Inhibition of Coagulation Activation and Inflammation by a Novel Factor Xa Inhibitor Synthesized from the Earthworm *Eisenia andrei*

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We have cloned an earthworm-derived Factor Xa (FXa) inhibitor, with an excellent inhibitory specificity from the midgut of the *Eisenia andrei*. We designate this inhibitor eisenstasin. An eisenstasin-derived small peptide (ESP) was synthesized and we examined whether ESP played an essential role in FXa inhibition. Compared to antistasin-derived small peptides (ASP) originating from leech, ESP primarily exhibited a high level of FXa inhibition in chromogenic peptide substrate assays and revealed an approximately 2-fold greater inhibition of FXa cleavage of a target protein than ASP. This suggests that ESP could be an effective anti-coagulant that targets FXa during the propagation step of coagulation. ESP also inhibited proteinase-activated receptor 2-mediated FXa activation, which may trigger endothelial inflammation. Endothelial nitric oxide (NO) was significantly reduced by ESP ($p<0.0001$), indicating that protease-activated receptor-2 (PAR-2) was effectively inactivated. We also found that ESP reduced the expressions of pro-inflammatory cytokines (IL-1α, IL-1β, IL-8, IL-16, MCP-1, MIP-1α and MIP-1β) by cultured cells treated with both ESP and FXa. Our results provide the first evidence that ESP might interrupt coagulation cascades by inhibiting FXa, and thereby may effectively control the bidirectional alternation between coagulation and inflammation.

Key words factor Xa; antistasin; eisenstasin; nitric oxide; inflammation; proteinase-activated receptor

Proteases from metazoan species play prominent roles in physiologically important processes, such as blood clotting, tissue reorganization and immune responses. These processes can be induced by proteolytic cascades that lead to very rapid and irreversible cellular responses. Bloodsucking animals have evolved mechanisms to interfere with proteases involved in the coagulation of a blood donor. For instance, leeches are parasites that derive their nutrition from blood. They produce potent inhibitors that interfere with proteases in the blood of their hosts.[1] In invertebrates, serine proteases and their inhibitors are involved in phagocytosis, coagulation, complement activation and fibrinolysis as seen in vertebrates.[2]

Factor Xa (FXa) is a critical serine protease situated at the confluence of the intrinsic and extrinsic pathways of the blood coagulation cascade; it catalyses the conversion of prothrombin to thrombin *via* the prothrombinase complex.[3] As serine protease activities are amplified at each step of the coagulation cascade, anti-coagulants, like FXa inhibitors, that target coagulation factors located earlier in the cascade might be more effective than those that directly target thrombin. In addition, several lines of evidence suggest that FXa also elicits other cellular responses, such as cytokine release, expression of adhesion molecules, expression of tissue factor genes and cell proliferation.[4—7]

The first FXa inhibitor (15 kDa) was isolated from the salivary glands of the Mexican leech *Haementeria officinalis*. This inhibitor was designated antistasin and has been discussed as a potential target for the design of new anti-thrombotics.[8,9] Several antistasin proteins from hematophagous animals have been identified and comprehensive characterization of their amino acid sequences has shown that they contain repeated cysteine-rich peptides each consisting of 6 conserved cysteine and 2 conserved glycine residues.[10] However, the number of internal repeats depends upon the species, and these internal repeats may play an important role in the inhibition specificity of the antistasin protease inhibitors against FXa.

In addition to its coagulation effects, FXa mediates a variety of other biological effects, such as stimulation of lymphocyte proliferation and induction of mitogenesis of endothelial cells.[11,12] Importantly, the mechanism by which coagulation proteases influence inflammation is by binding to protease activated receptors (PARs), of which 4 types (PAR-1 to -4) have been identified. These belong to the family of transmembrane, G-protein-coupled receptors. Unlike PARs 1, 3 and 4, PAR-2 cannot bind thrombin. Rather, it can be activated by FXa.[13] In particular, the endogenous protease, FXa, can activate endothelial PAR-2, which triggers formation of endothelial nitric oxide (NO).[14]

Thus, modulation of coagulation and inflammation may be beneficial in various situations in which these two processes appear in disease pathogenesis. Because small direct and indirect FXa inhibitors could inhibit further thrombus propagation, small peptides and the small molecule Xa inhibitors would be applicable in therapeutic approaches.[15,16] In particular, recombinant antistasin has shown equal or superior efficacy to that achieved by heparin for the inhibition of thrombin.[17,18] Despite this clinical potency as an FXa inhibitor, its strong immunogenicity has been a fatal defect. An alternative approach introduced the most potent synthetic peptide derived from antistasin. The shortest antistasin peptide (D-RCRVHCP) displaying anti-coagulant activity increased clotting times by 50% at micromolar concentrations.[19]

Recently, we cloned a new antistasin gene (eisenstasin) from the earthworm *Eisenia andrei*, a non-hematophagous

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animal, and deposited the mRNA sequences into GenBank (AY913947). In an *E. andrei* midgut cDNA library, we found 2 contigs that had similarities to leech antistasin. The 2 contigs exhibited possible N-X-S/T glycosylation sites and included 3 to 4 internal cysteine repeats. Moreover, the internal repeats of eisenstasin showed the conserved pattern of 6 cysteines and 2 glycines at the same positions, between the third and fourth cysteine residues, as in leech antistasin, suggesting that eisenstasin is a member of the antistasin family.

To detect other selective peptides, we synthesized several small peptides and selected the most promising peptides (RCRFGYR) from the sequences of the cloned eisenstasin gene on the basis of previous reports for synthesized peptides.[19] In this study, we examined whether small synthetic eisenstasin peptides produced more potent pharmacological benefits than those of antistasin, primarily through FXa inhibition and secondarily *via* inhibition of pro-inflammatory responses.

MATERIALS AND METHODS

Synthesis of Peptides Antistasin- or eisenstasin-derived small peptides (ASP, ESP) were synthesized by solid-phase technology (Peptron, Daejeon, Korea) (Table 1). The purity (>98%) of the synthetic peptides was assessed by reverse-phase high-performance liquid chromatography (HPLC), amino acid analyses and matrix-assisted laser desorption/ionization time-of-flight (TOF) mass spectrometry (Kratos, Manchester, U.K.).

Cell Culture The human umbilical vein endothelial cell (HUVEC) line, HUVE-12, and Ham's F-12K medium were obtained from the American Type Culture Collection (ATCC, VA, U.S.A.). HUVE-12 cells obtained at passage 13 were cultured in T-75 tissue culture flasks in Ham's F-12 K medium (Kaighn's modification of Ham's F-12 containing 2 mM L-glutamine and 1500 mg/l sodium bicarbonate) supplemented with 10% (v/v) fetal bovine serum (Cellgro, VA, U.S.A.), 1% penicillin–streptomycin–glutamine, 0.1 mg/ml heparin (Sigma, MO, U.S.A.) and 0.05 mg/ml endothelial cell growth supplement (ECGS) (BD Biosciences, MA, U.S.A.). The cells were passaged with split ratios of 1 : 2 or 1 : 3. For all experiments, the cells were grown to greater than 90% confluency and subjected to no more than 20 cell passages.

FXa Enzyme Assay A single-stage chromogenic assay was used to characterize the inhibitory activities of ASP and ESP. All procedures were carried out at room temperature in 96-well microtiter plates following methods described previously, and the concentration for FXa inhibition (5 μg/ml) was also adopted from previously reported studies for antistasin.[20,24] The human enzyme reaction was initiated either by addition of substrate (S-2222, Chromogenix, Milano, Italy) to FXa protein (Novagen, WI, U.S.A.) or to mixtures containing pre-incubated FXa-ASP or -ESP, and the color that developed from the release of *p*-nitroanilide from the chromogenic substrate molecules was monitored continuously for 5 min at 405 nm on a Spectra Max Plus340 (Molecular Devices, CA, U.S.A.). The progress curves were recorded (% value=[1−(ΔOD_{405} of each sample/ΔOD_{405} of control)]×100).

Factor Xa Cleavage Assay FXa preferentially cleaves at the C-terminal side of its recognition sequence (IleGluGlyArg↓).[21] This sequence can, therefore, be used to detect inhibitory effects of small peptides. In order to detect cleavage inhibition in the context of a native protein, 3 μg of a FXa cleavage control protein (Novagen) containing the FXa recognition site was treated with 5 μg/ml of FXa (Novagen) in the presence or absence of ASP or ESP. All cleavage reactions were conducted at room temperature for 3 h in FXa cleavage buffer (100 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 5 mM $CaCl_2$) and supernatants were collected to monitor cleavage of the control protein using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 0.01% Coomassie Blue staining. The FXa cleavage control protein is converted from a single 49 kDa band to two bands of 32 kDa and 17 kDa following FXa cleavage. Staining intensity was corrected for background and normalized to the total corrected staining intensity for each lane. Results are presented as the fractional intensity of each fragment.

Table 1. Synthetic Peptides

Peptide name	Amino acid sequence	Species
ASP	NH_2-RCRVHCP-OH	*Hementeria officinalis* (leech)
ESP	NH_2-RCRFGYR-OH	*Eisenia andrei* (earthworm)

Cytotoxicity Assay The cytotoxicity induced by ESP and ASP in HUVE-12 cells was evaluated by measurements of lactate dehydrogenase (LDH) release into the culture medium. Following 24 h exposure to both peptides at varying concentrations (0.001 to 100 μg/ml), the culture medium was harvested and centrifuged for 10 min at 3000 rpm to generate cell-free supernatants. The LDH activity in the medium was determined using a commercial non-radioactive LDH assay kit, CytoTox 96® (Promega, WI, U.S.A.). LDH is a stable, cytoplasmic enzyme that is present in all cells, and is rapidly released upon plasma membrane damage. LDH activity was evaluated *via* a coupled enzymatic reaction, in which tetrazolium salt was reduced to formazan, with a microplate spectrophotometry system, Spectra Max Plus340 (Molecular Devices) at a wavelength of 490 nm. The cytotoxicity percentage was calculated using the following formula: [(experimental LDH release (OD_{490})/maximum LDH release (OD_{490})×100].

Western Blot and NO Analysis HUVE-12 cells were lysed in 1% RIPA (Radio-Immunoprecipitation Assay) buffer containing protease and phosphatase inhibitors (Roche, Mannheim, Germany) and whole cell lysates were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and the membranes were blocked with 5% skim milk in Tris buffered saline (TBS) solution with 0.1% Tween-20. Immunblotting with anti-PAR-1, anti-PAR-2, anti-phospho-extracellular signal-regulated kinase1/2 (ERK1/2) or anti-ERK1/2 antibodies (Santa Cruz Biotechnology, CA, U.S.A.) followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Stressgen, CA, U.S.A.). Blots were developed using an enhanced chemiluminescence (ECL) solution (Amersham Bioscience, NJ, U.S.A). Densitometry was performed using Gelquant software (MiniBIS Pro, Jerusalem, Israel), and background was subtracted. Values for the protein of interest were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For NO as-

says, HUVE-12 cells (1×10^5 cells/ml) were seeded into the wells of a 96-well tissue culture plate and pre-incubated at 37 °C for 24 h to achieve stable attachment. Next, the wells were washed with 1% phosphate buffered saline (PBS), replenished with 2% FBS F-12K medium and incubated for 24 to 48 h after treatment with either FXa or ASP/ESP. NO production was monitored by measuring nitrite levels in the culture media using the Griess reagent (Sigma). After culture, each medium (50 μl) was mixed with an equal amount of Griess reagent and the absorbance was measured at 540 nm.

Cytokine Protein Array Multiple pro-inflammatory cytokines were simultaneously detected in cell lysates using a commercial enzyme-linked immunosorbent assay (ELISA)-based cytokine protein array (Ray Bio cytokine array; RayBiotech, GA, U.S.A.) containing 40 pairs of captured monoclonal antibodies spotted onto a nitrocellulose membrane according to the manufacturer's instructions. Briefly, cytokine array membranes were blocked in 2 ml of 1× blocking buffer for 30 min and then incubated with 1 ml of each sample (cell lysate) at room temperature for 1 to 2 h. Sample solutions were then decanted and the membranes were washed 3 times with 2 ml of 1× wash buffer I, followed by 2 washes with 2 ml of 1× wash buffer II at room temperature with shaking. Membranes were then incubated in 1 : 250-diluted biotin-conjugated primary antibodies at room temperature for 1.5 h and washed as described above before incubation in 1 : 1000-diluted horseradish peroxidase-conjugated streptavidin. After incubation in horseradish peroxidase-conjugated streptavidin for 1 h, membranes were washed thoroughly and exposed to a peroxidase substrate (RayBiotech) for 5 min in the dark before imaging. Membranes were exposed to X-ray film (Kodak X-OMAT AR film) within 30 min of exposure to the substrate. The positive control signals on each membrane were used to normalize the cytokine signal intensities. Their intensities were determined by densitometric analysis using Gelquant software (MiniBIS Pro, Jerusalem, Israel). For each spot, the net optical density was determined by subtracting the background optical density level from the total raw optical density level.

Statistical Analysis Results are given as means±S.D. Results were compared by one-way analysis of variance (1 way ANOVA) with Tukey's multiple comparisons test. A p-value <0.05 was accepted as statistically significant.

RESULTS

FXa Enzyme Assay Chromogenic substrates were used to assess the inhibitory effects of ASP and ESP on the coagulation enzyme FXa. The activities of both synthetic peptides were determined by their extents of FXa inhibition (Fig. 1A). As shown in Fig. 1B, ESP (33.3% inhibition) was a more potent FXa inhibitor than ASP (13.5% inhibition) when the OD values of the cleaved substrates were normalized to the absorbance at the zero time-point of the assay. In addition, ESP showed continuous FXa inhibition at time points up to 300 s as compared to the ASP reactions that had reached plateau levels at 120 s. This indicates that ESP could be an effective binding inhibitor of FXa.

Inhibition of FXa Cleavage FXa is a site-specific serine endoprotease that cleaves after the arginine residue in its preferred cleavage site, Ile-Glu-Gly-Arg, and it will occasionally

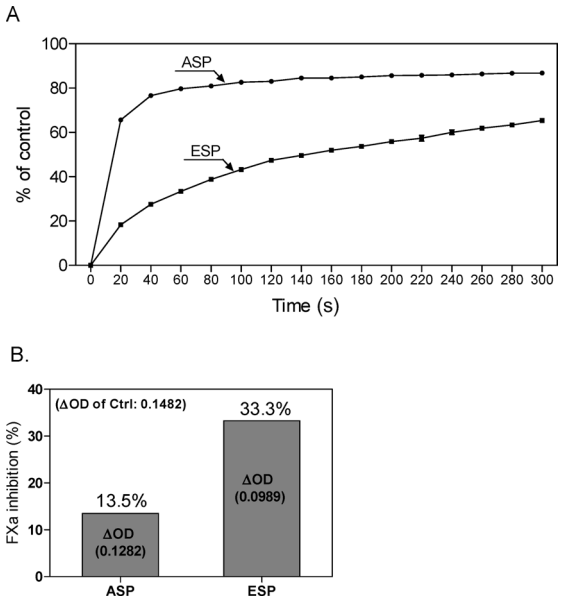


Fig. 1. Progress Curves for the Inhibition of FXa by ESP or ASP

(A) Enzyme reactions were initiated by the addition of substrate, and the color that developed from the release of p-nitroanilide was monitored for 5 min at 405 nm. The concentrations of ASP and ESP were adjusted to 5 μg/ml, and 2 mM substrate S-2222 was added to each reaction. (B) The % inhibition as compared to the control was calculated.

cleave at other basic residues. Thus, we assume that the inhibition of FXa cleavage by ESP and ASP may represent their relative levels of FXa-specific inhibition. In order to test this hypothesis, we utilized a 49 kDa FXa cleavage control protein that produced a cleavage form to two bands of 32 kDa and 17 kDa as determined by 12% SDS-PAGE analysis following FXa cleavage. This experiment revealed that ESP effectively inhibited FXa cleavage as compared to the control cleavage (lane 5) in which the cleavage control protein was maximally cleaved by FXa (Fig. 2, upper panel). Moreover, ESP showed an approximately 2-fold higher level of inhibition than ASP at 5 μg/ml (Fig. 2, lower panel). This result corresponds well with the excellent FXa inhibitory effect of ESP in the chromogenic substrate assay.

Cytotoxicity Assay Prior to determining whether ESP is coincidentally effective in inhibiting the inflammatory events induced by FXa along with its role in coagulation, cytotoxicity assays were performed in the human endothelial cell line, HUVE-12. As shown in Fig. 3, both ASP and ESP were not toxic at concentrations under 10 μg/ml. This indicated that ASP and ESP would have a similar impact on cellular responses at the same doses.

Western Blot and NO Analysis To identify the PAR-2 receptor by which FXa induced signaling, HUVE-12 cells were incubated overnight with FXa in the presence or absence of ASP or ESP. As shown in Fig. 4A, PAR-1 and PAR-2 were increased when treated with FXa (lane 2), whereas ASP and ESP inhibited the activation of FXa in a dose dependent manner. Interestingly, PAR-2 was remarkably reduced by a higher dose of ESP (5 μg/ml), suggesting that ESP would impart greater specificity for PAR-2 than that of PAR-1 when reacted with FXa. Moreover, we found that ERK1/2 phosphorylation was well inhibited by ESP at higher dose (Fig. 4B). These results strongly suggest that ESP may interfere with a PAR-2-mediated signal pathway and thereby

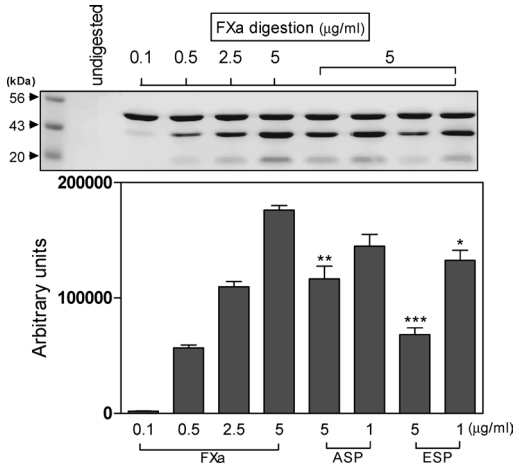


Fig. 2. Inhibition of FXa Cleavage

The FXa cleavage control protein (3 μg) was digested with 5 μg/ml FXa in the presence of ASP or ESP. To verify the cleavage, various concentrations of FXa (0.1 to 5 μg/ml) were reacted with target protein. Samples were analyzed on a 12% SDS-PAGE followed by staining with 0.01% Coomassie blue after a 3 h incubation at room temperature. 49 kDa control protein produced optimally digested forms to 32 kDa and 17 kDa at 5 μg/ml of FXa (lane 5), and lanes 6 to 9 show the inhibition of FXa cleavage by ESP or ASP. The band intensities of the separated proteins were compared using a gel documentation system (lower panel). Results are the mean±S.D. of three independent experiments (n=3). $*p<0.05$, $**p<0.001$, $***p<0.0001$ *vs.* lane 5.

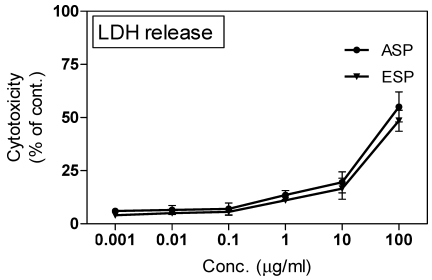


Fig. 3. Cytotoxicity of ASP and ESP in HUVE-12 Cell Line

Cells were treated with serial dilutions (0.001—100 μg/ml) of either ASP or ESP and incubated for 24 h. After incubation, supernatants were collected and the levels of LDH leakage from the cells were analyzed according to the manufacturer's instructions. Results are the mean±S.D. of three independent experiments (n=3).

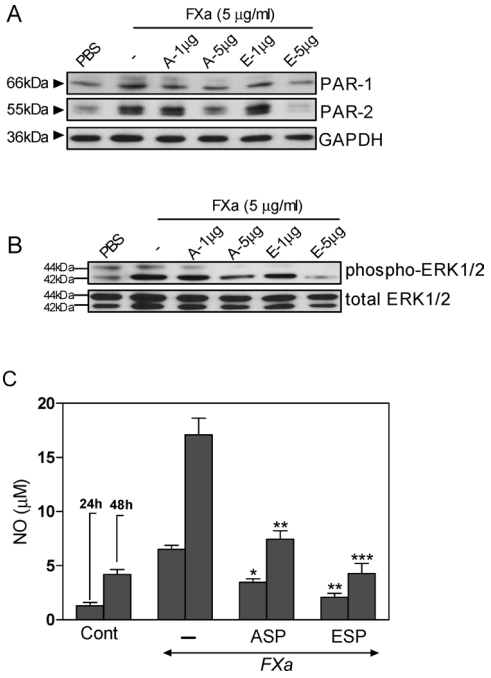


Fig. 4. Inhibition of Proteinase-Activated Receptor Activation, ERK1/2 Phosphorylation, and NO Measurements

HUVE-12 cells were pre-treated with ASP and ESP (1—5 μg/ml) before treating with FXa (5 μg/ml). After incubation for 24 h, cell lysates were prepared with RIPA buffer containing protease and phosphatase inhibitor cocktail. Equivalent amounts of protein lysates were resolved by 10% SDS-PAGE, transferred, and immunoblotted with (A) anti-PAR-1 or anti-PAR-2 antibodies and (B) anti-p-ERK or anti-ERK antibodies. The membrane was stripped and reprobed with anti-GAPDH antibody to control for loading. (C) For the cell-based NO inhibition assay, HUVE-12 cells were seeded into the wells of a 96-well culture plate and incubated for 24 h or 48 h in the presence of FXa (5 μg/ml) +/− ASP or ESP (5 μg/ml). NO levels were determined using the Griess reagent followed by OD measurements at 540 nm. Experiments were performed in triplicate (n=3) and the results are the mean±S.D. Asterisks indicate statistically significant differences relative to FXa treatment alone ($*p<0.05$, $**p<0.001$, $***p<0.0001$).

inhibits ERK1/2 phosphorylation. The effects of the peptides on NO production were examined in long-term incubations (24, 48 h) with FXa in the presence of 5 μg/ml ESP or ASP (Fig. 4C). The inflammatory response of HUVE-12 cells to FXa was significantly inhibited by ESP ($p<0.0001$), and this inhibition was maintained for 48 h.

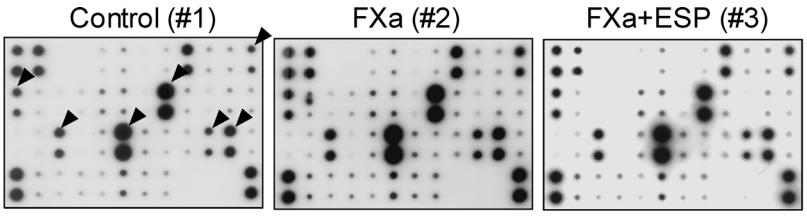
Inflammatory Cytokine Profiles in HUVE-12 Cells To determine differences in the spectra of inflammatory cytokines expressed by FXa-treated HUVE-12 cells in the presence or absence of ESP, we examined the levels of 40 inflammatory cytokines in HUVE-12 cell lysates using a cytokine antibody array. The relative variability in the cytokine spectrum of each group is shown in Fig. 5. Of the 40 inflammatory cytokines present on the array, interleukin-1α (IL-1α), IL-1β, IL-8, IL-16, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β levels were found to be reduced by the presence of ESP, compared to the group treated with FXa alone. It is interesting that ESP effectively attenuated the FXa-stimulated pro-inflammatory responses of endothelial HUVE-12 cells, suggesting that ESP may effectively interrupt the downstream pathway of PAR-2 signaling in endothelial cells by inhibiting FXa.

DISCUSSION

Inflammation and coagulation play pivotal roles in the pathogenesis of vascular disease. These two events are intimately linked, and cellular signaling by coagulation proteases through PARs may affect both pro- and anti-inflammatory responses.[13] Thus, coagulation inhibitor systems have anti-inflammatory activities along with their anti-coagulant functions and may provide dual pharmacological benefits during drug discovery.

Among the coagulation factors, FXa is generated following the stepwise activation of coagulation proteases of either the intrinsic or extrinsic pathway of the coagulation cascade. The intrinsic pathway is paramount in initiating coagulation, whereas the extrinsic pathway maintains coagulation.[22] Therefore, selective FXa inhibitors are attractive candidates as FXa is positioned at the convergence point of both the extrinsic and the intrinsic coagulation systems. Moreover, anti-coagulants that target coagulation factors located in the upstream cascade (*e.g.,* FXa) might be more effective than those that directly target thrombin. Thus, FXa has long been viewed as a target for anti-coagulation therapy.

We previously identified novel expressed sequence tags in the earthworm that may include anti-coagulant genes, or anti-



	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	POS	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1 α
2	POS	POS	NEG	POS	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1 α
3	IL-1 β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
4	IL-1 β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1 α	MIP-1 β	MIP-1 δ
6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1 α	MIP-1 β	MIP-1 δ
7	RANTES	TGF-β	TNF-α	TNF-β	sTNFRI	sTNFRII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS
8	RANTES	TGF-β	TNF-α	TNF-β	sTNFRI	sTNFRII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

Fig. 5. Representative Qualitative Levels of Cytokine Protein Expressions in HUVEC-12 Cells

HUVE-12 cells were exposed to FXa or ESP+FXa (5 μg/ml, each) for 24 h. Untreated cell cultures were used as a control and culture supernatants were applied to a cytokine antibody array. The array key represents the location of each antibody in duplicate on the membrane and black circles indicate the extents of the Ag–Ab reactions (upper panel). Decreased protein expression levels of IL-1α, IL-1β, IL-8, IL-16, MCP-1, MIP-1α and MIP-1β (boxed) were detected in ESP+FXa membrane (#3) compared to FXa-stimulated membrane (#2). $N=3$/treatment group.

stasins (FXa inhibitor), containing amino acid sequences highly similar to the hydra antistasin. The presence of these genes in the earthworm represents a novel finding.[23] Further, we determined that earthworm eisenstasin is a basic protein due to the frequent occurrence of arginine residues that may provide FXa with secondary and tertiary recognition sites after the primary recognition (unpublished data). Thus, the presence of multiple arginine residues indicated that earthworm antistasin would have greater FXa inhibitory capacity than other antistasins. Based on these backgrounds, we synthesized four short peptides (*e.g.*, RCRNECA, RCRFGYR, RCKSAAY, RCRCWKE) that were designed from the novel expressed sequence tags obtained from the *E. andrei* midgut. Among those peptides, NH_2-RCRFGYR-OH, ESP, was found to have an excellent FXa inhibitiory effect (33.3%) compared to other analogues (10.2—1.6%) (data not shown).

In the present study, a selected short peptide, ESP, was prepared. The FXa inhibitory effects of the peptide were analyzed and compared to those of the ASP peptide (derived from the sequence of leech antistasin) which showed a good anti-coagulant activity. The advantages of such a short peptide are that it would be non-immunogenic and provide higher efficacy in the coagulation cascade.[24] Our results demonstrated that ESP would be an effective, potent FXa inhibitor that might inhibit the bidirectional pathways mediating the coagulation and inflammatory signals. In particular, ESP appeared to directly bind to the active site of FXa, thereby blocking its interaction with substrate. The presumed direct action of ESP against FXa was confirmed by FXa cleavage assays, which showed a highly effective reduction of protein cleavage in the presence of ESP. Interestingly, we also found that ESP may inhibit inflammation resulting from FXa-mediated activation of PARs on endothelial cells.

PAR activation induces a wide variety of inflammatory conditions, such as sepsis, myocardial infarction, stroke, acute lung injury and glomerulonephritis.[25,26] In order to address potential roles of ESP in the PAR pathway, we examined whether ESP effectively inactivated FXa and thereby inhibited signaling pathways. The results from this experiment showed that ESP well inhibited ERK1/2 phosphorylation, followed by a dramatic reduction of NO production as compared to the ASP group. In addition, pro-inflammatory markers of the cytokine protein array provided clear evidence for PAR-2-mediated FXa signaling and the consequent anti-inflammatory effect of ESP.

PAR-2 activation by FXa increases the spectrum of PAR receptors triggered by coagulation factors. Reports for the effects of PAR-2 activation include induction of leukocyte rolling and adhesion, induction of inflammation in a rat hind paw, stimulation of a pro-inflammatory response *via* neurogenic mechanisms or stimulation of proliferation in vascular smooth muscle cells.[27—30] In the cytokine array, we found that ESP may exert anti-inflammatory effects on endothelial cells by regulating cytokines, including IL-1α, IL-1β, IL-8, IL-16, MCP-1, MIP-1α and MIP-1β, which are all correlated with cellular inflammation. Therefore, the inhibition of FXa-induced inflammatory cytokine and chemokine production by endothelial cells may provide a novel, rational therapeutic approach to various diseases during the initiation and propagation of inflammation-mediated coagulation activation.

In conclusion, it is worthwhile that we consider the presumed direct inhibitory role of ESP on FXa in mediating both anti-coagulation and anti-inflammation effects, as FXa is positioned at the center of multiple coagulation cascades. The findings from our research indicate that ESP can effectively bind to FXa, inhibit PAR-2-mediated activation, and thus may inhibit downstream signaling pathways in the circulatory, respiratory and central nervous systems during inflammation, although additional *in vivo* researches are necessary to confirm these findings.

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