

Serpins, the vasculature, and viral therapeutics

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1. ABSTRACT

Serine protease inhibitors, termed serpins, are key regulators of numerous biological pathways that initiate inflammation, coagulation, angiogenesis, apoptosis, extra-cellular matrix composition and complement activation responses. Viruses have encoded serpins to guard themselves from host immune attack. The myxoma virus which infects rabbits secretes a highly potent anti-inflammatory serpin, Serp-1, which targets thrombolytic and thrombotic proteases as a means to fend off coagulation and inflammatory reactions to viral infection. These reactions act as a defense, produced by the host, to counter viral infection and invasion. When infused in animals after vascular injury, Serp-1 elicits exceptional anti-inflammatory activity, whereas the mammalian serpin, plasminogen activator inhibitor-1 (PAI-1), which also targets thrombotic and thrombolytic proteases can induce a pro-thrombotic response. During arterial injury, PAI-1 is highly expressed and increased PAI-1 concentration can result in acute thrombosis after aortic transplant in mouse

models. The reactive center loop amino acid sequence is a fingerprint for serpin function and this function is highly sequence specific such that modification in this sequence can markedly alter activity. For instance, the alteration of the serpin reactive site loop P1-P1' amino acid sequence nullified the anti-inflammatory activity of Serp-1 and modification of P2-P7 initiated a pro-inflammatory response with vascular remodeling with aneurysm formation. Furthermore Serp-1 has demonstrated the capacity to utilize a mammalian serine protease receptor, the urokinase-type plasminogen activator receptor (uPAR), to alter cellular signaling in part through the actin binding protein cytoskeletal system (via filamin B). In this review, the molecular mechanisms relating inflammation and coagulation pathways to atherosclerosis and how the viral serpin, Serp-1, modifies these pathways in order to exhibit this profound anti-inflammatory activity without associated adverse thrombosis are discussed. Viral and vascular serpins targeting the thrombolytic cascade represent a potential new and untapped therapeutic resource.

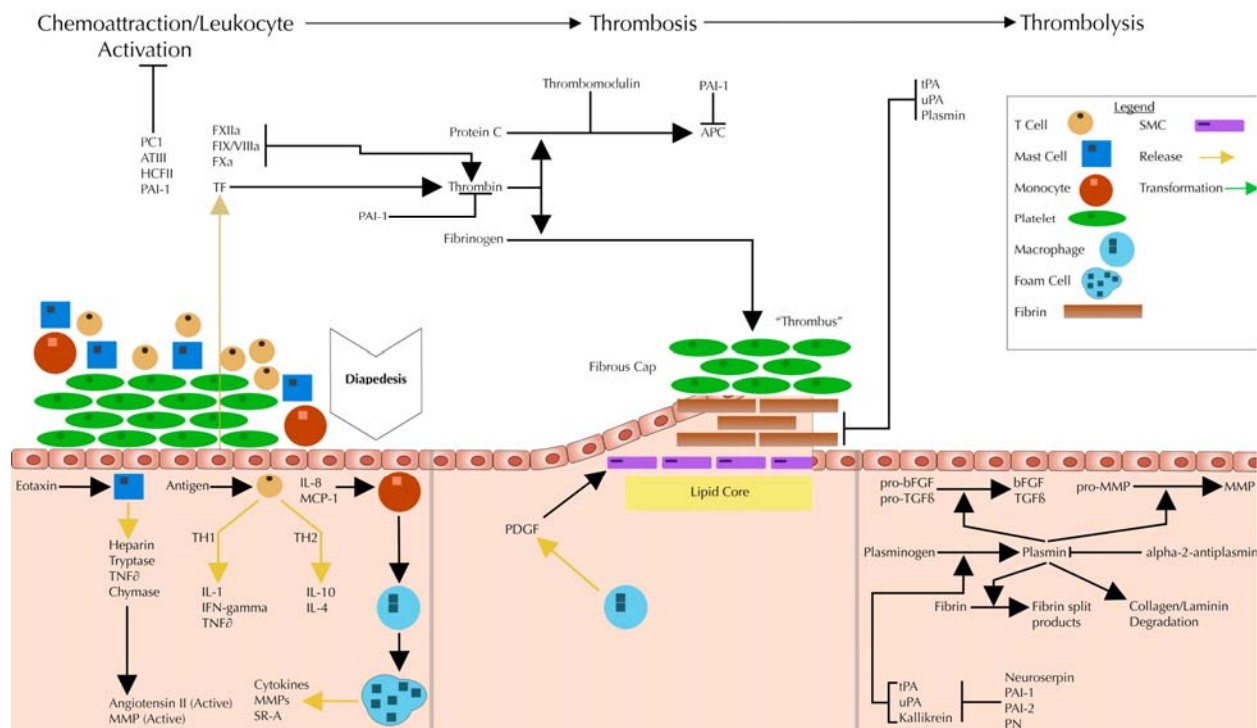


Figure 1. Inter-relation of coagulation and innate immune responses. Illustration of the processes of atherogenesis, from chemoattraction and leukocyte activation through to the development of an advanced plaque complicated by thrombosis and including the thrombolytic system and its inhibition of excessive arterial thrombosis and occlusion. The mechanisms displayed are highly simplified, however attention should be given to components (for example macrophages, foam cells, etc.) and systems (for example diapedesis, thrombolysis, etc.). PC1 (protein convertase-1), ATIII (anti-thrombin III), HCFII (heparin cofactor II), PAI-1 (plasminogen activator inhibitor-1), TF (tissue factor), TNF- α (tumor necrosis factor-alpha), MMP (matrix metalloproteinase), SR-A (scavenger receptor-A), IL (interleukin), IFN-gamma (interferon-gamma), TH (T helper cell), MCP-1 (monocyte chemotactic protein-1), PDGF (platelet derived growth factor), APC (activated protein C), bFGF (basic fibroblast growth factor), TGF- β (transforming growth factor-beta), PN (protease nexin), tPA (tissue plasminogen activator), uPA (urokinase plasminogen activator), and SMC (smooth muscle cells).

2. INTRODUCTION

For the developed world, cardiovascular disease, heart attack, stroke, and peripheral vascular disease remain the leading causes of death for adults. Several processes simultaneously converge on the vasculature having developed over different periods of time and culminate in vascular occlusion. Vascular injury triggers an inflammatory reaction resulting in the creation of a thrombotic lesion that occludes the arterial lumen, obstructing blood flow to vital organs. Injury can be caused by hyperlipidemia (high cholesterol), diabetes (high sugar), hypertension (high blood pressure), or even vascular surgery (angioplasty or bypass) designed to treat arterial blockages. Over-reach of the inflammatory responses can then result in excess growth of the lesion or rupture of the fibrous cap, exposing the hyperlipidaemic core. Erosion and rupture of the atheroma are the leading cause of acute vascular thrombosis and can be due to many factors such as collagen degradation, macrophage invasion or cellular apoptosis in the intimal layer, that may or may not be followed by a thrombotic mechanism. Thrombosis is a highly regulated response employing clot forming serine

proteases, the thrombotic enzymes, and regulating serine protease inhibitors (serpins) capable of preventing transformation of fibrinogen to fibrin by serine protease activity.

The formation of a clot is balanced by another series of activated thrombolytic serine proteases that break down fibrin. Both thrombotic and thrombolytic pathways can in turn up-regulate and be up-regulated by the inflammatory response that can lead to instability (Figure 1). Preventing plaque rupture and the liberation of the clot forming lipid-rich necrotic core is crucial for prevention of acute thrombotic vessel occlusion, the cause of heart attacks, strokes, and peripheral gangrene.

Serp-1, a viral serpin, has been demonstrated to profoundly modulate immune cell responses. This 55kDa secreted myxoma virus glycoprotein binds to and inhibits the thrombolytic serine proteases, urokinase and tissue-type plasminogen activators (uPA and tPA), plasmin, and factor Xa, inducing an anti-inflammatory state during host-rabbit infection. Serp-1 also displays exceptional anti-inflammatory activity in a wide variety of animal models of

Table 1. Summary of animal studies making use of Serp-1 treatment.

Animal	Artery	Delivery route	Outcome: plaque reduction	Reduced mononuclear cell invasion
Arterial angioplasty model				
New zealand white rabbit	Aorta	Local/Systemic (i.v./i.a)	+	+
Sprague-Dawley rat	Iliofemoral	Systematic (i.v.)	+	+
White leghorn rooster	Aorta	Systematic (i.v.)	+	+
Yucatan microswine	Iliac/Femoral	Local	+	+
Arterial stent implant model				
New zealand white rabbit	Aorta	Systemic (i.v.)	+	+
Yucatan microswine	Coronary	Local/Systemic (i.c./i.v.)	+	+
Bypass vein graft				
Sprague-Dawley rats	Renal Artery	Systemic (i.v.)	+	+
Carotid cuff compression model				
ApoE deficient mice	Carotid	Systemic (Osmotic pump - s.c.)	+	+
Allograft transplant				
Rats	Aorta/Renal/Cardiac	Systemic (i.v.)	+/+/+	+

Serp-1 reduced plaque growth in the majority these studies (83, 142).

angioplasty, stent implant, bypass surgery, organ transplant and arthritis (Table 1). Monocyte and T lymphocyte invasion into the arterial wall is generally reduced by Serp-1 infusion. This reduction in inflammatory cell invasion is mirrored by reduced activation of endothelial cells, macrophages, T cells, and platelets with associated changes in gene expression, calcium transport, and enzyme activity.

This review details serpin involvement in the thrombotic and thrombolytic responses (with emphasis on thrombolysis) and the association of serine proteases with catalysis of inflammation and plaque rupture. The unique interplay between the thrombolytic and inflammatory pathways and the regulation of these pathways by serpins will be illustrated by current studies of the myxoma viral serpin, Serp-1. The potential of Serp-1 as a curative measure is also discussed with regard to *in-vivo* and *in-vitro* data, suggesting an innate ability of this viral serpin to modulate the proteases that cause arterial thrombosis and occlusion (myocardial infarction, stroke, and peripheral gangrene). The study of a viral serpin and its interaction with human physiology has also provided new insights into the human vasculature and improved our understanding of its operation.

3. THROMBOSIS, THROMBOLYSIS AND FACTORS IN PLAQUE RUPTURE

Historically, the pathology of atherosclerosis was believed to begin with the “lipid” hypothesis or via smooth muscle cell proliferation (the benign tumor hypothesis). Due to advances in recent decades we now know that in addition, factors such as cholesterol transport, macrophage and T lymphocyte activation, proliferation and invasion as well as apoptosis of endothelial cells, macrophages, T lymphocytes and smooth muscle cells are critical players. Platelet activation and surface thrombus formation are also closely involved in plaque formation and rupture with vascular occlusion as proposed in the “Response to Injury” hypothesis put forward by Russell Ross (1). Because lines between the different phases of atherosclerotic plaque development have become increasingly blurred over time, the term “atherothrombosis” has been suggested to

incorporate the inflammatory and thrombotic components of developing plaque. To improve preventative and also pro-active medical procedures, a complete analysis of cross-talk between inflammation and both coagulation and fibrinolysis is ongoing (2-5).

3.1. Thrombosis and Factors Leading to Inflammation

3.1.1. Initiation and leukocyte recruitment

Vascular injury has been clearly identified as a catalyst of atherosclerosis (6). In this “Response to Injury” hypothesis, the vascular endothelial cell layer plays a central role in mediating interplay in the arterial response to damage by such agents as elevated serum cholesterol, hypertension, diabetes, infection, or more direct mechanical trauma such as invasive arterial surgery and percutaneous intervention. Pro-inflammatory cytokines, interleukin 1- β (IL-1 β) and tumor necrosis factor- α (TNF- α), are released inducing expression of vascular cell adhesion molecule-1 (VCAM-1) (7) and intercellular adhesion molecule-1 (ICAM-1) (8) on the endothelial cell surface (Figure 1). VCAM-1 has been observed binding subsets of leukocytes, monocytes and T-lymphocytes present in early atherosclerotic lesions (7). Characteristic expression of surface integrins, very late antigen-4 (VLA-4) on monocytes and lymphocyte function associated antigen-1 (LFA-1) on lymphocyte membranes, increases monocyte and T cell interaction with endothelial cell adhesion molecules (9). CD62P and CD62E selectins slow circulating lymphocyte rolling allowing cellular adhesion to the injured endothelium (10-11).

3.1.2. Leukocyte translocation and chemotraction

Upon adherence to the endothelial membrane surface, leukocytes migrate to the intima by diapedesis through the junctions of adjacent endothelial cells and even through the endothelial cell centre (12-15) in response to chemokines. Monocyte chemoattractant protein-1 (MCP-1), a CC chemokine (16), and IL-8, a CXC chemokine (17), have similar roles directing phagocytic mononuclear cell and leukocyte recruitment into early atheroma, respectively. *In vivo* work with murine knockout models deficient in MCP-1 and the MCP-1 cognate receptor CC chemokine receptor 2 (CCR2) has demonstrated a

pronounced decrease in mononuclear phagocyte accumulation and atherosclerotic plaque growth (16). Chemokines in three chemokine classes, namely CC, CXC, and CX₃C, have in fact been found to be clearly associated with plaque progression after vascular injury (18).

3.1.3. Leukocyte activation

Macrophages have been long understood to play major roles in both the innate and adaptive immune responses. They are capable of releasing oxygen radicals, complement, cytokines and growth factors but also have the ability to present antigen to T cells. Hematopoietic monocytes, already resident in intimal plaque, undergo differentiation to macrophages upon contact with macrophage colony stimulating factor (M-CSF) (19) which is over-expressed in atherosclerotic plaque lesions. Macrophages have numerous effects as part of the arterial response to injury with an increased release of growth factors and cytokines, an elevated expression of scavenger receptor-A (SR-A), which acts as a pro-survival agent and a mitogenic stimulus (20-21), and the release of cytokines such as interferon gamma (IFN- γ), tumour necrosis factor-alpha (TNF- α) (22-23), and IL-6 (24).

3.1.4. Atherosclerotic plaque progression

Monocyte/macrophage and T lymphocytes are in turn activated by, and activate, thrombotic enzymes either directly or via the exposure of pro-thrombotic plaque. Thrombin is a central serine protease in the coagulation cascade that has been observed as a critical and potent growth factor and chemoattractant in tissue repair in addition to the fibrin formation essential to clot formation. Thrombin has effects on platelets, macrophages, fibroblasts, and smooth muscle cells, all of which are known to have integral roles in plaque growth. Thrombin is produced either through the intrinsic or extrinsic coagulation pathways, both converging on factors Xa (a serine protease) and Va and forming the prothrombinase complex which cleaves pro-thrombin to release active thrombin. Thrombin then activates formation of fibrin from fibrinogen (Figure 1). Thrombin also has mitogenic effects with the release of growth factors from platelets, matrix proteins from fibroblasts, initiating proliferation of SMC's and fibroblasts, monocyte and SMC migration, and finally the differentiation of monocytes (25-28). Coupled with this, platelet derived growth factor (PDGF) is released from macrophages and platelets and acts as a chemottractant for SMC's (29-30).

Tissue factor (TF) and factor VIIa (FVIIa) have also been implicated in plaque progression. FVIIa is able to elicit several signaling effects in SMCs including upregulation of poly(A) polymerase (31) and urokinase type plasminogen activator receptor (uPAR) (32). Beyond its activation of coagulation and intracellular signaling, FVIIa binding to TF, a cell surface anchored glycoprotein, on SMCs also induces proliferation via a coagulation independent mechanism (33). In the context of vascular injury the result of this complex series of cellular interactions is the eventual termination of blood flow through tissue growth (the atheroma) and the creation of a stable, or unstable, thrombus plug (25-28).

3.1.5. Regulation of thrombosis

Functional redundancy is seen in the numerous pathways that act to inhibit the clotting cascade. One key regulatory pathway is composed of thrombolytic serine proteinases that dissolve clots (Figure 1). Both thrombotic and thrombolytic enzyme cascades are regulated by serpins. Thrombosis is regulated by the serpins antithrombin III (ATIII)(SERPINC1) and heparin cofactor II (HCII)(SERPIND1), as well as the Kunitz inhibitor, tissue factor pathway inhibitor (TFPI). As a second mechanism, activation of the protein C pathway (an anticoagulant cascade) that inactivates prothrombinase and the factor IXa/VIIIa ('ten-ase') complex also participates in the regulation of thrombosis and is, in turn, regulated by serpins (34-35).

3.2. Inflammation and Thrombolysis

Thrombolysis is a serine protease system that is upregulated whenever there is clot formation for the purpose of striking an innate balance. To prevent the over-activity of the thrombotic processes there is a counter-balancing thrombolytic cascade of sequential serine protease activators. The enzymes involved in clot production activate the thrombolytic system which ultimately inhibits excessive arterial thrombosis and occlusion (36-37). Thrombolysis begins with the activation of plasminogen through uPA and tPA to form plasmin which then goes on to cleave fibrin. Not only is the fibrin network degraded but additionally the pro-form of MMP's and growth factors are activated and released into the surrounding cellular substratum. Transformation of plasminogen to plasmin, the primary thrombolytic protease, is directly regulated by tPA, uPA, and thrombolytic plasminogen activated serine proteases. The thrombolytic proteases are secreted by endothelial cells, monocytes, and smooth muscle cells (SMCs) in the arterial wall along with their specific inhibitory serpin(s); plasminogen activator inhibitor-1 (PAI-1)(SERPINE1) being the main regulatory serpin for the plasminogen activators. The plasminogen activators, specifically tPA, are also activated by the thrombotic factors thrombin and fibrin thereby creating a positive feedback loop (38).

The uPA receptor (uPAR) has been identified as potentially playing a central role in inflammatory cell invasion. Elevated levels of uPAR, tPA, and uPA are detected in areas of atherosclerotic plaque growth (39-44). When uPA is bound to uPAR, this complex enhances inflammatory cell migration (40, 42, 46-48), cell to matrix to cell adhesion via vitronectin (45, 49-50), cell invasion through activation of matrix metalloproteases (MMPs) such as collagenase and elastase (39, 51), and the release and activation of growth factors (52). Furthermore, uPA bound to uPAR is found on the leading edge of invading macrophages at sites of fibrous cap degradation, one of the factors that are believed to compromise the plaque surface leading to an unstable prothrombotic (clot forming) plaque surface (39, 53). Inhibition of uPA and tPA by PAI-1 or PAI-2(SERPINE2) can be mediated either through a direct binding mechanism in the circulating blood (42), or via PAI-1 binding of the uPA-uPAR complex on the cell surface (Figure 1) followed by internalization and

degradation of this ternary complex (36, 38, 40). PAI-1 is considered the major player in regulation of the thrombolytic cascade in the vasculature.

Therefore, inhibiting the thrombolytic cycle may reduce cellular infiltration resulting in plaque stabilization. The thrombolytic inhibitors include serpins, such as PAI-1, PAI-2, and protease nexin (PN)(SERPINE2) that may function both to block clot lysis and to increase clot formation, but also to modify plasminogen activator and plasmin induced enhancement of inflammatory cell responses. PAI-1 may thus be a critical mediator in the above cascades as it determines whether processes that initiate vascular thrombosis and arterial occlusion proceed.

3.3. Factors in Plaque Rupture

A complex atheroma arises from the migration of smooth muscle cells and macrophages into the inner most arterial layer (intima) bordering the vascular lumen with subsequent cellular proliferation, deposition of lipid, and development of an extensive extra-cellular matrix. As inflammatory macrophages and T lymphocytes continue to invade the plaque this leads to plaque erosion, thrombosis, and arterial occlusion (54-55). The serine proteases tissue factor (TF), factor VIIa, thrombin, and fibrinogen play critical roles in the transition of atherosclerosis from a chronic state to an acute syndrome. The thrombolytic serine proteases are now believed to also trigger plaque surface degradation through pro-matrix metalloproteinase activation and to activate, as well as enhance, inflammatory cell adhesion and migration. Atherosclerotic plaque can rupture either through fracture of the fibrous cap (56) or superficial erosion of the intima (57). In both scenarios the end-result is the same with myocardial infarction (heart attack), peripheral vascular occlusion (gangrene), or cerebrovascular accident (stroke).

Plaque fracture can result from a structurally weakened fibrous cap which can be due to many factors. Collagen is responsible for providing the majority of the mechanical strength in the fibrous cap, however, it is susceptible to the effects of both catabolism and decreased synthesis. Cytokines such as IFN- γ released by T cells down-regulate collagen synthesis by SMC's (58). Furthermore, macrophages over-express MMPs and elastolytic cathepsins that are capable of breaking down collagen and elastin in the extra-cellular matrix. The same matrix degrading enzymes that are charged with aiding SMC migration and arterial remodeling represent the forces also responsible for weakening the fibrous cap (59-60).

A second factor leading to a structurally weakened fibrous cap is loss of SMC's through apoptosis or damage. Areas of local inflammation often contain T-lymphocytes and macrophages that associate with SMC's via membrane surfaces. This association can inadvertently initiate apoptotic mechanisms (61). As a result, a reduction in SMC's occurs in areas where rupture will later develop and because SMC's are primarily responsible for collagen synthesis, their absence leaves the region weakened (62) and prone to rupture.

Superficial erosion of the intima accounts for roughly a fourth of all myocardial infarctions (63-65), but the underlying biochemical mechanisms remain poorly understood. Apoptosis of endothelial cells can result in a locally desquamated area and the presence of MMP's, for instance gelatinases (MMP-1, MMP-2, MMP-3, and MMP-9) (66), can degrade collagen after their conversion to the pro-enzyme form by uPA, tPA, and plasmin. Removing the physical link between endothelial cells and the basal lamina also promotes endothelial cell apoptosis and desquamation (67). Tissue inhibitors of metalloproteinases (TIMPs) do not counteract MMP activity due to an excess of inflammatory mediators, such as IL-1 β , CD154, and TNF- α , that act on mononuclear phagocytes, endothelial cells, and smooth muscle cells to augment MMP expression (68-69). An inactive pro-MMP is subsequently released from the cell and activated through cleavage by an activating proteinase, for example tPA, uPA or plasmin (68).

Superficial erosion of the atheromatous plaque causally leads to microvessel formation. Not only is there ongoing production of growth factors for SMC's, but a simultaneous release of angiogenic mediators such as acidic and basic fibroblast growth factor (a and bFGF) and vascular endothelial growth factor (VEGF) also occurs, all of which are activated and released from matrix stores by thrombolytic serine proteases (70-71). Serpins have also been reported to regulate new vessel formation, for instance PAI-1 (72) and Human Kallikrein 5 (hK5) (73). It is understood that microvessel formation serves a nutritive function, but it also represents both a potential site of hemorrhage and potential plaque rupture (74). Angiogenic mediators and other agents released by inflammatory cells can go on to stimulate cells or promote particular activities conducive to vessel creation, however in the context of a fibrous plaque, vessel formation requires weakening the forces present to stabilize the plaque. Classical serpins (such as PAI-1 and PN) have been shown to alter cell migration and MMP activity whereas cross class serpins (serpins that bind and inhibit both serine and cysteine proteases) have been reported to alter cellular apoptotic responses.

3.4. Serpins

3.4.1. Introduction

Serpins are a large and growing superfamily of structurally homologous yet functionally diverse proteins. Their presence in the biological world has expanded from beyond mammals, specifically *Homo sapiens*, to include examples from kingdoms Plantae, Metazoa, Bacteria, and Viruses. Recently in an evolutionary study serpins that appear in unicellular organisms were found to be phylogenetically distinct when compared to clades from their multicellular eukaryotic counterparts. Serpins encoded by unicellular organisms are now believed to all generally possess an inhibitory capability, while organisms that encode several serpins within the same genome present a range of inhibitory action produced by variability in the RCL, particularly around the P1-P1' linkage (75).

Phylogenetic analysis has revealed 16 clades in the Serpin family with 10 divergent orphan sequences (76).

Functional classification is difficult due to the fact that the majority of serpins inhibit serine proteases of the chymotrypsin family thus making differentiation on the basis of inhibitory specificity somewhat more difficult. Some serpins also act as cross class inhibitors that inhibit both serine protease and cysteine protease (caspases) activity, for instance proteinase inhibitor-9 (PI9)(SERPINB9), Serp-2, and cytokine response modifier-A (CrmA) (77). Other serpins have lost their inhibitory mode of action performing other functions, for instance hormone transport. Overall, serpins can make up 2 to 10% of circulating plasma proteins with examples that include corticosteroid-binding globulin (CBG)(SERPINA6), blood pressure regulation (angiotensinogen, AGT, SERPINA8), and hormone transport (thyroxine binding globulin, SERPINA7) (78). Approximately 500 serpins have been identified thus far consisting of between 350 and 400 amino acids with molecular weights ranging from 40 to 55 kDa (79).

More recently a number of new serpin subsets have been identified, many with as yet undefined functions and mechanisms. One such group are the ov-serpins, so called because of amino acid sequence similarity to chicken ovalbumin (among others). Upon comparison with the prototypical serpin α_1 -antitrypsin (α_1 -AT)(SERPINA1), ov-serpins are shorter at both termini, lack a secretory signal peptide (this despite the fact that they have been observed secreted from cells), and have an additional polypeptide loop that may add additional effector functions (80). Interestingly, in one such loop in bomapin (SERPINB10) a nuclear localization signal is present (81). This group predominantly comprises serpins capable of inhibiting more than one target serine proteinase. For this reason, this serpin group has been tenuously assigned the primary role of barrier function where ov-serpins have been observed in host defense, inactivating foreign proteases that are of bacterial or viral origin (for instance PI9) (82-83).

PAI-1 is thought to serve a protective role through its' prevention of excessive serine hydrolysis. When PAI-1 is not expressed in genetically deficient mice, this protective role only becomes evident with the development of increasing plaque growth after induced vascular injury (84-91). Administering PAI-1 with an adenoviral vector blocked this increase in plaque (92). Other studies have however shown PAI-1 to accelerate thrombosis, angiogenesis, and plaque growth (93-96). Neuroserpins (SERPINI1) have also been found to provide a homologous role in protecting the brain from ischemic injury. *In vivo* work with the rat stroke model has revealed that injection of neuroserpin lowered both apoptotic cells and stroke volume significantly (91). Conversely, elevated levels of the serpin α_1 antichymotrypsin (ACT)(SERPINA3) have been observed in the plaques of individuals afflicted with Alzheimer's Disease. At elevated concentrations of ACT, head-to-tail complexes are formed between $A\beta_{1-42}$ peptides and multiple different ACT molecules thereby sequestering the neurotoxic $A\beta_{1-42}$ peptide in aggregates. This is believed to be an important determinant of the neurotoxic fibril load in the brain, however the mechanism by which this leads to symptoms

associated with Alzheimer's disease remains incomplete (92). The formation of serpin polymers may also accelerate neurological dysfunction. Serpins, such as PAI-1 and the neuroserpins, thus play vital roles in maintaining the homeostasis of vascular protease reactions (91, 97).

Another serpin, pigment epithelium derived factor (PEDF)(SERPINF1), has been noted previously to interfere with angiogenesis, specifically the survival and differentiation of retinal photoreceptors, cerebellar granule neurons, and spinal motor neurons. PAI-1 has a biphasic effect and has also been observed inhibiting angiogenesis at some doses and enhancing angiogenesis at others (98-100). *In vitro* work has revealed PEDF's ability to inhibit neovascularization and antagonize angiogenic factors such as VEGF, basic-fibroblast growth factor, platelet derived growth factor (PDGF), and IL-8 (101) reinforcing a protective role at physiological levels for serpins (100). Similar observations have been made for PAI-1, maspin (SERPINB5), and RCL cleaved ATIII, however both a pathological and physiological connection have yet to be established (102-105).

The study of human serpins over the years has led to the identification of a very key role for serpin regulation of blood coagulation, fibrinolysis, programmed cell death, development, and inflammation (106-108). Many contrasting functions of human serpins are seen, dependent upon the cell type or tissue system in which they are found. PAI-1, as mentioned earlier, is responsible for inhibiting plasminogen's catalysis to plasmin in the vasculature. This is in contrast to its' neuroprotective role under TGF- α stimulation in neuronal cells from N-methyl-D-aspartate (NMDA) induced excitotoxic neuronal death (109). From a clinical standpoint, because of their modulation and involvement in a myriad of biochemical processes, it would be imminently beneficial medically to define medical and therapeutic targets of serpins in order to perform a complete analysis of serpin function *in vivo*.

Serpins are also observed in genera with subfamilies of the vertebrate poxviruses and gamma-herpesviruses (46, 110). *Orthopoxvirus* (cowpox) is capable of encoding three serpins [Salmonella pathogenicity island 1, Spi-1, Spi-2/Crma, and Spi-3] with a highly conserved nature, while *Leporipoxvirus* (myxoma virus) also encodes three serpins (Serp1, Serp2, and Serp3) with, conversely, very little conservation. Viral serpin homology between subfamilies extends to the amino acids that appear in the RCL. Serp-1 has a P1-P1' sequence of Arg-Asn (R-N) while the sequence for PAI-1 is Arg-Met (R-M). All leporipoxviruses, orthopoxviruses, and fowlpox viruses encode serpins with a putative P1 arginine (R) residue and all genera of poxviruses encode serpins that have an asparagine (N) at the P1' position, again indicating some conservation. The cross class serpins, Serp-2 and CrmA/Spi2, conversely have aspartic acid at the P1 site providing the capacity and specificity for caspase (cysteine protease) inhibition (46, 111-112).

3.4.2. Mechanism of Inhibition, Structure, and Kinetics

Peptide bond hydrolysis is required for many functions, however, if allowed to occur promiscuously this

can prove imminently fatal. Hence, regulating protein inhibitors are employed. Serine protease inhibitors (serpins) utilize a sophisticated suicide inhibition mechanism. Deficiency or excess of serpins is associated with human diseases, for example excess thrombosis (Protein C Inhibitor deficiency, SERPINA5), angioedema (protein C1-esterase inhibitor deficiency, SERPING1), hemorrhage (α_1 -AT deficiency associated with an excess of the PiZ and PiS alleles or an arginine substitution at position 358) (113-114), emphysema (α_1 -AT deficiency) (115), and dementia (α_1 -ACT aggregates, neuroserpin) (116-117) among others. We are fortunate in our understanding of the structure, mechanism of inhibition, and kinetics of model serpins at both the biochemical and molecular level. Coupled with on-going genetic analysis, the role of both intracellular and extra-cellular serpins has demonstrated the existence of both functional and non-functional family members able to assume various structural states.

The mechanism of functional serpin inhibition is described as a branched pathway consisting of an inhibitory or non-inhibitory (cleaved) route. Residues flanking the P1-P1' linkage form a non-covalent Michaelis-like complex. After attack of the scissile bond by Ser-195 in the active site of the serine proteinase an ester linkage is formed between the active site residue and the carbonyl of P1 (118). This is followed by the insertion of the RCL into beta-sheet A to the P9 position resulting in the movement of the remainder of the serine protease across the serpin surface. The globular portion acts as a plug, preventing further insertion of the RCL and producing a locked, heterodimeric protein where the strain in the uncleaved RCL is transferred to the target protease. At this point the enzyme active site is so distorted, due to compression created by the serpin, that deacylation becomes impossible and an irreversible complex is created (119). A critical step in the cascade, that determines whether the inhibitory branch of the serpin/serine protease pathway is taken, is the movement of the covalently bound serpin across the surface of the protease. With unimpeded serpin movement across the protease surface the rate of reaction for the deacylation steps fall six to eight orders of magnitude. However, restricting this movement might provide a sufficient amount of time for the enzyme to complete the deacylation step leaving an active enzyme and a cleaved serpin (the other branch of the pathway). The essence of a successful inhibition then is the competition between the stages of loop insertion and ester hydrolysis (120).

Variations on the above mechanism have been identified that are essentially structural issues. For instance (as alluded to earlier) non-functional serpins exist, therefore no use of the RCL mechanism is made despite sharing all the structural hallmarks of functional serpins. Examples include ovalbumin, AGT, thyroxin binding globulin, and cortisol binding globulin (SERPINA6). Another variation of the RCL mechanism is one employed by PAI-1 that can assume different uncleaved inactive states as well as an inactive form that can be converted to an active form (118), the latter being a capacity also shared by the serpins plasminogen activator inhibitor-3 (PAI-3)(SERPINA5) (121) and alpha-2 antipplasmin (α_2 -AP)(SERPINF2)(122).

3.4.3. Viral Serpins

Viruses have evolved to express immune modulators that act to counter host defenses. Secreted viral immune modulators are transiently delivered into the host cell's surrounding microenvironment at very low concentrations acting upon specific target(s) and deflecting a focused response against infected tissue. Other mediators are present inside the infected cell, altering cell functions such as apoptosis or inflammatory responses. Viral-host co-evolution has resulted in the development of viruses with highly selective and powerful anti-immune and anti-inflammatory molecules.

Such proteins are found across the gamut of RNA and DNA viruses, however the greatest number appear in the large complex DNA viruses, specifically the herpes and pox virus families. The greater size of the DNA genomes allow viruses to encode proteins with functions beyond those required for replication alone (a typical restraint in RNA viruses that often employ compression tactics such as overlapping reading frames) (123-125). Viral DNA genomes have the ability to integrate into their hosts' genome through homologous recombination as part of the latent phase without actively beginning to produce viral progeny. Over time, this integration has allowed elements of the host's genome to be hijacked, more precisely, those genes responsible for encoding host immune mechanisms. Evolution has put selective pressure on the modification of these host-derived proteins providing maximal advantage to the virus (126-128).

Secreted, as opposed to intracellular or surface expressed viral immune modulator proteins, have been subdivided into four classes with a proposed fifth class: (1) cytokine inhibitors, (2) cytokine mimics, (3) complement inhibitors, (4) protease inhibitors, and (5) cross class inhibitors. Two classes (classes 4 and 5) include viral serpins (125).

With the demonstration of active protease involvement in inflammatory responses, for instance in apoptotic cysteine protease cascades, it seems a natural step in this coevolutionary process between viruses and mammals that viruses would make use of serpins (from the fourth and fifth classes mentioned above) (128). The best characterized are from the Pox virus family, specifically vaccinia, myxoma and cowpox viruses, the highly competent pathogens of humans, rabbits, and cows respectively. Eight serpins are encoded in the inverted terminal repeats of the Pox virus family genomes (the areas of the viral genome that encode virulence factors) demonstrating the importance that this anti-immune serpin-based strategy has in the pathogenicity and survival of these viruses (118).

Myxoma virus is responsible for encoding two such serine protease modulators, Serp-1, a secreted glycoprotein that binds thrombolytic factors (tPA, uPA, and plasmin) and thrombotic factors (Factor Xa and thrombin) and Serp-2, a cross-class intracellular inhibitor that binds granzyme-B and caspase-1 (130). Serp-1 inhibits tPA, uPA, plasmin, and Factor Xa, with the exception of thrombin that

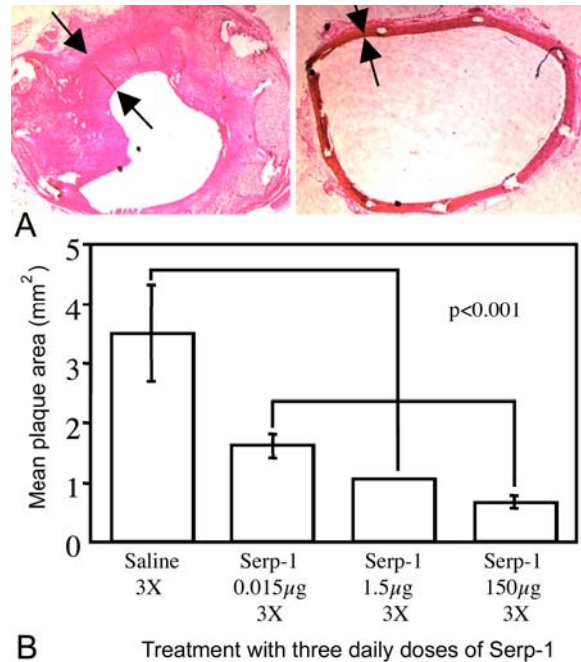


Figure 2. Plaque growth and Serp-1 treatment in vivo. A. Histological cross sections of aortas from cholesterol fed rabbit aorta stained with hematoxylin and eosin that had undergone angioplasty and bare metal stent implant. Left quadrant identifies control treated rabbits and the right identifies Serp-1 treated rabbits. B. Bar graph demonstrating reduced plaque growth (mean \pm standard error) after balloon angioplasty of the abdominal aorta and stent implant - four weeks follow up. Magnification at 25X where arrows show limits of intimal plaque.

requires a ten-fold molar ratio excess of Serp-1 in order for inhibition to take place. Otherwise, thrombin is able cleave Serp-1 under lesser molar ratios (130-131). Myxoma virus causes myxomatosis in old world, European rabbits producing fulminant primary and secondary lesions. Infected rabbits typically succumb within two weeks of the initial viral infection due to immunosuppression and secondary Gram-negative bacteria infections. The duration of viral infection is characterized as a period of systematic disabling of the innate and adaptive responses in the host (130). Upon infection with Myxoma in gene knockout studies in immune competent hosts, a greater inflammatory response followed by recovery from the infection was revealed demonstrating Serp-1's profound interaction with the host inflammatory response (110, 132). Furthermore, leukocyte inflammatory responses are greatly reduced in myxoma viral infection studies (132-133).

In recent work over the past 10 to 12 years, Serp-1 has been tested as the first native viral protein based anti-inflammatory therapeutic agent (Viron Therapeutics Inc., London, Ontario). In animal models of vascular injury and/or surgery, infusion of Serp-1 after angioplasty or allograft transplant in animal

models (Table 1) observed a markedly reduced number of monocytes and T lymphocytes in the arterial wall and associated significant reductions in atherosclerotic plaque growth (Figure 2A-B) (134-137). These studies have been reported in a wide range of animal models and research labs (Table 1).

3.4.4. *In vivo* Observations: Serpins that regulate thrombolysis

3.4.4.1. PAI-1 and the Regulation of the Vascular Response

PAI-1, as stated earlier, is a serpin that functions to impair fibrinolysis and inhibit plasmin production. The serine protease targets for PAI-1 are tPA, uPA, activated Protein C, and thrombin. After serpin suicide inhibition, the inactivated complexes are removed from circulation by the liver. As described in a preceding section, PAI-1 is associated with regulation of the neointimal growth observed after vascular injury where thrombosis and thrombolysis are critical events in vascular wall inflammation resulting in vascular occlusion. What follows is an introduction to the *in vivo* work demonstrating PAI-1's involvement in those events (138).

Making use of an adenoviral vector expressing PAI-1 and the rat arterial balloon injury models, it was demonstrated that neointima formation (arterial plaque growth) was reduced in PAI-1 transduced arteries compared to control transduced arteries (91). Two weeks later arteries were observed to have a significantly larger lumen in the PAI-1 transduced compared to control transduced arteries. This demonstrates that PAI-1 in conjunction with the plasminogen system provides a central component of the vascular wound repair response (91). Lutton et al also demonstrated that the absence of PAI-1 promoted the growth of atherosclerotic plaques in ApoE^{-/-} mice over a 25 week period. Increased extra-cellular matrix deposition and collagen fibre disorganization were also observed (139). This is in contrast to findings by Ploplis et al where decreased fibrin and collagen deposition in the adventitial compartments of PAI-1^{-/-} mice was observed with a copper induced arterial injury model (140). Significant PAI-1 expression increase has also been reported by numerous researchers in human atherosclerotic plaques (141-143). Carmeliet et al have also demonstrated the importance of uPA, but not tPA and uPAR, in scar tissue formation and plaque growth in vascular injury models. For example, uPA and plasminogen deficient knockout mice were observed to have reduced plaque growth (41, 43, 102, 144). It has been previously stated that PAI-1 is present to aid in the development of the neointimal layer by cell invasion through decreased growth factors and MMP activation. PAI-1 may also function to increase fibrin deposition by inhibiting fibrinolysis and actively partaking in the remodeling of the vasculature. In cell types predicted to interact with PAI-1 during remodeling, inhibition of the thrombolytic cascade is produced by forming the ternary PAI-1/uPA/uPAR complex that interferes with cellular adhesion and migration. PAI-1 may well therefore have a role in the chain of events leading to fibrous cap rupture, specifically cellular migration and vascular thrombosis.

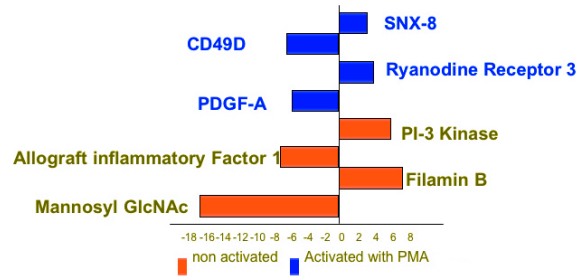


Figure 3. Microarray analysis of gene expression in THP-1 human monocyte cell line. Levels of expression reflect differences elicited by PAI-1 versus Serp-1 treatment.

3.4.4.2. Serp-1 and the Regulation of Vascular Responses

Serp-1, the only known secreted viral serpin, has demonstrated profound anti-inflammatory and anti-atherogenic function that clearly defines an elegant and powerful interaction between serpin regulation of thrombolysis and inflammation. Serp-1 and regulatory vascular serpins have been found to reduce inflammation and plaque growth in animal models after balloon injury and aortic allograft transplant. Treatment with Serp-1 for balloon injury in rats, rabbits, roosters, microswine and after stent implant (Figure 2) significantly decreased plaque growth in the vasculature was significantly decreased (Table 1). In models testing aortic, renal, and cardiac allograft transplants Serp-1 also markedly reduced transplant vasculopathy and scarring (Table 1). Associated with this reduction in atherosclerosis (no reduction showing decreased vascularization yet published) and vascularization was a constant and significant reduction in inflammatory cell invasion of the arterial wall.

Work with RCL mutant of Serp-1 that lacks plasminogen activator inhibiting activity (the Serp-1 mutant with Ala-Ala (A-A) P1-P1¹ sequence) demonstrates a loss of the inhibitory function for plaque growth (83) and indicates that the Serp-1 anti-inflammatory function is serpin function based, at least in part. Only minute (picogram to nanogram) amounts of Serp-1 have been required to reduce plaque growth in many of these animal models (134, 145). Furthermore, in many of the models tested (mice, rats, rabbits, roosters, and microswine) Serp-1 was administered as a single bolus immediately following mechanical injury with angioplasty wires, balloon angioplasty and stent implants (83, 145). A post-operative morphological follow-up at four weeks revealed significantly reduced plaque growth with Serp-1 treatment (Figure 2A-B). This work has clearly demonstrated Serp-1's ability to prevent chronic inflammation (145). Early (\approx 1 week) and late (4 weeks to 5 months) follow-up of cellular infiltration was performed in rats that had undergone segmental aortic renal and cardiac allograft transplantation where Serp-1 reduced macrophage and lymphocyte invasion into the medial and adventitial layers (136, 142). With doses at nanogram to microgram levels an inhibition of medial smooth muscle cell depletion with reduced macrophage invasion was

observed in ApoE^{-/-} mice, a risk factor for plaque rupture (136). Vascular injury was further simulated after aortic transplant in PAI-1 deficient mice while mice treated with Serp-1 had less vasculopathy development. Previously, Serp-1 treatment had been found to produce increases in PAI-1 expression in cells. Serp-1 also was capable of blocking plaque growth in PAI-1^{-/-} isografts demonstrating that PAI-1 expression is not required for the therapeutic benefits of Serp-1 to be apparent (145). In uPAR deficient mice, Serp-1 anti-inflammatory activity was negated demonstrating that Serp-1 requires uPAR for anti-inflammatory activity (145). More recently Serp-1 RCL chimeras (results not published) revealed the importance of the amino acids in the P1-P1¹ active site and in the second to seventh amino acid positions of the P arm. Altering the P1-P1¹ sequence to that of other serpins known to target thrombolytic proteases or serpins with cysteine protease inhibitory activity resulted in a loss of anti-inflammatory function for Serp-1. When substituted to alanine (Ala₆) at P2-P7 residues, increased thrombosis, monocyte invasion, and aneurysm formation was observed. It was also revealed that Serp-1 significantly reduced plaque growth and mononuclear cell invasion ($P < 0.01$) in contrast to the native human analog, PAI-1 that was found to significantly ($P < 0.0001$) increase thrombosis and early mortality (unpublished data, manuscript submitted).

The advantages of Serp-1 extend beyond cardiovascular disease. One study undertook evaluation of Serp-1 treatment in models of antigen-induced-arthritis in rabbits (147). Histological examination of the synovial fluid revealed a dose dependent response of reduced synovial lining cellular hyperplasia, cartilage erosion, and chronic inflammation (147).

From our own laboratory, we have recently detected Serp-1 binding to monocyte cell surfaces. Microarray data from activated and non-activated THP-1 monocytes in the presence of Serp-1 treatment revealed significant changes in expression for eight selected genes (Figure 3). Activated monocytes for instance demonstrated approximately six fold decreases for genes encoding PDGF-A and CD49D, the latter being an α -4 integrin responsible for associating with CD29 and binding fibronectin and VCAM-1, among others. In non-activated monocytes an approximately sixteen-fold decrease in a gene encoding mannosyl transferase was observed with an eight-fold increase in expression for filamin-B. Considering the previous *in vivo* observations of Serp-1 treatment coupled with Serp-1's proposed effects on monocyte migration, it comes as little surprise to observe increases in expression for genes such as filamin-B (Figure 3). Treatment with antibody to uPAR prevented this observed increase in filamin-B (Figure 4), further emphasizing the role of uPAR in Serp-1 mediated anti-inflammatory action. This capability is believed to be enabled through filamin-B's carboxy terminal cytoplasmic signaling domain and through associations with adjacent receptors, like the glycoprotein complex, in focal adhesion lipid raft receptor super-complexes.

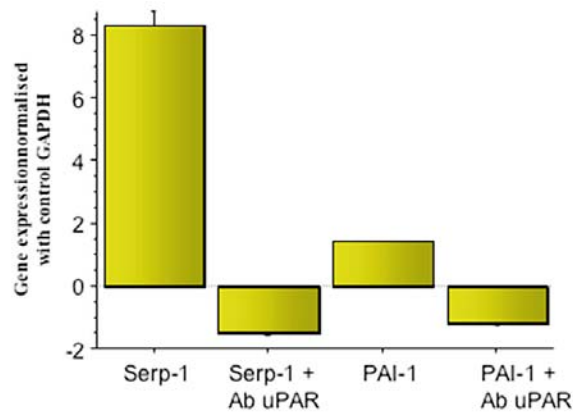


Figure 4. Effect of Serp-1 and PAI-1 on expression of Filamin-b in THP-1 monocytes treated with and without a blocking antibody to uPAR. All changes in gene expression were normalized to GAPDH expression and saline treatment. Treatment of THP-1 monocytes was done with 1µg/mL of PAI-1 or Serp-1 and 20µg/mL of anti-uPAR antibody. Cells were harvested after a one hour incubation with Serp-1 or PAI-1.

Literature is replete with *in vivo* data demonstrating the role played by PAI-1 in the vasculature, particularly in the thrombolytic cascade. What has also been demonstrated is PAI-1's limited number of targets, tPA, uPA, thrombin, and activated Protein C along with its presumed involvement in precipitating thrombosis and plaque rupture. Increased PAI-1 has certainly up-regulated expression in areas of vascular injury, inflammatory responses, and plaque growth. As seen in the preceding discussion PAI-1 may also have a protective anti-inflammatory role preventing cell migration, invasion, and proliferation. Medicine and the human physiology have reached a point where native immune mechanisms are simply inadequate to cope with the challenges posed by the modern clinical environment and autoimmunity. Our own immune system can precipitate lethal long-term effects that ironically enough, occur after a life saving invasive procedure. The high efficacy and specificity of Serp-1, a myxomal protein crafted by evolution to mask virally infected tissue through immune modulation presents the first of many next-generation drugs based on viral immunomodulators to treat diseases related to inflammation and cellular infiltration.

4. CONCLUDING REMARKS

The functional role that serpins play across a large biological spectrum is immensely varied. Serpins are capable of directing the thrombotic and thrombolytic cascades which are central events leading to plaque rupture. The thrombolytic cascade is said to have a significant impact as it accelerates macrophage invasion, exposing highly thrombotic substrates previously contained within the lipid filled thrombus to the rest of the system. Therefore, treatment with serine protease inhibitors may be able to forestall the chain of events that lead to plaque rupture and vascular occlusion.

The serpin, PAI-1, administered to mice in concentrations greater than those found physiologically was found to have adverse effects. The benefit PAI-1 is supposed to bring to the vasculature in the event of injury or aortic allograft transplants is lost at these levels, so clearly limits exist and caution must be maintained when proposing the use of serpins that target the thrombotic and thrombolytic pathways as new therapeutic approaches. We have also been recently demonstrated that Serp-1, a secreted myxomal virus protein that also targets the thrombolytic and thrombotic pathways, is capable of modulating inflammatory responses. Serp-1 is able to modify serine protease, serpin and receptor responses. Further, Serp-1 has been observed thus far operating seamlessly with both human and murine immune systems. The efficacy of Serp-1 at low concentrations and the number of potential cellular and molecular targets go beyond those of PAI-1. This work has opened up a therapeutic class of native viral serpin therapeutics, specifically naturally developed viral anti-inflammatory proteins with Serp-1 as the first in this class. This unique viral serpin Serp-1, thus represents a new direction in drug development.

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