HERPESVIRUS MEDIATED ACTIVATION OF COAGULATION AND FIBRINOLYTIC PROTEINS

by

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ABSTRACT

Vascular disease, a leading cause of death worldwide, is associated with multiple risk factors that include age, diet, lifestyle and genetics. Herpesviruses, highly prevalent in the general population, have also been linked to vascular disease. To investigate the molecular basis of this relationship, the interactions between virus surface proteins and host hemostatic plasma proteins, comprising both clot forming (coagulation) and clot dissolving (fibrinolytic) proteases, which can both contribute to vascular disease, were studied.

Previously, our laboratory demonstrated that purified herpes simplex virus type-1 (HSV1) and -2 (HSV2) and cytomegalovirus (CMV) contain cell-derived tissue factor (TF) and anionic phospholipids (aPL). Independent of cells, TF and aPL with factor (F) VIIa (FVIIa) initiate the extrinsic pathway of coagulation, activate FX to FXa, and lead to thrombin generation. This thesis identified additional herpesvirus-mediated coagulation pathway(s) and also demonstrated herpesvirus-mediated fibrinolysis.

In addition to TF, FVIII amplified HSV1-initiated coagulation through the intrinsic pathway. Alternatively, independent of TF, HSV1 initiated coagulation through the contact pathway, via FXII activation. The ability to exploit the extrinsic, intrinsic and contact pathway of coagulation should make herpesvirus infection a strong prothrombotic risk yet the clinical correlation to vascular disease is relatively weak.

To explain the in vitro versus clinical discrepancy, virus-mediated fibrinolysis was evaluated. Purified herpesviruses accelerated tissue plasminogen activator (tPA)-dependent plasminogen (Pg) activation to plasmin (Pn), the primary fibrinolytic protease responsible for fibrin clot dissolution. This Pn generation was independent of the physiological cofactor fibrin. Cell-derived annexin 2 (A2), previously identified on the

surface of CMV, is a known accelerator of tPA-dependent Pn generation. Although A2 was identified among several Pg binding partners associated with each herpesvirus, it was dispensable for Pn generation. Herpesvirus-mediated plasminogen activation enhanced fibrinolysis independent of exogenous tPA. The enhanced fibrinolysis may attenuate the prothrombotic risk of herpesviruses, as an independent predictor of vascular disease. Plasmin enhanced cell susceptibility to infection, a virus-survival advantage also known for thrombin, FVIIa and FXa.

Overall, for herpesviruses and other enveloped viruses, a mechanism is suggested where the envelope constituents initiate the activation of both pro-coagulation and pro-fibrinolytic proteins, modulating host cell susceptibility to infection and contributing to vascular disease.

PREFACE

This thesis work titled “Herpesvirus mediated activation and coagulation and fibrinolytic proteins” was carried out under the supervision of Dr Edward Pryzdial**,** in the Department of Pathology & Laboratory Medicine, at the Centre for Blood Research, UBC.

Chapters 3 and **4** are data chapters and are based on publications as described below

Gershom ES, Sutherland MR, Lollar P, Pryzdial ELG. Involvement of the contact phase and intrinsic pathway in herpes simplex virus-initiated plasma coagulation. Journal of Thrombosis and Haemostasis 2010; 8(5):1037-1043.
G.E.S. performed all of the experiments, analyzed results and wrote the first draft of manuscript; **S.M.R**. did the virus culture and purification; **L.P**. contributed vital reagents and **P.E.L.G**. conceived the project, reviewed and edited the manuscript.

Gershom ES, Vanden-Hoek AL, Sutherland MR, Lollar P, Pryzdial ELG. Herpesviruses enhance fibrinogen clot lysis.
G.E.S. performed all of the experiments (except the fibrinolysis), analyzed data and wrote the first draft of manuscript; **V.A.L.** performed the fibrinolysis experiments (Figure 32 and 38); **S.M.R** did the virus culture and purification and performed the plaque assay experiments (Figure 39) and **P.E.L.G**. conceived the project, reviewed and edited the manuscript.

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a	activated
A2	annexin 2
A2t	annexin 2 tetramer
aPL	anionic phospholipid
APTT	activated partial thromboplastin time
AT	antithrombin
BME	basal eagle media
Ca^{2+}	calcium
CMV	cytomegalovirus
gC	glycoprotein C
HBS	hepes buffered saline
HFF	human foreskin fibroblasts
HK	high molecular weight kininogen
HRP	horse radish peroxidase
HS	heparin sulfate
HSV	herpes simplex virus
HSV1	herpes simplex virus type-1
HSV2	herpes simplex virus type-2
HUVEC	human umbilical vein endothelial cells
kDa	kilodalton
KK	kallikrein
µg	microgram

µL	microliter
µM	micromolar
mAb	monoclonal antibody
mM	millimolar
nM	nanomolar
NS	HSV1 clinical isolate
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
PAR	protease activated receptor
PC	protein C
Pg	plasminogen
PK	prekallikrein
Pn	plasmin
PT	prothrombin time
RT	room temperature
S2251	H-D-Val-Leu-Lys-p-Nitroaniline dihydrochloride
TF	tissue factor
TM	thrombomodulin
tPA	tissue Plasminogen Activator
uPA	urokinase Plasminogen Activator
Vero cells	african green monkey kidney cells
vp	virus particles
vWF	von Willebrand Factor

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Lastly, and most importantly, I owe everything to my parents, Vatsala Gershom and Samuel Gershom. you bore me, raised me, supported me, taught me, and loved me.

To my mother,
Vatsala Gershom

1. INTRODUCTION

1.1 Overview

Blood cells and plasma proteins work in coordination to maintain hemostasis, ensuring that blood circulates in intact vasculature and preventing bleeding upon vessel damage. Hemostasis is a complex system that constitutes coagulation, the process of blood clotting, and subsequently fibrinolysis, clot dissolution, following repair of the damaged vessel (38).

Blood coagulation involves the sequential proteolytic activation of circulating plasma zymogens to functional proteases, leading to thrombin generation. Thrombin is the final protease that converts fibrinogen to fibrin and also functions as a potent cell agonist (179,288). The reactions leading to clot formation occur on cell surfaces, wherein the enzymes are recruited along with other components. Plasma contains the clotting factors necessary to form a clot, except the coagulation initiators on cell surfaces that are restricted to the site of vascular injury (181).

Upon vascular damage, exposure of plasma clotting factors to cell surface tissue factor (TF) and anionic phospholipids (aPL) initiate coagulation. TF, a transmembrane protein, acts as cofactor, and aPL provide a surface for localization and activation of coagulation enzymes (9). These initiate coagulation because their accessibility is triggered by vascular damage. This restricted accessibility of TF and aPL at sites of vascular damage prevents the possible development of occlusive thrombi. Ensuring hemostatic balance, the coagulation reactions leading to fibrin clot formation also initiate fibrinolysis. Plasminogen gets incorporated into the clot, binds to fibrin and is activated to plasmin (305). Plasmin is the major fibrinolytic protease that cleaves fibrin, dissolving the clot (63). Dissolution of clot and the ensuing repair process heal the wound and

restore vascular integrity.

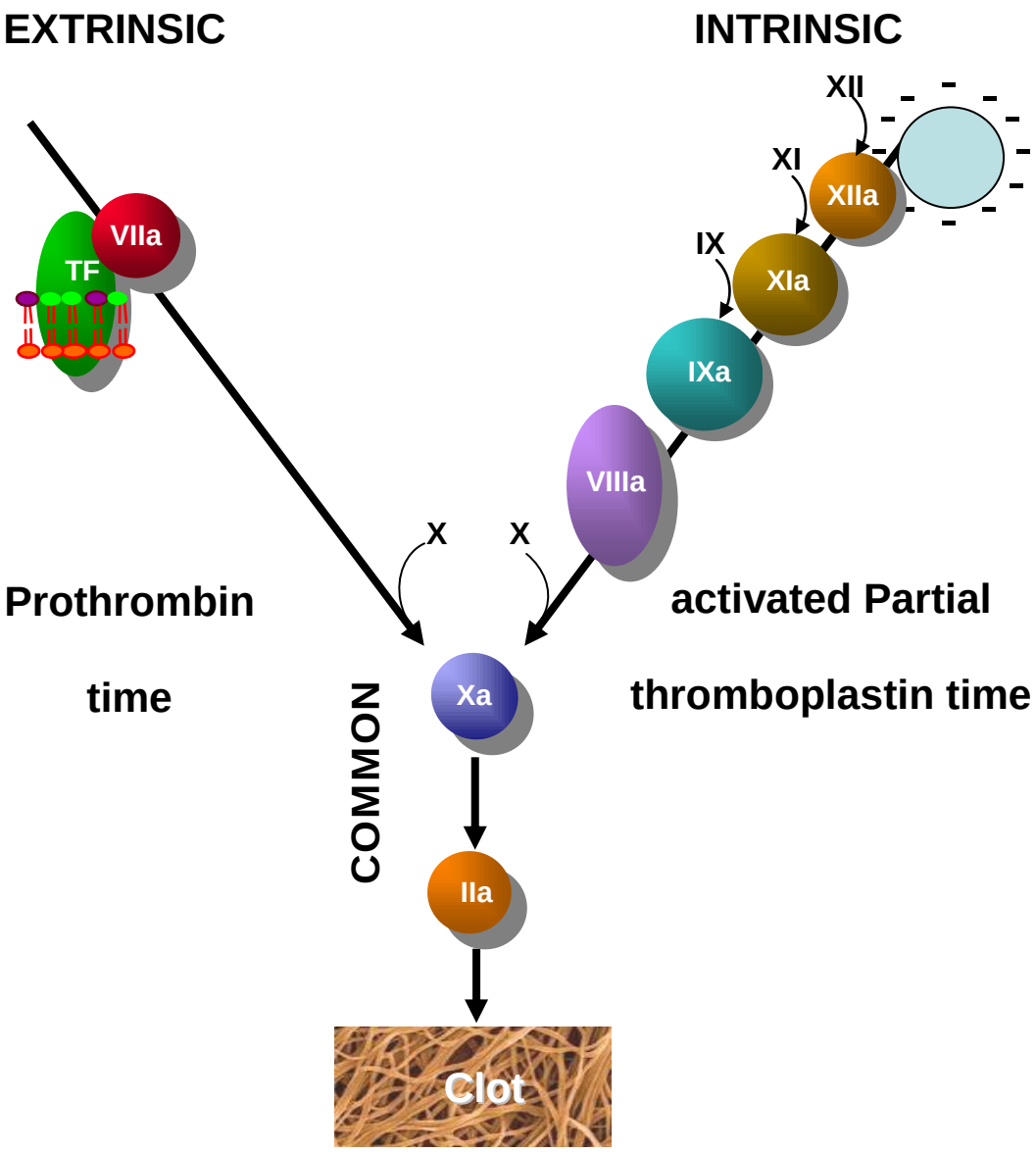
Herpesviruses such as herpes simplex type 1 (HSV1), herpes simplex virus type 2 (HSV2) and cytomegalovirus (CMV) are highly prevalent in the general population and expose the host to recurrent infections throughout life (405). Herpesvirus infections have been implicated in vascular disease, corroborated with multiple levels of evidence that include clinical correlations, biopsy reports, animal models, procoagulant transformation of cells and coagulation initiators on the surface of purified virus particles (156,307,309,339,457,487). Consequently, the hemostatic balance between coagulation and anticoagulation associated with the cell is altered by the virus to favor thrombin production. Thus, herpesvirus infections have been established as a weak risk factor for vascular disease. However, their contribution as risk factor is more significant when combined with other factors (341,438,526). The purpose of this thesis was to investigate the links between purified herpesviruses and hemostatic proteins belonging to both the coagulation and fibrinolytic pathways, and evaluate viral contribution to vascular disease. To have a better understanding of the work done, an overview of the foundations of hemostasis and virology, with emphasis on coagulation, fibrinolysis and the biochemistry of the herpesvirus infection mechanism follows.

1.2 Coagulation

Blood coagulation involves the sequential proteolytic activation of circulating plasma proteins (Table 1). A simplified schematic of coagulation is Y-shaped, as outlined in Figure 1. The two arms of the “Y” represent the extrinsic and the intrinsic pathways (80,181). The extrinsic pathway is facilitated by exposure of TF, the cellular coagulation initiator that is normally absent in plasma (335). The intrinsic pathway is

Factor number	Descriptive name	Mol. wt (kDa)	Plasma conc.	
			nM	µg/ml
I	Fibrinogen	340	7600	2600
II	Prothrombin	72	1400	100
III	Tissue factor (TF)	44	NA (Cell bound)	
IV	Calcium			
V	Proaccelerin	330	20	6.6
VII	Proconvertin	50	10	0.5
VIII	Antihemophilic factor	330	0.7	0.2
IX	Plasma thromboplastin (Christmas factor)	55	90	5.1
X	Stuart-Prower factor	58	170	10
XI	Plasma thromboplastin	160	30	4.8
XII	Hageman factor	80	500	40
XIII	Fibrin stabilising factor,	320	90	30
_	Prekallikrein (Fletcher factor)	85	500	42
_	High Molecular Weight Kininogen (Fitzgerald-Flaujeac factor)	120	670	80

Table 1. The coagulation factors. Mean plasma concentrations and properties of coagulation protein. The coagulation factors are assigned roman numerals also included in the table are their historical names. Note: NA, not applicable (membrane protein).



Correlations of coagulation test and defect in coagulation cascade			
Prothrombin time (PT)	prolonged	normal	prolonged
activated Partial thromboplastin time (APTT)	normal	prolonged	prolonged
Defect	extrinsic	intrinsic	common

Figure 1 The coagulation cascade. The cascade consists of the extrinsic and intrinsic pathways. The two pathways converge leading to formation of FXa, which generates thrombin (IIa) leading to clot formation. The two pathways are assayed using the prothrombin time (PT) and activated partial thromboplastin time (APTT) (80,181).

initiated, when plasma clotting factors are exposed to a negatively charged surface. The two pathways result in activation of factor X (FX) to FXa and feed into the common pathway of thrombin generation. Thrombin is the most important final protease generated in the coagulation system that leads to clot formation. This scheme is based on observations of in vitro plasma coagulation tests and does not adequately represent the hemostatic process in vivo. Nevertheless, it serves as a basic diagnostic chart to interpret plasma clotting time assays: prothrombin time (PT) and activated partial thromboplastin time (APTT). The PT determines the deficiencies of clotting factors of the extrinsic and common pathways whilst APTT of the intrinsic and common pathways (207). We now know that the intrinsic and extrinsic pathways do not operate in vivo as independent and redundant systems. As research progresses, coagulation is getting more complicated with evidence of numerous interconnections between the two pathways and anticoagulant mechanisms to regulate coagulation. In vivo coagulation is a cell based system with overlapping phases of initiation, amplification and propagation (181,399).

1.2.1 Initiation

In vivo coagulation takes place on the cell surface (Figure 2) and is initiated by the extrinsic pathway. Vascular damage exposes subendothelium, and cell surface TF and aPL from stimulated endothelial cells are exposed to circulating clotting factors in plasma. As TF is extrinsic to plasma, this branch of coagulation is referred to as the extrinsic pathway. TF and aPL are the normal initiators of coagulation (181). TF acts as a cofactor for factor VII (FVII) and the activated form FVIIa, which is believed to be circulating at <1% of the total FVII antigen. Exposure of aPL provides the negatively charged surface for the assembly of coagulation enzyme complexes (181).

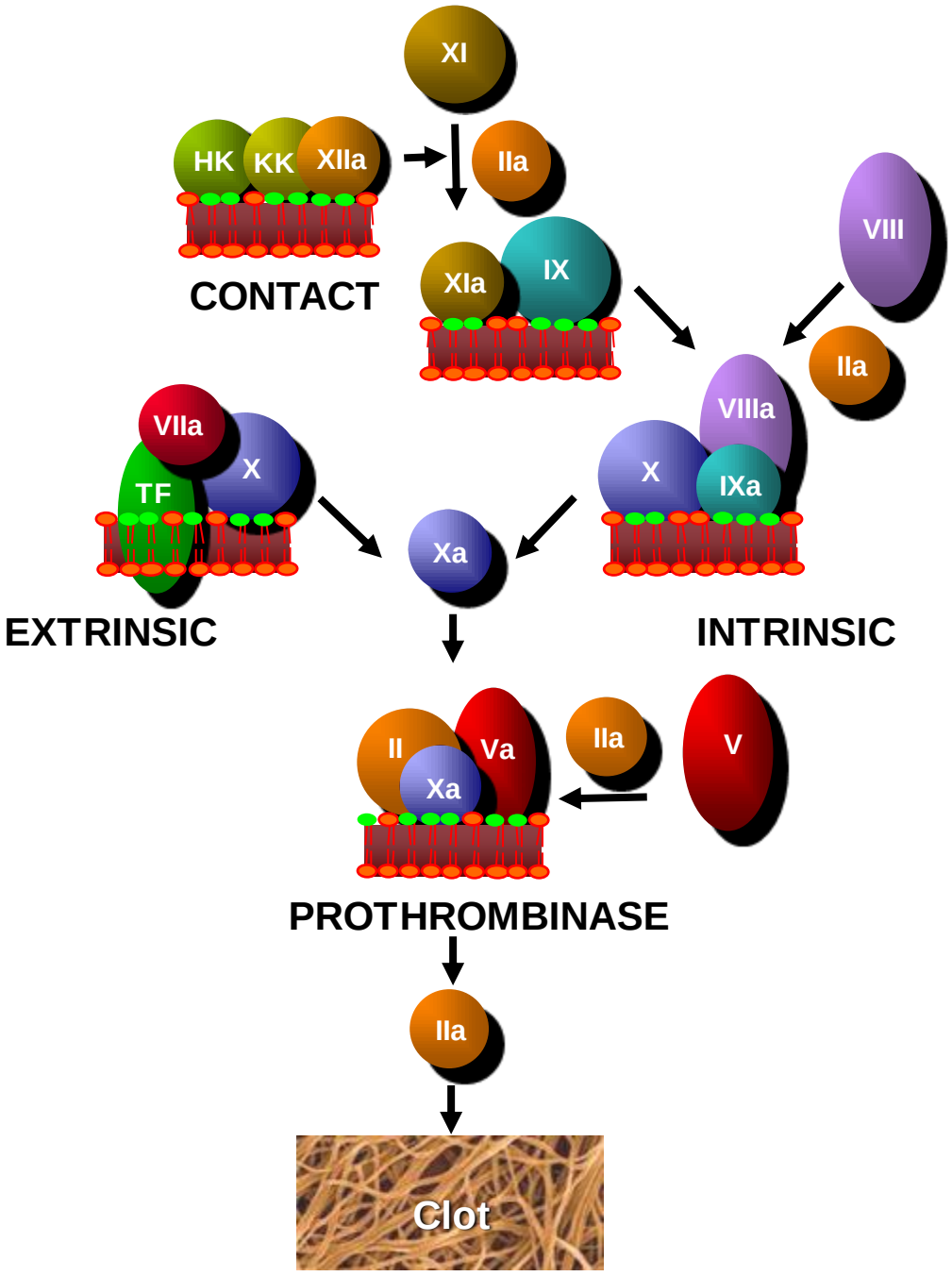


Figure 2 A cell based model of coagulation. The schematic depicts the assembly of extrinsic, intrinsic, prothrombinase and contact phase components. Exposure of cell surface tissue factor (TF) and anionic phospholipids (aPL) initiate coagulation through the extrinsic pathway. The small amount of thrombin (IIa) generated feeds back activating cofactors V and VIII and factor XI amplifying IIa generation leading to clot formation (80,181).

The initiating macromolecular interaction within the clotting cascade is the binding of TF to FVII or FVIIa, which are locked in a position suitable for subsequent enzymatic activation of FX, FIX, and FVII (181,361). The physiological basis of the first molecules of FVIIa is still under debate, however, it is established that TF-FVIIa can activate TF-FVII and FVII as a probable source. Other known activators of FVII are thrombin, FIXa and FXa, which may also contribute (31).

Encryption/decryption of TF activity has recently been suggested as a mode of regulating coagulation. Studies using cells that constitutively express TF demonstrated the existence of noncoagulant TF, which can bind to FVIIa but did not lead to activation of FX. This form of TF was termed ‘‘encrypted’’ and shown to be regulated (37,252). Treatment with certain cell-activating agents, such as calcium ionophores increased coagulation activity by stimulating intracellular disulfide isomerase leading to the introduction of a new disulfide bond in TF (4). The basis of this enhanced TF activity is controversial and later argued by other laboratories as attributed to the exposure of aPL on the outer leaflet of the plasma membrane (10). Another possible explanation is that TF dimerization and compartmentalization in lipid rafts might also limit enzymatic activity, which is liberated upon cell stimulation (37). There is likely a basis for the contribution of each of these mechanisms in the regulation of TF activity, which may additionally be complicated by circulating sources on microvesicles librated from stimulated cells (331).

TF-FVIIa together with aPL form the extrinsic tenase (Xase) complex and activate FIX to FIXa and FX to FXa (234,323,434). FXa then forms the prothrombinase complex with factor Va on the aPL surface, converting prothrombin to thrombin (206,336), the critical step in coagulation. The availability of aPL restricts thrombin

production to sites of vascular damage, where release of cell agonists induces the “flip” of aPL from the inner leaflet to the outer face of cells adjacent to the injury site (15). To avoid thrombin generation at other locations in the vasculature, aPL in the resting cell membrane is inaccessible and maintained on the inner leaflet (529). Thus, cells control assembly of coagulation enzyme complexes by providing accessible aPL and a mechanism to generate initial FXa. However, the amount of FXa is inadequate to drive the thrombin generation needed to form a clot (379).

1.2.2 Amplification

In a positive feedback loop (Figure. 2), a small amount of thrombin generated through the extrinsic pathway activates the non-enzymatic cofactor factor VIII (FVIII) to FVIIIa (29). Congenital deficiency of FVIII leads to a bleeding disorder called hemophilia A(187). FVIIIa binds factor IXa(FIXa) to form the intrinsic Xase complex activating FX (481). Additional FXa generated by intrinsic Xase complex amplifies thrombin generation. Thrombin also activates factor XI (FXI) (124), which in turn activates FIX (89) further amplifying FX activation by assembling more intrinsic Xase complex. TF-FVIIa complex activates not only FX but also FIX (228,251), showing that the pathways are linked. Deficiency of FXI might or might not be associated with bleeding risk depending on the individual (138), but deficiency of FIX results in hemophilia B (398). Once a sufficient concentration of thrombin has been generated to overcome the intrinsic anticoagulant barrier of plasma, thrombin directly converts fibrinogen to fibrin (11,208,325) leading to clot formation restoring vascular integrity. Thus, a hemostatic response to vascular injury is initiated by the extrinsic pathway of coagulation and amplified by the intrinsic pathway (181).

1.2.2.1 Factor VIII

Factor VIII is an essential blood clotting factor. A deficiency or functional defect in FVIII results in a serious bleeding disorder, haemophilia A (187). It is a large protein of about 330 kDa, is synthesized primarily in the liver (90), and circulates in plasma as an inactive molecule with no procoagulant activity. Once activated, it acts as a cofactor for FIXa accelerating activation of FX to FXa (110,468,481).

Structure

The FVIII gene is located on the X chromosome (135,468). It encodes a polypeptide of 2351 amino acids, 19 of which encode a signal peptide. The FVIII molecule consists of three homologous A domains, two homologous C domains and a unique B domain, arranged in the order starting from the N-terminus A1-A2-B-A3-C1-C2 to C-terminus (Figure 3) (485). At the time of secretion, the signal peptide is removed and there is cleavage in the B domain (135,468). Consequently, FVIII is released as a heterodimer composed of a 200 kDa heavy chain and an 80 kDa light chain linked by a metal ion (95,121). Once released into the circulation FVIII binds with high affinity to von Willebrand factor (vWF), which functions as a carrier protein to stabilize FVIII from proteolytic degradation (494).

Function

The initiation of the extrinsic pathway results in limited amounts of thrombin and FXa generation that activate FVIII (29,379). Thrombin and FXa both cleave FVIII at Arg 372, 740 and 1689 converting FVIII into its heterotrimeric activated form FVIIIa (111,270,365,366) (Figure 3) and releases FVIIIa from vWF. FVIIIa functions as a

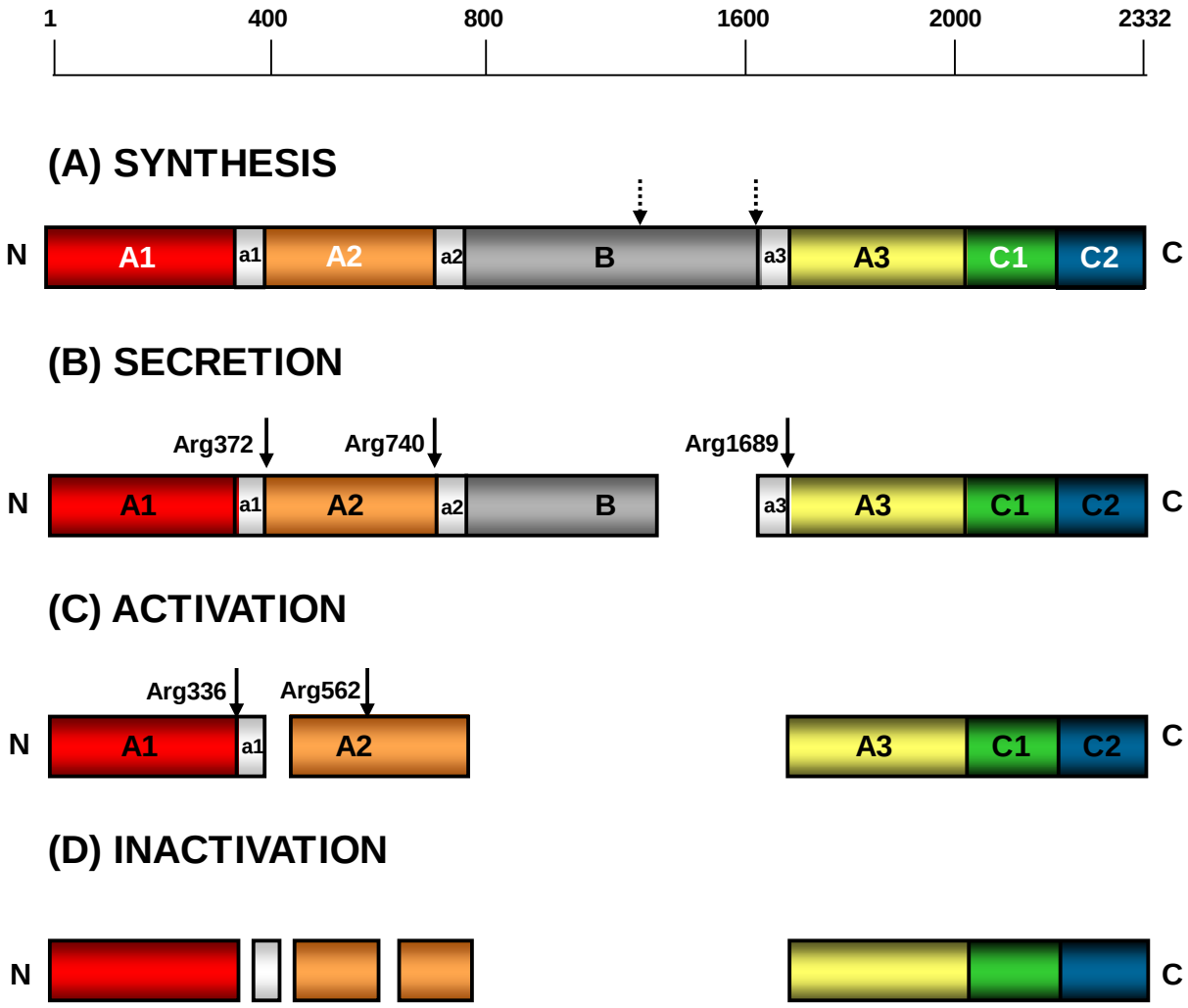


Figure 3 Factor VIII (FVIII) domain structure and processing. FVIII is synthesized as a single polypeptide chain of 330kDa. It constitutes three different domains: an A-domain which is repeated three times, a central B-domain and twice repeated C-domain. The "a1", "a2" and "a3"are short acidic regions (A). Within the Golgi compartment, FVIII is cleaved at two sites within the B-domain and secreted as an heterodimer of 160kDa heavy chain and 80 kDa light chain (B). Thrombin mediated cleavage at Arg 372, Arg 740 and Arg 1689 activates FVIII to FVIIIa (C). Activated protein C cleavage at Arg 336 and Arg562 leads to inactivation of FVIIIa (D) (366).

cofactor for the serine protease FIXa forming an aPL-dependent complex known as intrinsic Xase (481) (Figure 2). The intrinsic Xase complex converts additional FX to FXa amplifying thrombin generation leading to clot formation (481).

1.2.3 Contact pathway

Upstream of the intrinsic pathway, is the contact pathway. The contact pathway comprises factor XII (FXII), PK, and high molecular weight kininogen (HK) (223). In vitro on a negatively charged surface, assembly of contact pathway proteins activates FXII to FXIIa. FXIIa activates FXI (23,242,243) feeding into the intrinsic pathway (Figure 4). FXII and PK have no role in normal hemostasis as deficiencies do not lead to bleeding problems (397). However, recently FXII has been implicated in thrombotic pathology. FXII deficient (FXII-/-) mice are protected in models of arterial thrombosis (387), cerebral ischemia reperfusion injury (225) and pulmonary embolism (330).

1.2.3.1 Factor XII

Structure

Factor XII (FXII, Hageman factor) is an 80 kDa (143), single chain glycoprotein produced in the liver (141). FXII consists of several structural domains starting from the N-terminus, a fibronectin type II domain, an epidermal-growth-factor-like (EGF-like) domain, a fibronectin domain type I, a second EGF-like domain, a kringle domain, a proline-rich region and the catalytic serine protease domain (Figure. 5) (66). These domains are homologous to those found in other serine proteases (66) (Figure 13), except for the proline-rich region that is unique to FXII. The catalytic domain is the single largest domain and constitutes the active site of FXIIa (450).

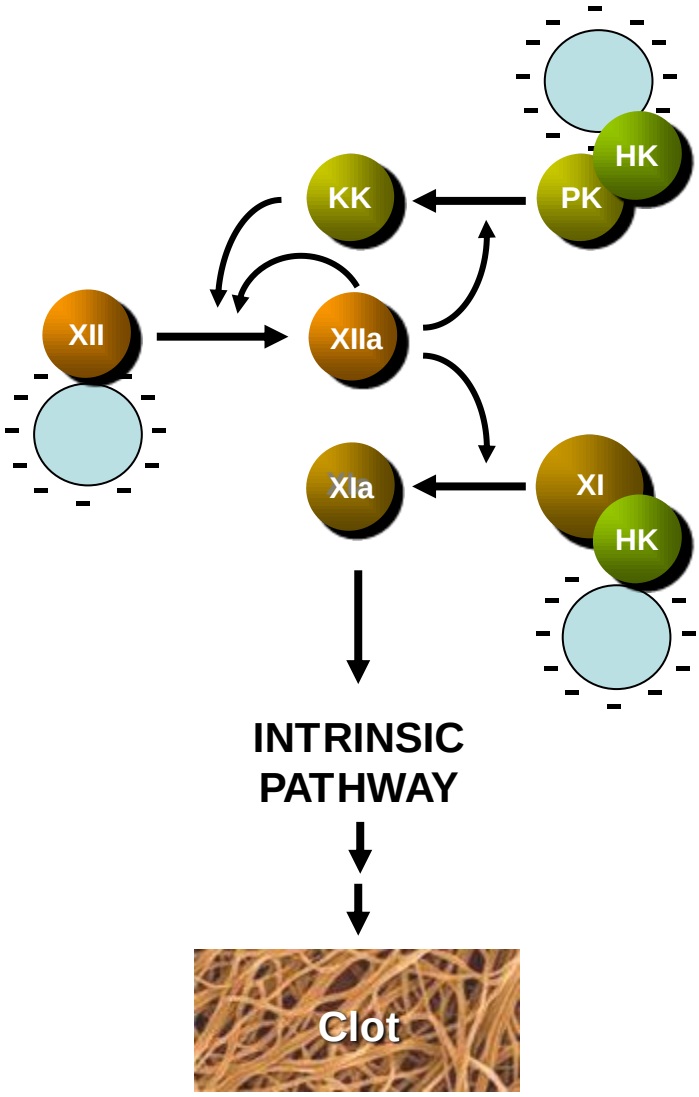


Figure 4 Contact pathway activation. Factor XII (FXII) is activated by contact with negatively charged surfaces. XIIa converts prekallikrein (PK) to kallikrein (KK) and can feed back to activate more XII. Similarly, XIIa also can feed back to amplify its own generation. About 75% of circulating PK is bound to high-molecular-weight kininogen (HK), which localizes it to anionic surfaces and promotes PK activation. XIIa propagates clotting by activating XI, feeding into intrinsic pathway

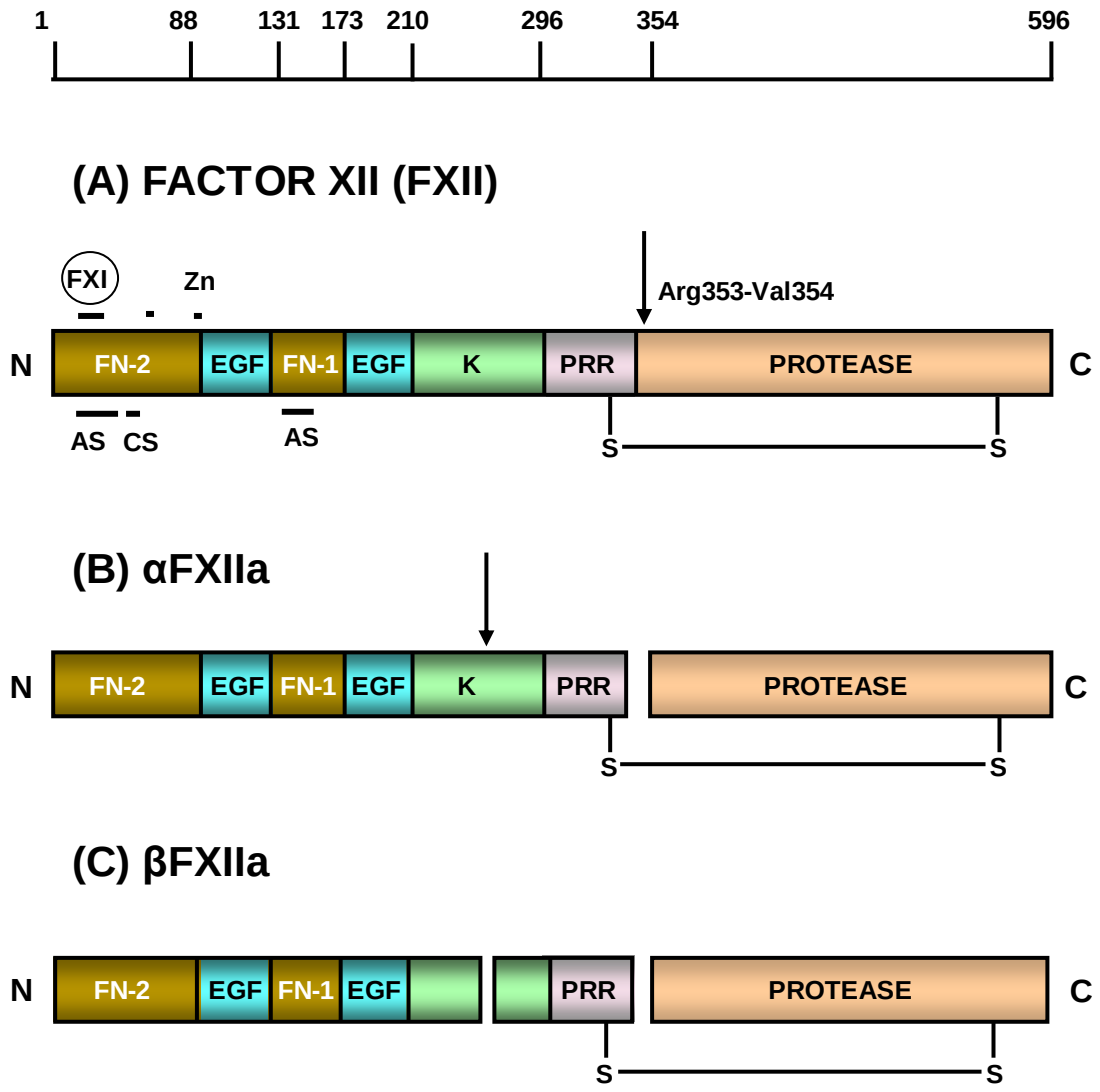


Figure 5 Factor XII (FXII) domain structure. FXII is divided into several domains. fibronectin type II (FN-2), EGF-like domain, fibronectin type I (FN-1), EGF-like domain, kringle domain (K), proline-rich region (PRR), catalytic protease domain or light chain. The binding sites for FactorXI, Zinc (Zn), artificial surface (AS) and cell surface (CS) are depicted. Cleavage of Arg353-Val354 by plasma kallikrein yields αFXIIa and further cleavage of αFXIIa outside the disulfide bond yields βFXIIa (450).

Activation

FXII activation is a result of proteolytic cleavage of the Arg353-Val354 bond that converts single chain zymogen FXII (80 kDa) into a two chain protease α-FXIIa (66). In vivo, α-FXIIa circulates as a two chain protein, a heavy chain of ~50 kDa (353 residues) and a ~30 kDa light chain (243 residues) held together by a disulfide bond. The heavy chain contains the anionic surface-binding portion (58,368) and the light chain constitutes the catalytic triad. Further proteolytic cleavage of α-FXIIa outside the disulfide bond yields the β-FXIIa fragment with a molecular weight of 28kDa. Upon disulfide bond reduction, the liberated β-FXIIa retains its proteolytic activity towards substrates but is unable to bind to negatively charged surfaces and no longer promotes clotting (388-390).

Activation of FXII may be through autoproteolysis upon contact with a negatively charged surface (101,317)(509). A variety of negatively charged substances, both physiological and non-physiological have been shown to promote XII activation. The non-physiological substances include glass, kaolin, celite, dextran sulfate(183,407,464) and ellagic acid (101). The physiological substances include articular cartilage, collagen (473), fatty acids (88), endotoxins, misfolded proteins (279), and polyphosphates (442). Additionally, FXII undergoes proteolytic activation on the surface of endothelial cells by PK/kallikrein and HK (202)(328,406). This involves reciprocal activation of FXII to FXIIa by kallikrein (59,390). Surface bound α-FXIIa in turn activates FXI to FXIa. Secondary cleavage of α-FXIIa by kallikrein yields β-FXIIa. The β-FXIIa is known to catalyze solution phase activation of PK and FVII. As a clinical evidence for the latter, an epidemiological study reported a correlation between increased FVIIa and FXIIa levels in plasma, a risk for coronary heart disease (CHD) (374,428,527). Binding to negatively

charged surfaces by itself is not sufficient to activate FXII. The auto activation of FXII was not observed in studies using highly purified preparations of FXII and plasma deficient in PK and HK (57,120,390). Binding to anionic surfaces induces a conformational change, making the FXII zymogen more susceptible to cleavage by a variety of proteases (144) (302) (14,419).

Inhibitors

The major inhibitor of FXIIa is complement component C1 esterase inhibitor (C1-INH) that binds and irreversibly inactivates FXIIa (115) (424) (84) (367). Antithrombin III is another inhibitor of FXIIa but not as effective as C1-INH (451), which accounts for 90% of FXIIa inhibition in plasma(115,450). Also, a specific non-physiological inhibitor used in the current work is corn trypsin inhibitor (CTI) (184). It affects plasma APTT, without affecting the PT (379). Therefore the specificity for FXIIa makes CTI useful for segregating and studying TF-dependent coagulation reactions (379) as done in the current thesis work.

1.2.3.2 Prekallikrein

Prekallikrein (PK) is a precursor of plasma kallikrein. In the circulation, most of it is bound to HK and only 25% exists as free PK.

Structure

The precursor form, PK is a single chain serine protease that consists of 619 amino acids. Starting from the N-terminus (Figure 6) it consists of four tandem repeats domains called apple domains (A1-A4) and the catalytic serine protease domain at the C-terminus. The A1 and A4 domain constitute binding regions for HK and for FXIIa the

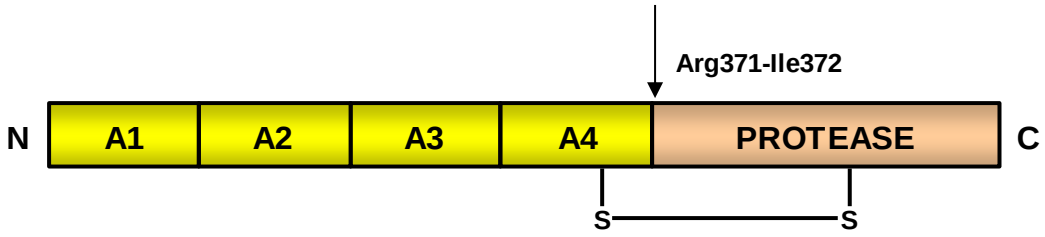


Figure 6 Prekallikrein (PK) domain structure. PK contains four apple domains (A1 to A4 from the N-terminus) and a C-terminal catalytic protease domain. The A1 and A4 domains constitute binding regions for high molecular weight kininogen (HK). Proteolytic cleavage of Arg371-Ile372 activates PK to kallikrein (453).

binding regions are located on A3 and A4 (356,357). FXIIa mediated proteolytic cleavage of Arg371-Ile372 converts the single chain PK into two chain kallikrein, composed of a heavy chain and a light chain, held together by a disulfide bond (286,426). The light chain constitutes the catalytic active site of Ser559, His415 and Asp464. A 1:1 stoichiometric complex of the kallikrein, light chain with C1-INH results in loss of its proteolytic activity (474).

Function

The major protein substrates of plasma kallikrein are FXII, HK and urokinase (uPA). Both α-FXIIa and β-FXIIa are able to convert PK into kallikrein (518), which in turn accelerates the activation of FXII (59) (Figure 4). Plasma kallikrein binding to HK is required for the surface-dependent procoagulant activity of plasma kallikrein (480). Through FXII activation, plasma kallikrein activates FVII of the extrinsic system contributing to coagulation(428). An epidemiological study found a direct correlation between FVIIa and FXIIa and an increased plasma FXIIa in middle aged men with high risk of CHD (527). Plasma Kallikrein has been shown to be an initiator of plasminogen activation through activation of uPA, a proteolytic enzyme of the fibrinolytic pathway (165,286). Plasma Kallikrein also cleaves HK to liberate bradykinin, an inflammatory mediator (217). Thus, PK may be involved in multiple biochemical systems.

1.2.3.3 Factor XI

Structure

Factor XI is a plasma glycoprotein that is synthesized in liver. In plasma, it circulates in a non-covalent complex with HK (467)(23). FXI and prekallikrein share 58% homology in amino acid sequence resulting in similar structural domains (Figure 7). FXI is a 160

kDa disulfide-linked dimer that constitutes two identical monomers of 80 kDa joined together by disulfide bonds. Starting from the N-terminus (Figure 8) it consists of four tandem repeats domains called apple domains (A1-A4) and the catalytic serine protease domain at the C-terminus(97). Similar to PK, the apple domains constitute HK binding regions. For FXI, the A1, A2 and A4 domain constitute binding regions for HK, A2 being the most significant (386). FXIIa cleaves the Arg369-Ile370 bond in each monomer of FXI converting it into FXIa (23) (242). FXIa consists of two N-terminal heavy chains, and two C-terminal light chains, all of which are held together by disulfide bonds. A major substrate for FXIa is FIX (89). The FIX binding site is localized to A3 domain of FXIa but not FXI (453). Arg184 connecting A2 and A3 is a critical residue for FIX activation (148), but probably remains buried under the protease domain in the zymogen FXI (97).

Function

FXIIa (23,242) and thrombin (334) have been shown to activate FXI to FXIa through cleavage of Arg369-Ile370 bond. FXIa participates within the intrinsic pathway of coagulation by catalyzing the conversion of FIX to FIXa (89). A recent study reported activation of procofactors FVIII and FV by FXIa in a purified system (507). FXI deficiency is associated with bleeding. In contrast to FVIII and FIX, bleeding manifestations for FXI deficiency do not correlate well with the FXI activity or antigen levels (8,222,254,375,380). The variable bleeding tendencies observed in FXI deficient patients may be related to the ability of the extrinsic pathway contributing to FIX activation (182,251,319) or variation in genetic backgrounds or FXI mutations, or coinheritance of other bleeding disorders (214).

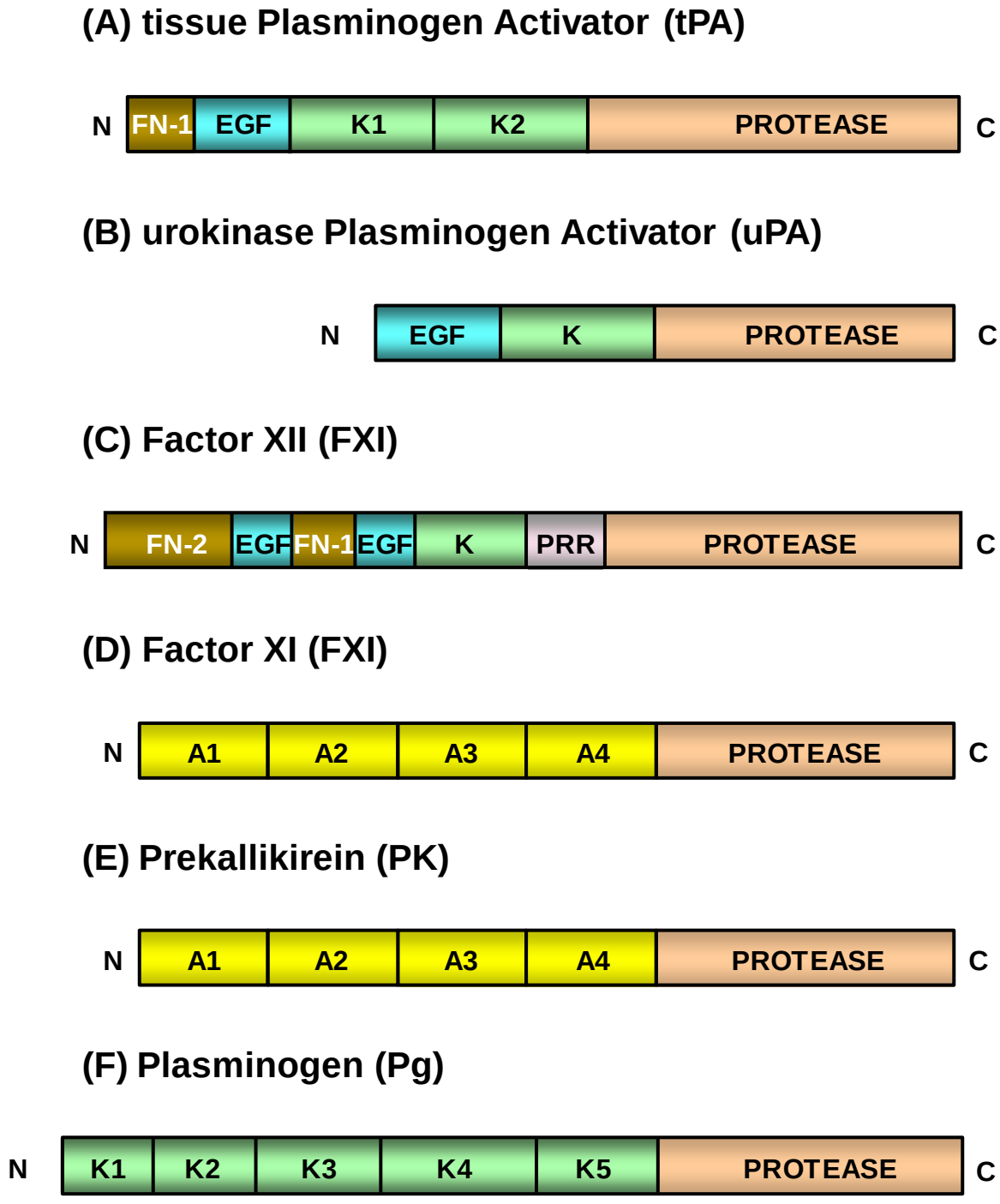


Figure 7 Structural domains of fibrinolytic and contact pathway proteins.A, apple domain; EGF, epidermal growth factor domain, FN, fibronectin domain; K, kringle domain; PRR, proline rich region; and protease domain (453).

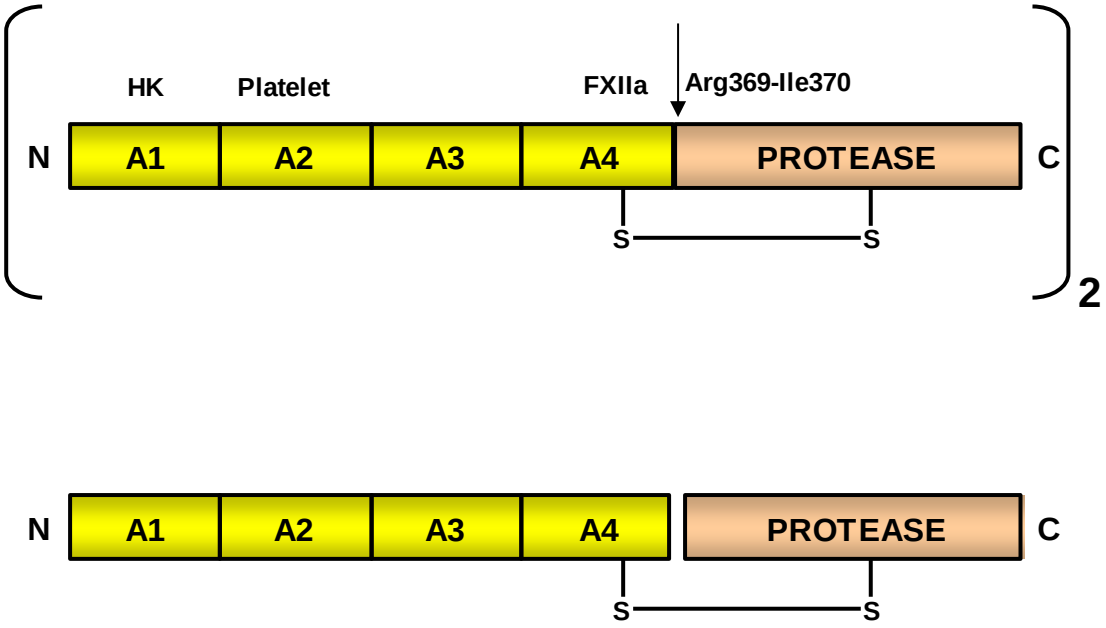


Figure 8 Factor XI (FXI) domain structure. FXI is a 160-kDa disulfide-linked dimer of identical 607 amino acid subunits. The domain structure of the FXI monomer is represented. FXI monomer contains four 90- or 91-amino acid repeats called apple domains (A1 to A4 from the N-terminus) and a C-terminal catalytic protease domain. The binding sites for high molecular weight kininogen (HK), platelets and FXII are localised to the apple domains. Proteolytic cleavage of Arg369-Ile370 activates FXI converting it into two chain; the heavy chain comprises the apple domains and the light chain comprises the catalytic protease domain. The heavy and light chains of FXIa remain associated through a disulfide bond (97).

1.2.4 Propagation of thrombin generation

The assembly of prothrombinase (IIase) complex converts zymogen prothrombin to active thrombin (Figure 2). IIase is a ternary complex composed of FXa, the cofactor FVa (FVa), and aPL (236,290) in the presence of calcium. Cells control IIase assembly, by providing accessible aPL and a mechanism to produce the first FXa from its inactive precursor, FX (264). However, the amount of thrombin generated is limited to convert fibrinogen to fibrin leading to a clot (379). Thrombin activates FV and as discussed earlier, it activates FVIII and FXI of the intrinsic pathway to generate more FXa essential for its amplification (29,364,481). Thrombin activates and localizes platelets to the site of clot. Activated platelets express aPL and glycoprotein receptors for coagulation proteins. At the site of vascular damage, the binding of glycoprotein receptor GP Ib-IX-V complex to VWF on exposed endothelium initiates platelet adhesion. The fibrinogen binding to platelet GPIIb/IIIa leads to platelet aggregation (429). Aggregation of activated platelets provides the surface for intrinsic Xase and IIase complex assembly resulting in a burst of thrombin leading to fibrin clot (349).

1.2.5 Thrombin converts fibrinogen to fibrin clot

1.2.5.1 Fibrinogen structure

Fibrinogen is a glycoprotein. It is made up of two identical subsets of three different polypeptide chains named Aα, Bβ and γ composed of 644, 491 and 453 amino acids, respectively (175,180) (Figure 9). At the centre is the nodular E domain that contains the N-terminal residues of all six chains. From the E domain, the chains branch out in two sets of α-helical coiled coils to the two distal domains, called D (162). The D

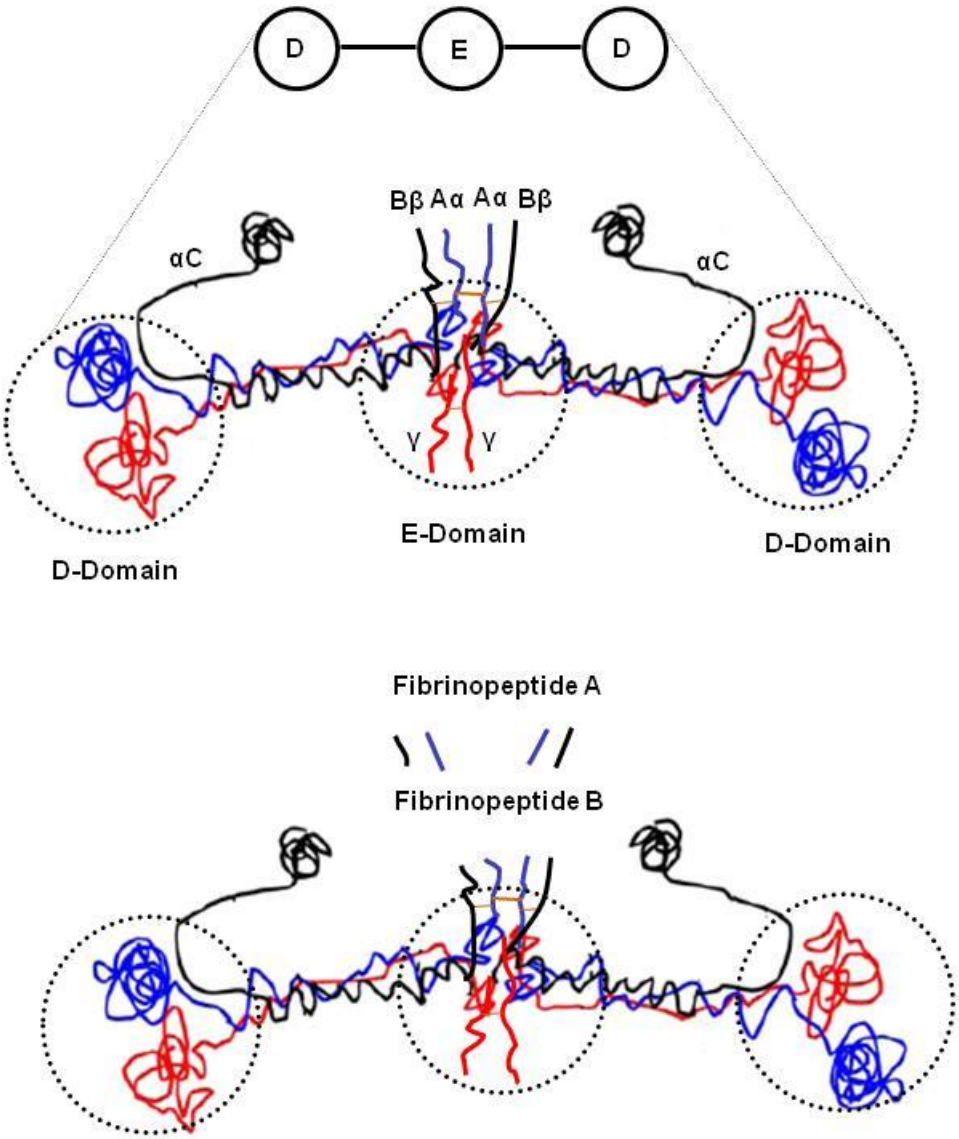


Figure 9 Schematic model of fibrinogen and fibrin showing the major domains (D and E). (A) Structure of fibrinogen. Fibrinogen is a dimer. Each dimer consists of three chains: Aα shown in blue, Bβ shown in red, and γ shown in black. The disulfides that link the two dimers are in the central E domain. The globular C-terminal domains of the Bβ and γ chains forming the D regions are shown, as well as the central E region, which contains the N-terminal portions of all three chains. Unlike the βC and γC domains, the C-terminal domains of the A α chain are flexible and tend to be noncovalently tethered in the vicinity of the central E region. (B) Thrombin cleaves the N-terminal regions of the Aαa and Bβ chains of fibrinogen at the peptide bonds Arg16-Gly17 and Arg14-Gly15, respectively, releasing fibrinopeptides A (FPA) and B (FPB) converting fibrinogen to fibrin. Adapted from (325).

nodules contain the C-terminal ends of Bβ and γ, as well as part of Aα. The C-terminal end of Aα then protrudes from each D domain as extensions (506). These extended portions of Aα, from each D domain can interact with each other and with the E domain during fibrin clot cross-linking. A total of 29 disulfide bonds hold the six chains together, creating the dimeric structure of the molecule.

1.2.5.2 Conversion of fibrinogen to fibrin clot

Thrombin cleaves at the N-terminus of fibrinogen Aα- and Bβ-chain releasing short sequences called fibrinopeptide A (FpA), and fibrinopeptide B (FpB) respectively (92). Consequently, the new N-termini of the α and β chains, called 'A' and 'B' knobs, respectively, become exposed and are polymerization sites. These sites form non-covalent interactions with pre-existing complementary "a" and "b" holes in the D domains of adjacent fibrin molecules. The resulting intermolecular A:a and B:b interactions produce half-staggered fibrin oligomers that lengthen into protofibrils. The protofibrils then aggregate laterally to form fibers and consequently form an elaborate, branched three-dimensional fibrin clot (267) (Figure 10).

Finally, thrombin converts fibrinogen to fibrin leading to clot formation and sealing of leaky vasculature (11,208,325). Thus a hemostatic response to vascular injury is initiated by the extrinsic pathway of coagulation and amplified by the intrinsic pathway, which are regulated by anticoagulants.

1.3 Anticoagulation

The regulation of coagulation is necessary to limit the amount of clot formation and restrict it to the site of damage to prevent thrombosis. The various components of

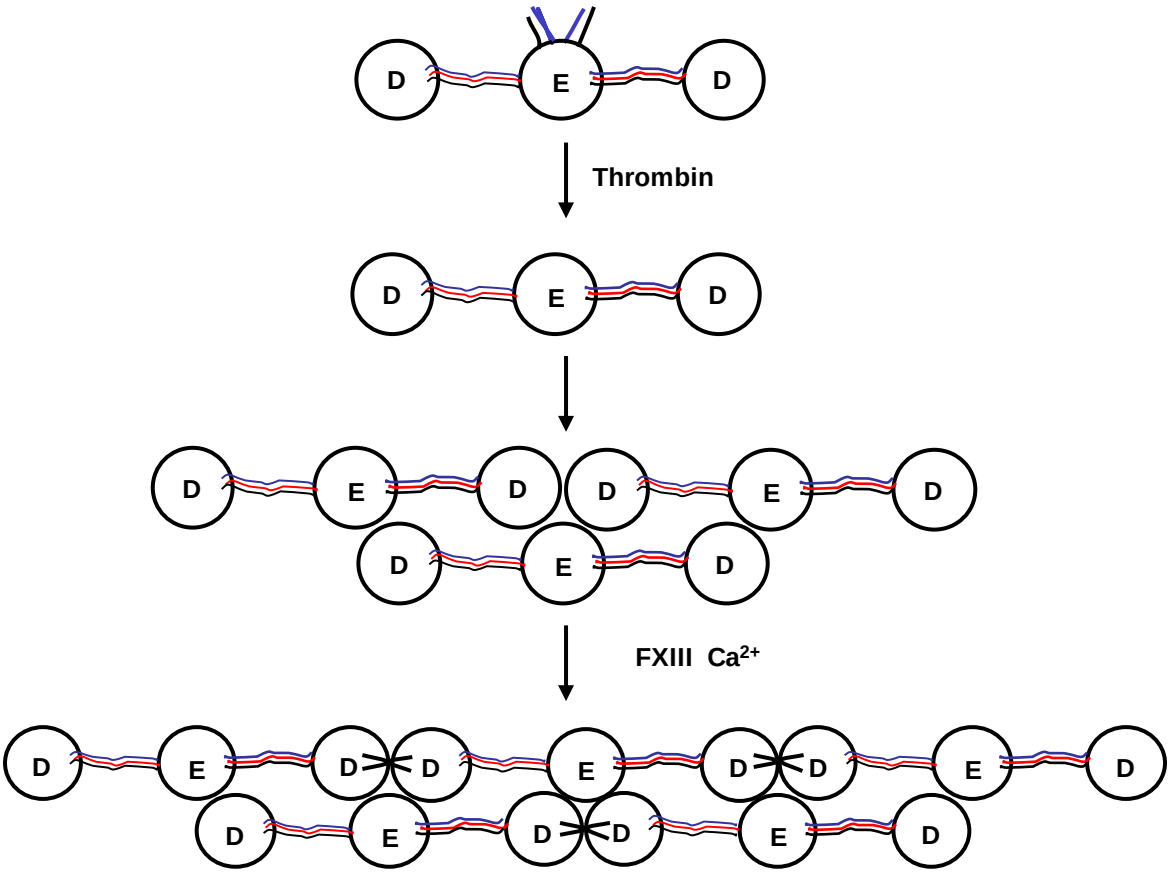


Figure 10 Thrombin induced fibrin polymerization and FXIIIa induced fibrin cross-linking. Thrombin cleaves fibrinogen releasing fibrinopeptides A (FPA) and B (FPB) converting it to fibrin, that undergo polymerisation. Thrombin in the presence of calcium activates FXIIIa that cross-links fibrin polymers (325).

coagulation are regulated by natural anticoagulants, which includes tissue factor pathway inhibitor (TFPI), antithrombin (AT), a member of the serpin family, and the protein C system including thrombomodulin (75,411).

1.3.1 Tissue factor pathway inhibitor

TFPI belongs to the Kunitz family of serine protease inhibitors and regulates the extrinsic pathway of coagulation initiation. TFPI inhibits the TF-FVIIa complex and is more potent when also bound to FXa. Thus the FXa generated by the extrinsic tenase is involved in feedback inhibition of its own synthesis. This involves two steps: First, TFPI binds to and inactivates FXa in a 1:1 stoichiometric complex by interacting at or near the active site of the enzyme. Second, the TFPI-FXa complex binds to and inhibits the TF–FVIIa complex. Alternatively, TFPI could bind to a preformed TF-FVIIa/FXa complex (28,278). This highly efficient simultaneous inhibition of TF, FVIIa and FXa by by TFPI explains the requirement of both the extrinsic and intrinsic pathways of the coagulation cascade.

The plasma concentration of TFPI is 2nM and much of the circulating TFPI is bound to plasma lipoproteins. TFPI is primarily synthesized by endothelial cells and to a lesser extent by megakaryocytes, precursor of platelets. Additionally, a small pool of TFPI is stored in platelets and secreted upon activation and degranulation. A significant portion of endogenous TFPI is bound to the endothelium that localizes its antithrombotic potential to the vascular wall for efficient function (71,278). To regulate clotting at the microenvironment level, thrombin generation can induce the release of TFPI from the endothelium leading to an increase in localized plasma levels of TFPI along with a simultaneous increase in endothelial cell surface TFPI (277). Increased levels of plasma

TFPI under septic conditions may represent endothelial dysfunction. Thus TFPI plays a key role in modulating TF-induced thrombogenesis, and is a probable moderator of virus mediated thrombin production as we have found involves virus-surface TF(457).

1.3.2 Antithrombin

AT is a member of the serpin family of homologous proteins. It is arguably the most important inhibitor of coagulation that regulates activated proteases of the intrinsic, extrinsic and common pathways. It binds irreversibly and directly inactivates thrombin and the other serine proteases FIXa, FXa, FVIIa and FXIa in the coagulation cascade. It is synthesized by liver and endothelial cells. AT circulates in plasma (along with other serpins) at vastly higher concentration than its target substrates, as even small amounts of thrombin may be sufficient to feedback amplify coagulation(75,198).

The inhibition mechanism mediated by AT involves formation of 1:1 complex between the active site of a serine protease and the scissile bond of AT. The rate of inhibition by AT is accelerated at least 1000 fold upon therapeutic administration of exogenous heparin. In vivo, vessel wall heparan sulfate functions like medicinal heparin as the antithrombin cofactor. Endothelial cells express heparan sulfate anchored to proteins (e.g. syndecan) which may enhance AT-mediated inactivation of nearby serine proteases, thus preventing the formation of fibrin clot. In the presence of heparin, the primary target of AT is thrombin. Heparin (or heparan) binding to AT alters its conformation and provides a scaffold on which both enzyme and substrate are colocalized, which greatly enhances its ability to inactivate the target enzyme (198,371,411).

1.3.3 Protein C

The PC system regulates coagulation by proteolytically inhibiting the cofactors FVIIIa and FVa (or their inactive procofactors) in the activation of FX and prothrombin, respectively. PC circulates as a zymogen. Thrombin cleaves PC to activated PC (APC), but only when bound to the endothelial membrane protein thrombomodulin (TM). TM functions as a cofactor and functional modulator of thrombin. The binding of thrombin to TM enhances PC activation by >1000-fold. The endothelial protein C receptor (EPCR) further enhances thrombin-TM activation of PC by 20-fold. APC together with its cofactor protein S inhibits coagulation by cleaving FVIIIa and FVa at discrete sites. Thus APC limits the generation of FXa and thrombin blocking the amplification of the coagulation system. Within the prothrombinase complex, FVa is protected from cleavage by APC. Thrombin regulates APC activity through inactivation of its cofactor protein S. APC is also regulated directly by a member of the serpin family, heparin-dependent protein C inhibitor (75,76).

1.3.4 Thrombomodulin

TM is an ~75 kDa transmembrane glycoprotein expressed on the surface of endothelial cells When bound to thrombomodulin, thrombin loses its known procoagulant functions, including cell stimulation activation, conversion of fibrinogen to fibrin, and activation of cofactors FV and FVII for feedback amplification. This is because TM occupies the functionally important exosite 1 on thrombin and thereby blocks interactions with other thrombin-binding proteins. Also, binding of TM to thrombin induces rapid endocytosis and degradation of the protein. Thus, TM has multiple anticoagulant properties: converting thrombin into an activator of protein C and accelerating the inhibition of

thrombin by AT(411). Further, thrombin-TM complex can also activate thrombin-activable fibrinolysis inhibitor (TAFI). Thus, TM confers many functions that are essential to control of hemostasis and therefore fundamental health.

1.4 Fibrinolysis

The free flow of blood in the systemic circulation is maintained through a dynamic and delicate balance between the coagulation and fibrinolytic systems (382). Upon vascular injury or in the case of trauma, there is exposure of the subendothelium and activated platelets get recruited to damaged sites. The platelet activation together with the exposure of the subendothelium activates the coagulation system leading to formation of a haemostatic plug composed of platelets and a fibrin clot network to prevent blood loss (129). When vascular damage has undergone repair and normal blood flow must be restored, the fibrinolytic system is activated, degrading the fibrin into soluble products for removal (81). Like the coagulation cascade, the fibrinolytic system comprises of proteases (Table 2) and inhibitors for regulation. The final zymogen to enzyme conversion in fibrinolysis is the proenzyme, plasminogen to active enzyme, plasmin. Plasmin generation is regulated by plasminogen activators and their specific inhibitors (261). In addition to fibrin degradation, plasmin also exhibits a range of protease activity against other extracellular matrix proteins and has been implicated in other biologic processes, that include wound healing, embryogenesis, angiogenesis, tumor growth and metastasis (77,145,284,340,355,414,452). The current overview will focus on the role of plasmin in fibrinolysis.

Property	Plasminogen	tPA	uPA
Molecular weight (kDa)	92	72	54
Amino acids	791	527	411
Site of synthesis	Liver	Endothelium platelets	Endothelium, platelets,
Plasma concentration			
nM	1500	0.075	0.150
µg/mL	140	0.005	0.008
Plasma half-life	48 h	5 min	8 min
Two-chain cleavage site	Arg560-Val561	Arg275-Ile276	Lys158-Ile159
Heavy chain domains			
Finger	No	Yes	No
Growth factor	No	Yes	Yes
Kringles (no.)	5	2	1
Light-chain catalytic triad	His602, Asp645, Ser740	His322, Asp371, Ser478	His204 Asp255, Ser356

Table 2 Properties of fibrinolytic proteins. tPA, tissue-type plasminogen activator; uPA, urokinase plasminogen activator.

1.4.1 Plasminogen

Plasminogen has a molecular weight of 92kDa. It is a single chain glycoprotein synthesized in the liver(383,413). It circulates as an inactive zymogen in plasma at a relatively high concentration of 200µg/ml. Increased plasminogen levels are associated with pregnancy (171), obesity and are seen in kidney and liver transplant patients(423). Low levels of plasminogen have been reported in sepsis (126) and thrombolytic therapy(166).

1.4.1.1 Structure

The plasminogen molecule contains a total of six structural domains with a preceding preactivation peptide (62,369) (Figure 11). Starting from the N-terminus, the preactivation peptide is followed by five kringle domains and the catalytic serine protease domain at the C-terminus. Kringle domains are also found in other proteins of the fibrinolytic system, such as tissue plasminogen activator (tPA) (360) and urokinase plasminogen activator (uPA) (149), and in the contact pathway protein, FXII (281,282,303). The lysine binding sites (LBS) on the kringle domains facilitate plasminogen binding to fibrin and cell surface receptors. Three of the five kringle domains in plasminogen possess functional LBS. Binding experiments using lysine and lysine analogues, epsilon aminocaproic acid (EACA) have shown that the plasminogen K1 domain contains a strong LBS (255,311) followed by K4 (255,296,344) and K5 (296,344). The K2 has a weak interaction with EACA but of no known functional significance. The native K3 has no interaction with lysine analogues (292), but showed a weak interaction upon mutation revealing a preformed non-functional LBS (30). Within the active site, Ala at 601 is essential for the normal function of plasminogen. The amino

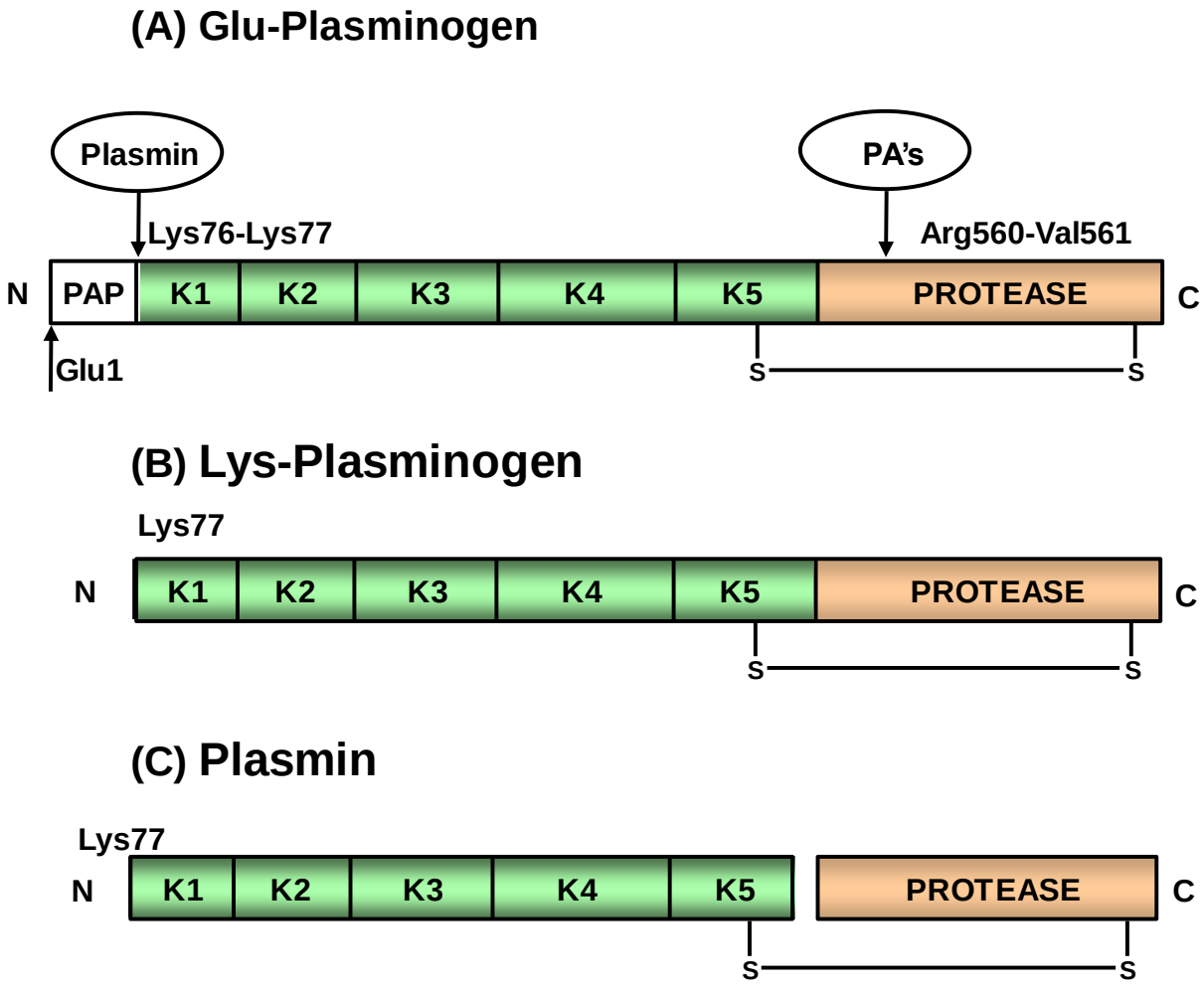


Figure 11 Domain structure of plasminogen (Pg). Starting from the N-terminus is a pre-activation peptide (PAP), five kringle (K) domains(K1-K5) and protease domain that contains the catalytic site of plasmin. The arrows indicate the sites of proteolytic cleavage by plasmin, and plasminogen activators (PA's) (A). Hydrolysis of Lys76-Lys77 bond mediated by plasmin converts the native Glu-Pg to Lys-Pg (B). The cleavage of Arg560-Val561 bond by PA's results in the two-chain plasmin molecule that remains covalently associated by a disulfide bond (C) (315)(396).

acid substitution of Ala601Thr resulted in the impairment of proteolytic activity and has been linked to an increased risk of thrombosis (318), testifying to the importance of balance between coagulation and fibrinolysis.

The native form of plasminogen in plasma has glutamic acid at the N-terminus and is consequently termed Glu-plasminogen. Cleavage at Lys76-Lys77 by plasmin results in plasminogen with lysine at the N-terminal position and is termed Lys-plasminogen (315). Glu-plasminogen can be thought of as a pro-zymogen, because it is activated ~10-fold more slowly than Lys-plasminogen by plasminogen activators(315). Upon binding to fibrin, the typical “closed” conformation of Glu-plasminogen is transformed into the open conformation of Lys-plasminogen (378), which is more readily activated to plasmin. Recently, mutational studies demonstrated conversion of Glu-plasminogen to Lys-plasminogen on cell surfaces as essential for optimal plasmin generation (139).

1.4.1.2 Activation to plasmin

The specific proteolytic cleavage at Arg560-Val561 converts single chain plasminogen into an active two chain enzyme, plasmin (396). The native Glu-plasminogen is converted to an active two chain Glu-plasmin with an N-terminal heavy chain that contains the lysine binding sites and a light chain that has the serine protease domain (315). Because of the “closed” conformation, the lysine binding sites in the heavy chain are buried and so this molecule is functionally inactive. Further, through an auto-proteolytic cleavage in the heavy chain at Lys76-Lys77, the Glu-plasmin is converted to Lys-plasmin (139). Consequently, the lysine binding sites get exposed and allow its binding to the fibrin cofactor. Interestingly, both Lys and Glu-plasmin can cleave the

Lys76-Lys77 bond in Glu-plasminogen to form Lys-plasminogen (42).

Plasmin degrades the fibrin clot into fibrin degradation products (FDP) (498) (Figure 12). Plasmin degradation of fibrin clot is selective. With the polymerization of fibrinogen to fibrin, plasminogen and plasminogen activator get incorporated into the fibrin clot. The initial plasmin generated on the fibrin clot primes the fibrin clot exposing C-terminal lysine residues for further incorporation off plasminogen and tPA leading to more plasmin. The binding sites on fibrin are both C-terminal lysine-dependent and -independent. Probably, the initial binding is independent of C-terminal lysine (394).

The fibrin clot promotes activation of fibrinolysis by catalyzing the activation of plasminogen by plasminogen activators to form plasmin (188) (Figure 13). Plasmin proteolytically degrades cross-linked fibrin, ultimately producing soluble fibrin degradation products of various sizes that include cross-linked fragments containing D-dimer (DD) epitopes (Figure 12). Plasmin also degrades fibrinogen to form fragments X, Y, D, and E. The FDP's include fragments X and Y (early cleavages) and D and E (late cleavages) (498).

Although fibrin is the physiological substrate of plasmin, plasmin can also degrade circulating fibrinogen, FV and FVIII, which are needed for subsequent clot formation. Therefore regulation of plasmin is necessary to avoid depletion of plasma fibrinogen. Thus any free plasmin is inactivated by inhibitors. Free plasmin in the circulation is rapidly inactivated by irreversible binding to α2-antiplasmin (415). α2-antiplasmin is a serpin that forms a complex with plasmin. In addition, plasminogen activation to plasmin is regulated by plasminogen activator inhibitor-1(PAI-1), which is released by endothelial cells and inactivates tPA. Thus plasmin is directly and indirectly inhibited by serpins.

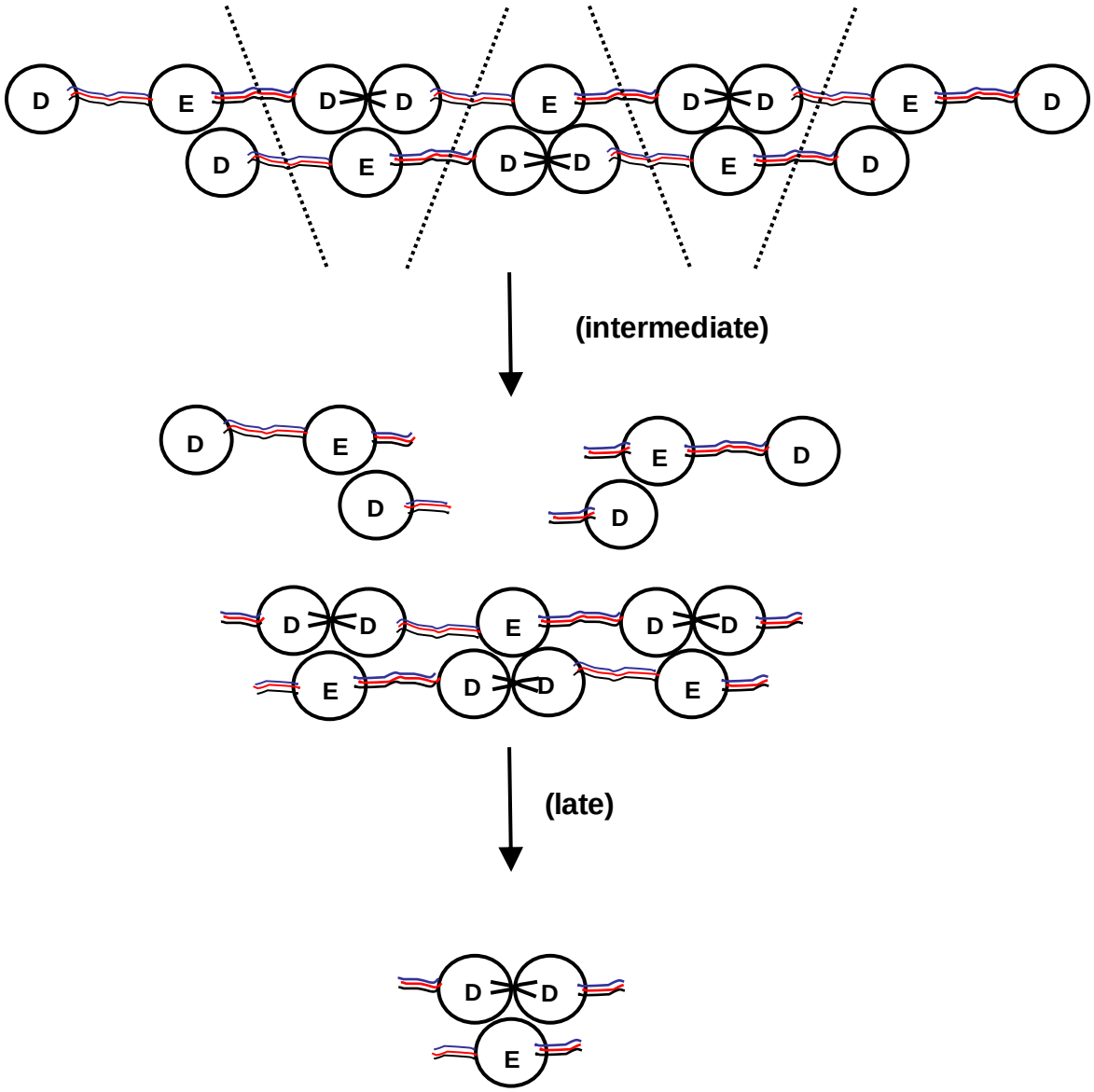


Figure 12 Plasmin induced fibrin degradation.Plasmin degrades fibrin at multiple sites to release fibrin degradation products. The initial fragments are high-molecular-weight complexes followed by further degradation to produce the terminal D-dimer–E complex, which contains the D dimer antigen (498).

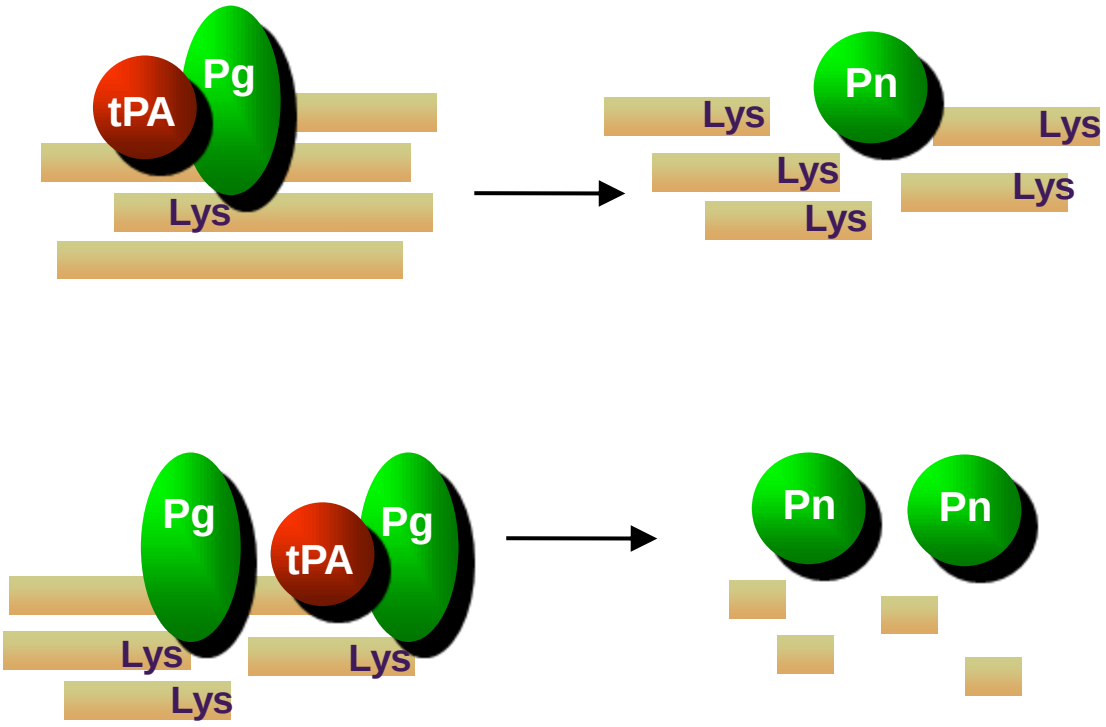


Figure 13 Fibrin as a cofactor for plasmin generation. An undegraded fibrin clot, contains the internal lysine residue, for binding of plasminogen (Pg), and the binding site for tPA, necessary to form the ternary activation complex. The binding of Pg and tissue plasminogen activator (tPA) results in plasmin (Pn) formation and the initiation of lysis, which exposes C-terminal lysine residues on fibrin. More Pg binds to these termini, and Pn generation is enhanced by tPA that lyses the fibrin clot to fibrin degradation products.

1.4.2 Plasminogen activators

A number of physiological and non-physiological molecules have been demonstrated to activate plasminogen and are termed plasminogen activators (PAs). The tissue-type plasminogen activator (tPA) (512) and urokinase-like plasminogen activator (uPA) (511) are the only known physiological plasminogen activators (Table 2). Other plasminogen activators have been identified in nature, which include streptokinase (297) and staphylokinase (262) isolated from streptococcus and staphylococcus respectively. Streptokinase and Staphylokinase are not enzymes, unlike tPA and uPA (Sec 1.4.2.3).

1.4.2.1 Tissue Plasminogen Activator (tPA)

Structure

tPA is a 70kDa, single polypeptide chain of 527 amino acids. A two-chain form is produced by proteolytic cleavage of the Arg275-Ile 276 peptide bond. It is a serine protease composed of five structural domains (Figure 14). The N-terminal heavy or A-chain consists of a fibronectin type 1 also called a finger domain, an EGF domain and two kringle domains. The C-terminal light or B-chain contains the catalytic serine protease domain and is homologous to the catalytic domains of other serine proteases (63) (Figure 7). The light chain is able to activate plasminogen, but requires the heavy chain for fibrin-binding and fibrin-stimulation. The finger domain and kringle 2 of the heavy chain are involved in the interaction with fibrin (33,415).

Function

tPA is synthesized and released by endothelial cells both constitutively and upon stimulation by a number of substances including thrombin (257,259). Protein kinase C has been implicated in tPA synthesis, but the mechanistic details are not clear (258). A

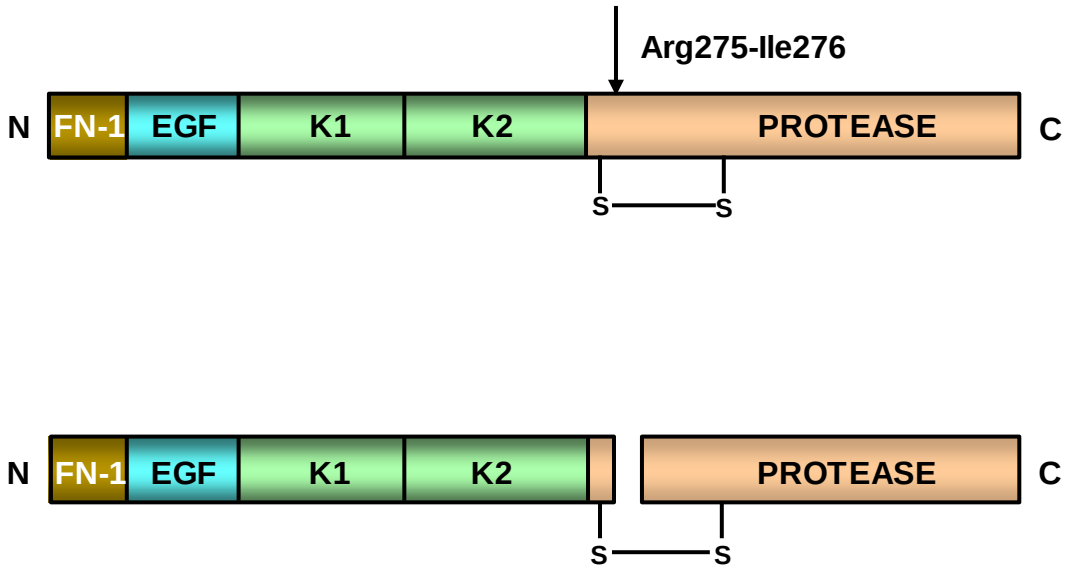


Figure 14 tissue Plasminogen Activator (tPA) domain structure. Starting from the N-terminus; FN-1, fibronectin type 1 domain; EGF, epidermal growth factor domain; K1 and K2, two kringle domain; and protease domain. The function for the various domains are as follows: K1, receptor binding (liver); K2, fibrin binding (low affinity); F, fibrin binding (high affinity) and protease domain for catalytic activity and plasminogen activator inhibitor 1–binding. Cleavage of the Arg275-Ile276 peptide bond by plasmin converts tPA to a disulfide linked, two-chain form (261).

recent in-vitro study suggested regulation of tPA synthesis in endothelial cells under epigenetic control(94). Endothelial cells store tPA in vesicles also called secretory organelles. Studies have suggested tPA is stored in Weibel-Palade bodies along with vWF and in vesicles distinct from Wieibel-Palade termed as tPA storage organelle (350). tPA is also found in platelet α-granules at low abundance (500). tPA is synthesized in megakaryocytes and is packaged into α-granules during platelet formation (54).

Upon plasmin-mediated proteolytic cleavage, tPA is converted to a two-chain form held together by a single disulfide bond. Unlike uPA, tPA does not require prior proteolysis to have enzymatic activity. In comparison to micromolar concentrations of plasminogen, tPA circulates at picomolar and most of it is bound to PAI-1. Consequently, the functional circulating tPA is very low and to add to it tPA is not very efficient without an accelerating cofactor such as fibrin (62). As a result, normal physiological tPA concentrations do not induce systemic plasmin generation. It is the fibrin which is generally thought to regulate plasminogen activation leading to fibrinolysis through recruitment and co-localization of both tPA and plasminogen on its surface. The stimulation of endothelial cells at the site of damage ensures rapid local release of active unbound tPA to be incorporated into the clot.(350) tPA binds to fibrin via its finger domain and through LBS of K2, and activates fibrin-bound plasminogen 3-orders of magnitude more rapidly than it activates plasminogen in the circulation (261).

The fibrin specific binding of tPA stimulates plasminogen activation to plasmin on the fibrin clot surface resulting in clot dissolution and maintaining vascular hemostasis. Further, binding sites for tPA have been identified on endothelial cells. This involves

annexin 2 (A2), a calcium and phospholipid binding protein on the plasma membrane and has been shown to enhance tPA-dependent plasminogen activation that is C-terminal lysine dependent (161). A2 has also been identified on the surface of at least one herpesvirus, CMV (384). For this reason, an important part of this thesis is tPA-dependent plasminogen activation on the virus surface.

1.4.2.2 Urokinase Plasminogen Activator (uPA)

Structure

uPA was originally found in human urine (511), and later in blood and in many cell types (78,510). It is single chain glycoprotein with a molecular mass 54 kDa. The single chain form is a prozymogen, which upon plasmin-mediated proteolytic cleavage is converted to an active two-chain form held together by a single disulfide bond. Similar to tPA, uPA is composed of two chains derived from the N terminus, termed A-chain or heavy chain, and the C-terminus, termed B-chain or light-chain. The N-terminus has an EGF domain and one kringle domain. The C-terminal region contains a serine protease domain (Figure 15). The growth factor domain constitutes a binding site for uPA receptor (uPAR) and the kringle domain lacks lysine binding sites (415).

Function

uPA provides a system for pericellular plasminogen activation. uPA binds with high affinity to uPAR on the cell surface. On the surface of extravascular cells, uPA/uPAR complex mediate plasminogen activation to plasmin. The cell surface plasmin generated is involved in proteolytic degradation of the extracellular matrix. This remodelling and other mechanisms contribute to regulating cell migration and proliferation (77).

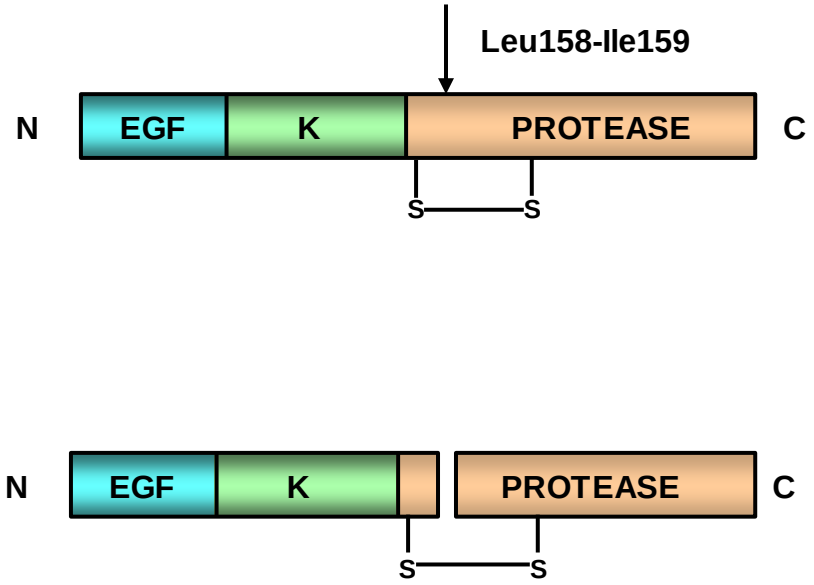


Figure 15 urokinase Plasminogen Activator (uPA) domain structure. Starting from the N-terminus; EGF, epidermal growth factor domain; K kringle domain; and protease domain. The cleavage of the Leu158-Ile159 bond converts the single uPA into an active two chain uPA (261).

The role of uPA in intravascular plasminogen activation and fibrinolysis is less well established. Unlike tPA, uPA is not fibrin specific and is relatively protected from PAI-1 inhibition. Plasmin is the physiological activator of uPA, which in turn activates plasminogen to plasmin, so there is reciprocal activation. However, the single chain form of uPA has very low intrinsic activity so that it does not lead to systemic activation of plasminogen and is also inactivated by thrombin cleavage(394). In vivo endothelial cells and platelets express uPA, but its regulation is not clear(151). In vitro studies have shown uPA expression in endothelial cell culture is regulated by a variety of cytokines, and growth factors (287).

1.4.2.3 Bacterial plasminogen activator

Invasive human pathogens such as streptococci and staphylococci have evolved plasminogen activators, such as streptokinase (297) and staphylokinase (262). Unlike tPA and uPA, these are not enzymes. Both streptokinase and staphylokinase form 1:1 complexes with human plasminogen configuring the uncleaved plasminogen into a functional enzyme enabling the proteolytic activation of other molecules of plasminogen to plasmin (230,385). In contrast to plasmin alone, these 1:1 complexes possess a remarkable specificity and efficiency to activate plasminogen. In vivo and in vitro experiments have demonstrated staphylokinase specificity for plasminogen activation on the fibrin clot surface (263,294). However, streptokinase is not fibrin specific, activating both circulating and fibrin-bound plasminogen (358).

1.4.3 Plasminogen activator inhibitors

The plasminogen activators uPA and tPA are regulated by inhibitors called

plasminogen activator inhibitor-1 (PAI-1) or PAI-2. PAI-1 and PAI-2 are glycoproteins that belong to the family of serine protease inhibitors called serpins (237). Serpins are known to adopt a variety of conformations under physiological conditions. They are only partially stable in their active form, but are more stable when they snap into their target protease. They have a reactive centre loop (RCL) that acts as bait. The proteases are trapped and destroyed when they take this bait. The RCL contains a scissile bond (termed the P1-P1´ peptide bond) that is recognized by target serine proteases. The P1 and P1´ denote the N- and C- terminal residues of the scissile bond respectively. The serpin-mediated inhibition of serine protease involves multiple steps. Foremost, a non-covalent complex is formed between the serine protease and the P1-P1´ scissile bond. Subsequently, the P1-P1´ scissile bond is cleaved resulting in a covalent acyl intermediate between the serine residue of the protease and the P1 residue of the serpin. Then the N-terminal portion of the RCL (from P1 residue) is inserted into the central β-sheet region of the serpin. As a result, the covalently linked protease is moved in a direction to the opposite of serpin followed with a disruption of its active site conformation. Consequently, the serpin is permanently consumed and the protease is also permanently inhibited. Hence, serpins are called suicide inhibitors(382).

1.4.3.1 Plasminogen activator inhibitor-1

PAI-1 is a 54kDa protein that can exist in its native inhibitory form, in an inactive latent form, in complexes with proteinases and in a cleaved substrate form (169). It assembles complexes both with single-chain and two chain tPA and with two-chain uPA (83), but is more selective for tPA. In plasma, there is a large molar excess of active PAI-1 over tPA. Consequently, most of tPA is in complex with PAI-1 to regulate constitutive

plasminogen activation (415). Endothelial cells as well as many other types of cells in culture secrete PAI-1 (74,229,483). Platelets contain a large pool of PAI-1, mostly in an inactive form (21). Platelets recruited into the fibrin clot are activated and release PAI-I from their α–granules (203). Although most of it inactive, there is enough active PAI-I to inhibit the plasminogen activator tPA and uPA and protect clot formation (26,112). In contrast, platelets are thought to provide a surface for enhanced plasminogen activation by promoting an interaction between tPA and plasminogen and for protection of plasmin from α2-antiplasmin (273). Plasma PAI-1 is elevated in atherosclerosis (395), diabetes (5), severe sepsis and in inflammatory conditions. PAI-1 is implicated in tumor cell invasion, metastasis, and neovascularization (300).

1.4.3.2 Plasminogen activator inhibitor-2

PAI-2 protein exists in two forms a nonglycosylated intracellular form, 42kDa and a glycosylated, secreted extracellular form, 60kDa (128). PAI-2 inhibits uPA (215) rapidly, but tPA very slowly (238). PAI-2 is expressed in placental trophoblasts (215), as an inflammatory response in macrophages (425). PAI-2 secreted by monocytes is involved in uPA-mediated cell migration (221), whereas intracellular PAI-2 prevents apoptosis (240) (87).

1.4.4 Cofactors in plasminogen activation

1.4.4.1 Fibrin

Fibrin acts as a cofactor in tPA-mediated activation of plasminogen to plasmin (188) (Figure 13). It increases the rate of activation by 1000-fold compared with tPA alone. With the polymerization of fibrinogen to fibrin, cryptic tPA and plasminogen binding sites

get exposed (22,306,418). Probably, the initial binding is independent of C-terminal lysine. Studies have demonstrated binding of the D domain of fibrin to tPA (478) and plasminogen. The γ312-324 sequence of the D domain binds the finger domain of tPA and through the aminohexyl site plasminogen binds to the intact lysine residues in the D domain(52). Fibrin brings both tPA and plasminogen into close proximity forming a ternary complex, enhancing plasmin generation. Subsequently, the plasmin that is formed cleaves fibrin exposing new carboxyl-terminal lysine residues, accelerating its cofactor activity over 3 fold that of intact fibrin, plus more binding sites are created for tPA and plasminogen(53,113,114).

Thrombin Activable Fibrinolysis inhibitor

TAFI is a pro-carboxypeptidase that circulates in plasma as a 60 kDa zymogen. It is synthesized in the liver and circulates in plasma at a concentration of 50nM. The thrombin-thrombomodulin complex activates it most efficiently. Thrombin cleaves the Arg92-Ala93 peptide bond in TAFI to generate active TAFI (TAFIa). TAFI activation by thrombin is enhanced in the presence of thrombomodulin, which requires a high concentration of thrombin (20). This is to ensure there is enough thrombin to form a fibrin clot, as thrombin bound to thrombomodulin is no longer procoagulant. The thrombin-thrombomodulin generated TAFIa is to stabilize the fibrin clot and protect it from lysis. TAFIa removes the C-terminal lysine residues from fibrin, which enhance binding of tPA and plasminogen to fibrin (501). Thus, fibrin loses its cofactor activity in tPA mediated plasminogen activation, resulting in less plasmin, leading to down regulation of fibrinolysis. Plasmin inactivates TAFIa activity through proteolytic cleavage of Lys327 to ensure clot lysis (293). Thrombin activation of TAFI and plasmin inactivation of TAFIa ensures a strategic balance of coagulation and fibrinolysis. The

antifibrinolytic role of TAFI explains elevated levels in thrombosis and TAFIa inhibitors are being considered to enhance the tPA-induced lysis of a thrombus(332).

1.4.4.2 Annexin 2

Structure

Annexin 2 (A2) is a calcium-dependent 36kDa aPL-binding protein with a C-terminal core domain and an N-terminal tail domain (326). The 34 kDa core domain consists of four sub-domains that are conserved within the annexin family, whereas the N-terminal tail domain is member specific (130,131,496). The C-terminal core constitutes the calcium binding site(s) in each of its four subdomains. The N-terminus consists of 36 amino acids, with binding sites for protein–protein interactions and post translational modification sites that include serine (504) and tyrosine phosphorylation (136) as well as N- terminal acetylation.

Annexin 2 is known to exist in two forms (132,496) (Figure 16). A monomeric form with one A2 unit of 36 kDa called A2 monomer (A2m). The monomeric form is typical of annexins. A2 also exists in a heterotetrameric form of two A2 units with two p11 proteins called A2 tetramer (A2t). The two forms differ in their calcium requirement for phospholipid binding leading to differential localization and functions(130).

Function

The A2 function is still being explored and has been implicated in cell membrane trafficking, fibrinolysis and signaling. A2 is expressed on a number of cell types that include epithelial, endothelial, fibroblast, macrophages and spleenocytes (55,60,241,489,496,496). A2 has been demonstrated on the cell surface though it lacks a secretory signal for secretion through the endoplasmic

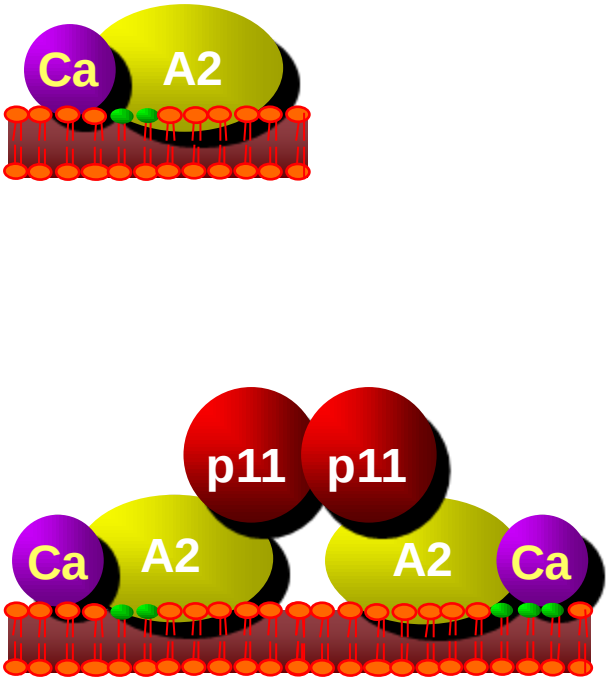


Figure 16 Annexin 2 (A2) exists in two different forms, a monomer form (A2m), and a heterotetrameric form (A2t) resulting from the non-covalent association of two A2m subunits with a p11 dimer.

reticulum pathway. In endothelial cells, A2 translocation to the cell surface is constitutive and can be induced by stimuli such as thrombin (362). With no induction, A2 is translocated to the cell surface within 16 hours of biosynthesis (158,161,521). The cell surface A2 is approximately 5% of the total cellular A2(343). The A2 distribution within the cell is dependent on the form in which it is found. A2m is mostly in the cytosol and nucleus (70), whereas the A2t is found on the inner leaflet of the plasma membrane. This is because A2t can bind aPL at intracellular calcium concentrations, but not A2m (353,466). Although, both A2m and A2t can mediate membrane aggregation and F-actin binding because of their association with aPL at low intracellular calcium concentrations, only A2t can participate in membrane fusion. A2m in the nucleus may be involved in DNA synthesis by regulating the activity of DNA polymerase α (199,490). The roles for A2m and A2t described have not yet been unequivocally established *in vivo.* Recent data have implicated A2 in fibrinolysis (43,159,161), viral infection (253,384) and tumor invasion (283).

A2 in fibrinolysis

Many studies have shown that A2 is involved as a cofactor in fibrinolysis (43,159,161). It participates in tPA mediated activation of plasminogen to plasmin (Figure 17). In vitro binding and kinetic studies using cultured endothelial cells identified surface A2 as a coreceptor for both tPA and plasminogen, accelerating tPA mediated plasmin generation(43). The C-terminal lysine analog EACA inhibited both binding of plasminogen and tPA and plasmin generation suggesting the participation of C-terminal lysine residues on A2 (43,161).

Further to endothelial cell culture evidence, animal models support a role for A2

Figure 17 Cell surface annexin 2 (A2) mediated plasmin generation. A2 has been identified as an endothelial cell surface coreceptor for plasminogen (Pg) and tissue plasminogen activator (tPA). A2 acts a cofactor in the tPA dependent Pg activation to plasmin (Pn) leading to clot dissolution.

as a cofactor in tPA-dependent plasmin generation. In these studies A2 deficient mice displayed fibrin deposition in the microvasculature, reduced clearance of injury-induced arterial thrombi and reduced cell surface plasmin generation of isolated endothelial cells (265). In a rat model of carotid artery injury, pretreatment with recombinant A2 prevented thrombosis (193). Overexpression of A2 by acute promyelocytic leukemia blast cells contributes to a hyperfibrinolytic hemorrhagic state in humans (310,351). Finally, in sickle cell disease patients, A2 polymorphism is linked as a risk factor for stroke (427). The recognition of a profibrinolytic cofactor role of A2 has improved our understanding of vascular pathology and continued work should lead to better treatment.

A2 in CMV infection

Numerous studies have implicated A2 in CMV infection. These include binding between cell surface A2 and CMV observed for endothelial cells (516), fibroblasts, cancer cell lines of epithelial, monocytes and lymphoid origin and increased A2 expression in cells was associated with CMV productive infection (345,516). Additionally, A2 was identified on the surface of CMV cultured in fibroblasts (384,515). Finally, a direct role of A2 in CMV infection is obtained from experiments using specific A2 antibodies and addition of exogenous purified A2. A2 antibody attenuated CMV infection of fibroblasts, and addition of exogenous A2 enhanced CMV binding to cells and membrane fusion. Further studies from our laboratory determined that addition of exogenous purified p11 and A2t caused a 4-fold enhancement in CMV cell entry (86). Antibody inhibition of endogenous p11 and A2 present on the surface of human foreskin fibroblasts lead to an 80% drop in CMV entry and infection (86). Thus, in addition to having a role in fibrinolysis that is relevant to my thesis, A2 also participates in viral infection

mechanism(s) (253,384), which may conceivably involve plasmin generation.

Other functions

A2 has been implicated in cell-cell, cell-matrix interactions, and matrix remodeling. It has been shown to bind extracellular matrix proteins including tenascin C (55)(45), collagen I (51,513)(148), and fibrin (51)(140) and modulate cells. A2 association with tenascin C has been shown to regulate cell migration and induce mitogenesis enhancing cell proliferation (56). A2 expression is enhanced in a number of cancers and cancer cell lines (60,118,489,521). Additionally, in comparison to non-metastatic cancer cells, metastatic cancer cells show increased A2 on their surface (521). The increased A2 expression along with their ability to colocalize with extracellular matrix proteins supports their role in tumor invasion (283).

Thus A2 has a wide cellular distribution and multiple roles in fibrinolysis, extracellular proteolytic activity leading to cellular proliferation, cellular migration and contributing to patho-physiology conditions. Its association with viruses leads to enhanced infection. Therefore, the role of virus-associated A2 contributing to plasminogen activation will be probed in this work.

1.5 Protease activated receptor

In the current work I have evaluated herpesviruses as a potential enhancer of plasmin generation. These viruses have been shown to activate coagulation serine proteases. Since plasmin and the coagulation serine proteases are known to modulate cells through activation of protease-activated receptor (PAR) (239) a short overview of PARs follows.

PARs belong to a family of seven-transmembrane domain G protein-coupled

receptors (GPCR) (322), with a unique activation mechanism, where they carry their own activating ligand. PARs are ubiquitously expressed in multiple cell types, including platelets, cardiomyocytes, endothelium, smooth muscle cells, epithelium, fibroblasts, hepatocytes, macrophage, lymphocytes, neutrophils, mesangial cells, keratinocytes, neurons, astrocytes, oligodendrocytes, and microglia. PARs mediate diverse signaling events in multiple systems, including the cardiovascular system, respiratory system, gastrointestinal system, immune system, renal system, and nervous system (354).

1.5.1 Structure

As in Figure 18(A) starting from the N-terminus is an extracellular domain followed by seven transmembrane helices and an intracellular C-terminal domain. The seven transmembrane (TM1-7) hydrophobic regions give rise to three intracellular loops (ICL1-3) and three extracellular loops (ECL1-3). The long N-terminal extracellular domain constitutes cleavage site for proteases leading to activation and subsequent signaling (495). The ECL-2 constitutes the binding site for the new N-terminus that is exposed upon cleavage. A disulfide bond between the ECL-2 and TM3 provides stability to PAR structure. They mediate signal transduction to extracellular serine proteases like thrombin and trypsin. So far, four members (PAR1-4) of this family have been identified (2,68,185).

1.5.2 Activation

Of the four PARs, PAR1 is the most extensively studied. The mechanism of PAR activation was initially established for PAR1 (495) and appears to be a general paradigm

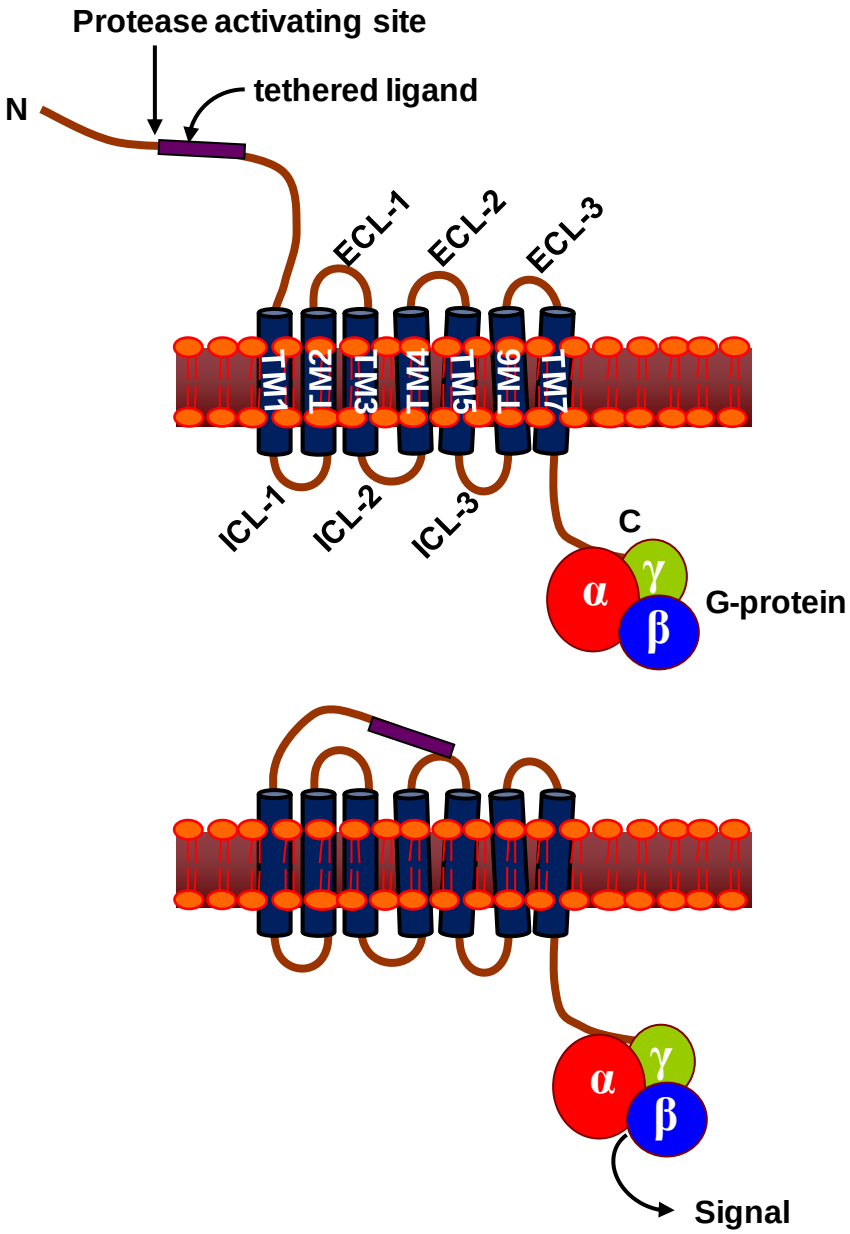


Figure 18 Protease activated receptor (PAR) structure (A) and activation (B). PARs contain seven transmembrane (TM) helices, an extracellular amino terminal domain encompassing a signal peptide and a pro-domain of, three intracellular loops (ICL1-3), three extracellular loops (ECL1-3) and an intracellular carboxy terminal domain couple to G-protein. Protease mediated cleavage unmasks a new N-terminus that serves as tethered ligand, binding intramolecularly to the ECL-2 which results in transmembrane signal transduction mediated by G- protein Adpated from (2)

for other PARs. PAR1 activation is mediated mainly by thrombin. The other serine proteases that activate PAR1 include FXa, trypsin, granzyme A and plasmin and a recent study reported, PAR1 activation by matrix metalloprotease-1 (MMP-1) in breast cancer cells (2).

The activating proteases cleave PARs to expose a new N–terminus, which functions as a tethered ligand (TL) (495) (Figure 18 (B)). The specificity of activation depends on the sequence of amino acids that are revealed upon receptor cleavage. The TL binds intramolecularly with ECL-2, leading to a conformational change in the receptor. This initiates downstream intracellular signaling. PARs can also be activated by a short synthetic peptide mimicking the sequence of the TL, called activating peptides (AP). PAR APs are a very useful reagent to study activation mechanisms in a scenario where more than one PAR is expressed and activated by a common protease (377). For example, in the case of human platelets, both PAR1 and PAR4 are expressed and activated by thrombin(205). Once activated, PARs are rapidly uncoupled from signaling and internalized by phosphorylation-dependent mechanisms. New PARs are then delivered to the surface from a preformed intracellular pool or from new protein synthesis (67).

Like other GPCR, PARs are associated with the heterotrimeric G-proteins α, β and γ subunits, which are bound together. In an inactive state, the α subunit is bound to guanosine diphosphate (GDP) and upon G protein activation, the GDP bound to the α subunit is replaced by guanosine triphosphate GTP (24). This causes a conformational change resulting in the dissociation of the α subunit from the γ subunit, activating them.

Upon activation, PARs signal through the activated G-protein subunits causing downstream activation of several signal transduction pathways (Figure19), which

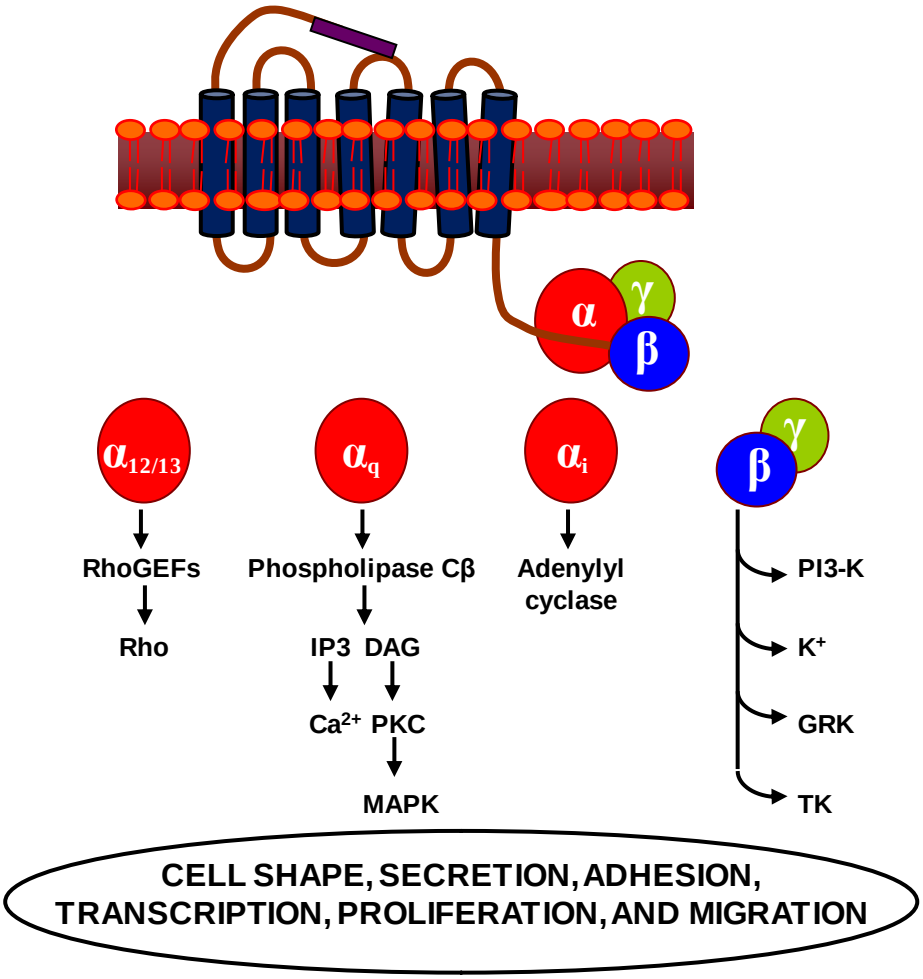


Figure 19 PAR1 signal transduction. PAR1 couples to $G\alpha_i$, $G\alpha_{12/13}$, and $G\alpha_q$. $G\alpha_i$ inhibits adenylyl cyclase, promoting platelet aggregation. $G\alpha_{12/13}$ couples to guanine nucleotide exchange factors (GEF), resulting in activation of Rho, mediating cytoskeleton responses such as shape changes in platelets. $G\alpha_q$ activates phospholipase Cβ to generate inositol trisphosphate (IP3), which mobilizes Ca^{2+}, and diacylglycerol (DAG), which activates protein kinase C (PKC) and subsequently activates mitogen-activated protein kinase (MAPK). MAPK cascades mediate a number of cellular responses ranging from granule secretion integrin activation to transcriptional responses regulating proliferation. $G_{\beta\gamma}$ activate phosphoinositide -3 kinase(PI3-K) and other pathways, such as activation of G proteins receptor kinases (GRKs), potassium channels (K^+), and nonreceptor tyrosine kinases (TK). PI3-K modifies the inner leaflet of plasma membrane providing attachment sites for a number of host signaling proteins Adpated from (67).

include multiple kinase pathways- as the phosphoinositide-3 (PI-3) kinase, mitogen-activated protein (MAPK) kinase, Rho kinases, c-Jun N-terminal kinase and protein kinase C (67)**.** PAR1 agonists also activate transcription factors such as nuclear factor-κB (NF-κB). Activation of NF-κB is mediated by G protein subunits through the kinase pathways (376). Consequently, NF-κB is translocated to the nucleus and initiates transcription of genes involved in metastasis, survival and angiogenesis, such as MMPs, the anti-apoptotic gene Bcl-2, and vascular endothelial growth factor. Of functional relevance, it has been shown that PAR1 dependent activation of NF-κB p65 promotes survival of cancer cells and increases expression of the pro-inflammatory cytokines IL-6 and IL-8 (465).

1.5.3 PAR and coagulation factors

Coagulation proteases are not only responsible for the generation of insoluble fibrin clot but are also potent cell modulators that can induce important intracellular and extracellular regulatory processes through PARs (96,152,289). Thrombin mediates its effects through cleavage of PAR1 and PAR4, but not PAR2. The upstream TF-FVIIa complex can activate PAR2 and FXa has been shown to activate PAR1 and PAR2 (35,36,392). Further, studies have established that the ternary complex TF-FVIIa-FXa can also signal through PAR2 (475). Thrombin released from the cell must rapidly re-associate in close proximity to the PAR scissile bond (409) to facilitate cleavage prior to efficient inactivation by circulating serpins. However, FVIIa or FXa remain cell surface-localized and concentrated through cofactor or phospholipid interactions proximal to PARs (393). In this way PAR activation by coagulation proteases remains localized.

Thrombin mediated PAR1 signaling (Figure 20) leads to shape changes in

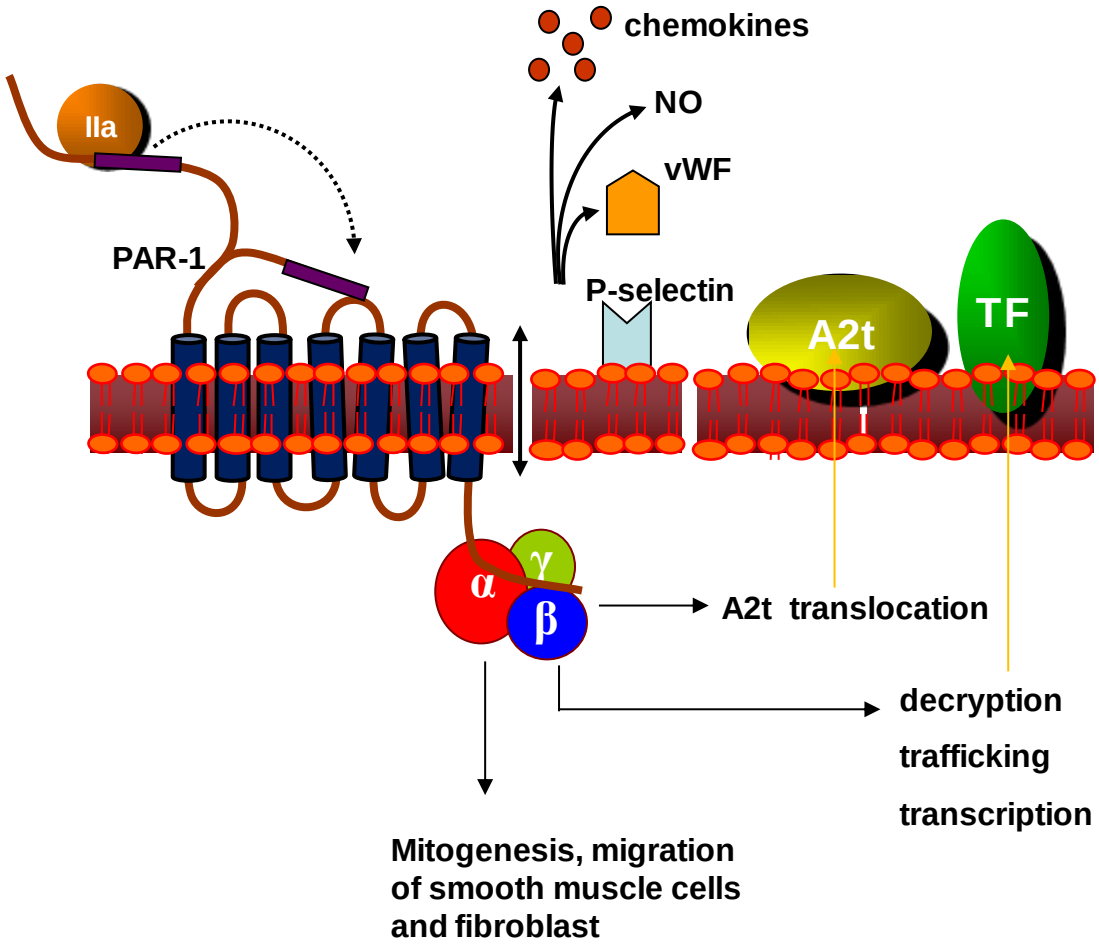


Figure 20 Thrombin mediated PAR-1 signaling. Thrombin increases the permeability of the endothelium allowing the exudation of plasma proteins and cellular proteins, modulates P-selectin on plasma membrane, vWF secretion, chemokine production, NO release, and increases cell surface expression of A2 by translocation and TF through decryption, trafficking and transcription. Thrombin is also mitogenic leading to proliferation and migration of smooth muscle cells. Adapted and modified from (69).

platelets as well as permeability and migration in endothelial cells. (69,164,224,231). PARI activation results in decreased levels of cAMP levels and inhibits adenylyl cyclase, an inhibitor of platelet activation. Thus, thrombin can activate platelets through PAR1 activation, through decreased levels of adenylyl cyclase and cAMP (69). PAR activation up-regulates the functional expression of adhesion molecules on the endothelial surface and platelet adhesion occurs (163,204). The platelet procoagulant activity that results enhances thrombin generation. Thus endothelial and platelet stimulation through PARs is a mechanism of positive feedback in which thrombin generation is accelerated (347). Our laboratory also reported thrombin-induced cell surface A2 expression that participates in plasmin generation (362). Evidence has also been reported that shows A2 presents FXa for more efficient stimulation of cells through PAR2 (16). Both may play a role in viral infection.

1.6 Herpesviruses

1.6.1 General characteristics

Herpesviruses are a family of DNA viruses with a core icosahedral nucleocapsid (that is approximately 100-300 nm in size). Inside the nucleocapsid is a linear double-stranded DNA genome. The nucleocapsid is surrounded by an amorphous layer of proteins termed the tegument, surrounded by a phospholipid bilayer envelope (Figure 21) (146,313).

The common biological features of the Herpesvirus family include the following: 1) the genome encodes enzymes that are involved in DNA synthesis, nucleic acid metabolism and processing of host and viral proteins. 2) The assembly of new progeny virus occurs in the nucleus with subsequent envelopment through cellular membranes;

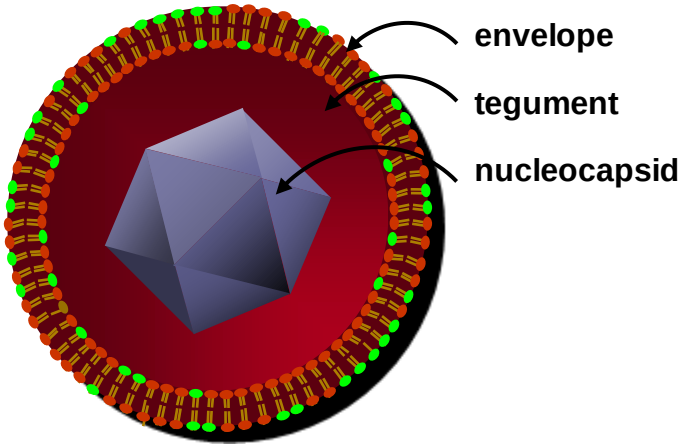


Figure 21 Herpesvirus virion architecture.The virion constitutes the envelope, the tegument and the nucleocapsid. The nucleocapsid is icosahedral in shape and enclosed within is the viral genome. The nucleocapsid is surrounded by the tegument, an amorphous layer of proteins globular structure. Finally, the outermost is the envelope, a phospholipid bilayer derived form the host cell that constitutes both viral and host genome encoded proteins.

3) successful infection results in cell destruction, and 4) the ability to form lifelong latent infections (404). In cells harboring latent virus, the viral genome is a circular molecule, with limited gene expression until the dormant state is broken by poorly understood stimulation mechanisms (82,402).

1.6.2 Herpes simplex virus

Herpes simplex virus type 1 (HSV1) and type 2 (HSV2) are members of the alpha herpesvirus family (19). They are highly prevalent human pathogens with 80-90% of the population expressing antibodies to HSV1 by the age of 50, while 20% are positive for antibodies to HSV2 by the same age (492). Predominantly, HSV1 is the causative agent of cold sores and HSV2 of genital herpes. HSV1 is transmitted mainly by contact with oral secretions; and HSV2 is transmitted by contact with genital secretions (519). The classification of HSV1 and HSV2 as exclusively non-genital and genital respectively is inaccurate. They are predominantly neurotropic and have the ability to establish latency in sensory ganglia (82,312).

HSV1 and HSV2 have common clinical manifestations that include gingivostomatitis labialis, whitlow, keratitis and eczema (432). In immunocompetent individuals, HSV infections are mostly restricted to their initial sites of entry i.e., muco-epithelial cells. However, serious complications can occur in immunosuppressed individuals and neonates, as a result of dissemination. HSV dissemination via blood to the vital organs can cause HSV hepatitis (7,324) and herpes simplex encephalitis (HSE) (219). A recent study reported HSV viremia in individuals with primary genital herpes (201). The dissemination is more common in imunocompromised individuals because of other preexisting infections, extreme age or congenital defect in immune system. Children with congenital disorders of the thymus are also likely to develop unusually

severe HSV infection. As a consequence of dissemination the infection may spread to the liver, spleen, lungs and in rare instances to the central nervous system (CNS). HSV hepatitis can occur, but confirmed cases are extremely rare (324). Herpes simplex encephalitis (HSE) is a relatively infrequent manifestation of HSV infection. It may result from a primary, but more commonly upon reactivation of latent infection. HSE is a serious complication and is associated with significant morbidity and mortality when there is CNS involvement in neonatal HSV infection (197,219).

HSV1 and HSV2 share genetic homology (91,298) and a high degree of similarity at the molecular level. The genome structure and morphological appearance of the viruses are almost identical.

1.6.2.1 Morphology

Nucleocapsid

Within the nucleocapsid lies the virus genome, which is predicted to encode approximately 80 genes. The nucleocapsid is a highly ordered structure consisting of repeating pentameric and hexameric subunits (525). The capsid itself is made up of five different virus-encoded proteins of which VP5 (the major capsid protein) is the most abundant (525). The assembly of the capsid proteins occurs in the nuclei of infected cells and coincides with replication of the progeny viral DNA (430). Prior to completion, the viral DNA is inserted into the capsid. Once completed, the nucleocapsid exits the nucleus by budding through the nuclear membrane and enters the cytoplasm (313,439).

Tegument

The tegument is the protein layer in between the viral capsid and the envelope. It is non-structured and contains several proteins (154). When the virus uncoats along

with nucleocapsid the tegument proteins are released in the cytoplasm. The tegument proteins include virion host shutoff (vhs) protein and VP16 protein that take control of the cell machinery and subvert it to virion production. The vhs protein rapidly degrades the cellular mRNAs disabling host protein synthesis. The VP16 is transcription activator that is transported to the nucleus and induces viral DNA transcription (276,327), and other tegument proteins are involved in capsid trafficking within the cell.

Envelope

The envelope is the outermost layer that surrounds the mature virions. It is made up of a phospholipid bilayer that is solely derived from the host cell membrane. Strikingly, the virus envelope has higher aPL content than the cell membrane from which it is derived (482). Additionally, embedded in the envelope are virus-encoded glycoproteins and host cell proteins acquired from the cell upon egress (456,457,515). The envelope proteins are important in virus-mediated interactions with the host cells and subvert the host defense systems, contributing to the pathology. The envelope proteins actively control biochemical processes of the host to facilitate viral replication. The viral glycoproteins identify and bind to receptor sites on the host's membrane followed by envelope fusion, allowing the capsid and viral genome to enter and infect the host (400). In this thesis, the role of herpesvirus envelope proteins in modulation of the host hemostatic system is investigated to understand the molecular basis of their clinical correlation to vascular diseases.

1.6.2.2 Infection

Attachment

The virus infection mechanism begins with attachment of the virus to the host cell.

Virus entry into the cell is subsequently mediated through multiple interactions between cell surface receptors and viral envelope proteins. The envelope constitutes more than a dozen virally-encoded glycoproteins, of which five of them gB, gC gD, gH and gL are known to participate in entry (400). The virus infection involves attachment and entry mediated through a sequential interaction of virus surface proteins with a binding receptor and entry coreceptor respectively. The first interaction is between virus-encoded gB (49) and/or gC with cell surface heparan sulfate(HS) (250). HS is a glycosaminoglycan (GAG) on cell surfaces, typically attached covalently to a core protein such as syndecan forming a HS proteoglycan. Cell surface HS mediates interactions with a variety of extracellular ligands such as growth factors and adhesion molecules. Cell surface HS can act as both a binding receptor and entry coreceptor to mediate virus infection(381). Infection can be inhibited in cells lacking HS either due to a mutation or upon enzymatic digestion. Further, lack of either gC or gB on the virus surface reduced HS-mediated binding to cells, while in cases of viruses lacking both gC and gB binding was completed inhibited (49,177,178,461). The initial interaction with HS facilitates the subsequent interaction of virus gD with entry coreceptors, which include herpesvirus entry mediator (HVEM)(508), nectins (170) and 3-O-sulfated heparan sulfate (3OS-HS), a modified form of HS (433). The entry coreceptors are structurally unrelated. HVEM belongs to the tumor necrosis-factor family (321) and nectins belong to the immunoglobulin super family. These unrelated molecules bind gD independently and do not act as coreceptors during entry. A list of interactions for HSV1, HSV2 and CMV between virus surface glycoproteins and cell surface molecules that acts as receptors and coreceptors for attachment and entry is included in Table 3(170). The molecular nature of the association of HSV1 and HSV2 with target cells depends on the host cell type, and is a complicated mechanism that is still incompletely resolved.

Virus type	Virus glycoprotein	Cell surface molecule	Function
HSV1	gC	HS	Attachment
	gB	HS	
	gH/gL	Integrin	
	gD	HVEM	Entry
	gD	Nectin-1	
	gD	3OS-HS	
HSV2	gC	HS	Attachment
	gB	HS	
	gD	Nectin-1	Entry
	gD	3OS-HS	
CMV	gB	HSPG	Attachment
	gB	TLR	Entry
	gB	Integrins	
	gB	EGFR	
	gO-gH/gL	Integrins	

Table 3 Functional interaction between virus surface glycoproteins and cell surface molecules.Numerous virus surface glycoproteins interact with cell surface molceules that act as binding receptor and entry coreceptors for attachment and entry respectively(170). herpesvirus entry mediator (HVEM), heparin sulphate proteolglycan (HSPG), 3-O-sulfate heparin sulphate (3OS-HS), epidermal growth factor receptor(EGFR) and toll-like receptor(TLR) (170)

Entry

The virus attachment to receptors and entry coreceptors triggers the fusion of virus envelope with the host cells plasma membrane, leading to cell entry. This fusion is mediated by four virus-encoded proteins: gB, gD, and a heterodimer of glycoprotein H (gH)/glycoprotein L (gL) (34,260). gB, gD and gH are integral in membrane fusion, while gL is required for proper conformation of gH (190). Consequently, the viral capsid containing the viral genome and tegument proteins are released into the cytoplasm. The capsid then travels along a microtubule towards the nucleus where it docks with a nuclear pore (444). The viral DNA enters the nucleus through the pore and circularises before replication (127)(195). Participating in both binding and fusion to the host cell, several of the virally encoded proteins are multifunctional and dissection of their individual functions has been an experimental challenge.

Replication

The viral vhs protein from the tegument rapidly degrades host cell mRNA and also promotes synthesis of viral mRNA and DNA (107)(246,441). Upon delivery to the nucleus, the viral DNA is ready to be transcribed and replicated. An important characteristic of herpesvirus genomes is that they encode most of the enzymes that are involved in DNA synthesis and replication, nucleic acid metabolism and processing of host and viral proteins(19). The transcription of herpesvirus proteins is temporal and sequential. It involves transcription of three classes of genes: immediate early (α), early (β) and late genes (γ) (45). The VP16 tegument protein activates the expression of α genes (247). The α genes encode DNA binding proteins and promote transcription of β genes. The β genes encode DNA synthesis proteins and promote transcription of γ genes. Upon completion of DNA replication, the γ genes encode capsid structural proteins and glycoproteins.

Assembly

The synthesized structural proteins are transported back to the nucleus for capsid assembly. The newly synthesized viral DNA is inserted into capsids and the nucleocapsid then travels into the cytoplasm where tegumentation occurs. An envelope is derived from the host cell prior to viral egress (404). Recent studies suggest that herpesviruses undergo a double envelopment process (439). The newly assembled nucleocapsid buds through the inner nuclear envelope gaining a temporary envelope. The temporary envelope then fuses with the outer nuclear envelope and the naked nucleocapsid is released into the cytoplasm. The nucleocapsid then buds into Golgi vesicles laden with viral proteins. Upon fusion with Golgi vesicle membrane and plasma membrane, mature virions are released (27,470)(200,301).

1.6.3 Cytomegalovirus

1.6.3.1 Morphology

CMV belongs to the beta herpesvirus family (401,403). It shares many attributes with other herpes viruses, including genome, virion structure, and the ability to cause latent and persistent infections. It has the largest genome of the herpesviruses (47). Amongst the herpesviruses, the CMV mature virions have the largest diameter of ~150-200 nm and are known to exist in non-infectious forms that include dense bodies and non-infectious enveloped particles (192). The dense bodies lack nucleocapsid with some reports suggesting they may also lack viral DNA. The non-infectious enveloped particles have nucleocapsid but no DNA. CMV infection characteristics are enlarged cells bearing intranuclear inclusions and a long reproductive cycle, which ultimately leads to cultured cell rupture and death.

1.6.3.2 Pathology

CMV exposure is reported in 50-80% of the adult population in North Americans and nearly 100% in the developing world (12,39,449). CMV is an opportunistic pathogen, typically not becoming viremic unless an individual is immunocompromised, as seen in AIDS patients, organ transplant recipients, immunosuppressive therapy recipients and neonates (93,172,197). In neonates, there is CNS involvement and severe sepsis that is often fatal. The other complications include severe retinitis in AIDS, and subclinical myocarditis in transplant recipients. In short, disseminated CMV infection in the immunodeficient state is a complication that increases morbidity and mortality.

1.7 Herpesviruses in vascular disease

1.7.1 Clinical correlations

There is abundant epidemiological data spanning over more than three decades associating herpesviruses to vascular disease. These include a number of retrospective and investigative cohort studies that suggests vascular disease is associated with the common herpesviruses, HSV1, HSV2 and CMV.

A 2-fold increased risk of myocardial infarction and coronary heart disease mortality in HSV1 seropositive patients as compared to HSV1 seronegative has been reported (438). A similar observation was made with an increased risk of 2 and 1.5 fold, respectively for CMV and HSV2 seropositivity (526). Fibrin deposits in the microvasculature of mucosal lesions were linked to HSV1 infection (304,417). In neonates, severe HSV1 infection was linked to disseminated intravascular coagulation. Prior CMV infection was linked to subclinical and clinical arterial thickening (50,341). In

immunosuppressed cardiac transplant recipients, a more frequent rejection was associated to an active CMV infection, correlated to an accelerated graft atherosclerosis. CMV infection was shown to be a strong risk factor for restenosis after angioplasty (99,524). Further, virus genomes and antigens from both HSV1 and CMV have been observed in endothelial and smooth muscle cells of atherosclerotic tissue (1,13,72,153,173,174,307-309). In patients with atherosclerosis, activation of both platelets and blood coagulation and an increase in fibrin turnover are detectable that lead to thrombotic complications. Further, virus genomes in biopsy specimens of cerebro-vasculitis of temporal artery have been detected (370).

When combined with well established risk factors for vascular disease such as, smoking (438), hypertension and hypercholesterolemia (341), herpesviruses strongly correlated to vascular disease. A report found an increased positive correlation of HSV1 infection as a risk to cardiovascular disease in smokers (438). Another study observed an increased risk of myocardial infarction and cardiovascular death in patients seropositive for CMV (odds ratio 1.31) that strongly increased in patients with diabetes and CMV seropositive (OR 2.58) (147). Additionally, reports have found that increased pathogen burden increased the risk of incident myocardial infarction or death in CHD patients and atherosclerosis (103,104,479,526). The pathogen burden is defined as multiple infections in an individual. Although significant, the clinical correlation of herpesvirus to vascular disease is relatively weak when evaluated as an independent variable. Nevertheless the prevalence of these viruses suggests that they may be an overlooked variable toward understanding how to predict the propensity of an individual's susceptibility to disease severity. Furthermore, herpesviruses offer an excellent model for the general contribution of enveloped viruses in vasculopathy, which

certain viruses may have even greater involvement.

The data demonstrating a serological or anatomic association of herpesviruses to vascular diseases are not entirely consistent. Although most studies show a more significant association with coronary artery disease (CAD) or restenosis, others do not (167,431,435). A large multiethnic study of atherosclerosis found that HSV1, HSV2 and CMV serostatus had no effect on development of atherosclerosis and incident myocardial infarction (459). Also within the studies that found association linking herpesvirus infection to adverse cardiac outcomes using serological evidence, at times the risk for a particular herpesvirus type was significant, but not so significant for other types (438,526). A reason for these discrepancies is assay method, where a negative serology does not always rule out prior infection that has cleared. The high seroprevalence of herpesviruses, multiple risk factors for vascular disease and in-built biases of epidemiological study designs are bound to result in conflicting observations. Thus, the independent contribution of herpesviruses to vascular disease may be weak, but is more significant in combination with other vascular risk factors and increased pathogen burden. Nevertheless, in animal models a direct cause and effect relationship of herpesvirus infection to vascular disease was evaluated.

1.7.2. Animal model correlations

A direct causality cannot be ascertained from the extensive retrospective clinical observations. The clinical studies are difficult to control because of the high seroprevalence of herpesviruses in the population and the multiple confounding risk factors of vascular diseases. Thus, in order to ascertain a direct role of virus contribution to vascular diseases, animal models were utilized. A distinct cause-and-

effect relationship has been established using several animal models of virus infection to confirm that herpesviruses accelerate thrombosis and atherosclerosis (109,446,488). Atherosclerosis may be considered to be the precursor of CHD. Atherosclerotic plaque ruptures lead to new thrombotic events that cause CHD. The pathologies of thrombosis and atherosclerosis are intimately connected.

An avian model of herpesvirus induced atherosclerosis in pathogen free chickens was first studied. High serum levels of cholesterol are associated with atherosclerosis. Briefly, two groups of normocholesterolemic and hypercholesterolemic chickens were infected with an avian herpesvirus, Marek's disease herpesvirus (MDV). Infection with MDV resulted in atherosclerosis in both normocholesterolemic and hypercholesterolemic chickens, while in the absence of this pathogen none of the two groups developed atherosclerosis. Further, MDV genome was detected in the arteries of infected chicken. Similar to lesions in human atherosclerosis, the lesions in chicken had extensive proliferation of smooth muscle cells within the intima and increased cholesterol ester in aortic smooth muscle cells(108,109).

In a rat model of induced vascular injury, CMV infection resulted in atherogenic lesions and lipid accumulation in the endothelium (446). In a rat solid organ transplantation model, acute infection with CMV accelerated transplant vascular sclerosis, leading to graft rejection (352). Finally in a mouse model, gamma herpesvirus infection resulted in thrombosis (488). Thus, animal models demonstrated herpesvirus infection lead to vasculopathy. These animal models allowed to focus on the contribution of herpesvirus infection to vascular diseases while controlling for other risk factors. Although the atherosclerotic lesions in these animal models bear resemblance to human lesions there are certain caveats. Atherosclerosis is typically a chronic

condition that develops over decades in humans with an average life span of 75 years, whereas, this is induced quickly in experimental animals The average life span of a mouse is about 2 years and they are naturally resistant to atherosclerosis. The two species differ in their major carrier of plasma cholesterol; low-density lipoprotein for humans and high-density lipoprotein in mice. In humans high-density lipoprotein is protective against atherosclerosis. Mice fed their normal low-fat chow diet do not get atherosclerosis, while it is a common disease in humans. An important consideration of these studies is usage of a relatively high viral dose, which usually exceeds that typifying viremia in humans Nevertheless; these independent animal studies demonstrate clearly that herpesvirus infection leads to atherosclerosis and thrombosis compelling more detailed work on interactions between herpesviruses and the haemostatic system.

1.7.3 Cellular basis of virus-induced vasculopathy

1.7.3.1 Induction of prothrombogenic changes to host cells

Numerous cell culture studies have reported the ability of herpesviruses to convert resting host cells, which are intrinsically anticoagulant to a prothrombotic phenotype. Upon CMV(471,472) and HSV1 (105,106,491) infection, resting vascular endothelial cells were transformed from a noncoagulant to a procoagulant state. The HSV1 induced procoagulant phenotypic changes in endothelial cells include, TF expression (486) and rearrangement of phospholipid membrane exposing aPL (491). Further, HSV1-infection of cells attenuated inherent anticoagulant properties of resting cells, including; 1) reduced expression of TM (486), a cofactor within the Protein C-dependent anticoagulant pathway; 2) reduced synthesis of HS (210,212), a cofactor

within the serpin-dependent anticoagulant pathway; and 3) decreased prostacyclin secretion (491), a platelet activation inhibitor. Compared to HSV1, CMV-induced phenotypic cellular changes have not been studied as extensively. The procoagulant changes due to CMV include TF expression, rearrangement of the membrane phospholipid and the secretion of vWF (471,472,476). Thus virus infection disrupts the hemostatic balance between coagulation and anticoagulation of cells to favor thrombin production.

1.7.3.2 Induction of atherogenic changes to host cells

The hallmarks of atherosclerotic plaque (196,309) include smooth muscle cell proliferation, adhesion of inflammatory cells and lipid accumulation (196,309). Atherogenesis is believed to follow a response to endothelial injury, which exposes the underlying arterial smooth muscle cells to stimuli causing them to proliferate excessively. The thrombin generated at the site of injury also act as a cell stimulant and links sub-clinical CMV or HSV infection to atherosclerosis (304,307,309,486). Research is ongoing to better understand abnormal lipid metabolism and the cellular abnormalities of lesions in the vascular wall that promote atherosclerosis.

Recent work has elucidated biochemical mechanisms associating atherogenesis and CMV. Expression of CMV gene products has been linked to smooth muscle cell proliferation and accumulation of oxidized low density lipoprotein. The CMV gene product, IE84, is capable of binding to the p53 tumor suppressor protein to induce smooth muscle cell proliferation (447). Furthermore, another CMV gene product, IE72, triggers the synthesis and expression of the “scavenger” receptor for oxidized low density lipoprotein deposition (448,523). The cumulative biochemical evidence provides a molecular explanation for the clinical studies.

1.7.4 The procoagulant herpesvirus surface

While highly informative, none of the studies that demonstrated herpesvirus-induced thrombogenic and atherogenic changes to host cells eliminated the possibility that the virus itself is capable of generating thrombin or other hemostasis proteases, with respective functional implications. Thus, our studies showing that the purified HSV1, HSV2 and CMV surfaces initiate the coagulation cascade (372,456,456,457) likely reveal the earliest step of the mechanism linking viruses to vascular pathology.

Our laboratory was the first to demonstrate that independent of cells, HSV1, HSV2 and CMV activated FX leading to thrombin generation (457). Through electron microscopy the demonstration of coagulation initiators TF and aPL on the purified virus surface confirmed the procoagulant ability of viruses. Additional studies revealed TF-independent FX activation on the virus surface (456) (Figure22). The HSV1-gC involvement in FX activation was implicated using a gC deficient virus strain (269). The gC involvement was confirmed in a purified system with recombinant gC enhancing FVIIa mediated activation of FX. Further, our laboratory also demonstrated that thrombin, FVIIa and FXa generation is advantageous for virus, as it enhanced cell susceptibility to virus infection (455). Moreover, thrombin induced expression of endothelial cells surface plasminogen A2, which has been shown to enhance membrane fusion leading to increased infection at least for CMV (362,384) (Figure 23). Thus, the herpesvirus surface constituents, host cell derived aPL and TF, and virus genome encoded gC may initiate coagulation as an early event in vasculopathy (457). This suggests that during viremia, especially in immunocompromised individuals, virus infection should be a strong independent predictor of vascular disease.

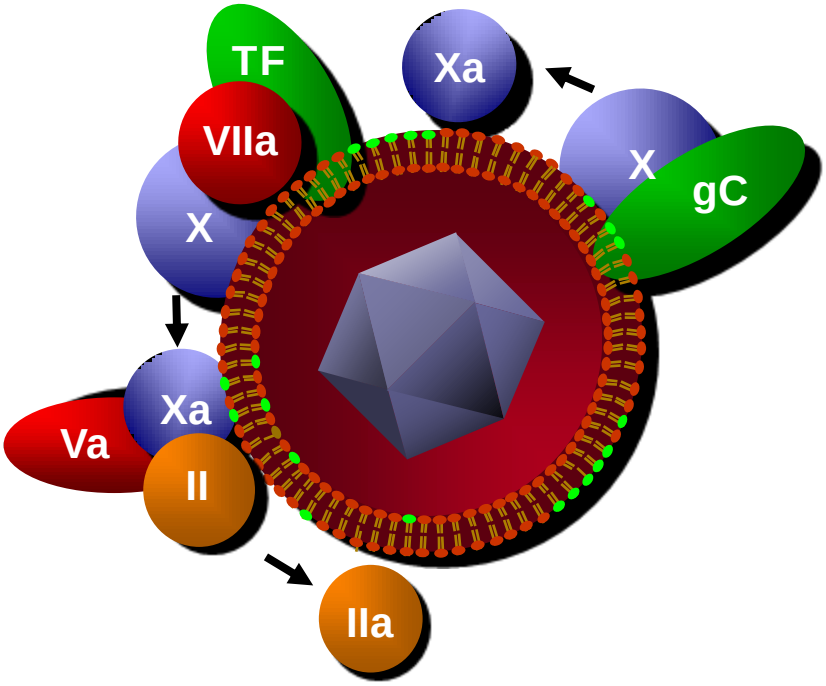


Figure 22 Coagulation initiated on herpesviruses. Thrombin generation on the surface of herpesviruses is initiated by two independent pathways. Tissue factor (TF) and anionic phospholipids (aPL) on the surface of herpesvirus acquired from the host cells and virus genome encoded glycoprotein C (gC) activate FX to FXa in the presence of FVIIa. Additionally the assembly of prothrombinase complex leading to thrombin generation.

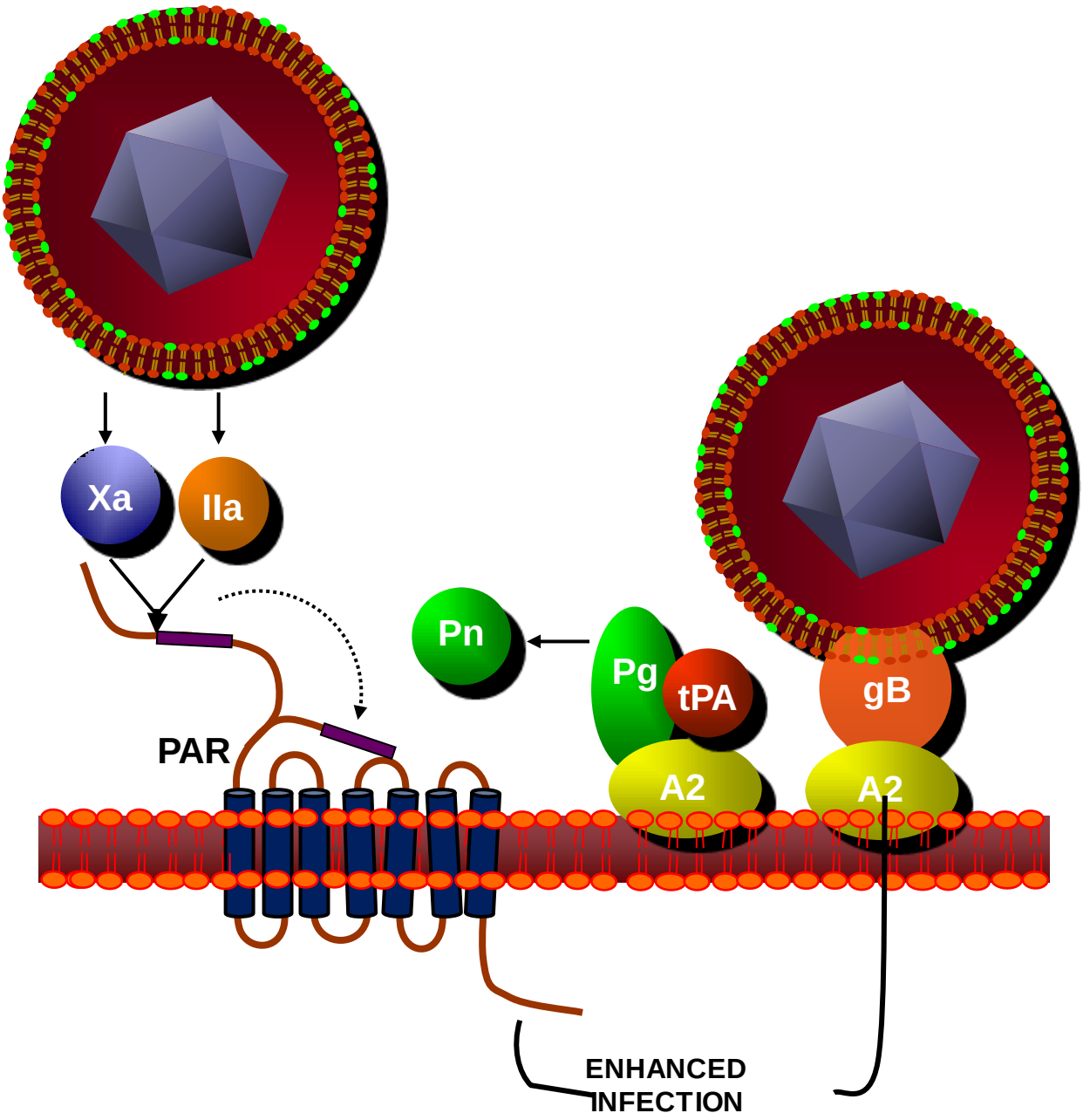


Figure 23 Herpesviruses initiate coagulation to enhance cell infection through cell modulation. Thrombin and FXa generated by herpesviruses enhanced cell infection through protease activated receptor (PAR) signaling. At least for CMV, the enhanced infection is due to increase cell surface expression of annexin 2 (A2), receptor for virus entry. A2 is also a fibrinolytic cofactor mediating tPA dependent plasminogen (Pg) activation to plasmin (Pn).

1.8 Rationale

Prior to work in our laboratory, herpesviruses had been implicated in vascular disease at three experimental levels; clinical, animal and cellular. The concept of herpesvirus infections causing vascular disease is intriguing because they are highly prevalent in the human population and establish a life long latent state. Therefore, these viruses have the potential to play a critical role in long-term chronic disease processes, which perfectly matches the etiology of vascular disease. As such, these pathogens can be found in the walls of affected vasculature and have been shown to modify the host cellular phenotype to a procoagulant state. While the pathogenesis of vascular disease is multifactorial, herpesviruses are a probable contributors that have been largely overlooked as predictors of severity and probability.

To understand the initiating links between viruses and vascular disease, our laboratory is studying direct links between viruses and hemostatic proteins. Previous work demonstrated that purified HSV1, HSV2 and CMV possess TF and aPL derived from host cells and these induced plasma clot formation by passing the normal host cell regulation of coagulation (457). This provided further biochemical evidence for the clinical observations of herpesvirus association with vascular disease. The robust in vitro procoagulant activity suggests that herpesvirus infection should be a strong independent risk factor for vascular diseases.

To explain the discrepancy between striking in vitro coagulation potential of herpesviruses and their relatively modest independent clinical correlation to vascular disease, this thesis work addressed the general hypothesis that the herpesvirus envelope constituents activate both coagulation and fibrinolysis, thereby attenuating the thrombotic effects of clot formation. The latter was based on the knowledge that the

tPA-enhancing cofactor A2 has been reported on at least one herpesvirus, CMV. Our earlier work furthermore suggested that thrombin produced by the virus enhanced infection by stimulating cells through PAR1. Therefore an extension of the hypothesis addressed here is that like thrombin, plasmin also enhances infection.

1.8.1 Coagulation pathway

To provide further insight into how herpesviruses initiate coagulation in addition to the TF-dependent pathway already established on the virus surface, the first part of this thesis investigates the intrinsic and contact pathways as modes by which the virus can activate and amplify coagulation. The specific goals were:

1. To determine the role of FVIII in amplifying virus-initiated coagulation.
2. To determine if the virus can facilitate contact pathway-mediated initiation of coagulation.

1.8.2 Fibrinolytic pathway

To explain why herpesvirus infection is only a weak independent predictor of vascular risk despite the strong in vitro procoagulant activity of purified viruses, herpesvirus mediated fibrinolysis was studied. The possibility that herpesvirus associated A2, known to be on at least CMV (384,515), or other virus constituents participate in fibrinolysis was investigated.. The specific goals were:

1. To determine if purified viruses enhance plasmin generation and whether A2 plays a role.
2. To determine whether purified viruses enhance fibrin clot lysis.

2. METHODS

2.1 Reagents

Normal plasma, pooled normal plasma, congenital FVIII deficient plasma and congenital FVII deficient plasma (George King BioMed, Overland Park KS), corn trypsin inhibitor (CTI), human factor XII (FXII) and anti-FXI monoclonal antibody (mAb) (5061; Haematologic Technologies Inc, Essex Junction VT), human PK (PK) (Enzyme Research Laboratories, South Bend, IN), anti-FXII heavy chain mAb (B7C9; Affinity Bioreagents, Rockford IL), anti-PK mAb (13G11; Abcam, Cambridge MA), and APTT reagent (Organon Teknika Corp., Dublin Ireland) were obtained commercially. Anti-A2 FVIII mAb (#413) (271) and purified recombinant FVIII (rFVIII) were produced as reported (168).

2.2 Proteins

Recombinant tPA (Genentech San Francisco, CA, USA), Human plasma derived Lys-plasminogen, plasmin, thrombin (Haemtologic Technologies, Essex Junction, VT, USA), fibrinogen (Enzyme Research Laboratories, South Bend, IN, USA), mouse monoclonal anti-A2 antibody (Invitrogen, Camarillo, CA, USA), aprotinin (Calbiochem, Gibbstown, NJ, USA), and epsilon aminocaproic acid (EACA) (Sigma, Oakville, ON, Canada) were purchased commercially. Recombinant A2 (Dr. David Waisman, Dalhousie University) and recombinant plasminogen activator inhibitor (PAI-1; Dr. Thomas Podor, University of British Columbia) were kind gifts.

2.3 Virus preparation

HSV1, HSV2 and CMV were cultured in various cell lines, purified by ultracentrifugal

density techniques and quantified by electron microscopy as follows. HSV1 NS is a low-passage clinical isolate (117). A strain of NS has been produced in which gC was removed and then restored (RNS) and is essentially identical to NS (116).

HSV1 NS strain was propagated in African green monkey kidney cells (Vero, ATCC CCL-81) (403), referred to as HSV1/V, and Human umbilical vein endothelial cells (HUVEC, ATCC CRL-1730) referred to as HSV1/E. HSV1 RNS was propagated in A7 melanoma cells (the cells were a kind gift from Dr. Wolfram Ruf, Scripps Research Institute, La Jolla, CA) and referred to as HSV1/M. A well-characterized laboratory strains of CMV (AD169) was propagated in human foreskin fibroblasts (HFF, ATCC CRL-2056), termed CMV/F. HSV2 strain G (Advanced Biotechnologies Inc., Columbia, MD, USA) was propagated in African green monkey kidney cells (Vero, ATCC CCL-81), referred to as HSV2/V.

Cells were grown to approximately 85% confluent in T175 culture flasks at 37°C in an atmosphere of 5% CO_2 and then inoculated with individual virus strains. Mature virus was harvested from the clarified infected cell supernatant at 23,000 X g. Virus pellets were then resuspended in hepes (N-[2-Hydroxyethyl] piperazine-N1-[2-ethanesulfonic acid]) buffered saline (HBS) and subjected to ultracentrifugation at 200,000 X g through a 10/30/60% stepwise sucrose gradient (456). The virus band was removed, pelleted and resuspended in HBS, then frozen at -80°C. All virus preparations were purified and evaluated for quality and quantified to derive virus particle number per millilitre (vp mL^{-1}) by negative staining electron microscopy (372). Less than 10% of particles in the virus preparations were attributed to cellular debris.

2.4 Cell culture

Vero cells were grown in Medium 199 supplemented with fetal calf serum (5%) and gentamycin (2 μg mL^{-1}) (Gibco). Once complete monolayers were formed (4 to 6 days), Experiments were conducted on Vero up to and including passage 10. Human foreskin fibroblasts (HFF, ATCC CRL-2056) were obtained from American Tissue Cell Collection (ATCC, Manassas, VA, USA), and grown in Basal Medium Eagle containing BCS (5%), glutamine (14 μM), and gentamycin (2 μg mL^{-1}) (complete media) (Gibco). For maintenance, cells were split at a ratio of 1:6 every 6–7 days. Experiments were conducted on HFF up to and including passage 13. HUVEC were obtained from ATTC (CRL-1730) and maintained in complete media: Iscove's Modified Dulbecco's Medium supplemented with FBS (20%), glutamine (14 μM), gentamycin (2 μg mL^{-1}), fungizone (2.5 μg mL^{-1}) (Invitrogen), heparin (20 U mL^{-1}), and endothelial cell growth supplement (ECGS; 20 μg mL^{-1}, BD, Mississauga, ON, Canada). Cells were split at a ratio of 1:4 every 3 days into 0.2% gelatin-coated flasks or 24-well plates. Experiments were conducted on HUVEC up to and including passage 10. A7 were grown in Earl's minimum essential medium (EMEM) supplemented with newborn calf serum (8 %), FBS (2%), glutamine (2 mM), HEPES (10 mM) and geneticin (500 μg mL^{-1}) (Invitrogen).

2.5 Clotting assays

FVIII: In manual tilt clotting assays, purified HSV1 (at indicated concentrations) and constant aPL (50 μM; small unilamellar vesicles (SUV) composed of 75%:25%, L-alpha-phosphatidylcholine:L-alpha-phosphatidylserine) (235) were incubated for 5 minutes at 37°C with either 1) congenital FVIII deficient plasma in the presence or absence of

rFVIII or 2) normal plasma in the presence or absence of anti-FVIII mAb. At this point pre-warmed $CaCl_2$ (20 mM) at 37°C was added to initiate clotting. All reagents were diluted to final concentrations in Hepes buffered saline (HBS; Hepes, 20 mM; NaCl, 150 mM; pH 7.2).

Contact pathway: To preclude the participation of the extrinsic pathway of coagulation, congenital FVII-deficient plasma was used in manual tilt clotting assays as above except HSV1 was kept constant (3.3×10^{10} vp mL^{-1}) in the presence or absence of CTI, anti-FXII mAb (1 µM), anti-PK mAb (0.45 µM), anti-FXI mAb (13.3 nM) or isotype control mAb. TF on the HSV1 surface was inhibited using anti-TF mAb to determine whether trace amounts of FVII in congenital FVII deficient plasma contribute to procoagulant activity. aPL was excluded from the reaction mixture to avoid virus-independent autoactivation of FXII activation.

2.6 Blotting assay

2.6.1 Antibody blotting for FXII

Purified FXII (0.5 µM) was incubated with or without HSV1 (3.3×10^{11} vp mL^{-1}) in the presence or absence of PK (0.3 µM) at 37ºC. At zero and 15 minutes incubations, 2 µL were added to Laemmli sample buffer and were electrophoresed on 8% SDS-PAGE under reducing conditions. The electrophoretic pattern was transferred to a polyvinylidine difluoride (PVDF) membrane (Millipore, Mississauga, ON, Canada), blocked with 5% skim milk, 0.05% Tween-20, pH 8.0 in HBS and then incubated with 100 ng mL^{-1} anti-FXII. After washing, the PVDF membrane was incubated with a secondary Ab, a horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove PA) for 60 minutes and again washed. Antigen was

detected using ECL-Plus chemiluminescent reagent (GE Healthcare, Piscataway, NJ, USA) followed by documentation using a ChemiGenius^2 image analyzer (PerkinElmer, Woodbridge, ON, Canada). Molecular weight markers (Precision Plus) were from Bio-Rad, Mississauga, ON, Canada.

2.6.2 Antibody blotting for A2

A 10 µl final volume of purified herpesvirus ($5x10^{11}$ vp mL^{-1}) in HBS and Laemmli sample buffer were subjected to 10% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) under reducing conditions, transferred to PVDF membrane and blocked as above and then incubated with 0.6 nM anti-A2 Ab (Invitrogen) for 60 minutes at room temperature. The PVDF membrane was washed, incubated with a secondary antibody, again washed and antigen was detected as described earlier. The electrophoretic location of A2 was confirmed by comparison to purified rA2 and molecular weight markers.

2.6.3 Ligand blotting for plasminogen

Purified virus ($5x10^{11}$ vp mL^{-1}) was subjected to SDS-PAGE under reducing conditions, transferred to PVDF and blocked, as above. After washing, the PVDF membrane was incubated with horseradish peroxidase-conjugated plasminogen (Plasminogen-HRP, 1 nM) using Lightning Link reagent (Innova Biosciences, Cambridge, UK), for 60 minutes and again washed. Plasminogen-HRP binding to bands was detected and the apparent molecular weights of bands were estimated using molecular weight markers, as above.

2.7 Plasmin generation assay

The tPA mediated activation of plasminogen was followed at room temperature in HBS; with 0.1% polyethyleneglycol (PEG) (HBS/PEG) in flat bottom 96 well microplates (Corning Incorporated, Corning NY, USA) using a Amax micro plate reader (Molecular Devices). Purified virus at various concentrations was incubated with Lys-plasminogen (0.5 µM), tPA (10 nM) and $CaCl_2$ (2mM) in HBS/PEG in a final reaction volume of 10 µL. The amount of plasmin generated after 30 minutes incubation at room temperature was monitored using the chromogenic substrate, (H-D-Val-Leu-Lys-p-Nitroaniline dihydrochloride) (S-2251; 200 µM) (Chromogenix, Milano, Italy). Standard curves of S-2251 cleavage by known amounts of purified plasmin were used to determine the concentration of plasmin formed in each experiment. To evaluate the contribution of tPA, varying amounts of tPA (0-10nM) were incubated with Lys-plasminogen (0.5 µM), $CaCl_2$, (2 mM) with or without virus (10^{11} vp mL^{-1}). In control experiments, each component of the reaction mixture was individually omitted and the amount of plasmin generated was determined. To investigate the involvement of A2 in plasmin generation by viruses, virus were preincubated with anti-A2 antibody (50 nM) (Invitrogen) or non-immune mouse Ig (Sigma) at 37˚C for 1 hour. To determine virus-mediated plasminogen activation independent of exogenous tPA, virus (10^{11} vp mL^{-1}) and Lys-plasminogen (0.5 µM) together or alone were preincubated at 37˚C for 2 hours and plasmin generated was measured. In this and other experiments, EACA was used to assess the dependence of plasmin generation on C-terminal lysine.

2.8 Fibrinolysis assay

The final concentration of reagents in fibrinolysis assays using purified proteins was fibrinogen (3 μM), Lys-plasminogen (0.6 μM), $CaCl_2$ (5 mM), tPA (1 pM), and virus (10^{11} vp mL^{-1}) in HBS/PEG. Clotting was initiated with thrombin (3 nM). The experiments were conducted at room temperature in Costar 96 well flat bottom microplates, which were sealed to prevent evaporation. The clot turbidity was monitored at 405 nm using a kinetic microplate reader (VMax, Molecular Devices). The time required to achieve 50% lysis was determined by an inverse sigmoidal fit of the averaged data using GraphPad Prism 4 software(462).

2.9 Plasmin-dependent plaque assays

The effect of plasmin on HSV infectivity was determined by standard plaque assays in human umbilical vein endothelial cells (HUVEC, ATTC, CRL-1730) as previously described (455). Prior to inoculation, cells were washed once with phosphate buffered saline (PBS) and serum free media (SFM) supplemented with 1 mg mL^{-1} BSA (SFM/BSA). After washing, cells were simultaneously inoculated with a fixed amount of virus and varying amounts of plasmin diluted in SFM/BSA (200 μL per well). Following 90 minutes at 37^{o}C, the inoculum was removed and cells were washed and replaced with reduced serum media. After 24 hours post infection, the cells were stained to derive the number of productive infectious events (plaques). To maintain the integrity of the cell monolayer the initial number of plaques for each virus was relatively low (2 - 5). Alternatively, the effect of plasmin on CMV infection was monitored using human foreskin fibroblasts (HFF, ATTC, CRL-2056) because HUVEC are not permissive to this strain. The data were corrected for the number of plaques detected in the absence of added enzyme, taking into account any enzyme-independent infection.

3. INVOLVEMENT OF THE CONTACT PATHWAY AND INTRINSIC PATHWAY IN HERPES SIMPLEX VIRUS-INITIATED PLASMA COAGULATION.

3.1 Background

Herpesviruses establish a life long latency, exposing the host to recurrent infections (405) and have been implicated in vascular disease (100). The considerable clinical evidence (104,304,341,363,417,438,524) is corroborated by animal studies (109,446,505) and has been attributed to the cumulative biochemical changes of infected cells (156,492). Thus, the hemostatic balance between coagulation and anticoagulation associated with the cell is altered by the virus to favor thrombin production,

The thrombin generation is initiated by the "*extrinsic*" pathway of coagulation. The pathway is initiated upon exposure of extrinsic TF and aPL to plasma clotting FVIIa. As a ternary complex, the FVIIa/TF/aPL tenase functions to activate FX to FXa. FXa is the only known physiological protease that converts prothrombin to thrombin, the critical step in coagulation. Availability of TF and aPL are restricted to sites of vascular damage by the cell. In contrast, TF and aPL acquired from the host cell are constitutively accessible on the envelope surface of several herpesviruses, including herpes simplex virus type 1 (HSV1) (457). These viruses consequently initiate thrombin production through an endogenous extrinsic pathway (269,455-457). FX activation by FVIIa on the virus is furthermore accelerated by another transmembrane protein, glycoprotein C (gC) (456). Unlike TF, gC is encoded by the HSV1 genome. These mechanisms bypass the pivotal cell-mediated control of the extrinsic pathway of coagulation and may be the

earliest stage connecting these viruses to vascular pathology.

While the extrinsic pathway of coagulation initiates normal hemostatic thrombin production, the "*intrinsic*" pathway is essential for amplification over the anticoagulant threshold of plasma. The fundamental cofactor within the intrinsic pathway is factor VIIIa (FVIIIa), which accelerates FXa generation by factor IXa (FIXa) as part of the respective tenase complex, FVIIIa/FIXa/aPL. It is generally accepted that products of the extrinsic pathway, FVIIa, and thrombin, feedback activate the non-functional precursors, factor VIII (FVIII), factor IX (FIX) and factor XI (FXI). However, under certain conditions the intrinsic pathway can also be triggered by the contact pathway through autoproteolytic activation of factor XII (FXII) or reciprocal activation of FXII and PK (PK) on a negatively charged surface (391). The functional form of FXII (FXIIa) produces activated factor XI (FXIa), which feeds into the intrinsic pathway by generating FIXa. However, FXII deficiency is not linked to spontaneous bleeding (233). The contact pathway activation of FXII normally has no role in normal hemostasis but is thought to contribute during severe trauma and thrombosis (125,186,387). Unlike for other protein complexes in coagulation, aPL is not the obligate anionic surface for assembly of the contact pathway proteins. Diatomaceous earths, kaolin or glass may facilitate surface-dependent FXII activation and are the basis of the activated partial thromboplastin time (APTT) clotting assay. Thus extrinsic and intrinsic pathway function can be dissected in plasma depending on the type of initiator.

3.2 Rationale and hypothesis

Previous work in our laboratory demonstrated the FVIIa-dependent coagulation activity on the virus surface as a molecular level explanation of early virus contribution in the development of vascular disease. Additionally, the study provided a direct evidence for

the presence of extrinsic pathway constituents; TF and aPL on the surface of herpesviruses. However, it is unknown whether the intrinsic pathway and the upstream contact pathway are also involved in HSV1-initiated plasma clotting. Hence, the hypothesis that in addition to the already established TF dependent pathway FVIII and contact pathway proteins are utilized by viruses to trigger plasma clotting.

3.3 Results

3.3.1 FVIII enhances HSV1-mediated plasma clotting

In normal hemostasis, FVIII is the essential cofactor that functions to augment FXa generation initiated by the extrinsic pathway. To evaluate whether FVIII is also important to enhance coagulation triggered by the known TF on the HSV1 surface, the effect of adding rFVIII to congenital FVIII deficient plasma was evaluated. During viremia, the HSV1 genome copy number ranges from approximately 10^6 to greater than 10^9 mL^{-1} (220,497) and may by locally concentrated at primary sites of propagation or when bound to the host cell surface. Figure 24(A) shows that at less than ~10^8 vp mL^{-1}, rFVIII enhanced HSV1-mediated clotting in FVIII-deficient plasma. Above ~10^8 vp mL^{-1}, clotting was independent of FVIII, which is likely due to the TF on the virus surface and resultant overwhelming contribution of the extrinsic pathway of coagulation.

A similar observation was made when normal pooled plasma was added to multi-donor plasma immuno-depleted of FVIII (Figure 25). Immuno-depleted plasma are prepared using multiple antibodies to FVIII. Commercial FVIII immuno-depletion may result in incomplete removal of FVIII. Additionally, other proteins may be concomitantly removed that include vWF, carrier of FVIII and non-specific binding of non-targeted proteins to the specifically depleted protein and to the depletion matrix. Studies have

reported that immuno-depletion of FVIII results in 25% dilution for other plasma factor

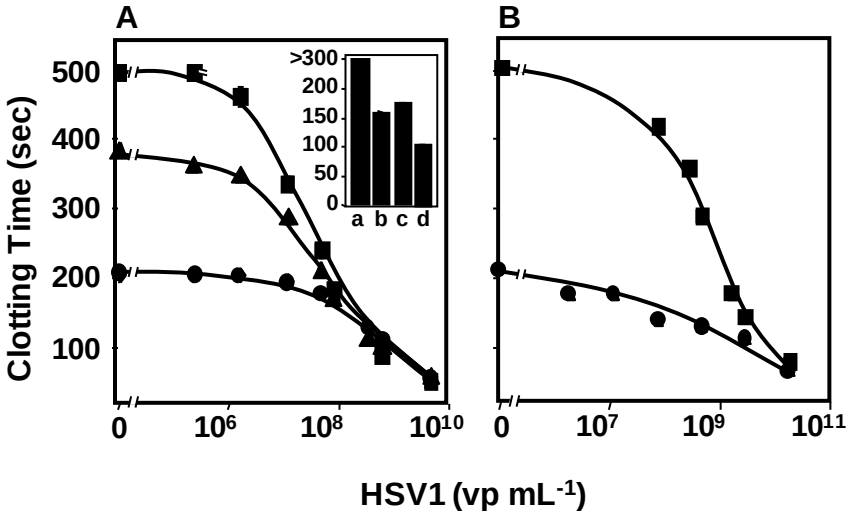


Figure 24 FVIII enhances HSV1-initiated plasma clotting. HSV1-initiated clotting times were determined for (A) congenital-deficient FVIII plasma (■), with 0.5 nM (▲) or 2 nM (●) rFVIII and (B) normal plasma in the absence (●) or presence of inhibitory FVIII mAb (50 nM) (■). HSV1/V was incubated with the respective reagents and SUV (50 μM) for 5 min at 37°C followed by the addition of Ca^{2+}(20 mM). Timing was stopped at 500 s (n = 3; ± standard deviation (SD) is smaller than the size of symbols.) Inset: Without additional aPL, clotting times were determined (as above) for immuno-depleted FVIII pooled plasma (a, b) and normal pooled plasma (c, d) in the absence (a, c) or presence (b, d) of 10^9 vp mL^{-1} as initiator. Timing was stopped at 300 s (n = 2, ± SD).

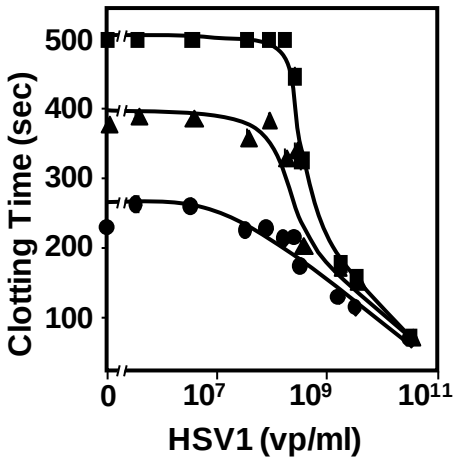


Figure 25 Plasma enhances HSV1-initiated clotting of FVIII-immunodepleted plasma. Increasing concentrations of purified HSV1/V were added to normal human plasma (●) and phospholipid (50µM). Following a 5 minute preincubation at 37°C, calcium (20 mM) was added and the time to clot formation was determined. Identical experiments were also conducted using either normal plasma or immuno-depleted FVIII plasma (■) or FVIII-immunodepleted plasma plus 20% normal plasma as a source of FVIII (▲). (n=3; ± standard deviation).

levels (348) and also that fibrinogen levels are reduced (460). In short, these observations raise concerns about the validity of the quantitative representation of the other plasma factor when only the immuno-depleted plasma is analyzed. Therefore, congenital FVIII deficient plasma from a severe hemophilia A patient with FVIII <1% and no FVIII inhibitor is ideal, but is difficult to have a steady supply from the same individual at all times. The hemophilia patients receive frequent FVIII replacement and consequently FVIII inhibitors develop in 80%of them.

Effective formation of t he FVIIIa/FIXa complex requires aPL, which is known to be on the virus envelope surface (457). To avoid limiting the available aPL at low HSV1 concentrations, SUV was added in excess. In the absence of added SUV, the inset to Figure 24(A) shows that FVIII continues to enhance HSV1-initiated clotting. Since our previous report showed that aPL is associated only with the virus in our purified preparations (457), these results imply that assembly of intrinsic pathway constituents must be directly on HSV1.

To corroborate the FVIII-deficient plasma experiments, the effect of an inhibitory anti-FVIII mAb in normal pooled plasma was evaluated. Figure 24(B) further demonstrates the involvement of FVIII in HSV1-initiated clotting in normal plasma by the prolongation of clotting times due to anti-FVIII mAb. As in Figure 24(A), concentrations of HSV1 less than ~10^8 vp mL^{-1} demonstrated dependence on FVIII.

3.3.2 HSV1 initiates the contact pathway

The intrinsic pathway of coagulation can be initiated by activation of the contact pathway in addition to the extrinsic pathway. To investigate a possible contribution of the contact pathway in HSV1-mediated clotting, the involvement of the extrinsic

pathway of coagulation was precluded by using congenital FVII deficient plasma. In these experiments, no SUV were added so that contact pathway protein complex assembly was forced to the negatively charged virus surface. With a 5 min reaction preincubation, HSV1 concentrations greater than 3.3×10^{10} vp mL^{-1}, clotting was initiated independent of FVII (Figure 26A). Under these conditions, HSV1 produced a titratable decrease in clotting time, demonstrating extrinsic pathway-independent enhancement of coagulation.

The contact pathway of coagulation requires activation of FXII to FXIIa. CTI is an inhibitor selective for FXIIa (184) as it is shown to prolong APTT but not PT in normal plasma (379). Therefore, the effect of CTI (184), on HSV1-initiated clotting in FVII-deficient plasma in the absence of added SUV was investigated. As shown in Figure 26(B), increasing concentrations of CTI resulted in a dose-dependent increase in the clotting time. These results indicated that HSV1 promotes initiation of the contact activation pathway.

To confirm that the effect of CTI was due to contact pathway inhibition during HSV1-triggered clotting of FVII-deficient plasma, immuno-inhibition was conducted. Commercial FVII deficient plasmas obtained from individual donors with congenital deficiency and have considerably different clotting times. Because the same donor plasmas were not available for all of our experiments, the results shown in Figure 27 compare the clotting time of no mAb subtracted from that of anti-FXII mAb, anti-PK mAb or anti-FXI mAb. Each of the three mAbs we evaluated significantly prolonged clotting times ($P<0.05$) induced by HSV1 in FVII-independent plasma. In each case non-immune isotype control mAb had an insignificant effect compared to no mAb ($P>0.1$). Furthermore, TF on the HSV1 surface was inhibited using an anti-TF mAb to exclude

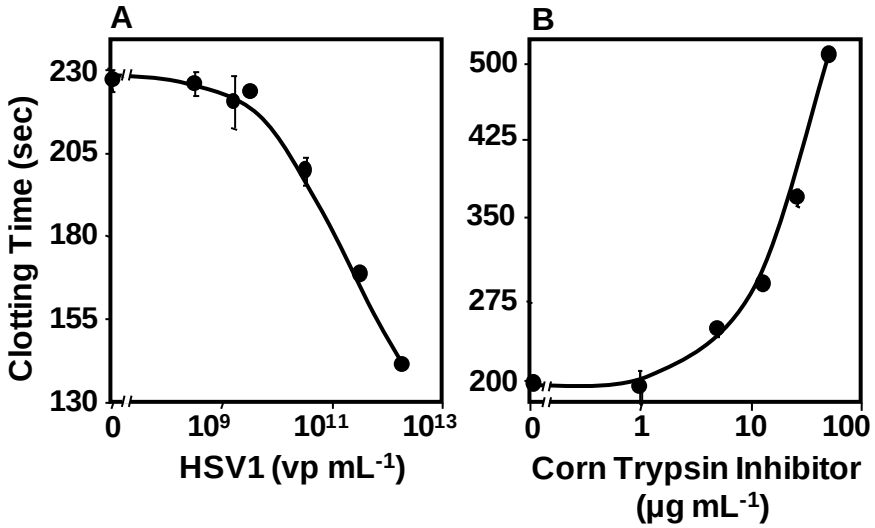


Figure 26 HSV1 initiation of plasma clotting through the intrinsic pathway is attenuated by corn trypsin inhibitor (CTI). Clotting times were determined by (A) preincubating congenital FVII-deficient plasma with HSV1/V for 5 min at 37 °C in the absence of SUV followed by the addition of Ca^{2+} (20 mM) and (B) incubating HSV1/V (3.3×10^{11} vp mL^{-1}) with congenital FVII-deficient plasma as in A, except in the presence of CTI (n = 3; ± SD was smaller than the size of symbols).

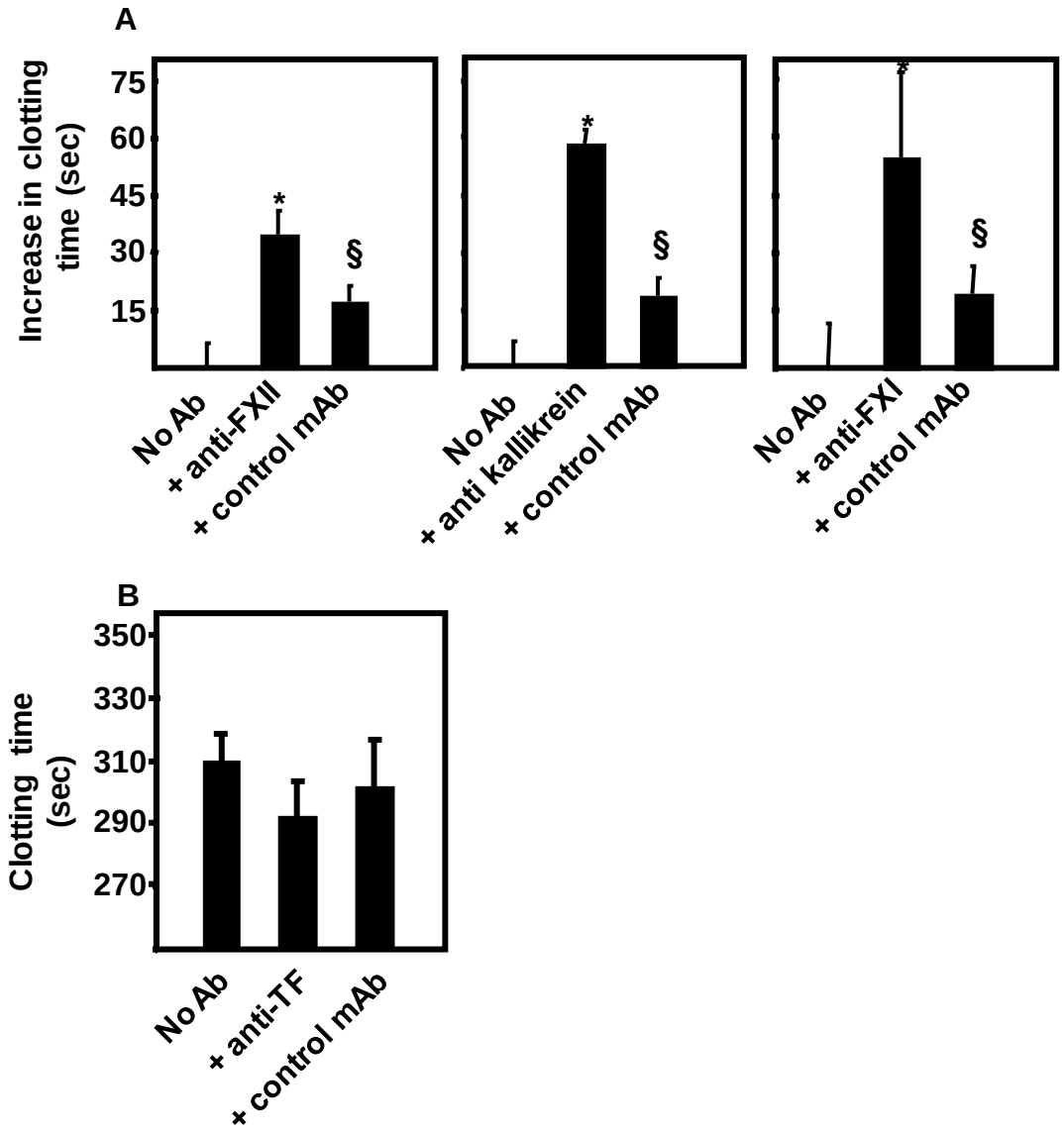


Figure 27 HSV1 initiates the contact phase of coagulation. (A) HSV1/V (3.3×10^{11} vp mL^{-1}) was used to initiate clotting of congenital FVII-deficient plasma as described in Fig. 26, except in the absence or presence of inhibitory anti-FXII (1 μM), anti-PK (0.45 μM) or anti-FXI (13.3 nM) antibody (n = 3; ± SEM). P-values; * indicates significant increase in clotting time over no Ab (n=3, mean ±SEM, t-test, *P* <0.05) and § indicates no significant difference in clotting time for control antibody over alone (n=3, mean±SEM, t-test, P>0.1) (B) HSV1/V (3.3×10^{11} vp mL−1) preincubated with anti-TF antibody (0.25 μM) was used to initiate clotting of congenital FVII-deficient plasma as described above. The absolute time are represented with no significant difference between anti-TF, control antibody and no antibody (n=3, mean±SD, t-test, P>0.1).

the possible contribution of trace amounts of FVII in congenital FVII deficient plasma. The TF inhibition resulted in clotting times lower than that seen with no antibody but not significant (P>0.1). The absolute clotting times for these are represented in Figure 27 (B). These findings provide additional evidence that the contact pathway activation complex between FXII and PK and activation of FXI is involved in HSV1-mediated plasma clotting.

To directly demonstrate that HSV1 facilitates proteolytic conversion of FXII to FXIIa, purified FXII, with or without PK and HSV1 were incubated together at 37ºC. In plasma it has been estimated that <0.1% of total FXII requires activation to initiate clot formation (46,150). Due to the sensitivity of the mAb used to detect FXII/XIIa in our purified experiment, it was necessary to allow much more to accumulate. Under these conditions, the FXII/XIIa-specific western blot presented in Figure 28 shows that HSV1 significantly enhanced FXII activation, and that enhancement by PK was required.

3.4 Discussion

Blood coagulation is initiated when vascular damage triggers the exposure of cellular TF and aPL. Accessibility of these to plasma enables recruitment and activation of the protease FVIIa and assembly of the ternary FVIIa/TF/aPL extrinsic pathway tenase. The extrinsic tenase is required for initial FXa production (179). Thus, it is well established that cells regulate and localize the normal hemostatic response to vascular injury (399). In contrast to the cell-based dogma for initiating coagulation, we previously reported that HSV1 has endogenous TF and aPL on its envelope surface that facilitate constitutive FX activation in the presence of FVIIa (457). Additional to host-derived TF, we have also identified virus-encoded gC as contributing to FVIIa-dependent FXa

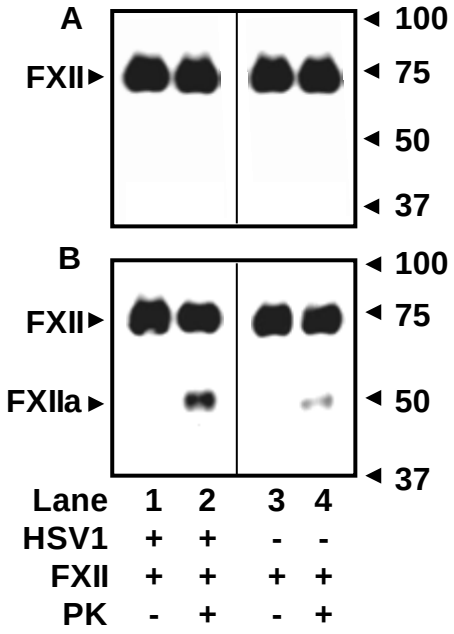


Figure 28 HSV1 mediates activation of FXII in the presence of prekallikrein. Purified FXII (0.5 µM) alone or with prekallikrein (PK) (0.3 µM) in the presence or absence of HSV1/V (3.3×10^{11} vp mL^{-1}) was incubated at 37 °C. At time zero (A) and at 15 min incubation (B), 2 µL of the reaction mixture was run on SDS-PAGE under reducing conditions and evaluated for FXIIa generation by Western blot. Time zero reaction components were directly added to the SDS-PAGE sample buffer. The positions of molecular weight markers, FXII and FXIIa are shown.

generation on HSV1 (456). Through TF, aPL and gC, the virus can consequently bypass the important barrier to clot formation that is imposed by cells (339,487). In normal plasma, the TF-independent intrinsic tenase, consisting of the cofactor FVIIIa and protease FIXa bound to aPL, is known to be critical to amplify FXa production beyond the physiological anticoagulant barrier. Our previous studies revealing FVIIa-dependent FX activation on HSV1 precluded a potential contribution of FVIIIa because purified proteins (269,456,457) or a relatively high concentration of virus in plasma were used (457). Therefore, in the current study we addressed the hypothesis that HSV1 not only activates clotting through the extrinsic pathway in plasma, but also promotes intrinsic pathway activation. Indeed, we now report that HSV1-induced clotting is further elaborated by contributions from the pivotal intrinsic pathway cofactor, FVIII. This conclusion was supported by enhanced HSV1-triggered clot formation in FVIII-deficient plasma when purified FVIII was added, and attenuation in normal plasma by an inhibitory FVIII mAb (Figure 24). As predicted, the FVIII-dependence of HSV1-initiated clotting was obscured at high concentrations of HSV1 when the extrinsic pathway originating from viral TF can generate sufficient FXa without the need of amplification by the intrinsic pathway. Assembly of the intrinsic FVIIIa/FIXa complex requires the availability of aPL. HSV1-induced clotting was enhanced by FVIII without supplementing the aPL. Therefore, assembly of the intrinsic tenase must occur directly on the aPL-containing virus envelope (457). These results demonstrate that assembly of both the intrinsic and extrinsic tenases are initiated on HSV1 leading to clot formation. A model combining our current and previously reported observations is presented in Figure 29.

The intrinsic pathway is generally accepted as being activated by proteases produced through the extrinsic pathway; FVIII is feedback-activated by either thrombin

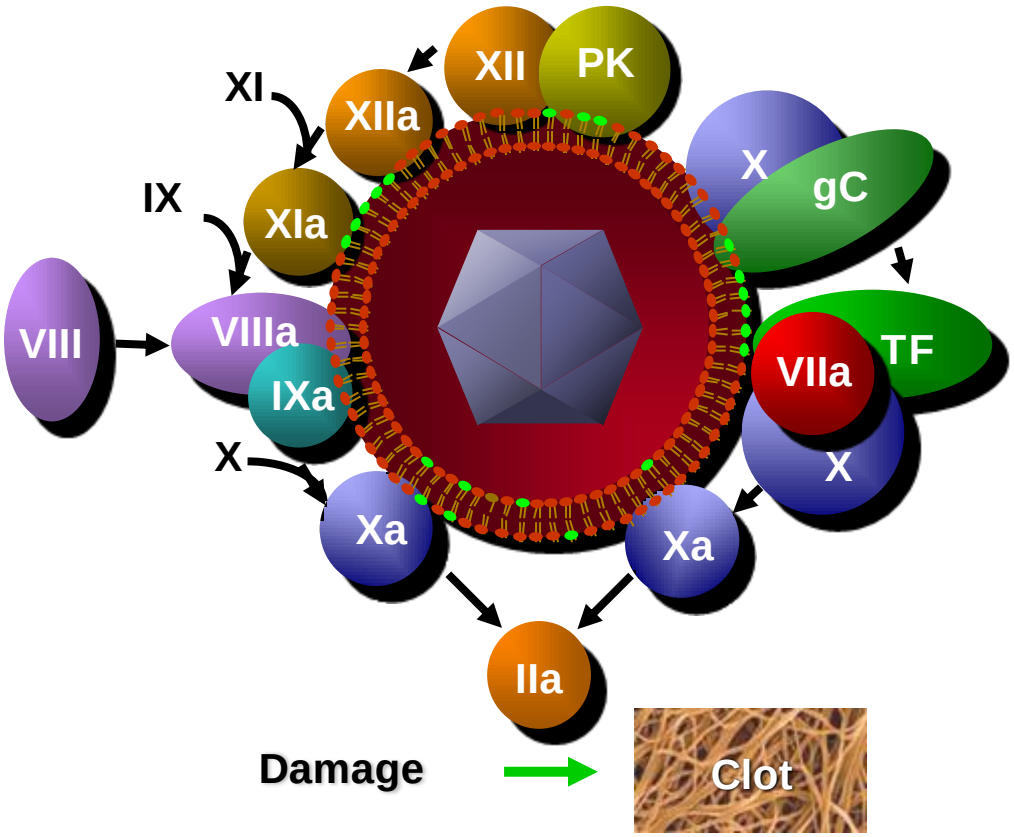


Figure 29 Coagulation initiated by HSV1 involves the contact, intrinsic and extrinsic pathways. Our previous work has shown that host cell-derived TF and aPL and virus-encoded gC enhance FXa generation by FVIIa on the HSV1 envelope. The current results show that coagulation can also be triggered by the virus independent of FVIIa through contact activation involving FXII and PK, and formation of the FVIII-dependent intrinsic tenase.

or FXa, and FVIIa crossing into the intrinsic pathway by directly activating FIX. FIX can also be indirectly feedback-activated by thrombin through activation of FXI. However, the upstream contact pathway can completely circumvent the extrinsic pathway by initiating the intrinsic pathway independent of TF and FVIIa. The contact pathway is initiated when FXII and PK bind to an appropriate anionic surface, such as aPL. FXII can then be activated by autoproteolysis or by activated PK, to kallikrein (KK), which ultimately leads to consecutive FXI and FIX activation(421,499). The results presented here show that purified HSV1 can initiate coagulation even in the absence of FVII and therefore independent of the tenase cofactor function of TF on its surface. Attenuation of FVII-deficient plasma clotting by an inhibitor of FXIIa (CTI) (Figure 26B) and a specific FXII mAb (Figure 27) demonstrated the involvement of FXII. FXIIa activates FXI, whose involvement in the virus model was confirmed by an increased clotting time in the presence of inhibitory anti-FXI mAb (Figure 27).

To determine if HSV1 can directly activate FXII, purified protein was combined with HSV1. No FXIIa was detectable unless purified PK was also present. Since the purified virus was the only added source of anionic surface in these experiments, a model (Figure 29) is proposed wherein the FXII and PK complex formation directly on the virus. The involvement of PK was further supported by prolongation of clotting by a PK mAb. These observations demonstrate that the surface of HSV1 can initiate clotting through the contact pathway.

Contact activation of coagulation is unlikely to be involved in normal hemostasis because deficiencies do not result in a spontaneous bleeding diathesis (17). However, an involvement in coagulation has undergone recent re-evaluation. Animals deficient in proteins responsible for contact activation of the intrinsic pathway are protected from

experimental induction of thrombosis (125,387), suggesting a role in pathology. Contact activation may also contribute to thrombotic risk in surgical procedures involving medical polymers (e.g. catheters), which expose plasma to a surface suitable for FXIIa generation (186). Thus contact activation may not play a critical role in a hemostatic response to vascular injury, but appears to be a criterion in development of thrombotic occlusion. The current report shows that the HSV1 surface facilitates intrinsic pathway complex assembly and suggests that blood-borne enveloped viruses may be a further route for participation of the contact pathway in pathology.

Our previous work has provided an explanation for why HSV1 has evolved to initiate coagulation (269,456,457). Extrinsic pathway-mediated thrombin production due to the virus was found to increase infection through PAR1 on host cells (455). The model emerging from the current work suggests the virus envelope has been designed to efficiently initiate coagulation protease activation through multiple routes including the contact, intrinsic and extrinsic pathways and a pathway involving virus-encoded gC (Figure 29). FVII-independent stimulation of plasma clotting required a relatively high virus concentration. Therefore the observed acceleration of coagulation by FVIII at low HSV1 is conceivably through assembly of extrinsic tenase on the virus surface, the products of which cross-over to activate FVIII and FIX. As virus load increases during viremia, direct contact pathway activation would predictably further augment coagulation. In addition to our studies on HSV1, HSV2, and CMV (372,456,457), enveloped viruses, have also been implicated in vascular disease by others, including HSV1 (438), CMV (445,522), human immunodeficiency viruses 1 (HIV1) and 2 (HIV2) (503,514), hepatitis C virus (HCV) (194), influenza virus (329) and further examples (18,48,142,477). Based on these reports it is conceivable that the HSV1 studies

presented here may constitute a general model for enveloped viruses.

4. HERPESVIRUSES ENHANCE FIBRINOGEN CLOT LYSIS

4.1 Background

Herpesviruses such as HSV1, HSV2 and CMV are highly prevalent in the general population and expose the host to recurrent infections throughout life (405). Herpesvirus infections have been implicated in vascular disease (156,307,309,339,487), corroborated by multiple experimental levels of evidence. Clinical reports include virus genetic material associated with atherosclerotic plaque(13,173,174,189,517), fibrin deposition in microvasculature correlating to infection(50,153,304,308,363,417), and an increase in mortality following myocardial infarction(438,526) or adverse cardiac outcomes(103,342) in seropositive patients. A direct cause and effect relationship was established in both avian and rodent animal models where virus inoculation leads to rapid development of thrombosis and atherosclerosis (109,446,505). On a cellular level, virus infection transforms a resting anticoagulant phenotype to procoagulant by inducing TF availability and membrane phospholipid rearrangement, the normal physiological initiators of coagulation (156,492). Furthermore, HSV1 may attenuate the anticoagulant properties of resting cells by reducing expression of TM (218), reducing synthesis of HSPG (211,212), and decreasing prostacyclin secretion (491). Consequently, the hemostatic balance between coagulation and anticoagulation associated with the cell is altered by the virus to favor thrombin production, providing an explanation for the clinical and animal reports.

In addition to modulating the procoagulant properties of cells, studies from our laboratory have demonstrated that purified herpesviruses initiate and propagate thrombin production through multiple mechanisms directly on their surface(133,457).

Being independent of the host cell, these likely constitute the first link between the virus and vasculopathy. All members of the herpesvirus family and many other virus families have an envelope as part of their architecture, which consists of a phospholipid bilayer containing host- and virus-encoded membrane proteins. We have shown that the envelope of HSV1, HSV2 and CMV comprises TF and aPL, the normal physiological initiators of coagulation, and can activate FX in the presence of FVIIa (457). Presumably because of the presence of aPL and consequent assembly of intrinsic tenase, FVIII has also been shown to accelerate virus-triggered clotting in plasma (133). Furthermore, virus surface-dependent initiation of coagulation also occurs through the FXII/PK-dependent contact pathway (133). Implying evolutionary pressure to initiate coagulation, HSV1-encoded gC provides an additional mode of interplay between virus and hemostasis, which has been shown to enhance FVIIa-dependant FX activation (105,456). Thus, the highly procoagulant phenotype in vitro predicts that herpesviruses should be a strong predictor of clinical vascular disease. However, although statistically significant alone, the contribution of herpesviruses to vascular disease is modest until combined with other risk factors(341,438). To help understand this paradox, we rationalized that these viruses may also contribute to clot dissolution, because earlier studies from our laboratory and others' reported host cellular A2, a known accelerator of fibrinolysis, on the CMV surface (384,515).

A2 participates in fibrinolysis by accelerating tPA-mediated activation of plasminogen to plasmin. Both tPA and plasminogen interact with C-terminal lysines on heterodimeric A2 (43,161). Plasmin directly solubilizes the clot by proteolysis(61). In this study, we addressed the hypothesis that herpesviruses enhance tPA-mediated plasmin generation and this mechanism correlates to the presence of A2 on the virus.

4.2 Rationale and hypothesis

HSV1, HSV2 and CMV have been implicated in vascular disease. Our laboratory demonstrated that purified herpesviruses contain aPL derived from host cells, and proteins encoded by the host genome as well as the virus genome, which initiate coagulation. Consequently, the virus surface is able to initiate coagulation activating the extrinsic and contact pathway and amplification of clot formation through assembly of intrinsic pathway. However, the prothrombotic potential of these viruses does not explain the weak correlations, observed clinically. To explain this discrepancy, herpesvirus-mediated fibrinolysis was investigated because of A2, known to exist on CMV surface, a cofactor for tissue tPA-mediated activation of plasminogen to plasmin leading to clot dissolution (Figure 30). **This study hypothesized that herpesviruses enhance tPA-mediated plasmin generation, correlating to virus associated A2.**

4.3 Results

4.3.1 Herpesviruses enhance tPA-mediated plasmin generation

In normal hemostasis, tPA mediates activation of plasminogen to plasmin resulting in degradation of fibrin clot. Fibrin or a number of cell surface tPA accelerators including A2 have been identified (114,157,160). To evaluate whether herpesvirus act as cofactors to enhance tPA-mediated plasmin generation, we used a chromogenic substrate to follow plasminogen conversion to plasmin. Figure 31 (A) shows that three different members of the herpesvirus family enhanced tPA-mediated plasmin

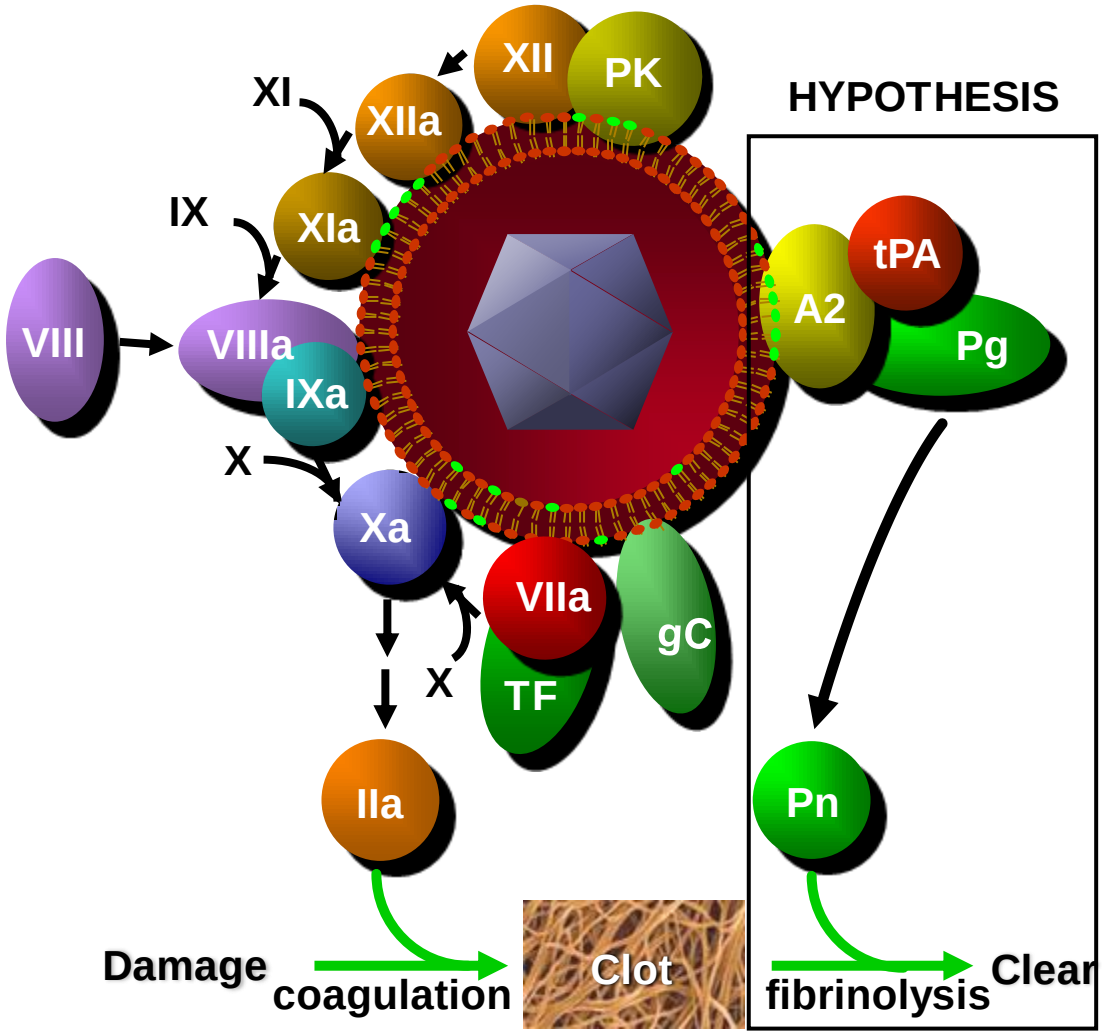


Figure 30 Herpesvirus associated annexin 2 (A2) mediate tissue plasminogen activator (tPA) dependent plasminogen (Pg) activation to plasmin (Pn) leading to clot dissolution. Herpesvirus initiates coagulation through extrinsic, contact and intrinsic pathways. Double arrows denote multiple steps omitted for simplicity. A2, a cell surface coreceptor for Pg and tPA has also been identified on the surface of at least one herpesvirus, CMV. It acts as a cofactor in the tPA dependent conversion of Pg to Pn and potentially play a similar role on the virus . This could explain why herpesviruses are not a strong independent predictor of vascular diseases.

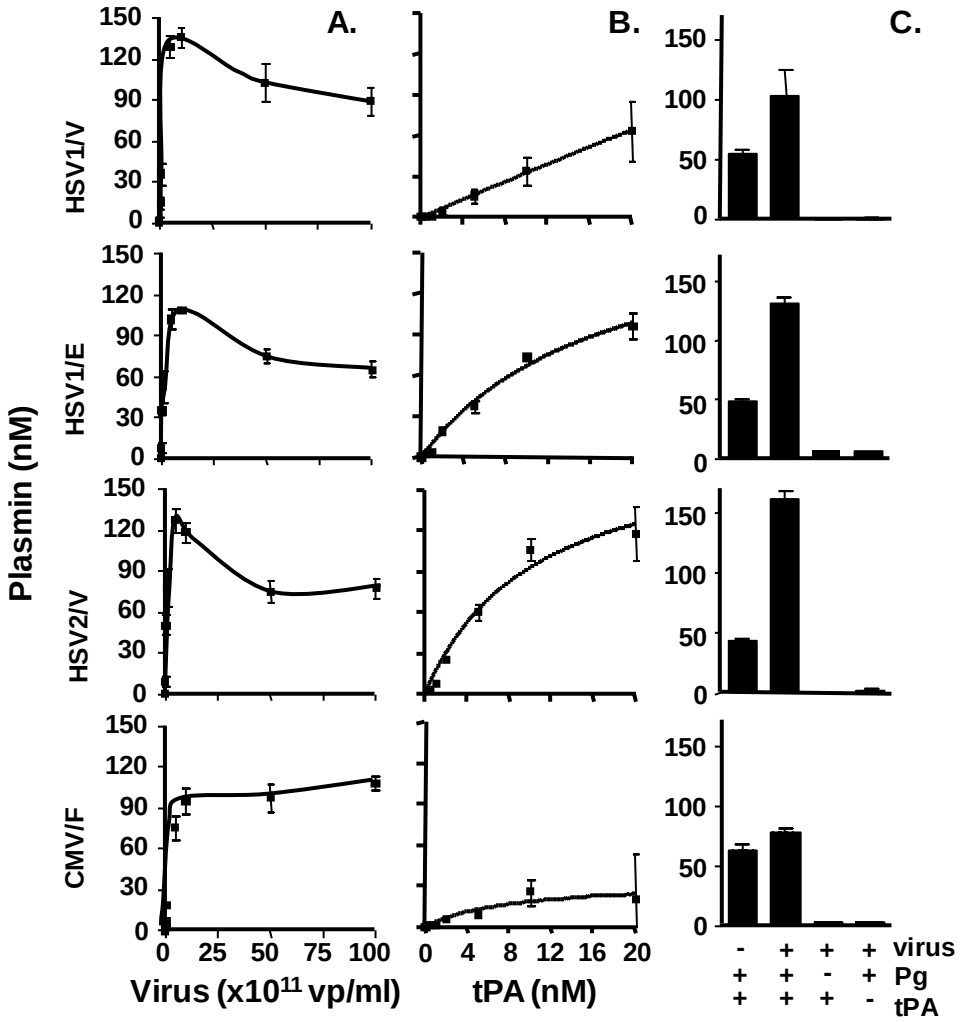


Figure 31 Herpesviruses enhance plasmin generation. (A) Purified HSV1, HSV2 or CMV were added to tPA (10nM), Lys-plasminogen (0.5μM) and calcium (2mM) in HBS/PEG for 30 minutes at 22ºC. (B) A reaction mixture of Lys-plasminogen (0.5μM), calcium (2mM), and virus (10^{11}vp mL^{-1}) were combined with tPA in HBS/PEG were incubated for 30 minutes at 22ºC. After the incubation period, chromogenic substrate (S2251; 200μM) was added and the amount of plasmin generation was measured at 405nm. (n=3; ± standard deviation). The data in A and B were corrected for plasmin generation in the absence of viruses. (C) Virus (10^{11}vp mL^{-1}), tPA (20nM), Lys-plasminogen (0.5μM) and calcium (2mM) in HBS/PEG were incubated for 30 minutes at 22ºC, then plasmin was determined by chromogenic substrate cleavage.

generation, including HSV1, HSV2 and CMV. This was independent of cell type as each virus was propagated in a different type of cell (HUVEC, Vero, HFF). Furthermore, HSV1 was produced in two different cell types (Vero, HUVEC) shows very similar plasmin-generation profiles, suggesting that cell-derived constituents may be playing a minor role. At high virus concentration, there was a plateau or decrease in plasmin generation, due to a probable partitioning of substrate and enzyme onto different particles. At a fixed low virus particle number, Figure 31 (B) confirms this idea by showing that plasminogen activation is tPA-dose dependent. Figure 31 (C) illustrates the extent of plasminogen activation enhancement conferred by each herpesvirus and the dependence on addition of plasminogen and tPA for color development in the assay.

4.3.2 Herpesviruses enhance fibrinogen clot lysis

The observations that purified herpesviruses accelerated tPA-dependent plasminogen activation to plasmin lead us to investigate whether the virus mediated plasmin generation accelerated clot lysis. tPA is used at 1pM to reflect its functional concentration in the absence of PAI-1 that is consistent with the combination of tPA and PAI-1 at physiological concentration (462). Figure 32 shows that when tPA and plasminogen were combined in the absence of virus during thrombin-induced clot formation, the time to reach 50% fibrinolysis was ~900 minutes representing the intrinsic tPA mediated plasminogen activation without fibrin. Fibrin itself is a known accelerator of tPA-mediated clot lysis that allows localization of tPA and plasminogen because of C-terminal lysine residues (63). However, the addition of HSV1/V, HSV1/E, HSV2/V or CMV/F accelerated fibrinolysis and consequently the 50% fibrinolysis time was

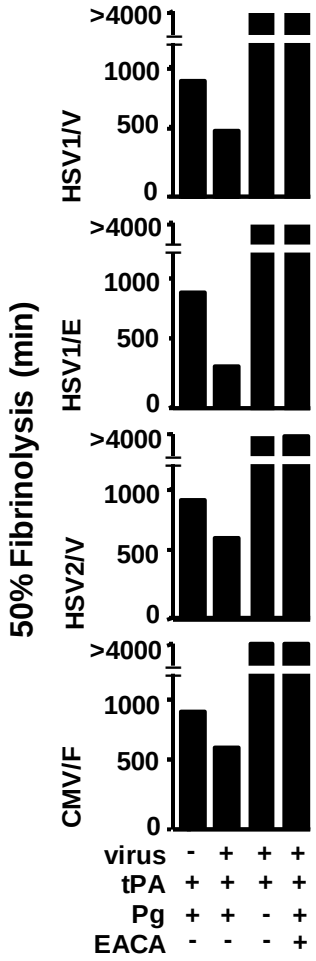


Figure 32 Herpesviruses enhance clot lysis. Fibrinogen (0.3μM), Lys-plasminogen (0.6 μM), calcium (5mM) and tPA (1pM) were combined with purified herpesvirus particles at 10^{11}vp mL^{-1}. A complete reaction mixture along with epsilon-aminocaproic acid (EACA; 5mM) was also included. Clotting was induced with thrombin (3nM) and the extent of clot formation and dissolution were followed by turbidity at room temperature. The time to reach 50% fibrinolysis was determined by an inverse sigmoidal fit of the averaged raw data. (n=3; ± standard deviation).

reduced to ~200-500 minutes. The virus had no direct effect on dissolving the clot, since adding plasminogen was obligate. EACA, a lysine analogue, completely inhibited clot lysis, demonstrating the viral mechanism was dependent on C-terminal lysines.

4.3.3 A2 associated with Herpesviruses binds plasminogen

As a possible candidate C-terminal lysine-dependent protein associated with virus that may bind plasminogen on the virus, host cell-derived A2 tetramer has been previously shown by our laboratory to be on the surface of CMV (384,515). Prior to evaluating the role of A2 in plasminogen binding to viruses, purified HSV1, HSV2 and CMV cultured in the various cell lines were probed for A2 antigen and also uninfected cell lines were evaluated. HUVECs (161) and fibroblasts (515) have been previously shown to express A2. The western blot in Figure 33(A) and 34(A) demonstrate A2 association with each herpesvirus, which was consistent with detection of A2 in the respective uninfected parental cell line. In comparison to the other viruses HSV/M had visibly less than 5% A2 antigen (Figure 34 A) and the melanoma cell line that was used for propogation had no detectable A2 based on the amount that could be loaded, suggested the virus may sequester A2 during the envelopement process.. To increase the sensitivity the blots in Figure 34(A) were overexposed, consequently HSV1/M showed a faint band. Thus purified HSV1/M had negligible A2 and was used as a control in subsequent experiments for plasminogen binding. In addition to antigenicity, purified recombinant A2 (rA2, 36kDa) was used to positively identify the mobility of A2 on these blots.

To identify virus-associated proteins that may bind to plasminogen, purified herpesvirus particles were run on 15% SDS PAGE, transferred to PVDF and probed

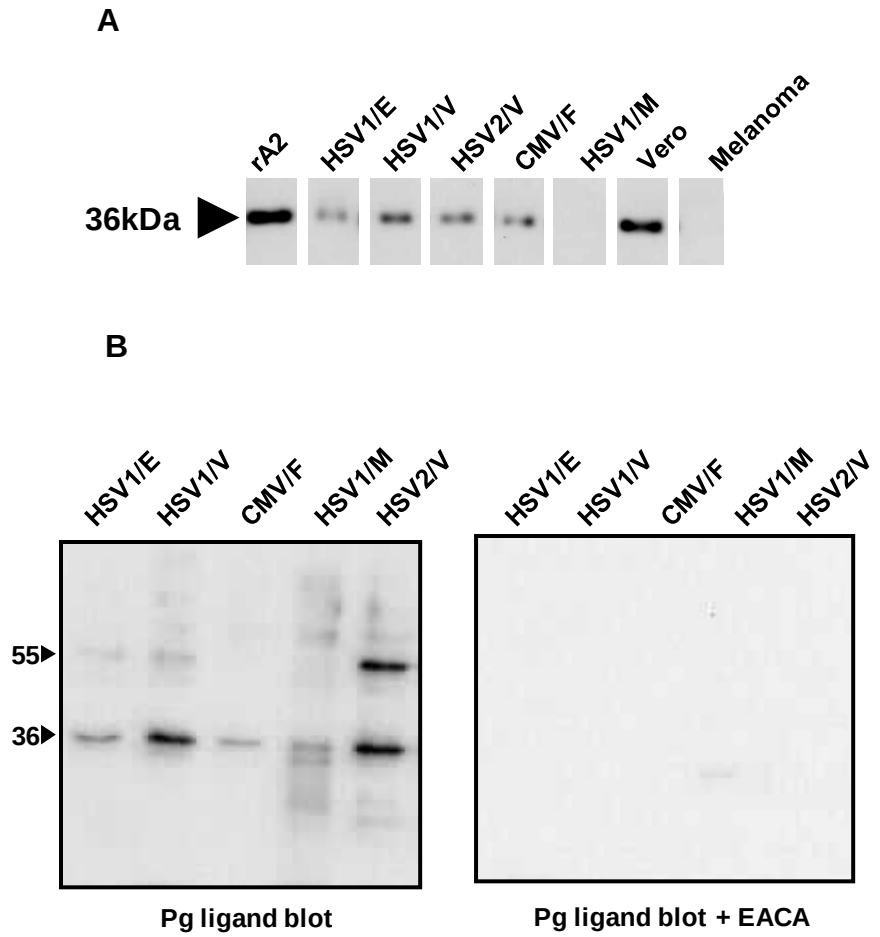


Figure 33 Annexin 2 (A2) is associated with herpesviruses. (A) Purified HSV1, HSV2 or CMV cultured in different cells at 10^{11}vp/ml were run on SDS-PAGE under reducing conditions and subsequently analysed by Western blot for the presence of A2. The uninfected cells were also evaluated (not shown). Arrow indicates the location of purified A2(36kDa) after blotting. The nomenclature of viruses and cells used to culture is as follows: HSV1/V- HSV1 in Vero cells, HSV1/E- HSV1 in Endothelial HUVEC cells, HSV2/V- HSV2 in Vero cells, CMV/F- CMV in Fibroblast and HSV1/M- HSV1 in Melanoma A7 cells (B) Plasminogen binding to herpesvirus particles is EACA inhibitable. Purified herpesvirus particles at $5x10^{11}$vp mL^{-1} were run on SDS-PAGE under reducing conditions, transferred to PVDF and subsequently analysed by ligand blot for binding of HRP-conjugated plasminogen (1nM) and in the presence of epsilon aminocaproic acid (EACA) (1mM)

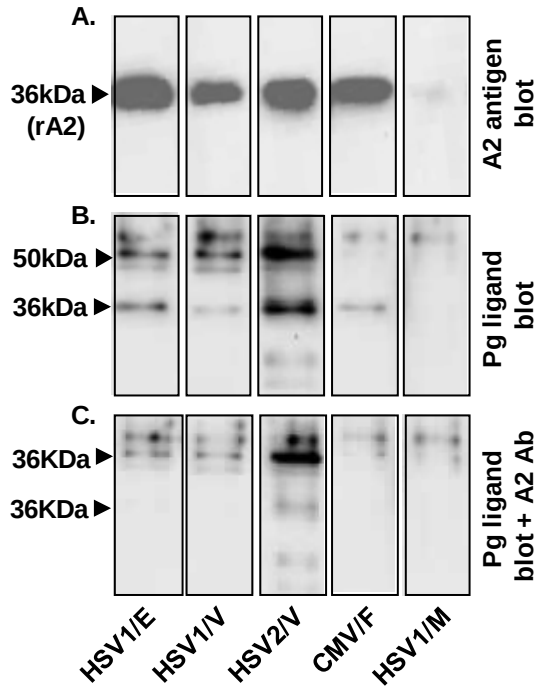


Figure 34 Annexin 2 (A2) associated with herpesviruses binds plasminogen. Purified herpesvirus particles at $5x10^{11}$vp mL^{-1} were run on 15%SDS-PAGE under reducing conditions, transferred to PVDF and subsequently analysed by (A) western blot for the presence of A2 or (B) ligand blot for binding of HRP-conjugated plasminogen (1nM) and (C) in the presence of A2 antibody. The arrows indicate the mobility of molecular weight markers and purified recombinant A2 (rA2) runs at 36kDa.

using a plasminogen-HRP as a ligand. Figure 33(B) and 34(B) reveals several herpesvirus-associated proteins that bind to plasminogen-HRP, one of which lines up precisely with the rA2 marker. No plasminogen-HRP binding in this position was detectable for HSV1/M, which is lacking A2. Further confirming the 36 kDa plasminogen-binding protein is A2, the anti-A2 Ab inhibited detection of this band by plasminogen-HRP, Figure 34(C). The interaction of plasminogen-HRP with all virus proteins was completely inhibited by the addition of EACA (1 mM) as shown in Figure 33(B). The bands above A2 at ~50kDa could be other plasminogen receptors with C-terminal lysine binding site, such as alpha enolase(6), actin or cytokeratin (316).

4.3.4 A2 inhibition of herpesvirus enhancement of tPA mediated plasmin generation.

The role of A2 in virus-enhanced plasmin generation was evaluated using an anti-A2 Ab, which was shown previously to inhibit plasminogen binding to cells (362). The anti A2 Ab inhibited plasminogen-HRP binding to herpesvirus associated-A2 (Figure 34). However, the A2 antibody had no effect on plasmin generation (Figure 35), possibly because of the redundancy due to the other detected plasminogen-binding species. Furthermore, HSV1/M, which has little detectable A2 antigen, had significant plasmin-enhancing capability. Thus despite viral A2 participating in plasminogen binding, it is not required for the enhancement of tPA-mediated plasmin generation facilitated by each virus.

4.3.5 Herpesviruses activate plasminogen independent of exogenous tPA

Viruses and bacteria have evolved mechanisms to activate plasminogen independent of tPA (85,253,320). To evaluate if such a mechanism is associated with members of the

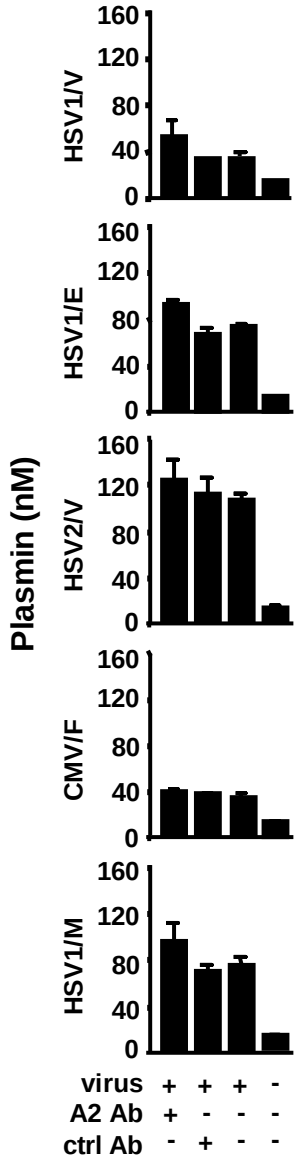


Figure 35 Annexin 2 (A2) is not required for herpesvirus-mediated plasmin generation. Purified herpesvirus particles 10^{11}vp mL^{-1} were preincubated with anti-A2 Ab (50nM) or an isotype matched control Ab, followed by addition of tPA (10nM), Lys-plasminogen (0.5μM) and calcium (2mM). The mixture was incubated for 30 minutes at 22ºC and S2251 chromogenic substrate (200μM) was then added to measure the amount of plasmin generation at 405nm. (n=3; ± standard deviation).

herpesvirus family, plasminogen activation was monitored independent of exogenous tPA. To increase the experimental sensitivity for such a pathway, plasminogen and/or herpesviruses were incubated at 37ºC for 2 hours and plasmin generation was measures using chromogenic substrate. Figure 36 shows that when a purified source of tPA is not added, the HSV1/V, HSV1/E, HSV2/V, CMV/F and HSV1/M all enhance plasmin generation. The incubation of virus particles alone with chromogenic substrate confirmed that the virus preparations did not contain plasminogen or plasmin-like activity. Plasminogen by itself accounted for autoactivation or endogenous plasmin activity detected over the prolonged incubation time of 2 hours, and was also insignificant. Addition of EACA inhibited herpesvirus-dependent plasminogen activation to the level of virus alone (Figure 37).

4.3.6 Herpesviruses enhance fibrinolysis independent of exogenous tPA

HSV1-mediated plasminogen activation leading to fibrinogen clot lysis was investigated independent of exogenous tPA. The assay was as described above, except that fibrinolysis was permitted to proceed for an extended duration to account for slower plasmin generation. As shown in Figure 38, when there is no virus, the time to reach 50% fibrinolysis was ~5700 minutes. When purified HSV1 (10^{11}vp mL^{-1}) cultured in endothelial cells or vero cells was added, the half lysis time was reduced by 9-fold and nearly 2-fold, respectively. The well-documented fibrinolysis inhibitor PAI-1 (0.5 nM) partially attenuated the effect of virus, while EACA (1mM) reverted fibrinolysis to approximately the same 50% time as no virus addition.

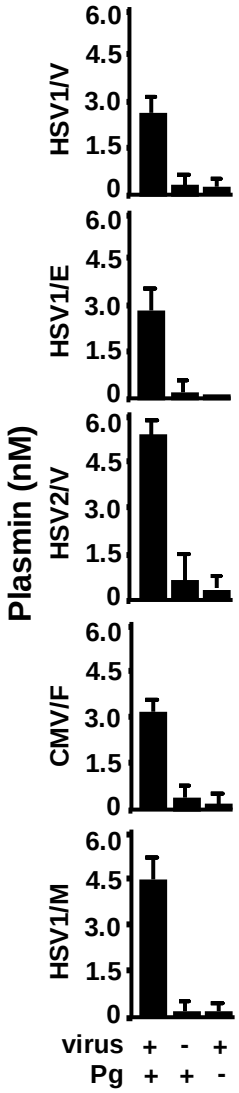


Figure 36 Herpesviruses activate plasminogen independent of exogenous tPA. Purified herpesvirus particles 10^{11}vp mL^{-1} and plasminogen (0.5μM) together or independently were preincubated at 37ºC for 2 hours. S2251 chromogenic substrate (200μM) was added and the amount of plasmin generated was measured at 405nm. (n=3; ± standard deviation).

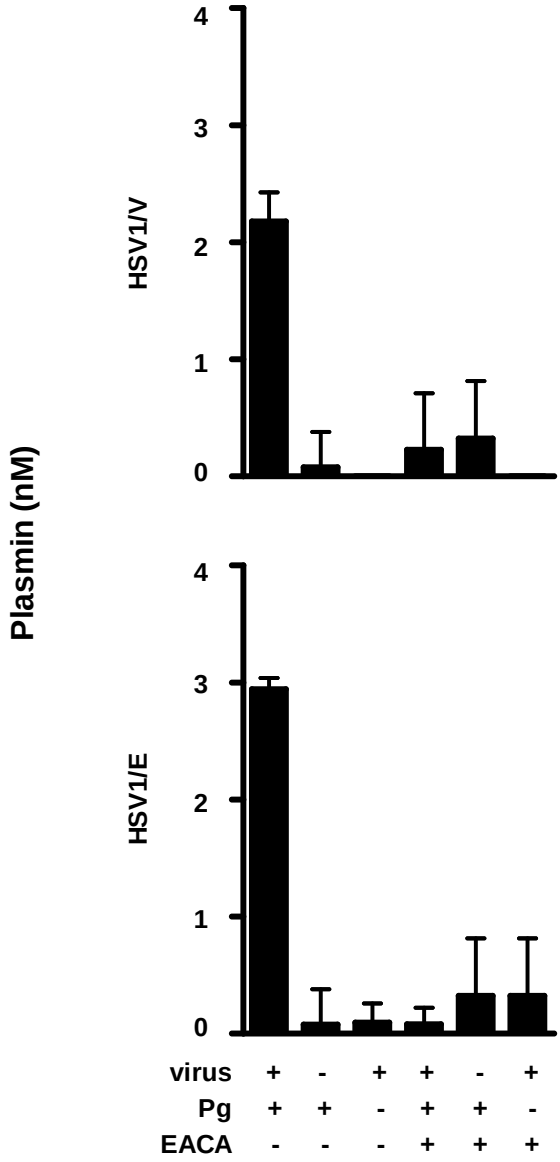


Figure 37 Herpesviruses activate plasminogen independent of exogenous tPA. Purified herpesvirus particles 10^{11}vp mL^{-1} and plasminogen (0.5μM) together or independently were preincubated in the presence or absence of epsilon-aminocaproic acid (EACA;5mM) at 37ºC for 2 hours. S2251 chromogenic substrate (200μM) was added and the amount of plasmin generated was measured at 405nm. (n=3; ± standard deviation).

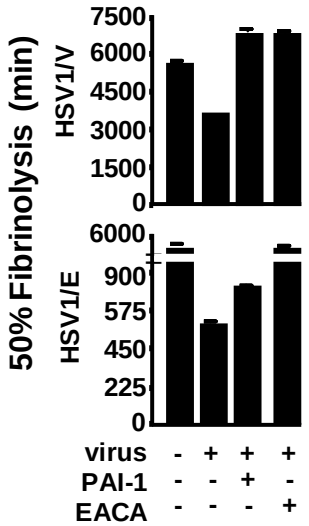


Figure 38 Exogenous tPA-independent enhancement of fibrinolysis by herpesviruses is attenuated by PAI-I. Fibrinogen (0.3μM), Lys-plasminogen (0.6 μM) and calcium (5mM) were combined with virus particles at 10^{11}vp mL^{-1} in the absence or presence of PAI-1 (500pM), epsilon-aminocaproic acid (EACA; 5mM). Clotting was induced with thrombin (3nM) and the extent of clot formation and dissolution were followed by turbidity. The time to reach 50% fibrinolysis was determined by an inverse sigmoidal fit of the averaged raw data (n=3; ± standard deviation).

4.3.7 Plasmin enhances herpesvirus mediated cell infection

Previously, herpesvirus-mediated thrombin generation was shown to increase the susceptibility of cells to infection (454). To evaluate the possible cell-modulatory effects of plasmin on herpesvirus infection, a serum-free plaque assay was used. Because endothelial cells are a potential site for herpesvirus infection and may contribute toward initiating vascular effects, infection of HUVEC by HSV1 and HSV2 was evaluated. The direct involvement of purified plasmin in HSV1 infection of HUVEC is demonstrated in figure 39. Plasmin enhanced infection by HSV1 strains by ~300% regardless of the parental cell type. HSV2/V infection was enhanced ~500% by treating HUVEC with plasmin. HUVEC are not permissive for infection by the laboratory strain of CMV (AD169) used in this study, therefore HFF were used instead of HUVEC. In contrast to infection of HUVEC by HSV1 and 2, there was no effect of plasmin on CMV plaque formation in HFF monolayers. This is similar to our prior observation comparing the relative effect of thrombin on infection of HUVEC versus HFF by CMV (455). Here, plasminogen had no effect on any virus infection.

4.4 Discussion

For significant plasmin generation, tPA requires a physiological cofactor. Fibrin is the accepted principal tPA cofactor(61). It functions optimally in fibrinolysis after partial cleavage exposes C-terminal lysine-containing binding sites that co-localize plasminogen and tPA (43,44). Other C-terminal lysine-dependent tPA accelerators have been reported, such as A2(245). Here, the three different members of the herpesvirus family, which include HSV1, HSV2 and CMV, are shown to enhance plasminogen

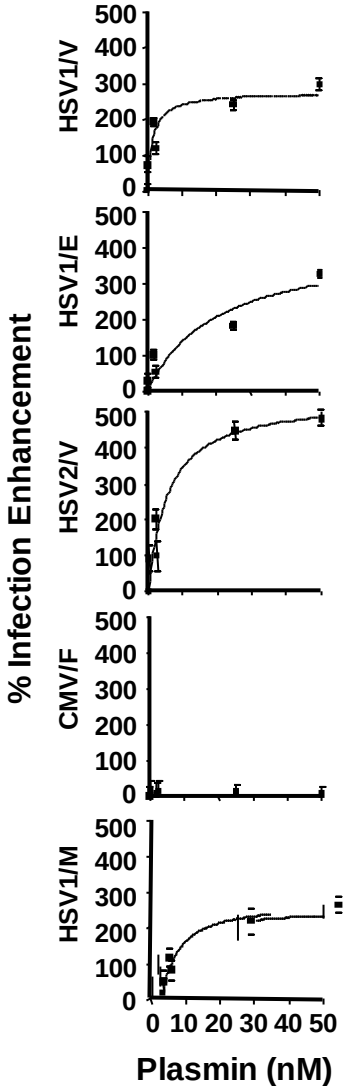


Figure 39 Purified plasmin enhances herpesvirus cell infection. HUVEC in serum- free media were simultaneously inoculated with a constant amount of plaque-forming units for each herpesviruses (HSV1/V, 1.2 x 10^6vp mL^{-1}; HSV1/E, 5.8 x 10^6vp mL^{-1}; CMV/F, 9.72 x 10^6vp mL^{-1}; HSV2/V, 1.10x 10^8vp mL^{-1} and HSV1/M, 1.0 x 10^7vp mL^{-1}) at the indicated concentration of plasmin. The cells were stained after 24 hours and the amount of infection (number of virus plaques) was determined. The data is presented as the % enhancement of infection and were corrected for the number of plaques detected in the absence of added plasmin (n=5; ± standard error of the mean)

activation to plasmin by tPA independent of fibrin. In addition to increasing the rate of plasminogen activation, these herpesviruses also accelerate fibrinolysis, despite the overwhelming concentration of fibrin in these experiments. The effect on tPA activity conferred by HSV1, HSV2 and CMV was inhibited by EACA, which indicated contributions from C-terminal lysine containing proteins on the virus surface.

To identify a putative viral protein that participates in enhanced plasmin generation, we investigated a role for the host cell-derived plasminogen receptor and tPA cofactor, A2, because it has been identified on the surface of CMV(384,515). Here we show that A2 is also associated with purified HSV1 and HSV2. Ligand blots demonstrated that viral A2 is a prominent plasminogen-binding protein, which was confirmed by an inhibitory A2-specific Ab and an A2-deficient virus. However, numerous additional virus proteins were found to bind to plasminogen that could be virus surfaces glycoprotein or additional plasminogen receptors such as as alpha-enolase, actin or cytokeratin (316) derived from the cultures cells or virus structural proteins. These are likely the basis for A2 not being obligate for virus-mediated enhancement of plasminogen activation by tPA. Interestingly, a recent report showed that A2-mediated plasminogen activation enhanced the replication of influenza virus(253). Although a role for plasminogen activation was not investigated, A2 has also been shown to increase CMV infection of cells (86), HIV replication(410) and entry of rabbit vesivirus into cells(140). Thus A2 may have multiple roles in infection depending on the virus.

Our laboratory previously reported activation of FX with subsequent thrombin generation on the surface of HSV1, HSV2 and CMV(133,457). The advantage of thrombin generation to the virus is that infection of cells is enhanced through PAR1 signaling (455,457). Additionally, a preliminary report from our laboratory observed

that HSV1 also utilizes FXa and FVIIa to significantly increase infection through PAR2 (458). Here, the data for another hemostatic protease, plasmin, whose generation is facilitated by the virus that also, enhanced infection of cells. Like thrombin, plasmin is known to cleave PAR1 leading to cell stimulation (239,373). Thus a general mechanism is emerging that suggests these viruses activate and exploit numerous hemostatic serine proteases to amplify cell infection through PARs.

Direct mechanisms of plasminogen activation by microorganisms are well documented (85,253,320). Here we show that independent of exogenous tPA, herpesviruses purified from different types of host cells were capable of activating plasminogen demonstrating a plasminogen activator associated with the virus. This activity was inhibited by EACA and PAI-1. While the former eliminates host cell-derived urokinase plasminogen activator as a candidate (261), both are inhibitors of tPA. Based on the amount of activity observed, the estimated concentration of putative endogenous tPA that could account for the observed activity would be ~ 5 pM. While we cannot exclude host-cell derived tPA as a possible virus-associated plasminogen activator because it is below our physical detection capabilities at this time, influenza virus has been suggested to have an intrinsic plasminogen activating mechanism(253)[].

Virus infections may cause an imbalance between coagulation and fibrinolysis. Increases in both pathways have been reported for HCV (359), hantavirus(248) and dengue virus infections(291,320). A hyperfibrinolytic state in dengue infection has been observed, mostly in the acute stage corresponding to a higher number of virus particles and a high ratio of tPA/PAI-1(291). We have reported that the purified herpesvirus surface is highly procoagulant and initiates coagulation reactions leading to clot formation by generating FVIIa, FXa and thrombin (455,457,458). Yet, herpesviruses are

a relatively weak predictor of vasculopathy unless combined with other variables (341,438). This work demonstrates that these viruses also enhance the fibrinolysis pathway, may attenuate the intrinsic procoagulant viral phenotype and help to explain the weak overall clinical vascular risk.

5. GENERAL DISCUSSION

5.1 Overview

Of the Herpesviridae family, HSV1 and CMV have been investigated most extensively within the context of vascular disease because they infect a majority of the otherwise healthy population(463) whose age and lifestyle places them at risk of thrombosis or atherosclerosis(520). Herpesviruses expose the host to recurrent but usually subclinical infections throughout life. They induce procoagulant and proinflammatory (102) cellular changes leading to activation of hemostatic proteases resulting in enhanced virus infection. This explains their predisposition to vascular disease (156,309,486). Thus, confined recurrent infections combined with additional risk factors may establish sensitized areas of vasculature that are especially susceptible to pathology.

Although insightful, the above studies of herpesvirus infection causing thrombogenic and atherogenic changes to host cells didn't account for virus contribution independent of cells. Nevertheless, the plausibility of virus itself being able to generate thrombin and other hemostasis proteases as cell modulators is being explored in our laboratory. This study combined with our previous reports; demonstrate that the purified HSV1, HSV2 and CMV surfaces can effectively initiate the activation of coagulation and fibrinolytic proteases leading to enhanced cell infection as the earliest of the mechanisms linking viruses to vascular pathology.

5.2 Herpesviruses are prothrombotic

Members of the Herpesvirus family which include HSV1, HSV2 and CMV have been implicated in vascular disease and shown to promote a procoagulant phenotype within the host vasculature(338). Upon vascular damage, exposure of TF and aPL expressed on cells surfaces initiate coagulation. TF and FVIIa together with aPL form extrinsic tenase complex activating FX to FXa. FXa converts prothrombin to thrombin, leading to clot formation(399). To explain the molecular basis of clinical correlations, our laboratory is studying the interactions between herpesviruses and plasma proteins. Previously, our laboratory demonstrated purified HSV1, HSV2 and CMV constitute extrinsic pathway TF and aPL on their surface derived from the host cell, and can consequently bypass strict cellular control of coagulation(457). The availability of TF and aPL on the virus surface enables them to convert FX to FXa in the presence of FVIIa. The current work demonstrated that in addition to the TF dependent extrinsic pathway HSV1-induced plasma coagulation involves intrinsic pathway, FVIII, and upstream contact activation pathway, FXII (Figure 29).

5.2.1 FVIII contribution

Physiologically, the initial FXa generated from the extrinsic pathway is limited; consequently not enough thrombin is generated to form a clot. In a positive feedback loop, thrombin activates the nonenzymatic cofactor FVIII to FVIIIa (29,181). FVIIIa binds FIXa on an aPL surface to form the intrinsic tenase complex activating FX(481). Additional FXa generated by the intrinsic tenase complex amplifies thrombin generation that is necessary to form a clot. Utilizing FVIII-deficient plasma and inhibitory antibodies to FVIII, a role of FVIII contribution in plasma clotting at low virus concentration was

determined in this project. At low virus concentration, the limited amount of FXa and thrombin generated through the extrinsic pathway activated FVIIIa for feedback amplification. At high virus concentration, the viral TF-mediated extrinsic pathway overwhelms FVIII contributions. FVIII enhanced HSV1 plasma clotting with or without addition of aPL vesicles, implying the aPL on the virus surface is not limiting. The aPL on the virus envelope has been shown to facilitate the surface assembly of prothrombinase complex(372). Likewise but not yet directly established with purified proteins, the assembly of intrinsic tenase, FVIIIa-FIXa, is also likely to occur on the virus surface because it requires aPL for assembly and the virus is the only source.

FVIII contributes in accelerating plasma clot formation initiated by low levels of HSV1. Elevated FVIII levels are associated with conditions such as trauma(79) and infection(244,416). Elevated FVIII has been identified as a risk for venous and also arterial thrombosis(209). FVIII activity in thrombotic patients is often above 1.5 IU/ml and might reach levels of 4-5 IU/ml(346). In patients with myocardial infarction and ischemic cerebrovascular lesions, increased FVIII has been associated with adverse outcomes of increased mortality or earlier death (155,314). The high levels of FVIII persist over time in thrombotic patients and are not just a response to acute phase reactions. Increased plasma FVIII is associated with obesity, diabetes, infections and advanced age, all of which are risk factors for vascular thrombosis(41,65,73). Additional support of a role for FVIII in arterial thrombosis comes from the observation that in hemophilia A patients ischemic heart disease mortality is much lower than in the general male population(408,469). FVIII involvement in virus initiated clot formation would predict that hemophilia A patients are protected from infection relative to healthy individuals. This possibility is supported by the finding that Kaposi Sarcoma virus,

another herpesvirus is the less prevalent in patients with hemophilia A compared to other subpopulations(436). An increased FVIII level predisposes to a hypercoagulable state that leads to thrombin production, which by our findings enhances cell susceptibility to infection. The availability of higher FVIII levels would be predicted to augment the virus-initiated coagulation leading to an enhanced prothrombotic state with a dual effect on pathology.

5.2.2 Contact pathway

This study demonstrated that herpesviruses initiate plasma clotting through the contact pathway to support thrombin formation. The contact pathway is initiated through activation of FXII. FXII has no role in normal hemostasis as congenital deficiency of FXII does not lead to bleeding problems(223). However, recent studies have implicated FXII activation contributing to thrombotic pathology. FXII deficient mice are protected from experimental induction of thrombosis(387). Additional studies have identified pathophysiologic activators of FXII, collagen(473), extracellular RNA(213), protein aggregates(279) and polyphosphate(442), a highly anionic polymer found in microorganisms and secreted by activated platelets. Further evidence of a pathological role for FXII in thrombosis comes from the current work where herpesvirus-mediated activation of purified FXII in the presence of PK. In the case of HSV1 the aPL on the virus envelope provides the negatively charged surface for activation of purified FXII in the presence of PK. aPL, a characteristic of enveloped viruses is a new addition to the list of FXII activators. The in vitro experiments and animal studies support a thrombogenic role of FXII, however there is still an ambiguity over FXII contribution to human thrombosis (98,134,227,528). A downstream contribution of FXII is activation of

FXI, which activates FIX leading to thrombin generation. Inhibition of FXI prolonged HSV1 mediated plasma clotting. In sharp contrast to FXII, FXI deficiency is associated with bleeding diathesis(8) and is an established risk factor for thrombosis in humans(274). The segregation of thrombosis and hemostasis at the FXII and FXI level and interactions between them is interesting and provides opportunity to target new anti-thrombotic therapy.

Overall, the herpesvirus envelope is composed of host encoded, TF and virus genome encoded gC and aPL derived from the host cells. TF and gC enable herpesvirus mediated activation of extrinsic pathway(456,457). The aPL on the virus surface facilitates direct activation and assembly of coagulation enzyme complexes – extrinsic, intrinsic, contact pathway and prothrombinase components leading to thrombin generation. The herpesvirus-mediated thrombin production has a dual effect. The thrombin generation will lead to clot formation contributing to vascular disease and enhance host cell susceptibility to viral infection through activation of PARs(455). Thus, the virus-mediated thrombin generation is likely one of the earliest events of the infection mechanism. The robust thrombin generation by the virus with subsequent amplification of virus replication suggests that herpesvirus infection should be a much stronger clinical prothrombotic risk factor.

5.3 Herpesviruses enhance fibrinolysis

The data presented here demonstrate HSV1, HSV2 and CMV enhance tPA-dependent generation of plasmin and fibrinolysis. In the absence of added tPA, these viruses demonstrated a low level of fibrinolytic activity, possibly suggesting a tPA-independent mechanism. This indicates an alternate pathway for plasminogen

activation that involves virus-associated proteins either acquired from the host or encoded by the viral genome that acts as plasminogen activator. The most probable plasminogen activator associated with the virus would be tPA or uPA derived from host cells. EACA inhibition of plasmin generation and fibrinogen clot lysis excludes uPA, which unlike tPA is not a lysine-binding species (261,415). PAI-I, an inhibitor of both uPA and tPA, but more selective for tPA (83), inhibited the herpesvirus-mediated fibrinogen clot lysis that was independent of exogenous tPA. Experiments to exclude the possibility of host cell derived tPA and identify the plasminogen activator associated with herpesviruses are discussed in the section on future work (below).

In addition to its role in fibrinolysis (181), plasmin degrades extracellular matrix proteins, that allows endothelial, smooth muscle, inflammatory or cancerous cell migration (232,232). Further, plasmin also activates cytokines stimulating inflammatory response. In the current study, addition of purified plasmin amplified herpesvirus mediated endothelial cell infection. Explaining the advantage to the virus of activating plasminogen, the infectivity of cells was enhanced by plasmin. Thus, initiation of plasmin generation may attenuate the potential vasculopathic effects of the highly procoagulant herpesvirus surface, while at the same time enhancing virus replication.

Plasmin has been shown to stimulate cells through PARs (285). Also, other hemostasis proteases, FXa and thrombin generated on the herpesvirus surface have been shown to signal cells through PARs resulting in enhanced cell infection(458). Thus, plasmin generation could enhance virus replication and also increase invasiveness possibly leading to metastasis when the virus is known to be associated with cancer(300). For example Kaposi sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV) and CMV have been linked to a number of cancers (440),

wherein increased plasminogen activators is a hallmark (337).

The herpesvirus-mediated plasmin generation may help to explain why infected individuals do not always develop thrombosis due to viral thrombin production. However the number of virus particles required for plasmin generation is at least 1000 fold more that required for clot formation tilting in favor of thrombin production. Therefore, the herpesvirus-mediated plasmin generation demonstrated here using purified systems needs to be explored further at the cellular level using HUVEC cells. It is known that stimulated HUVEC cells secrete tPA that will initiate plasmin generation (257). However, at the same time there is increased PAI-1 secreted from platelets that would shut off the fibrinolytic pathway leading to clot stabilization(176). Therefore, to substantiate which way herpesviruses shift the balance of coagulation and fibrinolysis, all the cellular complexities of vasculature should be studied together and is discussed in the section on future work.

5.4 Herpesviruses- a model enveloped virus

Virus infections induce alterations in hemostatic balance and contribute as a risk factor to vascular disease. In addition to the Herpesvirus family, studies have reported the implication of HIV1 and HIV2 (32,122,503,514), Hepatitis B (194), Hepatitis C(484), influenza A and B (329,502), vaccinia and rubella virus(299) in vascular disease. A common characteristic of all of the above mentioned viruses is that they are enveloped viruses that constitute aPL derived from the host cell (443). HSV1 and CMV have been investigated most extensively within the context of vascular disease. Here, HSV1, HSv2 and CMV were used as model enveloped viruses to follow the interaction between purified herpesviruses and hemostatic proteins.

The herpesvirus envelope constitutes host and virus genome encoded proteins and host cell derived aPL (456,457) that contribute to activating host coagulation and fibrinolysis proteases. These act as cell modulating agents and have been shown to enhance host cell susceptibility to viral infection (455) (Figure 40) . Cumulatively, these data provide insight into the early interaction between viruses and hemostatic host proteins contributing to vascular pathology. Thus, the ability of herpesviruses to activate hemostatic proteins is an evolutionary advantage and may be a generalized virus phenomenon. A mechanism is emerging wherein the virus envelope constituents initiate the activation of coagulation and fibrinolysis enzymes that contribute to vascular disease and modulate host cells to become more susceptible to infection. Therefore, although this study focuses on HSV-1, HSV-2 and CMV other enveloped viruses are also likely to increase an individual's risk of developing vascular disease.

Upon viral infection, endothelial cell perturbation is a common feature that alters hemostasis. HSV1, CMV, influenza virus(493), measles virus, mumps virus, human T-cell lymphoma virus (HTLV) type I, HIV and other viruses have been shown to infect endothelial cells(216). Infection of endothelial cells by HSV1 induces a hypercoagulant phenotype resulting in enhanced thrombin generation and fibrin deposition (156). HIV infection increases cell surface expression of TF leading to increased thrombotic risk(122). Influenza virus infection acts as a prothrombotic factor(493) and has been correlated to the occurrence of acute myocardial infarction in individuals with other preexisting coronary artery disease(280,333). Also, influenza virus–induced endothelial dysfunction supports the role of virus infection as a cardiovascular risk factor(266). Host cells infected with measles virus, or murine hepatitis virus display increased thrombogenesis (123,295). At least for the viruses studied in the current work, once

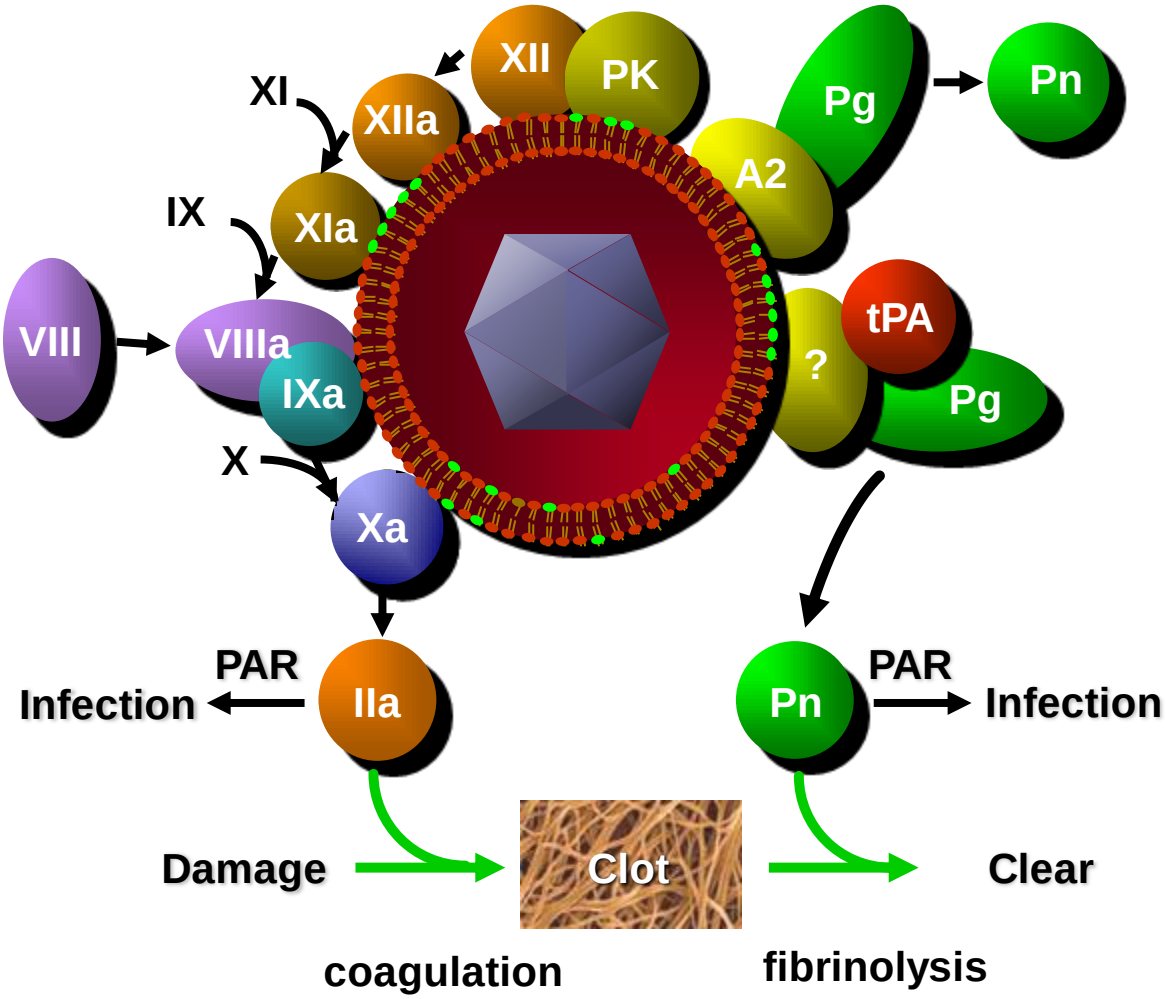


Figure 40 Herpesviruses activate hemostatic proteins. Herpesviruses initiate coagulation and fibrinolysis for enhanced cell infection through protease activated receptor signaling

thrombin is generated, PAR activation enhances virus infection(455). For CMV this may involve cell-surface exposure of A2(86). Thus, it's a vicious cycle wherein hypercoagulability predisposes to infection and infection leads to hypercoagulation. Thrombin induces cell surface expression of A2 (362), and A2 has been shown to increase CMV infection of cells(86) and replication of HIV(410). A2 is furthermore a cofactor for tPA-mediated plasminogen activation to plasmin(161), providing a link between coagulation and fibrinolysis. This work has demonstrated A2 association with HSV1 and HSV2, herpesvirus-mediated plasminogen activation and fibrinolysis, and plasmin enhancement of cells susceptibility to herpesvirus infection. Recently, plasmin has also been shown to stimulate cells through PAR1 (285) and enhance cell surface TF activity of endothelial cells. Thus evidence is accumulating that numerous enveloped viruses may trigger the activation of both coagulation and fibrinolysis enzymes with impact on health, that may enhance the ability of the virus to replicate.

In summary, we now know that herpesviruses mediate activation of coagulation serine proteases FVIIa, FXa and thrombin and the fibrinolytic serine protease plasmin to enhance cell infection. Figure 40 presents an in vitro model which aims to explain the interactions between virus surface proteins and host plasma proteins and their contribution to vascular disease. A general mechanism for enveloped viruses that suggests how these viruses activate and exploit numerous hemostatic serine proteases to amplify cell infection through PARs contributing to vascular pathology is emerging.

5.5. Future work

5.5.1 Plasminogen activator associated with herpesviruses

The molecular details of herpesvirus-mediated plasminogen activation independent of

exogenous tPA is yet unknown. In the past, one study reported increased plasminogen activation for herpesvirus transformed cells over non-transfomed cells (3). Minute amounts of cell-derived tPA or uPA being associated with these viruses cannot be ruled out. The virus-mediated fibrinogen clot lysis independent of exogenous tPA was inhibited by both EACA and PAI-1. EACA inhibition of virus-mediated fibrinogen clot lysis excludes uPA. EACA would not affect uPA, as it lacks lysine binding sites (415). PAI-1 is an inhibitor of plasminogen activator, more selective for tPA but not exclusive (83). Therefore, to exclude that it is cell derived tPA, foremost the different cell lines used in the current study should be evaluated for tPA production before and after virus infection.

The estimated amount of tPA-like species that is associated with virus particle number used in the current study is 1000 times below the detection limit of currently available techniques. Correspondingly, an increased number of herpesvirus particles should be evaluated for tPA using western blotting. Further, the virus particles could be resolved on SDS-PAGE and fractionated based on molecular weight, followed by trypsin digestion and analyzed using mass spectrometry for tPA. Along with virus fractionated proteins, purified tPA at levels above and 1000 fold below the detection limit should be used. Alternatively, the herpesvirus particles could be incubated with or without PAI-1. PAI-1 would bind to virus associated tPA/tPA-like species forming a 1.1 stoichiometric complex. Following that fluorophosphonate (FP)-Biotin, a biotinylated active site directed serine protease inhibitor(268) would be added. The biotinylated inhibitor-virus protease complex would be resolved by SDS-PAGE and probed using avidin blot. The binding between biotin and avidin is very specific and strong (Kd = 10^{-15} M)(249). FP-Biotin binding to virus associated tPA/tPA-like species would be blocked in

the presence of PAI-1. The corresponding bands in the absence of PAI-1 would be analyzed through mass spectrometry to rule out tPA as described above. The identification of mechanistic details of the tPA like species associated with herpesvirus-mediated plasminogen activation would aid in better design of a therapeutic fibrinolytic agent.

5.5.2 Herpesvirus-mediated alteration of coagulation and fibrinolysis

Under resting conditions the endothelial cell surface is anticoagulant because of constitutive expression of thrombomodulin, heparan sulphate cofactors for the anticoagulant pathway and secretion of plasminogen activator to mediate fibrinolysis (216). In vitro cell culture studies have been well documented that show herpesvirus infection converts cells from a resting anticoagulant to a procoagulant state (218,412,491), but no reports assessing effects on fibrinolytic pathway have appeared.

Plasminogen activation by cultured endothelial cells upon herpesvirus inoculation would be evaluated first using purified proteins as an extension of the current work. As a further extension, platelets would be added to the endothelial cell system, because in vivo the platelets provide procoagulant and anti-fibrinolytic properties. Moving further in complexity, plasma would be used to evaluate simultaneous virus-mediated clot formation and clot lysis using light scattering. In this experiment, purified virus particles would be titrated to determine their relative effect on coagulation and fibrinolysis. The rate of clot formation and clot lysis is expected to correlate to the number of virus particles. The clot turbidity restricts the measurement of plasmin generated. Applying a simple modification of ionic strength as described (272) would result in a transparent fibrin thereby facilitating the evaluation of plasmin generation using a chromogenic

substrate. A more recent study described an assay to determine simultaneous thrombin and plasmin (STP) generation in plasma using a mixture of TF and tPA (437). The individual fluorometric substrates were used to measure the amount of thrombin and plasmin. The surface of herpesviruses constitutes activators for both coagulation and fibrinolysis. Additionally, our laboratory has developed viruses that are devoid of coagulation initiators TF and/or gC. Using a wild type HSV1 strain and a gC deficient strain and culturing them in A7 melanoma cell (TF gene missing)/ A7III cell (transected with TF), these new viruses have been designated according to the presence of TF/gC, respectively (i.e. HSV1+/+, HSV1+/-, HSV1-/+ and HSV1-/-). These viruses would help to determine whether the activation of coagulation and fibrinolytic is independent. For e.g., in the case of TF/gC-deficient HSV1-/- it is expected to reduce the rate of plasma clot formation but without an effect on the rate of clot lysis, in other words a reduction in thrombin but not plasmin generation. The STP assay described earlier would be employed to measure herpesvirus-mediated thrombin and plasmin generation in plasma. And, addition of exogenous tPA along with herpesviruses would resemble a therapeutic scenario. To account for platelet contributions, both platelet poor and platelet rich plasma will be employed.

Finally, herpesvirus infection of $tPA^{-/-}$ mice would be evaluated to determine if tPA deficiency augments vascular fibrin deposition in comparison to wild type. Mice deficient in tPA develop normally as wild type but demonstrate reduced fibrinolytic activity in plasma clot lysis and develop extensive thrombosis upon endotoxin challenge (40). A dose response of herpesvirus infection in $tPA^{-/-}$ and wild type mice leading to development of thrombosis would be studied. It is predicted that an initial increase in virus particles would enhance thrombosis and further increase would initiate fibrinolysis.

The plasma level of thrombin, plasmin and FDP in these animals would be measured, before and after infection and correlated to virus infective dose. All these experiments would help us to understand the interaction between herpesviruses and the hemostatic system and their relative contribution to vascular disorders.

5.5.3 Herpesvirus-mediated FXII activation contributing to coagulation and/or fibrinolysis

In humans and in mice, FXII deficiency has no effect on normal hemostasis. However, in an arterial injury model, mice deficient in FXII were protected from pathological thrombus formation (387). In case of humans, FXII pathophysiology is ambiguous; FXII deficiency has been identified as a risk factor for thrombosis (134,227) and reduced FXII levels protected against development of acute coronary disease (98,528).

FXII activation contributes to coagulation and inflammation through activation of FXI and PK respectively (421). Interestingly, FXIIa has been implicated in fibrinolysis (226) through activation of PK, which is known to activate plasminogen activators, uPA (191,275). The kallikrein cleaves HK to liberate bradykinin, an inflammatory mediator. Both kallikrein and bradykinin have been shown to induce tPA secretion in vivo (119). Plasma kallikrein, FXIIa, FXIa are able to activate plasminogen directly and indirectly through activation of urokinase plasminogen activator to uPA. The direct FXII dependent activation of plasminogen (137) is not very efficient when compared to uPA. When taken into account that the plasma concentrations of FXII is four fold of uPA, FXII could be equally potent as uPA in activating plasminogen(64).

The FXII-dependent activation of plasminogen has been demonstrated both in vivo (256) and in vitro. In vivo evidence is from reduced fibrinolytic activity in FXII

deficient individuals and incomplete inhibition of fibrinolysis with specific antibodies to tPA and uPA in normals (256). In vitro in a purified system and plasma, the presence of a negatively charged surface enhanced FXIIa-mediated plasminogen activation (25,420,422). Here, we have shown that purified herpesviruses potentiated FXII activation by PK and additionally, these viruses accelerated plasminogen activation leading to fibrinolysis. Therefore the possibility of herpesvirus-mediated activation of FXII contributing to plasminogen activation (Figure 41) would be evaluated in a purified system and plasma settings in a chromogenic assay as described in methods. Further, FXII contribution in herpesvirus-mediated fibrinolysis will also be determined using purified fibrinogen and plasma clot as described earlier. Purified FXII will be added to FXII deficient plasma and inhibitors of FXII, CTI and anti-FXII Ab, will be added to normal plasma. Finally, thrombosis development upon herpesvirus infection in FXII deficient mice will be evaluated. The herpesvirus mediated FXII activation contributing to plasminogen activation would mean that these viruses initiate simultaneous activation of coagulation and fibrinolytic pathways and in turn explain their weak clinical correlation to vascular disease.

5.5.4 Herpesvirus-mediated coagulation regulation by anticoagulants

The coagulation pathways are subject to regulation by soluble and cell surface-associated anticoagulants. In particular, TM and HS may be of relevance in the current study. As these may be derived by the virus from the host cell, it is possible that their anticoagulant function may attenuate herpesvirus-mediated clinical effects thereby helping to explain their modest contribution to clinical vascular disease when evaluated as an independent risk. To test this idea, virus particles would be titrated in plasma

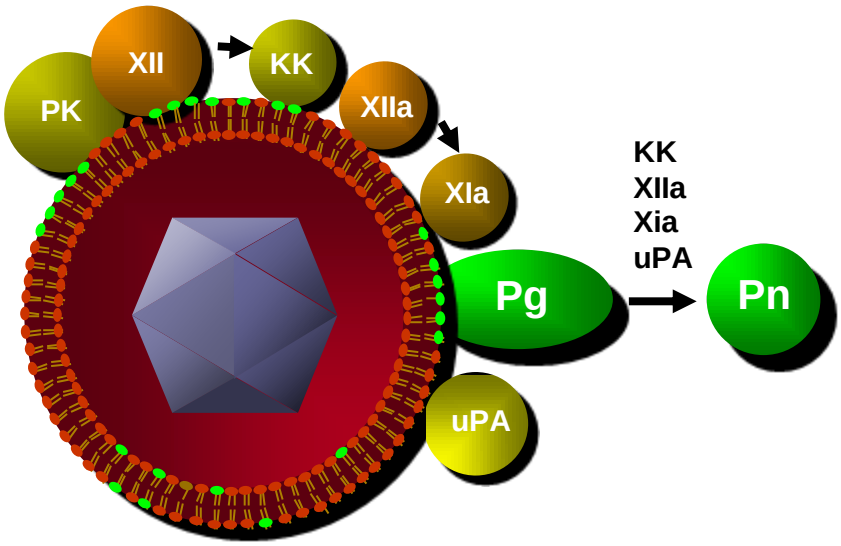


Figure 41 The activation of contact pathway on the surface of herpesvirus contributing to plasmin generation. Factor XII (FXII) and prekallikrein (PK) recruited on the surface lead to activation of FXII to FXIIa. FXIIa activates PK to kallikrein (KK). Both FXIIa and KK reciprocally activate each other. FXIIa activates FXIa. All three activated proteases KK, FXIIa, FXIa are able to activate plasminogen (Pg) to plasmin (Pn), directly and indirectly through activation of urokinase plasminogen activator (uPA).

deficient in AT or PC and the effect on clotting followed when these are added back. Purified herpesviruses especially cultured in HUVEC will be evaluated antigenically forTM and HS using antibodies by western blot analysis. These experiments may reveal an additional link between the virus and the hemostatic system, in this case further explaining the attenuated effect of a strong intrinsic and extrinsic procoagulant mechanism we have discovered on the virus surface.

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APPENDIX

Appendix A: Clinical studies correlating herpesviruses to vascular pathology

Melnick J, 1983 Lancet	CMV antigen detected in cells cultured from arterial tissue surgically removed from atherosclerotic patients.
Gyorkey F 1984 Exp Mol Pathol.	Herpesvirus particles were identified by electron microscopy in aortic tissue from 10 of 60 atherosclerosis patients.
Adam E 1987 Lancet	In patients undergoing cardiovascular surgery 57% of them had high level of CMV titers versus 26% in control group. (Population size=157 pairs)
Petrie B, 1987 J Infec Dis	CMV antigen in cultured SMCs from arterial plaques
Yamashiroya H 1988 Am J Pathol	HSV or CMV antigen detected in eight of 20 specimens from coronary arteries of young trauma victims.
Grattan MT 1989 JAMA	In transplant recipients; CAD incidence was 10% in patients with no evidence of CMV versus 30% in those with CMV infection Increased graft rejection, as a consequence of accelerated atherosclerosis was 69% in patients with CMV infection. CMV seropositive patients, who received a seronegative organ, had higher risk of transplant atherosclerosis. (Population size=301 pairs)

McDonald K 1989 Am J Cardiol	In transplant recipients, CMV infection occurred in 62% of patients with Coronary Artery Disease (CAD) and in only 25% of those without. (p = 0.007) (Population size=102)
Havlik RJ 1989 Arteriosclerosis.	The Framingham Heart Study of 658 male and 919 female (ages 58 to 89) found no association of "fever blisters or cold sores" to coronary heart disease. However in a subgroup of women with recurrent cold sore infection, a very mild risk 1.5 was noted.
Hendrix M, 1990 Am J Pathol.	CMV genome in 90% of samples obtained from patients with severe atherosclerosis compared with only 53% in patients with minimal or no atherosclerosis.
Tanaka S, 1992 J Vasc Surg.	CMV DNA was detected in 88% (7 of 8) of inflammatory aortic lesions, including 5 of 6 aortic aneurysms, and 61% (20 of 33) of atherosclerotic aneurysms, but only 31% (5 of 16) autopsy samples without inflammation or atherosclerosis.
Koskinen P 1994 Am J Pathol.	CMV infection associated with subendothelial inflammation in atherosclerosis of coronary allografts.
Koskinen P 1993 J Heart Lung Transplant.	29/53 recipients developed CMV infection during the first post transplant year. CMV-infection was associated to increases intimal thickening and accelerated cardiac allograft vasculopathy in comparison to CMV negative
Paavonen T 1993 J Heart Lung	CMV infection contributed to the development of endothelialitis and accelerated arteriosclerosis in heart

Transplant.	transplant recipients.
Koskinen P, 1993 J Heart Lung Transplant	CMV infection occurred in 27 of 46 patients, shown by CMV-antigenemia test. Acute rejection episodes were diagnosed in 15 of 27 patients with CMV and in 9 of 19 patients free of CMV.
Skowronski EW, 1993 J Heart Lung Transplant	8/15 heart transplant recipients had CMV exposure of which 3 had atherosclerosis. CMV genome detected in coronary artery specimens in a single patient with known CMV exposure, but no evidence of atherosclerosis.
Melnick JL 1994 J Med Virol	In a study of 135 patients with atherosclerosis, 89% had CMV DNA present in the arterial tissue
Sorlie PD 1994 J Med Virol	The case-control odds ratio for CMV antibodies was 1.55 (P = .03), for HSV1 1.41 (P = .07), and for HSV2 0.91 (P = .63) as a marker for carotid artery thickening. (Population size=340 pairs)
Speir E 1994 Science	CMV genome detected in 60% of human restenosis lesions
Gulizia J1995 Am J Pathol	A low frequency of CMV genome in accelerated coronary artery disease. (Study size = coronary artery segments from 41 allograft recipients and 22 donor matched controls.
Zhou FY 1996 N Eng J Med	In a study of 75 patients undergoing coronary atherectomy for CAD, prior CMV infection 21/23 (91%) of restenosis patients were CMV seropositive
Nieto FJ 1997	CMV seropositivity correlated to hypercoagulability and

ATVB	reduced fibrinolysis in a large study of Atherosclerosis Risk in Communities.
Chiu B 1997 Circulation	CMV in atherosclerotic plaques for 35.5% patients with carotid artery stenosis.
Siscovick 2000 Circulation	A 2-fold increased risk of myocardial infarction and coronary heart disease mortality in HSV1 seropositive patients as compared to HSV1 seronegative in 213 cases and 405 matched controls
Zhu J 2001 Circulation	Increased risk for myocardial infarction and coronary heart disease mortality of 2 and 1.5 fold, respectively for CMV and HSV2 seropositive. (Population= 809 coronary angiography patients)
Klein E 2002 Circulation	CMV and HSV-2 seropositivity associated with advanced atherosclerosis ($P<0.01$) (Population=572)
PowerJF 2005 Am J Clin Pathol	HSV genome detected in 21/24 biopsy specimens of cerebro-vasculitis of temporal artery.
Sheehan J 2005 Heart.	No significant association between chronic infection and cumulative burden of infection and acute coronary syndrome. Cases and controls did not differ significantly in seropositivity to CMV and HSV. there was no evidence of an increasing risk for acute coronary syndrome with increasing burden of infection.
Ibrahim AI 2005 J Clin Virol	HSV-1 DNA was detected significantly more frequently in plaques (35%) than in control veins (9%, $P = 0.006$). CMV

	and EBV DNA were exclusively found in plaques but not in controls, with 10% for CMV (P = 0.06 versus veins, P = 0.17 versus graft arteries) and 2% for EBV (P = 1.0), respectively. No HSV-2 detected in plaques or in controls. Herpes viral DNA was significantly associated only with arterial hypertension but not with other classical risk factors (P = 0.02)
Cankovic 2006 Cardiovasc Pathol	No herpesvirus genome associated with cerebro-vasculitis of temporal artery. (Population=35 biopsy specimens)
Kilic A, 2006 Pol J Microbiol	CMV DNA was found in 37.9% atherosclerotic and 32.7% non-atherosclerotic vascular wall specimens with no statistically significant differences (P > 0.05).
Ozdemir FN 2007 Transplantation Proceedings	CMV infection may be a triggering factor for AE in renal transplant recipients.
Lijfering 2008 Thromb Haemost	Prior CMV infection (2- fold risk) and seroconversion (1.7-fold) in renal transplantation recipients was associated with recurrent venous thrombosis. (Population size = 606)
Y Ono 2008 Transplantation Proceedings	15/34 renal transplantation recipients were positive for CMV DNA
Yi L 2008 Acta Virol. 2008.	Levels of HCMV IE gene/protein were significantly higher in the stroke group than in control group detected by the three methods (IHC 34.3% vs. 10.0%; HIS 40.0% vs. 10.0; PCR

	60.0% vs. 30.0%). Population= 35 patients with ischemic stroke and from 20 control
Szklo 2009 J Cadriovasc Med	No association between atherosclerosis and herpesviruses (HSV and CMV). (Multi- Ethnic Study of Athersclerosis = 1056 individuals
Elkind 2010 Stroke	CMV seropositivity (P=0.009) was strongly associated to carotid plaque thickness but not HSV1 or HSV2 (Population= 3298)
Roberts et al. 2010 Am J Epidemiol.	Population size = 1,468. High CMV IgG antibody titers(highest quartile v/s with lower quartiles) in adjusted models all-cause mortality was 1.43 times higher over 9 years and, the hazard of CVD mortality was also elevated (hazard ratio = 1.35)
Simanek 2011 PLoS One.	CMV seropositive together with high CRP levels showed a 30.1% higher risk for all-cause mortality and 29.5% higher risk for CVD-related mortality compared to CMV seropositive individuals with low CRP level. Population Size= 33994 subjects more than 25 years of age (range 24-90years of age)