Modulation of Atherothrombotic Factors: Novel Strategies for Plaque Stabilization

Bot, I.

3

Viral Cross-Class Serpin Inhibits Apoptosis, Inflammation and Atherosclerosis

Kasinath Viswanathan*, Ilze Bot, Liying Liu*, Erbin Dai*, Peter C. Turner[$], Jide Togonu-Bickersteth*, Ben Pang*, Yue Li*, Erik A.L. Biessen, Richard Moyer[$] and Alexandra Lucas*

Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands,
*John P. Robarts Research Institute, Vascular Biology Research Group, University of Western Ontario, London, Ontario, Canada,
[$]Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida, USA.

Manuscript in preparation

Abstract

Apoptosis can initiate innate immune responses in the artery, which could lead to atherosclerotic plaque progression and vascular occlusion, the cause for heart attacks and strokes. T lymphocytes secrete granzyme B and perforin, which induce intra-cellular caspase activity. Two viral intra-cellular serine protease inhibitors (serpins), Serp-2 and CrmA, have cysteine protease inhibitory activity targeting granzyme B and caspases. The capacity for cross-class serpins to bind granzyme B indicates a potential extracellular anti-apoptotic function. We have assessed the effects of Serp-2 and CrmA in animal models of atherosclerosis and in human endothelial, monocyte and T lymphocyte cells. Serp-2 markedly reduced inflammation and plaque growth, whereas CrmA and two reactive center mutants did not. Serp-2 bound the T cell surface membrane, blocking up-regulation of caspase activity and increasing expression of anti-apoptotic genes. This work defines previously unknown extra-cellular granzyme B- and perforin-dependent anti-inflammatory and anti-apoptotic activities for the intracellular myxoma virus cross-class serpin, Serp-2.

Introduction

The vasculature acts as the first line of defense in innate immune responses. Monocyte and T cell invasion from the circulating blood and endothelial cell dysfunction along the arterial lumen are closely associated with atherosclerotic plaque growth, scarring and vessel occlusion, leading causes for heart attack, stroke and sudden death[1-3]. Apoptosis, also termed programmed cell death, can lead to microparticle formation with increased expression of inflammatory mediators[3-7]. In endothelial cells, apoptosis leads to a prothrombotic, inflammatory state[3-7], while in monocytes and smooth muscle cells[3-7] apoptosis has been implicated in plaque rupture[3,4,7]. Plaque rupture exposes the collagenous, fatty plaque core causing clot formation and vascular occlusion. Increased numbers of cytotoxic, perforin-positive T lymphocytes are present in acute unstable coronary syndromes and in the accelerated vasculopathy of transplanted hearts[8-16]. Interference with T cell apoptosis in rats[5] leads to a tolerant state and granzyme B deficiency in mice[12] reduces transplant vasculopathy. Cellular apoptosis thus contributes to accelerated plaque development and progression to plaque rupture[3-14]. Two key pathways to cellular apoptosis are mediated by serine and cysteine proteases[3-7,17]. Caspases are cysteine proteases that initiate intracellular apoptotic pathways while granzyme B is a serine protease released by T cells into the surrounding medium, initiating extracellular apoptotic responses either via interaction with perforin or through cellular uptake via less well-defined pathways[3-14,17]. Poxviruses encode cross-class inhibitory serpins (*se*rine *p*roteinase *in*hibitors) that target granzyme B and caspases[17-25]. Serp-2 from Myxoma virus and CrmA (Cytokine response modifier A) from cowpox virus are two such viral cross class inhibitors. CrmA is a more potent inhibitor, binding caspase 1 (Interleukin-1β Converting Enzyme, ICE), caspase 8 and granzyme B, with greater inhibition of inflammation in chicken chorioallantoic membranes[20,24,25], whereas Serp-2 binds ICE and Granzyme B with lower affinity *in vitro*[18,19,21,24,25] and has greater effects on myxoma virulence in rabbits[21,25]. We present here a series of studies in rat and mouse models of accelerated atherosclerotic plaque growth[26-29] demonstrating significant differential effects of two intracellular viral cross class serpins, Serp-2 and CrmA, on apoptosis, mononuclear cell invasion and vasculopathy development, *in vivo*, and specific cellular anti-apoptotic activities, *in vitro*.

Methods

Animal Models

Effects of each serpin on cellular invasion and plaque growth were assessed in three animal models, the first being angioplasty injury in 250-300 g male Sprague Dawley (SD) rats[26-28] (Charles River, Wilmington, Mass USA).

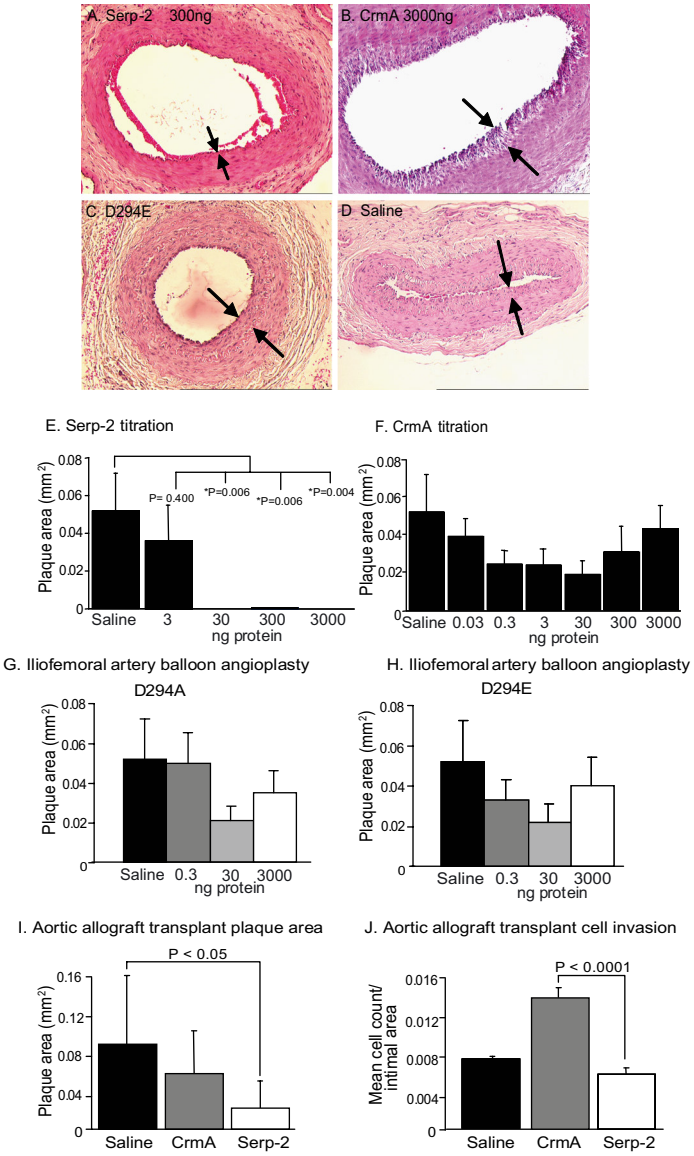

Figure 1. Hematoxylin and eosin stained sections of rat iliofemoral arterial sections at 4 weeks demonstrate reduced plaque growth with Serp-2 treatment at doses ≥ 30 ng (A, 300 ng Serp-2, P<0.006). CrmA at similar or higher doses (B, 3000 ng CrmA, P=0.612), the Serp-2 reactive center loop mutant D294E (C, 3000 ng D294E, P=0.567) and saline treatment (D) did not inhibit plaque growth. Arrows bracket intimal plaque limits. Magnification 50X. A clear dose response to Serp-2 treatment with reduced plaque growth at doses greater than 30 ng is seen in the titration curve (E). CrmA (F) and the two Serp-2 active center loop mutants, D294A (G) and D294E (H) did not display a reduction in plaque growth, although a non-significant decrease was seen at 0.3-30 ng (P=not significant at all concentrations). Reductions in plaque area were also detected in the ACI to Lewis aortic allograft transplant model with Serp-2 treatment, but not with CrmA (I) with associated inhibition of mean mononuclear cell counts per area (J).

The second model was the aortic allograft transplant from inbred 250-300 g ACI to Lewis rats[27,28] (Charles River Laboratories) and third, we used a model of spontaneous atherosclerosis in the aortic root and of collar-induced carotid artery atherosclerosis in 12-14 week old western type diet fed ApoE$^{-/-}$ mice[29] (obtained from the local animal facility, Gorlaeus Laboratories, Leiden, the Netherlands) with all surgeries performed as previously described[26-30]. All research protocols and general animal care were approved by University laboratory animal ethics and conformed to national guidelines. All surgeries were performed under general anesthetic, 6.5 mg per 100 g body weight Somnotrol (MTC Pharmaceuticals, Cambridge, Canada) intra-muscular injection for rats and subcutaneous 60 mg/kg ketamine (Eurovet Animal Health), 1.26 mg/kg Fentanyl, and 2.0 mg/kg fluanisone (Janssen Animal Health) for carotid collar placement in ApoE$^{-/-}$ mice[29]. Viral serpins were infused intravenously (i.v.) immediately after surgery in rats at doses of 0.3 ng–3000 ng (0.001-10 ng/g), with follow up at 4 weeks (Table 1). Daily subcutaneous injections of PBS, CrmA (240 ng/mouse/day, 12 ng/g/day) and Serp-2 (1800 ng/mouse/day, 90 ng/g/day) were started two weeks after collar placement in ApoE$^{-/-}$ mice and continued for 4 weeks until sacrifice. ^{125}I labelled CrmA and Serp-2 were injected on the first day and the last two days in two ApoE$^{-/-}$ mice detecting serum concentrations of 0.16 nM for CrmA and 1.72 nM for Serp-2. A separate group of 120 rats had angioplasty injury with 300 ng by i.v. injection of 300 ng of each serpin for early follow up at 0.12, or 72 hours to assess early apoptotic pathway enzyme activity (6 animals/treatment group). Body weight was measured weekly.

Table 1. Animal models

Strain	Number	Treatment
STUDY 1 - Rat Iliofemoral angioplasty - 28days		
SD	6	Saline
SD	6	Serp-2 3ng
SD	6	Serp-2 30ng
SD	6	Serp-2 300ng
SD	6	Serp-2 3000ng
SD	6	CrmA 0.3ng
SD	6	CrmA 0.03ng
SD	12	CrmA 3ng
SD	12	CrmA 30ng
SD	12	CrmA 300ng
SD	6	CrmA 3000ng
SD	6	D294A 0.3ng
SD	6	D294A 30ng
SD	6	D294A 3000ng
SD	6	D294E 0.3ng
SD	6	D294E 30ng
SD	6	D294E 3000ng
Total	**120**	

Strain	Number	Treatment
STUDY 2 – Rat Aortic transplant		
A/L	6	Saline
A/L	6	Serp-2 12ng
A/L	6	Serp-2 12µg
A/L	6	CrmA 12ng
A/L	6	CrmA12µg
A/L	6	D294A 12ng
A/L	6	D294A 12µg
A/L	6	D294E 12ng
A/L	6	294E 12µg
L/L	6	Saline
Total	**60**	
STUDY 3 – ApoE$^{-/-}$ Mice		
ApoEKO	11	saline
ApoEKO	11	Serp-2
ApoEKO	11	CrmA
Total	**33**	

* (L/L) Lewis to Lewis isograft, (A/L) ACI to Lewis allograft, SD – Sprague Dawley rat, NZW – New Zealand white rabbit

Histological, Morphometric and Fluorescence Analysis

At the designated study end, 4 weeks for plaque analysis and 0-72 hours for enzyme activity analysis, rats and mice were sacrificed with Euthanyl (Bimenda MTC animal Health company, Cambridge, Ontario, Canada). For rat models, arterial sections were fixed, processed, paraffin embedded, and cut into 5 µm sections (2-3 sections per site) for histological analysis. For the $ApoE^{-/-}$ mice, the aortic valve area (10 µm sections throughout the valve area) and the carotid artery from the bifurcation through the site of collar placement (5 µm sections at 25 µm intervals) were assessed. Sections were stained with hematoxylin/eosin, trichrome and Oil-red-O for analysis of plaque area, thickness and invading monocuclear cells as previously described[26-30].

For spectroscopic analysis of Serp-2 and CrmA binding to cells, $1*10^6$ cells/mL were treated with 1 µg/mL of FITC labeled protein for two hours, lysed with cell lysis buffer and fluorescence emission at 527 nm quantified during excitation at 485 nm. For fluorescence microscopy, cells were fixed with 2% formaldehyde, mounted with 10% glycerol mounting solution and viewed with a Zeiss fluorescence microscope. For FACS analysis, $1*10^6$ cells/mL were treated with 1 µg/mL of FITC labelled Serp-2 or CrmA for two hours and analyzed using FACS (fluorescence activated cell sorting, FACScalibur, BD Falcon), acquiring data for 20,000 events with three replicates (CellQuest data analysis program).

Cell culture

Human umbilical vein endothelial cells (HUVEC, CC-2519 Clonetics, Walkersville, MD, passages 2-5), THP-1 cells (American Type Culture Collection, Rockville, MD, USA, ATCC TIB-202), or Jurkat cells (E6.1 clone, ATCC TIB-152) ($0.5 – 1.0 *10^6$ cells/mL) were incubated with saline control, one of the apoptosis inducing agents (3 ng/mL membrane bound Fas ligand, 0.5 µM staurosporine, or 2-10 µM camptothecin) together with individual serpins (500 ng/mL). Medium was supplemented with 10% Fetal Bovine Serum (Invitrogen Canada Inc., Burlington, ON), Penicillin (100 units/mL) and Streptomycin (100 µg/ml, Gibco BRL).

Source and Purification of Serp-2, CrmA, D294A and D294E

All serpins were His-tagged at the amino-terminus, expressed in vaccinia/T7 vector in HeLa cells, and purified by immobilized metal affinity using His-Bind resin (Novagen)[25]. The D294A protein is a site-directed mutant of Serp-2 with P1 Asp 294 changed to Alanine to inactivate the serpin, while the D294E protein has P1 Asp 294 replaced by Glutamic acid to alter the inhibition spectrum[25]. Eluted proteins were judged ≥90% pure by SDS-12% PAGE, silver staining and immunoblotting. Serp-2 and CrmA were tested for ICE and granzyme B inhibitory activity, CrmA displaying greater (≥5-6 fold) caspase 1 inhibition than Serp-2 (data not shown), as previously reported[25].

Serp-2 and CrmA were labeled with Fluorescein Isothiocyanate (Fluorotag FITC conjugation kit, Sigma-Aldrich Canda Ltd., Mississauga, Ontario) and passed through streptavidin column to separate unbound FITC. The F/P (FITC/protein) ratio was 2.8 and 2.1, for Serp-2 and CrmA respectively. The caspase 1 inhibitory activity of FITC labeled proteins was assayed, displaying normal activity.

Enzyme activity and messenger RNA expression analyses

For whole arterial lysates, Serp-2, CrmA, 294A, or 294E treated rat femoral arteries (2-3 cm length) were excised at 0, 12 and 72 hours after angioplasty injury. The tissues were homogenized, lysed and extracted in PBS containing 1 mM EDTA buffer, centrifuged at 10,000 rpm for 10 min (8°C) to remove undissolved solids and supernatant stored at -80°C. For cell lysates, $1*10^6$ cells per mL (HUVEC, THP-1, or T-cells) were treated with saline, apoptosis inducers (2 µM camptothecin for THP-1 and 10 µM for T cells, 0.5 µM staurosporine from Sigma, Oakville, ON, Canada, or FasL (3 ng/mL) from Upstate solutions, Charlottesville, VA, U.S.A.), and each inducer in combination with either Serp-2, CrmA, 294A or 294E (500 ng/mL). Cells were collected at 6 hours, washed with cold PBS and treated with 60 µL lysis buffer (150 mM NaCl, 20 mM Tris base, 1% (v/v) Triton-X 100 at pH 7.2, for 10 min, 4°C) followed by centrifugation at 10,000 rpm for 10 min (8°C). Supernatant was collected and stored at -80°C until use. Protein concentration was measured (Bio-Rad Protein assay, Bio-Rad Laboratories, Hercules, CA, U.S.A.).

A subset of T cell cultures were treated with phorbol myristic acid (PMA, 1 µg/mL) and Ionophore A23187 (1 µg/mL)) to induce a cytotoxic T lymphocyte (CTL) state. Medium from treated T cell cultures containing granzyme B and perforin was removed after 2 hours incubation and applied to fresh, untreated T cell cultures together with Serp-2, CrmA, D294A or E with and without antibody to granzyme B or perforin (Sigma) for 12 or 24 hours.

For mRNA expression, total cellular RNA was extracted from cultured THP-1 and Jurkat T cells (treated for 6 hours with saline, 2 µM camptothecin or camptothecin with 500 ng/mL Serp-2 or CrmA) using TRIzol reagent (Invitrogen Canada Inc., Burlington, ON) and purified using the RNeasy kit (Qiagen Inc., Mississauga, ON). Semi-quantitative RT-PCR analysis was performed using Superscript one-step RT-PCR with platinum Taq (Invitrogen Canada Inc.), as previously described (primers listed in Table 2)[27] in an Eppendorf Scientific Inc. thermocycler (Westbury, NY). Bands run on 1.5% Agarose gel containing ethidium bromide were quantified (Molecular analyst program 2.1.2, Bio-Rad laboratories Canada, Ltd., Mississauga, ON). For the cell death ELISA assay, fragmented nucleosomes were determined using quantitative sandwich-enzyme immunoassay (Cell Death ELISA kit, Roche Diagnostics, Germany) with conjugated peroxidase measured photometrically at 405 nm with ABTS (2,2'-azino-di[3-ethylbenzthiazoline sulfonate) as substrate using a Multiscan Ascent Spectrophotometer

(Thermo labsystems Oy Inc., Beverly, MA, US).
For the DEVDase, 10 µL of the cell/tissue lysate was incubated at 37°C for one hour in 90 µL of reaction buffer (100 mM Acetyl-DEVD-AFC (Bachem, Torrance, CA, U.S.A.), 100 mM HEPES, 0.5 mM EDTA, 20% (v/v) Glycerol and 5 mM DTT, pH 7.5). for IEPDase, 90 µL of reaction buffer (100 mM Acetyl-IEPD-AFC (Kamia biomedical company, Seattle, WA), 50 mM HEPES, 0.05% (w/v) CHAPS, 10% (w/v) sucrose and 5 mM DTT, pH 7.5)[31]. For the cathepsin K, S, L, and V assays, 10 µL of the cell/tissue lysate was incubated in 90 µL of reaction buffer (5 mM Rhodamine 110, bisCBZ-L-Phenylalanyl-L-arginine amide, 50 mM Sodium acetate, 1 mM EDTA and 4 mM DTT, pH 5.5 (Bachem, Torrance, CA, U.S.A.). For DEVDase and IEPDase hydrolysed fluorochrome, 7-amino-4-trifluoromethyl Coumarin was measured using a Spectrofluorometer (Fluoroskan; excitation 405 nm, emission 527 nm) Thermolabsystems, Oy, (Thermolabsystems Inc., Beverly, MA, US). For the cathepsin assay, hydrolysed fluorochrome Rhodamine110 was measured using excitation 485 nm, emission at 527 nm). Final values were corrected for protein concentration.

Table 2. Primer sequences for RT-PCR analysis.

Genes	Primer sequence	Product length
GAPDH	5' –CCACCCATGGCAAATTCCATGGCA 3' –TCTAGACGGCAGGTCAGGTCCACC	598
Bcl-2	5' –CGACGACTTCTCCCGCCGCTACCGC 3' –CCGCATGCTGGGGCCGTACAGTTCC	373
NFkB	5' –TAACCAACCACCACCACCAC 3' –CAGGTCACTGGCATTCTTAG	396
cFLIP	5' –CAGAGTGAGGCGATTTGACC 3' –CTTGTCCCTGCTCCTTGAAC	363
Mcl-1	5' –TAACCAACCACCACCACCAC 3' –CAGGTCACTGGCATTCTTAG	394
XIAP	5' –CTTGCATACTGTCTTTCTGAGC 3' –ACACCATATACCCGAGGAAC	818
Bcl-XL	5' –CATGGCAGCAGTAAAGCAAG 3' –CTGCTGCATTGTTCCCATAG	356

Statistical Analysis
Significance was assessed by a 2-tailed Student's t-test or Mann-Whitney analysis[32]. Mean plaque area or cell count for experimental animals was used for all analyses[26-30]; P values ≤ 0.05 considered significant.

Results

Reduced plaque growth in rat vascular surgery models
Infusion of a single dose of Serp-2 immediately after angioplasty injury significantly reduced plaque growth (Figure 1A). Treatment with CrmA (Figure 1B) and the two active site mutations of Serp-2, D294A and D294E (Figure 1C), conversely, did not reduce plaque growth when compared to

saline control (Figure 1D) treatment. There was a clear dose-dependent, titrated response with reduced plaque at doses of 30 ng or higher of Serp-2 (P=0.006, Figure 1E). CrmA (Figure 1F), D294A (Figure 1G) and D294E (Figure 1H) produced non-significant trends toward plaque reduction at 0.3-30 ng (0.001 to 0.1 ng/g). Testing of increased numbers of animals (12 animals per dose tested) did not alter the overall significance of inhibitory effects of CrmA on plaque growth. Glutamic acid substitution at the P1 site in the reactive center loop (RCL, D294E) was predicted to increase inhibitory activity due to a preserved negative charge with P1 glutamic acid substitution. However, no anti-atherogenic activity was detected with either D294E or D294A where P1 is substituted by alanine.
The rat aortic allograft model exhibits an extensive inflammatory response with mononuclear cell invasion in intimal and adventitial arterial layers. The aortic transplant model was used as a rigorous test of anti-inflammatory activity. Serp-2 again reduced plaque growth in the aortic allograft transplant model (P<0.05), while CrmA did not (Figure 1I). Mononuclear cell invasion was reduced after Serp-2 treatment, when compared to CrmA treatment, in both intimal (P<0.0001) and adventitial layers in the rat aortic transplant (Figure 1J) and angioplasty (data not shown) models.

Reduced plaque development in Apolipoprotein E deficient mice
The effects of each cross class serpin on atherosclerotic lesion development was also independently assessed in Western type diet fed $ApoE^{-/-}$ mice, both at the aortic root and after collar placement at both carotid arteries. Serp-2 significantly reduced plaque area in the atherosclerotic aortic root (P=0.001, Figures 2A and D) and also tended to inhibit plaque development at sites of collar-induced carotid artery atherosclerosis (P<0.06, Figure 2E), 42% versus 44%, respectively, when compared to PBS control (Figures 2C, D and E). CrmA treatment had no effect on plaque development in $ApoE^{-/-}$ mice (Figure 2B, D and E). Lipid content of collar-induced atherosclerotic plaques was significantly reduced on Oil-Red-O stained sections with Serp-2 treatment (P=0.01, Figure 2F), while CrmA again had no effect when compared to PBS control infusions. Neither CrmA, nor Serp-2, infusion altered body weight or plasma triglyceride levels (data not shown), however cholesterol levels were slightly reduced at six weeks after collar placement (P=0.03, data not shown).

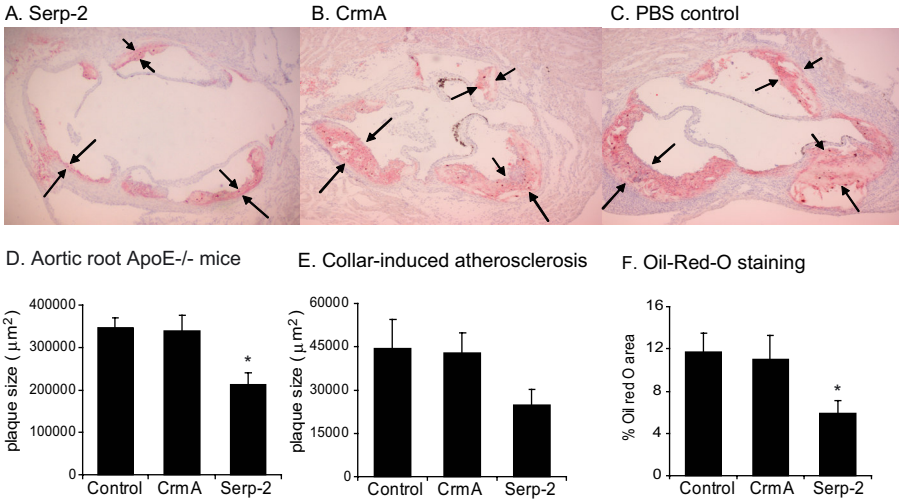

Figure 2. Cross sections of arteries from ApoE$^{-/-}$ mice taken at the aortic valve level (Oil-red-O staining) demonstrate a reduction in plaque area with Serp-2 (A), but not with CrmA treatment (B) when compared to PBS treated controls (C). Morphometric analysis of plaque area at the aortic root in ApoE$^{-/-}$ mice demonstrated that Serp-2 inhibited spontaneous aortic plaque development (P=0.001) (D) as well as collar-induced atherosclerotic plaque formation in this mouse model (P=0.06) (E). Oil-red-O staining confirmed a reduction in fatty plaque in the carotid artery plaques (P=0.01) (F), indicating a reduction in foam cell formation. Arrows indicate intimal plaque limits; Magnification - 50X. Error bars represent SEM.

Apoptotic responses with serpin treatment

At early times post angioplasty injury in rats, caspase 3, 7 and 8 and granzyme B enzyme activity were increased at 12 hours for whole arterial section extracts after saline treatment (P<0.0001). Treatment with individual serpins Serp-2, CrmA, D294A or D294E reduced caspase and granzyme B activity (P<0.017, P<0.0001, P<0.037 and P<0.008 for caspase 3/7 activity, respectively, data not shown) with no detectable significant differences between the proteins. Effects of Serp-2 and CrmA on apoptotic responses in individual cells, Jurkat T lymphocyte, THP-1 monocyte and HUVEC cultures were assessed. Apoptotic responses were induced through three differing pathways using Fas ligand (extrinsic pathway), staurosporine (intrinsic pathway) and camptothecin (topoisomerase). In T cells, caspase 3 and 7 activity (as measured by DEVDase assay) were significantly increased after treatment with camptothecin (P<0.001, Figure 3A) and staurosporine (P<0.0001, Figure 3B). Granzyme B and caspase 8 activity (as measured by IEPDase assay) were significantly increased by staurosporine and by Fas ligand (P<0.0001, data not shown). Serp-2 reduced the levels of caspases 3 and 7 in T cells after camptothecin (P<0.013, Figure 3A) and after staurosporine induced apoptosis (P<0.033, Figure 3B), while CrmA did not significantly block activation of these apoptotic pathways in T cells. Serp-2 however, did not significantly alter granzyme B and caspase 8 activity in T cells after inducing apoptosis with camptothecin, staurosporine or Fas ligand

(data not shown). Serp-2 also reduced apoptosis measured by cell death ELISA assay in T cells after camptothecin treatment (data not shown). In THP-1 monocytes, camptothecin (Figure 3C, P<0.001) and staurosporine (P<0.0009, data not shown) both significantly increased caspase 3 and 7 activity, but had little effect on caspase 8 and granzyme B activity (not shown).

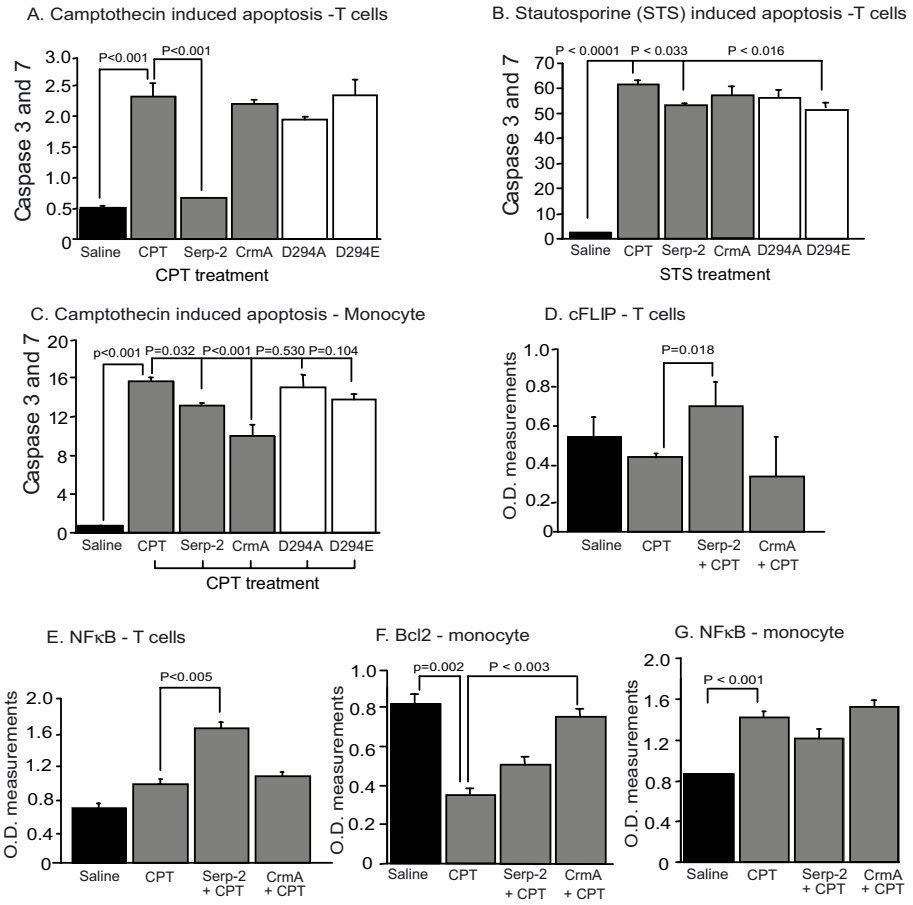

Figure 3. Serp-2, but not CrmA nor D294A (and D294E) treatment of Jurkat T cells reduced caspase 3 and 7 activity (DEVDase) after camptothecin (A) or staurosporine (B) apoptosis actuator treatment. Serp-2 and CrmA both significantly reduced camptothecin induced elevations in caspase 3 and 7 activity in monocytes (C). Semi-quantitative RT-PCR analysis demonstrated increased cFLIP (D) and NFκB (E) gene expression in Serp-2 treated Jurkat T cells with camptothecin induced apoptosis, but CrmA had no effect. In THP-1 monocytes, camptothecin treatment significantly reduced Bcl2 (F) and increased NFκB (G) mRNA expression. CrmA and Serp-2 both significantly increased Bcl2 levels (F) whereas neither CrmA nor Serp-2 significantly altered NFκB levels in THP-1 cells (G).

In monocytes, caspase 3 and 7 activity was significantly reduced by both CrmA ($P<0.0001$) and Serp-2 ($P<0.035$) after camptothecin treatment (Figure 3C); CrmA producing greater inhibition. Neither Serp-2 nor CrmA reduced caspase activity in THP-1 after staurosporine treatment (data not shown).
The Serp-2 RCL mutants, D294A and E, did not alter caspase activity in T cells after camptothecin treatment (Figure 3A, $P=0.11$), but D294E (not D294A) did reduce caspase 3,7,8 and granzyme B activity after staurosporine treatment ($P<0.016$, Figure 3B), indicating that this RCL mutant retained some anti-apoptotic activity that differed from Serp-2. The RCL mutants, D294A and D294E had no inhibitory activity when tested in THP-1 monocytes after camptothecin or staurosporine treatment (Figure 3C, $P=0.104$).
In HUVEC cultures, serum deprivation, staurosporine and camptothecin all increased caspase 3, 7, 8 and granzyme B activity ($P\leq0.0012$, data not shown). Serp-2 and CrmA both significantly reduced caspase 8 and granzyme B activity after treatment with camptothecin ($P<0.0001$) in HUVEC, but had no effect on caspase 3 and 7 activity (data not shown). Staurosprine induced apoptosis was not altered by Serp-2, CrmA, D294A or D294E in HUVEC (data not shown). In all cell lines tested after Fas ligand treatment CrmA, Serp-2 and D294A had no inhibitory activity (data not shown). The Serp-2 RCL mutant, D294E did, however, reduce caspase 3, 7, 8 and granzyme B activity after Fas ligand treatment of T cells ($P<0.0005$, data not shown). Cathepsins K, S, L and V activity in T cells was not affected by Serp-2, CrmA, D294A or E treatment ($P = 0.386$, data not shown).

Differential effects of Serp-2 and CrmA on gene expression
Based upon the differential effects of Serp-2 and CrmA on human T cell apoptotic responses, expression of genes that regulate apoptosis was measured in camptothecin treated T lymphocyte and monocytic cell lines. Camptothecin significantly reduced Bcl2 gene expression, but had no significant effect on cFLIP, NFκB and Mcl1 expression (Figures 3D and E). In camptothecin treated T cells Serp-2 increased cFLIP (Figure 3E, $P<0.018$) and NFκB (Figure 3F, $P<0.005$) expression while CrmA had no effect on cFLIP or NFκB (Figures 3D, E). Expression of Mc11, Bcl2, Bcl-XL and XIAP (data not shown) were not altered in T cells with Serp-2 or CrmA treatment (data not shown). Camptothecin markedly reduced the expression of Bcl2 ($P<0.002$, Figure 3F) and increased expression of NFκB ($P<0.001$, Figure 3G) in THP-1 monocytes. In THP-1 cells, CrmA significantly increased Bcl2 gene expression ($P<0.003$, Figure 3F), whereas Serp-2 produced a borderline increase ($P=0.061$). Neither CrmA nor Serp-2 significantly altered expression of NFκB in camptothecin treated THP-1 cells, although Serp-2 caused a non-significant decrease (Figure 3G).

Differential Effects of Serp-2 and CrmA on activated T cell responses

Treatment of Jurkat T cells in culture with phorbol myristic acid (PMA) and ionophore induces an activated, cytotoxic T lymphocyte (CTL) state with release of granzyme B and perforin. Naïve T cell cultures were treated with medium from PMA and ionophore treated T cells, inducing a significant increase in caspase 3 and 7 activity (P<0.0001, DEVDase assay, Figure 4A) and caspase 8 and granzyme B activity (P<0.0001, IEPDase activity, Figure 4B)[30]. Serp-2 reduced CTL mediated increases in caspase 3/7 (P=0.064) and caspase 8/granzyme B (P=0.0009, Figures 4A and B) activity.

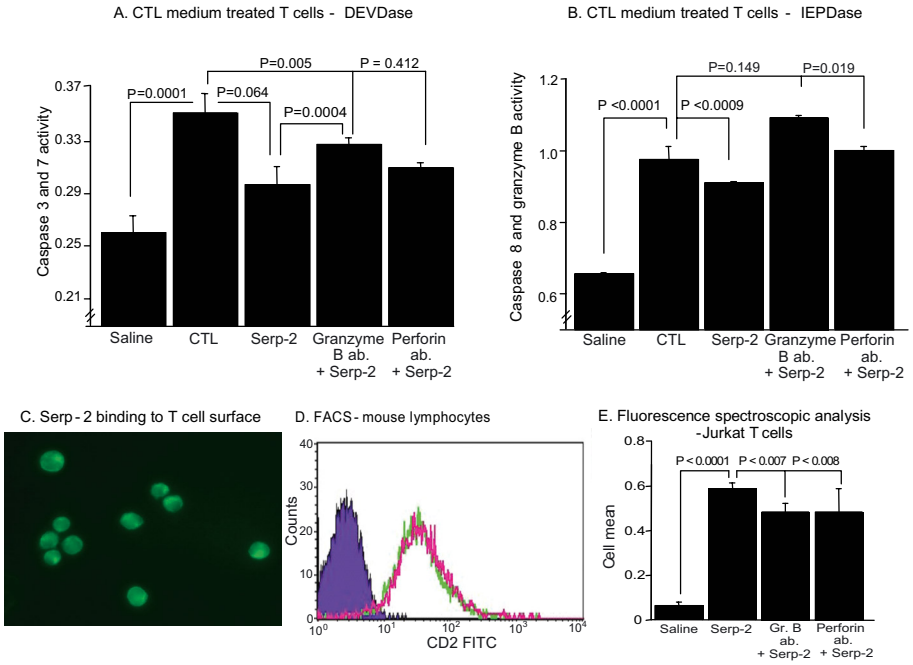

Figure 4. Medium from PMA and ionophore treated Jurkat T cell cultures with increased granzyme B and perforin when applied to naïve T cells in culture increased caspase 3 and 7 (A, DEVDase) and caspase 8 and granzyme B (B, IEPDase) activity. Treatment with Serp-2 reduced both caspase 3/7 (A) and caspase 8/granzyme B (B) activity. This inhibition was blocked by incubation of cells with antibody to granzyme B and perforin. Fluorescence microscopy (C, Magnification 65X) and FACS analysis (D) demonstrated that FITC labeled Serp-2 bound to T cells. Treatment with antibody to granzyme B or perforin partially blocked Serp-2 binding (E, P<0.007 and P<0.008 respectively).

Concomitant treatment with antibody to granzyme B and perforin blocked Serp-2 inhibition of caspase 8/granzyme B (P=0.0009) and caspase 3/7 (P=0.0004) activity (Figures 4A and B) at 24 hours, but not at 12 hours. FITC labelled Serp-2 (Figure 4C) and CrmA (not shown) bound to T cells in culture (fluorescence emission microscopic examination). Binding of Serp-2 and CrmA was further confirmed by FACS analysis of mouse peritoneal

lymphocytes (Figures 4D) and Jurkat T cells (not shown) and as well as spectroscopic analysis (Figure 4E) indicating that cross class viral serpins have the capacity to bind the T cell plasma membrane. Fluorescence emission from FITC labeled Serp-2 associated with Jurkat cells was reduced after treatment with antibody to granzyme B ($P<0.007$) and perforin ($P<0.008$, Figure 4E), further supporting an association between Serp-2 mediated inhibition of T cell apoptotic responses and granzyme B.

Discussion

Infusion of Serp-2, an intracellular viral cross class serine and cysteine protease inhibitor, effectively inhibited atherosclerotic plaque growth in a wide range of animal models, markedly reducing plaque growth at sites of vascular surgery in rats and significantly reducing both spontaneous diet-induced atherosclerosis in the aortic root as well as collar-induced plaque development in the carotid arteries of $ApoE^{-/-}$ mice. CrmA and two RCL mutants of Serp-2 did not effectively block plaque growth, indicating the specificity of Serp-2 anti-atherogenic activity. Serp-2 bound to the T cell surface, also selectively inhibited caspase 3 and 7 activity in Jurkat T cells with associated increase in cFLIP and NFκB gene expression, Serp-2 binding and inhibitory activity was blocked by antibodies to granzyme B and perforin. We have postulated that Serp-2 selectively binds to and inhibits T cell apoptosis, effecting a generalized reduction in arterial inflammation through a granzyme B/perforin dependent pathway.

The detectable capacity of Serp-2 to block camptothecin and staurosporine induced apoptosis in T cells, suggests a central role for T cells in Serp-2 mediated anti-inflammatory and anti-atherogenic actions. Serp-2 mediated inhibition of T cell apoptosis may prolong T cell function, allowing subsets of T cells to provide anti-inflammatory actions or may initiate apoptotic responses in macrophages and smooth muscle cells[3-17]. Serp-2 also inhibited camptothecin induced apoptosis in monocytes, but to a lesser extent than CrmA. Thus Serp-2 mediated inhibition of camptothecin induced apoptosis in both T cells and monocytes may further reduce inflammation. The lack of differential functions for Serp-2 and CrmA in monocytes further suggests that blockade of monocyte/macrophage apoptosis is not sufficient to alter vascular inflammation and plaque growth. A generalized non-specific reduction in apoptotic responses in the vessel wall was detected with all serpins tested, however, whole arterial extracts represent an average of all cells in the arterial wall and are thus not representative of individual cellular responses as evidenced by the studies in cell cultures (Figures 3, 4).

Camptothecin binds to topoisomerase I, an enzyme class that alters DNA topography, and interferes with DNA re-ligation[33], creating persistent DNA breaks. Resistance to camptothecin activity has been linked to topoisomerase mutations and to altered expression of p53, Bax and Bcl2. Inhibition of topoisomerase by camptothecin also leads to caspase activation

while caspase 3 can cleave topoisomerase providing feedback control[31]. Inhibition of camptothecin-induced apoptosis implies that Serp-2 blocks initiation of apoptosis in T lymphocytes at a very basic level, potentially through altered expression of cFLIP, NFκB, Bcl-2 or caspase activity.
Unexpected dual functions for intracellular proteins displaying extracellular actions have been previously reported for numerous mammalian and viral proteins including calreticulin with calcium binding, chaperone, anti-thrombotic and anti-atherogenic activities[34], cathepsins, chymases and granzymes that alter apoptosis and cellular adhesion[35], histatidyl tRNA synthetase interaction with CCR5 chemokine receptor[36] and myxoma viral, M-T7, with interferon γ and chemokine binding activity[26,28]. Given the economy and potency of function of these viral anti-inflammatory proteins, a secondary extra-cellular function for these intracellular viral cross-class serpins is proposed after release from infected cells. The inhibitory effects of Serp-2 on generalized atherosclerotic plaque growth in the aorta of hyperlipidemic ApoE$^{-/-}$ mice and at sites of collar-induced atherosclerosis indicate potentially broader applications for Serp-2 in treatment of vascular disease.

Acknowledgments

We would like to thank Joan Fleming for her help with typing and collating this manuscript and Dr. Grant McFadden for many thoughtful discussions. This work is funded by research grants from the Canadian Institutes of Health research (CIHR) and the Heart and Stroke foundation of Ontario (HSFO).

References

1. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 2005;352:1685-1695.
2. Hansson GK, Libby P, Schonbeck U, Yan Z-Q. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res.* 91, 281-291 (2002).
3. Littlewood TD, Bennett MR. Apoptotic cell death in atherosclerosis. *Curr Opin Lipidol.* 2003;14:469-475.
4. Rossig L, Dimmeler S, Zeiher AM. Apoptosis in the vascular wall and atherosclerosis. *Bas Res Cardiol.* 2001;96:11-22.
5. Akyurek LM, Johnsson C, Lange D, Georgii-Hemming P, Larsson E, Fellstrom BC, Funa K, Tufveson G. Tolerance induction ameliorates allograft vasculopathy in rat aortic transplants. Influence of fas-mediated apoptosis. *J Clin Invest.* 1998;101:2889-2899.
6. Stoneman VEA, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. *Clin Sci.* 2004,107:343-354.
7. Geng Y-G, Libby P. Progression of Atheroma: A struggle between death and procreation. *Arterioscler Thromb Vasc Biol.* 2002;22:1370-1380.
8. Nakajima T, Goek O, Zhang X, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. De novo expression of killer immunoglobulin-like receptors and signaling proteins regulates the cytotoxic function of CD4 T cells in acute coronary syndromes. *Circ Res.* 2003;93,106-113.
9. Fox WM 3rd, Hameed A, Hutchins GM, Reitz BA, Baumgartner WA, Beschorner WE, Hruban RH. Perforin expression localizing cytotoxic lymphocytes in the intimas of coronary arteries with

transplant – related accelerated arteriosclerosis. *Hum Pathol.* 1993;24,477-482.
10. Hruban RH, Beschorner WE, Baumgartner WA, Augustine SM, Ren H, Reitz BA, Hutchins GM. Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. *Am J Pathol.* 1990;137,871-882.
11. Nakajima T, Schulte S, Warrington KJ, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. T-cell –mediated lysis of endothelial cells in acute coronary syndromes. *Circulation.* 2002;105, 570-575.
12. Choy JC, Cruz RP, Kerjner A, Geisbrecht J, Sawchuk T, Fraser SA, Hudig D, Bleackley RC, Jirik FR, McManus BM, Granville DJ. Granzyme B induces endothelial cell apoptosis and contributes to the development of transplant vascular disease. *Am J Transplant.* 2005;5,494-499.
13. Littlewood TD, Bennett MR. Apoptotic cell death in atherosclerosis. *Curr Opin Lipidol.* 2003;14,469-475.
14. Alcouffe J, Therville N, Segui B, Nazzal D, Blaes N, Salvayre R, Thomsen M, Benoist H. Expression of membrane-bound and soluble FasL in Fas- and FADD-dependent T lymphocyte apoptosis induced by mildly oxidized LDL. *FASEB J.* 2004;18,122-124.
15. Buffon A, Biasucci LM, Liuzzo G, D'Onofrio G, Crea F, Maseri A. Widespread coronary inflammation in unstable angina. *N Engl J Med.* 2002;347,5-12.
16. Viles-Gonzales JF, Fuster V, Badimon JJ. Atherothrombosis: A widespread disease with unpredictable and life-threatening consequences. *Eur Heart J.* 2004;25,1197-1207.
17. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nature Rev.*2002; 2,401-409.
18. Turner PC, Moyer RW. Serpins enable poxviruses to evade immune defenses. *Am Soc Microbiol News.* 2001;67,201-209.
19. McFadden G, Barry M. How poxviruses oppose apoptosis. *Semin Virol.* 1998;8,429-448.
20. Komiyama T, Ray CA, Pickup DJ, Howard AD, Thornberry NA, Peterson EP, Salvesen G. Inhibition of interleukin-1-beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J Biol Chem.* 1994;269,19331-19337.
21. Messud-Petit F, Gelfi J, Delverdier M, Amardeilh MF, Py R, Sutter G, Bertagnoli S. Serp2, an inhibitor of the interleukin-1β-converting enzyme, is critical in the pathobiology of myxomavirus. *J Virol.* 1998;72,7830-7839.
22. Roy M, Sapolsky RM. The neuroprotective effects of virally-derived caspase inhibitors p35 and crmA following a necrotic insult. *Neurobiol Dis.* 2003;14,1-9.
23. Fujino M, Kawasaki M, Funeshima N, Kitazawa Y, Kosuga M, Okabe K, Hashimoto M, Yaginuma H, Mikoshiba K, Okuyama T, Suzuki S, Li XK. CrmA gene expression protects mice against concanavalin-A-induced hepatitis by inhibiting IL-18 secretion and hepatocyte apoptosis. *Gene Ther.* 2003;10,1781-1790.
24. Turner PC, Sancho MC, Thoennes SR, Caputo A, Bleackley RC, Moyer RW. Myxoma virus Serp2 is a weak inhibitor of granzyme B and interleukin-1β-converting enzyme in vitro and unlike crmA cannot block apoptosis in cowpox virus-infected cells. *J Virol.* 1999;73,6394-6404.
25. Nathaniel R, MacNeill AL, Wang YX, Turner PC, Moyer RW. Cowpox virus CrmA, Myxoma virus SERP2 and baculovirus P35 are not functionally interchangeable caspase inhibitors in poxvirus infections. *J Gen Virol.* 2004;85,1267-1278.
26. Liu L, Lalani A, Dai E, Seet B, Macauley C, Singh R, Fan L, McFadden G, Lucas A.. A viral anti-inflammatory chemokine binding protein, M-T7, reduces intimal hyperplasia after vascular injury. *J Clin Invest.* 2000;105,1613-1621.
27. Dai E, Guan H, Liu L, Little S, McFadden G, Vaziri S, Cao H, Ivanova IA, Bocksch L, Lucas A. Serp-1, a viral anti-inflammatory Serpin, regulates cellular serine proteinase and serpin responses to vascular injury. *J Biol Chem.* 2003;278,18563-18572.
28. Liu L, Dai E, Miller L, Seet B, Lalani A, Macauley C, Li X, Virgin HW, Bunce C, Turner P, Moyer R, McFadden G, Lucas A. Viral chemokine binding proteins inhibit inflammatory responses and aortic allograft transplant vasculopathy in rat models. *Transplantation.* 2004;77,1652-1660.
29. von der Thüsen JH, van Berkel TJC, Biessen EAL. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation.* 2002;103:1164-1170.
30. Bot I, von der Thusen JH, Donners MM, Lucas A, Fekkes ML, de Jager SC, Kuiper J, Daemen MJ, van Berkel TJ, Heeneman S, Biessen EA. Serine protease inhibitor Serp-1

strongly impairs atherosclerotic lesion formation and induces a stable plaque phenotype in ApoE$^{-/-}$ mice. *Circ Res.* 2003;93:464-471.
31. Korkmaz B, Attucci S, Hazouard E, Ferrandiere M, Jourdan ML, Brillard-Bourdet M, Juliano L, Gauthier F. Discriminating between the activities of human neutrophil elastase and proteinase 3 using Serpin-derived fluorogenic substrates. *J Biol Chem.* 2002;277,39074-39081.
32. Johnson RA, Bhattacharyya GK. Statistics: Principles and Methods, ed. John Wiley, New York. Chapter 14, 2001.
31. Rasheed ZA, Rubin EH. Mechanism of resistance to topoisomerase I – targeting drugs. *Oncogene.* 2003;22,7296-7304.
33. Dai E, Stewart M, Ritchie B, Mesaeli N, Raha S, Kolodziejczyk D, Hobman ML, Liu LY, Etches W, Nation N, Michalak M, Lucas A. Calreticulin, a potential vascular regulatory protein, reduces intimal hyperplasia after arterial injury. *Arteroscler Thromb Vasc Biol.* 1997;17:2359-2368.
34. Michel J-B. Anoikis in the cardiovascular system: Known and unknown extracellular mediators. *Arterioscle. Thromb Vasc Biol.* 2003;23,2146-2154.
35. Howard OM, Dong HF, Yang D, Raben N, Nagaraju K, Rosen A, Casciola-Rosen L, Hartlein M, Kron M, Yang D, Yiadom K, Dwivedi S, Plotz PH, 36. Oppenheim JJ. Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells. *J Exp Med.* 2002;196,781-791.