ELASTASE INHIBITOR

Characterization of the Human Elastase Inhibitor Molecule Associated with Monocytes, Macrophages, and Neutrophils

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Preservation of the integrity of local organ function requires a delicate balance of the activities of phagocytic cell proteinases and the action of proteinase inhibitors. Loss of this balance may be a major causative factor in the pathogenesis of asthma, chronic bronchitis, emphysema, sarcoidosis, respiratory distress syndromes, arthritis, and certain skin diseases. Ultimately, to monitor and manipulate the proteinase-proteinase inhibitor balance of human phagocytes within a pharmacological context will require that the relevant molecules be identified and their interactions defined at the molecular level.

Of the phagocytic cell proteinases, the quantitatively most important is the serine active site proteinase commonly called "neutrophil elastase." Neutrophil elastase is 218-amino acid glycosylated protein of known sequence (1) that is particularly abundant in human neutrophils (0.5% of total protein) and is also found in monocytes and macrophages (2–4). Neutrophil elastase is contained in granules and functions optimally at neutral pH; its multiple documented activities all involve extracellular action (5, 6). Elastase cleaves extracellular matrix proteins such as elastin, proteoglycans, fibronectin, type III and type IV collagen (7–10), and certain soluble proteins (11). It is required by neutrophils for their migration through cell barriers in vitro (12, 13).

The continuous action of elastase inhibitors in vivo is evident from the neutrophil turnover rate. Despite the fact that neutrophils enter most body sites, turnover of $\sim 10^{11}$ neutrophils (14) with a content of ~50 mg elastase (2) occurs daily in humans without evidence of uncontrolled tissue degradation. Attention has focused primarily on the prevalent soluble blood protein α1-antitrypsin (α1-AT)[1], which is a fast-acting elastase inhibitor in vitro. α1-AT enters extravascular sites and inhibits elastase in vivo, at least in some situations, since individuals with genetically reduced levels

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[1] *Abbreviations used in this paper:* α1-AT, α1-antitrypsin; DFP, diisopropylfluorophosphate; EI, elastase inhibitor of monocytes, neutrophils and macrophages; PAI 1, plasminogen activator inhibitor of endothelial cells, hepatoma cells, and platelets; PAI-2, plasminogen activator inhibitor of placenta and monocytes; PNGase F, peptide *N*-glycosidase F; PVDF, polyvinylidine difluoride.

(homozygous Z variant) are predisposed to develop pulmonary emphysema in the third or fourth decade of life due to uncontrolled elastase action (15, 16).

On the other hand, four individuals have been identified who totally lack α1-AT. Surprisingly, these individuals have had a disease course similar to that associated with reduced α1-AT levels, remaining relatively disease free and displaying no clinical evidence of uncontrolled elastase action at the local organ levels for two to three decades (17–20). The finding that α1-AT null/null individuals remain relatively disease free for two to three decades despite daily release of ~50 mg neutrophil elastase is strong evidence that molecules other than α1-AT are involved in physiological control of elastase activity.

A molecule fulfilling the requirements for a physiological regulator of neutrophil elastase activity has been detected in monocytes and neutrophils in several species. In humans, an endogenous protein-like elastase inhibitor was detected in the cytosolic fraction of blood leukocytes and lung macrophages (21). The cytosolic fraction from the macrophages prevented the degradation of tissue elastin when neutrophil granules were used as the source of elastase (22). Cytosolic proteins that inhibit elastase were identified and purified from horse blood leukocytes (23, 24), pig blood leukocytes (25), and bovine lung macrophages (26). In guinea pigs, an elastase inhibitor was detected in the extracellular fluid of peritoneal macrophages based on its ability to form a covalent complex with elastase (27). When the macrophages were lysed, larger quantities of the elastase inhibitor were detected. In the human system, the technique of complex formation was used to detect and quantify a prevalent, fast-acting elastase inhibitor in mature human monocytes and monocyte-like cells (28).

This work demonstrates the presence of high levels of elastase inhibitor molecules of identical apparent molecular weight in human monocytes, macrophages, and neutrophils, and in a monocyte-like cell line, U937. The elastase inhibitor has been purified from the monocyte-like cell line, and its composition and characteristics are presented.

Materials and Methods

Cells. Neutrophils and monocytes were purified from anticoagulated blood as described (28). Freshly isolated monocytes were examined and, also, monocytes were matured in culture for 6–7 d (28). Pulmonary macrophages were obtained by broncholavage (29) of healthy, nonsmoking volunteers with normal pulmonary function and no symptoms of chronic or acute (prior 2 wk) respiratory disease. Pulmonary macrophages were >95% viable (trypan blue exclusion), >95% positive for latex bead injestion, and contained <1% neutrophils. U937 human histiocytic lymphoma cells (30), donated by Dr. David Y. Liu in 1984 (28), were grown in RPMI 1640 medium or DMEM with 4.5 mg/ml glucose with 10% FCS and 50 μg/ml gentamycin. The lymphoblastoid cell line CEM (31) was grown in the latter medium with 100 μg/ml streptomycin and 100 U/ml penicillin. All cells were washed, preincubated in HBSS at ~22°C to release adsorbed α1-AT, and lysed as described (28).

Purification of Elastase Inhibitor. U937 cells from 12-liter cultures (1.8×10^{10} cells) grown by the Massachusetts Institute of Technology Cell Culture Center (Cambridge, MA) were washed twice at 4°C in PBS containing Ca^{2+}/Mg^{2+}. The cells at 2×10^7/ml in HBSS were incubated at ~22°C for 15 min; this treatment releases adsorbed α1-AT (27, 28). The cells were brought to 4°C and pelleted. Lysates (2.5×10^7 cells/ml) were prepared by extraction with 0.5% NP-40 in PBS for 4 min at ~22°C and 10 min at 4°C, and clarified by centrifugation in a Sorvall SS34 rotor (DuPont Co., Wilmington, DE) at 18,000 rpm for 30 min at 4°C.

In preliminary purification experiments, elastase inhibitor activity was lost concomitant with the formation of actin-containing precipitates. To avoid this loss, the cell lysates were immediately chromatographed on DNase-Sepharose, which specifically adsorbs actin (32).

The lysate (720 ml) was incubated with 180 ml DNase-Sepharose-6B equilibrated against PBS at 4°C in a roller bottle. The mixture was transferred to a 6.5-cm-diameter column, and the nonadherent fraction, together with a 0.8-column volume wash with 0.5% NP-40 in PBS, was stored at −70°C.

The DNase-nonadherent fraction (900 ml) was incubated with 30 ml Thiopropyl-Sepharose-6B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated against 0.5% NP-40, 10 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1 mM EDTA (NP-40/Tris/150-NaCl/EDTA) at ~22°C for 30 min. The mixture was transferred to a 3-cm column and washed sequentially with one column volume of NP-40/Tris/150-NaCl/EDTA, NP-40/Tris/500-NaCl, Tris/500-NaCl, and Tris/150-NaCl. The column was eluted with 50 mM mercaptoethanol in Tris/150-NaCl to yield a single 70-ml "Thiol-eluate" fraction.

The Thiol-eluate was applied at ~22°C to a 3.5-cm column of Phenyl-Sepharose-CL4B (70 ml; Pharmacia Fine Chemicals) equilibrated against 10 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1 mM mercaptoethanol (Tris/150-NaCl/ME). The nonadherent fraction was collected together with ~20 ml wash with Tris/150-NaCl/ME.

The Phenyl-nonadherent fraction (~110 ml) was diluted with 0.5 volume Tris/ME and applied at 4°C to a 2-cm column of 20 ml Matrex gel red A (crosslinked 5% agarose with covalently coupled dye; Amicon Corp., Danvers, MA) equilibrated against Tris/100-NaCl/ME. The nonadherent fraction (~180 ml), including one column volume wash with Tris/100-NaCl/ME, was collected, dialyzed against Tris/50-NaCl/ME for 3 h at 4°C, and stored at −70°C.

Portions (50 ml) of the dialyzed Red A-nonadherent fraction were filtered through 0.2-μm nylon membranes (Schleicher & Schuell, Inc., Keene, NH) and applied at 0.8 ml/min to the polymer-base, anion exchange, HPLC column DEAE-5PW (7.5 × 75 mm; Waters Associates, Milford, MA) equilibrated against Tris/50-NaCl/ME at ~22°C. The column was washed with equilibration buffer. To elute the elastase inhibitor, Tris/85-NaCl/ME was applied, and fractions absorbing at 280 nm were collected.

To concentrate the purified molecule, active fractions from 3–4 DEAE fractionations were pooled, diluted with Tris/ME, and reapplied to the DEAE-5PW column in Tris/50-NaCl/ME. A single active fraction of 1–2.5 ml was eluted with Tris/140-NaCl/ME.

Portions of the concentrated active DEAE-5PW fraction were chromatographed at 0.7 ml/min on the HPLC gel filtration resin Protein-Pak I-125 (Waters Associates) (two columns totalling 7.8 × 600 mm).

Compositional Analyses. Portions of concentrated active elastase inhibitor from DEAE-5PW were gel filtered (described above) in 50 mM NH_4HCO_3. The elastase inhibitor peak was pooled and lyophilized, and an aliquot was hydrolyzed in 6 N HCl at 110°C for 24 h. The amino acid composition was determined on a D-500 analyzer (Dionex Corp., Sunnyvale, CA). Cys/2 was determined as cysteic acid after performic acid oxidation. Protein concentration was calculated by integrating amino acid determinations. The carbohydrate content was determined by methanolysis of the lyophilized sample followed by gas-liquid chromatography of the per(trimethylsilyl) derivatives (33).

Laemmli SDS-PAGE. Cell fractions were separated by Laemmli SDS-electrophoresis (34) as described (28). For microgram amounts of polypeptides, the gels were stained with Coomassie brilliant blue (Schwartz/Mann Biotech, Cleveland, OH). When greater sensitivity was required, the polypeptides were "gold stained" after transfer to polyvinylidine difluoride (PVDF) membranes (0.45 μm; Millipore Continental Water Systems, Bedford, MA) (constant 70 mamps; 160 V/1.6 A power supply; Bio-Rad Laboratories, Richmond, CA) with 42 mM Tris/190 mM glycine buffer, pH 8.3, for 18 h at ~22°C (Transphor Cell; Hoefer Scientific Instruments, San Francisco, CA). The PVDF membranes were washed seven times with 0.1% Tween-20 (Janssen Products, Piscataway, NJ) in PBS (twice for 15 min; five times for 5 min) and twice with water, and were incubated with 0.2–0.3 ml/cm^2 of Aurodye protein stain (Janssen Products) at ~22°C for 4 h. Apparent M_r was determined by SDS-electrophoretic mobility (35) relative to previously described marker proteins (28).

Covalent Complex Assay. Elastase inhibitor activity was measured by incubating cell fractions (10–100 μl) with 30–200 ng of ^{125}I-labeled porcine pancreatic elastase (Elastin Products, Pacific, MO) at 37°C for 10 min (27, 28). The covalent elastase-elastase inhibitor com-

plex was detected by autoradiography after SDS-electrophoresis using the Fairbanks/Laemmli gel system. This system uses relatively low pH and low primary amine concentration to minimize hydrolysis of the complex during electrophoresis (28).

Elastinolysis Inhibition Assay. Elastase inhibitor fractions were preincubated with porcine pancreatic elastase in PBS for 5 min at 22°C and assayed for elastinolytic activity using a modified diffusion assay (36). 5-μl portions were incubated at 37°C in 3-mm wells of agar gels containing 0.24% fluorescein-elastin (400 mesh; Elastin Products). The diameters of the lysis rings surrounding the wells were measured after 48 h and compared with lysis rings of parallel standard elastase dilutions.

DNase-Sepharose. DNase I (bovine pancreas; 1,800 Kunitz U/mg protein; Sigma Chemical Co., St. Louis, MO) was treated with 2 mM diisopropylfluorophosphate (DFP) in PBS for 30 min at ~22°C, and was coupled at 3 mg/ml in 0.1 M $NaHCO_3$, pH 8.5, to Sepharose-6B activated by CNBr (37) (18 h at 4°C; >90% coupling efficiency). The resin was treated with 100 mM Tris-HCl buffer, pH 8.0, for 2 h at ~22°C; three times with 100 mM sodium acetate buffer, pH 4.0, followed by 100 mM $NaHCO_3$, pH 8.5; once with PBS; and once with 2 mM DFP in PBS at ~22°C for 30 min.

Results

Demonstration of Elastase Inhibitor in Monocytes, Macrophages, and Neutrophils. Elastase inhibitor activity can be detected in cell lysates by the ability of the molecule to form a covalent complex with ^{125}I-elastase, a reaction that is the basis of a semiquantitative assay (28). Elastase inhibitor activity was detected by this approach in human monocytes that had matured in culture and also in the monocyte-like cell line U937, but it was not detected in freshly isolated human monocytes or neutrophils (28). However, when the cells were incubated with the serine proteinase active site reagent DFP and were lysed in the presence of DFP, elastase inhibitor activity was readily detected in fresh monocytes as well as neutrophils (Fig. 1), but not in the lymphoblastoid cell line CEM (not shown). These findings indicate that the elastase inhibitor molecule is present in neutrophils and fresh monocytes, but its activity is rendered nondetectable in lysates by endogenous serine proteinase. The endogenous proteinase that prevents detection of elastase inhibitor activity may be elastase, which is present in neutrophils and fresh monocytes and decreases to negligible levels when monocytes mature (38). Elastase inhibitor activity was also detected in lysates of pulmonary macrophages, and DFP treatment was not required (Fig. 2). Taken together, these findings show that elastase inhibitor molecules with identical apparent molecular weight are present in human neutrophils, fresh monocytes, mature monocytes, macrophages, and in a monocyte-like cell line.

Isolation of Elastase Inhibitor. The U937 cells (12-liter cultures) were used as the

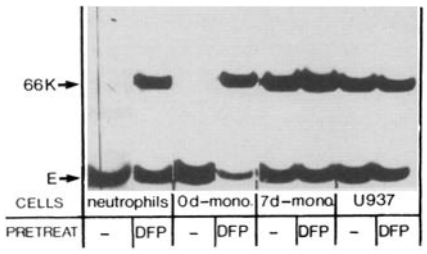

FIGURE 1. Detection of elastase inhibitor activity in monocytes and neutrophils by the covalent complex assay. Lysates of 1.5 × 10^6 neutrophils, freshly isolated monocytes (0-d monocytes), monocytes matured in culture (7-d monocytes), or U937 cells were incubated with ^{125}I-elastase. *DFP-pretreat,* cells incubated with DFP (2 mM in HBSS) and lysed in the presence of DFP (2 mM); unreacted DFP was removed by dialysis. Shown is an autoradiograph of a Fairbanks/Laemmli SDS electrophoresis gel. *E,* ^{125}I-elastase (M_r 26,000); *66K,* the ^{125}I-elastase-elastase inhibitor complex of M_r 66,000.

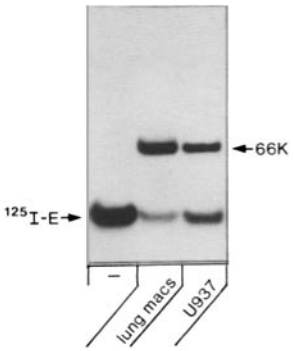

FIGURE 2. Detection of elastase inhibitor activity in pulmonary macrophages. Lysates of 1.5×10^6 pulmonary macrophages or U937 cells (examined as a control) were incubated with ^{125}I-elastase. Formation of the ^{125}I-elastase-elastase inhibitor complex is shown in the autoradiograph with details as in Fig. 1.

source of elastase inhibitor. An initial step, DNase-Sepharose, was developed to remove actin, which interferes with chromatography of the elastase inhibitor (see Materials and Methods). Actin adheres to DNase-Sepharose (32) (Fig. 3 *b*, *first and second lanes*), and elastase inhibitor activity is recovered with the bulk of cellular proteins in the nonadherent fraction (Figure 3 *a*, *first and second lanes*).

The active fraction was applied to the disulfide resin Thiopropyl-Sepharose. Elastase inhibitor adheres and is quantitatively eluted with mercaptoethanol (Fig. 3 *a*; *Thiol*). Since the bulk of proteins are nonadherent, Thiopropyl-Sepharose chromatography is a very effective purification step (Fig. 3 *b*, *compare second and third lanes*). The active fraction was further purified by Phenyl-Sepharose chromatography (nonadherent fraction) and Red A-agarose (nonadherent fraction) (Fig. 3, *a* and *b*).

The active fraction was then adsorbed (at 50 mM NaCl) on the HPLC anion ex-

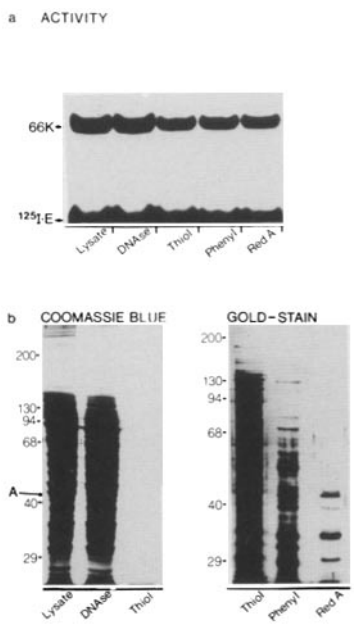

FIGURE 3. (*a*) Chromatography fractions from the purification assayed for elastase inhibitor by formation of the covalent complex with ^{125}I-elastase. The autoradiograph shows sequential chromatography fractions with volumes equivalent to 1.5×10^6 cells as follows: Lysate, the clarified U937 lysate; DNAse, the nonadherent fraction from DNase-Sepharose; Thiol, Thiopropyl-Sepharose eluate; Phenyl, the Phenyl-Sepharose nonadherent fraction; and Red A, the Red A-agarose nonadherent fraction. Details as in Fig. 1. All other chromatography fractions contained negligible activity. (*b*) The same active sequential chromatography fractions examined by protein staining of Laemmli SDS-electrophoresis gels. The left panel shows Coomassie blue staining. Actin (*A*; *arrow on left*) is removed by DNase chromatography. The right panel shows the more sensitive technique of gold staining of an electrophoretic transfer of the gel. The volumes of all fractions are equivalent to 1.5×10^6 cells. Note that the Thiol fraction was examined by both techniques.

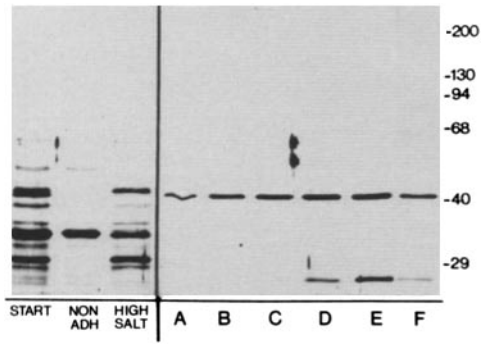

FIGURE 4. DEAE-HPLC fractions examined on a gold-stained transfer of a Laemmli SDS gel. The first three lanes show volumes of fractions equivalent to 1.5×10^6 cells. Start is the Red A fraction applied to the column; and Non-ad, the nonadherent fraction. (*A–F*) 10 μl of sequential fractions eluted with 85 mM NaCl; these contained all the elastase inhibitor activity (not shown). High salt is the inactive fraction released by 400 mM NaCl after recovery of the active fractions. The arrow on the left indicates the predominant polypeptide that fractionates in the active fractions; it is the faster-migrating band in an apparent doublet.

change resin DEAE-5PW. Application of 85 mM NaCl elutes an 280-nm absorbing peak that contains all elastase inhibitor activity (Fig. 4, *A–F*); the bulk of inactive proteins remains on the column (Fig. 4, *High Salt*). Typically, elastase inhibitor–containing fractions from 3–4 chromatographs were pooled and concentrated by repeat DEAE HPLC chromatography.

The active concentrated DEAE fraction consists of a M_r 42,000 polypeptide (>85–90%) and an M_r 27,000 polypeptide. These apparent molecular weights were determined by comparative SDS-mobility (reference 35; not shown). The polypeptides can be separated by HPLC gel filtration, in which elastase inhibitor activity coelutes with the M_r 42,000 polypeptide in the second of two 214-nm absorbing peaks (Fig. 5).

The average yield of the M_r 42,000 elastase inhibitor molecule (see next paragraph) was 480 μg from 1.8×10^{10} cells (12-liter culture). Based on the estimated content of this number of cells (2,100 μg) (28), this yield represents 23% overall recovery.

Characteristics of Purified Elastase Inhibitor: Complex Formation. To demonstrate that the M_r 42,000 polypeptide is the elastase inhibitor, the DEAE-purified fraction was incubated with nonlabeled elastase and examined on silver-stained SDS-electrophoresis gels. On co-incubation for 1 min, the M_r 42,000 polypeptide and elastase disappeared concomitant with the formation of a M_r 66,000 elastase-elastase inhibitor complex (Fig. 6). This finding demonstrates, first of all, that the M_r 42,000 polypeptide is the elastase inhibitor. It also shows that the bulk of the purified molecules

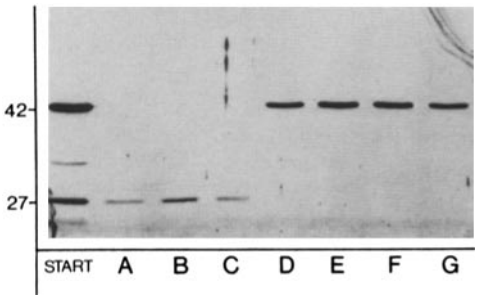

FIGURE 5. HPLC gel filtration. The active DEAE fraction (200 μl) was chromatographed at 0.7 ml/min on the HPLC gel filtration resin Protein-Pak I-125 (Waters Associates) (2 columns in series totalling 7.8 × 600 mm) in 10 mM Tris-HCl buffer, pH 7.4, 90 mM NaCl. Mercaptoethanol (1 mM) was added to the fractions after chromatography. Shown is a gold-stained transfer of a Laemmli SDS gel showing the Start fraction and sequential 214-nm-absorbing fractions. Elastase inhibitor activity (not shown) chromatographed with the M_r 42,000 polypeptide.

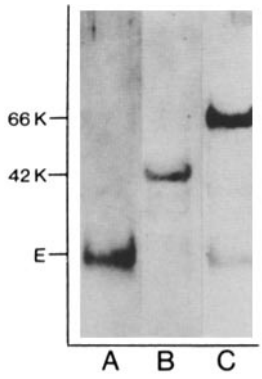

FIGURE 6. Demonstration that the purified M_r 42,000 polypeptide is elastase inhibitor. Shown is a silver-stained (39) Fairbanks/Laemmli gel of (*A*) 1.2 μg nonradiolabeled pancreatic elastase; (*B*) 40 μl active DEAE fraction; and (*C*) 40 μl active DEAE fraction plus 1.2 μg elastase incubated at 37°C for 1 min.

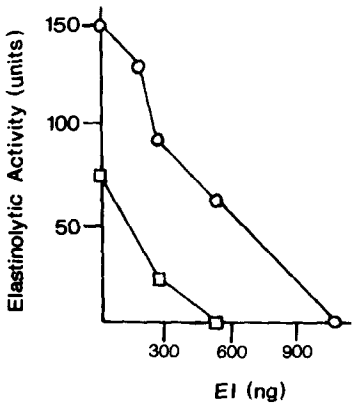

FIGURE 7. Inhibition of elastinolysis by purified elastase inhibitor. Varying amounts of elastase inhibitor (pooled, concentrated DEAE fraction) were combined with 150 ng (○) or 75 ng (□) pancreatic elastase for 5 min at ~22°C, and then incubated in wells of fluorescein-elastin-agar plates for 48 h at 37°C. The extent of elastinolysis, measured as the diameter of the lysis rings (average of duplicate determinations), was converted to units (1 U = the activity of 1.0 ng elastase) by reference to a parallel standard curve.

have retained complex-forming activity and that the reaction of the purified molecule with elastase is rapid (complete at 1 min), as was previously shown for the elastase inhibitor activity in cell lysates (28). The molecular weights of the reactants and the complex clearly suggest that the reaction has 1:1 stoichiometry.

Inhibition of Elastinolysis. It was necessary to formally demonstrate that the purified molecule selected by virtue of its ability to form a complex with elastase will also function as an inhibitor in an elastase assay. Elastinolytic activity of 75 and 150 ng elastase was assayed by generation of lytic zones in an elastin-containing agar gel (36). The purified elastase inhibitor preparation caused dose-dependent inhibition of elastinolysis (Fig. 7), thereby demonstrating that the purified molecule inhibits elastase. The molar ratio of elastase inhibitor/elastase required for inhibition in this assay was ~3:1, rather than the anticipated 1:1, possibly due to the long duration of the assay (48 h), which favors dissociation of the complex and inactivation of elastase inhibitor.

Amino Acid Composition. The amino acid composition of the elastase inhibitor (gel-filtered fraction) is presented in Table I.[2] For comparison, the mean composition

[2] The gel filtered fraction was used for compositional analyses. The ~10-fold dilution produced on gel filtration makes this fraction unsuitable for functional analyses that require elastase inhibitor as a stoichiometric reagent at high concentration. The DEAE fraction has the requisite activity and concentration for functional studies.

TABLE I

Amino Acid Composition

Residue	No. residues/100 amino acids			No. of residues/molecule	
	Average of 200 proteins*	EI‡	α1-AT§	EI‡	α1-AT§
Asx	10.7	11.1	10.9	40	43
Glx	10.6	12.3	12.7	44	50
His	2.2	1.9	3.3	7	13
Lys	6.5	7.1	8.6	26	34
Arg	4.4	3.8	1.8	14	7
Ser	6.3	8.1	5.3	29	21
Thr	5.7	5.7	7.6	21	30
Pro	4.8	5.4	4.3	20	17
Ala	8.5	8.1	6.1	29	24
Cys	2.3	1.5	0.2	5	1
Gly	8.1	7.0	5.6	25	22
Tyr	3.3	2.1	1.5	8	6
Val	6.8	5.2	6.1	19	24
Ile	5.0	3.0	4.8	11	19
Leu	8.1	9.2	11.4	33	45
Phe	3.7	4.9	6.8	18	27
Met	1.9	2.0	2.3	7	9
Trp	1.3	ND	0.5	ND	2
Total				~360	394

* The mean amino acid composition of >200 proteins is from reference 40.

‡ EI (elastase inhibitor) values are means of data from three HPLC-gel filtered preparations. The number of residues per molecule is a calculated value based on M_r 42,000.

§ α1-AT composition is from reference 41.

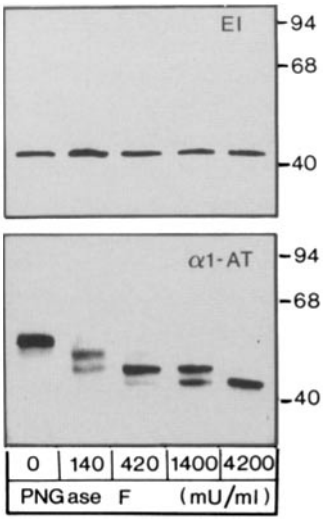

FIGURE 8. Effect of PNGase F on elastase inhibitor and α1-AT. Elastase inhibitor (EI, *upper panel*) (100 ng of concentrated DEAE fraction) and α1-AT (*lower panel*) (250 ng; Chemicon Co., El Segundo, CA) were denatured at 100°C for 1 min in 0.5% SDS, 2 mM mercaptoethanol. The denatured proteins were incubated with PNGase F (peptide:N-glycosidase F from Flavobacterium meningosepticum; Genzyme, Boston, MA) for 3 h at 37°C in 30 mM Tris HCl buffer, pH 8.6, 1% NP40, 0.14% SDS, 1 mM DFP, 10 mM 1,10 phenanthroline. Shown is a gold stained transfer of a Laemmli SDS-gel with molecular weight marker positions indicated on the right.

of 200 purified proteins and that of α1-AT, the elastase inhibitor of plasma, are also presented. The elastase inhibitor composition differs only slightly from the mean of 200 proteins. The molecule does not have the low arginine content of α1-AT. Its cysteine content, five residues per molecule, although lower than the mean value for 200 proteins, is, nonetheless, much higher than the α1-AT value of one residue per molecule.

Two attempts to determine NH_2-terminal amino acid sequence of elastase inhibitor yielded no sequence, suggesting that the NH_2 terminus is blocked. The elastase inhibitor is stable to reducing agents. Mercaptoethanol was used at 50 mM in the purification without loss of elastase inhibitor activity and 200 mM mercaptoethanol does not adversely affect the ^{125}I-elastase complex formation assay (not shown). It thus appears that the elastase inhibitor molecule has no disulfide bonds that are essential for activity.

Carbohydrate Composition. The carbohydrate content was determined by gas-liquid chromatography. Per molecule of elastase inhibitor, 3.6 residues of xylose (possibly a contaminant) and 0.5 residues mannose (average values for two preparations) were detected. Galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid were not detected. These findings strongly indicate that the elastase inhibitor is a nonglycosylated protein.

Treatment with PNGase F. Additional evidence for the nonglycosylated status was provided by treating the purified molecule with peptide *N*-glycosidase F (PNGase F), which cleaves all classes of *N*-linked carbohydrate units (42). On treatment with 140–4,200 mU/ml PNGase F, no change was detected in the apparent M_r of elastase inhibitor, whereas denatured α1-AT, treated in parallel, was converted in stepwise decrements from apparent M_r 51,000 to apparent M_r 44,000 (Fig. 8).

Evidence for an Essential Cysteine Residue. Addition of the sulfhydryl reagent iodoacetamide to pure elastase inhibitor or U937 cell lysates causes almost complete loss of covalent complex activity with elastase (Fig. 9). Destruction of unreacted iodoacetamide by addition of mercaptoethanol or removal by dialysis did not restore activity, demonstrating that the elastase inhibitor molecule is the sensitive component. This finding suggests that the elastase inhibitor has a cysteine residue that is essential for formation of the covalent elastase-elastase inhibitor complex.

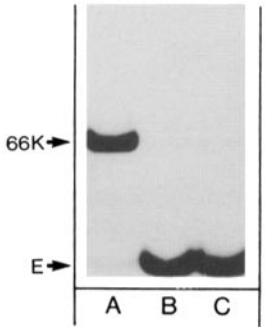

FIGURE 9. Inactivation of elastase inhibitor activity by iodoacetamide. The autoradiograph shows the covalent complex (66K) formed from ^{125}I-labeled pancreatic elastase and pure elastase inhibitor (DEAE fraction) after the latter was preincubated at ~22°C for 10 min without (lane *A*) or with (lane *B*) 3 mM iodoacetamide. Lane *C* shows iodoacetamide-treated elastase inhibitor that was reacted with excess mercaptoethanol. Mercaptoethanol does not adversely affect the assay (not shown). The ability of pure α1-AT to form a covalent complex with ^{125}I-elastase was not affected by parallel treatment with iodoacetamide (not shown).

Discussion

A unique, abundant, cell-associated inhibitor of the serine active site proteinase elastase has been purified from the monocyte-like line U937. The purified molecule (*a*) reacts with elastase to form the covalent elastase-elastase inhibitor complex; and (*b*) inhibits the elastinolytic activity of elastase. The elastase inhibitor is a single polypeptide of apparent M_r 42,000. The NH_2 terminus appears to be blocked. The negligible levels of carbohydrate detected by gas-liquid chromatography and the insensitivity to PNGase F strongly indicate that the elastase inhibitor is nonglycosylated.

In terms of cellular distribution, this elastase inhibitor, like elastase, is found in neutrophils, monocytes, and macrophages. The finding of colocalization, together with the demonstrated capacity of the two molecules to interact rapidly in vitro (28) (Fig. 6), suggests that this elastase inhibitor functions as a physiological regulator of elastase, thus bringing to three the number of inhibitor molecules thought to regulate elastase activity in physiological settings. The other two molecules are α1-AT and the low molecular weight acid-stable inhibitors (described below). The high levels of elastase, particularly in neutrophils of humans, and the multiplicity of susceptible target structures indicate the need for close regulation in vivo. The cases of early onset emphysema associated with genetically low α1-AT levels indicate a critical role for α1-AT in protecting the lower respiratory tract from elastase-induced injury (15, 16), but beyond that, the relative contribution of each of these molecules to elastase regulation at particular sites and situations is not delineated. In view of the destructive potential of elastase, its regulation by several molecules with overlapping function would not be a surprising finding.

The three molecules are all effective against elastase in vitro and each is abundant in vivo. A major feature suggesting different physiological contributions for the three inhibitors is their partially overlapping, but principally different, localization. The low molecular weight, acid-stable inhibitors are closely related or identical M_r ~12,000 polypeptides with two inhibitory domains that are produced by secretory cells and found in bronchial mucus, cervical mucus, seminal plasma, and salivary gland secretions (43–45). These inhibitors might function in vivo to protect the important cell surface mucin molecules lining airway luminal surfaces from degradation by elastase (46).

α-AT is found in plasma and enters tissues and inflammatory sites (16). Although α1-AT effectively inhibits elastase in solution, it is not effective in preventing the degradation of matrix proteins by elastase delivered by live neutrophils (47, 48). This finding has been attributed to oxidative inactivation of α1-AT by the neutrophils (47, 49, 50), and/or spatial exclusion of α1-AT from the contact areas where neutrophils encounter matrix proteins (48). Against this background, we hypothesize that the newly identified elastase inhibitor functions in vivo to regulate elastase activity in the immediate vicinity of monocytes, neutrophils, and macrophages, particularly in regions where these cells migrate, localize, and spread on matrix proteins.

In this context, the well-known sequence of recruitment of phagocytic cells to extravascular sites may consitute an orchestrated program for proteolytic action. Neutrophils with high elastase and low elastase inhibitor content are recruited first, and proteolytic action predominates at inflammatory sites for several days until recruitment of the monocytes/macrophages with high ratios of elastase inhibitor to elastase.

This U937 elastase inhibitor was previously shown to function by forming a complex requiring the active site serine of the proteinase, i.e., it does not react with elastase inactivated with the active site reagent DFP (28). The elastase inhibitor-elastase complex is stable in boiling SDS and susceptible to base-catalyzed cleavage (28), suggesting that the new bond is an ester. This mechanism of action (51) characterizes serine proteinase inhibitors of the serpin family (52, 53). Although sequence information will be required to confirm the assignment, the mechanism of action strongly suggests that the purified elastase inhibitor is a serpin molecule.

The purified molecule is unique, i.e., sufficient properties have been documented to distinguish it from all other proteinase inhibitors. Thus, although it shares function (rapid inhibition of elastase) with α1-AT, the two differ in electrophoretic mobility, reactivity with antisera (28), composition (Table I), and sensitivity to iodoacetamide (Fig. 9). The elastase inhibitor can be distinguished from protease nexin (inhibitor of thrombin and plasminogen activator found in fibroblasts), which does not react with elastase (54), and from PAI-1 (plasminogen activator inhibitor of endothelial cells, hepatoma cells, and platelets) (55, 56), which, unlike elastase inhibitor (Remold-O'Donnell, E., unpublished results), adheres to Con A and is stable to denaturing agents (57).

The elastase inhibitor can be distinguished from the plasminogen activator inhibitor of placenta (58–60), monocytes, and U937 cells (61) (PAI-2), since PAI-2 does not inhibit elastase and the elastase inhibitor does not react with anti-PAI-2 antiserum (62). Moreover, PAI-2 is present in only low quantity in monocytes except when the cells are stimulated by PMA or LPS (63, 64), whereas the elastase inhibitor is abundant in unstimulated monocytes.

The purified molecule appears to be the cytosolic protein of blood leukocytes and lung macrophages whose activity as an elastinolysis inhibitor was described by Janoff and Blondin in 1971 (21, 22). The purified molecule is thought to be the human counterpart of the elastase inhibitor of guinea pig macrophages called macrophage proteinase inhibitor (27). Shared properties of the human and guinea pig elastase inhibitors include rapid reactivity with elastase, identical apparent molecular weight, distribution in neutrophils, as well as macrophages, and similar sensitivity to iodoacetamide (27, 28) (Remold-O'Donnell, E., unpublished results).

For elastase, all of its documented functions occur after the molecule is secreted from the granules to the extracellular space (7–11), and it is presumed that the reaction of elastase with elastase inhibitor also occurs extracellularly. The human elastase inhibitor has not yet been examined in extracellular fluids, but, in the guinea pig system, active elastase inhibitor molecules with identical apparent molecular weight have been detected in extracellular fluid as well as cell lysates. The mechanism and signals for externalization of elastase inhibitor are unknown.

The apparent presence of an essential cysteine will be more rigorously examined now that the pure molecule is available, in particular to determine whether the putative essential cysteine is part of the active site. From a biological viewpoint, one could predict that a proteinase inhibitor with an essential sulfhydryl residue would be subject to inactivation in vivo by oxidants including those released by phagocytes (65). Thus, elastase inhibitor might be active for only a brief period after release from the phagocytes. From the reciprocal perspective, the intracellular location may pro-

tect the reservoir of elastase inhibitor molecules from inactivation by phagocyte oxidants and by components of cigarette smoke, an exposure known to reduce the activity of α1-AT (66).

Summary

A fast-acting inhibitor of serine elastase has been detected at high levels in human neutrophils, fresh monocytes, matured monocytes, and macrophages. The elastase inhibitor was isolated from large scale cultures of the monocyte-like cell line U937 by DNase chromatography, disulfide exchange, Phenyl-Sepharose, Red A–agarose, and DEAE HPLC chromatography with an average yield of 480 μg from 1.8×10^{10} cells. The isolated polypeptide was verified as elastase inhibitor by its ability to (*a*) form a covalent complex with elastase; and (*b*) inhibit the elastinolytic activity of elastase.

The purified elastase inhibitor molecule is unique, i.e., physicochemical and/or functional properties distinguish it from all other serine proteinase inhibitors. Treatment with iodoacetamide abrogates the ability of the molecule to form a complex with elastase, thereby providing evidence for the presence of an essential cysteine residue. Based on functional criteria, this elastase inhibitor has been grouped with the proteinase inhibitors of the serpin superfamily.

The purified elastase inhibitor is a single polypeptide of M_r ~42,000. The NH_2 terminus appears to be blocked. Compositional analyses indicates five cysteine residues per molecule of ~360 amino acid residues. Negligible levels of carbohydrate were detected on gas-liquid chromatography. This finding and the insensitivity of the molecule to peptide *N*-glycosidase F treatment strongly indicate that the elastase inhibitor is a nonglycosylated protein.

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