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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 signaling responses. By contrast to AT, α_1 -proteinase inhibitor (α_1 -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 α_1 -PI, we replaced the D-helix of human α_1 -PI with the corresponding sequence of human AT and expressed the chimeric serpin (α_1 -PI/D-helix) in a bacterial expression system. High molecular weight heparin bound to α_1 -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, α_1 -PI/D-helix exhibited antiinflammatory properties in both cellular and animal models. Thus, α_1 -PI/D-helix inhibited the barrier-disruptive effect of proinflammatory cytokines and inhibited the activation of NF- κ B transcription factor in LPS-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 α_1 -PI confers antiinflammatory properties on the serpin and that the chimeric serpin may have therapeutic utility for treating inflammatory disorders.

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

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Disclosure of conflict of interests

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pocket of target serine proteases through a P1-Arg residue present on its reactive center 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 2 to 4 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 Ca^{2+} 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 antiinflammatory signaling 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 hypothesized 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 signaling 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 ischemia/reperfusion injury models (10–12,14–16). Protective signaling activity of AT has been demonstrated to effectively inhibit NF- κ B 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, α_1 -proteinase inhibitor (α_1 -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). α_1 -PI exhibits poor reactivity with coagulation proteases and possesses no HSPG-dependent vascular protective signaling activity. To determine whether the antiinflammatory signaling specificity of AT can be transferred to α_1 -PI, we replaced the heparin binding D-helix of α_1 -PI with the corresponding loop of AT by recombinant DNA methods. Characterization of the chimeric serpin indicates that similar to AT, the α_1 -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 α_1 -PI

The cDNA coding for human α_1 -PI was cloned in the *E. coli* expression/purification vector, pQE-30 with a His tag, and expressed in *Escherichia coli* SG13009 cells (Qiagen, Valencia, CA) as described (20,21). The α_1 -PI chimera (α_1 -PI/D-helix) in which the D-helix sequence of the serpin (⁸³NLTEIPEAQIHEGFQELLRTL-NQPDSQ¹⁰⁹) (22) was replaced with the corresponding D-helix sequence of human AT (¹¹²SEKTSQIHFFFAKLNCRLYRKANKS¹³⁷) was prepared by PCR methods and expressed using the same expression/purification vector system. An α_1 -PI/D-helix mutant was constructed in which Lys-114 of D-helix was replaced with Glu (α_1 -PI/D-helix-K114E). The P1-Met to Trp mutants of α_1 -PI in the background of either wild-type (α_1 -PI-Trp) or α_1 -PI/D-helix (α_1 -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 α_1 -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). 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 dialyzed 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) 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⁻¹cm⁻¹ for α_1 -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) 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 stoichiometric 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). The chromogenic substrate for elastase (MeOSuc-AAPV-pNA) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). Human plasma-derived proteins thrombin, FXa and antithrombin were purchased from Haematologic Technologies (Essex Junction, VT) and porcine pancreatic elastase was from Worthington Biochemical Corp. (Lakewood, NJ).

Kinetic characterization

The inhibitory activities of α_1 -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 α_1 -PI and increasing concentrations of heparin (0–100 μ gM) for 30–120 min at room temperature in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 and 2.5 mM Ca^{2+} (TBS/ Ca^{2+}) containing 0.1 mg/mL BSA and 0.1% PEG-8000. All reactions were carried out in 50 μ L volumes in 96-well plates and at different time points, 50 μ L 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). Observed 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 3 independent measurements $\pm$ S.D.

Fluorescence measurements

Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY) 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 μ L of high concentration of stock solution of unfractionated therapeutic heparin into 250 nM of wild-type α_1 -PI or α_1 -PI derivatives containing a Trp at P1 positions (α_1 -PI-Trp and α_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 3 experiments were analyzed 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 α_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) were cultured to confluence in a humidified atmosphere at 37°C in Dulbecco modified eagle medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) 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 α_1 -PI) (0.67–5.0 μ M for 4h) 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 μ m pore size and 12-mm diameter) for 2 days. The confluent monolayers were then incubated with different concentrations of serpin derivatives (AT, α_1 -PI or α_1 -PI/D-helix) in serum free growth media containing 0.5% BSA for 4h followed by stimulation of by cells by either thrombin (10 nM) for 10 min or HMGB1 (40 μ g/mL for 16h) as described (26,27). Inserts were washed with PBS, pH 7.4 before

adding 0.5 mL Evans blue (0.67 mg/mL) (Sigma, St. Louis, MO) 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 colorimetric kit (Genescript) according to the manufacturer's instruction. Briefly, EA.hy926 endothelial cells were first incubated with serpin derivatives (1–5 μ M for 4h) followed by their treatment with lipopolysaccharide (LPS) (10 ng/mL) for 16h. 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 μ L of the cell lysate was incubated on ice for 5 min before centrifugation at $10,000 \times g$ 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- κ B and IL-6 ELISA

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

In vivo permeability and leukocyte migration assays

Eight to ten weeks old male C57bl/6 mice were used for in vivo studies after a 5-day acclimatization 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 μ L of each serpin (0.01–0.04 mg/g body weight) or 200 μ L of normal saline as a control. After 3h, 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 μ g/g bodyweight) or 0.7% acetic acid as a positive control. Thirty minutes later, mice were sacrificed, and peritoneal exudates were collected (after being washed with 5 mL of normal saline) and centrifuged at $200 \times g$ for 10 min. The absorbance of the supernatant was read at 650 nm. The vascular permeability was expressed in terms of dye (μ g/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. Four hours later, mice were sacrificed and peritoneal surfaces were washed with 5 mL of normal saline. Next, 20 μ L 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) 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 3 different mice.

Survival rate

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

Statistical analysis

Data were expressed as means $\pm$ S.D. 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 α_1 -PI/D-helix

Thrombin reacts slowly with α_1 -PI both in the absence and presence of heparin. The concentration-dependence of α_1 -PI inhibition of thrombin yielded second-order rate constants (k_2) of $4 \times 10^1 \text{ M}^{-1}\text{s}^{-1}$ and $6 \times 10^1 \text{ M}^{-1}\text{s}^{-1}$ for the serpin inhibition of the protease in the absence and presence of therapeutic high molecular weight heparin, respectively (Fig. 1A). The α_1 -PI/D-helix chimera exhibited a slightly lower k_2 value of $1 \times 10^1 \text{ M}^{-1}\text{s}^{-1}$ with thrombin in the absence of heparin. However, heparin accelerated the reactivity of the chimeric serpin with thrombin ~ 100 -fold ($k_2 = 1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$), suggesting that grafting D-helix of AT to α_1 -PI renders the chimeric serpin capable of interacting with heparin (Fig. 1A). Analysis of inhibition rate constants of porcine pancreatic elastase by α_1 -PI and α_1 -PI/D-helix ($7.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $2.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, respectively) suggested that grafting D-helix of AT on α_1 -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 utilized to evaluate affinities of polysaccharides for interaction with the serpin (25). However, heparin titration experiments with α_1 -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 α_1 -PI and α_1 -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 K_D of $\sim 84 \pm 10$ nM for the interaction (Fig. 1B). In contrast to α_1 -PI/D-helix-Trp, heparin titration of α_1 -PI-Trp did not result in any change in intrinsic fluorescence (Fig. 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 K_D 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-catalyzed 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 α_1 -PI/D-helix is presented in Fig. 1C. Similar to heparin-catalyzed AT inhibition of thrombin, α_1 -PI/D-helix inhibition rates exhibited a bell-shaped dependence on heparin concentration (Fig. 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 α_1 -PI/D-helix inhibition of an exosite II mutant of thrombin (thrombin-3A) (Fig. 1C, empty squares) which has been shown to not interact with heparin (29).

The reactivity of α_1 -PI derivatives with FXa was also evaluated in both the absence and presence of heparin. Interestingly, heparin accelerated the reactivity of wild-type α_1 -PI with FXa ~ 5 -fold, thus yielding k_2 values of $4.6 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ and $2.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ in the absence and presence of heparin respectively. On the other hand, heparin accelerated the inhibition of FXa by α_1 -PI/D-helix ~ 37 -fold ($k_2 = 1.6 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ and $6.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ in the absence and presence of heparin, respectively). The concentration-dependence of FXa inhibition by α_1 -PI and α_1 -PI/D-helix revealed that while the heparin-catalyzed inhibition of FXa by α_1 -PI is saturable, the inhibition rates with α_1 -PI/D-helix exhibit a bell-shaped dependence on heparin concentration (Fig. 1D). These results suggest that, similar to thrombin, heparin accelerates the α_1 -PI/D-helix inhibition of FXa by a template mechanism. The mechanism by which heparin accelerates the α_1 -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.

α_1 -PI/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 (Fig. 2A shown for thrombin only). Unlike AT, pretreatment of endothelial cells with α_1 -PI did not protect cells from the barrier disruptive effect of either thrombin (Fig. 2B) or HMGB1

(Fig. 2C), however, similar to AT, α_1 -PI/D-helix exerts a protective effect thus reversing proinflammatory effects of both mediators by a concentration-dependent manner (Fig. 2B,C). These results suggest that binding of D-helix of AT to endothelial HSPGs is responsible for protective effects of the serpin and that grafting it to α_1 -PI endows a similar protective function on the chimeric serpin.

α_1 -PI/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 (Fig. 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 (Fig. 3A). By contrast to AT, both wild-type α_1 -PI and the α_1 -PI/D-helix chimera exhibited a cytoprotective activity in LPS-stimulated endothelial cells (Fig. 3B). Analysis of the concentration dependence of protective effects indicated that α_1 -PI/D-helix is markedly more potent than wild-type α_1 -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 observed 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 α_1 -PI/D-helix inhibit LPS-mediated NF- κ B activation in endothelial cells

AT inhibited the activation of NF- κ B in endothelial cells in response to LPS by a concentration dependent manner as has been reported (Fig. 3C) (12). While wild-type α_1 -PI exhibited no modulatory effect on the NF- κ B pathway in response to LPS in endothelial cells, the α_1 -PI/D-helix chimera inhibited LPS-mediated activation of the nuclear transcription factor to an extent similar to that observed with AT (Fig. 3D). Similar to AT, α_1 -PI/D-helix but not wild-type α_1 -PI inhibited thrombin-mediated synthesis of IL-6 in endothelial cells (Fig. 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 α_1 -PI/D-helix chimera, we replaced this residue with Glu and evaluated properties of the chimeric serpin mutant in signaling assays. Similar to AT-K114E (12), the barrier protective activity of the K114E mutant of α_1 -PI/D-helix in response to thrombin was markedly decreased (Fig. 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 (Fig. 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 α_1 -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 μ g/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 (Fig. 5A). While α_1 -PI exhibited no barrier protective activity in this model, α_1 -PI/D-helix protected vasculature from the disruptive effect of HMGB1 (Fig. 5B). At an optimal concentration of 0.04 mg/g both AT and α_1 -PI/D-helix exhibited similar maximal protective activities, though at lower concentrations, AT exhibited slightly higher potency (Fig. 5A,B). Similarly, both AT and α_1 -PI/D-helix, but not α_1 -PI, markedly enhanced HMGB1-mediated binding of leukocytes to the vascular endothelium and their subsequent migration to the peritoneal cavity (Fig. 5C,D), suggesting that interaction of D-helix of AT with cell surface HSPGs is solely responsible for the antiinflammatory 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 Fig. 6. Relative to negative control (A), both CMC-Na (B) and HMGB1 (C) recruited inflammatory cells into peritoneal tissues of experimental animals. AT effectively inhibited infiltration of inflammatory cells into the peritoneal tissues of HMGB1-treated mice (Fig. 6D). However, α_1 -PI did not have any detectable effect on the HMGB1-mediated recruitment of inflammatory cells into peritoneal tissues (Fig. 6E). Consistent with all other data presented above, α_1 -PI/D-helix exhibited a potent protective effect and effectively inhibited the migration of inflammatory immune cells into peritoneal tissues in response to HMGB1 (Fig. 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 5 days. There was a 60% survival rate for the LPS-treated group (10 mice) in this model (Fig. 7). The same survival rate was obtained with the wild-type α_1 -PI-treated group (Fig. 7), thus 4 mice from both groups were dead after 3 days. By contrast only one mouse from the α_1 -PI/D-helix treated and 2 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 (Fig. 7). Given the modest mortality rate with untreated mice and the limited sample size, the observed beneficial effects with AT and α_1 -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 α_1 -PI endows antiinflammatory properties on the chimeric serpin, thus enabling it to interact with cell surface HSPGs and elicit protective signaling 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. α_1 -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- κ B and down-regulating the expression of cell adhesion molecules in response to proinflammatory stimuli (10–12,14–16). The results presented above suggest that α_1 -PI/D-helix can exert a protective effect through the same mechanism. The comparable efficacies of both AT and α_1 -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 signaling 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 signaling 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 signaling 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 signaling in these two alternative pathways. Thus, it has been found that neutralization of the basic charge of Lys-114 in the native AT 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 similarly 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 α_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 α_1 -PI/D-helix exhibited both barrier protective activity and anti-apoptotic activities, though with 2–3-fold lower efficiency (Fig. 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 α_1 -PI. Direct binding studies further support this proposal as evidenced by heparin binding to engineered D-helix of the α_1 -PI chimera with a K_D of 84 nM. Relative to AT which binds heparin with low-nanomolar affinity, the α_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 the 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 α_1 -PI.

It is worth noting that α_1 -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 α_1 -PI is the neutralization of active neutrophil elastases that are released by activated neutrophils at sites of infection and inflammation. The observation of this study that α_1 -PI is not capable of eliciting intracellular signaling activities in endothelial cells in response to potent inflammatory mediators such as LPS, HMGB1 and thrombin, suggests that α_1 -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, α_1 -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 α_1 -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 α_1 -PI with coagulation proteases and is responsible for the inability of the serpin to have a major influence on the regulation of hemostatic pathways during activation of coagulation and inflammation. In support of this hypothesis, substitution of P1-Met of α_1 -PI with Arg by

recombinant DNA methods or its occurrence in the natural Pittsburgh variant of α_1 -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 α_1 -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 optimize its interaction with thrombin (36). Results presented in this study now suggest that it is possible to use similar strategies and develop α_1 -PI derivatives which have altered signaling specificity and can directly elicit protective intracellular signaling 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 α_1 -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 α_1 -PI/D-helix chimera to evaluate its therapeutic utility in inflammatory disorders.

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Figure 1.

Dependence of rate constants for inhibition of thrombin on concentration of α_1 -PI derivatives in the absence and presence of heparin. A, k_{obs} values for the inhibition of thrombin (2 nM) by wild-type α_1 -PI in the absence (○) and presence (●) of heparin or by α_1 -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 α_1 -PI-Trp derivatives. The spectral changes were monitored by addition of 1–2 μL of a concentrated stock solution of heparin to 250 nM α_1 -PI-Trp (○) or α_1 -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 secondorder rate constants for inhibition of wild-type thrombin and the thrombin heparin-binding exosite II mutant (thrombin-3A) by α_1 -PI derivatives on heparin concentration. The same as panel A except that the inhibition of thrombin by a fixed concentration (250 nM) of either α_1 -PI (○) or α_1 -PI/D-helix (●) and of thrombin-3A by α_1 -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 α_1 -PI (○) or α_1 -PI/D-helix (●) on heparin concentration. The same as panel C except that the inhibition of FXa by α_1 -PI derivatives was monitored. All values are average of at least 3 independent measurements $\pm$ S.D.

Figure 2.

Effects of serpin derivatives on the barrier permeability function of endothelial cells in response to proinflammatory stimuli. A, cell permeability in response to thrombin was measured by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional endothelial cell monolayer in the absence and presence of increasing concentrations of AT as described in “Materials and methods”. B, the same as A except that cell permeability in response to thrombin was measured in the presence of increasing concentrations of either α_1 -PI or α_1 -PI/D-helix. C, the same as B except that cell permeability in response to HMGB1 was measured on cells pretreated with different concentrations of either α_1 -PI or α_1 -PI/D-helix. All results are shown as means $\pm$ S.D. of three different experiments. Control, cells incubated with growth media only (with or without serpin). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 3.

Analysis of cytoprotective and antiinflammatory effects of serpin derivatives. A, endothelial cells were incubated with different concentrations of AT (4h) followed by stimulation with LPS (16h) and measurement of the caspase-8 activity by a colorimetric assay. B, the same as A except that the caspase-8 activity of endothelial cells pretreated with either α₁-PI or α₁-PI/D-helix was measured. C, analysis of NF-κB activation in LPS-stimulated endothelial cells pretreated with different concentration of AT. D, the same as C except that NF-κB activation was measure on cells pretreated with either α₁-PI or α₁-PI/D-helix. E, analysis of IL-6 expression in thrombin-stimulated endothelial cells pretreated with different serpins (AT, α₁-PI or α₁-PI/D-helix). All results are shown as means ± SD of three different experiments. Control, cells incubated with growth media only (with or without serpin). * p < 0.05, ** p < 0.01.

Figure 4.

Analysis of barrier protective and cytoprotective effects of α_1 -PI/D-helix and α_1 -PI/D-helix-K114E serpins. A, cell permeability in response to thrombin was measured by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional endothelial cell monolayer in the absence and presence of different concentrations of either α_1 -PI/D-helix or α_1 -PI/D-helix-K114E. B, endothelial cells were incubated with different concentrations of the serpin mutants (4h) followed by stimulation with LPS (16h) and measurement of the caspase-8 activity by a colorimetric assay. Control, cells incubated with growth media only (with or without serpin). * $p < 0.05$, ** $p < 0.01$.

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 3h, 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, the same as A except that the vascular protective effect of α₁-PI or α₁-PI/D-helix in response to HMGB1 was monitored. C, the inhibitory effect of AT on the migration of leukocytes to peritoneal cavity in response to HMGB1 was analyzed. D, the 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.

Figure 6.

Inhibitory effect of serpin derivatives on infiltration of inflammatory cells into peritoneal tissues. Representative images of the peritoneal tissue histology (hematoxylin-eosin stain) are shown for the untreated negative control group (A), CMC-Na-treated positive control group (B), HMGB1-treated group (C), HMGB1 + AT treated group (D), HMGB1 + α_1 -PI treated group (E), and HMGB1 + α_1 -PI/D-helix treated group (F).

Figure 7.

Analysis of survival rates of LPS-treated mice. Each group (10 mice/group) were injected intraperitoneally with 15 mg/kg LPS in 200 μ L saline. The serpin derivatives (AT, α_1 -PI, or α_1 -PI/D-helix) (0.04 mg/g body weight) was injected through the same route 3h prior to administration of LPS. Mortality was assessed at days 1, 2, 3, 4 and 5. $p < 0.05$ for both α_1 -PI/D-helix and AT as compared to both non-treated control and α_1 -PI-treated groups.