Engineering D-helix of antithrombin in alpha-1-proteinase inhibitor confers antiinflammatory properties on the chimeric serpin

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Summary

Antithrombin (AT) is a heparin-binding serpin in plasma which regulates the proteolytic activity of procoagulant proteases of the clotting cascade. In addition to being an anticoagulant, AT also exhibits antiinflammatory activities when it binds to cell surface heparan sulfate proteoglycans (HSPGs) on the endothelium via its basic residues of D-helix to elicit intracellular signalling responses. By contrast to AT, α1-proteinase inhibitor (α1-PI) is a non-heparin-binding serpin that exhibits very slow reactivity with coagulation proteases and possesses no HSPG-dependent antiinflammatory properties. To determine whether the antiinflammatory signaling specificity of AT can be transferred to α1-PI, we replaced the D-helix of human α1-PI with the corresponding sequence of human AT and expressed the chimeric serpin α1-PI/D-helix in a bacterial expression system. High molecular weight heparin bound to α1-PI/D-helix and accelerated the inhibition of thrombin by the serpin mutant by a template mechanism reminiscent of the cofactor effect of heparin on inhibition of thrombin by AT. Like AT, α1-PI/D-helix exhibited antiinflammatory properties in both cellular and animal models. Thus, α1-PI/D-helix inhibited the barrier-disruptive effect of proinflammatory cytokines and inhibited the activation of nuclear factor-κB transcription factor in lipopolysaccharide-stimulated endothelial cells by a concentration-dependent manner. Furthermore, the chimeric serpin reduced lipopolysaccharide-mediated lethality, elicited a vascular protective effect and inhibited infiltration of activated leukocytes to the peritoneal cavity of mice in an HMGB1-mediated inflammatory model. These results suggest that grafting the D-helix of AT to α1-PI confers antiinflammatory properties on the serpin and that the chimeric serpin may have therapeutic utility for treating inflammatory disorders.

Keywords

Antithrombin, coagulation inhibitors, endothelial cells, inflammation, SERPINs

Introduction

Antithrombin (AT) is a plasma inhibitor of the serpin superfamily which regulates the proteolytic activity of trypsin-like coagulation proteases of the clotting cascade in both intrinsic and extrinsic pathways (1-3). Similar to other serpins, AT binds to the active-site pocket of target serine proteases through a P1-Arg residue present on its reactive centre loop (4, 5). The binding induces a large scale conformational change in the serpin which is accompanied by the distortion of the catalytic pocket and entrapment of the protease as an acylated inactive complex (5-7). AT is a heparin-binding serpin whose reactivity with coagulation proteases is dramatically accelerated by the polysaccharide (1, 4). Heparin can bind to basic residues of the D-helix on AT to accelerate the reactivity of the serpin with coagulation proteases by two to four orders of magnitude (1, 4). Depending on its molecular size, heparin can accelerate the AT inhibition of coagulation proteases by two distinct mechanisms. The binding of a distinct 3-O-sulfate containing pentasaccharide fragment of heparin to the D-helix of AT conformationally activates the serpin to accelerate its reactivity with vitamin K-dependent coagulation proteases (factors VIIa, IXa and Xa) by ~200-500-fold (3, 6). In addition to acceleration of the protease inhibition by the serpin activation mechanism, high-molecular-weight heparins can also bind simultaneously to basic exosites of vitamin K-dependent coagulation proteases in the presence of Ca2+ to promote the AT inhibition of these proteases by a bridging (template) mechanism (1, 8). The latter mechanism of protease inhibition is the primary mechanism through which high-molecular-weight heparins accelerate the AT inhibition of thrombin since pentasaccharide-mediated conformational activation of AT makes a minor contribution to the serpin inhibition of thrombin (~2-fold) (9).

In addition to its anticoagulant activity through direct inhibition of procoagulant proteases, AT also elicits potent antiin-
flammatory signalling responses when it binds to heparan sulfate proteoglycans (HSPGs) on endothelial cells which line the vasculature (10-12). It has been demonstrated that a small subpopulation of vascular HSPGs contains the characteristic 3-O-sulfate containing pentasaccharide which can support high-affinity interaction of AT with the vessel wall (13). It has been hypothesised that interaction of AT with this population of vascular HSPGs not only improves reactivity of the serpin with vitamin K-dependent coagulation proteases, but it also renders AT competent of eliciting protective signalling responses in endothelial cells (12). Thus, AT has been shown to exhibit potent antiinflammatory activities in both in vitro and in vivo inflammatory models including severe sepsis and different ischaemia/reperfusion injury models (10-12, 14-16). Protective signalling activity of AT has been demonstrated to effectively inhibit nuclear factor (NF)-κB-dependent expression of proinflammatory cytokines and cell adhesion molecules on endothelial cells in both cellular and animal models (10-12). Nevertheless, protective activity of AT has been found to require supraphysiological concentrations of the serpin, thus limiting its potential therapeutic utility as an antiinflammatory drug due to the inhibitor increasing the risk of bleeding (15).

By contrast to AT, α1-protease inhibitor (α1-PI) is a non-heparin-binding serpin which is specific for neutrophil elastase and, as an acute-phase protein, its concentration is dramatically elevated during inflammation (17-19). α1-PI exhibits poor reactivity with coagulation proteases and possesses no HSPG-dependent vascular protective signalling activity. To determine whether the antiinflammatory signalling specificity of AT can be transferred to α1-PI, we replaced the heparin-binding D-helix of α1-PI with the corresponding loop of AT by recombinant DNA methods. Characterisation of the chimeric serpin indicates that similar to AT, the α1-PI chimeric serpin binds to heparin to elicit potent antiinflammatory responses in both cellular and animal models. Thus, the chimeric serpin may have potential therapeutic utility for treating inflammatory disorders.

Materials and methods
Expression of α1-PI

The cDNA coding for human α1-PI was cloned in the Escherichia coli expression/purification vector, pQE-30 with a His tag, and expressed in Escherichia coli SG13009 cells (Qiagen, Valencia, CA, USA) as described (20, 21). The α1-PI chimera (α1-PI/D-helix) in which the D-helix sequence of the serpin (5′NLTEIPAEQL-HEGFQELLRTL-NQPDSQ) was replaced with the corresponding D-helix sequence of human AT (112SEKTDSQHFF-FAKLNCRLYKANS) was prepared by PCR methods and expressed using the same expression/purification vector system. An α1-PI/D-helix mutant was constructed in which Lys-114 of D-helix was replaced with Glu (α1-PI/D-helix-K114E). The PI-Met to Trp mutants of α1-PI in the background of either wild-type (α1-PI-PI-Trp) or α1-PI/D-helix (α1-PI/D-helix-Trp) were prepared by PCR methods and expressed using the same expression/purification vector system. Following confirmation of mutations by DNA sequencing, recombinant α1-PI derivatives were prepared from 1-2 liters of bacterial cultures as described (20, 21). Briefly, the cell pellet was dissolved in 50 mM sodium phosphate buffer containing 300 mM NaCl, 10 mM imidazole containing complete inhibitor cocktail (Clontech, Mountain View, CA, USA). After sonication and centrifugation, the supernatant of cell lysate was loaded on Ni-NTA Sepharose column (Qiagen) and bound proteins were eluted using 250 mM imidazole in sodium phosphate buffer. Fractions containing the eluted protein were pooled and dialysed against 50 mM NaCl and 20 mM Tris-HCl (pH, 7.4) overnight at 4°C and applied on HiTrap Q (GE Healthcare, Piscataway, NJ, USA) column followed by elution of the protein by a linear sodium chloride gradient of 50 mM to 1.0 M. Protein concentrations were calculated based on the absorbance at 280 nm assuming an extinction coefficient of 27,000 M⁻¹ cm⁻¹ for α1-PI derivatives (23) and by stoichiometric titration of serpins with known concentrations of porcine pancreatic elastase. The homogeneity of recombinant serpin derivatives were ensured by the SDS-PAGE. To eliminate possible endotoxin contamination of recombinant serpins, the purified proteins were applied through a column packed with Pierce High-Capacity Endotoxin Removal Resin (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instruction.

Unfractionated therapeutic heparin (average MW ~15 kD) was purchased from Pharmacia and Upjohn Company (USA). The concentration of heparin was based on the AT-binding sites and was determined by stochiometric titration of the serpin with the polysaccharide, with monitoring of the interaction by changes in protein fluorescence as described (1). The chromogenic substrates, Spectrozyme TH (SpTH) for thrombin and Spectrozyme FXa (SpFXa) for factor Xa (FXa) were purchased from American Diagnostica (Greenwich, CT, USA). The chromogenic substrate for elastase (MeOSuc-AAPV-pNA) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Human plasma-derived proteins thrombin, FXa and antithrombin were purchased from Haematologic Technologies (Essex Junction, VT, USA) and porcine pancreatic elastase was from Worthington Biochemical Corp. (Lakewood, NJ, USA).

Kinetic characterisation

The inhibitory activities of α1-PI derivatives were evaluated in an inhibition assay under pseudo-first order rate conditions in both the absence and presence of a high molecular weight heparin (injectable therapeutic heparin) using thrombin or FXa as the heparin binding proteases as described (24). Briefly, either thrombin (2 nM) or FXa (1 nM) was incubated with 250-2000 nM α1-PI and increasing concentrations of heparin (0-100 μM) for 30-120 minutes (min) at room temperature in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 and 2.5 mM Ca²⁺ (TBS/ Ca²⁺) containing 0.1 mg/ml bovine serum albumin (BSA) and 0.1% PEG-8000. All reactions were carried out in 50 μl volumes in 96-well plates and at different time points, 50 μl chromogenic substrate (SpTH for thrombin and SpFXa for FXa) in TBS was added to each reaction and remaining enzyme activities were measured at 405 nm using a Vmax Kinetic Plate Reader (Molecular Devices, Menlo Park, CA, USA). Ob-
served pseudo-first-order rate constants ($k_{obs}$) and second-order association rate constants ($k_2$) were calculated as described (24). All values are presented as averages of at least three independent measurements ± SD.

**Fluorescence measurements**

Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY, USA) was used for protein fluorescence measurements at 25°C. Excitation and emission wavelengths were 280 and 340 nm, respectively. Bandwidths were set at 4 nm for excitation and 8 nm for emission. Titration was performed by addition of a 1-2 µl of high concentration of stock solution of unfractionated therapeutic heparin into 250 nM of wild-type $\alpha_1$-PI or $\alpha_1$-PI derivatives containing a Trp at P1 positions ($\alpha_1$-PI-Trp and $\alpha_1$-PI/D-helix-Trp) in 0.1 M NaCl, 0.02 M Tris–HCl (pH 7.4) containing 0.1% PEG-8000. Data from at least three experiments were analysed as the ratio of change in the fluorescence intensity of the sample containing heparin to the initial intensity of the control protein lacking the cofactor. Affinity of $\alpha_1$-PI derivatives for heparin was calculated by nonlinear least-squares computer fitting of the data by the quadratic binding equation as described (25).

**Permeability assay**

EA.hy926 endothelial cell (provided by Dr. C. Edgell from University of North Carolina at Chapel Hill, NC, USA) were cultured to confluence in a humidified atmosphere at 37°C in Dulbecco modified eagle medium supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and antibiotics (penicillin G and streptomycin) as described (26, 27). Cell permeability in response to either thrombin or the proinflammatory nuclear cytokine high mobility group box 1 (HMGB1) in the absence or presence of increasing concentrations of serpin derivatives (AT or $\alpha_1$-PI) (0.67-5.0 µM for 4 hours [h]) was assessed by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional cell monolayers by a modified two-compartment chamber model as described (26, 27). Briefly, endothelial cells were plated (2 × 10^5/well) in trans-well plates (3 µm pore size and 12-mm diameter) for two days. The confluent monolayers were then incubated with different concentrations of serpin derivatives (AT, $\alpha_1$-PI or $\alpha_1$-PI/D-helix) in serum-free growth media containing 0.5% BSA for 4 h followed by stimulation of by cells by either thrombin (10 nM) for 10 min or HMGB1 (40 µg/ml for 16 h) as described (26, 27). Inserts were washed with phosphate-buffered saline, pH 7.4 before adding 0.5 ml Evans blue (0.67 mg/ml) (Sigma, St. Louis, MO, USA) diluted in growth medium containing 0.5% BSA. Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 min, the optical density at 650 nm was measured in the lower chamber. Experiments were performed in duplicate and repeated three times.

**Caspase-8 assay**

The activity of caspase-8 was measured using a colourimetric kit (Genescript) according to the manufacturer's instruction. Briefly, EA.hy926 endothelial cells were first incubated with serpin derivatives (1-5 µM for 4 h) followed by their treatment with lipopolysaccharide (LPS) (10 ng/ml) for 16 h. Cells were lysed with 50 mM HEPES, pH 7.5, 100 mM NaCl, 0.1% 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate [CHAPS], 1 mM dithiothreitol, 0.1 mM EDTA and 50 µl of the cell lysate was incubated on ice for 5 min before centrifugation at 10,000 × g for 10 min at 4°C. Supernatants were incubated with the caspase-8 specific Ac-LEHD-pNA substrate for 4h in the dark and the enzyme activity was measured at 405 nm.

**NF-κB and interleukin (IL)-6 ELISA**

The activation of NF-κB in nuclear lysates of different treated groups was determined using an ELISA-based non-radioactive transcription factor assay kit (Abcam, Cambridge, UK) according to manufacturer's protocol. The secretion of IL-6 in different treated groups was also analysed by an ELISA assay (eBioscience Inc., San Diego, CA, USA) according to manufacturer's protocol.

**In vivo permeability and leukocyte migration assays**

Eight- to 10-week-old male C57bl/6 mice were used for in vivo studies after a five-day acclimatisation period. All animals were treated in accordance with Guidelines for Care and Use of Laboratory Animals in Saint Louis University. The vascular permeability assay was conducted as described (27, 28). Briefly, each group of mice was injected intraperitoneally with 200 µl of each serpin (0.01-0.04 mg/g body weight) or 200 µl of normal saline as a control. After 3 h, 1% BSA-bound Evans blue dye in normal saline was injected intravenously to each mouse, followed by immediate intraperitoneal injection of the proinflammatory cytokine HMGB1 (15 µg/g bodyweight) or 0.7% acetic acid as a positive control. After 30 min, mice were sacrificed, and peritoneal exudates were collected (after being washed with 5 ml of normal saline) and centrifuged at 200 × g for 10 min. The absorbance of the supernatant was read at 650 nm. The vascular permeability was expressed in terms of dye (µg/mouse), which leaked into the peritoneal cavity according to a standard curve constructed with Evans blue as described (27, 28).

For assessing leukocyte migration, animals were intraperitoneally injected with above concentrations of the stimuli dissolved in normal saline. After 4 h, mice were sacrificed and peritoneal surfaces were washed with 5 ml of normal saline. Next, 20 µl of peritoneal fluid was mixed with 0.38 ml of Turk’s solution (0.01% crystal violet in 3% acetic acid) and the number of leukocytes was counted under a light microscope. In these studies, CMC-Na (1.5%) was administrated to mice as a positive control for leukocyte migration as described (27, 28). All measurements in mice were performed for three mice in each group of the study.
Histological examination

Peritoneal tissues were fixed in fixative (American Master Tech Scientific Inc., Lodi, CA, USA) and processed using paraffin embedding procedures. Sections (5 mm thickness) were cut and stained with hematoxylin and eosin and stained sections were observed under a light microscope to assess extravasation of the immune cells. Images were captured by an Olympus DP25 camera. Each group of images in the figures show representative micrographs of more than 20 images obtained from peritoneal tissue sections of at least three different mice.

Survival rate

Wild-type C57bl/6 mice (8-10 weeks old) were injected intraperitoneally with 15 mg/kg LPS in 200 μl saline. For serpin treatment groups (10 mice/group), each serpin (0.04 mg/g bodyweight) was injected through the same route 3 h prior to administration of LPS. Mortality was assessed at days 1, 2, 3, 4 and 5.

Statistical analysis

Data were expressed as means ± SD from at least three independent experiments. Group data were compared using analysis of variance (ANOVA) followed by Bonferroni post-hoc tests (for comparisons among three or more groups) or Student's t-test, as appropriate. Survival rate was assessed using the Fisher Exact test for the evaluation of differences in survival rates. P-values of <0.05 were considered statistically significant.

Results

Heparin binds to α1-PI/D-helix

Thrombin reacts slowly with α1-PI both in the absence and presence of heparin. The concentration-dependence of α1-PI inhibition of thrombin yielded second-order rate constants (kₐ) of 4 x 10⁻⁶ M⁻¹s⁻¹ and 6 x 10⁻⁶ M⁻¹s⁻¹ for the serpin inhibition of the protease in the absence and presence of therapeutic high-molecular-weight heparin, respectively (Figure 1A). The α1-PI/D-helix chimera exhibited a slightly lower kₐ value of 1 x 10⁻¹ M⁻¹s⁻¹ with thrombin in the absence of heparin. However, heparin accelerated the reactivity of the chimeric serpin with thrombin ~100-fold (kₐ = 1 x 10⁻¹ M⁻¹s⁻¹), suggesting that grafting D-helix of AT to α1-PI renders the chimeric serpin capable of interacting with heparin (Figure 1A). Analysis of inhibition rate constants of porcine pancreatic elastase by α1-PI and α1-PI/D-helix (7.5 x 10⁻⁶ M⁻¹s⁻¹ and 2.2 x 10⁻⁶ M⁻¹s⁻¹, respectively) suggested that grafting D-helix of AT on α1-PI reduces its reactivity with elastase ~3-fold.

The binding of heparin to D-helix of AT is associated with an enhancement in the intrinsic fluorescence intensity of AT which can be utilised to evaluate affinities of polysaccharides for interaction with the serpin (25). However, heparin titration experiments with α1-PI/D-helix did not yield any change in the intrinsic protein fluorescence to be able to estimate its affinity for heparin (data not shown). Thus, we decided to introduce a Trp residue to the P1 position of the serpin to determine whether it can act as a reporter for the serpin binding to heparin. We replaced the P1-Met of both α1-PI and α1-PI/D-helix with a Trp and monitored their intrinsic fluorescence upon titration with heparin. Interestingly, fluorescence of the P1-Trp residue was sensitive to heparin binding to D-helix of the chimeric serpin, yielding a Kₐ of ~84 ± 10 nM for the interaction (Figure 1B). In contrast to α1-PI/D-helix-Trp, heparin titration of α1-PI-Trp did not result in any change in intrinsic fluorescence (Figure 1B, empty circles), suggesting heparin-mediated fluorescence change is specific for the chimeric serpin.

It is known that high-molecular-weight heparins promote the AT inhibition of thrombin by a template mechanism (1). In this mechanism of protease inhibition, heparin simultaneously binds to D-helix of AT and exosite II of thrombin, thereby decreasing Kₐ for interaction of the serpin with the protease by holding the two proteins together by a template (bridging) mechanism (1, 8). In the template mechanism of heparin-catalysed protease inhibition by AT, the inhibition rate constants exhibit a characteristic bell-shaped dependence on the heparin concentration (1, 8). This heparin concentration-dependence of thrombin inhibition by α1-PI/D-helix is presented in Figure 1C. Similar to heparin-catalysed AT inhibition of thrombin, α1-PI/D-helix inhibition rates exhibited a bell-shaped dependence on heparin concentration (Figure 1C), suggesting that heparin binds to both the serpin and the protease to form a ternary complex, thus accelerating the reaction by a template mechanism. In support of this hypothesis, heparin did not accelerate α1-PI/D-helix inhibition of an exosite II mutant of thrombin (thrombin-3A) (Figure 1C, empty squares) which has been shown to not interact with heparin (29).

The reactivity of α1-PI derivatives with FXa was also evaluated in both the absence and presence of heparin. Interestingly, heparin accelerated the reactivity of wild-type α1-PI with FXa (5-fold, thus yielding kₐ values of 4.6 x 10⁻⁶ M⁻¹s⁻¹ and 2.5 x 10⁻⁶ M⁻¹s⁻¹ in the absence and presence of heparin, respectively. On the other hand, heparin accelerated the inhibition of FXa by α1-PI/D-helix (37-fold (kₐ = 1.6 x 10⁻⁶ M⁻¹s⁻¹ and 6.0 x 10⁻⁶ M⁻¹s⁻¹ in the absence and presence of heparin, respectively). The concentration-dependence of FXa inhibition by α1-PI and α1-PI/D-helix revealed that while the heparin-catalysed inhibition of FXa by α1-PI is saturable, the inhibition rates with α1-PI/D-helix exhibit a bell-shaped dependence on heparin concentration (Figure 1D). These results suggest that, similar to thrombin, heparin accelerates the α1-PI/D-helix inhibition of FXa by a template mechanism. The mechanism by which heparin accelerates the α1-PI inhibition of FXa was not investigated. However, the saturable dependence of inhibition rates on heparin concentrations suggests that polysaccharide may alter the conformation of the protease and/or serpin to accelerate the inhibition reaction.
α₁-PL/D-helix protects the barrier permeability function of endothelial cells in response to proinflammatory stimuli

Thrombin and HMGB1 are known to disrupt the barrier permeability function of endothelial cells (26, 27), and AT exerts a protective effect to reverse this proinflammatory process (Figure 2A shown for thrombin only). Unlike AT, pretreatment of endothelial cells with α₁-PI did not protect cells from the barrier disruptive effect of either thrombin (Figure 2B) or HMGB1 (Figure 2C), however, similar to AT, α₁-PL/D-helix exerts a protective effect thus reversing proinflammatory effects of both mediators by a concentration-dependent manner (Figure 2B, C). These results suggest that binding of D-helix of AT to endothelial HSPIs is responsible for protective effects of the serpin and that grafting it to α₁-PI endows a similar protective function on the chimeric serpin.

α₁-PL/D-helix protects against LPS-mediated apoptosis in endothelial cells

Analysis of caspase-8 activity in LPS-stimulated endothelial cells showed an increased enzymatic activity, suggesting that LPS induces apoptosis in endothelial cells as has been reported (Figure 3A) (12). Pretreatment of cells with increasing concentrations of AT before stimulation with LPS did not provide any evidence of cytoprotective activity for AT, but rather the serpin appeared to promote the apoptotic pathway in endothelial cells at high concentrations (Figure 3A). By contrast to AT, both wild-type α₁-PL and the α₁-PL/D-helix chimera exhibited a cytoprotective activity in LPS-stimulated endothelial cells (Figure 3B). Analysis of the concentration dependence of protective effects indicated that α₁-PI/D-helix is markedly more potent than wild-type α₁-PI in inhibiting LPS-induced apoptosis in endothelial cells. The basis for a lack of cytoprotective activity for AT in this assay is not known but results are consistent with the literature and the reason for that may relate to antiangiogenic properties of AT which have been ob-

Figure 1: Dependence of rate constants for inhibition of thrombin on concentration of α₁-PI derivatives in the absence and presence of heparin. A) Kobs values for the inhibition of thrombin (2 nM) by wild-type α₁-PI in the absence (○) and presence (●) of heparin or by α₁-PI/D-helix in the absence (●) and presence of heparin (■) (0.1 µM) were determined by an amidolytic activity assay as described in Materials and methods. B) Binding of heparin to α₁-PI-Trp derivatives. The spectral changes were monitored by addition of 1–2 ml of a concentrated stock solution of heparin to 250 nM α₁-PI-Trp (○) or α₁-PI/D-helix-Trp (●) in TBS (pH 7.4) containing 0.1% PEG-8000 and the dissociation constant was calculated from the changes of the intrinsic protein fluorescence as described under Materials and methods.

C) Dependence of second-order rate constants for inhibition of wild-type thrombin and the thrombin heparin-binding exosite II mutant (thrombin-3A) by α₁-PI derivatives on heparin concentration. The same as panel A except that the inhibition of thrombin by a fixed concentration (250 nM) of either α₁-PI (○) or α₁-PI/D-helix (●) and of thrombin-3A by α₁-PI/D-helix (●) was monitored in the presence of increasing concentrations of heparin (x-axis) as described in Materials and methods. D) Dependence of second-order rate constants for inhibition of FXa by α₁-PI (○) or α₁-PI/D-helix (●) on heparin concentration. The same as panel C except that the inhibition of FXa by α₁-PI derivatives was monitored. All values are average of at least three independent measurements ± SD.
served for latent and/or cleaved conformers of the serpin (30-32). A population of latent AT is present at equilibrium with the native AT in most preparations and its level may increase with storage and exposure to elevated temperatures.

AT and $\alpha_1$-PI/D-helix inhibit LPS-mediated NF-κB activation in endothelial cells

AT inhibited the activation of NF-κB in endothelial cells in response to LPS by a concentration dependent manner as has been reported (Figure 3C) (12). While wild-type $\alpha_1$-PI exhibited no modulatory effect on the NF-κB pathway in response to LPS in endothelial cells, the $\alpha_1$-PI/D-helix chimera inhibited LPS-mediated activation of the nuclear transcription factor to an extent similar to that observed with AT (Figure 3D). Similar to AT, $\alpha_1$-PI/D-helix but not wild-type $\alpha_1$-PI inhibited thrombin-mediated synthesis of IL-6 in endothelial cells (Figure 3E).

Previous results have indicated that Lys-114 of AT D-helix is one of the key heparin binding residues of the serpin. Intriguingly, it has been found that the charge reversal mutagenesis of this residue in AT not only impairs affinity of the serpin for heparin but also confers a potent pro-apoptotic activity on AT (12). To determine whether Lys-114 of AT plays a similar role in $\alpha_1$-PI/D-helix chimera, we replaced this residue with Glu and evaluated properties of the chimeric serpin mutant in signalling assays. Similar to AT-K114E (12), the barrier protective activity of the K114E mutant of $\alpha_1$-PI/D-helix in response to thrombin was markedly decreased (Figure 4A). However, unlike AT-K114E (12), the chimeric mutant did not acquire pro-apoptotic activity but rather exhibited impaired anti-apoptotic activity in response to LPS (Figure 4B), suggesting that the interaction of D-helix with endothelial
cells is responsible for both barrier protective and cytoprotective activity of the serpin. The basis for the pro-apoptotic activity of AT-K114E remains unknown.

AT and α₁-PI/D-helix inhibits the proinflammatory effect of HMGB1 in vivo

AT has been demonstrated to exert protective effects in several in vivo inflammatory models (10, 14-16). Here we set up an HMGB1-mediated vascular permeability model by intraperitoneal injection of the nuclear cytokine into mice, followed by measuring vascular permeability based on extravasation of BSA-bound Evans blue dye from plasma into the peritoneal cavity. An HMGB1 concentration of 15 µg/g dramatically increased endothelium leakiness, similar to the effect of using a solution of 0.7% acetic acid as a positive control in this model. AT inhibited HMGB1-mediated vascular permeability by a concentration-dependent manner (Figure 5A). While α₁-PI exhibited no barrier protective activity in this model, α₁-PI/D-helix protected vasculature from the disruptive effect of HMGB1 (Figure 5B). At an optimal concentration of 0.04 mg/g both AT and α₁-PI/D-helix exhibited similar maximal protective activities, though at lower concentrations, AT exhibited slightly higher potency (Figure 5A, B). Similarly, both AT and α₁-PI/D-helix, but not α₁-PI, markedly enhanced HMGB1-mediated binding of leukocytes to the vascular endothelium and their subsequent migration to the peritoneal cavity (Figure 5C, D), suggesting that interaction of D-helix of AT with cell surface HSPGs is solely responsible for the antiinflammat
tory effect of the serpin in this model. A 1.5% carboxymethylcellulose-sodium (CMC-Na) solution was used as a positive control for leukocyte migration in this model as described (27, 28).

**Histological evaluation**

Histology of peritoneal tissues for untreated negative control (A), CMC-Na-treated positive control (B) and HMGB1-treated (C) groups are presented in Figure 6. Relative to negative control (Figure 6A), both CMC-Na (Figure 6B) and HMGB1 (Figure 6C) recruited inflammatory cells into peritoneal tissues of experimental animals. AT effectively inhibited infiltration of inflammatory cells into the peritoneal tissues of HMGB1-treated mice (Figure 6D). However, α1-PI did not have any detectable effect on the HMGB1-mediated recruitment of inflammatory cells into peritoneal tissues (Figure 6E). Consistent with all other data presented above, α1-PI/D-helix exhibited a potent protective effect and effectively inhibited the migration of inflammatory immune cells into peritoneal tissues in response to HMGB1 (Figure 6F).

**LPS-induced mortality**

Protective effects of serpin derivatives were evaluated in an LPS-induced mortality model by intraperitoneal injection of 15 mg/kg LPS to 10 mice in each group followed by monitoring the survival rate of the mice for five days. There was a 60% survival rate for the LPS-treated group (10 mice) in this model (Figure 7). The same survival rate was obtained with the wild-type α1-PI-treated group (Figure 7), thus four mice from both groups were dead after three days. By contrast only one mouse from the α1-PI/D-helix-treated and two mice from the AT-treated groups were found dead during the same time period, suggesting a 90% and 80% survival rate, respectively, for the two serpins (Figure 7). Given the modest mortality rate with untreated mice and the limited sample size, the observed beneficial effects with AT and α1-PI/D-helix should be considered as trend rather than a firm conclusion in this model.

**Discussion**

We have demonstrated in this study that engineering the basic D-helix of AT in α1-PI endows antiinflammatory properties on the chimeric serpin, thus enabling it to interact with cell surface HSPGs and elicit protective signalling responses in vascular endothelial cells. This conclusion is derived from the observation that the chimeric serpin inhibits the barrier disruptive effect of proinflammatory mediators, thrombin and HMGB1, in both cellular and animal models. α1-PI/D-helix also inhibits HMGB1-mediated migration of inflammatory cells to the peritoneal cavity. Several previous studies have demonstrated that AT elicits D-helix dependent antiinflammatory responses in endothelial cells through interaction with HSPGs, thereby inhibiting the activation of NF-κB and down-regulating the expression of cell adhesion molecules in response to proinflammatory stimuli (10-12, 14-16). The results presented above suggest that α1-PI/D-helix can exert a protective effect through the same mechanism. The comparable efficacies of both AT and α1-PI/D-helix in the inflammatory models described above suggest that the engineered D-helix of AT contains all essential structural information to effectively elicit protective signalling responses in endothelial cells, thus D-helix of AT is primarily responsible for the HSPG-dependent antiinflammatory activities of the serpin.
In addition to its antiinflammatory activities, AT also possesses antiangiogenic properties (30-32). Both activities of AT require its interaction with cell surface HSPGs on endothelial cells (12, 32). However, in contrast to the antiinflammatory function of AT which is mediated by the native serpin having high affinity for heparin (12), the antiangiogenic activity of AT is specific for latent AT and/or an AT derivative in which the reactive center loop (RCL) of the serpin has been cleaved (30-32). Unlike native AT, both latent and RCL-cleaved AT have low affinity for heparin and neither derivative possesses barrier protective and antiinflammatory activities in endothelial cells (12). Thus, the signalling specificity of AT in two alternative pathways is determined by different AT conformers: the high-affinity heparin conformer eliciting antiinflammatory activity, the low-affinity heparin conformer mediating antiangiogenic activity. The mechanism through which the two different heparin conformers of AT exert distinct signalling functions is not known since both activity-types require AT interaction with cell surface HSPGs on endothelial cells (12, 32). An intriguing previous observation has been that Lys-114 of AT (a residue immediately outside the D-helix) (22) is involved in modulating the specificity of AT signalling in these two alternative pathways. Thus, it has been found that neutralisation of the basic charge of Lys-114 in the native serpin by substituting it with Ala confers a potent antiangiogenic activity on the mutant serpin (32). Interestingly, in a previous study we also found that the charge reversal of Lys-114 by substituting it with Glu not only dramatically reduced the affinity of the mutant (AT-K114E) for heparin and abrogated the barrier protective activity of the serpin but also conferred a potent pro-apoptotic activity on the mutant serpin (12). This activity was specific for Lys-114 and not solely due to the mutant serpin losing its high affinity for heparin since the mutation of other key residues of the D-helix including Lys-125 which simi-

Figure 5: In vivo analysis of protective effects of serpins on HMGB1-mediated vascular leakage and leukocyte infiltration. A) Each group of mice (n=3 for each group) were intraperitoneally injected with different concentrations of AT (0.01–0.04 mg/g body weight). After 3 h, mice were intravenously injected with 1% BSA-bound Evans blue dye followed by an immediate intraperitoneal injection of HMGB1 (15 µg/g body weight) or 0.7% acetic acid as a positive control and normal saline (as a negative control). Vascular permeability was determined from the extent of extravasation of Evans blue to the peritoneal cavity as described in Materials and methods. B) Same as A except that the vascular protective effect of α₁-Pi or α₁-Pi/D-helix in response to HMGB1 was monitored. C) Inhibitory effect of AT on the migration of leukocytes to peritoneal cavity in response to HMGB1 was analysed. D) Same as C except that the inhibitory effect of α₁-Pi or α₁-Pi/D-helix in response to HMGB1 was studied. All results are shown as means ± SD of three different experiments. * p < 0.05, *** p < 0.001.

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larly reduced the affinity of AT for heparin did not alter its activity in the apoptotic pathway (12). To further investigate this question, we replaced Lys-114 in the $\alpha_1$-PI/D-helix chimera and monitored its activity in both permeability and apoptosis assays. In contrast to the pro-apoptotic effect of AT-K114E, the homologous mutant of $\alpha_1$-PI/D-helix exhibited both barrier protective activity and anti-apoptotic activities, though with two- to three-fold lower efficiency (Figure 4), possibly suggesting that the pro-apoptotic activity of low-affinity heparin conformers of AT involves interactions with cell surface receptors other than those engaging the D-helix residues of AT and that the mutation of Lys-114 in AT directs the serpin to another binding site on endothelial cells. Thus, the anti-apoptotic (and antiangiogenic) activity of AT is not due to the mutagenesis of Lys-114 simply lowering the affinity of D-helix for heparin (or HSPGs), but it is a property that is exclusive for the low-affinity heparin conformer of AT (latent or cleaved) interacting with a specific glycoprotein receptor on endothelial cells (12, 32).

The observation that, similar to AT, heparin interacted with the chimeric serpin and promoted inhibition of thrombin (and FXa) by a template mechanism, supports the hypothesis that the mutagenesis strategy has transferred the D-helix-dependent heparin-binding property of AT to $\alpha_1$-PI. Direct binding studies further support this proposal as evidenced by heparin binding to engineered D-helix of the $\alpha_1$-PI chimera with a $K_D$ of 84 nM. Relative to AT which binds heparin with low-nanomolar affinity, the $\alpha_1$-PI chimera exhibited somehow weaker affinity for heparin, suggesting that in addition to residues of D-helix, other basic residues of AT also contribute to its high-affinity interaction with heparin. This hypothesis is consistent with reports in the literature that several basic residues of AT other than those on D-helix including...
N-terminus loop residues (Lys-11 and Arg-13) and A-helix residues (Arg-46 and Arg-47) of AT also contribute to high affinity interaction of the serpin with heparin (33). It should be noted that as indicated above, Lys-114, a basic residue immediately outside the N-terminus side of D-helix, also makes a significant contribution to binding of AT to heparin (12, 32). Nevertheless, this residue together with Lys-136 of AT, another basic residue immediately outside D-helix on the C-terminus end, were both included in the sequence that has been engineered in α1-PI.

It is worth noting that α1-PI is an acute phase protein circulating in plasma with a high concentration of 1-2 mg/ml and its plasma level increases several fold during inflammation (17-19). The most important known physiological function of α1-PI is the neutralisation of active neutrophil elastases that are released by activated neutrophils at sites of infection and inflammation. The observation of this study that α1-PI is not capable of eliciting intracellular signalling activities in endothelial cells in response to potent inflammatory mediators such as LPS, HMGB1 and thrombin, suggests that α1-PI does not have direct receptor-mediated barrier protective activities and that its protective effect is primarily mediated through the serpin inhibiting the proteolytic activity of neutrophil elastases which can cause severe tissue damage at sites of inflammation. Unlike AT, α1-PI has a canonical RCL that optimally fits into the catalytic pocket of the neutrophil elastase (3, 18), thereby rapidly inhibiting the protease with a diffusion-limited rate constant independent of a cofactor. The basis for the slow reactivity of α1-PI with coagulation proteases is the presence of a Met residue instead of an Arg (as in AT) at the P1 position of the RCL on the protease recognition site of the serpin (22). This residue modulates the specificity of α1-PI with coagulation proteases and is responsible for the inability of the serpin to have a major influence on the regulation of haemostatic pathways during activation of coagulation and inflammation. In support of this hypothesis, substitution of P1-Met of α1-PI with Arg by recombinant DNA methods or its occurrence in the natural Pittsburgh variant of α1-PI dramatically alters the reactivity of the mutant serpin with thrombin, thus improving it by more than 3-4 orders of magnitude and explaining the fatal bleeding tendency of a patient carrying this mutation (34-36). The optimal canonical conformation of α1-PI has prompted investigators to employ this serpin as a starting template for developing potent antithrombotic molecules by altering the RCL residues of the serpin to optimise its interaction with thrombin (36). Results presented in this study now suggest that it is possible to use similar strategies and develop α1-PI derivatives which have altered signalling specificity and can directly elicit protective intracellular signalling activities in response to inflammatory stimuli. Such a serpin derivative having normal anti-elastase activity and direct vascular protective property may have excellent therapeutic utility in treating inflammatory disorders like severe sepsis and acute respiratory distress syndrome where neutrophils can play important roles in causing severe tissue damage and inflammation. It should be noted that glycosylation of α1-PI is required for its stability in plasma (36, 37) and the serpin chimera produced in bacteria is likely to have a short plasma half-life. Thus, suitable mammalian expression systems, capable of proper protein glycosylation, need to be developed to prepare the α1-PI/D-helix chimera to evaluate its therapeutic utility in inflammatory disorders.

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Conflicts of interests
None declared.

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