Journal of Cellular Biochemistry 96:137–144 (2005)

Characterization of Serine/Cysteine Protease Inhibitor α_1-Antitripsin From Meconium-Instilled Rabbit Lungs

A.M. Zagariya,[1,3] R. Bhat,[1] E. Zhabotynsky,[3] G. Chari,[1] S. Navale,[3] Q. Xu,[2] T.A. Keiderling,[2] and D. Vidyasagar[1]*

[1]Department of Pediatrics, Division of Neonatology, The University of Illinois, Chicago, Illinois
[2]Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois
[3]Department of Pediatrics, M. Reese Hospital and Medical Center, Chicago, Illinois

Abstract We have recently purified from meconium-instilled rabbit lungs a novel serine protease inhibitor, with an apparent molecular mass of 50 kDa, which we assign to be α_1-antitripsin. We hypothesize that serpin may attenuate pulmonary inflammation and improve surfactant function after meconium aspiration. α_1-antitripsin is a member of the proteinase inhibitor (serpin) superfamily and inhibitor of neutrophil elastase, and it can be identified as a member of the family by its amino acid sequence due to the high degree of conserved residues. α_1-antitripsin is synthesized by epithelial cells, macrophages, monocytes, and neutrophils. Deficiency in α_1-antitripsin leads to exposure of lungs to uncontrolled proteolytic attack from neutrophil elastase or other damaging factors culminating in lung destruction and cell apoptosis. We hypothesize that accumulation of α_1-antitripsin in the lungs serves as a predisposed protection against meconium-induced lung injury. In this paper, we show how this knowledge can lead to the development of novel therapeutic approaches for treatment of MAS. J. Cell. Biochem. 96: 137–144, 2005.

Key words: meconium; lungs; serine; cysteine; proteases; apoptosis

Meconium aspiration syndrome (MAS) is a severe respiratory disease in full-term infants. It is still a frequent neonatal condition despite an intensive prevention strategy. It induces a pulmonary inflammation, cell apoptosis, and acute lung cell injury. MAS is a complex of events, leading to pulmonary complications of the newborn. Its treatment presently is limited to airway cleaning and ventilation support, which is surely not enough to achieve a significant success. In our preliminary experiments, we studied a solid content of meconium, its debris, as well as bile acids, lipids, polysaccharides, inflammatory cells, and even compliment in MAS, but so far detailed about the specific protein content is missing. The present study evaluated the protein content of the meconium-instilled newborn lungs and compared it with saline-instilled. We hypothesize that protein content is directly involved in meconium induced lung injury (Table I).

Serine/cysteine proteases are involved in protein degradation and inducing of apoptosis. They can be activated in response to endotoxin or inflammatory cytokines [Dickinson et al., 1995]. Usually, they play a therapeutic role, and apoptosis-induces caspases are not activated in the presence of one or more protease inhibitors [Ikari et al., 2001]. Most serpins have a similar amino acid sequence loop: Gly-Asp-Ser-Gly-Gly-Pro in the middle of the molecule, which can recognize target proteases in apoptosis [Grabarek et al., 2002]. A similar loop is found in chymotrypsin, trypsin, elastase, thrombin, and plasmin. Mutation in this loop results in loss of protease activity [White et al., 1978]. Cell survival was assured when serum-free medium was supplemented with serine protease inhibitors. In contrast, cells were sensitive to apoptosis when cultured in a medium containing serum from which protease inhibitors were

Grant sponsor: Thrasher Research Foundation.

*Correspondence to: Dr. D. Vidyasagar, MD, Division of Neonatology, Department of Pediatrics, The University of Illinois at Chicago, 840 South Wood Street, Chicago, IL 60612. E-mail: dsagar@uic.edu

Received 9 December 2004; Accepted 15 February 2005
DOI 10.1002/jcb.20492

TABLE I. Mass Spectroscopy of Different Areas of Protein

Mass (M)	Mass	Start	To	Peptide sequence
1,089.5	1,089.5	218	225	WERPFEVK
1,091.5	1,091.6	259	267	LSSWVLLMK
1,109.6	1,109.5	315	324	LSITGTYDLK
1,204.7	1,204.6	150	159	LVDKFLEDVK
1,219.7	1,219.6	258	267	KLSSWVLLMK
1,274.7	1,274.6	216	225	GKWERPFEVK
1,478.7	1,478.7	180	192	QINDYVEKGTQGK
1,640.8	1,640.8	50	63	ITPNLAEFAFSLYR
1,802.9	1,802.9	284	298	LGHLENELTHDIITK
1,862.7	1,862.8	226	241	DTEBEDFHVDQATTVK
2,089.0	2,089.0	199	215	ELDRDTVFALVNYIFFK
2,184.9	2,185.0	160	178	KLYHSEAFTVNFGDTEEAK
2,184.9	2,185.0	161	179	LYHSEAFTVNFGDTEEAKK

M/Z is a ratio of molecular mass to molecular charge. 1,640.87 is a very aromatic area of protein, which located between 50 and 63 amino acids from NH_2 tail.

removed [Ikari et al., 2001]. Antiapoptotic effect was proportional to protease inhibitory activity. Without inhibitors, extra cellular matrix was degraded and cells could not attach to the matrix. In the presence of a small amount of inhibitors, cells detached, but did not die [Ikari et al., 2001].

The α_1-antitripsin is the most abundant circulating protease inhibitor and the archetypal member of the serine protease inhibitors (serpins) superfamily [Dickinson et al., 1995]. The serpin superfamily is divided into 16 different classes, which are responsible for regulating a variety of proteolytic processes through a unique irreversible suicide substrate mechanism. Serpins are single chain (glyco-) proteins comprising about 400 amino acid residues. Physiological functions of serpins are not well understood, however, studies suggest their direct roles in disease formation. Serpins can inhibit apoptosis process [Bird et al., 1998; Buzza et al., 2001] and can be mediated by interleukins [Morgan and Kalsheker, 1997]. Cell survival is dramatically higher in the presence of serpins [Nakashio et al., 2000]. RNA of α_1-antitripsin is expressed in lung cells preferentially in cytoplasm. α_1-antitripsin is a highly polymorphic 50-kDa acute phase glycoprotein encoded by a single gene on the long arm of chromosome 14. It is synthesized primarily by monocytes reaching the lungs by passive diffusion. In addition, there is a local production of α_1-antitripsin within the lung by alveolar macrophages and epithelial cells. The main role of α_1-antitripsin is to protect the alveolar matrix from proteolytic attack by neutrophil elastase that has been liberated from activated neutrophils. It's function is a critical survival factor in apoptotic cells. But α_1-antitripsin after self-polymerization also can attenuate lung disease [Silverman et al., 2001]. The secondary structure of α_1-antitripsin is dominated by a five-stranded β-sheet, which supports a mobile reactive center loop [Silverman et al., 2001]. This loop presents key methionine-serine residues as bait for the target protease, neutrophil elastase. Proteases bind to this loop and cleave it. After that α_1-antitripsin becomes inactive. This binding distorts and inactivates protease that makes it significantly more degradable. The α_1-antitripsin/protease complex is then cleared by circulating macrophages.

In this study, we describe and characterize a novel 50 kDa protein, which was expressed in meconium-instilled lungs. We describe and identify this protein as a member of the serpin family, and provide structural data and analysis to show that it is α_1-antitripsin. It is thought that this novel protein might play a crucial role in embryogenesis and lung cell apoptosis through binding to serine/cysteine proteases in the lungs.

MATERIALS AND METHODS

Study Design

Two-weeks-old New Zealand white rabbit pups (Kuiper Rabbit Ranch, Gary, IN, USA) were used in the study. The animals were handled according to the National Institute of Health guidelines. Pups were housed before each experiment with the mother in stainless steel rabbit cages. The mothers were given regular Purina rabbit chow (Scientific Animal Feed Co., Arlington Heights, IL, USA). The Animal Care and Use Committee of Michael Reese Hospital, Chicago approved the experimental protocol.

Two groups of animals were studied (four rabbit pups in each group): Group I, meconium-instilled rabbits; Group II, saline-instilled rabbits.

Meconium solution was prepared according to previously published procedures [Zagariya et al., 2000]. In brief, first pass human meconium samples were obtained from full-term, healthy human neonates and pooled. Before each experiment, 1 g of fresh meconium was homogenized on ice in a blender with 9 ml of 0.9% NaCl to a 10% (weight/volume) final concentration and centrifuged at 5,000 rpm for 20 min (4°C). The supernatant was filtered using a glass filter and then sterilized by 0.2 μm filter filtration (both filters were from Millipore Co., Bedford, MA). This debris free supernatant of meconium solution was used in the study.

A control group (four rabbit pups) received normal saline. The experimental group (four rabbit pups) was instilled with 1.2-mg/kg meconium. Prior to lavage, rabbits were anesthetized by intraperitoneal injection of 10 mg/kg Ketamine and 1 mg/kg Xylazine. A small midline incision was made on the ventral aspect of the neck to expose the trachea, and 1.2 ml/kg of the 10% sterile meconium supernatant or an equivalent volume of 0.9% NaCl was instilled with a 1 ml syringe and a needle followed by a 5 ml bolus of air to disperse the meconium into the lungs. The skin incision was closed with 4-0 nylon suture, and the pups were allowed to breathe room air spontaneously. Pups were euthanized using Nembutal (100 mg/kg, i.p.) at 0, 2, 4, 8, and 24 h after meconium or saline injection. Immediately thereafter the chest was opened by a midline incision, lungs were isolated, and lung lavage was performed. The lavage fluid was used to study alveolar cell death.

Bronchoalveolar Lavage, Recovery of Lung Cells, and Histology

Bronchial lavage was performed on left lung only according to previously published procedures [Kang et al., 1996]. After i.p. Nembutal the lungs were removed, and split into two parts. The right lung ligated at the hilus and excised for a 2 μm microtome cuts preparations, which was used for immunohistochemistry. The left lung was used for isolation of lavage cells. Left lung was washed by 10 ml of normal saline three times. Aliquots were pooled together, centrifuged at 3,000 rpm for 5 min at room temperature, supernatant was discarded and cells were collected. After that cells were homogenized and lyzed in 10 mM of Tris-HCl (pH 7.6) in ice using UV sonicator and spin down to separate walls debris at 10,000 RMP. Protein supernatant was stored in the −80°C freezer in the presence of proteolysis inhibitor phemilmethylsulphonilsluorin (PMSF). The right lung was cannulated at the bronchus and instilled for 2 h with 4% formaldehyde in PBS at 20-cm H_2O constant pressure. The fixed tissue was washed in PBS three times for 15 min and embedded in paraffin. All lung sections were cut into 0.6 μm thickness and mounted on glass slides as previously described [Zagariya et al., 2000].

Basic staining with hematoxylin-eosin (H&E) of freshly resected lungs was performed and analyzed by light microscopy under ×20 and ×40 magnification.

Purification of the Enriched Protein From Meconium-Instilled Lungs

Total lung lavage protein extracts were prepared by grinding frozen lavage cells in liquid nitrogen, resuspending them in ice-cold buffer (20 mM Mes, pH 7.0, 50 mM NaCl, and 1 mM EDTA), and centrifuging them twice at 4°C at 21,000*g* for 15 min.

α_1-antitripsin was isolated using ammonium sulphate fractionation, acetone precipitation and Sephadex G-100 (Amersham Pharmacia Biotech, Uppsala, Sweden) column chromatography. After each purification step, protein purity was monitored by SDS-PAGE, and fractions corresponding 50 kDa were pulled and used for the next step. The latter step resulted in the complete separation of serine proteinase, α_1-antitripsin. This enzyme was purified to homogeneity on a Sephadex G100 column (2×50 cm^2), equilibrated with 0.05M sodium phosphate buffer at pH 7.0, with the enzyme eluted in 0.1M sodium chloride. The enzyme was collected in eight successive tubes (2.5 ml in each) and dialyzed overnight against 20 mM Tris-HCl, pH 7.5. The dialysate was concentrated using Centricon −30 (Amicon, Beverly, MA) and was stored at −80°C. The yield of the protein was about 0.3 mg/ml in a total volume of 20 ml (8 tubes × 2.5 ml). The dialysate was used for evaluation of the protein and its structure. The purity and molecular weight was determined by 10% SDS gel electrophoresis as described below.

SDS Gel Electrophoresis

Protein titrations were performed using a fluorescence spectrophotometer (F-3010, Hitachi Instruments, Inc., San Jose, CA) with a 500 nm wavelength. Assays were performed in assay buffer (50 mM Tris-HCl, 20 mM $CaCl_2$, 100 mM NaCl). A final concentration of serpin was determined by using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) using the Bradford method [Bradford, 1976].

Sodium dodecyl sulfate (SDS) homogenates were prepared from the eight samples as described earlier [Zhou et al., 1992]. Briefly, equal amounts of purified protein from each sample (10 μg) were mixed with two times gel loading buffer (4% SDS, 20% Glycerol, 120 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue, 2% β-mercaptoethanol), heated to 95°C for 5 min and separated by 10% SDS gel electrophoresis at constant voltage of 100 V according to the method of Laemmli [1970]. The running buffer (pH 8.3) was 25 mM Tris-base, 250 mM glycine, and 0.1% SDS. Protein bands were visualized after staining in a solution containing 0.25% Coumassie Brilliant Blue R-250, 45% methanol, and 10% acetic acid.

For protein sequencing, 100 pmol of the 50 kDa protein, excised from the gel, was incubated at 37°C for 30 min, followed by the addition of 3,4-dichloroisocoumarin or PMSF to a final concentration of 1 mM to neutralize uninhibited elastase. The reaction mixture was subsequently desalted and washed by centrifugation on a ProSpin sample preparation cartridge (Perkin-Elmer/ABI, Foster City, CA), and sequenced in a Beckman LF3000 protein sequencer (Rockefeller University, New York, NY).

Fourier Transform Infrared (FTIR) Spectroscopy

A pre-exchanged (H/D) protein sample was dissolved directly into 10 mM phosphate (D_2O) buffer at resulting on a protein solution with $pD = 7.4$. This solution was transferred to a demountable homemade cell composed of two CaF_2 windows separated by a 50 μm spacer sealed in a circular brass mount. The IR absorption spectra were recorded at a nominal resolution of 4 cm^{-1} as an average of 940 scans using a Digilab FTS-60A FTIR spectrometer. For the temperature variation studies, the sample cells were tightly fit into a homemade double-walled brass jacket, which was temperature controlled with a Neslab RTE-7 water bath. A thermocouple placed in the outer jacket of the cell was used to regulate the bath.

DATA ANALYSIS

We compared our measurements for multiple groups of rabbits using ANOVA. Results of each parameter within a group were expressed as mean ± standard deviations. Paired evaluations were made for experimental and control groups, and the significance was determined. Statistical significance was taken as $P < 0.05$.

RESULTS

In this paper we evaluated a protein of about 50 kDa from the lung lavage after meconium-induced lung injury. This protein was collected in four independent experiments and its presence in meconium-instilled lungs is statistically significant (ANOVA $P < 0.05$). After repeated evaluations we here describe results of an analysis and evaluation of purification and structural data for this protein.

Fractionation of Lung Lavage Proteins From Meconium- and Saline-Instilled Rabbits

Using 10% SDS gel electrophoresis, we found enrichment of the 50 kDa protein fraction in meconium-instilled rabbit lungs compared to saline-instilled lungs (Fig. 1, top panel. Lane 1, saline-instilled lungs. Lane 2, meconium-instilled lungs). We performed a detailed analysis and characterization of the 50 kDa protein is mentioned. For this purpose, we purified this protein on Sephadex G100 (Fig. 1, bottom panel). The protein was relatively stable in water and saline. Eluted protein was seen in the SDS gel as two slightly separated protein bands that move together corresponding to a molecular weight of about 50 kDa.

Different fractions were compared using 10% SDS gel electrophoresis, as shown in Figure 1. Finally, purified protein was judged to correspond to two bands close to each other with a molecular mass of about 50 kDa. These two bands always co-fractionated during the purification process and appeared to represent the same protein possibly with different degrees of glycosylation. When protein was heat-inactivated to 85°C, its structure was changed irreversibly.

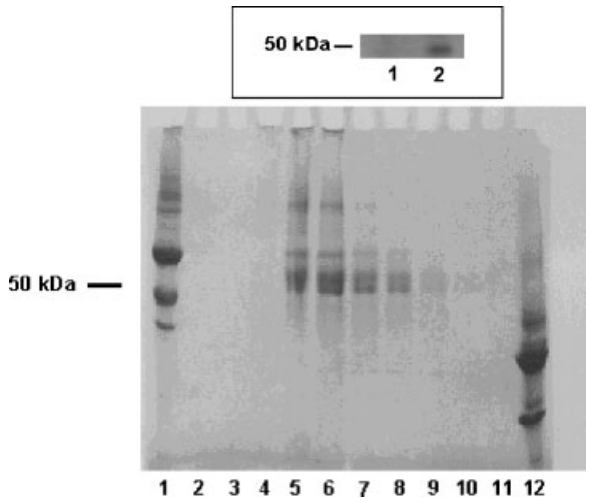

Fig. 1. Enrichment of meconium-instilled lungs by 50 kDa protein (top panel, lane 2), compared to saline-instilled lungs (top panel, lane 1) as seen in 10% SDS gel Sephadex G100 gel filtration of lung lavage proteins from meconium and saline-instilled rabbits (bottom panel, lanes 1–12). Protein lyzate was loaded on the Sephadex column in normal saline and collected in several eluants (2.5 ml in each). Every fifth fraction was analyzed in 10% SDS gel. Protein of interest (50 kDa) was eluted in several fractions (lanes 5–8), with maximal concentration 0.3 μg/ml in line 6. Then it was dialyzed against water and concentrated using Amicon Concentrators. Lanes 1 and 12 are mixture of reduced standard proteins. Lane 1 is a high molecular weight marker, include phosphorylase b (94 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa). Lane 2 is a low molecular weight marker, includes carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

Results of Protein Sequencing Analysis

The 50 kDa band, enriched in meconium-instilled lungs was cut out of the gel and sequenced at the Rockefeller University Sequence Core Facility. Then an amino acid homology search was performed using a protein sequence database (NBRF). The sequence analysis results demonstrate that our 50 kDa protein is most likely a serine/cysteine proteinase inhibitor, α_1-antitripsin. To identify in detail the sequence of our serpin, we screened the genomic sequence database of GenBank™ using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST/).

Using a combination of gene prediction programs, MetaGene (www.ares.ifrc.mcw.edu/MetaGene/) and simple pairwise BLAST, alignments between the 50 kDa protein and known serpin genes containing at least seven exons were identified. It is possible that calculated molecular weight for certain proteins can be smaller then the weight identified by gel electrophoresis [Zhou et al., 1992]. Because serpins are glycoproteins, this difference can be attributed to glycosylation of the native protein at four potential sites, which were located with two consensus sequences. These are theoretical sites and not confirmed by experiment. The translated amino acid sequence of this meconium-induced serpin was compared to other proteins and serpins. The sequence identity analysis is present in Figure 2.

Figure 3 shows the temperature variation of the FTIR spectra of protein in the amide I region. At $T = 5^{\circ}C$, an amide I peak centered at 1,630 cm^{-1} is observed, which is consistent with mainly β-sheet conformation [Stuart, 2000]. The shoulder at ~1,590 cm^{-1} is due to COO^{-} groups on the residue side-chains. With

Protein Information and Sequence Analyze Tools:	%	kDa
15300686 ref XP 028358.2 serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitripsin	32	40.46

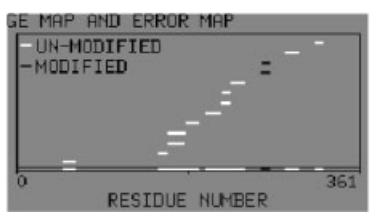

Fig. 2. Sequence of 50 kDa serine/cysteine proteinase inhibitor. It shows that observed serine/cysteine proteinase inhibitor is a α1-antitripsin. It is a 361 amino acids protein (top panel). Note two modified amino acids present in the studied inhibitor in the area of about 300 amino acids from NH_2 tail.

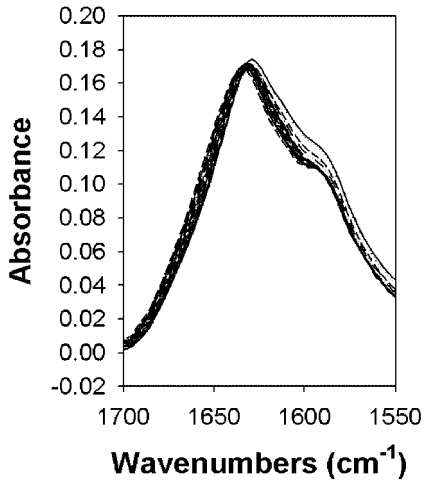

Fig. 3. Temperature dependent Fourier transform infrared (FTIR) spectra in the amide I region for protein in 10 mM phosphate buffer (pH 7.8).

increase of temperature, a peak shift to somewhat higher wave number is observed with only a small intensity decrease. At $T = 85°C$, the amide I is $\sim$1,635.5 cm^{-1}, which could still be due to β-sheet conformation with some small degree of denaturing of the native state (coil formation), but no aggregation peak is observed as high as 85°C. When the temperature is decreased from 85 to 5°C, the original spectrum is recovered, indicating that the thermal change was reversible.

Factor analysis of sets of spectra for both temperature increase and decrease were carried out [Baumruk et al., 1996]. We will focus here on the second and third subspectra with their corresponding temperature dependent loadings, which are presented in Figure 4A,B, respectively.

The first spectral component is basically the average of all spectra measured, and changes a little in loading, while the fourth is primarily noise. The second spectral component (Fig. 4A) accounts for the amide I band shift, which is due to the growth of a peak at $\sim$1,648 cm^{-1}, which could be due to increased random coil conformation at higher temperatures. The corresponding loading indicates a roughly linear change with temperature instead of a sigmoidal curve, which suggests that this change is far from a complete denaturation, and the overlapped values for temperature increase (open rectangle) and decrease (filled circle) demonstrate that it is reversible. The third spectral component loading (Fig. 4B) suggests the formation of an intermediate, as temperature increases with the β-sheet decreasing.

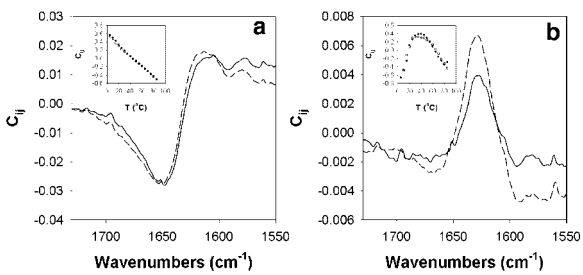

Fig. 4. The factor analysis results for FTIR thermal denaturation of protein. **A**: Second and (**B**) third spectral components with temperature increase (dash) and decrease (solid). Their corresponding loadings are shown as insets with temperature increase (open) and decrease (filled).

DISCUSSION

We hypothesize that α_1-antitripsin expression is a self-response of the lung to meconium. In the presence of α_1-antitripsin, lung cells are less accessible to apoptosis as observed by reduced cell death. By contrast, our preliminary data did not show a direct impact of lipopolysaccharides, bile acids, and carbohydrates on the apoptotic process. On this basis, we decided to evaluate the importance of the protein fraction in generating meconium-induced apoptosis.

Activation of proteases can play an important role in apoptotic cell death, and their inhibitors can significantly prevent it. These effects are associated with protection of extra cellular matrices from being degraded by cell-derived proteases. Also, in the presence of such inhibitors, internucleosomal DNA ladder formation in apoptosis was not observed [Kim et al., 2001]. It is unclear how the apoptotic signal is transmitted to protease inhibitors to activate their effect. It is clear that a broad spectrum of proteases have apoptotic activity, and it is reasonable to assume that many protease inhibitors are potentially antiapoptotic factors, acting via a mechanism similar to what we have described in the present work.

Results of our work show that α_1-antitripsin, a serine/cysteine proteinase inhibitor or serpin may play an important role in the response of newborn rabbit lungs to injury by meconium. This conclusion is also based on our preliminary data about the blocking of cell apoptosis by pretreatment of human A549 cells with a very low 5 μM dose of α_1-antitripsin (unpublished

data). It is also possible that some other types of proteinases are involved in meconium-induced injury as well.

Cysteine proteases are a class of proteases which are able to degrade elastin. Serpins regulate proteolytic events by binding to target proteases with a 1:1 stoichiometry to form stable and denaturation-resistant inactive complexes [Potempa et al., 1994]. There is a growing evidence that after docking with its target protease, the reactive loop of the serpin is cleaved and fully inserts into a β-sheet, breaking the stabilizing hydrogen bonds and forming strong covalent link with protease. This would then swing a still connected (suicide inhibited) protease from the upper to the lower pole of the protein [Stratikos and Gettings, 1999]. The serpin inhibitory mechanism depends on the flexibility of the reactive loop. Recent studies suggest that this mechanism involves several steps between the formation of the initial Michaelis complex and that of the irreversible final complex [Meller et al., 1998].

Self-polymerization of α_1-antitripsin can cause a significant lung injury. Polymerization would not only diminish the anti-protease shield but may also lead to recruitment of neutrophils to increase lung inflammation and injury. As a result of polymerization, inclusion bodies could form in the lung cells. Polymerization of serpins then could rapidly lead to a plasma deficiency of α_1-antitrypsin. This would expose lung tissue to uncontrolled proteolytic attack from neutrophil elastase, culminating in alveolar destruction [Parfrey et al., 2003].

CONCLUSION

We found that α1-antitripsin is expressed in meconium-instilled rabbit lungs as a self-defense against acute lung injury and lung cell apoptosis. We found that α1-antitripsin molecule appears to be very stable to degradation and to the heat denaturation. The α1-antitripsin has two modification sites about 300 amino acids from sequence start site (from the NH_2 terminus). The structure of the α_1-antitripsin molecule has a predominantly β-conformation. This novel activity in meconium-instilled lungs may suggest that protease inhibitors could play a protective role in lung disease. Additional experiments are needed to determine whether blocking α_1-antitripsin will increase lung damage and lung cell apoptosis. The answers to this question should clarify the mechanism of meconium-induced lung injury in a newborn and may lead to novel therapeutic approaches for the treatment of MAS.

REFERENCES

Baumruk V, Pancoska P, Keiderling TA. 1996. Predictions of secondary structure using statistical analyses of electronic and vibrational circular dichroism and fourier transform infrared spectra of proteins in H_2O. J Mol Biol 259:774–791.

Bird CH, Sutton VR, Sun J, Hirst CE, Novak A, Kumar S, Trapani JA, Bird PI. 1998. Selective regulation of apoptosis: The cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. Mol Cell Biol 18:6387–6398.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Buzza MS, Hirst CE, Bird CH, Hosking P, McKendrick J, Bird PI. 2001. The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses. Cell Immunol 210:21–29.

Dickinson JL, Bates EJ, Ferrante A, Antalis TM. 1995. Plasminogen activator inhibitor type 2 inhibits tumor necrosis alpha-induced apoptosis. Evidence for an alternate biological function. J Biol Chem 270(46):27894–27904.

Grabarek J, Du L, Johnson GL, Lee BW, Login DJ. 2002. Sequential activation of caspases and serine proteases (serpases) during apoptosis. Cell Cycle 1(2):124–131.

Ikari Y, Mulvihil E, Schwartz SM. 2001. α1-proteinase inhibitos, α1-antichymotrypsis, and α2-macroglobylin are the antiapototic factors of vascular smooth muscle cells. J Biol Chem 276(15):11798–11803.

Kang BU, Manderschield BD, Huang YCT, Crapo JD, Chang Li Y. 1996. Contrasting response of lung parenchimal cells to instilled TNFα and INFγ: The inducibility of specific cell ICAM-1 in vivo. Am J Resp Cell Mol Biol 15:540–550.

Kim R, Inoue H, Tabane K, Toge T. 2001. Effect of inhibitors of cysteine and serine proteases in anticancer drug-induced apoptosis in gastric cancer cells. Int J Oncol 18(6):1227–1232.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(259):680.

Meller P, Boudier C, Mely Y, Bieth JG. 1998. DNA strongly impairs the inhibition of cathepsin G by alpha(1)-antichymotrypsin and alpha(1)-proteinase inhibitor. J Biol Chem 273:9119–9123.

Morgan K, Kalsheker NA. 1997. Regulation of the serine protease inhibitor (SERPIN) gene α1-antitrypsin: A paradigm for other SERPINs. Int J Biochem Cell Biol 29(12): 1501–1511.

Nakashio A, Fujita N, Rokydai S, Sato S, Tsuruo T. 2000. Prevention of Phosphatidylinositol 3′-Kinase-akt survival signaling pathway during topotecan-induced apoptosis. Cancer Res 60(18):5303.

Parfrey H, Mahadeva R, Lomas DA. 2003. α_1-antitrypsin deficiency, liver disease and emphysema. Int J Biochem Cell Biol 35(7):1009–1014.

Potempa J, Korzus E, Travis JJ. 1994. The serpin superfamily of proteinase inhibitors: Structure, function, and regulation. J Biol Chem 269:15957–15960.

Silverman GA, Bird PI, Carrell RW, Coughlin PB, Gettins PG, Irving IJ, Lomas AD, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnel P, Salvesen GS, Travis J, Whisstock JC. 2001. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem 276:33293–33296.

Stratikos E, Gettings PGW. 1999. Formation of the covalent serpin-proteinase complex involves translocation of the proteinase by more than 70 A and full insertion of the reactive center loop into beta-sheet A. Proc Natl Acad USA 96:4808–4813.

Stuart BH. 2000. Infrared spectroscopy of biological applications. In: Meyers RA, editor. Encyclopedia of analytical chemistry. Chichester: John Wiley & Sons Ltd. pp 529–559.

White A, Handler P, Smith EL, Hill RL, Lehman IR. 1978. Principles of biochemistry. Sixth edition. New York: McGraw-Hill Book Company.

Zagariya A, Bhat R, Uhal B, Navale S, Freidine M, Vidyasagar D. 2000. Cell death and lung cell histology in meconium aspirated newborn lungs. Eur J Ped 159: 819–826.

Zhou GX, Chao L, Chao J. 1992. Kallistatin: A novel human tissue kallikrein inhibitor. Purification, characterization, and reactive center sequence. J Biol Chem 267:25873–25880.