Characterization of the
Fgl2-Prothrombinase Binding Protein

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science,
Graduate Department of Laboratory Medicine and Pathobiology.
University of Toronto

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Characterization of the Fgl2-Binding Protein

Master of Science 2001

Jennifer Crookshank

Department of Laboratory Medicine and Pathobiology
University of Toronto

Abstract

The inflammatory cascade and the coagulation system are intimately connected; several coagulation proteins have been demonstrated to regulate inflammatory events. Studies have shown that fibrinogen is able to promote cellular adhesion and transendothelial migration through interactions with ICAM-1 and Mac-1, prompting speculation that related proteins may have similar roles. Fgl2 prothrombinase shares significant homology to the fibrinogen-γ chain; in particular the P1 domain of fibrinogen, which is responsible for interactions with Mac-1, is highly conserved in Fgl2. We hypothesized that soluble Fgl2 has an immunomodulatory effect through interactions with a cell-surface receptor. We further speculated that Fgl2 interacts with this putative receptor through its fibrinogen-related domain, and may bind to Mac-1 through the conserved P1 region. We found that Fgl2 binds to RAW 264.7 cells in a specific and dose-dependent manner (K_d = 185 nM), and that this binding is not dependent on Mac-1.
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List of Abbreviations

Adult T-cell leukemia  
American Type Culture Collection  
Autographa californica multiple nuclear polyhedrosis virus  
D-Biotinyl-ε-aminocaproic acid-N-hydroxy-succinimide ester  
Bovine Serum Albumin  
Complementary Deoxyribonucleic Acid  
Concanavalin A  
Deoxyribonucleic Acid  
Dulbecco’s Modified Eagle’s Medium  
Dulbecco’s Phosphate-Buffered Saline  
Effector Cell Protease-1  
Ethylenediaminetetraacetic Acid  
Fetal Bovine Serum  
Glycosylphosphatidylinositol  
Granulocyte-Macrophage-Colony Stimulating Factor  
High Molecular Weight Kininogen

ATL  
ATCC  
AcMNPV  
Biotin-7-NHS  
BSA  
cDNA  
ConA  
DNA  
DMEM  
DPBS  
EPR-1  
EDTA  
FBS  
GPI  
GM-CSF  
Hka
<table>
<thead>
<tr>
<th>Term</th>
<th>Acronym</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human T-cell Leukemia virus type 1</td>
<td>HTLV-1</td>
</tr>
<tr>
<td>Inserted Domain</td>
<td>I_domain</td>
</tr>
<tr>
<td>Intercellular Adhesion Molecule-1</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Interleukin</td>
<td>IL</td>
</tr>
<tr>
<td>Leukocyte Adhesion Deficiency</td>
<td>LAD</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>LPS</td>
</tr>
<tr>
<td>Lymphocyte Function-Associated Antigen</td>
<td>LFA-1</td>
</tr>
<tr>
<td>Mean Fluorescence Intensity</td>
<td>MFI</td>
</tr>
<tr>
<td>Messenger Ribonucleic Acid</td>
<td>mRNA</td>
</tr>
<tr>
<td>Modified Eagle’s medium</td>
<td>MEM</td>
</tr>
<tr>
<td>Monoclonal Antibodies</td>
<td>mAbs</td>
</tr>
<tr>
<td>Mouse Hepatitis Virus-3</td>
<td>MHV-3</td>
</tr>
<tr>
<td>Natural Killer</td>
<td>NK</td>
</tr>
<tr>
<td>Nucleocapsid</td>
<td>N</td>
</tr>
<tr>
<td>Phosphate-Buffered Saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Platelet-Endothelial Cell Adhesion Molecule-1</td>
<td>PECAM-1</td>
</tr>
<tr>
<td>Polymerase Chain Reaction</td>
<td>PCR</td>
</tr>
<tr>
<td>Procoagulant activity</td>
<td>PCA</td>
</tr>
<tr>
<td>Protease-Activated Receptor-1</td>
<td>PAR-1</td>
</tr>
<tr>
<td>P-Selectin Glycoprotein Ligand-1</td>
<td>PSGL-1</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Streptavidin-Phycoerythrin</td>
<td>SA-PE</td>
</tr>
<tr>
<td>Transforming Growth Factor-β</td>
<td>TGF-β</td>
</tr>
<tr>
<td>Tumor Necrosis Factor-α</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Urokinase Type Plasminogen Activator</td>
<td>uPA</td>
</tr>
<tr>
<td>Urokinase Receptor</td>
<td>uPAR</td>
</tr>
<tr>
<td>Vascular Cell Adhesion Molecule-1</td>
<td>VCAM-1</td>
</tr>
<tr>
<td>Very Late Antigen</td>
<td>VLA</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1: Inflammation

Inflammation is a critical component of the host defense against invading pathogens and tissue injury. However, this innate response can result in significant damage to the host when unregulated or inappropriately invoked, as demonstrated in auto-immune diseases such as rheumatoid arthritis and multiple sclerosis. The inflammatory response also presents a significant barrier to successful transplantation, as it is significantly involved in the rejection response (1).

Several events occur on a cellular level to trigger the inflammatory cascade. First, endothelial cells become activated in response to tissue injury. This results in increased expression of P-selectin on the endothelial cell surface. P-selectin in turn interacts with its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), a molecule found on the surface of monocytes, neutrophils, natural killer (NK) cells and memory T lymphocytes (2). The
interaction of P-selectin with its ligand instigates leukocyte rolling and tethering, of the leukocytes along the activated endothelial cell layer.

The activated endothelial cells release chemokines that interact locally with their receptors (members of the seven-transmembrane-spanning, G-protein-coupled receptor family) on the surface of the rolling leukocytes (3), triggering an inside-out signaling pathway that leads to integrin activation (4). Firm adhesion is mediated by interactions between the leukocyte integrins and members of the immunoglobulin superfamily, such as intercellular adhesion molecule-1 (ICAM-1) (5). Leukocyte adhesion is followed by the transendothelial cell migration of effector cells into the site of tissue injury. While the molecular mechanism of transendothelial migration is still being investigated, it has been demonstrated it is marked by a disruption of endothelial cell adherens junctions, specifically the dissociation of β-catenin, p120/p100, and plakoglobin from VE-cadherin (6).

1.1.2: Role of Mac-1 in inflammation

Integrins are a large family of heterodimeric molecules that have been implicated in a wide range of cellular functions, including signal transduction and adhesion to the extracellular matrix. Attachment of leukocytes to the endothelial cell layer during inflammation is dependent on interactions between members of the β1, β2 and β7 subfamilies of integrins and members of the immunoglobulin superfamily. Leukocyte Adhesion Deficiency (LAD) syndrome is caused by a lack or greatly reduced expression
of the β2 integrins, underscoring the importance of this integrin subclass in inflammation (7). Four β2 integrins (also known as the leukocyte integrins, due to their restricted cellular distribution) have been identified to date: LFA-1, Mac-1, CD11c/CD18 and the recently discovered CD11d/CD18. In particular, Mac-1 has been shown to be critical to the inflammatory process.

Mac-1, also known as CD11b/CD18, is perhaps the best-characterized member of the integrin family. Mac-1 is a highly promiscuous receptor, whose ligands include ICAM-1 (8), high molecular weight kininogen (Hka) (9), urokinase receptor (uPAR) (10), C3bi (11) and fibrinogen (12). Mac-1 also interacts with a wide variety of microbial ligands (13). Many of these ligands bind to the Mac-1 inserted (I) domain, a 200 amino acid region that is found in several proteins, including the remainder of the β2 integrins, Very Late Antigen-1 (VLA-1), VLA-2 and αEβ7 integrin (14). Both the Mac-1 and LFA-1 I-domain contain a metal ion-dependent adhesion site (MIDAS), indicating that ligand binding is somewhat dependent on the presence of divalent cations. Studies have shown that Mac-1 mediated-leukocyte adhesion is positively regulated by divalent cations, in particular by Mn$^{2+}$ (15).

A variety of studies have demonstrated that, in addition to its ability to recognize a variety of diverse ligands, Mac-1 is able to associate with several glycosylphosphatidylinositol (GPI)-linked receptors, including FcγRIIIB, uPAR, and CD14 (16). These interactions provide a means by which the GPI-linked receptors may participate in transmembrane signaling, despite the lack of an intracellular domain (17).
Interactions between Mac-1 and uPAR have been extensively discussed in the literature and there is increasing evidence that the actions of the β1 and β2 integrin families are coordinated through the actions of uPAR.

Similar to Mac-1, uPAR is a promiscuous receptor; its ligands include urokinase type plasminogen activator (uPA) and vitronectin (17). uPAR was first shown to interact with Mac-1 in 1996, and it was demonstrated that this association resulted in an increase in cellular adhesive properties (18); further studies have shown that uPAR clustering initiates neutrophil activation and increases Mac-1 expression (19). Recently, a pathway has been elucidated by which uPAR mediates activation of Mac-1 and LFA-1 by the α4β1 integrin (VLA-4). Briefly, the clustering of α4β1 integrin is able to induce adhesion through the activation of both Mac-1 and LFA-1; this reaction was inhibited by treatment with an anti-uPAR antibody (20). The result of this study suggests that uPAR plays a critical role in regulating cellular adhesion, in addition to its other roles.

1.1.3: The link between inflammation and coagulation

The inflammation and coagulation cascades are intimately connected, with cross-talk occurring between the two pathways. Inflammatory molecules are known to increase expression of procoagulants on the surface of monocytes and macrophages, while coagulation proteins can in turn regulate inflammation. Thrombin has been shown to activate endothelial cells, to increase P-selectin expression (2), and to induce the expression of ICAM-1 and Vascular Cell Adhesion Molecule-1 (VCAM-1) on the
CHAPTER 1: INTRODUCTION

endothelial cell surface (21). Thrombin is thought induce these effects through a Protease-Activated Receptor-1 (PAR-1)-mediated pathway (22).

Other coagulation proteins are also involved in the regulation of the inflammatory response. Factor X has been demonstrated to inhibit leukocyte-endothelial interactions, (23) while its derivative, factor Xa, has been demonstrated to increase cytokine production and adhesion molecule expression on endothelial cells via interactions with Effector Cell Protease-1 (EPR-1) (24). Tissue factor has been shown to promote inflammation in conjunction with factor VIIa (25) and to play a role in the regulation of the inflammatory response by promoting the reverse transmigration of mononuclear phagocytes (26). Tissue factor as has also been implicated in the activation of PAR-2 (27), a serine protease believed to be involved in early inflammatory events.

1.2: Role of Fibrinogen and Related Proteins in Inflammation

Fibrinogen is multi-functional protein with several important biological roles. In addition to its well-characterized role in coagulation, fibrinogen has also been demonstrated to mediate cell adhesion, transendothelial migration, and to increase cell proliferation. Mac-1 was first identified as a fibrinogen-receptor in 1988. Studies by Wright and colleagues (12) demonstrated that, when coated onto plates, fibrinogen could mediate the binding and spreading of polymorphonuclear leukocytes and that this could be blocked using antibodies against the α and β chains of Mac-1. ICAM-1 was later identified as a second fibrinogen-receptor (28) and studies have since demonstrated that
fibrinogen mediates interactions between Mac-1 and ICAM-1 (29). Further research has localized the sites of fibrinogen-Mac-1 interaction to amino acid residues 190-202 (P1) (30) and residues 377-395 (P2) (31) of the fibrinogen γ chain and the Mac-1 I-domain (32). Fibrinogen-ICAM-1 interactions occur between amino acid residues 117-133 of the fibrinogen γ chain and the first immunoglobulin-like domain of ICAM-1 (33; 29; 34). These interactions have been shown to promote both cell-cell adherence and transendothelial migration (28; 29).

The role of fibrinogen in promoting cell adhesion has prompted speculation that related proteins may also be involved in adhesion and migration. Tenascin, which contains a fibrinogen-related domain at the C-terminus, is an extracellular matrix protein that is mainly expressed during embryo morphogenesis; it has also been implicated during tumor growth, inflammation and tissue repair. Studies have shown that this molecule to have immunoregulatory functions in that it can inhibit integrin-dependent adhesion of monocytes to fibronectin, induce Epstein-Barr virus-transformed B cell aggregation, and alter some pathways of T cell activation (35). It has also been demonstrated that tenascin interacts with endothelial cells (36) and aortic smooth muscle cells (37) through its fibrinogen-related domain. Further studies have shown that endothelial attachment to tenascin is mediated through the integrins α2β1 and αvβ3 (38). Interestingly, the αvβ3 integrin also binds to the P1 site and to amino acid residues 246-358 of the fibrinogen γ chain (39).
M-ficolin is also a member of the fibrinogen-related family of proteins, and contains a fibrinogen-related domain at the C-terminus. The Pl region, responsible for mediating interactions between fibrinogen and Mac-1, is conserved in this protein (table 1). M-ficolin is found on peripheral blood monocytes but not on monocyte-derived dendritic cells and at low levels on monocyte-derived macrophage cells. Its function on circulating monocytes has yet to be fully elucidated, however a recent report has shown that M-ficolin is able to mediate adhesion of the monocyte-derived cell line U937 to immobilized F(ab')² fragments of an anti-fibrinogen antibody and thus may be involved in monocyte migration (40).
**Table 1: Conservation of the P1 Domain in Fibrinogen-related Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Integrins Interactions</th>
<th>Conserved P1 region</th>
<th>% Conservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>Coagulation protein, immunoregulation</td>
<td>αMβ2, αVβ3</td>
<td>GWTVFQKRLDFSV</td>
<td></td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>Angiogenesis, anti-apoptotic factor</td>
<td>αvβ5 β1</td>
<td>GWTVIQHREDGSL</td>
<td>71%</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>Angiopoietin-1 antagonist</td>
<td>αvβ5 β1</td>
<td>GWTVIQHREDGSV</td>
<td>77%</td>
</tr>
<tr>
<td>M-Ficolin</td>
<td>Unknown; may play role in cellular adhesion</td>
<td>-</td>
<td>GWTVFQRRVDGSL</td>
<td>77%</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>Embryogenesis, wound healing inflammation</td>
<td>αVβ3, α2β1, α8β1, α9β1</td>
<td>Not conserved</td>
<td>NA</td>
</tr>
<tr>
<td>Fgl2 prothrombinase</td>
<td>Procoagulant with roles in viral hepatitis, fetal loss, graft rejection</td>
<td>?</td>
<td>GWTVLQARLDGST</td>
<td>77%</td>
</tr>
</tbody>
</table>

Both angiopoietin-1, and its antagonist angiopoietin-2, contain fibrinogen-related domains at the C-terminus and thus are members of the fibrinogen-related family of proteins. As with M-ficolin, the P1 region is conserved (table 1). Studies have shown that angiopoietin-1 is an apoptosis survival factor, and that it inhibits the apoptotic processes through interactions with the Tie-2 receptor (41). Binding of angiopoietin-1 to Tie-2 has been localized to the fibrinogen-related domain (42). A recent study has shown that angiopoietin-1 inhibits endothelial cell permeability by promoting the recruitment of Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM-1) to cellular junctions. In
addition, angiopoietin-1 expression inhibited E-selectin expression on the surface of endothelial cells, and prevented Tumor Necrosis Factor-α (TNF-α)-induced transmigration of leukocytes (43). Other studies have shown that angiopoietin-1, and to a lesser degree angiopoietin-2, binds to endothelial cells through interactions with αvβ5 integrin and members of the β1 integrin subfamily (44). This suggests that in addition to its role in angiogenesis and as an anti-apoptotic factor, angiopoietin-1 also functions as an anti-inflammatory molecule and may be involved in cellular adhesion.

1.3: Fgl2 Prothrombinase

The fibrinogen-related protein Fgl2 prothrombinase (fibroleukin; pT49 gene product; (mus)fblp protein) has a molecular weight of 70 kilodaltons (kDa) and shares 36% homology with the fibrinogen γ chain. The gene was first isolated from a T-cell minus B-cell subtractive complementary deoxyribonucleic acid (cDNA) library (45) and has been localized to the proximal region of chromosome 5 in mice (46) and to chromosomal region 7q11.23 in humans (47). Constitutive expression of this protein has been reported by both CD4+ and CD8+ cells (48) and can be induced on macrophages by treatment with Interferon-γ (IFN-γ) (49). In addition, Fgl2 messenger ribonucleic acid (mRNA) transcripts have been detected in mouse endothelial cells following infection with Mouse Hepatitis Virus-3 (MHV-3), suggesting a wider cellular and tissue distribution (50). To date the functions of this protein have not been fully elucidated, however studies have shown Fgl2 to have a role in MHV-3 infection in mice (51) fulminant hepatitis in humans
(52) and spontaneous abortion (53). There are indications that Fgl2 may also be involved in transplant rejection (54) and adult T cell leukemia (55).

1.3.1: Role of Fgl2 prothrombinase in viral hepatitis

The majority of individuals who contract acute hepatitis recover completely. However, a small percentage (less than 0.1%) go on to develop fulminant hepatitis, a condition characterized by an influx of inflammatory cells resulting in extensive liver necrosis. Previous studies have utilized MHV-3 infection in susceptible mouse strains to investigate the pathogenesis of fulminant hepatitis.

MHV-3 is a member of Coronaviridae, a family of positive, single-stranded RNA viruses. The outcome of infection with this virus in mice is strain-dependent; A/J and SJL strains are fully resistant to the disease, while the Balb/cJ and C57BL/6J stains are susceptible and inevitably die of fulminant hepatic failure (56). The disparity in the manifestation of the disease cannot be accounted for by differences in virus susceptibility as both susceptible and resistant strains have similar viral loads (57). Rather, variable stimulation of the immune coagulation system, specifically the induction of macrophage procoagulant activity, by MHV-3 appears to be the key factor in the development of fulminant hepatitis (58).

A panel of monoclonal antibodies (mAbs), directed against the MHV-3-induced procoagulant activity (PCA), was produced in order to investigate the role of immune coagulants in fulminant hepatitis. The mAb 3D4.3 reacted against a 70 kDa protein by
Western blot analysis and was shown to inhibit PCA expression in a one-stage clotting assay and to inhibit the conversion of prothrombin to thrombin in vitro (59). Further studies demonstrated mAb 3D4.3 increased the survival of susceptible mice when used in vivo (60). In order to identify the gene encoding the protein responsible for the MHV-3-induced PCA activity, a cDNA library was prepared from MHV-3-infected macrophages and screened using mAb 3D4.3 (51). A reactive clone was identified; sequencing of this cloned showed that it was identical to a portion of exon 2 of the previously identified musfblp gene (45).

The identification of the gene encoding the MHV-3-induced PCA protein (Fgl2 prothrombinase) allowed for investigation into the molecular pathogenesis of fulminant hepatitis. Studies demonstrated that Fgl2 mRNA transcripts were not detectable in the liver, spleen, lungs kidneys or brain prior to MHV-3 infection, nor was Fgl2 protein expressed (50). Following MHV-3 infection, Fgl2 mRNA transcripts were detected in the liver (8 hours post-infection), spleen and lungs (6 hours post-infection), but not in the kidneys or the brain. Fgl2 transcripts were further localized to nonparenchymal cells. Recent studies have shown that the MHV-3 Nucleocapsid (N) protein is able to induce transcription of the Fgl2 gene, and investigations have been undertaken to identify elements in the Fgl2 gene promoter that are responsive to the N protein (61).
1.3.2: Role of Fgl2 prothrombinase in Fetal Loss Syndrome

The successful pregnancy represents an immunologic paradox; as a composite of both maternal and paternal antigens, it should be recognized as 'foreign' by the maternal immune system, and thus be rejected. However, while a number of embryos are lost throughout gestation, a high proportion are carried through until term; the question therefore arises as to how these immunologically foreign embryos survive. The uterus constitutes a unique immune environment that is adapted to supporting pregnancy while maintaining its role as a mucosal barrier to infection (62). Several variables within this environment have been identified as factors contributing to the success or failure of a fetus, however the role of T cells and their associated cytokine profiles is of particular interest. Studies have shown that a Th2 response is associated with a successful pregnancy, while a Th1 cytokine profile contributes to the loss of a fetus.

CD4+ cells can be grouped into several subsets based on their function. Th1 cells secrete cytokines that induce cell-mediated immunity, such as interleukin-2 (IL-2) and IFN-γ. Th2 cells induce B cell proliferation and antibody production primarily through the interaction of CD40 on B cells and CD40L on T cells (63) and also produce cytokines, such as IL-4 and IL-10, which enhance antibody production. The recently described Th3 cell subset (64) is thought to induce tolerance via the secretion of Transforming Growth Factor-β (TGF-β). The factors that cause T cell differentiation have yet to be fully elucidated, however there is evidence that cytokines secreted by antigen-presenting cells such as dendritic cells, B cells and macrophages play a critical
role (65; 66). Th1/Th2 paradigm is now known to affect the outcome of several pathological conditions, such as tuberculosis, and MHV-3 infection (56). Typically, a Th1 response is associated with a resistant phenotype and a Th2 response is associated with a susceptible or tolerant phenotype.

Cytokine-mediated abortion has been investigated using the CBA x DBA/2 mouse model of fetal loss, in which fetal rejection is believed to be mediated by activated NK cells and macrophages (67) and the cytokines TNF-α and IFN-γ. Fetal resorption is marked by thrombosis and hemorrhage in this model, suggesting a role for coagulation proteins in fetal loss. Fgl2 was first implicated as an important mediator of fetal rejection by a study that demonstrated that treatment with an anti-Fgl2 antibody significantly reduced fetal rejection in the CBA x DBA/2 after cytokine treatment (53). In situ hybridization studies demonstrated that Fgl2 mRNA expression was increased at the junction of the decidua and myometrium and in trophoblast after cytokine treatment with TNF-α and IFN-γ as compared to control mice (68). Similar levels of Fgl2 mRNA expression were observed in human decidua and trophoblast in cases of recurrent spontaneous abortion with normal fetal karyotypes (68). A recent study has shown that the effects of Fgl2 in the decidua and trophoblast may be countered by the molecule OX-2, suggesting a mechanism by which the uterus regulates fetal success (69).
1.3.3: Other roles of Fg12 prothrombinase

Currently Fg12 has been identified as an important factor in viral hepatitis and fetal loss syndrome; however the biological role of this protein has yet to be fully understood. Several studies have been undertaken to gain a more complete picture as to the total functionality of this protein, including its role in adult T-cell leukemia and transplant rejection.

Adult T-cell leukemia (ATL) is one of several diseases caused by the human retrovirus Human T-cell Leukemia virus type 1 (HTLV-1). It exhibits a wide spectrum of clinical features, and thus can be further classified into acute, lymphomatous, chronic and smoldering types (70). ATL cells are characterized by several unique features, including their ability to infiltrate into a variety of organs, including the liver, lung, spleen and gastrointestinal tract; this is made possible by interactions between the leukemic cells and the vascular endothelium. OX-40 and its ligand pg34 have been identified as the major mediators of interactions between ATL leukemic cells and the endothelium (71). A recent study has shown that Fg12 expression is decreased in chronic and acute ATL (55); it is suggested by the authors that this may be partially responsible for the malignant features of afflicted cells.

Transplantation is now widely utilized as an effective treatment for end-stage organ failure. General immunosuppressive agents, such as cyclosporin A, have been demonstrated to greatly enhance post-transplant survival, however the use of these drugs also increases the graft recipients risk of bacterial and viral infection. Thus current
research efforts have focused on dissecting the mechanism of rejection at both a molecular and immunological level in order to determine a more specific method of promoting graft survival.

Graft rejection can be sub-divided into three types: hyperacute rejection, acute rejection and chronic rejection. Hyperacute rejection occurs rapidly following transplant, and is characterized by neutrophil accumulation, the deposition of pre-existing antibodies along the vessel walls, and the activation of the complement and coagulation cascades. Acute rejection occurs somewhat later than hyperacute rejection, and is mediated by T cells. Long-term organ loss following transplantation is referred to as chronic rejection, and is mediated by both cellular and humoral mechanisms (72). Several studies are currently being undertaken to examine the role of Fgl2 in both allograft and xenograft loss. Preliminary studies have shown that Fgl2 prothrombinase expression is increased during allograft rejection, and have localized expression to endothelial cells, CD3+ T cells and CD11b+ macrophages. Fgl2 expression was correlated with thrombin formation and fibrin deposition within the graft (54). Studies using an Fgl2 knockout mouse have been undertaken to determine whether Fgl2 prothrombinase constitutes the key pathway of thrombin formation and fibrin deposition during transplantation.

In addition to its role as a membrane bound procoagulant, Fgl2 prothrombinase may have additional functions as an immune-modulator. Recently Marazzi and colleagues (73) reported that fibroleukin (also known as Fgl2 prothrombinase) was secreted as a 250-300 kDa complex from freshly isolated peripheral blood mononuclear cells and from
purified T lymphocyte; the authors speculated that this soluble form of Fgl2 may have immuno-modulatory activity. Work in our laboratory has demonstrated that Fgl2 prothrombinase inhibits T cell proliferation, and promotes a Th2 cytokine response (74), suggesting that Fgl2 does have immuno-regulatory activity.

The question remains as to how a protein can be both a membrane-bound procoagulant and a secreted immune modulator. Studies are currently being performed in our laboratory to resolve this paradigm. Analysis of the DNA sequence has revealed the presence of multiple Kozak consensus sequences. These translation start sites are often preceded by transcription start sites, thus raising the possibility that multiple mRNA species exist. This hypothesis is currently being investigated using 5' RACE (75). We suspect that analysis will reveal the presence of at least two mRNA species, leading to the production of both a membrane-bound Fgl2 molecule, and a secreted Fgl2 molecule that lacks the N-terminal transmembrane domain.
Chapter 2

Rationale and Statement of Hypothesis

Fgl2 prothrombinase has been shown to be a unique procoagulant that is able to directly cleave prothrombin to thrombin. The role of this protein in both fulminant hepatitis and fetal loss syndrome has been characterized and a role in transplant rejection is currently being investigated. Both membrane-bound and soluble forms of this protein have been reported (59; 48: 73), suggesting that, in addition to its function as a procoagulant, Fgl2 may act as an immune modulator as well. We therefore hypothesize that soluble Fgl2 binds to a cell surface receptor, and that this ligand-receptor interaction causes an immuno-modulatory effect.

Fibrinogen and several members of the fibrinogen-like family of proteins have been shown to mediate adhesive events, such as leukocyte adhesion and transendothelial migration. Fibrinogen has been shown to mediate interactions between Mac-1 and ICAM-1 to promote cellular adhesion and migration (28; 29). Fibrinogen-Mac-1 interactions occur in part through the P1 domain of the fibrinogen γ chain (30); this site
has also been implicated in fibrinogen-αvβ3 interactions (39). Analysis of the amino acid sequence has shown that this region is highly conserved in the Fg12, and several other fibrinogen-related proteins (table 1). Therefore, we hypothesize that Fg12 may regulate leukocyte-endothelial interactions in a similar manner to fibrinogen and other fibrinogen-related proteins. Furthermore, we suspect that Fg12 may directly interact with Mac-1 and ICAM-1 through the conserved P1 domain.

We therefore hypothesize that:

- Soluble Fg12 binds to a cell-surface receptor, and is an immune modulator in this form
- Binding of Fg12 to its receptor is mediated through the fibrinogen-related domain
- Fg12 binds to both Mac-1 and ICAM-1, and acts as a regulator of the inflammatory response
Chapter 3

Materials and Methods

3.1: Recombinant Virus Production

A vector containing the fgl2 gene under control of the polyhedrin promoter was constructed using the pBlueBacHis2a vector (Invitrogen, Carlsbad, California). A 1.4 kilobase cDNA was amplified by Polymerase Chain Reaction (PCR) using the forward primer 5'-TGCCGCACTGGATCCATGAGGCTTCCTGGT-3' and the reverse primer 5'-AAGGCTGAAGGAATTCATCGTCC-3'. A total of 25 amplification cycles were performed (2 min at 96 °C, 2 min at 55 °C and 3 min at 72 °C). The resulting PCR product was cloned into the EcoR1 and the BamH1 sites of the pBlueBacHis2a vector.

A recombinant baculovirus was generated by co-transfecting Sf9 cells with 2 μg of the pBlueBacHis2a vector containing the fgl2 insert and 1 μg linearized Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) DNA using the Bac-N-Blue transfection kit (Invitrogen). Three rounds of plaque purification were then performed;
plaquecontaining putative recombinant viruses were analyzed by PCR, and the resulting product was sequenced using an automated DNA sequencer (model 377, PE Applied Biosystems, Foster City, California).

3.2: Recombinant Protein Production

Recombinant Fgl2 prothrombinase was produced under denaturing conditions using the Xpress® Baculovirus Expression System (Invitrogen) according to the manufacturers directions. HighFive cells (Invitrogen) were infected with the recombinant Baculovirus containing the Fgl2 gene under the control of the polyhedrin promoter. Cell pellets were harvested at 3 days post-infection and lysed in guanidinium lysis buffer (6M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) by repeated passage through an 18-gauge needle. Cellular debris was removed by centrifugation, and the cell lysate was applied to a ProBond resin nickel column (Invitrogen) and incubated at 4°C for 1 hour with gentle rotation. The column was then washed 2X with binding buffer (8 M Urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8), 2X with wash buffer (8 M Urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0) and 2X with wash buffer (8 M Urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 5.3). The protein was then eluted from the column with elution buffer (8 M Urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 4.0) and dialyzed against decreasing concentrations of urea (6M, 4M, 2M, 1M, pH 7.8) for 2 days. The protein solution was then dialyzed against 10 mM Tris, pH 7.8 to remove traces of urea.
3.3: Biotinylation of Fgl2, BSA and Fibrinogen

A 1 mg/mL solution of purified Fgl2 and immunoglobulin-free bovine serum albumin (BSA) (Sigma, Oakville Ontario) were incubated with a 1:10 molar reaction mixture of D-Biotinyl-ε-aminocaproic acid-N-hydroxy-succinimide ester (Biotin-7-NHS) (Roche Diagnostics, Laval Quebec) for 1 hour at room temperature with gentle stirring. The reaction mixture was then applied to a Sephadex G-25 column (Roche Diagnostics) and washed with 1.5 mL of PBS, and eluted with 3.5 mL of PBS. The elutant was collected in 0.5 mL aliquots and the protein concentration was determined using a BCA assay (BioLynx, Brockville Ontario). Murine fibrinogen (Sigma) was labeled in a similar manner, however the molar reaction mixture was 1:100.

3.4: Cells

3.4.1: Preparation of Macrophages

Macrophage production in Balb/c mice was stimulated by intraperitoneal administration of 1.5 mL of a 5% thioglycolate solution. Cells were harvested after 4 days, and washed twice with serum-free RPMI (Life Technologies, Burlington Ontario). The cells were then stimulated with 20 µg/mL lipopolysaccharide (LPS) (Sigma) for 6 hours in RPMI media supplemented with 10% fetal bovine serum (FBS) (Life Technologies) at 37°C.
3.4.2: Preparation of B cells and T cells

Splenic B cells and T cells were collected from Balb/c mice as follows: the spleen was minced and passed through a nylon mesh filter to obtain a cell suspension. The cells were then washed with serum-free α-modified Eagle’s medium (MEM) media, and resuspended in a hypotonic ammonium chloride solution. The red blood cells were lysed by incubating the cells with the ammonium chloride solution for 3 minutes at 37°C. The cells were then washed with α-MEM media, and applied to a nylon wool column (76). The non-adherent T cells were removed by washing the column with α-MEM media after a 1 hour incubation at 37°C; the adherent B cells were then collected by gently squeezing the nylon wool and collecting the media. In some experiments, the cells were then cultured in α-MEM with 10% fetal bovine serum (Life Technologies) and 2% Penicillin-Streptomycin (Life Technologies) at 37°C and 5% CO₂. T cells were stimulated with 2 μg/mL Concanavalin (ConA) (Sigma) for 72 hours, and B cells were stimulated with 20 μg/mL of LPS (Sigma) for 48 hours.

3.4.3: Preparation of Dendritic cells

Dendritic cells were prepared as described elsewhere (77; 78). Bone marrow cells were isolated from Balb/c mice by flushing α-MEM media through the extracted femur bone using a 22-gauge needle. The cells were then collected by centrifugation, and resuspended in a hypotonic ammonium chloride solution. The red blood cells were then lysed by incubating the cells with the ammonium chloride solution for 3 minutes at 37°C.
The cells were then washed with α-MEM, and incubated with anti-CD4 antibody (Cedarlane Laboratories, Hornby Ontario) on ice for one hour. The cells were then washed with α-MEM and incubated with rabbit anti-mouse complement for one hour at 37°C and 5% CO₂. The cells were then washed with α-MEM supplemented with 10% fetal bovine serum and 2% Penicillin-Streptomycin, and cultured with IL-4 (10 ng/mL) (Cedarlane Laboratories) and Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) (10 ng/mL) (Cedarlane Laboratories) for 6 days, with media exchange occurring every 48 hours. The mature dendritic cells were obtained by stimulating the culture with 20 μg/mL for 2 days. The cells were confirmed to be dendritic cells based on morphology and cell surface staining for CD80 as assessed by flow cytometry.

3.4.4: Cell lines

The murine macrophage-like cell line RAW 264.7 (79) was obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 2% Penicillin-Streptomycin at 37°C and 5% CO₂. For some experiments the cells were stimulated with 10 μg/mL of LPS for 12 hours. The murine endothelial-like cell line SVEC4-10 (80) was obtained from the ATCC and were cultured in DMEM with 10% fetal bovine serum and 2% Penicillin-Streptomycin at 37°C and 5% CO₂. Murine fibroblast cells were a kind gift of Dr. Andres Kapus (University of Toronto, CIHR Transplantation Research Group) and
CHAPTER 3. MATERIALS AND METHODS

were cultured in DMEM with 10% fetal bovine serum and 2% Penicillin-Streptomycin at 37°C and 5% CO₂.

3.5: Flow Cytometry Analysis

Cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS) (Life Technologies), and then blocked for 5 minutes with 5% mouse serum (Cedarlane Laboratories) at room temperature. The cells (1*10^6) were then incubated with biotin-labeled Fgl2, biotin-labeled immunoglobulin-free BSA (Sigma) or biotin-labeled fibrinogen (Sigma) for 30 minutes at 4°C. The cells were washed again, and incubated with a Strepavidin-phycoerythrin (SA-PE) conjugate (BD Pharmingen, Mississauga, Ontario) for 20-30 minutes at 4°C. ExtrAvidin-phycoerythrin conjugate (Sigma) was used in some experiments. After an additional two washes, the cells were analyzed by flow cytometry.

3.6: Inhibition Studies

Cells were washed twice with DPBS and blocked for 5 minutes with 5% mouse serum at room temperature. The cells (1*10^6) were then incubated with 10X molar excess of unlabelled fibrinogen or fibronectin, anti-CD18 antibody or anti-CD11b antibody or 10X molar excess of unlabelled Fgl2 protein for 30 minutes at 4°C. Cells were then washed and stained according to the above protocol. In control experiments, cells were incubated with 10X molar excess of unlabelled fibrinogen, anti-CD18 antibody
or anti-CD11b antibody for 30 minutes at 4°C and then incubated biotinylated fibrinogen for 30 minutes at 4°C. In some experiments cells were pre-treated with 10mM EDTA (Sigma) for 30 minutes at 4°C prior to being stained in accordance with the above protocol.

3.7: Association of Fgl2 with RAW 264.7 cells over time

RAW 264.67 cells were washed twice with DPBS and blocked for 5 minutes with 5% mouse serum at room temperature. The cells (2*10⁵) were then incubated biotinylated Fgl2 protein (119 nM) for increasing amounts of time (10 minutes-210 minutes) at 4°C. The cells were then washed, and stained for flow cytometry analysis as described.

3.8: Saturation Binding Curve

RAW 264.7 cells were washed twice with DPBS and blocked for 5 minutes with 5% mouse serum at room temperature. The cells (2*10⁵) were then incubated with increasing concentrations of biotinylated Fgl2 protein (23.8 nM-476.2 nM) for 2.5 hours at 4°C. The cells were then washed and analyzed by flow cytometry as described previously. Non-specific binding was assessed in the presence unlabelled Fgl2 protein (2.38 µM). Specific binding was calculated by subtracting non-specific binding from total binding. The K_d value was determined by non-linear regression analysis, using the GraphPad Prism Software version 3.02 (GraphPad Software, San Diego California).
Chapter 4

Results

4.1: Hypothesis: Fgl2 prothrombinase acts to mediate adhesion between leukocytes and endothelial cells, and does so through interactions with the integrin Mac-1 and ICAM-1

4.1.1: Fgl2 binding to Macrophages and Raw 264.7 cells

In order to investigate the possible alternative role of Fgl2 prothrombinase as an adhesion molecule, we first examined the binding of Fgl2 to macrophages. Cells were harvested from Balb/c mice 4 days post-injection of 5% thioglycolate, and stimulated with LPS for 6 hours. The cells were then incubated with biotinylated Fgl2, stained with strepavidin-PE and analyzed by flow cytometry. An increase in the fluorescent signal was apparent in cells that had been stained with biotin-Fgl2 compared to cells stained with Strepavidin-PE alone or with biotinylated BSA (figure 1).
Figure 1a) Murine macrophages were harvested 4 days after the intraperitoneal injection of 1.5 mL of thioglycolate; 10^6 cells were incubated with biotinylated Fg12 protein (840 nM) for 30 minutes on ice; the cells were then stained with 1 μg of SA-PE and analyzed by flow cytometry. (b) RAW 264.7 cells were cultured in DMEM+10% FBS and 0.2% penicillin-streptomycin; the cells were harvested at 95% confluency, and analyzed as with macrophage cells.
In addition, the macrophage-like cell line RAW 264.7 was also found to bind biotinylated Fgl2 in a similar manner to the thioglycolate-elicited macrophage cells (figure 1). This binding was dose-dependent (figure 2). These results suggested the presence of an Fgl2-binding protein on the surface of macrophage and macrophage-like cells.
10^6 RAW cells were incubated on ice with increasing concentrations of biotinylated Fgl2 protein (a) 42 nM, (b) 84 nM, (c) 420 nM, (d) 820 nM, (e) 1260 nM, (f) 1681 nM, (g) 2521 nM) for 30 min; cells were then stained with 1 μg of SA-PE and analyzed by flow cytometry. Cells incubated with biotinylated Fgl2 and SA-PE shown in black; cells incubated with SA-PE alone shown in grey.
The effect of LPS stimulation on Fgl2 binding to RAW 264.7 cells was then investigated. Cells were either unstimulated or stimulated with 10 μg/mL of LPS for 12 hours, and incubated with biotinylated Fgl2 for analysis by flow cytometry. Analysis showed that binding of biotinylated Fgl2 was decreased in cells treated with LPS as compared to untreated cells. In contrast, binding of biotinylated fibrinogen to RAW 264.7 cells increased with LPS stimulation (figure 3).
Figure 3) Effect of LPS Stimulation on (a) Fgl2 and (b) Fibrinogen binding to RAW cells: RAW 264.7 cells were cultured in DMEM + 10% FBS and 0.02% Penicillin-Streptomycin, and in some cases stimulated with 10 μg/mL LPS for 12 hours. Cells were then either incubated with biotinylated Fgl2 protein (420 nM) or biotinylated fibrinogen protein (170 nM) for 30 minutes on ice and analyzed as described by flow cytometry.
4.1.2: Binding of Fgl2 to SVEC4-10 cells

The endothelial-like cell line SVEC4-10 is derived from BALB/c mice and does not express ICAM-1. This cell line was therefore used to investigate the binding of Fgl2 to endothelial cells and to determine whether this binding was ICAM-1 dependent. Cells were harvested by treatment with 2 mM EDTA, washed and stained for flow cytometry analysis as described. An increase in fluorescent signal was apparent for cells stained with biotinylated Fgl2 as compared to cells that had been stained with biotinylated BSA or strepavidin-PE alone (figure 4a), suggesting that Fgl2 binds to endothelial cells, and that this binding is independent of ICAM-1. Binding occurred in a dose-dependent manner (figure 4b).
Figure 4) Fgl2 binding to SVEC4-10 cells: a) cells were cultured in DMEM + 10% FBS and 0.2% penicillin-streptomycin until 95% confluent; cells were harvested by treatment with 2mM EDTA in PBS for several minutes at room temperature and detached by gentle pipetting; cells were then stained with various concentrations of biotinylated Fgl2 and analyzed as described previously; (b) binding was dose-dependent.
4.1.3: Fgl2 binding to RAW 264.7 cells is inhibited by pre-treatment with unlabelled Fgl2, but not by known Mac-1 ligands or by anti-Mac-1 antibody.

In order to investigate the role of Mac-1 as a potential Fgl2-binding protein, a series of inhibition studies were carried out using known Mac-1 ligands and anti-Mac-1 antibody. Cells were pre-treated with unlabelled Fgl2, unlabelled fibrinogen or unlabelled anti-Mac-1 antibody for 30 minutes at 4°C. The cells were then stained as described previously for flow cytometry analysis. Cells treated with unlabelled Fgl2 showed a dose-dependent inhibition of biotinylated Fgl2 binding, whereas cells treated with unlabelled fibrinogen or unlabelled anti-CD11b antibody showed no inhibition (figure 5); in contrast, binding of biotinylated fibrinogen was inhibited by pretreatment with unlabelled fibrinogen, anti-CD11b antibody and anti-CD18 antibody (figure 6). These results suggested that the Fgl2 binding site on macrophages is therefore unique compared to that of fibrinogen and may be a protein other than Mac-1.

In order to investigate the role of other integrins as Fgl2 binding proteins, a series of inhibition studies were undertaken using the extracellular matrix protein fibronectin and anti-CD18 antibody. Cells were pretreated with unlabelled fibronectin or anti-CD18 for 30 minutes on ice and then stained for flow cytometry analysis as described above. Pretreated cells failed to show any inhibition of biotinylated Fgl2 binding as compared to untreated cells, suggesting that the Fgl2-binding protein is distinct from the fibronectin receptors, and is not a member of the β2 integrin family (figure 5).
CHAPTER 4. RESULTS

Inhibition of Fgl2 binding

Figure 5) Inhibition of Fgl2 binding to RAW cells: RAW cells were cultured and harvested as described previously; 10^6 cells were incubated with an excess of unlabelled Fgl2, fibrinogen, fibronectin, Mac-1 antibody or CD18 antibody for 30 minutes on ice; the cells were then washed and incubated with 420 nM biotinylated Fgl2 and analyzed by flow cytometry as described previously. The MFI value for each sample was compared to non-pretreated cells. Results were statistically analyzed using the students T test.
CHAPTER 4. RESULTS

Figure 6) Inhibition of Fibrinogen binding to RAW 264.7 cells: RAW cells were cultured and harvested as described previously; \(10^6\) cells were incubated with an excess of unlabelled fibrinogen, antiCD11b antibody or antiCD18 antibody for 30 minutes on ice; the cells were then washed and incubated with biotinylated fibrinogen (210 nM) and analyzed by flow cytometry as described previously. The MFI was for each sample was compared to the non-pretreated cells. Results were analyzed using the student’s T-test.
4.1.4: Chelation of divalent cations inhibits Fgl2 binding to RAW 264.7 cells, but not to SVEC4-10 cells

As the binding of ligands to members of the integrin family is known to be cation dependent, the effect of pretreatment with EDTA on Fgl2 binding to RAW 264.7 cells and SVEC4-10 cells was investigated. Cells were washed with PBS without divalent cations. The cells were pre-treated with 10 mM EDTA for 30 minutes at 4°C, and were then stained as described previously for analysis by flow cytometry. RAW 264.7 cells that had been pre-treated with EDTA demonstrated inhibited Fgl2 binding compared to cells that had not been pre-treated with EDTA. In contrast, SVEC4-10 cells that had been pretreated with EDTA did not demonstrate inhibited Fgl2 binding (figure 7). Therefore the dependency of Fgl2 binding on the presence of divalent cations varies with cell type.
Figure 7) Cells were cultured, harvested and analyzed as described previously with the following exception: cells were pre-treated (30 minutes on ice) and analyzed in the presence of 10 mM EDTA in PBS. Results were statistically analyzed using the students T test.
CHAPTER 4. RESULTS

4.2: Characterization of the Fgl2 binding protein

4.2.1: Distribution of the Fgl2-binding protein

A number of cell types were investigated to determine the cellular distribution of the Fgl2-binding protein. Macrophages, RAW 264.7 cells, SVEC4-10 cells, murine fibroblasts, T cells, B cells, and dendritic cells were isolated and cultured as described previously. Cells were then analyzed by flow cytometry as described. The results of these studies indicated that the Fgl2-binding protein is expressed constitutively on macrophages and macrophage derived cells (RAW 264.7 cells), SVEC4-10 cells, fibroblast cells, and dendritic cells. Binding of Fgl2 prothrombinase was not evident on unstimulated B cells, but was demonstrated after LPS-stimulation. Similarly, Fgl2 did not bind to unstimulated T cells, but a population of T cells demonstrated Fgl2 binding after ConA stimulation. (figure 8).
Figure 8) Cellular Distribution of FgI2-binding protein: Cells were cultured, harvested and washed as described. Cells were then incubated with biotinylated FgI2 protein (840 nM) for 30 minutes on ice, and then analyzed by flow cytometry as described.
4.2.2: Association of Fgl2 prothrombinase with RAW 264.7 cells over time

The association of Fgl2 prothrombinase with RAW 264.7 cells over time was investigated. Cells were incubated a constant concentration (119 nM) of biotinylated Fgl2 protein for increasing amounts of time, and then analyzed by flow cytometry. Maximum binding of Fgl2 to RAW 264.7 cells was achieved at 150 minutes at 4°C. This time point was used for subsequent investigation of binding affinity (figure 9).

Figure 9) Association Kinetics: Cells were harvested and blocked as described previously. $2\times10^5$ cells were incubated with biotinylated Fgl2 protein (119 nM) for increasing time intervals up to 210 minutes on ice. The cells were then analyzed by flow cytometry as described ($n\geq3$).
4.2.3: Saturation Curve and Assessment of Affinity

The binding of Fgl2 prothrombinase to RAW 264.7 cells was assessed. Cells were incubated with increasing concentrations of Fgl2 for 2.5 hours on ice; non-specific binding was assessed in the presence of an excess of unlabelled Fgl2 protein. Binding of the Fgl2 protein to RAW 264.7 cells was saturable and specific (figure 10). The $K_d$ value was determined by non-linear regression analysis using the GraphPad Prism software version 3.02, and calculated 185 nM (figure 9).

![Saturation Curve: Fgl2 binding to RAW 264.7 cells](image)

Figure 10) RAW cells were cultured and harvested as described previously; $2 \times 10^5$ cells were incubated with increasing concentrations of biotinylated Fgl2 at 4°C for 2.5 hours on ice; for non-specific binding, cells were incubated with an excess of unlabelled Fgl2. Specific binding was calculated by subtracting non-specific binding from total binding ($n=4$).
Non-linear Regression analysis of FgI2 prothrombinase binding to RAW 264.7 cells

Figure 11) Non-linear Regression Analysis of FgI2 prothrombinase binding to RAW 264.7 cells: Nonlinear regression analysis of specific binding in MFI units was performed using GraphPad Prism Software version 3.02. The $K_d$ value was determined to be 185 nM.
Chapter 5

Discussion and Future Directions

The relationship between coagulation and inflammation has been well characterized. Damage to the endothelial cell layer triggers both the coagulation and the inflammatory pathways, resulting in the cleavage of coagulation zymogens to an active state, and the recruitment of leukocytes to the site of tissue damage. It is therefore not surprising that coagulation factors and proinflammatory molecules, such as adhesion factors, are able to reciprocally regulate each other. Several recent publications have investigated the relationship between inflammation and coagulation, and found that many coagulation factors, including factor Xa, thrombin and tissue factor are important inflammatory mediators. Moreover, there is increasing evidence that serine protease receptors, such as the PAR family of receptors and EPR-1, are the bridge that spans the divide between coagulation and inflammation (81).

The diverse biological functions of fibrinogen have been well documented; in addition to its role as a coagulation factor, fibrinogen exerts pro-adhesive and pro-
migratory effects through interactions with the integrin Mac-1 and the immunoglobulin superfamily member ICAM-1 (28; 29), and participates in αvβ3-mediated clot retraction (39). Several other fibrinogen-related proteins, such as tenascin and M-ficolin, have recently been shown to mediate cellular adhesion (36, 37; 40). In addition, angiopoietin-1 has been demonstrated to inhibit endothelial permeability and leukocyte transendothelial cell migration (43). This raises the possibility that other fibrinogen-related proteins may also act to regulate adhesion and migration, and may be important immune regulators.

Fgl2 prothrombinase was originally identified in cytotoxic T lymphocytes. Studies have shown a pivotal role for Fgl2 prothrombinase in the pathogenesis of fulminant hepatitis, and have also demonstrated its role in fetal loss syndrome. Current research is focused on examining the role of Fgl2 in both allo- and xenotransplantation, and preliminary results suggest that it may also mediate graft rejection. A secreted form of the molecule has also been reported, leading to speculation that Fgl2 may act as an immuno-regulatory molecule, however no work has been published to support this possibility. Amino acid sequence analysis has demonstrated that Fgl2 shares 36% homology with the fibrinogen γ chain, and that the P1 site is highly conserved. This site has been shown mediate interactions between fibrinogen and Mac-1, and to promote cellular adhesion and migration. We therefore hypothesized that soluble Fgl2 may regulate cellular adhesion and migration by binding to Mac-1 on leukocytes and ICAM-1 on endothelial cells.
True ligand-receptor interactions are characterized by both saturability and specificity (82). We therefore investigated the binding of Fgl2 to RAW 264.7 cells, a macrophage cell line, to determine whether or not this interaction met the criteria of a true ligand-receptor relationship. We found that Fgl2 prothrombinase fully associated with the putative receptor in 150 minutes on RAW 264.7 cells at 4°C. Fgl2 binds with high affinity to its putative receptor \( K_d = 185 \text{ nM} \). This is comparable to several other ligand-receptor interactions (see table 2). Therefore we conclude that the binding of Fgl2 prothrombinase on RAW 264.7 cells does represent a true ligand-receptor interaction.

**Table 2:** Comparison of the calculated \( K_d \) value for the interaction of Fgl2 prothrombinase with the Fgl2-binding protein and the reported \( K_d \) values for other ligand-receptor interactions.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor or Cell Type</th>
<th>( K_d )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>Monocytes</td>
<td>High affinity site: 4 nM Low affinity site: 1.3 ( \mu \text{M} )</td>
<td>83</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Neutrophils</td>
<td>170 nM</td>
<td>84</td>
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<td>Vitronectin</td>
<td>UPAR</td>
<td>30 nM</td>
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<td>Angiopoietin-1</td>
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<tr>
<td>Fibronectin</td>
<td>Platelets</td>
<td>300 nM</td>
<td>87</td>
</tr>
<tr>
<td>Fgl2 Prothrombinase</td>
<td>Unidentified receptor on RAW 264.7 cells</td>
<td>185 nM</td>
<td></td>
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</table>

In order to test our hypothesis regarding the identity of the Fgl2-binding protein, we first investigated whether Fgl2 was able to bind to cell types known to express the Mac-1 molecule. Monocytes and macrophages are known to express Mac-1, and studies have shown that fibrinogen binds to monocytes in a Mac-1 dependent fashion (88). We have demonstrated that Fgl2 is able to bind to peritoneal macrophages and to the macrophage-
like cell line RAW 264.7 in a dose-dependent and specific manner. Furthermore, we have shown that binding of Fg12 to RAW 264.7 cells is dependent on the presence of divalent cations, a common requirement of integrins for ligand binding.

To examine the possibility that Mac-1 is the receptor for Fg12, we performed a series of inhibition experiments using fibrinogen and antibodies for the Mac-1 α and β subunits. The rationale for these experiments was that if Fg12 was binding to Mac-1 through the P1 region then pre-treatment of the cells with fibrinogen should inhibit binding of Fg12, as should pre-treatment with the Mac-1 antibodies. However, our results demonstrated that Fg12 binding was not be inhibited by pre-treatment with fibrinogen or with antibodies to the Mac-1 α and β subunits. Furthermore, we have demonstrated that Fg12 binding to RAW 264.7 cells is decreased when the cells are stimulated with LPS, while binding of fibrinogen is increased. Therefore, we have concluded that Fg12 prothrombinase binds to RAW 264.7 cells in a Mac-1-independent fashion. Furthermore, the failure of the anti-CD18 antibody to inhibit Fg12 binding while inhibiting the binding of fibrinogen suggests that none of the β2 integrins are involved in the binding of Fg12 to RAW 264.7 cells.

Several other integrins have been shown to be important inflammatory mediators. The nine β1 integrins function primarily to mediate cell-cell adhesion, and cell-extracellular matrix adhesion; some members have been shown to be involved in T cell signaling (89). Our data indicated that binding of Fg12 to RAW 264.7 cells was cation dependent, suggesting that the Fg12 binding protein shares properties with members of the integrin family. We therefore attempted to Fg12 binding to RAW 264.7 cells with
fibronectin, a common ligand for several members of the β1 integrin family. We found that pretreatment of RAW 264.7 cells with fibronectin did not inhibit Fg12 binding, suggesting that Fg12 and fibronectin do not share a common cellular binding site and that the Fg12 binding protein may not be a member of the integrin family.

We concurrently investigated binding of Fg12 to the endothelial-like cell line SVEC4-10. Fibrinogen has been shown to facilitate leukocyte adhesion and migration by binding to both Mac-1 and ICAM-1 and by promoting interactions between these two molecules. We therefore hypothesized that Fg12 may also bind to ICAM-1 on endothelial cells. The SVEC4-10 cell line has been previously shown to not express ICAM-1 in either a constitutive or inducible fashion (80), and we therefore used this cell line as a preliminary means to test our hypothesis. We demonstrated that Fg12 bound to the SVEC4-10 cells in a dose-dependent manner. We have also shown that this binding is not dependent on the presence of divalent cations. Therefore, we can conclude that binding of Fg12 prothrombinase to SVEC4-10 cells is ICAM-1 independent.

Having established that Fg12 prothrombinase did not bind to Mac-1 on macrophage and macrophage-like cells, and that Fg12 did not bind to ICAM-1 on endothelial cells, we then examined the cellular distribution of the Fg12 receptor. We chose to look at the distribution of the binding protein on both leukocytes, and fibroblast cells. We found that the Fg12 receptor is expressed on several cell types. It is expressed constitutively on macrophages and dendritic cells; while not constitutively expressed lymphocytes, it can be induced on both LPS-stimulated B cells and population of ConA activated T cells. It
is also expressed on endothelial and fibroblast cells; Fgl2 binding to these cells is cation-independent.

**Future Directions**

Although we have determined that Fgl2 does not bind to Mac-1 or ICAM-1, the cellular distribution on leukocytes, endothelial cells and fibroblast cells does suggest that Fgl2 prothrombinase may be involved in the regulation of the immune system. As stated previously, coagulation proteins have been shown to have a broad spectrum of activities in addition to their primary function as procoagulants. These functions range from promoting adhesion and migration and increasing the expression of pro-inflammatory cytokines to inhibiting the interactions between leukocytes and endothelial cells. In addition, related proteins such as tenascin, M-ficolin and angiopoietin-1 have been shown to have immuno-modulatory effects; this strongly suggests that Fgl2 is involved in immune regulation.

Several recent findings in our laboratory support this hypothesis. Ongoing studies have demonstrated that Fgl2 prothrombinase promotes a Th2 cytokine response in vitro, inhibits T cell proliferation and suppresses cell surface expression of the co-stimulatory molecules CD80 and CD86 (74), suggesting that Fgl2 negatively regulates the T cell response. An anti-invasive role for Fg2 is suggested by recent studies utilizing an fgl2 -/- mouse as the donor animal in a heterotopic heart xenotransplant model (90). Animals lacking the fgl2 gene demonstrated an increase in the infiltration of mononuclear cells as
compared to the control animal. Although the mechanism has yet to be elucidated, one possible suggestion is that Fgl2 inhibits cellular adhesion and/or migration through the endothelial cell layer, and that its absence results in increased permeation. This hypothesis is supported by the finding that expression of Fgl2 is decreased in ATL leukemic cells, which have increased adhesive properties. Also, Fgl2 shares a high degree of homology with angiopoietin-1, which was recently shown to be an anti-invasive molecule (43).

However, in order to fully elucidate the mechanism by which Fgl2 causes these effects, the identity of the Fgl2-binding protein must be established. There are several methods that could be utilized to realize this goal. The conventional approach for determining protein-protein interactions is the yeast two-hybrid system (91). In this system, a known gene is cloned into vector to create a “bait” protein, where the protein of interest is linked to a DNA binding domain. This protein is then used to screen a library of “prey” proteins, in which unknown proteins are linked to an activation domain. When the DNA binding domain and activation domain are brought together by the interactions of the “bait” and “prey” proteins, a reporter gene is transcribed.

Several more traditional, biochemical methods exist for determining protein-protein interactions, such as photoaffinity or chemical crosslinking and immunoprecipitation. Photoaffinity crosslinking has been used for the characterization of many receptor-ligand interactions, including the mapping of the integrin αvβ3-ligand interface (92). In this method, a photactivatable molecule is chemically linked to the ligand of interest. The
crosslinker is activated in the presence of ultraviolet light, causing the ligand to be irreversibly bound to the receptor molecule (93). The ligand-receptor complex can then be analyzed on a sodium dodecyl-sulfate (SDS) gel, either as a crude cellular lysate or as a purified complex.

The field of proteomics has been defined as "the study of protein properties...on a large scale to obtain a global, integrated view of diseases processes, cellular function and networks at the protein level" (94). This emerging field illuminates protein structure and function, and identifies protein-protein interactions using complex, computer-based algorithms. We are currently investigating this method in our laboratory as a means of identifying potential Fgl2-prothrombinase receptors. We will then test the target molecules to determine if they bind purified Fgl2. Once the receptor has been identified, we will also carry out more extensive kinetic studies using $^{125}$I labeled protein.

Our data has also indicated that Fgl2 prothrombinase has a variable requirement for divalent cations when binding to its receptor. We have shown that Fgl2 binding to RAW 264.7 cells is significantly reduced in the presence of EDTA, while binding to SVEC4-10 cells and fibroblast cells is not affected. This suggests that Fgl2 binds to two distinct receptors. More work must be done to investigate this possibility. Other leukocyte cell types should be investigated to determine if they require the presence of divalent cations to bind Fgl2 prothrombinase. Furthermore, the kinetics of Fgl2 binding to SVEC4-10 cells should be investigated. If the results of these experiments support the theory that
Fgl2 prothrombinase binds to two receptors, the second receptor will also have to be identified.

Following the identification of the receptor molecule, the site of interaction must then be identified. We have hypothesized that the binding of Fgl2 prothrombinase to its receptor is mediated by the conserved P1 site; this hypothesis could be tested by using a synthetic P1 peptide to block Fgl2 binding to RAW 264.7 cells. If interactions with the receptor are not mediated by the P1 site, truncated proteins could be utilized to determine which region of Fgl2 prothrombinase mediates interactions with the Fgl2-binding protein. Once the binding of Fgl2 prothrombinase to its receptor is fully characterized, functional assays can be performed to further elucidate the biological role of this protein in vivo.

In summary, we have demonstrated that Fgl2 does bind to a specific cell surface receptor on RAW 264.7 cells. Our data indicates that this molecule is not the Mac-1 integrin. We have also determined that Fgl2 binding to SVEC4-10 cells in a dose-dependent manner, but that the putative receptor on these cells is not ICAM-1. We have determined that Fgl2-binding protein is expressed on several leukocyte cell types, and also on endothelial cells and fibroblast cells. In the future, studies will be carried to determine the identity of the Fgl2 receptor, to localize the site of interaction between Fgl2 and its receptor, and to explore the biological functions of this molecule.
References


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