is faster than that of Glu\textsuperscript{1}-Pg. This illustration is courtesy of Dr. Francis J Castellino.
Figure 3.4 Mechanism of activation of hPg to Pm by SK. SK forms stoichiometric complexes with hPm and hPg and these complexes act as Pg activators (PAs). Initially, a stoichiometric complex of SK and hPg forms, within which conformational rearrangement of hPg takes place allowing an active site to form (SK-hPg*). Next, another complex forms, finally yielding the most stable of the complexes (SK^--hPm). SK undergoes degradation at both its amino-terminal and carboxy-terminal regions, leading to several forms of SK, globally designated here as SK^-. This latter complex is also formed from SK and hPm. In the second step of the activation, the SK-hPg*, SK-hPg’, and SK^-hPm, become catalytic activators of hPg. This illustration is courtesy of Dr. Francis J Castellino.
3.7. References


CHAPTER 4:
THE GENERATION OF A STREPTOKINASE SENSITIVE MOUSE PLASMINOGEN

4.1. Introduction

The mechanisms of invasiveness of many extracellular human pathogens rely on the productive interactions with the host proteolytic systems. Plasminogen activation is a critical component in establishing group A streptococci invasive infections. Pathogenic microorganisms have evolved plasminogen activators, such as staphylokinase and streptokinase which are not enzymes themselves but form bimolecular complexes with plasminogen or plasmin that in turn catalyze the conversion of free plasminogen into plasmin. Detailed mechanisms of the activation of hPg by SK have been elucidated through years of intensive research. SK has been shown to be composed of three distinct domains, designated α, β, and γ, separated by two coiled coils, and small regions at the N- and C-termini of the protein with disordered, flexible structures. While the regions at the two ends are known to be dispensable for the biological activity of SK, selective deletions of α or γ domains, significantly reduce hPg activation. However, the exact manner in which each domain contributes toward the high rates of Pg activation characteristic of full-length SK remains unclear (1-3). Moreover, several questions regarding the nature of the mechanism of activation of hPg by SK remain unanswered. First, what specific regions within hPg determine SK sensitivity? Although studies have shown that the locus within hPg that interacts with SK is limited to the Pg catalytic
domain, referred to as microplasminogen (μPg) (4, 5) the detailed regions within this domain that mediate such interactions remained unknown. Second, what are the mechanisms underlying the species selectivity of human derived SK in the activation of other mammalian Pgs? In further refining the regions within the Pg catalytic domain wherein SK binding sites reside, it is useful to compare the amino acid sequence of hPg and mPg. The amino acid sequence of these two proteins are very similar, however some amino acid clusters in mPg that differ from hPg suggest areas of the protein that may alter its reactivity with human derived SK. The purpose of this study was to address these issues by generating and characterizing recombinant Pgs carrying human and mouse sequences. This approach allowed for the identification of specific regions of hPg that determine SK sensitivity and their role in binding and activation. In addition, the results presented herein provide novel findings regarding the mechanistic steps towards the formation of the activation complex SK-Pg.

4.2. Materials and Methods

4.2.1. PCR mutagenesis: Cloning and exon exchange between hPg and mPg

All the chimeric and active site Pg mutants (mHhLPg, hHmLPg, hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], hPg [mE19], mPg [hE16], mPg [hE18], mPg [hE16+hE18], and hPgS741A, mPgS741A, mHhLPgS741A, and hHmLPgS741A) were designed by Dr. Takayuki Iwaki. WT hPg and WT mPg expression plasmids, hPg-Puro-pMT and mPg-Puro-pMT were generated by insertion of the cDNA encoding these proteins into the multiple cloning site of the Drosophila S2 parent expression plasmid,
Puro-pMT. This latter plasmid was generated by inserting the pCo-Puro cassette into the commercial plasmid, pMT/BiP/V5-His(A) (Invitrogen) (6, 7). mPg cDNA (cloned from mouse genomic DNA) and the plasminogen mutants mHhL (mouse plasminogen heavy chain-human plasminogen light chain), hHmL (human plasminogen heavy chain-mouse plasminogen light chain), hPgS741A, mPgS741A, mHhLPgS741A, and hHmLPgS741A were generated by Dr. Iwaki. The following human and mouse plasminogen mutants, hPg[mE14], hPg[mE15], hPg[mE16], hPg[mE17], hPg[mE18], hPg[mE19], mPg[hE16], mPg[hE17], and mPg[hE16+hE18] were generated by the polymerase chain reaction (PCR). The primer sets and templates used for mutagenesis purposes are listed in Table 4.1 and 4.2. Polymerase chain reaction (PCR) was used to generate the different hPg mutants. The different PCR fragments were generated by overlapping PCR. The conditions for the first PCR reaction were: template DNA 50 ng; primer set 25 pmol/µl each; dNTPs 2.5 mM; 5X Phusion HF buffer (Mg+); Phusion DNA polymerase (New England Biolabs) 2 U in 10 µl reaction. The steps for the amplification cycle were: 1) 98°C/30 sec, 2) 98°C/5 sec, 3) 60°C/15 sec, 4) 72°C/15 sec (15 sec/kb) according to the fragment size (steps 2-4 were repeated 25 times); 5) 72°C/5 min; 6) 10°C/end. The PCR products were purified using the QIAquick gel extraction kit (Qiagen). A second PCR reaction was set up under the following conditions: dNTPs 2.5 mM; 10X ExTaq buffer (Mg+) (Takara, Japan); PCR fragment 1 0.5 µl; PCR fragment 2 0.5 µl in 10 µl reaction. The reaction mixture was heated for 10 min at 94°C, RAMP to 25°C (-0.1°C/sec), and held at 25°C for 10 min. Next, ExTaq DNA polymerase (Takara, Japan) was added to the mixture, and the reaction continued under the following conditions: 1) 72°C/3 min, 2) 94°C/hold. Finally, a primer set mixture (25 pmol/µl) was added to the reaction. The
steps of the amplification cycle were: 1) 98°C/30 sec, 2) 98°C/5 sec, 3) 60°C/15 sec, 4) 72°C/60 sec (60 sec/kb) followed by 25 cycles, 5) 72°C/3 min, 6) 10°C/end. The PCR products were then digested with Mfe I-Xba I, or Eag I-Xba I restriction enzymes and transferred to a digested pBs-T3 vector (generated by Dr. Iwaki) for blue and white colony selection. Positive colonies were cultured in 20 mL of LB/ampicillin medium. The cultures were shaken overnight at 37°C, and the plasmids were purified using the QIAGEN Plasmid Mini Kit purification system. The purified plasmids were then digested with Mfe I-Xba I or Bgl I-Xba I, and ligated into freshly digested Bgl II- Mfe I fragment from hPg-pMT-puro, or a Bgl II- Bgl I fragment from mHhL-pMT-puro, into the Puro-pMT expression vector. Correct DNA sequence and reading frame of each one of the constructs generated was confirmed by sequencing at the W. M. Keck Biotechnology Center (University of Illinois, Urbana-Champaign, IL).

4.2.2. Expression of recombinant proteins

All plasminogen variants (hPg, mPg, mHhLPg, hHmLPg, hPgS741A, mPgS741A, mHhLPgS741A, hHmLPgS741A, hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], hPg [mE19], mPg [hE16], mPg [hE18], and mPg [hE16+hE18]) were expressed in the pMT vector utilizing Schenider 2 (S2) cells (6, 7). Briefly, cells were transferred to 24-well plates and transfected employing the calcium phosphate transfection kit as described by the manufacturer (Invitrogen, Carlsbad, CA). Cells were placed in individual wells of a 24-well plate at a density of 1x10^4 cells/ml, and incubated overnight at room temperature in complete medium (Ex-Cell 400, supplemented with 2 mM glutamine and 10% heat inactivated fetal bovine serum). The
transfection mixture was prepared using 4 µg of DNA. The calcium phosphate-DNA precipitate was incubated with the cells for 10-12 hr. The cells were pelleted gently for 3 min at 1500 rpm and resuspended in 1 ml of fresh complete medium and incubated for 2 days. The cells were then pelleted for 5 min at 1000 rpm and resuspended in 1 ml of fresh complete medium containing puromycin at a final concentration of 5 µg/ml. Fresh selection medium was replaced every 4–5 days, each time replating the cells in the original 24-well plate. Once cells reached a density of 1x10⁶ cells/ml, they were pelleted as before, resuspended in 3 ml of selection media, and transferred to a 6-well plate. After 2-3 weeks of selection, protein expression was induced with 600 µM copper sulfate. Recombinant protein expression was examined by Western blot analysis using a polyclonal antibody raised against plasma hPg. Stable cell lines expressing the proteins were maintained on puromycin selection in T-flasks and were expanded for large-scale expression in 1-liter spinner flasks. SK was expressed into the pMAL vector (New England Biolabs, Ipswich, MA) in *Escherichia coli* as a fusion protein (with maltose binding protein, MPB).

4.2.3. Protein Purification

All plasminogen variants were purified using a Sepharose CL4B-Lysine as previously described by Deutsch and Mertz, 1970. The SK fusion proteins were purified by affinity chromatography on an amylose resin as described by the manufacturer (New England Biolabs, Ipswich, MA).
Cyanogen Bromide Activation of Sepharose

Sepharose CL-4B resin was activated by cyanogen bromide (CNBr). A 20 ml volume of packed resin was washed with approximately 10x the resin volume of ice-cold 0.1M NaCl solution. The washed resin was resuspended in 2x its volume with ice-cold water and transferred to a small beaker. The resin was stirred constantly at low speed, and the pH was monitored continuously. In a hood, CNBr (6g/20 ml resin to be activated) was dissolved in a minimal volume of acetonitrile and added to the resin/water slurry. Immediately following CNBr addition, 6 N NaOH was titrated into the reaction at rate fast enough to maintain the pH between 11-12. The reaction was carried out for 2-5 minutes until the pH was maintained at 12 with no further addition of NaOH. CNBr activated resin was immediately filtered through a Buchner funnel and washed with 10x the resin volume of ice-cold 1 mM HCl, followed by 2x the resin volume of 0.1 mM HCl.

Coupling Activated Sepharose to Lysine

Activated sepharose was coupled to L-lysine. CNBr-activated sepharose was immediately washed through a Buchner funnel with 2x the resin volume of pre-chilled 0.2 M sodium bicarbonate, pH 8.5. The activated sepharose was added to 2x the gel volume of pre-chilled 20% L-lysine solution in 0.2 M sodium carbonate, pH 8.5. The activated resin was mixed with the lysine solution overnight at 4° C on an orbital shaker. In order to block any activated groups on the resin that had not been occupied by lysine, the resin was washed with 0.2 M sodium bicarbonate, pH 8.5, resuspended in 2x the gel volume of blocking solution (1 M ethanolamine, pH 8.2) and shaken on an orbital shaker at 4° C for 4-6 hours. After blocking, the resin was washed in 50 mM Tris-HCl/50 mM NaCl, pH 7.8, and was used to pack 10-ml lysine sepharose syringe columns. Unused
resin was stored in 50 mM Tris-HCl/50 mM NaCl, pH 7.8, containing 0.02% sodium azide.

**Isolation of recombinant Plasminogen**

Human, mouse, and mutant plasminogen proteins were purified according to the method of Brockway and Castellino (1972). Stable S2 lines were grown in 150 cm² T-flasks in incomplete medium to a cell density of 1x10⁷ cells/ml. These cells were pelleted and seeded into 200 ml fresh medium in a 1-liter spinner flask. After reaching a minimal cell density of 1 x10⁹ cells/ml, the cells were induced with 600 µM copper sulfate. Two to three days after induction, the cells were pelleted and the medium collected. The medium was immediately concentrated at 4° C in an Amicon concentration cell using a YM 30 membrane. Following dialysis in 100 mM sodium phosphate, pH 7.4, the recombinant proteins were purified by affinity chromatography on lysine–Sepharose. The concentrated medium was applied to the column that was pre-equilibrated in the same buffer. Following loading, the column was washed with 100 mM sodium phosphate, pH 7.4, until the absorbance at 280 nm was 0.05. The proteins were then eluted from the column using a 0–0.1 M ε-aminocaproic acid (EACA) gradient in the above buffer. Pooled plasminogen fractions were dialyzed 3X against 4 liters of 100 mM sodium phosphate buffer and stored at −80° C for future use.

**Isolation of recombinant SK**

SK was produced employing the pMAL™ Protein Fusion and Purification System (New England Biolabs, Ipswich, MA). The cloned SK gene was inserted into a pMAL vector downstream from the malE gene, which encodes the maltose-binding protein (MBP) (8). The MBP-SK was isolated following the manufacturer’s
instructions. Briefly, MBP-SK was expressed intracellularly in *E. coli* TB1 cells. The cells were induced with 0.3 mM IPTG after initial growth until mid-log phase. The bacterial pellets were obtained by centrifugation from 11 shaker flask cultures. The pellets were resuspended in 50 ml of column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) and subjected to ultrasonication for 2 min with pulses of 15 sec. Protein release was monitored by the Bradford assay. After centrifugation at 9,000 × g for 30 minutes, the supernatant (crude extract) was diluted 1:5 with column buffer. The dilute crude extract was loaded at a flow rate of 1ml/min onto an amylose resin column. The column was then washed with 12 column volumes of column buffer. The fusion protein was elute with column buffer + 10 mM maltose. SK was cleaved from the maltose binding protein by incubation for 3 hours at room temperature with FXa. Following cleavage, FXa was either inactivated by the addition of dansyl-glu-gly-arg-chloromethyl ketone (Calbiochem, San Diego, California) to a final concentration of 2 µM, or removed by passing the reaction mix over a small benzamidine-agarose column (Amersham, UK). The maltose binding protein was then removed by hydroxyapatite chromatography and domain separation was achieved by rebinding to the amylose resin. SK concentration was then quantified by Bradford assays.

**Determination of Protein Concentrations**

Protein concentration of samples used in kinetic and binding assays was determined according to the following $\varepsilon_{280}$ values: 152200 for hPg; 162630 for mPg, 159650 for mHhLPg; 155180 for hHmLPg; 152200 for hPg [mE14] and hPg [mE15]; 153690 for hPg [mE16] and hPg [mE17]; 152200 for hPg [mE18] and hPg [mE19]; 161140 for mPg [hE16] and for mPg [hE16+hE18]; and 162630 for mPg [hE18].
extinction coefficients were calculated on http://ca.expasy.org/ (The proteomics server for in-depth protein knowledge and analysis Nucleic Acids Res.).

4.2.4. Activity assays

**Activation of Pg variants by SK**

The activation of the different Pg constructs by SK, was monitored using the plasmin-specific chromogenic substrate S2251 (H-D-Val-L-Leu-L-Lys-p-nitroanilide) as previously described (9). Plasminogen activation was measured in a 96-well plate using 200 µl of the reaction buffer containing 0.250 mM of the chromogenic substrate S2251 in 10 mM HEPES/150 mM NaOAc, pH 7.4. The concentration of each individual plasminogen variant was 0.2 µM. The reaction was initiated by the addition of 0.0025 µM of SK. The amidolytic activity generated by Pm hydrolysis of the substrate S-2251 was monitored by measuring A₄₀₅ for 30 min.

**Activation of Pgs by uPA**

The assay conditions were as for SK. The reaction was initiated by addition of 10 IU of uPA (Abbokinase, Abbot Laboratories, N. Chicago, IL) to 25 nM of the plasminogen variants. The hydrolysis of S2251 was monitored continually at 405 nm for 30 min.

**Activation of Pg by equimolar SK-hPg**

The activation of the different Pg constructs by pre-formed SK-hPm complexes was monitored in a similar manner as previously described (9). The complexes were formed by the incubation of SK with hPg in a 1:1 molar ratio. A stock solution containing 0.2 µM of hPg was incubated with 0.2 µM of SK in 10 mM HEPES/150 mM NaOAc, pH
7.4 for 30 sec at room temperature. Following this, 0.250 mM of the chromogenic substrate S2251 and 0.2 µM of Pgs, were mix together in a reaction volume of 200 µl. The activation assay was initiated with the addition of 0.0025 µM of SK:hPg complex, and monitored spectrophotometrically on a plate reader.

**Activation of Pg by SK-hPg and/or SK-mPg [hE16+hE18]**

The activation of Pg to Pm by either SK-hPg or SK-mPg [hE16+hE18] was monitored in a similar manner to that described for the activation of Pg by SK. The complexes were formed by the incubation of SK with hPg or mPg [hE16+hE18] in a 1:1 molar ratio. A stock solution containing 0.2 µM of hPg or mPg [hE16+hE18] was incubated with 0.2 µM of SK in 10 mM HEPES/150 mM NaOAc, pH 7.4 for 30 sec at room temperature. In a 200 µl reaction volume containing 0.250 mM of the chromogenic substrate S2251 and 0.2 µM of hPg, mPg, and mPg [hE16+hE18], the activation assay was initiated with the addition of 0.0025 µM of SK-hPg or SK-mPg [hE16+hE18] complex, and monitored spectrophotometrically on a plate reader.

4.2.5. Amidolytic Assays of hPm, mPm, mPm[hE16+hE18], and Stoichiometric Complexes of SK-hPg, and SK-mPg[hE16+hE18]

**Titration of plasmin with p-nitrophenyl-p’-(guanidinium) benzoate (NPGB)**

Active site titrations using p-nitrophenyl-p’-(guanidinium) benzoate (NPGB) were performed as previously described (10). Briefly, a stock solution of 0.04 M NPGB in dry dimethylformamide was diluted to 5x10⁻⁵ M with 0.05 M sodium veronal, pH 8.3. One hundred µl of diluted substrate (200 µM) was added to 100 µl of sample containing plasmin. Release of p-nitrophenolate was monitored immediately at 410 nm. To
determine the plasmin concentration, a standard curve was generated from 200 µM to 3.125 µM of NPGB.

Kinetics of hPm, mPm, mPm [hE16+hE18], and Stoichiometric Complexes of SK-hPg, and SK-mPg[hE16+hE18] towards S2251.

The kinetics of hydrolysis of S2251 by Pm derived from hPg, mPg, mPg [hE16+hE18], and preformed stoichiometric SK-hPm, and SK-mPg[hE16+hE18] complex (final concentration of 1 µM) was determined. Different concentrations (0.125 mM-1 mM) of the chromogenic substrate (S2251) were added into a 96-well plate, followed by a buffer consisting of 10 mM Hepes/150mM NaOH, pH 7.4. The hydrolysis of the substrate was accelerated by the addition of pre-formed hPm, mPm, and h16h18Pm (1 µM). The rate of hydrolysis of S2251 by Pm was monitored as the change in absorbance over 30 min at 405 nm in a plate reader. The absorbancies were converted to initial activation rates, and the rate data was analyzed according to the usual Lineweaver-Burk plots. The enzyme complexes were generated by incubation at 25° C of stoichiometric amounts of SK and the desired plasminogen.

4.2.6. Surface Plasmon Resonance

Association and dissociation between the different plasminogen variants and SK were measured in real time by surface plasmon resonance with a BIAcore X biosensor (BIAcore Inc., Sweden). In these experiments, full length SK was immobilized onto the surface of a carboxylated dextran matrix (CM-5) sensor chip using the amine coupling kit following the manufacturer’s instructions. For each injection, 10 µg/mL of protein solution in 10 mM acetate, pH 4.25, was used at a flow rate of 5 µl/min. Sequential
injections were performed until a surface density of 5000-8000 resonance units (RU) of protein was coupled to the sensor surface. A reference cell was coupled using buffer alone to obtain a control surface for the measurement of the nonspecific refractive index component. After coupling, the sensor surface was blocked using 1M ethanolamine, pH 8.5. Binding experiments were performed as previously described (11). The kinetic constants were then calculated from the sensorgrams by nonlinear fitting of the association and dissociation curves according to a 1:1, model A+B=AB, using BIAevaluation software version 4.1 (BIAcore, Sweden). Specifically, $k_{obs}$ and $k_{off}$ rate constants were determined through nonlinear regression fitting of the equations for monophasic association and dissociation, $RU=RU_{max}(1-e^{-k_{obs}t})$ and $RU=\Delta RU(e^{-k_{off}t})$. The association rate constants ($k_{on}$) were determined from the equation $k_{on}=(k_{obs}-k_{off})/\lbrack P\rbrack$.

**Binding of hPg, mPg, Pg variants, and active site Pg mutants to SK**

Various concentrations (20-2.5 nM) of hPgS741, mPgS743A, mHhLPgS741A and hHmLPgS741A were separately injected over the sensor chip in a running buffer of 10 mM Hepes, pH 7.2, 150 mM NaCl, and 0.005% surfactant P-20, using a flow rate of 20 µl/min. Associations were measured during injection time (240 sec for mPgS743A, and 270 sec for the rest of the plasminogen variants); then dissociations were measured during injection of running buffer alone (260 sec for mPgS743A, and 290 sec for the rest of the proteins). After each cycle, the sensor surface was regenerated with 100 mM Tris buffer, pH 7.2, 3.5 M urea. Similar experiments were performed with WT hPg, mPg, and different Pg variants (hPg[mE14]-hPg[mE19], mPg[hE16],mPg[hE18], and mPg[hE16+hE18]). Pg samples were injected over the sensor surface at 20 µl/min in 10 mM Hepes, pH 7.2, 150 mM NaCl, and 0.005% surfactant P-20. All the injected
plasminogen samples contained 5x10^{-5} M p-Nitrophenyl p-guanidinobenzoate (Sigma, Chemical Co., St. Louis, MO) to prevent plasmin-mediated degradation of the immobilized protein. Binding constants were calculated as specified above.

4.2.7. Competition assay between hPg and mPg by surface plasmon resonance

In these experiments, full length human plasminogen (hPg_{S741}) and full length mouse plasminogen (mPg_{S743A}) were immobilized onto the surface of two carboxyalted dextran matrix (CM-5) sensor chips using the amine coupling kit following the manufacturer’s instructions. hPg (5 \mu g/mL), or mPg (5 \mu g/mL) in 10 mM acetate buffer, pH 4.0, was injected at a flow rate of 5 \mu l/min until 8000 to 10000 resonance units of protein was coupled to the sensor surface. To evaluate the possibility that hPg and mPg share the same binding site to SK, different concentrations (12.5-400 nM) of either protein in combination with 100 nM of SK, were injected over the sensor chips in a running buffer of 10 mM Hepes, pH 7.2, 150 mM NaCl, and 0.005% surfactant P-20, using a flow rate of 20 \mu l/min. Full length SK (100 nM), mPg_{S743A} (100 nM) or hPg_{S741} (100 nM) were separately injected over the sensor chips as controls. Associations were measured during injection time (240 sec). Dissociations were measured during injection of running buffer alone (260 sec). After each cycle, the sensor surface was regenerated with 100 mM Tris buffer, pH 7.2, 3.5 M urea.

4.2.8. Determination of binding of hPg and mPg to SK

These assays were performed in an ELISA-type manner. Ninety six-well plates were coated with 100 \mu l of hPg or mPg at a concentration of 100 nM in 0.05 M
carbonate/bicarbonate buffer, pH 9.6, overnight at 4° C and blocked with 1% BSA in phosphate-buffered saline (PBS) for 1 hr. Native SK (100 nM) was then added to the wells and incubated for 1 hr at 4 °C. After washing with PBS containing 0.05 % Tween 20, HRP-labeled hPg was added at different concentrations and incubated for another hour at 4° C. After washing with the same buffer, bound protein was detected by adding 100 µl of TMB (Tetramethylbenzidine) as the substrate and incubated for 5 min. The reaction was stopped by the addition of 100 µl of 2 N sulfuric acid. Changes in the optical density were determined with a microplate reader set at 450 nm with a wavelength correction at 540 nM. Nonspecific binding was determined by using BSA instead of hPg or mPg. To test the effect of additional Pg on the stability of the primary binding site between SK-Pg, HRP-labeled SK (100 nM) was added to the wells coated with hPg or mPg and incubated for 1 hr at 4° C. After washing with PBS 0.05 % Tween 20, hPg or mPg was added at a concentration of 800 nM. Following 1 hr incubation, different concentrations (6.4 µM-100 nM) of additional hPg or mPg were added, and experiments were performed with the same procedures described above.

4.3. Results

4.3.1. Cloning and expression of Pg constructs

All Pg constructs (Figure 4.1) used in this study were cloned and mutated (employing PCR techniques). For each r-Pg, ten clones were selected from hundreds of Escherichia coli transformants, and the plasmids were purified. Positive clones were then selected by restriction enzyme digestion. After selection, each purified cDNA insert was
subjected to nucleotide sequencing and one clone with the correct sequence was selected for plasmid purification and expression in Drosophila S2 cells. These cells were stably transfected using the Puro-pMT vector. Following transfection and selection with puromycin, a small-scale expression tests was carried out with those cells positively selected, in order to determine the level of protein expression and the quality of the product. Protein expression was confirmed by SDS-PAGE and Western-blot analysis using the polyclonal rabbit anti-hPg C35 as the primary antibody. Once the quality and the potential production level was confirmed, S2 cell cultures were expanded, and purification carried out. Final yields of the proteins after purification ranged from 1-10 mg/liter.

4.3.2. Activation of hPg, mPg, mHhLPg, hHmLPg, hPg[mE14], hPg[mE15], hPg[mE16], hPg[mE17], hPg[mE18], hPg[mE19], mPg[hE16], mPg[hE18], and mPg[hE16+hE18] by SK

Each plasminogen construct was examined for its ability to be activated by SK using amidolytic assays. Catalytic levels of SK were used to activate the plasminogen variants. We observed rapid activation of hPg by SK. In the case of mPg, and the chimeric protein hHmLPg, no measurable amidolytic activity was observed. The chimera mHhLPg recovered its ability to be activated by SK. This shows that amino acid sequences within the human plasminogen light chain mediate SK-hPg interactions and activation (Figure 4.2). Since the previous results had established that regions within the Pg light chain dictate the species specificity of SK, the amidolytic assays were carried out to assess the catalytic activation of hPg and mPg variants. The following hPg and mPg
mutants were tested: hPg[mE14], hPg[mE15], hPg[mE16], hPg[mE17], hPg[mE18], hPg[mE19], mPg[hE16], mPg[hE18], and mPg[hE16+hE1 8]. For most cases, the ability of the hPg constructs to be activated by SK was not significantly different from the parent hPg. However, in the case of hPg[mE16] and hPg[mE18] no activity was found (Figure 4.3). On the other hand, when the mPg mutants were assayed with SK, no measurable activity was detected in the case of mPg[hE16], mPg[hE18], but amidolytic activity generation was detected for the mutant mPg[hE16+hE1 8], which partially recovered its sensitivity to SK (Figure 4.4).

4.3.3. Activation of hPg, mPg, mHhLPg, hHmLPg, hPg[mE14], hPg[mE15], hPg[mE16], hPg[mE17], hPg[mE18], hPg[mE19], mPg[hE16], mPg[hE18], and mPg[hE16+hE1 8] by uPA.

In order to validate our previous results and to determine if all proteins had proteolytic activity, each of the plasminogen variants, as well as hPg and mPg, were activated to plasmin with uPA and their amidolytic activity towards S2251 was measured. No significant differences were found in the activities of each Pg variant and the respective parent hPg (Figures 4.5 and 4.6) or mPg (Figure 4.7).

4.3.4. Activation of hPg, mPg, mHhLPg, hHmLPg, hPg[mE14], hPg[mE15], hPg[mE16], hPg[mE17], hPg[mE18], hPg[mE19], mPg[hE16], mPg[hE18], and mPg[hE16+hE1 8] by SK-hPg

SK-hPg complexes were pre-formed by incubation of equimolar concentrations of SK and hPg for 30 sec at room temperature. The data shown in Figure 4.8 indicates that
SK-hPg had the same ability to activate mPg than hPg. No differences in the activability by SK-hPg complex of the various hPg and mPg constructs were observed as compared to hPg (Figures 4.9 and 4.10).

4.3.5. Active site titration of plasmin (Pm) with p-Nitrophenyl-p’-Guanidino-Benzolate (NPGB)

Pm was titrated with NPGB substrate in order to obtain an accurate Pm concentration for further experiments. After the incubation period of 30 min with uPA (~2 µg), approximately 70% of the original Pg (~50 µg) was converted to Pm (Figure 4.11).

4.3.6. Kinetic studies for the amidolytic activity toward S2251

Kinetic studies were performed to examine whether mPg[hE16+hE18] plasmin presented an altered capacity to hydrolyze S2251. The kinetic parameters of the cleavage of the tripeptide substrate, S2251, at pH 7.4 and 25° C by human plasmin, mouse plasmin and mPg[hE16+hE18] are shown in Table 4.3. The values of the apparent Michaelis-Menten constants, $K_m$, as compared to hPm ($K_m=0.301$ mM) were slightly decreased with mPm ($K_m=0.122$ mM) and mPm[hE16+hE18] ($K_m=0.212$ mM). The $k_{cat}$ values were also lower for mPm ($k_{cat}=15.1$ sec$^{-1}$) and mPm[hE16+hE18] ($k_{cat}=29.3$ sec$^{-1}$) by ~1.3-2.6 fold than hPm ($k_{cat}=40$ sec$^{-1}$). However, the overall catalytic efficiencies ($k_{cat}/K_m$) were comparable with that of hPm. The kinetic parameters of the stoichiometric complexes SK-hPg and SK-mPg[hE16+hE18] are presented in Table 4.4. No significant differences in the amidolytic efficiency were observed between the SK-hPg and SK-
mPg[hE16+hE18] complexes, indicating that this latter complex had the same capability
to process the chromogenic substrate S2251 as SK-hPg. These results indicated that Pm
from hPg and mPg[hE16+hE18], and SK-Pg complexes possess identical steady-state
kinetic constants toward S2251.

4.3.7. Plasminogen activation by SK-hPg and SK- mPg[hE16+hE18]

The ability of the SK-hPg and SK- mPg[hE16+hE18] complexes to activate full-
length hPg, mPg, and mPg[hE16+hE18] was examined using activation assays. The
results indicated that SK-mPg[hE16+hE18] displayed reduced Pg activator capability
compared to SK-hPg (Figure 4.12). SK- mPg[hE16+hE18] activates Pg at rates slightly
delayed as compared to those observed with SK-hPg. These results indicated that the
delay in the activation of mPg[hE16+hE18] by SK, observed in previous experiments,
was due to decreased efficiency of the complex SK-mPg[hE16+hE18] in processing Pg.

4.3.8. Binding of hPg, mPg, mHhLPg, hHmLPg to SK

The binding of SK to the Pg variants was assessed quantitatively by surface
plasmon resonance (SPR). The curves obtained are illustrated in Figure 4.13, and the
corresponding kinetic binding values obtained are listed in Table 4.5. For the different Pg
constructs used in this part of the study, the active site Ser741 was replaced by an alanine
to prevent proteolysis during the binding studies. The $K_D$ for interaction of hPg$^{S741A}$,
mPg$^{S743A}$, mHhLPg$^{S741A}$, and hHmLPg$^{S743A}$ with SK was assessed at different
concentrations of Pg. The $k_{on}$ and $k_{off}$ rate constants obtained from the data were used to
calculate the $K_D$ values as a global fitting of the entire curve. The sensorgrams and the
calculated rate constants indicated that the binding between hPg^{S741A} and SK \((K_D=17 \times 10^{-9} \text{M})\) is about the same as the binding between mPg^{S743A} and SK \((K_D=12.2 \times 10^{-9} \text{M})\). While mHhLPg^{S741A} \((k_{on}=4.57 \times 10^5 \text{ M}^{-1} \text{s}^{-1})\) had an association rate constant about the same as hPg^{S741A} \((k_{on}=5.75 \times 10^5 \text{ M}^{-1} \text{s}^{-1})\) and mPg^{S743A} \((k_{on}=6.15 \times 10^5 \text{ M}^{-1} \text{s}^{-1})\), hHmLPg^{S743A} \((k_{on}=0.35 \times 10^5 \text{ M}^{-1} \text{s}^{-1})\) presented an association rate constant more than 10-fold lower than hPg^{S741A} and mPg^{S743A}. This lower association rate may be due to conformational restraints resulting from the combination of human elements of the plasminogen heavy chain (Hc) and mouse elements from the plasminogen light chain (Lc). These observations suggested that SK has the ability to form complexes with plasminogen in a non-species specific manner.

4.3.9. Binding of hPg[mE14], hPg[mE15], hPg[mE16], hPg[mE17], hPg[mE18], hPg[mE19], mPg[hE16], mPg[hE18], and mPg[hE16+hE18] to SK

The interactions between immobilized SK and various concentrations of the human and mouse plasminogen variants were further studied (Figures 4.14 and 4.15). To prevent undesired proteolysis the active site acylating reagent, ρ-nitrophenol (NPGB), was added to the Pg constructs sensitive to SK. The results of Table 4.5 showed that NPGB addition did not affect the \(K_D\) values for SK binding. We observed that SK was bound to all chimeric human and mouse plasminogens, with virtually the same \(K_D\) as for hPg and mPg (Tables 4.6 and 4.7).
4.3.10. Competition assays between hPg and mPg

In order to determine if hPg and mPg may be sharing the same binding site to SK, hPg${}^{S741A}$ and mPg${}^{S743A}$ were immobilized onto the surface of CM-5 sensor chips. SK (100 nM), and SK-hPg (100 nM:12.5-400 nM) or SK-mPg (100 nM:12.5-400 nM) complexes pre-formed in solution were then passed over the sensor surface. The results are shown in Figure 4.16. We observed full binding when 100 nM of SK was injected onto the sensor chip coupled to either hPg or mPg. Approximately, 300 RU and 250 RU were reached in each case (Figure 4.16 A and C). Incubation of SK with Pg will rapidly lead to the generation of stoichiometric complexes. We hypothesized that if hPg and mPg are binding to the same site on SK, no binding will be observed when a pre-formed SK-Pg (1:1) complex is injected over the sensor chips. We found that the binding capacity of hPg and/or mPg to the SK-Pg complex decreased proportionally to the increased concentration of Pg (12.5-100 nM) in combination with a fixed concentration of SK (100 nM). Interestingly, no return to baseline occurred when the SK-Pg 1:1 complex was injected onto the sensor chip, we only observed a decreased in the RU to about half of what was obtained when SK was injected alone (Figures 4.16 B and D). These results suggested the possibility of the existence of additional binding sites. In order to further validate these observations, SK (100 nM) was incubated with an excess amount of Pg (200 nM and 400 nM), to assure that all available SK will be in complex with Pg. If only one binding site exists, no further binding will be observed. Results in Figure 4.16, clearly indicate that the SK-Pg complex has the ability to bind to Pg suggesting the existence of a ternary complex Pg:SK:Pg.
4.3.11. Sandwich binding assays

Assays were performed to study the formation of the ternary complex Pg:SK:Pg. For these experiments, 96-well plates were coated with hPg\textsubscript{S741A} and mPg\textsubscript{S743A}. SK was added to induce the formation of the SK-Pg complex. Horseradish peroxidase (HRP) labeled hPg\textsubscript{S741A} or mPg\textsubscript{S743A} were then added to the plates. The amount of Pg bound to the SK-Pg-coated plate was determined spectrophotometrically on a plate reader. We observed an increasing formation of the Pg-SK-Pg complex in a dose-dependent and saturable manner. Both hPg and mPg formed ternary complexes with hPg-SK and/or mPg-SK (Figure 4.17). No binding of Pg was observed when BSA was used instead of SK. Therefore, our results confirmed that a Pg-SK-Pg ternary complex existed.

4.4. Discussion

Invasive bacteria have evolved a series of pathogenic mechanisms to disseminate across tissue barriers, and penetrate from superficial areas to deep tissues and circulation. Tissue barriers are composed of ECMs and BMs, which are mainly formed of insoluble collagen fibers. Consequently, penetration and degradation are major problems in bacterial metastasis. Proteolysis is a key factor in the pathogenesis of invasive bacterial infections. Some bacteria produce ECM-degrading proteases, such as collagenases that aid in tissue degradation and invasiveness. However, the majority of bacterial pathogens produce very low numbers of proteases. They must rely on other mechanisms to achieve invasiveness (12). Bacteria have developed strategies to manipulate the host proteases, activators or inhibitors for their own benefit. The fibrinolytic pathway is a very attractive target for bacteria to exploit. Several bacterial species intervene with the fibrinolytic
pathways, specifically with the Pg system (13). Studies have demonstrated that Pm can degrade several ECM and BM components leading to tissue destruction and bacterial dissemination (14-16). Pathogenic bacteria, such as Gram-positive GAS produce Pg activators, e.g. SK and staphylokinase, which have the ability to activate Pg to the serine protease Pm (17). The mechanism by which SK activates Pg has been studied in depth, revealing that SK is not an enzyme and its activation of Pg is rather indirect. SK forms 1:1 complexes with Pg. These complexes are catalytic activators of Pg that catalyze the hydrolysis of the peptide bond Arg\textsuperscript{561}-Val\textsuperscript{562} of the remaining Pg molecules (18-20). One important aspect about SK and Pg interactions is that Pg activation by SK is highly species-specific (21). The basis for this species selectivity of SK is still unclear. In normal conditions, GAS infects only humans, and the SKs produced by these bacteria activate only hPg, with no measurable activity against mPg (22). A recent study employing a transgenic mouse in which the mPg gene was replaced by hPg, demonstrated that hPg expression highly increases GAS virulence in mice (23). Although the role of SK and Pg in GAS infections has been confirmed by this study, the restricted host specificity has limited studies of streptococcal pathogenesis in mouse models. To overcome this limitation, the identification of the loci within hPg that interact with GAS derived SK will provide important information for the future generation of a more accurate GAS infection murine model. We employed a straightforward biochemical approach to identify the regions within hPg responsible for the SK sensitivity. Early studies suggest that elements within the protease chain of hPg productively interact with SK (5). In the current study, using the knowledge that mPg is not activated by SK, we constructed chimeric proteins sharing human and mouse sequences. Activity assays
demonstrated that the amino acid sequences within the hPg light chain are responsible for Pg activation by SK. We showed that hPg activation by SK is eliminated by replacing the hPg light chain (hL) with the mPg light chain (mL). Based on these results, the activation of hPg by SK is accelerated by elements within the hL. To further redefine this region, we performed individual exon substitutions within this area in hPg for those of mPg. We generated six different hPg constructs: hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], and hPg [mE19]. We tested the ability of each construct to be activated by SK and found that replacing the hE16 for mE16, and hE18 for mE18 eliminates Pg activation by SK. To validate these results we generated three mPg mutants: mPg [hE16], mPg [hE18], and mPg [hE16+hE18] and by activity assays we showed that mPg became capable of activation by SK when mE16 and mE18 were replaced by their human counterparts. Sequence comparisons showed important amino acid differences between hPg and mPg in the regions encoded by E16 and E18 (Figure 4.18 A). The crystal structure of the catalytic domain of hPg complexed with SK revealed that the carboxyl-terminal domain of SK binds near the activation loop of Pg. It is speculated this binding may be responsible for the induction of conformational rearrangements that lead to Pg activation (24). hE16 and mE16 present differences at 18 of the 47 amino acids, which can be grouped in four clusters of amino acids. One of these clusters of 3 amino acid difference is: Thr\textsuperscript{643}, Arg\textsuperscript{644}, and Lys\textsuperscript{645} in human to Asn\textsuperscript{645}, Asn\textsuperscript{646} and Arg\textsuperscript{647} in mouse (Figure 4.18B). These sequences are localized next to Asp\textsuperscript{646} in hPg, Asp\textsuperscript{648} in mPg, which is a member of the catalytic triad of Pm (Figure 4.18A). A second cluster of amino acids, located in the “calcium binding loop”, present important chemical differences to the human counter part, is also localized in the vicinity of the
catalytic triad. This cluster is composed by 7 amino acids: Gln$^{622}$, Val$^{624}$, Asn$^{625}$, Leu$^{626}$, Glu$^{627}$, Pro$^{628}$, and His$^{629}$ in human to Glu$^{624}$, Tyr$^{626}$, Ile$^{627}$, Arg$^{628}$, Gly$^{629}$, Ser$^{630}$, and Asp$^{631}$ in mouse (Figure 4.18B). The x-ray crystal structure of the catalytic domain of hPg complexed with SK indicated that residues 622 to 628 participate in the binding of the SK $\gamma$ domain to microplasminogen ($\mu$Pg). Extensive charged and hydrophobic interactions have been detected. In addition, residues 625 to 629 interact with SK by forming salt bridges, and hydrogen bonds with the $\beta\beta$2 strand of SK (24, 25). hE18 and mE18 are homologous in amino acid sequence, with only 7 of 49 amino acids being different (Figure 4.18A). There are four amino acid differences clustered together in E18: Tyr$^{713}$, Phe$^{715}$, Gly$^{718}$, and Gln$^{721}$ in hPg to Val$^{715}$, Tyr$^{717}$, Asn$^{720}$, and Lys$^{723}$ in mPg (Figure 4.18C). Crystallography data showed that residues 713 to 721 of $\mu$Pg interact with the SK $\alpha$ domain. hPg -Arg$^{719}$ forms salt bridges with SK (amino acids Glu$^{39}$ and Glu$^{134}$) and displays van der Waals contact interactions with SK-Val$^{19}$ (25). The participation of amino acid sequences encoded by E16 and E18 in the interactions between SK and Pg, and the considerable differences observed in sequence between hPg and mPg, suggest that residues 622-629 and 713-721 may contribute to the inability of SK to activate mPg.

The binding of SK to Pg is a key mechanistic step towards the formation of the SK-Pg catalytic complex. We tested the ability of mPg, and each Pg construct to bind to SK. Our results showed that mPg binds to SK with a similar strength to that of hPg: $K_D$=17.0 $\times 10^{-9}$ M and $K_D$=12.2 $\times 10^{-9}$ M, respectively. Our $K_D$ value for hPg to full-length SK is in agreement with previous studies, with $K_D$ values of 7.88 $\times 10^{-9}$ M (4, 11). The replacement of the human heavy chain (hH) with mouse heavy chain in hPg did not
significantly affect the overall $K_D$ for binding to SK. The substitution of hL for mL reduced the overall binding to SK by 10-fold through alterations in the $k_{on}$ rate. This reduced binding may be the consequence of unfavorable interactions between elements within the hH and the mL. No significant effects were found in the binding of SK to the different hPg and mPg mutants. Our results indicate that SK binds to mPg, suggesting that the first step towards Pg activation is viable. However, no active site formation is induced.

To study the nature of the binding between mPg and SK, SPR experiments and ELISA type assays were performed. hPg and mPg display similar capacity to bind to SK-Pg pre-formed complexes, even at saturating concentrations of Pg combined to SK. SK seems to have the ability to bind to more than one molecule of Pg, with no distinction between hPg or mPg. These findings suggested that SK has more than one binding site for Pg. These results were further confirmed by sandwich binding assays that demonstrated direct evidence that one SK molecule can interact with two Pg molecules at the same time. In addition, mPg was activated equally well as hPg by the addition of catalytic levels of the pre-formed SK-hPg complex. Based on our results, we proposed the existence of a primary binding site on SK that displays very high affinity towards Pg, most likely in the picomolar range. This idea is support by early experiments where $K_D$ values of 28 pM for the formation of the SK-hPg complex were reported (26). Following primary binding, a secondary binding site becomes available. This second binding site is most likely is the one detected by SPR experiments with $K_D$ values in the nM range. We proposed that the first step towards Pg activation by SK is the formation of a 1:1 complex with Pg which displays a very tight binding, followed by an enzyme-substrate
intermediate, Pg:SK:Pg. Previous experimental data supported the idea of the existence of the ternary complex. Sandwich binding assays employing radiolabeled protein and cross-linking experiments suggested the formation of hPg-SK-hPg complexes in the process of Pg activation (27).

In conclusion, we show here that SK-Pg interactions are mediated by hLc, specifically by the sequences encoded by hE16 and hE17. We hypothesize, based on sequence comparisons between hPg and mPg, that amino acid differences within the calcium-binding loop (residues 622-629), encoded by E16, and residues 713-721, encoded by E18, may account for hPg activation by SK, and the species specificity. Although future mutagenesis studies will be required to narrow the amino acids responsible for hPg sensitivity to SK. Currently, five Pg mutants, with specific amino acid substitutions, have been constructed and expressed in Drosophila S2 cells. The proteins will be processed and experiments will be performed. Finally, our SPR experiments demonstrated that SK-Pg complex formation is not species specific. However, structural constraints within the complexes limited the induction of an active site formation. We found new evidence that the activation of hPg by SK might involve the formation of different intermediates, and that SK has more than one binding site for Pg. Overall, our results provide novel insights into the mechanisms associated with Pg activation by bacterial pathogens.
Figure 4.1 Plasminogen constructs. Nineteen exons are contained within the Pg gene. Exons 2-13 encoded the Pg heavy chain. Exons 14-19 encoded the Pg light chain. A) Pg heavy chain consists of an activation peptide (AP) followed by five homologous regions termed kringles (K). Pg light chain contains the protein catalytic domain (CD). The Pg catalytic triad consist of the amino acids His\(^{603}\) (H), Asp\(^{646}\) (D), and Ser\(^{741}\) (S). hPg and mPg were generated by the insertion of their cDNA into an expression vector. mHhL (mouse heavy chain-human light chain) and hHmL (human heavy chain-mouse light chain) were generated by PCR. Ser\(^{741/743}\) was substituted for Ala in order to generate the active site mutants, hPg\(^{S741A}\), mPg\(^{S743A}\), and mHhL\(^{S741A}\), hHmL\(^{S743A}\). B) Six hPg mutants, hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], and hPg [mE19], were constructed by the replacement of human exons 14, 15, 16, 17, 18 and 19 for those of mPg. C) mPg mutants were generated by the substitution of mE16, mE18, and mE16+mE18 for those of human.
Figure 4.2 Activation of hPg, mPg, mHhLPg and hHmLPg by SK. Activation of 0.2 μM of hPg (blue), mPg (red), mHhLPg (aqua), or hHmLPg (green) by 0.0025 μM of SK. The generation of amidolytic activity at 25°C was followed at A405 using 0.25 mM of S2251.
Figure 4.3 Activation of hPg, hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], and hPg [mE19] by SK. Activation of 0.2 µM of hPg (blue), hPg [mE14] (red), hPg [mE15] (green), hPg [mE16] (black), hPg [mE17] (orange), hPg [mE18] (purple), or hPg [mE19] (light green) by 0.0025 µM of SK. The generation of amidolytic activity at 25°C was followed at A405 using 0.25 mM of S2251.
Figure 4.4 Activation of hPg, mPg, mPg [hE16], mPg [hE18], and mPg [hE16+hE18] by SK. Activation of 0.2 μM of hPg (blue), mPg (red), mPg [hE16] (green), mPg [hE18] (black), or mPg [hE16+hE18] (orange) by 0.0025 μM of SK. The generation of amidolytic activity at 25°C was followed at A405 using 0.25 mM of S2251.
Figure 4.5 Activation of hPg, mPg, mHhLPg and hHmLPg by uPA. Activation of 25 nM of hPg (blue), mPg (red), mHhLPg (aqua), or hHmLPg (green) by 10 IU of uPA. The generation of amidolytic activity at 25°C was followed at A405 using 0.25 mM of S2251.
Figure 4.6 Activation of hPg, hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], and hPg [mE19] by uPA. Activation of 25 nM of hPg (blue), hPg [mE14] (red), hPg [mE15] (green), hPg [mE16] (black), hPg [mE17] (orange), hPg [mE18] (purple), or hPg [mE19] (light green) by 10 IU of uPA. The generation of amidolytic activity at 25°C was followed at A405 using 0.25 mM of S2251.
Figure 4.7 Activation of hPg, mPg, mPg [hE16], mPg [hE18], and mPg [hE16+hE18] by uPA. Activation of 25 nM of hPg (blue), mPg (red), mPg [hE16] (green), mPg [hE18] (black), mPg [hE16+hE18] (orange) by 10 IU of uPA. The generation of amidolytic activity at 25° C was followed at A405 using 0.25 mM of S2251.
Figure 4.8 Activation of hPg, mPg, mHhLPg, and hHmLPg by SK-hPg. Activation of 0.2 µM of hPg (blue), mPg (red), mHhLPg (aqua), or hHmLPg (green) by 0.0025 µM of pre-formed SK-hPg complex. The generation of amidolytic activity at 25° C was followed at A405 using 0.25 mM of S2251.
Figure 4.9 Activation of hPg, hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], and hPg [mE19] by SK-hPg. Activation of 0.2 µM of hPg (blue), hPg [mE14] (red), hPg [mE15] (green), hPg [mE16] (black), hPg [mE17] (orange), hPg [mE18] (purple), or hPg [mE19] (light green) by 0.0025 µM of pre-formed SK-hPg complex. The generation of amidolytic activity was followed at 25° C at A405 using 0.25 mM of S2251.
Figure 4.10 Activation of hPg, mPg, mPg [hE16], mPg [hE18], and mPg [hE16+hE18] by SK-hPg. Activation of 0.2 μM of hPg (blue), mPg (red), mPg [hE16] (green), mPg [hE18] (black), or mPg [hE16+hE18] (orange) by 0.0025 μM of pre-formed SK-hPg complex. The generation of amidolytic activity at 25° C was followed at A405 using 0.25 mM of S2251.
Figure 4.11 Plasmin generation. SDS-PAGE analysis of mixtures of Pg (50 µg) with uPA (2 µg). Each Pg was incubated with uPA. Aliquots were removed at 15 and 30 min. NPGB titration indicated that approximately 35 µg of hPm and/or mPm was obtained. hPg (lanes 1-3), mPg (lanes 4-6).
Figure 4.12 Activation of hPg and mPg by SK-hPg and SK-mPg[hE16+hE18] complexes. Equimolar amounts of SK (0.2 µM) and hPg or mPg[hE16hE18] (0.2 µM) were incubated for 30 sec at room temperature. Activation of hPg, mPg, and mPg [hE16+hE18] was initiated by addition of 0.0025 µM of SK-hPg or SK-mPg[hE16+hE18]. The generation of amidolytic activity at 25° C was followed at A405 using 0.25 mM of S2251.
Figure 4.13 Overlay plots of various concentrations of hPg$^{S741A}$, mPg$^{S743A}$, mHhL$^{S741A}$ and hHmL$^{S743A}$, hPg, and mPg binding to immobilized SK. Approximately 5000 RU of SK (10 µg/ml) was immobilized through use of amine coupling. Different amounts of Pgs (2.5-20 nM) were then injected onto the sensor chip at a flow rate of 20 µl/min for 7 min and binding levels were detected in real-time by Surface plasmon resonance (SPR). Sensograms were generated by subtractions of the nonspecific refractive index component from the total binding. The addition of NPGB (50 µM) did not affect binding characteristics. Blue (20nM), red(10nM), green (5nM), and brown(2.5nM).
Figure 4.14 Biacore curves of different concentrations of hPg [mE14], hPg [mE15], hPg [mE16], hPg [mE17], hPg [mE18], and hPg [mE19] binding to SK. hPg [mE14], hPg [mE15], hPg [mE17], hPg [mE18], and hPg [mE19] were mixed with 50 nM NPGB to prevent proteolysis. Sensograms were generated by subtraction of the nonspecific refractive index component from the total binding. Blue (20nM), red (10 nM), green (5 nM), and brown (2.5 nM).
Figure 4.15 Biacore curves of different concentrations of mPg [hE16], mPg [hE18], and mPg [hE16+hE18] binding to SK. mPg [hE16+hE18] was mixed with 50 nM NPGB to prevent proteolysis. Sensograms were generated by subtraction of the nonspecific refractive index component from the total binding. Blue (20nM), red (10 nM), green (5 nM), and brown (2.5 nM).
Figure 4.16 Competition assays between hPg and mPg. Approximately, 5 µg/ml of Pg was immobilized onto the surface of a CM5 sensor chip at a flow rate of 5 µl/min for seven min. One hundred nM of SK was injected at 20 µl/min. Various concentrations (12.5 nM-400nM) of mPg$^{S743A}$ or hPg$^{S741A}$ were incubated with SK (100 nM). The SK-Pg complexes were then injected at 20 µl/min over immobilized hPg$^{S741A}$ or mPg$^{S743A}$. A) hPg$^{S741A}$ was immobilized on the chip. SK (dark blue), SK-mPg$^{S743A}$ complexes [100 nM:12.5 nM (red), 100 nM:25 nM (green), 100nM:50 nM (brown), 100 nM:100 nM (purple), 100 nM:200 nM (pink),100 nM:300 nM (blue)], and mPg$^{S743A}$ (gray) were injected onto the surface of the chip. B) Response units (RU) decreased proportional to increasing concentrations of mPg$^{S743A}$ combined with a fixed amount of SK. C) mPg$^{S743A}$ was immobilized on the chip. SK (dark blue), SK-hPg$^{S741A}$ complexes [100 nM:12.5 nM (red), 100 nM:25 nM (green), 100nM:50 nM (brown), 100 nM:100 nM (purple), 100 nM:200 nM (pink),100 nM:300 nM (blue)], and hPg$^{S741A}$ (gray) were injected onto the surface of the chip. D) Decreases in response were proportional to increasing concentrations of added hPg$^{S741A}$ combined fixed amounts of SK. Saturation levels were reached at approximately 200 nM of mPg$^{S743A}$ or hPg$^{S741A}$. Even at saturating concentrations, binding was observed in all cases.
Figure 4.17 Binding of horseradish peroxidase (HRP) labeled Pg to SK-Pg complexes. Pg (100 nM) was immobilized on wells of a 96-well plate. SK (100 nM) was then added and incubated for 1 h at 4 °C. Concentrations ranging from 0.195 nM to 3200 nM of horseradish peroxidase (HRP) labeled Pg was then added. The formation of the Pg-Sk-Pg complexes was assessed as changes in optical density at A450 nM. hPgS741A coating (A and B), HRP-hPgS741A (A), HRP-mPgS741A (B). mPgS741A (C and D) coating, HRP-hPgS741A (C) or HRP-mPgS741A (D). All experiments were performed in triplicate.
Figure 4.18 Amino acid sequence comparisons between hPg exons 16 and 18 with those of mPg.  A) hPg light chain (hL) is composed of 230 amino acids. Members of the catalytic triad of plasmin consisting of His$^{603}$, Asp$^{646}$, and Ser$^{741}$ are displayed in blue. The amino acid sequence encoded by hPg exon 16 (hE16) is encircled by a red dotted line (…), and the amino acid sequence encoded by hPg exon 18 (hE18) is encircled by a green dotted line (…). Red letters indicate amino acids that are different in mPg. B) Representation of hE16. Amino acid differences in hE16 as compared to mPg exon 16 (mE16) are shown in red, mPg amino acids are displayed in purple. C) Representation of hE18. Amino acid differences in hE18 as compared to mPg exon 18 (mE18) are indicated in red, mPg amino acids are displayed in purple.
**TABLE 4.1**

PRIMERS AND TEMPLATES USED FOR MUTAGENESIS AND CLONING OF HUMAN AND MOUSE PLASMINOGENS (1)

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<td>mPg-Puro-pMT</td>
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<tr>
<td></td>
<td>(F): 5’- aac cca agg gac ttt cgg tgc cgg tc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R): 5’- ctg tca ccc tgg cag ctc cgg cag c</td>
<td>hPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(F): 5’- gtc cgc agg gtt aca cca ggt gag ctc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R): 5’- cct tag aag gca cag tca agg</td>
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# TABLE 4.2

PRIMERS AND TEMPLATES USED FOR MUTAGENESIS AND CLONING OF HUMAN AND MOUSE PLASMINOGENS (2)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPg [hE16]</td>
<td>(F): 5’-gac tct gag aca gag tgc atg</td>
<td>mPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- ttg ggg act tct cca aac agt ggg ca</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- ttg gag aag tcc cca agg cct tca tc</td>
<td>hPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- tgg ctc ggc tgc tta gct tta gca agg caa tat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- cta agc age cca ggc acc acc atc aeg g</td>
<td>mPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- cct tag aag gca cag tgc agg</td>
<td></td>
</tr>
<tr>
<td>mPg [hE16]</td>
<td>(F): 5’-gac tct gag aca gag tgc atg</td>
<td>mPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- ttg ggg act tct cca aac agt ggg ca</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- ttg gag aag tcc cca agg cct tca tc</td>
<td>hPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- tgg ctc ggc tgc tta gct tta gca agg caa tat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- cta agc age cca ggc acc acc atc aeg g</td>
<td>mPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- cct tag aag gca cag tgc agg</td>
<td></td>
</tr>
<tr>
<td>mPg [hE16+hE18]</td>
<td>(F): 5’-gac tct gag aca gag tgc atg</td>
<td>mPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- ttg ggg act tct cca aac agt ggg ca</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- ttg gag aag tcc cca agg cct tca tc</td>
<td>hPg-Puro-pMT</td>
</tr>
<tr>
<td></td>
<td>(R): 5’- tgg ctc ggc tgc tta gct tta gca agg caa tat c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- gtt gcc agg gcg aca gtc gag gac c</td>
<td>mPg-Puro-pMT</td>
</tr>
<tr>
<td>Mutation</td>
<td>Primer</td>
<td>Template</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>mPg [hE16+hE18]</td>
<td>(R): 5’- cct tag aag gca cag tgc agg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- gac tca agg tac ttt tgg age tgg cct tct c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R): 5’- ctg tgg ccc tgg caa ctg tca gtt cct c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F): 5’- cta agc age cca gcc acc atc aeg g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R): 5’- aag aeg tgg agt ctc tcc cca gcc</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>hPg-Puro-pMT</td>
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<tr>
<td></td>
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<td>mPg-Puro-pMT</td>
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</tbody>
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TABLE 4.3
KINETIC CONSTANTS AT 25 °C FOR THE AMIDOLYTIC ACTIVITY TOWARDS S2251 WITH PLASMINs

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (A405/min)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPm</td>
<td>0.301 ± 0.050</td>
<td>0.923 ± 0.085</td>
<td>40.0 ± 3.70</td>
<td>136.1 ± 10.69</td>
</tr>
<tr>
<td>mPm</td>
<td>0.122 ± 0.034</td>
<td>0.349 ± 0.017</td>
<td>15.1 ± 0.75</td>
<td>137.4 ± 25.16</td>
</tr>
<tr>
<td>mPm[hE16+hE18]</td>
<td>0.212 ± 0.006</td>
<td>0.675 ± 0.010</td>
<td>29.3 ± 0.44</td>
<td>137.3 ± 4.65</td>
</tr>
</tbody>
</table>

TABLE 4.4
KINETIC CONSTANTS AT 25 °C FOR THE AMIDOLYTIC ACTIVITY TOWARDS S2251 OF EQUIMOLAR COMPLEXES OF SK WITH PLASMINOGEN

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (A405/min)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-hPg</td>
<td>0.363 ± 0.039</td>
<td>0.769 ± 0.034</td>
<td>33.4 ± 1.46</td>
<td>93.27 ± 5.48</td>
</tr>
<tr>
<td>SK-mPg</td>
<td>0.209 ± 0.015</td>
<td>0.612 ± 0.009</td>
<td>26.6 ± 0.38</td>
<td>128.1 ± 7.21</td>
</tr>
<tr>
<td>[hE16+hE18]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# TABLE 4.5

## KINETIC AND AFFINITY DATA FOR VARIOUS HUMAN AND MOUSE PLASMINOGENS

<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$ (x10^5) (M⁻¹s⁻¹)</th>
<th>$k_{off}$ (x10⁻³) (sec⁻¹)</th>
<th>$K_D$ a (x10⁻⁹) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPg[S741A]</td>
<td>5.75 ± 0.10</td>
<td>9.77 ± 0.01</td>
<td>17.0 ± 0.32</td>
</tr>
<tr>
<td>hPg + NPGB</td>
<td>5.90 ± 0.01</td>
<td>12.6 ± 0.25</td>
<td>21.3 ± 0.50</td>
</tr>
<tr>
<td>mPg[S743]</td>
<td>6.15 ± 0.11</td>
<td>6.64 ± 0.18</td>
<td>12.2 ± 0.49</td>
</tr>
<tr>
<td>mPg + NPGB</td>
<td>5.40 ± 0.11</td>
<td>5.87 ± 0.30</td>
<td>10.9 ± 0.37</td>
</tr>
<tr>
<td>mHhLPg[S741A]</td>
<td>4.57 ± 0.38</td>
<td>7.35 ± 0.18</td>
<td>16.0 ± 0.34</td>
</tr>
<tr>
<td>hHmLPg[S743A]</td>
<td>0.35 ± 0.01</td>
<td>5.77 ± 0.41</td>
<td>167.3 ± 11.90</td>
</tr>
</tbody>
</table>

a The value of the $K_D$ was calculated from software fits of the entire binding curve. Standard error values are based on the global fitting of association and dissociation data.
<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$ (x10^5) (M^{-1}s^{-1})</th>
<th>$k_{off}$ (x10^{-3}) (sec^{-1})</th>
<th>$K_D$ (x10^{-9}) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPg[mE14] + NPGB</td>
<td>5.62 ± 0.19</td>
<td>12.5 ± 0.06</td>
<td>22.2 ± 0.83</td>
</tr>
<tr>
<td>hPg[mE15] + NPGB</td>
<td>5.85 ± 0.33</td>
<td>11.7 ± 0.15</td>
<td>19.5 ± 0.85</td>
</tr>
<tr>
<td>hPg[mE16]</td>
<td>14.1 ± 0.12</td>
<td>20.8 ± 0.05</td>
<td>17.0 ± 0.32</td>
</tr>
<tr>
<td>hPg[mE17] + NPGB</td>
<td>4.76 ± 0.11</td>
<td>9.52 ± 0.10</td>
<td>20.0 ± 0.31</td>
</tr>
<tr>
<td>hPg[mE18]</td>
<td>16.9 ± 0.86</td>
<td>19.6 ± 0.33</td>
<td>11.7 ± 0.82</td>
</tr>
<tr>
<td>hPg[mE19] + NPGB</td>
<td>4.19 ± 0.06</td>
<td>11.1 ± 0.10</td>
<td>26.2 ± 0.65</td>
</tr>
</tbody>
</table>

* The value of the $K_D$ was calculated from software fits of the entire binding curve. Standard error values are based on the global fitting of association and dissociation data.
TABLE 4.7
KINETIC AND AFFINITY DATA FOR VARIOUS MOUSE PLASMINOGEN MUTANTS

<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$ ($\times 10^5$) (M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ ($\times 10^{-3}$) (sec$^{-1}$)</th>
<th>$K_D$ a ($\times 10^{-9}$) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPg[hE16]</td>
<td>14.7 ± 0.53</td>
<td>24.4 ± 0.08</td>
<td>16.7 ± 0.65</td>
</tr>
<tr>
<td>mPg[hE18]</td>
<td>22.2 ± 0.08</td>
<td>20.1 ± 0.08</td>
<td>9.23 ± 0.41</td>
</tr>
<tr>
<td>mPg[hE16+hE18] +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPGB</td>
<td>6.23 ± 0.03</td>
<td>14.9 ± 0.49</td>
<td>23.4 ± 0.70</td>
</tr>
</tbody>
</table>

a The value of the $K_D$ was calculated from software fits of the entire binding curve. Standard error values are based on the global fitting of association and dissociation data.
4.5. References


Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. *Science* 305:1283-1286.


APPENDIX A:
IDENTIFICATION OF THE AMINO ACID SEQUENCES RESPONSIBLE FOR
HUMAN PLASMINOGEN ACTIVATION BY STREPTOKINASE

In the present dissertation we showed that replacing mE16 and mE18 by their human counterparts accelerate mPg activation by SK. Sequence comparison between hPg and mPg, suggested important amino acid differences in the regions encoded by E16 and E18. We hypothesized that amino acid differences between hPg and mPg within the denminate “calcium-binding loop” (residues 622-629), encoded by E16, and residues 713-721, encoded by E18, are the key amino acid sequences responsible for hPg activation by SK. To test this hypothesis, amino acid sequences within hE16 and hE18 of mPg [hE16+hE18] were substituted for the residues found in mouse. Each mPg [hE16+hE18] variant was then examined for its ability to be activated by SK employing amidolytic assays. The results are shown herein.

A.1. Construction, expression and purification of the mPg [hE16+hE18] variants

The mPg [hE16+hE18] constructs were cloned and mutated by Dr. Takayuki Iwaki employing PCR techniques. Five mPg [hE16+hE18] mutants were generated: CL1, CL2, CL3, CL4, and CL5 (Figure A1). Each construct was expressed in Drosophila S2 cells, and purified using a Sepharose CL4B-Lysine column, as described in the Materials and Methods section in Chapter 4.
A.2. Activity assays

**Activation of mPg CL1, mPg CL2, mPg CL3, mPg CL4, and mPg CL5 by SK, uPA and SK-hPg complexes**

The activation of CL1, CL2, CL3, CL4, and CL5 by SK, uPA, and SK-hPg complexes was monitored using the chromogenic substrate S2251. The assays conditions are described in detail in the Materials and Methods section in Chapter 4. Catalytic levels of SK were used to activate the different constructs. As expected the ability to be activated by SK was importantly affected in CL2 and CL5 (Figure A2). To validate these results and to determine if the proteolytic activity of the proteins was compromised due to structural alterations, each construct was assayed with uPA and pre-formed SK-hPg complexes. No significant difference in the Pg constructs activability by uPA (Figure A3) or, by pre-formed SK-hPg complexes (Figure A4) was observed, as compared to hPg.

A.3. Conclusion

The results shown in the present work provide for the first time direct evidence of the role of amino acid sequences 622-629 (Gln$^{622}$, Val$^{624}$, Asn$^{625}$, Leu$^{626}$, Glu$^{627}$, Pro$^{628}$, and His$^{629}$), and residues 713-721 (Tyr$^{713}$, Phe$^{715}$, Gly$^{718}$, and Gln$^{721}$) in hPg activation by SK. We demonstrated that replacement of the human sequences by the murine counterparts in mPg [hE16+hE18] abolished its ability to be activated by SK. These findings correlated with existing structural studies (1) that pointed out the importance of these amino acid sequences in the interactions between hPg and SK. In conclusion, our results clearly identified the regions within hPg responsible for Pg activation and sensitivity to bacterial SK.
Figure A.1 mPg [hE16+hE18] variants. A) Representation of the mPg [hE16+hE18] light chain. mPg [hE16+hE18] was generated by the substitution of the mouse exons 16 and 18 for those of human. The amino acid sequence encoded by human exon 16 (hE16) is encircled by an orange dotted line (…), and the amino acid sequence encoded by human exon 18 (hE18) is encircled by a yellow dotted line (…). Amino acid differences with mPg are displayed in red letters. The members of the catalytic triad are displayed in green letters. B) Five mPg [hE16+hE18] constructs were generated by the replacement of human amino acid sequences (red letters) within hE16 and 18 for their murine counterparts (purple letters): CL1 (Pro→Ser, Ser→Glu, and Ser→Phe), CL2 (Gln→Glu, Val→Tyr, Asn→Ile, Leu→Arg, Glu→Gly, Phe→Ser, and His→Asp), CL3 (Glu→Ser, Ser→Ala, Arg→Lys, and Phe→Ile), CL4 (Thr→Asn, Arg→Asn, and Lys→Arg), and CL5 (Tyr→Val, Phe→Tyr, Gly→Asn, and Gln→Lys).
Figure A.2 Activation of hPg, mPg, mPg[hE16+hE18], CL1, CL2, CL3, CL4, and CL5 by SK. Activation of 0.2 µM of hPg (blue), mPg (red), mPg [hE16+hE18] (green), CL1 (black), CL2 (orange), CL3 (purple), by 0.0025 µM of SK. The generation of amidolytic activity at 25° C was followed at A405 using 0.25 mM of S2251.
Figure A.3 Activation of hPg, mPg, mPg[hE16+hE18], CL1, CL2, CL3, CL4, and CL5 by uPA. Activation of 25 nM of hPg (blue), mPg (red), mPg [hE16+hE18] (green), CL1(black), CL2 (orange), CL3 (purple), by 10 IU of uPA. The generation of amidolytic activity at 25° C was followed at A405 using 0.25 mM of S2251.
Figure A.4 Activation of hPg, mPg, mPg[hE16+hE18], CL1, CL2, CL3, CL4, and CL5 by pre-formed SK-hPg. Activation of 0.2 μM of hPg (blue), mPg (red), mPg [hE16+hE18] (green), CL1(black), CL2 (orange), CL3 (purple), by 0.0025 μM of pre-formed SK-hPg. The generation of amidolytic activity at 25° C was followed at A405 using 0.25 mM of S2251.
A.4. References