Chapter 11

The Serpin Saga; Development of a New Class of Virus Derived Anti-Inflammatory Protein Immunotherapeutics

Alexandra Lucas,* Liying Liu, Erbin Dai, Ilze Bot, Kasinath Viswanathan, Ganesh Munuswamy-Ramunujam, Jennifer A. Davids, Mee Y. Bartee, Jakob Richardson, Alexander Christov, Hao Wang, Colin Macaulay, Mark Poznansky, Robert Zhong, Leslie Miller, Erik Biessen, Mary Richardson, Collin Sullivan, Richard Moyer, Mark Hatton, David A. Lomas and Grant McFadden

Wisdom in infinite thought,
Expanding time, endlessly sought,
An ancient star journeying here
So far, so near, bright seer
This unseen dance
A brilliant chance
—Anonymous

Abstract

Serine proteinase inhibitors, also called serpins, are an ancient grouping of proteins found in primitive organisms from bacteria, protozoa and horseshoe crabs and thus likely present at the time of the dinosaurs, up to all mammals living today. The innate or inflammatory immune system is also an ancient metazoan regulatory system, providing the first line of defense against infection or injury. The innate inflammatory defense response evolved long before acquired, antibody dependent immunity. Viruses have developed highly effective stratagems that undermine and block a wide variety of host inflammatory and immune responses. Some of the most potent of these immune modifying strategies utilize serpins that have also been developed over millions of years, including the hijacking by some viruses for defense against host immune attacks. Serpins represent up to 2-10 percent of circulating plasma proteins, regulating actions as wide ranging as thrombosis, inflammation, blood pressure control and even hormone transport. Targeting serpin-regulated immune or inflammatory pathways makes evolutionary sense for viral defense and many of these virus-derived inhibitory proteins have proven to be highly effective,

*Corresponding Author: Alexandra Lucas—University of Florida, 1600 SW Archer Road, PO Box 100277, Gainesville, FL 32610-0277, USA.
Email: alexandra.lucas@medicine.ufl.edu, alexluc1@ufl.edu

Pathogen-Derived Immunomodulatory Molecules, edited by Padraic G. Fallon.

working at very low concentrations—even down to the femptomolar to picomolar range. We are studying these viral anti-inflammatory proteins as a new class of immunomodulatory therapeutic agents derived from their native viral source. One such viral serpin, Serp-1 is now in clinical trial (conducted by VIRON Therapeutics, Inc.) for acute unstable coronary syndromes (unstable angina and small heart attacks), representing a 'first in class' therapeutic study. Several other viral serpins are also currently under investigation as anti-inflammatory or anti-immune therapeutics. This chapter describes these original studies and the ongoing analysis of viral serpins as a new class of virus-derived immunotherapeutic.

Innate Immunity

Many investigators have studied the antigen-dependent, antibody-mediated immune response, which is only found in vertebrates. Over a century ago, however, Ilya Ilyich Mechnikov described a more ancient and yet extraordinarily powerful immune response, known as the innate immune system. Mechnikov studied the response of a transparent starfish (bipinanaria) to wood splinters and recorded the early massing of cells around these splinters inside this organism. Mechnikov thus provided the first description of the cell-based innate immune response that forms the first defense response to injury or infection.[1] This 'inflammatory', cell-based immune system recognizes and then eradicates or blocks pathogen and parasite infection, invasion and dissemination long before antibodies are formed and the acquired, antibody dependent immune response is activated.[2-6] The innate immune response also orchestrates the first stages of tissue repair after other forms of injury produced by physical or chemical insults.

The vascular endothelium, together with the circulating inflammatory blood cells, monocytes/macrophages, T-lymphocytes and polymorphonuclear leukocytes (also called neutrophils), recognize patterns of microbial molecular expression through pattern recognition receptors (PRR) forming the prelude to this innate response. The PRRs now recognized include toll-like receptors (TLRs), nucleotide binding and oligomerization domain-like receptors (NLRs), C-type lectin-like receptors (CLRs), cytoplasmic double stranded RNA (dsRNA) helicase-like receptors and cytoplasmic dsDNA receptors.[7,8] These receptors comprise an alarm system that alerts inflammatory cells to danger or infection, signaling through MyD88, NFκB and MAPK signal-transduction pathways. The endothelial cell layer is the innermost layer of cells in the arterial tree and is composed of miles of interconnected cells, a living carpet of cells that encompasses the vasculature, the cardiac valves and the inner chambers of the heart. This endothelial cell layer is in constant contact with the circulating blood. Injury or infection of the endothelium causes loss and/or activation of endothelial cells with increasing expression of selectins on the activated cells. These selectins, when expressed, slow down circulating leukocytes that pass by in the blood stream. Once slowed, mononuclear cells (leukocytes composed of neutrophils, monocytes and lymphocytes) can then recognize cell adhesion molecules and adhere to the endothelium and in turn become activated.[9] Circulating and activated inflammatory cells also can recognize connective tissue and lipids exposed under areas of damaged endothelium. The activated endothelium expresses increased amounts of selectins and adhesion molecules that further stimulate cell adherence and activation. Once activated, inflammatory mononuclear cells, together with endothelial cells, begin to release chemoattractant proteins, particularly chemokines, which bind to surface glycosaminoglycans (GAGs). Also induced are pro-inflammatory immune signaling molecules, cytokines and growth factors that signal cells to migrate through the vessel wall and into the surrounding tissue, become further activated, proliferate and then release more inflammatory cytokines. Damaged cells also can become apoptotic and act as small cytokine release factories, further stimulating this inflammatory response.

Platelets, small clotting cell fragments derived from megakaryocytes, are also activated at sites of damaged or apoptotic endothelium. Platelets carry reserves of proteins in storage granules that are released upon platelet activation. Initially, platelets adhere to areas of arterial or tissue damage, secreting pro-inflammatory proteins from storage granules into this mix of cells and proteins. This activated and inflamed milieu then further stimulates cell invasion and activation. The clot forming

(thrombotic) and clot dissolving (thrombolytic) cascades are made up of sequentially activated serine protease enzymes. These protease cascades are initiated by intrinsic and extrinsic clotting factors (factor VII and tissue factor complex and thrombin) that cleave fibrinogen to form fibrin (Fig. 1). Thrombosis and fibrin formation occurs on the surface of platelets and the fibrin mesh that is deposited on and around the activated platelets, endothelial cells and leukocytes. Clot deposition will then stimulate fibrinolysis, the serine protease cascade that acts to break up the forming clot and maintain a natural homeostasis in the arterial wall, the balance between the clot forming (coagulation) and clot dissolving (fibrinolytic) pathways. When cells become apoptotic and engage in cell suicide, there is again a change in activation and in some cases, such as apoptotic endothelial cells and monocytes/macrophages, there is an increased release of cytokines and clot activating serine proteases into the local tissues which leads to a crescendo in the inflammatory responses.[10,11] Extrinsic apoptotic pathways can also be driven by serine proteases, particularly granzyme B.

At one further level of complexity, the connective tissue components, specifically collagen, elastin and glycosaminoglycans (GAGs) have many roles only now being uncovered. Connective tissues surrounding cells provide storage sites for release of growth factors and cytokines. Thus breakdown of these cellular-embedding and tensile-building materials can lead to a local increase in released inflammatory factors. The connective tissue layers also form adhesive platforms on which chemokines and cells adhere and through which cells migrate and invade tissues. Additionally GAGs directly regulate serine protease and serpin activation. The serpin antithrombin III is well known to have a 1000 fold or greater increase in activity when exposed to infusions of the GAG heparin, a drug commonly used in vascular patients.[12,13] When cells lose adhesion to connective tissue or basement membranes, an apoptotic state can be induced further activating these pathways. Serine proteases activate the proteases then that break down collagen, elastin and GAGs, the matrix metalloproteinases (MMPs). The fibrinolytic serine proteases, tissue- and urokinase-type plasminogen activators (tPA and uPA, respectively), plasmin and the thrombotic protease thrombin also directly cleave collagen and elastin. Thus the serine protease pathways in the coagulation and fibrinolytic pathways interact on many levels with the inflammatory and apoptotic responses.[11,12]

In summary, inflammatory cells and activating factors perform in concert to initiate host innate immune/inflammatory responses that heal sites of infection and injury but can also damage tissues when present in excess. Serine protease pathways are regulated by serpins, and many viruses have acquired and developed their own serpins over many millions of years of evolution that are designed to modulate host immune responses. These viral serpins probably target a variety of innate sensors, particularly PRR signals such as the extracellular TLRs and intracellular NLRs that trigger innate immune responses.

Serine Protease Inhibitors/Serpins

Serpin Structure and Function

The symphony of interactive responses between cells, cytokines, serine proteases, connective tissue and growth factors acts as a composite, and it is unclear if there is a single, controlling conductor for the inflammatory pathways. Several of these pathways, however, are known to be regulated by serpins (serine protease inhibitors), guiding these factors to play in concert. The term serpin was first introduced by Carrell and Travis in 1985[14] and describes a family of proteins with up to 30% sequence identity (ranging up to 70% when limited to hydrophobic sequences) that is believed to have arisen from countless gene duplications of an ancestral gene. Serpins are large complex proteins that exist in strained, latent and cleaved (inactive) conditions (Fig. 1A). The serpin basic protein secondary structure is fairly well conserved consisting of a 350 amino acid core with of 3 β-sheets (A, B and C) and 7 to 9 α-helices, labeled hA-hI.[15] The reactive site loop and the β-sheet A are labile and have key roles in serpin inhibitory function. The reactive site loop (RSL) sits exposed above the serpin folding framework, thus presenting the P1-P1′ site as bait. The β-sheet opens to incorporate the RSL loop after cleavage by a target protease.[15] Of course, each of the serpins has a variable inhibitory activity for a range of proteases and thus can

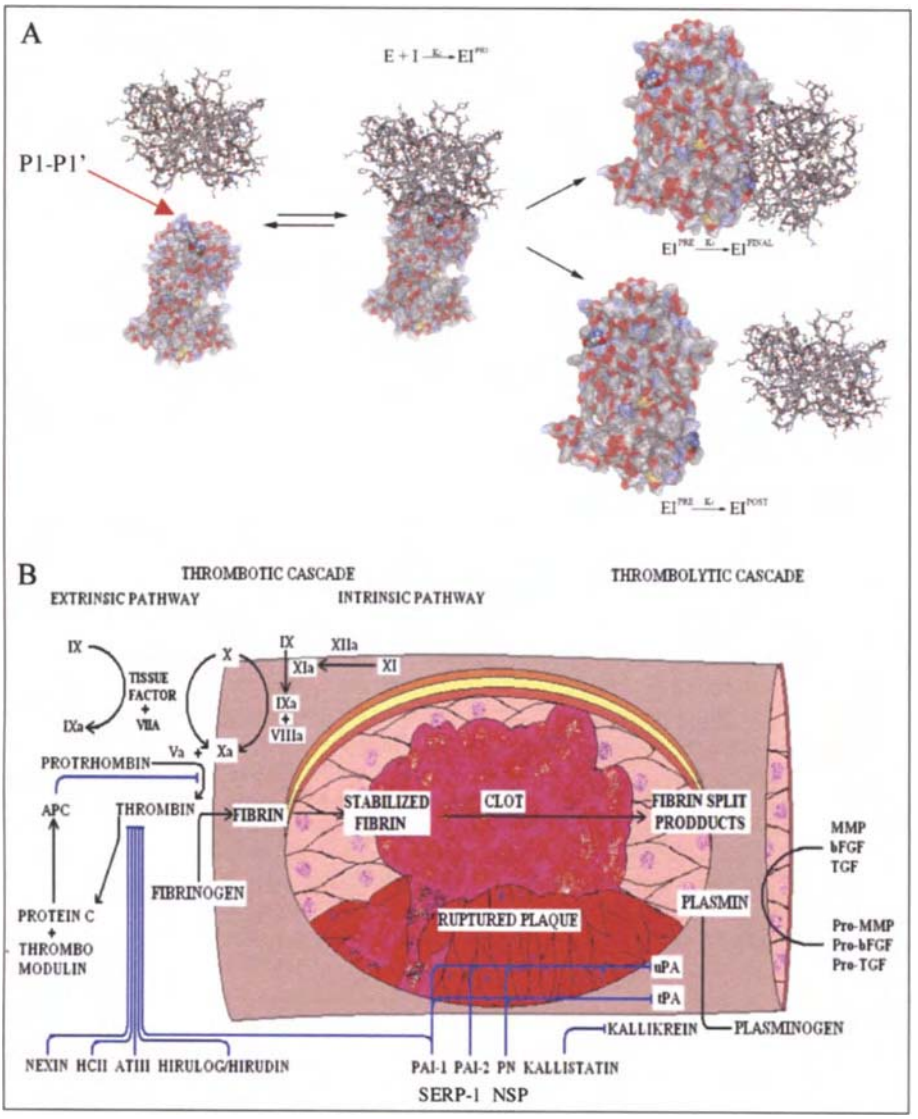

Figure 1. A) Serpin binding to target proteases occurs via interaction of the protease with the RSL P1-P1′ scissile bond. This interaction can result in either (1) a form of suicide inhibition wherein the serpin RSL is cleaved, but the protease remains bound to the serpin and is dragged across the face of the serpin to remain stuck to the opposite pole of the serpin (top serpin/protease interactive pathway) or (2) the serpin RSL is cleaved and the serpin rendered inactive (the bottom serpin/protease interactive pathway). B) Diagram of thrombotic and thrombolytic pathways as well as potential targeted pathways for the mammalian, PAI-1 and viral, Serp-1, serpins in an injured arterial wall.

be a true inhibitor for selected proteases or can be a target for protease cleavage (Fig. 1A). The earliest evolved serpins are found in simple arachaea and bacteria. Other serpin-related proteins are found in the more complex horseshoe crab, unchanged since the time of the dinosaurs are still used for clot lysis assays.[16] Serpins represent a large proportion of circulating proteins in the blood

of all vertebrates, with estimates ranging up to 2-10% of human serum proteins. Serpins have been classified into clades A-I based upon structure and function and these complex and highly effective inhibitors are now known to regulate processes ranging from thrombosis (clot formation), thrombolysis (clot dissolution), complement activation, inflammatory responses, sperm development, to hormone transport.[17,18] There are also serpin family members that, while having classical serpin folding structure, lack functional serpin-like inhibitory activity.[17] These serpins have been found to function in a noninhibitory capacity in diverse roles such as hormone transport, tumor suppression and as molecular chaperones[15,17,18] Three notable examples of these serpin family members include chicken ovalbumin, angiotensinogen the angiotensin (blood pressure controlling) precursor and the corticosteroid (SERPIN A6) and thyroxin (SERPIN A7) binding globulin.[15,18]

The basis for the inhibitory activity of true serpins lies in the fact that the active, inhibitory molecules exist in a metastable intermediate state, instead of their more stable latent conformation.[19] Of the more than 80 crystal structures that have been resolved for serpins to date, there is a clear indication that the inhibitory serpins prefer a metastable native state instead of their latent, lower energy folding.[15] Although it is surprising that the serpins avoid a more thermodynamically favorable conformation, this state is necessary for the inhibitory activity of the serpin. The folding pathway that yields the most stable state for serpins can be triggered by the cleavage of the RSL by the target protease (Fig. 1A). This protease-response mechanism relies on the serpin metastable structure and conformational mobility.[20] Within this pathway, there are two possible outcomes, inhibition or noninhibition of the substrate protease.[21] However, they both begin with the approach of the protease to the RSL and interaction with the residues surrounding the P1-P1′ scissile bond, thereby resulting in the formation of a noncovalent Michaelis-like complex in which neither the serpin nor the protease are conformationally changed.[15,22] Hydrolysis of the scissile bond by the protease results in the cleavage of the RSL and the formation of an acyl intermediate; the serpin thus efficiently traps the targeted protease and drags it across to the face of the serpin following cleavage at its P1′ position with the aid of the covalent acyl bond.[15,20] This changes the native conformation of both the serpin and the protease, causing loss of activity in both of them. It is at this point that the two potential pathways diverge. In the case of the noninhibitory pathway, the protease is able to deacylate the acyl intermediate complex before loop insertion and trapping can occur, resulting in the release of the active protease along with the inactive cleaved serpin.[15,23] In the case of the inhibitory pathway, both the serpin and the protease remaines entrapped in a 1:1 inhibitory complex exhibiting an extremely long half-life that effectively removes both parties from further biological roles.[15]

The RSL is also a key factor in serpin function consisting of a region of ~20 residues that projects above the body of the serpin, presenting the P-P1′ bond as a protease bait.[24] Any conformational change in the overall structure of the RSL will also significantly affect the serpin's activity. This has been observed in the case of antithrombin, which by itself is a poor inhibitor of thrombin, as a result of a poorly exposed RSL.[18,25] Upon interaction with heparin cofactor, the RSL flips out from its native partially inserted (into beta-sheet) confirmation thus exposing and greatly enhancing its activity.[26] Similarly alteration of the amino acids in the P1-P1′ position also results in the loss of activity as observed in the case of viral serpin Serp-1 mutant where replacing the P1-P1′ R-N sequence with A-A results in a total loss of serpin protease inhibitory activity and anti-inflammatory activity.[27,28] Interestingly, mutation of the Serp-1 P2-P7 arm of the RSL to a series of Ala (A) residues in the Serp-1 (Ala_6) mutant, leads to a complete reversal of anti-inflammatory actions and instead creates a now highly pro-inflammatory and pro-thrombotic protein.[27]

As noted above, the structural scaffold of the serpin has some advantages, but the thermodynamically unfavorable state means the active serpin can transition to spontaneous conformational changes as observed for PAI-1.[24] The large energetic barrier between the serpin's native state and its latent state prevents the inactive protein from spontaneously attaining this activated state.[24] This makes serpins less forgiving to mutations which can cause them to fold into their more stable, noninhibitory or latent state, in which the RSL is inserted into β-sheet A.[29] Such mutations can lead to disorders known as serpinopathies that are characterized by the misfolding of the serpins

and formation of serpin oligomers, i.e., polymeric forms of the protein.[13] The polymers are formed by insertion of the RSL of one serpin into the β-sheet of another serpin, known as the "trans" or foreign insertion.[24,29] Thus, misfolding of serpins can lead to an inactive conformation of the serpin whose thermodynamic stability is comparable with that of the cleaved serpin.

Thrombotic and Thrombolytic Serpins

At sites of injury to any tissue in the body, damaged blood vessels respond to limit blood loss through vasoconstriction and clot formation (thrombosis). With endothelial cell damage there is a loss of anticoagulant signals and the exposed inner connective tissue layers of arterial and venous walls stimulate platelet activation. These activated platelets form a surface on which serine proteases in the clot thrombotic pathways create a clot. Such clots are driven by factor VII, factor IX and tissue factor complexes in the extrinsic cascade and factors IX and VIII in the intrinsic cascade all of which lead to the activation of factor X and thrombin formation (Fig. 1B). Thrombin in turn activates fibrinogen to form fibrin and factor XIII leads to cross linking to form a fibrin and platelet mesh. This clot forms when there is damage, whether caused by outside physical trauma or internal damage such as high cholesterol, diabetes or smoking. The sudden formation of a thrombus on the damaged inner arterial surface occludes the arterial lumen and blocks blood flow. This causes heart attacks (myocardial infarctions) and strokes (cerebrovascular accidents) and/or gangrene with peripheral vascular occlusions. Antithrombin III (AT III, SERPIN C1) and heparin cofactor II (HC II, SERPIN D1) are the main inhibitors of the thrombotic protease pathway.

The thrombolytic pathway is best known for its role in clot breakdown or dissolution. 'Clot busting' or thrombolytic therapies are used to treat acute heart attacks and strokes where thrombosis in coronary and cerebrovascular arteries occludes blood flow to the heart or brain. These thrombolytic agents include streptokinase and tPA or uPA (urokinase). The clot dissolving drugs are mammalian or bacterium derived serine proteases, plasminogen activators that cleave the pro-form of plasminogen to form active plasmin. The clot dissolving thrombolytic cascade also has a central role in acute inflammation. tPA, uPA, plasmin, the uPA receptor (uPAR) and the mammalian serpin that inhibits these serine proteases, plasminogen activator inhibitor-1 (PAI-1) are all up-regulated at sites of tissue injury (Fig. 1B). Thus the thrombolytic pathway is part of an acute phase, inflammatory response to tissue injury. The uPA/uPAR complex also regulates this pathway, as described by Blasi.[30] Cellular invasion, whether inflammatory cells responding to tissue damage or invasive tumor cells (Fig. 1B), can be initiated by the uPA/uPAR complex. S Collen and P Carmeliet's groups have utilized mouse genetic 'knock out' models to demonstrate the key roles of uPA, tPA, uPAR and PAI-1 in arterial responses to injury in vascular disease.[31]

tPA and uPA have chemoattractant activities attracting cells into areas of damage. The uPA/uPAR complex sits at the leading edge of invading cells where uPA activates plasminogen to form plasmin, and the plasminogen activators together with plasmin also activate matrix degrading pro-enzymes, the pro form of matrix metalloproteinases (pro-MMPs), to form active MMPs that degrade local connective tissue, collagen and elastin. These actions are believed to allow cells to invade damaged or infected tissues by creating a path in the connective tissue layers through which cells migrate. The plasminogen activators (PAs) also activate growth factors and can release growth factors from connective tissue stores.

Native mammalian serpins regulate these pathways, with PAI-1 (SERPIN E1) functioning as the major serpin regulator for the tPA and uPA pathways. Other serpins that target and regulate this system include PAI-2 (SERPIN B2), PAI-3 (SERPIN A5), α-2 antiplasmin (SERPIN F2), protease nexin-1 (PN-1, SERPIN E2) and neuroserpin (SERPIN I1). These serpins exhibit differing degrees of inhibitory activity where PAI-1 has a 20 fold faster interaction with tPA than neuroserpin, whereas neuroserpin has a 20 fold faster inhibition rate than protease PN-1.[32,33] PAI-1 can also alter its regulatory patterns in the presence of vitronectin to become a stronger inhibitor of thrombin. PAI-1 is up-regulated increased amounts in inflammatory disorders such as unstable arterial plaque (unstable angina and impending heart attacks). PAI-1 forms a tripartite complex with uPA and uPAR which is then internalized, effectively blocking uPA/uPAR complex activity.

PAI-1 can also act on circulating serine proteases and under varied conditions, as with the presence of the GAG heparan sulfate, PAI-1 can inhibit thrombin. uPAR is a GPI linked nontransmembrane protein and relies upon a large array of associated membrane proteins that exist associated with other proteins in a large and complex lipid raft. Proteins associated with uPAR in the lipid raft complex include integrins, lipoprotein related proteins (LRP or alpha 2 macroglobulin), chemokine receptors, as well as many other proteins that can provide relays for signaling into the cell. The uPA/uPAR complex has been reported to modify cell signals through the intracellular signaling pathways (Fig. 1B).

PAI-1 binding blocks uPA/uPAR mediated actions, and the inhibition or knock out of PAI-1 has been shown to reduce plaque growth in animal models of vascular injury.[33,34] Similarly, excessive expression of uPA in the rabbit carotid after angioplasty led to increased plaque growth.[35] This observation has however been variable, with PAI-1 demonstrating exacerbation of vascular plaque growth in other models.[36] Similar variability has been seen in studies assessing PAI-1 up-regulation. For example, one study reported that elevated PAI-1 levels were associated with reduced restenosis after angioplasty[37] whereas many other reports have associated elevated PAI-1 with increased inflammation and risk of vascular disease.[38] Neuroserpin is up-regulated at sites of cerebral injury and is reported to reduce cerebral ischemic scarring in mouse stroke models. In preliminary work our lab has detected reductions in plaque growth in rodent models after neuroserpin infusions.[39] Thus, overall, native mammalian serpins have had varied and sometimes contradictory effects in both animal models and in patient clinical trials on inflammatory responses and vascular disease.

Serine Proteases and Serpins in Apoptotic Pathways

Apoptosis, or cellular suicide, can be activated by proteases. Aspartate specific cysteine proteases, named caspases, are considered the predominate mediators of classical cellular apoptosis (Fig. 2). The proteolytic cleavage and activation of a series of cysteine proteases is considered a key pathway to apoptosis, wherein caspases 8, 9 and 10 are believed to initiate the activities of caspases 3, 6, 9 that execute the apoptotic command through cleavage of a large number of cellular substrate proteins.[40] Extrinsic apoptotic pathways are mediated via death receptors (e.g., TNF and Fas receptors) and activate caspase 8 and 3, while activators, such as many anti-cancer drugs, trigger intrinsic mitochondrial pathways that induce apoptosis through Apaf-1 and recruitment of caspase 9 (Fig. 2). However, other diverse proteases such as the cysteine proteases calpain and cathepsin B, the threonine protease of the proteosome and other serine proteases have more recently also been linked to cell death. Cellular serine proteases that can initiate apoptotic responses include granule enzymes (granzymes) that interact with intracellular caspase pathways and others that associate with the bcl-2 pathways.[41] Granzyme B is a serine protease secreted by Cytotoxic T Lymphocytes (CTLs) or Natural Killer (NK) cells. Granzyme B is reported to have the strongest apoptotic activity of all the known granzymes. Granzyme B enters cells through pores formed on the cell membrane caused by perforin, which is also secreted by activated CTLs and NKs, and initiates apoptosis through direct cleavage and activation of caspases and also cleavage of the anti-apoptotic Bid. The cleaved form of Bid inserts into the outer mitochondrial membrane to induce the intrinsic apoptotic/death pathway. Granzyme has also been reported to enter cells through pathways separate from perforin pore structures. Another serine protease in the thrombotic pathway, thrombin, also activates apoptosis.[42] Once inside the cell, granzyme B cleaves the "pro" form of caspase 3 which then activates the apoptotic machinery (Fig. 2). The caspase enzymes are proven activators of the inflammasome complex through activation of the pro-forms of the interleukin-1β and IL18 by interleukin converting enzyme (ICE, also named caspase 1). Caspase 1 (ICE) drives inflammation through the activity of a cellular structure named the inflammasome.[43,44] Other researchers have reported direct-protease driven inhibition of poly-ADP-ribose polymerase (PARP) and iCAD that can act to protect cells against DNA damage and apoptosis.

The first apoptotic inhibitor identified was a viral cross-class cysteine and serine protease inhibitor isolated from Cowpox virus, called Cytokine response modifier A (CrmA) or Serine protease inhibitor-2 (Spi-2).[45] CrmA will be described in greater detail in a later section of this

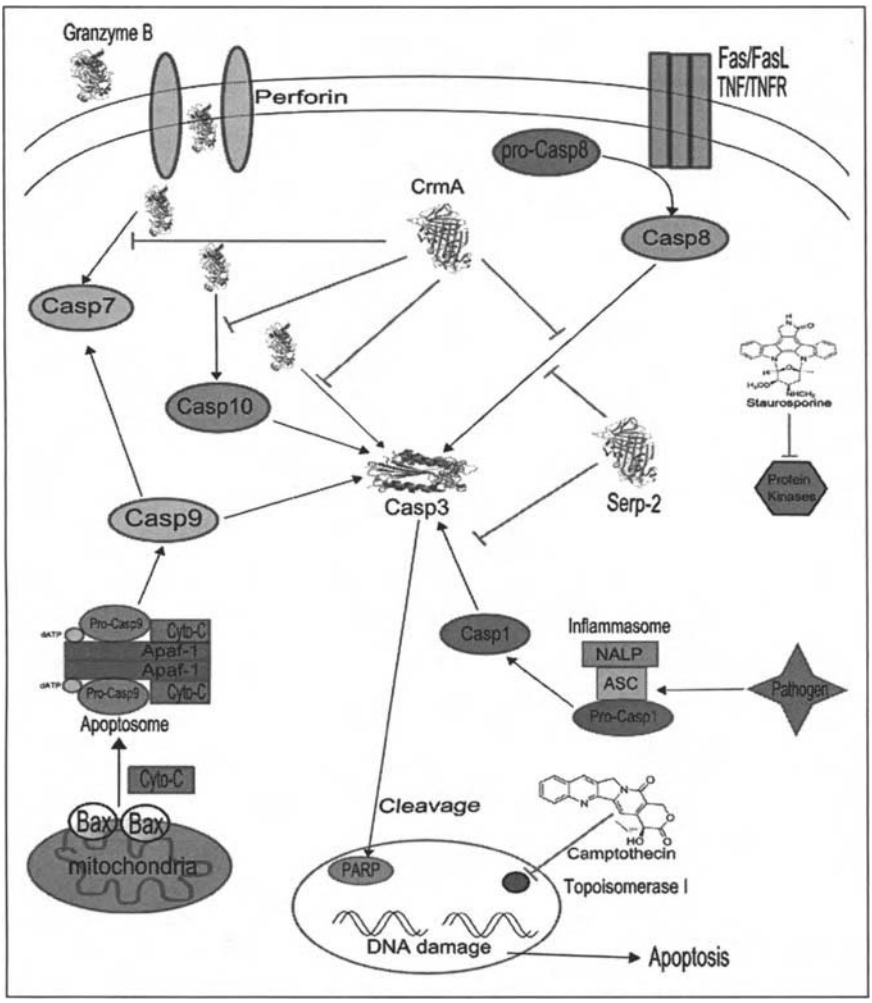

Figure 2. Apoptotic pathways-serine and cysteine proteases. Intrinsic and extrinsic Fas and granzyme B apoptotic pathways that are potentially targeted by Serp-2 and Crm A are illustrated. Staurosporine activates mitochondrial apoptotic pathways, Fas ligand and Granzyme B activate extrinsic apoptotic pathways; Camptothecin inhibits topoisomerase and blocks DNA repair.

chapter. Several mammalian serpins have more recently been identified as playing a part in the regulation of apoptotic responses.[46] CTLs also synthesize their own serpin inhibitors of granzyme B. Among these serpins are Protease Inhibitor-9 (PI-9, SERPIN B9), Plasminogen Activator inhibitor-2 (PAI-2) and maspin. Each of these cellular serpins has proven unusual, having both known extracellular actions as well as in many cases intracellular and even nuclear localization and function. PI-9 is a mammalian serpin that binds to and inhibits the actions of granzyme B effectively blocking cellular apoptosis mediated by Granzyme B.[47] PAI-2 preferentially binds uPA but is also localized in intracellular compartments and is poorly secreted. PAI-2 has been shown to alter apoptotic pathways and has been reported to have similarity in structure to Bcl-2 with potential for overlapping functions in this cell suicide pathway.[48] This potential overlap in function with Bcl-2

has potential impact in that Bcl-2 itself represents a larger family of anti-apoptotic intracellular proteins. Neuroserpin is a mammalian serpin that preferentially binds to and inhibits tPA and has now also been reported to alter apoptotic cell responses.[49] Maspin (mammary serine protease inhibitor, SERPIN B5) was first discovered as a breast cancer suppressor and also has both intracellular and extracellular functions by binding widely divergent proteins that include uPA, uPAR, HSp79 and 90, glutathione peroxidase, β-1 integrin and collagens I and III, among others.[50] In addition, the trypsin-like serpin PI-6 (SPI 3, SERPIN B6) in mouse brain can bind granzyme B and block cathepsin and downstream caspase 7 activation in neurons.[51-53] PI-6 also incidentally binds thrombotic and thrombolytic proteases, thrombin, uPA and plasmin. Three serpins that are best known as regulators of the thrombotic and thombolytic pathways, AT-III, PAI-1 and protein C inhibitor (PCI also named PAI-3, SERPIN A5), have also been found to alter apoptosis in human cells.[54] Nitric oxide nitrosylation of α-1 anti-trypsin (AAT) serpin which targets the trypsin protease modifies AAT such that it becomes an inhibitor of cysteine proteases.[55] Serpin activity is blocked through specific serine protease cleavage, nonspecific metalloproteinase (MMP) cleavage, oxidation and polymerization. Some of these cleaved serpins have additional activities as has been reported for AT-III. The precise mechanisms through which some of these serpin-driven changes in apoptotic responses are under investigation and are not yet fully defined.

These serine and cysteine protease cellular pathways are both preferentially targeted by virus-engineered serpins which are now known to block protease activity in the thrombotic/thrombolytic and apoptotic pathways and exhibit amazingly potent anti-inflammatory activities.

Viral Serpins and Their Anti-Inflammatory Activities

Viral Serpins That Target the Thrombotic and Thrombolytic Pathways; Serp-1 and Spi3

Myxoma virus is a member of the leporipoxvirus family of poxviruses that infects only rabbits. Myxoma virus induces a lethal infection in European rabbits with over 99% mortality.[56] The initial observation of the profound pathogenicity of this virus in rabbits was made in the late 1800s by Dr G Sanarelli in South America who had imported the European rabbit to his lab where they became inadvertently infected with myxoma virus.[56] Infection with myxoma virus in the European rabbit was lethal, causing a rapidly disseminated infection, immune dysfunction and overwhelming sepsis with over 99% mortality in the animals (European rabbits). In the early 1990s the McFadden lab reported that targeted genetic knockout of the myxoma virus Serp-1 gene resulted in a virus that could only cause a benign infection in normally susceptible European rabbits.[57] This Serp-1 gene knockout of myxoma virus produced only mild local dermal lesions that were eradicated within 1-2 weeks, similar to what is observed in rabbit infections with the closely related Shope fibroma virus, which has a naturally inactivated Serp-1 gene and also produces a benign, self-limiting infection in rabbits. Each of these infections, rabbits infected with wild type myxoma virus, myxoma virus with engineered Serp-1 knockout, or the Shope fibroma variant, differed greatly in lesion pathology. The Serp-1 knockout myxoma virus and the natural variant Shope fibroma virus, exhibit a more effective host inflammatory response to viral infection, while with the wild type myxoma infection, inflammatory cells do not properly migrate to tissue sites of viral infection.

In later studies, McFadden and Lomas demonstrated that the myxoma virus-encoded Serp-1 has sequence similarity to serpins, and the protein inhibits tPA, uPA and plasmin in the thrombolytic pathways as well as factor Xa in the thrombotic pathway.[58] However, despite the marked effects of Serp-1 on viral pathogenesis, the K_{ass} for Serp-1 is a lower affinity reaction at $7\text{-}8.6 \times 10^4$ $M^{-1}s^{-1}$, whereas the mammalian serpin PAI-1 has higher affinity and activity with K_{ass} on the order of $1.1\text{-}2.3 \times 10^7$ $M^{-1}s^{-1}$.[59] Serp-1 also binds human thrombin but acts as a protease target and is cleaved by thrombin under physiological conditions.[60] In the presence of heparin, however, Serp-1 becomes a more potent thrombin inhibitor with potential to become more anti-thrombotic than anti-thrombolytic in character.[61]

Spi-3 is a related but distinct viral protein expressed by orthopoxviruses like vaccinia virus and rabbitpox, which unfortunately has a confusing nomenclature with similarity to the unrelated PI6/SPI3 mammalian serpin nomenclature. The poxviral Spi-3 binds to and inhibits uPA and tPA and plasmin.[62,63] Spi-3 is also capable of forming weaker complexes with thrombin and factor Xa as measured by gel shift assays.[63] Spi-3 from vaccinia/rabbitpox and Serp-1 from myxoma virus share only 30% sequence similarity despite targeting similar host protease pathways. The K_i's for Spi-3 was measured as 0.51, 1.9 and 0.64 nM for uPA, tPa and plasmin, respectively. The K_i's for Serp-1 were similar at 0.16, 0.14 and 0.44 nM, respectively for uPa, tPa and plasmin.[63] However, whereas Serp-1 is secreted into the surrounding environment, Spi-3 remains tethered to the cell surface and exhibits a secondary function in the inhibition of cell fusion that is independent of its serpin-based activities.

Preclinical Analysis of Serp-1

Pilot Studies—Rabbit Model

Our founding hypothesis was that native, virus-derived, immunomodulatory proteins can be developed as a new class of protein therapeutics to treat inflammatory-based diseases. Rather than using a live virus or a viral vector in animal models or in patients and thus risk reactions to the viral construct itself, we followed the lessons of natural evolution and the selection pressures exerted by the immune system. Large DNA viruses, like many parasites, have evolved highly effective defenses against the host and inflammatory systems and thus such viruses have already accomplished the necessary research and development that created these viral immune evasion proteins. In other terms, rather than relying on man's imperfect knowledge and understanding of the inflammatory and immune responses, we utilized viral proteins already discovered, engineered, developed and proven effective as immune modifying agents and used these naturally-derived reagents in the form of the expressed and purified protein. We directed our initial studies to express secreted proteins encoded and engineered by complex poxvirus DNA genomes. Indeed, native viral immunomodulatory proteins that are often highly potent and naturally function at very low concentrations to divert the host immune response away from the invading viral organisms.

For the first preclinical animal studies, the viral serpin, Serp-1 protein, was expressed from a vaccinia virus expression system and the secreted serpin protein was purified on FPLC columns (J Macen, McFadden lab). Using our first generation purification strategy, only submicrogram (picogram to nanogram) quantities of purified protein were isolated, and it is these early preparations of secreted Serp-1 that were used for the first studies in 74 cholesterol fed New Zealand white rabbits. It was reasoned that the intact virus only expressed and secreted very low levels (femtomolar amounts) of Serp-1 protein into the surrounding tissues and thus the viral serpins might be capable of anti-inflammatory action even at very low dosages. Lower concentrations of therapeutic protein would also reduce the risk of inducing antibody and immune responses as well.

As this serpin was derived from a rabbit virus, the first disease model we tested was a rabbit angioplasty injury model, where the timing of initial injury and inflammatory response activation would be known, e.g., initiated with specific angioplasty injury. In the first studies, one bolus of 30-3000 picograms of Serp-1 protein per rabbit was then infused locally at sites of balloon injury in studies performed by LY Liu and E Dai in the Lucas lab.[64] The Serp-1 protein was infused locally using a Wolinsky catheter, which is an angioplasty balloon catheter with small perforations on the balloon that allowed the protein to be sprayed on the arterial surface while inflating the balloon (Fig. 3A). Later analyses with Evans blue dye delivered with the same device demonstrated that the majority of this presumed local infusion was in fact delivered in both trans-arterial and systemic fashion, with 90% of the delivered dose spilling into the blood stream. Efficacy of intravenous and intra-arterial infusion of 0.3 to 300 ng of Serp-1 protein were then tested, again demonstrating effective inhibition of plaque growth at 4 weeks follow-up after a single bolus injection given at the time of balloon angioplasty.[64]

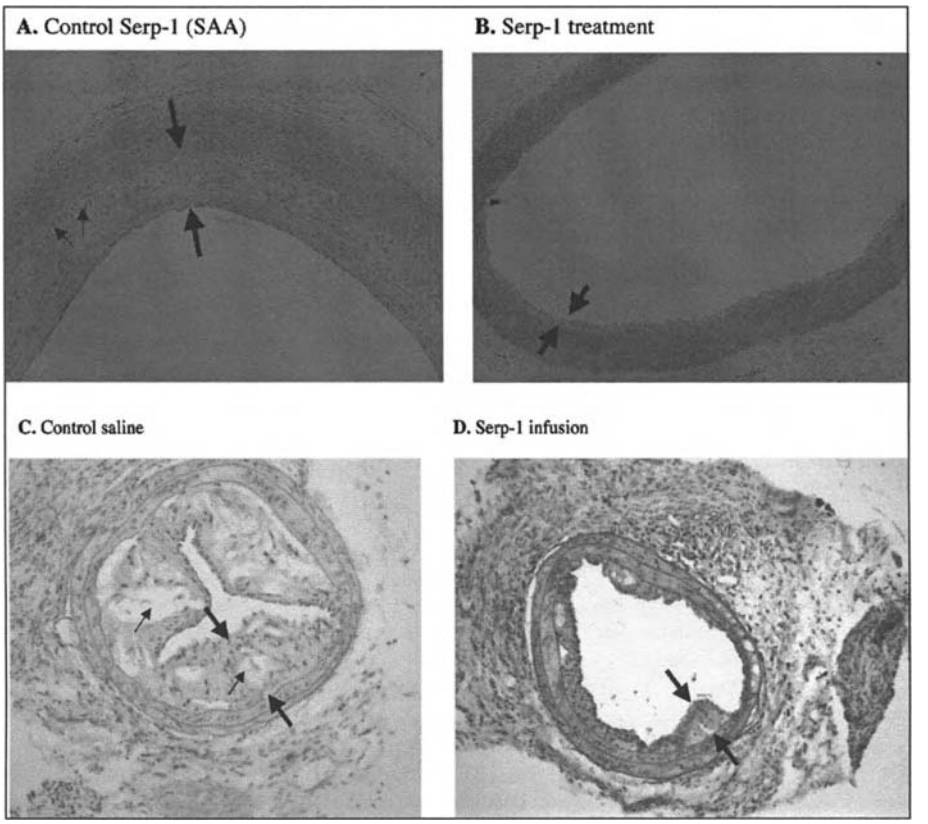

Figure 3E-F. In the first two panels representative histological cross sections of rabbit arteries taken at 4 weeks follow-up after balloon angioplasty injury and treatment with either inactive control Serp-1 (SAA) control (A) or active wild type Serp-1 (B) demonstrate the marked reduction in plaque area after a single bolus injection with Serp-1 (B) when compared to control (A). In this study rabbits were fed a high cholesterol diet and the drug was given as a single embolus injection immediately after balloon angioplasty. In the second two panels cross sections of ApoE null mouse carotid arterial histology sections are shown at follow-up after treatment with control saline treatment (C) or Serp-1 (D) infused by osmotic pump continuously. Again a marked reduction in plaque area was demonstrated with Serp-1 infusions. Large arrows bracket plaque growth areas in the intimal layer. Small arrows indicate areas of inflammatory cell invasion. Figure 3, continued on following page.

In these initial studies, early (i.e., 24 hrs to 7 days after angioplasty injury) inflammatory cell, macrophage and T-cell invasion was effectively blocked. Atherosclerotic plaque growth was significantly reduced at sites of angioplasty injury at 4 weeks follow-up, following injection of a single dose of Serp-1 protein (Fig. 3B). Smooth muscle cell and B cell invasion were not significantly altered in this model. Injection of a mutated Serp-1 protein, bearing a genetic replacement of the normal P1-P1′ Arg-Asn (R-N) scissile bond with Ala-Ala (A-A), produced an inactive serpin that was no longer able to block either inflammatory cell invasion or plaque growth.[64] In this original study there were no adverse effects observed; specifically no increased bleeding or clotting, infection or sepsis, no delay in wound healing, no increased mortality and overall no side effects. Inhibition of inflammation and plaque development was observed even after a single injection of picogram to nanogram doses of Serp-1 up to 4 weeks follow-up with no further bolus injections.[64] The half

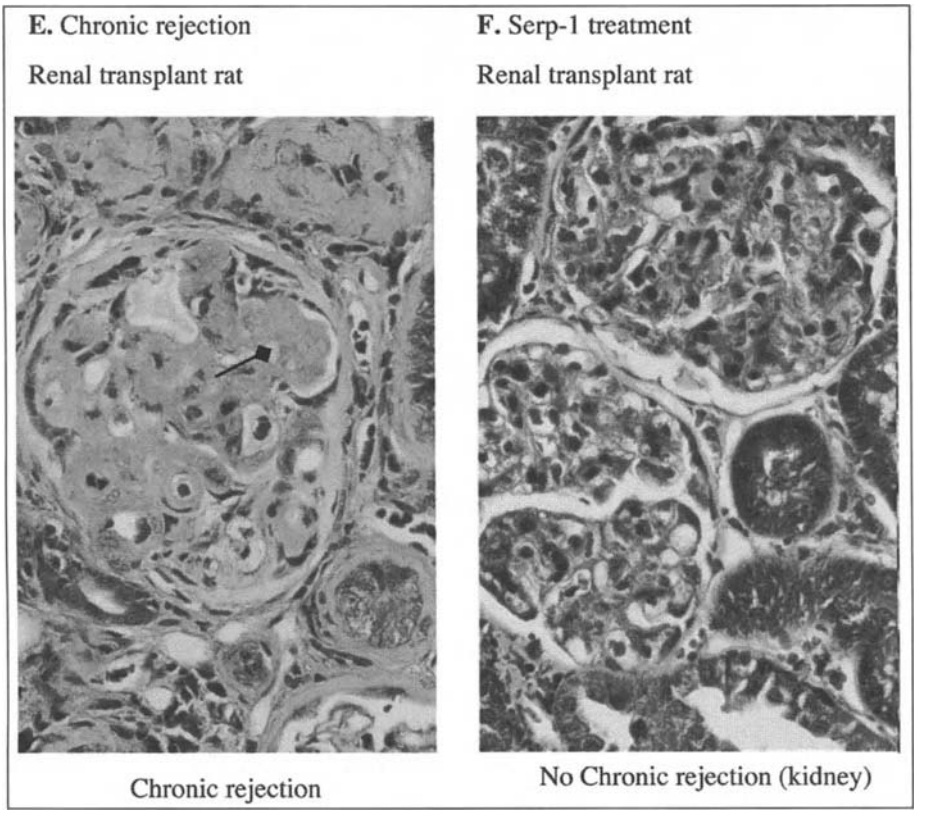

Figure 3, continued. Panels E and F illustrate treatment of rats after allograft renal transplant (F344 to Lewis rat) with either cyclosporine alone (E) or cyclosporine plus Serp-1 (F) at 5 months follow-up. Treatment was given as daily bolus injection i.v. for the first 10 days after renal transplant with no additional treatment. Areas of renal transplant scarring and loss of cellularity are indicated by the marker.

life of Serp-1 in normal rabbits was reported by Dr M Hatton (McMaster U, Hamilton, ON) to be less than 24 hours.[65]

Pre-Clinical Animal Models Studies of Angioplasty Injury

In subsequent work, the efficacy of Serp-1 therapy was tested across species and in differing models to assess the range of action and to confirm the anti-inflammatory and anti-atherogenic actions (blockade of atherosclerotic plaque growth) of Serp-1 in angioplasty injury models (Table 1). Both in the Lucas lab and in a collaborating lab (J Stroney, Case Western Reserve, OH) angioplasty-induced plaque was reduced at 4 weeks follow-up in cholesterol fed Yucatan microswine after peripheral arterial balloon angioplasty injury.[66] Plaque development was also significantly reduced after balloon angioplasty in rat iliofemoral and rooster aortic models (Lucas lab). In each case, early inflammatory mononuclear cell (macrophage and T-cell) invasion was significantly reduced with Serp-1 treatment at the site of injury. In these studies, doses of Serp-1 protein ranged from picogram/kg up to nanogram/kg body weight. Injections were given by either local Wolinsky perforated balloon injection (microswine) or by intravenous bolus (rabbit, rat and rooster), with Serp-1 infused immediately after angioplasty. No further injections were given until the time of follow-up at 4 weeks after angioplasty. While initial work was limited by

the amount of purified Serp-1 protein available from the original small protein preps produced, for use later studies a CHO cell expression system was developed in collaboration with Biogen (Leona Ling, Boston, MA USA and Viron Therapeutics, Inc, London, ON, Canada). The Serp-1 protein expressed and purified after secretion from CHO cells was produced at much higher quantities and allowed for higher dose infusions. In the most recent work, Serp-1 protein used for preclinical animal models was expressed in CHO cells and secreted Serp-1 protein was purified under good manufacturing practice (GMP) conditions, in a format suitable for clinical use. In all of the initial studies in rabbits and Yucatan microswine models, the perforated Wolinsky balloon was used for Serp-1 delivery at the site of angioplasty injury, based upon the original rabbit studies.[64] However, later work demonstrated efficacy on an equivalent level with systemic intravenous (i.v.) or intra-arterial (i.a.) systemic blood infusions. Many of these studies assessing the local perforated balloon vs systemic i.v. injection routes proceeded in parallel such that local infusions were still utilized in the swine models while work was ongoing in rabbits demonstrated efficacy when Serp-1 was infused as an i.v. bolus infusion. Once proven effective, animal studies were in general performed using i.v., dosing of Serp-1 protein.

Subsequent to these initial studies, Serp-1 was tested for efficacy in preventing plaque growth after both balloon angioplasty injury and bare metal stent implant.[66] Plaque was reduced after 2, 3 or 11 daily injections of Serp-1 given i.v. and starting immediately after stent implant in cholesterol fed rabbit aorta.[66] However, Serp-1 was not effective when given as a single bolus after stent implant in either cholesterol fed New Zealand white rabbit or Yucatan microswine models.[66] With repeated balloon angioplasty injury in microswine there was a trend toward reduced plaque when Serp-1 was infused after the final angioplasty injury in a series of three angioplasty injuries over 1.5 months, but this trend did not reach significance.[66] In none of these cases was baseline preformed plaque reduced by subsequent Serp-1 treatment. Specifically, only plaque at the site of balloon angioplasty or stent implantation was reduced, but plaque caused by cholesterol feeding outside the range of the vascular injury was not affected by Serp-1 treatment.[66] Thus, with ongoing or recurrent injury, as with repeated balloon angioplasty or with indwelling arterial stent implants one observes less effective reductions in plaque with single i.v., bolus injections. Multiple Serp-1 injections, however, did reduce plaque significantly in the rabbit stent implant model.[66] With repeated Serp-1 dosing (2 up to 11 daily i.v. boluses) starting on the day of aortic stent implant in cholesterol fed New Zealand rabbits, there was effective plaque reduction at 4 weeks follow-up.[66] In each animal model assessed, when early histological specimens were taken, there was evidence for Serp-1 mediated reductions in monocyte and nonspecific T-cell invasion at sites of vascular injury.[66]

Spi-3 Treatment in Balloon Angioplasty Models

Spi-3 protein from Vaccinia virus binds uPA and tPA, with weaker binding to fXa and plasmin as described in preceding sections. Spi- 3 was also tested in parallel with Serp-1 in a limited study after balloon angioplasty injury in rat models. Spi-3, as for Serp-1, reduced plaque growth significantly when a single injection was given i.v. after balloon injury (A. Lucas and R. Moyer, unpublished observations).

Preclinical Animal Models Study of Inflamed Plaque in ApoE^{null} Mouse Carotid Cuff Injury

The capacity of Serp-1 to reduce plaque inflammation and growth after carotid cuff compression in hyperlipidemic ApoE^{null} mouse models was also assessed. In this study, Serp-1 was infused at 2 μg/kg/day subcutaneously by continuous osmotic pump over 4 weeks. A markedly significant 67.7% reduction in plaque size and a reduction in histological markers for plaque instability (Fig. 3C, D) were both detected following Serp-1 treatment starting one week after carotid cuff placement. Serp-1 plasma levels were measured at approximately ng/mL concentrations.[67] When Serp-1 treatment was started at 5 weeks post cuff placement, a nonsignificant trend toward a reduction in plaque size (30% reduction) was observed. The plaque development in Serp-1 treated mice at the site of carotid cuff compression displayed reduced numbers of invading macrophage with increased smooth muscle cells and increased collagen deposition suggesting improved stability of

Table 1. Viral anti-inflammatory serpins assessed for anti-inflammatory activity in preclinical animal models

Viral Serpin	RSL/P1-P1' Sequence	Protease Target	Viral Origin	Route/X No Doses	Preclinical Model	Findings
Serp-1	EADERGTTAS SDTAITLIPRN ALTAIVANKP FMFLIYHKP	tPA, uPA, plasmin, FXa	Myxoma virus	Wolinsky local × 1	Balloon angioplasty-mouse, rat, rabbit, rooster, microswine	Inflammation-D, plaque-D
				V. X1, X2, X3 or X11	Balloon angioplasty and stent implant-rabbit, microswine	Inflammation-D, plaque-D plaque-NE
				Wolinsky Local × 1 Wolinsky Local × 1	Repeat balloon angioplasty injury-microswine	Plaque-D plaque-NE
				S.C. pump 30 days	Carotid cuff-mouse	Inflammation-D, plaque-D inflammation-D, plaque-D,
				I.V. X1	Renal artery vein bypass	Inflammation-D, plaque-D, scar-D
				I.V. X10	Aortic transplant-mouse, rat renal transplant-rat	Scar-D plaque-D chronic rejection-D
				I.V. X10	Cardiac transplant-mouse, rat	Prolonged allograft survival-I, acute rejection-D
				I.J. X1 I.V. X10	Antigen induced arthritis-rabbit,-rat	Inflammation-D, erosion-D
				Surface × 1	CAM-chicken	Angiogenesis-D
Spi-3	DVDEQGTVA EASTIMVAT ARSSPEQLEF NTPFIIRHDI	tPA, uPA, plasmin, thrombin, factor Xa	Vaccinia virus, cowpox virus	I.V. × 1	Balloon angioplasty-rat	Inflammation, plaque-D

continued on next page

Table 1. Continued

Viral Serpin	RSL/P1-P1' Sequence	Protease Target	Viral Origin	Route/X No Doses	Preclinical Model	Findings
Serp-2	... VTDFGG	Granzyme B, caspase 1 (ICE)	Myxoma virus	I.V. X1	Balloon angioplasty-rat	Inflammation, plaque-D
			Capripox virus,	S.C. pump x 30 days	Carotid cuff-mouse	Inflammation, plaque-D
			Yatapox virus	I.V. X1	Aortic transplant-rat, mouse	Inflammation, plaque-D
CrmA/ Spi2 Serpin B13R	ATCALVAD-CAST	Granzyme B, caspase	Cowpox virus,	I.V. X1	Balloon angioplasty-mouse, rat	NE
		1 (ICE), caspase 8, 10, 6, 3, 7	Vaccinia virus	S.C. pump x 30 days I.V. X1	Carotid cuff-mouse	NE
					Aortic transplant-mouse, rat	NE
					Prostate cancer leukemia	Cell growth-I
						Resistance to chemotherapy

uPA—urokinase type plasminogen activator, uPA receptor—uPAR, D—decrease, I—increase, NE-null or equivocal effects, Rb—rabbit, Rt—rat, Ms—mouse, MSw—microswine.

1. Srikanth S, Kraft AS. Inhibition of caspases by cytokine response modifier A blocks androgen ablation-mediated prostate cancer cell death in vivo. Cancer Res 1998; 58:834-839.
2. Antoku K, Liu Z, Johnson DE. Inhibition of caspase proteases by CrmA enhances the resistance of human leukemic cells to multiple chemotherapeutic agents. Leukemia 1997; 11:1665-1672.
3.Boomker JM, Luttikhuizen DT, Veniga H. The modulation of angiogenesis in the foreign body response by the poxviral protein M-T7. Biomaterials. 2005; 26: 4874-4881.
4.Richardson M, Liu L, Dunphy L, et al. Viral serpin, Serp-1, inhibits endogenous angiogenesis in the chicken chorioallantoic membrane model. Cardiovasc Pathol. 2007; 16: 191-202.

the plaque and reduced risk of plaque rupture.[67] Unstable atherosclerotic plaque is characterized by a necrotic lipid core with highly active macrophage and T-cell components that release factors that lead to thinning of the surface cap covering the inner plaque core. The core has activated macrophage and T-lymphocytes as well as smooth muscle cells, but can also contain apoptotic cells. The apoptotic macrophage cells in particular can act as small cytokine factories releasing large quantities of inflammatory activators. Proteases, such as the thrombolytic serine proteases (tPA, uPA and plasmin) and the matrix metalloproteinases (MMPs), are also released that breakdown the local connective tissue. In addition to these activated and apoptotic cells, layers of cholesterol deposits and connective tissue also can serve to activate cells and initiate inflammatory reactions.

With erosion, this thinned protective cap can rupture, exposing the underlying connective tissue, inciting thrombus formation and leading to heart attacks and strokes. Mice treated with Serp-1 in this study displayed reduced macrophage content and increased SMC and collagen content suggesting a more stable plaque phenotype.[67]

Preclinical Animal Model Studies of Transplant Rejection—Acute and Chronic Rejection

Aortic Transplant Models

Marked reductions were detected in both inflammatory cell invasion into the arterial wall along with associated reductions in late plaque growth in the balloon angioplasty and stent implant models, and thus the capacity of Serp-1 to reduce chronic inflammation in aortic allograft and renal transplant models was examined in rat models. In later studies mouse aortic transplant models were also utilized in order to analyze the effects of selected genetic 'knock out' on serpin mediated anti-inflammatory activities. Chronic transplant rejection of solid organ transplants is characterized by chronic vascular occlusion and associated end organ ischemic damage. Although inhibition of T-cell mediated rejection is efficacious in reducing chronic transplant vasculopathy and organ damage, it has been estimated that approximately 50% of chronic rejection responses are not adequately blocked and some of this chronic rejection and vasculopathy is now attributed to ongoing smoldering excesses in inflammation and scarring. Two collaborating labs, H Wong with R Zhong at the University of Western Ontario (ON, Canada) and R Morris at Stanford (CA, USA), examined the capacity of Serp-1 treatment to reduce the long term inflammation that contributes to chronic rejection.

The initial rat aortic transplant work was performed by Miller and Dai in the Lucas lab.[68] With a single i.v., bolus infusions of either ACI rat donor to Lewis rat recipient or Lewis rat donor to Sprague Dawley (SD) recipient rat aortic transplants, a significant reduction in early monocyte/macrophage and nonspecific CD2 positive T-cell invasion as well as a significant reduction in later plaque growth were detected.[68] Serp-1 was infused via i.v., bolus injection immediately after completion of an end-to-end aortic anastomotic connection once visible blood flow (aortic pulsation) was detectable. No further bolus doses of serpin were infused after this initial dose and no other drugs were given in this aortic transplant model study.

Renal and Heterotopic Cardiac Transplants

E Bedard in the Zhong lab subsequently examined F344 (RT^{1v1}) donor to Lewis (RT^{1}) recipient rat renal transplants at 5 months follow-up.[69] In this study Serp-1 i.v., plus cyclosporine A (0.75 mg/kg/day s.c.) treatment reduced both scarring and vascular stenosis at 5 months after giving Serp-1 treatment for only the first 10 days after transplant (Figs. 3E, F). Serp-1 treatment with and without cyclosporine A was compared to cyclosporine A alone.[69] When given together with cyclosporine A, Serp-1 at the higher 50 μg/kg i.v., dose markedly reduced all the classical histological markers for chronic rejection including tubular and glomerular atrophy, vascular hyalinization and cortical scarring in the renal transplant model at 5 months follow-up.[69]Additional bolus injections of Serp-1 at 1 month posttransplant did not produce further reductions in scarring and vasculopathy, indicating that the anti-inflammatory and anti-rejection activity of Serp-1 is realized during the first 7-10 days after transplant. Unlike the simple aortic transplant model, Serp-1 given alone, without concomitant cyclosporine injections, did not reduce transplant vasculopathy or scarring.[68] The work with the renal transplant rejection model provided several important advances in our work. First, treatment with Serp-1 together with cyclosporine for the first 10 days after transplant markedly reduced both scarring and vasculopathy at 5 months follow-up when compared to cyclosporine treatment alone.[68] Second, Serp-1 treatment could be limited to the first 10 days after transplant and result in continued protection of the allograft renal transplant even as late as 5 months follow-up with no further boluses. Finally, the renal transplant in this model was the only functioning kidney in the recipient rat and there was no evidence for toxicity, adverse events or adverse effects on renal function with Serp-1 treatment.

A similar study was performed by B Hausen in the Morris lab, examining the percentage of coronary vessels with evidence for vasculopathy and narrowing at 3 months follow-up in a rat cardiac transplant model following treatment with Serp-1 and cyclosporine treatment for the first 10 days post transplant.[70] The number of vessels displaying significant plaque and narrowing was significantly reduced with Serp-1 treatment at 3 months follow-up.

Acute rejection was more recently studied in a Brown Norway rat to Lewis rat heterotopic heart transplant model. In this work (H Wang in the Zhong lab) detected significantly reduced acute rejection following Serp-1 treatment.[71] Improved graft survival was also detected in rats treated with a lower dose of cyclosporin. Indefinite heterotopic heart allograft survival was demonstrated with greater than 100 days follow-up.[71] The Zhong lab also reported reduced xenograft rat to mouse cardiac transplant loss with Serp-1 treatment when given together with two other immunosuppressants. With this work one can propose that Serp-1 treatment together with low dose cyclosporine or other agents should reduce the toxicity of these acute rejection treatments.[70]

Preclinical Animal Model Study of Arthritis

In an early pilot study of 15 rabbits with ovalbumin antigen-induced arthritis, W Maksymovich and A Russell (University of Alberta, Edmonton, Canada) demonstrated reduced joint swelling and inflammation and reduced joint cartilage erosion.[72] In this study, Serp-1 protein was infused in nanogram doses via intra-articular injection. In a larger subsequent work, E Brahn (UCLA, CA, USA) detected marked reductions in joint swelling, erosions and an associated improvement in motility in a rat treated with Serp-1 prior to antigen challenge.[72]As this represented a preventative model and thus is an unlikely scenario in the clinic for arthritic patients, the Brahn group also proceeded to examine the effects of Serp-1 given after arthritis was already established in the rat model. In this follow-up study Serp-1 was infused either alone or together with cyclosporine. When given alone Serp-1 was not effective at reducing inflammation in this collagen induced arthritis model, whereas when dosed together with cyclosporine, Serp-1 protein treatment resulted in a synergistic reduction.[73]

Chicken Chorioallantoic Membrane (CAM) Model of Angiogenesis

In a chicken (*Gallus gallus*) chorioallantoic membrane (CAM) model of angiogenesis, Serp-1 treatment reduced new vessel creation significantly.[74]An inactive Serp-1 mutant Serp-1 (SAA), with an amino acid replacement of R-N to A-A at the P1-P1′ site, was ineffective. Treatment with the mammalian serpin, PAI-1, in the same model also did not reduce new vessel formation.[74] Treatment with Serp-1 reduced vascular endothelial growth factor and laminin gene expression at 6 hrs and 24 hrs after treatment in this model which has the potential to alter neovascular proliferation. Collagen IV expression was also altered but varied from reduced levels at 6 hours to increased at 14 hours follow-up. The role of Serp-1 in potentially controlling dysregulated angiogenesis merits further investigation.[74]

SERP-1 Mechanism of Action

Initial studies with Serp-1 used immunohistochemical analysis of tissue specimens from animal models of cellular invasion at early times after injury. In the rat and rabbit models there was a consistent early reduction in the invasion of macrophage and nonspecific CD2-positive, nonspecific lymphocyte (specifically NK cell) invasion from 24 hours up to 72 hours after Serp-1 treatment post angioplasty injury or aortic, cardiac and renal transplant. Similar reductions in macrophage cells and CD2-positive lymphocytes were detected at early follow-up after acute cardiac (<48 hours) and chronic renal transplant models. This reduction was greater than the effects of Serp-1 alone or cyclosporine treatment alone in these same models. This reduction in inflammatory cell invasion correlated closely with later reductions in plaque growth and vasculopathy development.

It should be noted that many viral proteins exhibit more than one function, often targeting two or more host response pathways. Serp-1 targets tPA, uPA, plasmin in the thrombolytic cascade and also factor Xa in the thrombotic cascade which represent more than one receptor and signaling pathway. It is certainly possible that this highly potent viral serpin may have acquired other

functions during evolution as a host immunomodulator, functions that are as yet undisclosed. As the uPA/uPAR complex has been clearly demonstrated to play an important role in inflammatory cell responses after vascular injury, other cellular responses and changes in gene expression in human endothelial cells, monocytes and T-cells were examined in Serp-1 treated cultured human cells. These studies performed by K Viswanathan (Lucas lab) demonstrated that Serp-1 treatment was capable of reducing cell activation as measured by calcium content and membrane fluidity in all cell types tested. As one of the earliest cell responders to i.v. Serp-1 infusions, it is possible that endothelial cells mediate the first inhibitory actions of Serp-1 during vascular injury.[75] Gene expression was also noted to be altered in endothelial cells and monocytes after Serp-1 treatment and this activation pattern differed for Serp-1 and mammalian PAI-1 treatment. Of particular interest, in rat arteries tPA, PAI-1 and uPAR exhibited increased expression following Serp-1 treatments, while for human endothelial cells in culture, the genes for PAI-1 as well as the ITPR2 receptor for calcium underwent altered levels of expression.[28,75] In human monocytes, Serp-1 treatment, differed from PAI-1 as measured by microarray analysis, thereby causing an increase in an actin binding protein and a reduction in CD18, beta 2 integrin expression (unpublished observation).[108] In the CAM model of angiogenesis, Serp-1 treatment reduced VEGF and laminin expression and increased collagen IV. In the acute rejection model, when Brown Norway rat donor to Lewis rat recipient heterotopic heart transplants were performed, Serp-1 treatment significantly reduced TLR2, TLR 4 and myD88 gene expression.[71] Also, in the early stages after chronic renal allograft transplant with Serp-1 treatment, TGFβ gene expression was reduced. Associated with these changes in inflammatory gene expression, there was a reduction in macrophage and dendritic cell invasion. An $ApoE^{null}$ mouse study similarly detected reduced macrophage invasion and associated increases in collagen and SMC in the plaque, all of which suggest increased plaque stabilization.[67] Serp-1 was also found to bind to the surface of endothelial cells, monocytes and T-cells in vivo using fluorescent microscopy and FACS (fluorescence activated cell sorting) analysis.

In mouse targeted gene knockout models, Serp-1 lost all inhibitory activity in uPAR deficient ($uPAR^{-/-}$) mouse model of aortic transplant. However, Serp-1 retained its potent anti-inflammatory activity in PAI-1 deficient ($PAI\text{-}1^{-/-}$) mice after aortic transplant, PAI-1 deficient C57Bl/6 background donor to PAI-1 expressing ($PAI\text{-}1^{+/+}$) Balb/C recipient mouse.[28] Conversely PAI-1 treatment only reduced plaque in PAI-1 knock out C57Bl/6 isograft transplants, but not in allograft transplants.[28] In the PAI-1 deficient allograft transplant model PAI-1 deficient C57Bl/6 background donor to PAI-1 expressing Balb/C recipient mouse, treatment with a single dose of PAI-1 caused a local excessive thrombotic activation and early mortality.[28] In stark contrast, a single injection of Serp-1 protein markedly reduced inflammation and plaque growth and caused no alteration in thrombosis or bleeding.[28] Treatment with an array of Serp-1/serpin RSL chimeras did not reduce inflammation or plaque growth indicating that Serp-1 requires an intact R-N sequence at the P1-P1′ site. Alteration of the adjacent P2-P7 amino acids, by replacing these residues with 6 alanines led to a loss of Serp-1 anti-inflammatory and anti-atherogenic activity.

Viral Serpins That Target Apoptotic Pathways: Preclinical Analysis of CRMA and SERP-2

Cowpox virus's cytokine response modifier A (CrmA) was the first viral serpin to be identified and has been intensely studied. CrmA is a stable intracellular protein synthesized early during cowpox viral infection that mediates the formation of characteristic red hemorrhagic pocks on the chorioallantoic membrane (CAM) of fertile hen eggs. A CrmA-like protein called Spi-2 is also expressed by certain vaccinia virus strains. This 38 kD intracellular protein is expressed early in viral infection and inhibits both caspases 1 (interleukin converting enzyme-1β or ICE) and 8 as well as Granzyme B, key players in apoptotic pathways. CrmA inhibits caspases-1 (Ki = 4-10 pM) and 8 (Ki = <340 pM) most effectively, but also inhibits caspase s10 (Ki = 4-17 nM), 6, 3 and 7 with decreasing effectiveness.[40] CrmA blocks apoptosis induced via death receptor signaling (Fas receptor and TNF), but is not effective at preventing cell death induced by stress or genotoxic damage induced through mitochondrial apoptotic (caspase 9) signaling. These inhibitions help the

virus to replicate, as the primary way to eliminate a viral infection is for T-cells to induce apoptosis in infected cells. A chicken chorioallantoic membrane model demonstrated a strong reduction in inflammation when treated with CrmA; however, recent research in animal models of orthopoxvirus infection has failed to confirm CrmA's anti-apoptotic capabilities in vivo.

CrmA (cytokine response modifier A) a cowpox viral protein and Serp-2 (an intracellular myxoma viral serpin) are two viral cross class serpins that bind and inhibit both granzyme B, a serine protease and caspase 1 (cysteine proteases). The presence of Asp (D) in the P1 site of the RSL of these two serpins allows these proteins to function both as serine and as cysteine protease inhibitors, which is why they are called cross class serpins (Fig. 2). Of interest, work by Moyer and Turner have demonstrated that while CrmA binds to both caspases 1 and 8 as well as granzyme B with greater affinity (Kass)[76,77] than Serp-2, whereas, Serp-2 displays greater effects on viral virulence in vivo during viral infections.[78] CrmA inhibits extrinsic apoptosis mediated through the Fas and TNF pathways, but does not block granzyme B mediated cell death after cytotoxic T-lymphocyte release of granules.[79] Serp-2 cannot block apoptosis in cowpox virus infected cells, but conversely Serp-2 deficiency in myxoma virus infection markedly attenuates virus infection in European rabbits with a reduction in mortality from 100% to 10% with inactivation of the Serp-2 gene.[76] CrmA cannot replace Serp-2 and results in only 70% mortality in myxoma virus rabbit infections.

Like CrmA, myxoma poxvirus 34kD Serp-2 is able to inhibit ICE. Serp-2 shares 35% similarity with CrmA. However, despite displaying a lower binding affinity in vitro for ICE and caspase 8, Serp-2 demonstrates a more robust anti-inflammatory activity in vivo during viral infections. Serp-2 is capable of inhibiting apoptosis in CAM models of infection but did not block inflammation in the CAM model.[79] Although both Serp-2 and CrmA are cross-class serpins and target some of the same proteases, in infected cells the insertion of Serp-2 in place of CrmA does not cause similar effects.[79] Thus these two cross class serpins are not functionally interchangeable and studies in animal models have confirmed a marked difference in their potential as anti-inflammatory agents.

Spi-1 is a less well described rabbit poxvirus intracellular protein with circumscribed anti-apoptotic activity. This viral serpin binds cathepsin G similar to the reports for the mammalian intracellular PI-6 protein. Spi-1 has been reported to inhibit a caspase independent form of apoptosis in selected cells.[80]

SERP-2 Preclinical Studies

In rat and mouse models of angioplasty injury and aortic transplant, effective reductions in inflammatory cell invasion and in plaque growth were observed following Serp-2 treatment.[81] CrmA conversely had no effect on plaque growth in these models, nor did two Serp-2 RSL mutants (D294A and E) provided by P Turner and R Moyer for these studies. Work by I Bot and E Biessen similarly detected significant reductions in plaque in the ApoE null mouse model with carotid cuff compression injury after Serp-2 treatment but not with CrmA.[82] While Serp-2 showed a trend toward reducing plaque at the site of carotid cuff compression injury, this trend did not reach significance. But, unlike Serp-1 treatment, Serp-2 was able to reduce the generalized increase in plaque detected at the aortic root of $ApoE^{null}$, mice suggesting that this protein can target and reduce systemic plaque buildup rather than plaque growth only at sites of vascular surgical injury.[82]

Other Mammalian Serpins

These studies with Serp-1 and other viral serpins provide a guide to the development of agents targeting pathways identified as having potential for high impact in the regulation of inflammatory and apoptotic pathways. Thus, mammalian serpins that inhibit the thrombolytic serine proteases, more specifically the uPA and tPA pathways, were studied to assess how close an analogy one might find for mammalian serpin inhibition of inflammation. PAI-1 and neuroserpin were tested in similar rodent models and compared to Serp-1 for anti-inflammatory and anti-atherogenic activity in the preclinical models. PAI-1 binds and inhibits tPA, uPA and thrombin as well as activated protein C (APC) with a higher K_{ass} than Serp-1 (often with an increase of K_{ass} on the order of two logs (10^7

versus 10^4)).[59] Neuroserpin binds and inhibits tPA and uPA, but with a greater predilection for tPA. As noted in prior reports, infusion of PAI-1 demonstrated anti-inflammatory and anti-atherogenic actions in some preclinical models, while other studies found a marked pro-inflammatory activity for PAI-1.[35-38] The work by Carmeliet demonstrated an increase in plaque after iliac injury in PAI-1 deficient mice,[34] while work by Plopis demonstrated reduced plaque in $FeCl_3$ injured arteries in PAI-1 deficient mice.[36] In our lab, PAI-1 infusion did reduce cell invasion and even plaque growth in PAI-1 deficient mice in both cell migration assays and in isograft aortic transplants where the donor and recipient mice both lacked PAI-1 expression.[28] In aortic allografts, however, with transplant of a PAI-1 deficient C57Bl/6 mouse aorta into a PAI-1 expressing Balb/c mouse there was excess local thrombosis and 100% mortality.[27] Thus, adding this natural mammalian serpin to a mouse already expressing background levels of PAI-1 leads to excess clotting and death. This pro-coagulant property is not seen following Serp-1 treatment.

Mammalian neuroserpin preferentially inhibits two chain tPA ($Ki = 6.2 \times 10^5\ M^{-1}s^{-1}$), but also inhibits single chain tPA, trypsin, uPA, nerve growth factor-γ, plasmin and thrombin (Ki ranging from 2.1×10^2 to $8.0 \times 10^4\ M^{-1}s^{-1}$).[32] Neuroserpin has also been implicated in neurological diseases such as dementia with neuroserpin mutation and polymer formation, cerebrovascular diseases, epilepsy, multiple sclerosis and schizophrenia. In mouse cerebral ischemia models neuroserpin appeared to have a protective role as evidenced by the finding that delivery of neuroserpin to the affected area (or overexpression of neuroserpin) decreased the ischemic territory and reduced the numbers of apoptotic cells in the setting of cerebral infarction.[33] Neuroserpin has also been reported to play a central role in development of the visual cortex in axonal growth and in the regulation of synaptic plasticity. We thus tested neuroserpin in a collaborative study with D Lomas (U Cambridge, Cambridge, UK). In our rat model of angioplasty injury and in the mouse aortic transplant models, neuroserpin injection did significantly reduce plaque growth (unpublished observations).

In summary, we have assessed two mammalian serpins that target thrombolytic pathways, plasminogen activators, in preclinical vascular surgery models. One, PAI-1, induced excess thrombosis and mortality but the second, neuroserpin, reduced inflammation. Analysis of these cellular serpin proteins and discovery work on the mechanism of action of these viral serpins has therefore provided insights into new therapeutic targets for drug discovery and treatment of inflammation based disorders. This work in effect adds to the serpin chorus as potential therapeutic agents and new pathways to target in treating inflammation based diseases.

Other Parasite Derived Serpins

Investigation of diverse genomes has yielded a wide range of organisms that encode serpins, from poxviruses to mammals to more recently discovered bacterial and unicellular serpins. The function of the majority of these proteins are unknown, although most are thought to have inhibitory activity based on sequence homology of known serine protease inhibitors. In this section, serpins from various organisms are presented and their importance in possible immunotherapies discussed.

Insects are hosts to a wide variety of disease causing organisms and act as vectors of transmission to man. Serpins in haematophagous (blood-feeding) insects are expressed for two different purposes; immune evasion/invasion for feeding from their host and defense against infection.[83] Tsetse flies are responsible for harboring and transmission of African sleeping sickness in humans. These insects express the serpins Tsal1 and Tsal2 in their saliva, which are important for transmission of Trypanosomes by suppression of B and T-cell activity.[84]

Mosquitoes serve as an intermediary in the transmission of malaria-causing Plasmodium parasites into humans. There are mechanisms in the mosquito gut designed to inhibit the invasion of the parasites.[83] *Anopheles* mosquitoes express several serpins that have important actions for the killing and/or clearing of parasites from the insect. Thus the mosquito can carry and transmit the infecting parasite to other hosts such as man without succumbing to infection itself. For example, in *A. gambiae*, SRPN10 is expressed as four isotypes, with variations in the RSL.[85] Invasion of the midgut of *A. gambiae* mosquitoes by *Plasmodium berghei ookinetes* up-regulates the mRNA of a

specific subset of isotypes, KRAL and RCM, possibly regulating the apoptosis of infected cells.[85] Progression of the timeline of infection can be observed just by the localization and expression of SRPN10. SRPN10 normally resides in the nucleus, but when invaded by *ookinetes*, SRPN 10 translocates to the cytoplasm.[86] Once a parasite is ready to exit from the gut basolateral membrane, SRPN10 protein expression is then increased.[86] Down-regulation of another mosquito serpin, SRPN2, by RNAi decreases oocyst formation after infection with *Plasmodium berghei* in the midgut,[87] however, in field isolates of *Plasmodium falciparum*, this did not reduce oocyst formation.[88] SRPN6, another *Anopheles* expressed serpin, is expressed in both *Anopheles gambiae* and *stephensi* and has an identical 28 residues for their RSL yet result in slightly different activities in the organisms when faced with Plasmodium infection.[89] In *A. stephensi,* SRPN6 is thought to have parasite killing activity whereas in *A. gambiae*, Plasmodium *berghei* SRPN6 possibly has a role in the clearance of parasites by delaying the progression of infection.[90] Interestingly, in *A. gambiae*, SRPN6 is also up-regulated upon exposure of the gut to *Escherichia coli*,[90] as observed in *Drosophila melanogaster.*[91]

Insects are not the only organisms to express serpins that allow these organisms to invade their hosts and evade the host inflammatory responses. Helminthes, such as *Schistosomes*[92,93] and *Brugia*[94,95] express serpins that dampen the immune responses of their hosts in not one, but several pathways.[96-102] In blood feeding, ticks also secrete serpins in their saliva to inhibit the local inflammatory response and blood clotting to prolong their ability to feed without detection.[103-107]

Thus these disease-causing organisms over time have developed an arsenal of proteins to facilitate successful invasion of their host organisms. By understanding the mechanism of action, serpins from parasitic organisms could be refined and utilized in therapeutics as anti-inflammatory and antithrombotic compounds. Serpins have potential uses other than specific protease activity. For example, serpin-coupled peptides, such as ovalbumin (a serpin that lacks classical serpin inhibitory functions) and coupled Tc52 peptides from *T. cruzi*, have been shown to inhibit T-cell activity.[108] By using ovalbumin as a carrier protein to reduce immunogenicity, specific host pathways can be targeted. Beneficial activities from proteins with more than one function can be dissected and isolated for coupling with ovalbumin, to minimize nonspecific activities. Utilizing already known mechanisms of immune modulation can be an efficient method of therapeutic development.

Clinical Study of SERP-1 Treatment in Acute Unstable Coronary Syndromes; Unstable Angina and Non-ST Elevation Myocardial Infarction (NSTEMI)

Like any new discovery in the drug field and clinical testing of a new class of protein therapeutics, this work could not have been done without the efforts of many investigators. A small biotech company was established in 1997 by Drs Lucas and McFadden, called Viron Therapeutics Inc, (London, ON, Canada). Viron was initially established around the Lucas and McFadden research laboratories and founded with the guidance of Dr M Ponansky, the then Director of the Robarts Research Institute, University of Western Ontario, London, ON, Canada. Viron coordinated the expertise and funding necessary to produce viral proteins according to good manufacturing practices (GMP) and to set up the preclinical animal toxicity screening according to good lab practice (GLP) mandates. With this foundation Serp-1 has been successfully taken through a Phase 1 safety trial in man and is being tested for safety and efficacy in a Phase IIa clinical trial conducted in the US and Canada.

For the Phase 1 study, single doses of Serp-1 protein were infused in normal volunteers as mandated by the FDA. This was the first trial in man with a new class of virus-derived native protein therapeutic. No changes in cardiac, renal or hepatic function, as well as no changes in clotting parameters were detected in this study. This Phase 1 safety study demonstrated that Serp-1 infusion was safe with no adverse events detected or reported. A Phase IIa study is currently ongoing in which the effects of a Serp-1 infusion is given for three days starting immediately after balloon angioplasty and stent implant in patients with acute unstable angina and non-ST elevation myocardial infarction (NSTEMI). This trial is still ongoing at 7 sites in Canada and the US. The

results of this study have yet to be reported. This Phase 2a study represents a first in man clinical trial of a native anti-inflammatory viral serpin. Clinical efficacy and safety thus remain to be finally determined for this protein, but, if safe, this study potentially opens the door to testing of other viral anti-inflammatory proteins in inflammation driven diseases.

While taking Serp-1 to clinical trial is a first step in the path toward using viral anti-inflammatory proteins as a new therapeutic, this work really represents only the first step in our symphony and our first passage in the study of serpins as guides to new therapeutic modalities.

References

1. Sulek K. Prize in 1908 awarded to P Erlich and E Metchnikoff for their work in immunology. Wiad Leuk 1967; 20:1117-1118.
2. Opitz B, Hippenstiel S, Eitel J et al. Extra and intracellular innate immune recognition in endothelial cells. Thromb Haemost 2007; 98:319-326.
3. Mariathasan S, Monack DM. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nature Rev Immunol 2007; 7:31-40.
4. Brown KL, Cosseau C, Gardy JL et al. Complexities of targeting innate immunity to treat infection. Trends Immunol 2007; 28:260-266.
5. Lin WW, Karin M. A cytokine mediated link between innate immunity, inflammation and cancer. J Clin Invest 2007; 117:1175-1183.
6. Ware C. Network communications: Lymphotoxins, light and TNF. Annu Rev Immunol 2005; 23:787-819.
7. Roy CR, Mocarski ES. Nature Imm 2007; 8:1179-1187.
8. Parker LC, Prince LR, Sabroe I. Translational minireview series on toll-like receptors: Networks regulated by toll-like receptors mediate innate and adaptive immunity. Clin Exp Immunol 2007; 147:199-207.
9. Methe H, Weis M. Atherogenesis and inflammation—was Virchow right? Nephrol Dial Transplant 2007; 22:1823-1827.
10. Schneider DF, Glenn CH, Faunce DE. Innate lymphocyte subsets and their immunoregulatory roles in burn injury and sepsis. J Burn Care and Research 2007; 28:365-379.
11. Opal SM, Esmon CT. Bench-to-bedside review: Functional relationships between coagulation and the innate immune response and their respective roles in the pathogenesis of sepsis. Crit Care 2003; 7:23-38.
12. Jancaiuskiene S. Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. Biochem Biophys Acta 2001; 1535:221-235.
13. Lomas DA, Belorgey D, Mallya M et al. Molecular mousetraps and the serpinopathies. Biochem Soc Transaction 2005; 33:321-330
14. Carrell RW, Travis J. α1-Antitrypsin and the serpins: Variation and countervariation. Trends Biochem Sci 1985; 10:20-24.
15. Irving JA, Pike RN, Lesk AM et al. Phylogeny of the serpin superfamily: Implications of patterns of amino acid conservation for structure and function. Genome Res 2000; 10:1845-1864.
16. Iwanaga S, Kawabata S. Evolution and phylogeny of defense molecules associated with innate immunity in horseshoe crab. Front Biosci 1998; 3:D973-984.
17. Law RHP, Zhang Q, McGowan S et al. An overview of the serpin superfamily. Genome Biol 2006; 7:216.2-216.11
18. Silverman GA, Bird PI, Carrell RW et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. J Biol Chem 2001; 276:33293-33296.
19. Marszal E, Shrake A. Serpin crystal structure and serpin polymer structure. Arch Biochem Biophys 2006; 453:123-129.
20. Huntington JA, Read RJ, Carrell RW. Nature 2000; 407:923-926.
21. Patston PA, Church FC, Olson ST. Serpin–ligand interactions. Methods 2004; 32:93-109.
22. Ye S, Cech AL, Belmares R et al. The structure of a michaelis serpin-protease complex. Nat Struct Biol 2001; 8:979-983
23. Lawrence DA, Olson ST, Muhammad S et al. Partitioning of serpin-proteinase reactions between stable inhibition and substrate cleavage is regulated by the rate of serpin reactive center loop insertion into β-sheet A. J Biol Chem 2000; 275:5839-5844.
24. Whisstock JC, Bottomley SP. Molecular gymnastics: serpin structure, folding and misfolding. Curr Opin Struct Biol 2006; 16:761-768
25. Li W, Johnson DJ, Esmon CT et al. Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. Nat Struct Mol Biol 2004; 11:857-862.
26. Johnson DJ, Li W, Adams TE et al. Antithrombin-S195A factor xa-heparin structure reveals the allosteric mechanism of antithrombin activation. EMBO J 2006; 25:2029-2037.

27. Dai E, Viswanathan K, Sun YM et al. Identification of myxomaviral serpin reactive site loop sequences that regulate innate immune responses. J Biol Chem 2006; 281:8041-8050
28. Dai E, Guan H, Liu L et al. Serp-1, a viral anti-inflammatory serpin, regulates cellular serine proteinase and serpin responses to vascular injury. J Biol Chem 2003; 278:18563-18572.
29. Ewa Marszal, Andrew Shrake. Serpin crystal structure and serpin polymer structure. Arch Biochem Biophys 2006; 453:123-129.
30. Blasi F. Proteolysis, cell adhesion, chemotaxis and invasiveness are regulated by the uPA-uPAR-PAI-1 system. Thromb Haemost 1999; 82:298.
31. Carmeliet P, Collen D. Molecular analysis of blood vessel formation and disease. Am J Physiol Heart Circ Physiol 1997; 273:H2091-H2104
32. Yepes M, Lawrence DA. Neuroserpin: a selective inhibitor of tissue-type plasminogen activator in the central nervous system. Thromb Haemost 2004; 91:457-464.
33. Yepes M, Sandkvist M, Wong MK et al. Neuroserpin reduces cerebral infarct volume and protects neurons from ischemia-induced apoptosis. Blood 2000; 96:569-576.
34. Carmeliet P, Moons L, Lijnen R et al. Inhibitory role of plasminogen activator inhibitor-1 in arterial wound healing and neointima formation. Circulation 1997; 96:3180-3191
35. Falkenberg M, Tom C, DeYoung MB et al. Increased expression of urokinase during atherosclerotic lesion development causes arterial constriction and lumen loss and accelerates lesion growth. PNAS 2002; 99:10665-10670.
36. Ploplis VA, Castellino FJ. Attenuation of neointima formation following arterial injury in PAI-1 deficient mice. Ann NY Acad Sci 2001; 936:466-468
37. Strauss BH, Lau HK, Bowman KA et al. Plasma urokinase antigen and plasminogen activator inhibitor-1 antigen levels predict angiographic coronary restenosis. Circulation 1999; 100:1616-1622
38. Vaughan DR. PAI-1 and atherothrombosis. J Thromb and Haemostasis 2005; 3:1879-1883
39. Munuswamy Ramunujam G, Lucas A. Mammalian serine protease inhibitor (serpin), neuroserpin, targets thrombolytic proteases to reduce inflammation, atherogenesis and T helper lymphocyte activation. Poster. American Heart Association. Orlando, FL 2007
40. Bortner CD, Cidlowski JA. Cellular mechanisms for the repression of apoptosis. Annu Rev Pharmacol Toxicol 2002; 42:259-281.
41. Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. Genome Biol 2001; 2:3014.1-3014.7
42. Roemisch J, Gray E, Hoffmann JN et al. Antithrombin: a new look at the actions of a serine protease inhibitor. Blood Coagul Fibrinolysis 2002; 13:657-670.
43. Mariathasan S, Monack DM. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nature Rev Immunol 2007; 7:31-40
44. Taniguchi S, Sagara J. Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. Semin Immunopathol 2007; 29:231-238.
45. Zhou Q, Snipas S, Orth K et al. Target proteases of the viral serpin CrmA: Analysis of five caspases. J Biol Chem 1997; 272:7797-7800.
46. Moffitt KL, Martin SL, Walker B. The emerging role of serine proteases in apoptosis. Biochem Soc Transactions 2007; 35:559-560.
47. Bird CH, Sutton VR, Sun J et al. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. Mol Cell Biol 1998; 18:6387-6398.
48. Medcalf RL, Stasinopoulos SJ. The undecided serpin: the ins and outs of plasminogen activator inhibitor Type 2. FEBS J 2005; 272:4858-4867.
49. Cinelli P, Maadani R, Tsuzuki N et al. Neuroserpin, a neuroprotective factor in focal ischemic stroke. Mol Cell Neurosci 2001; 18:443-457.
50. Khalkhali ellis Z. Maspin: The new frontier. Clin Cancer Res 2006; 12:7279-7283.
51. Kato K, Kishi T, Kamachi T et al. Serine proteinase inhibitor 3 and murinoglobulin I are potent inhibitors of neuroserpin in adult mouse brain. J Biol Chem 2001; 276:14562-14571.
52. Sun J, Ooms L, Bird CH et al. A new family of 10 murine ovalbumin serpins includes two homologs of proteinase inhibitor 8 and two homologs of the granzyme B inhibitor (proteinase inhibitor 9). J Biol Chem 1997; 272:15434-15441.
53. Charron Y, Madani R, Nef S et al. Expression of serpinb6 serpins in germ and somatic cells of mouse gonads. Mol Repro Develop 2006; 73:9-19.
54. Suzuki K, Hayashi T. Protein C and its inhibitor in malignancy. Sem Thromb Haem 2007; 33:667-672.
55. Miyamoto Y, Akaike T, Alam MS et al. Novel functions of human alpha(1)-protease inhibitor after S-nitrosylation: inhibition of cysteine protease and antibacterial activity. Biochem Biophys Res Commun 2000; 267:918-923.

56. Fenner F, Woodroffe GM. The pathogenesis of infectious myxomatosis: the mechanism of infection and the immunological response in the European rabbit (oryctolagus cuniculus). Br J Exp Pathol 1953; 34:400-411.
57. Upton C, Macen JL, Wishart DS et al. Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. Virology 1990; 179:618-631.
58. Macen JL, Upton C, Nation N et al. SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. Virology 1993; 195:348-363.
59. Xing Li, Schneider H, Peters A et al. Heparin binding alters viral serine protease inhibitor (serp-1) activity. Open Biochem J 2008; 2:6-15.
60. Nash P, Whitty A, Handwerker J et al. Inhibitory specificity of the anti-inflammatory myxoma virus serpin, serp-1. J Biol Chem 1998; 273:20982-20991.
61. Lucas A, Li X, Schneider H et al. Heparin alters viral serpin, serp-1, anti-thrombolytic activity to anti-thrombotic activity. Open Biochem J 2007; 2:6-15
62. Turner PC, Moyer RW. The cowpox virus fusion regulator proteins SPI3 and hemagglutinin interact in infected and uninfected cells. Virology 2006; 347:88-99.
63. Turner PC, Baquero MT, Yuan S et al. The cowpox virus serpin spi-3 complexes with and inhibits urokinase-type and tissue-type plasminogen activators and plasmin. Virology 2000; 272:267-280.
64. Lucas A, Liu LY, Macen J et al. Virus-encoded serine proteinase inhibitor serp-1 inhibits atherosclerotic plaque development after balloon angioplasty. Circulation 1996; 94:2890-2900.
65. Hatton MW, Ross B, Southward SM et al. Metabolism and distribution of the virus-encoded serine proteinase inhibitor serp-1 in healthy rabbits. Metabolism 2000; 49:1449-1452.
66. Lucas A, Dai E, Liu L et al. Transplant vasculopathy: Viral anti-inflammatory serpin regulation of atherogenesis. J Heart Lung Transplant 2000; 19:1029-1038.
67. Bot I, von der thusen JH, Donners MMPC et al. Serine protease inhibitor serp-1 strongly impairs atherosclerotic lesion formation and induces a stable plaque phenotype in ApoE-/- mice. Circulation 2003; 93:464-471.
68. Miller LW, Dai E, Nash P et al. Nation, Robert Zhong, Grant Mcfadden and Alexandra Lucas Inhibition of transplant vasculopathy in a rat aortic allograft model after infusion of anti-inflammatory viral serpin. Circulation 2000; 101:1598-1605.
69. Bedard ELR, Jiang J, Arp J et al. Prevention of chronic renal allograft rejection by serp-1 protein. Transplantation 2006; 81:908-914.
70. Hausen B, Boeke K, Berry GJ et al. Viral serine proteinase inhibitor (serp-1) effectively decreases the incidence of graft vasculopathy in heterotopic heart allografts. Transplantation 2001; 72:364-368
71. Jiang J, Arp J, Kubelik D et al. Induction of indefinite cardiac allograft survival correlates with toll-like receptor 2 and 4 downregulation after serine protease inhibitor-1 (serp-1) treatment. Transplantation 2007; 84:1158-1167.
72. Maksymowych WP, Nation N, Nash P et al. Amelioration of antigen induced arthritis in rabbits treated with a secreted viral serine proteinase inhibitor. J Rheumatol 1996; 23:878-882.
73. Brahn E, Do L, Lee S et al. Suppression of collagen-induced arthritis with a serin proteinase inhibitor cloned from a myxoma viral sequence. Arthritis and rheumatism 2000 annual scientific meeting, Philadelphia, PA. Abstract Supplement, American College of Rheumatology 2000; abstract no. 1000:43(9).
74. Richardson M, Liu L, Dunphy L et al. Viral serpin, serp-1, inhibits endogenous angiogenesis in the chicken chorioallantoic membrane model. Cardio Path 2007; 16:191-202.
75. Visinathan K, Liu L, Vaziri S et al. Myxoma viral serpin, serp-1, a unique interceptor of coagulation and innate immune pathways. Thromb Haemost 2006; 95:499-510.
76. MacNeill AL, Turner PC, Moyer RW. Mutation of the myxoma virus serp-2 P1-site to prevent proteinase inhibition causes apoptosis in cultured RK-13 cells and attenuates disease in rabbits, but mutation to alter specificity causes apoptosis without reducing virulence. Virology 2006; 356:12-22.
77. Turner PC, Sancho MC, Thoennes SR et al. Myxoma virus serp-2 is a weak inhibitor of granzyme B and interleukin-1beta-converting enzyme in vitro and unlike CrmA cannot block apoptosis in cowpox virus—infected cells. J Virol 1999; 73:6394-6404.
78. Messud Petit F, Gelfi J, Delverdier M et al. Serp-2, an inhibitor of the interleukin-1beta-converting enzyme, is critical in the pathophysiology of myxoma virus. J Virol 1998; 72:7830-7839.
79. Nathaniel R, MacNeill AL, Wang YX et al. Cowpox virus CrmA, myxoma virus serp-2 and baculovirus P35 are not functionally interchangeable caspase inhibitors in poxvirus infections. J Gen Virol 2004; 85:1267-1278.
80. Luttge BG, Moyer RW. Suppressors of a host range mutation in the rabbitpox virus serpin spi-1 map to proteins essential for DNA replication. J Virol 2005; 79:9168-9179.
81. Viswanathan K, Bot I. Liu L et al. Viral cross-class serpin inhibits apoptosis, inflammation and atherosclerosis. In: Modulation of Atherothrombotic Factors: Novel Strategies for Plaque Stabilization. Print Partners Ipstamp Enschede, The Netherlands 2005; pg 61-76.

82. Viswanathan K, Bot I, Liu L et al. Viral cross-class serpin inhibits apoptosis, inflammation and atherosclerosis. In modulation of atherothrombotic factors: Novel strategies for plaque stabilization. Print Partners Ipstamp Enschede The Netherlands 2005:61-76.
83. Abraham EG, Jacobs Lorena M. Mosquito midgut barriers to malaria parasite development. Insect Biochem Mol Biol 2004; 34:667-671.
84. Caljon G, Van Den Abbeele J, Stijlemans B et al. Tsetse fly saliva accelerates the onset of trypanosoma brucei infection in a mouse model associated with a reduced host inflammatory response. Infect Immun 2006; 74:6324-6330.
85. Danielli A, Kafatos FC, Loukeris TG. Cloning and characterization of four anopheles gambiae serpin isoforms, differentially induced in the midgut by plasmodium berghei invasion. J Biol Chem 2003; 278:4184-4193.
86. Danielli A, Barillas Mury C, Kumar S et al. Overexpression and altered nucleocytoplasmic distribution of anopheles ovalbumin-like SRPN10 serpins in plasmodium-infected midgut cells. Cell Microbiol 2005; 7:181-190.
87. Michel K, Budd A, Pinto S et al. Anopheles gambiae SRPN2 facilitates midgut invasion by the malaria parasite plasmodium berghei. EMBO Rep 2005; 6:891-897.
88. Michel K, Suwanchaichinda C, Morlais I et al. Increased melanizing activity in anopheles gambiae does not affect development of plasmodium falciparum. Proc Natl Acad Sci USA 2006; 103:16858-16863.
89. Abraham EG, Pinto SB, Ghosh A et al. An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. Proc Natl Acad Sci USA 2005; 102:16327-16332.
90. De Gregorio E, Spellman PT, Rubin GM et al. Genome-wide analysis of the drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci USA 2001; 98:12590-12595.
91. Perlmutter DH, Joslin G, Nelson P et al. Endocytosis and degradation of alpha 1-antitrypsin-protease complexes is mediated by the serpin-enzyme complex (SEC) receptor. J Biol Chem 1990; 265:16713-16716.
92. Pleass RJ, Kusel JR, Woof JM. Cleavage of human IgE mediated by schistosoma mansoni. Int Arch Allergy Immunol 2000; 121:194-204.
93. Zang X, Atmadja AK, Gray P et al. The serpin secreted by brugia malayi microfilariae, Bm-SPN-2, elicits strong, but short-lived, immune responses in mice and humans. J Immunol 2000; 165:5161-5169.
94. Manoury B, Gregory WF, Maizels RM et al. Bm-CPI-2, a cystatin homolog secreted by the filarial parasite brugia malayi, inhibits class II MHC-restricted antigen processing. Curr Biol 2001; 11:447-451.
95. Newlands GF, Skuce PJ, Knox DP et al. Cloning and expression of cystatin, a potent cysteine protease inhibitor from the gut of haemonchus contortus. Parasitology 2001; 122:371-378.
96. Peanasky RJ, Bentz Y, Paulson B et al. The isoinhibitors of chymotrypsin/elastase from ascaris lumbricoides: isolation by affinity chromatography and association with the enzymes. Arch Biochem Biophys 1984; 232:127-134.
97. Schonemeyer A, Lucius R, Sonnenburg B et al. Modulation of human T-cell responses and macrophage functions by onchocystatin, a secreted protein of the filarial nematode onchocerca volvulus. J Immunol 2001; 167(6):3207-3215.
98. Pfaff AW, Schulz Key H, Soboslay PT et al. Litomosoides sigmodontis cystatin acts as an immunomodulator during experimental filariasis. Int J Parasitol 2002; 32:171-178.
99. Dainichi T, Maekawa Y, Ishii K et al. Nippocystatin, a cysteine protease inhibitor from nippostrongylus brasiliensis, inhibits antigen processing and modulates antigen-specific immune response. Infect Immun 2001; 69:7380-7386.
100. Rhoads ML, Fetterer RH, Hill DE et al. Trichuris suis: a secretory chymotrypsin/elastase inhibitor with potential as an immunomodulator. Exp Parasitol 2000; 95:36-44.
101. Zang X, Maizels RM. Serine proteinase inhibitors from nematodes and the arms race between host and pathogen. Trends Biochem Sci 2001; 26:191-197.
102. Limo MK, Voigt WP, Tumbo Oeri AG et al. Purification and characterization of an anticoagulant from the salivary glands of the ixodid tick rhipicephalus appendiculatus. Exp Parasitol 1991; 72:418-429.
103. Joubert AM, Crause JC, Gaspar AR et al. Isolation and characterization of an anticoagulant present in the salivary glands of the bont-legged tick, hyalomma truncatum. Exp Appl Acarol 1995; 19:79-92.
104. Mulenga A, Sugimoto C, Ingram G et al. Characterization of two cDNAs encoding serine proteinases from the hard tick haemaphysalis longicornis. Insect Biochem Mol Biol 2001; 31:817-825.
105. Prevot PP, Adam B, Boudjeltia KZ et al. Anti-hemostatic effects of a serpin from the saliva of the tick ixodes ricinus. J Biol Chem 2006; 281(36):26361-26369.
106. Leboulle G, Crippa M, Decrem Y et al. Characterization of a novel salivary immunosuppressive protein from ixodes ricinus ticks. J Biol Chem 2002; 277:10083-10089.
107. Borges M, Da Silva AC, Sereno D et al. Peptide-based analysis of the amino acid sequence important to the immunoregulatory function of trypanosoma cruzi Tc52 virulence factor. Immunology 2003; 109:147-155.
108. Richardson J, Viswanathan K, Lucas A. Serpins, the vasculature and viral therapeutics. Front Biosci 2006; 11:1042-56.