Plasma Levels of Elastase-specific Fibrinopeptides Correlate with Proteinase Inhibitor Phenotype

Evidence for Increased Elastase Activity in Subjects with Homozygous and Heterozygous Deficiency of α_1-Proteinase Inhibitor

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Abstract

There is indirect evidence that unopposed human neutrophil elastase (HNE) is responsible for emphysema in patients with α_1-proteinase inhibitor (Pi) deficiency. To directly explore this possibility, we developed an assay for fibrinopeptide Aα1-21 and its degradation products and used it to measure HNE activity in 128 subjects of known Pi phenotype. The mean elastase-specific fibrinopeptide (ESF) level in 49 deficient PiZ individuals is significantly higher than that in 56 PiMZ heterozygotes (4.5 and 1.5 nM, respectively; $P < 0.01$), while the mean ESF value in heterozygotes is significantly elevated over that in 23 normal PiM subjects (1.5 and 0.6 nM, respectively; $P < 0.01$), consistent with increased HNE activity in those deficient in the major regulator of the enzyme. These results are not due to differences in smoking history because after correction for pack-years of smoking, ESF values in PiZ subjects are fourfold higher than those in PiMZ individuals ($P = 0.005$), while the ESF levels in heterozygotes are threefold higher than those in PiM subjects ($P = 0.02$). In addition, this analysis suggests that cigarette smoking and α_1-proteinase inhibitor deficiency have additive effects on ESF levels thereby explaining why PiZ and some PiMZ individuals are at especially high risk for the development of lung disease if they smoke. Finally, the observation that ESF levels in nonsmoking PiZ subjects are inversely related to the percent of predicted forced expiratory volume in 1 s (FEV$_1$%) provides direct support for the concept that unregulated HNE activity causes alveolar septal destruction in patients with α_1-proteinase inhibitor deficiency. (*J. Clin. Invest.* 1991. 88:766–773.) Key words: emphysema • cigarette smoking • neutrophils

Introduction

Human neutrophil elastase (HNE)[1] has been implicated in the pathogenesis of a wide variety of diseases (1). In particular, extracellular matrix injury resulting from the catalytic activity of HNE may be the pivotal event in the pathogenesis of pulmonary emphysema (2). Support for this concept comes from two lines of investigation. First, studies have demonstrated that congenital deficiency of α_1-proteinase inhibitor, the major plasma regulator of HNE (3), is associated with the early development of severe, panlobular emphysema (4). Second, several investigators have shown that the intrapulmonary instillation of HNE into experimental animals leads to anatomic derangements in lung architecture with the characteristics of human emphysema (5, 6). These observations have led to the proposal that unregulated HNE activity in the lungs is responsible for the alveolar septal destruction that occurs in patients with α_1-proteinase inhibitor deficiency.

In the past, it has been difficult to evaluate HNE activity in vivo because the enzyme rapidly binds to its substrates or its inhibitors (3), and because pathogenetically important HNE activity may be confined to localized microenvironments within tissues (7–15). Recently, we have developed an assay for in vivo HNE activity based on the unique capacity of the enzyme to cleave the Aα21 (Val)-Aα22 (Glu) bond on the amino-terminal region of the Aα-chain of fibrinogen (16). Hydrolysis of this bond results in release of the fibrinopeptide A-containing fragment Aα1-21. The original radioimmunoassay for the quantification of Aα1-21 was indirect (17), and used a specific antiserum against fibrinopeptide A (FPA or Aα1-16). This antiserum has its antigenic determinant at the carboxy-terminal region of the FPA molecule (17–20). Since this epitope is inaccessible in larger FPA-containing fragments, the antibody reacts poorly with Aα1-21 (16, 20). When Aα1-21–containing samples are incubated in vitro with thrombin, however, the FPA portion of the peptide is released. This results in an ~ 1,000-fold increase in immunoreactivity (20) which we designated thrombin-increasable FPA immunoreactivity (TIFPA) (16).

Using plasma levels of TIFPA as an index of in vivo HNE activity, higher peptide values were found in cigarette smokers than in nonsmokers (21). Further, individuals with congenital deficiency of α_1-proteinase inhibitor had plasma levels of TIFPA considerably higher than those in smokers (16, 21). The purpose of this study was to extend these observations in two ways. First, we set out to develop a specific assay for Aα1-21 to obviate the need for in vitro treatment of the plasma samples with thrombin. Second, using this test as an index of unopposed HNE activity, we set out: (*a*) to compare the effect of cigarette smoking on HNE activity in subjects with congenital deficiency of α_1-proteinase inhibitor (phenotype PiZ) with that in heterozygotes (type PiMZ) and in normals (type PiM); and (*b*) to explore directly the link between unregulated enzyme activity and lung destruction in PiZ individuals.

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1. *Abbreviations used in this paper:* ESF, elastase-specific fibrinopeptide; FEV$_1$%, percent of predicted forced expiratory volume in 1 s; FPA, fibrinopeptide A; HNE, human neutrophil elastase; Pi, proteinase inhibitor; TIFPA, thrombin-increasable FPA.

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Methods

Reagents. Human α-thrombin (sp act 2,850 U/mg) was generously provided by Dr. J. Fenton II, NY State Dept. of Health, Albany, NY. Carboxypeptidases A (36 U/mg) and B (68 U/mg) were from Worthington Biochemical Co., Freehold, NJ, while carboxypeptidase Y (35 U/mg) was from Pierce Chemical Co., Rockford, IL. The activities of carboxypeptidases A, B, and Y were tested immediately before use by measuring hydrolysis of hippuryl-L-phenylalanine, hippuryl-L-arginine, and acetyl tyrosine ethyl ester, respectively (Sigma Chemical Co., St. Louis, MO). MeO-Suc-Ala_2-Pro-ValCH_2Cl, a potent and specific inhibitor of neutrophil elastase (22), was purchased from Enzyme Systems Products, Livermore, CA, while aprotinin was from American Diagnostica Inc., Greenwich, CT. The heterobifunctional cross-linking agent, m-maleimidobenzoic acid *N*-hydroxysuccinimide ester, was from Pierce, while Keyhole Limpet hemocyanin was from Sigma. The peptides Aα1-21 and Aα1-20, and their analogues with an amino-terminal tyrosine residue were synthesized by Dr. G. Wilner, American Red Cross, Albany, NY, using the solid-phase method of Merrifield (23). Homologues of the amino-terminal region of the Aα-chain of human fibrinogen (Aα1-23, Aα1-19, Aα1-18, Aα1-15, Aα1-14, Aα1-13, and Aα1-12) were synthesized by R. Mumford, Merck Sharp and Dohme Research Laboratories, Rahway, NJ. Synthetic human FPA (Aα1-16) was purchased from Bachem Inc., Torrance, CA, and Aα14-21 with an amino-terminal cysteine residue at position Aα (13) was synthesized by the Pharmaceuticals Division of Imperial Chemical Industries Ltd., Cheshire, UK.

Preparation of immunogens. To promote the development of antibodies directed against the carboxy-terminal region of Aα1-21, an Aα14-21 homologue with a cysteine residue at position Aα13 was synthesized. Following the manufacturer's instructions, the heterobifunctional cross-linking agent, m-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Pierce), was then used to couple the amino-terminal cysteine residue of this peptide to Keyhole Limpet hemocyanin. As another immunogen, synthetic Aα1-20 was coupled to BSA (crystallized, grade V; Sigma Chemical Co.) using the glutaraldehyde method previously described for the conjugation of FPA (24).

Immunization procedure. The immunogens were suspended in 0.9% sterile NaCl at a concentration of 1 mg/ml and vortexed thoroughly with three parts of complete (primary immunization) or incomplete (booster immunizations) Freund's adjuvant (Calbiochem-Behring Corp., San Diego, CA) until a thick emulsion formed. Four sheep were each immunized with 500 μg of the Aα14-21 derivative using the multiple intradermal injection technique (25). Booster immunizations consisting of 500 μg of immunogen were given intramuscularly every 3 wk thereafter, and the animals were bled at the same time. Four white male New Zealand rabbits were each immunized with 200 μg of the Aα1-20 conjugate again using the multiple intradermal injection technique (25). 10 wk after primary immunization, each rabbit was given 100 μg of immunogen intramuscularly into the haunches. Blood was collected at 8 wk, and every 2 wk thereafter.

Radioimmunoassays. TIFPA was assayed as previously described (16) using antiserum R2. This antibody is specific for FPA and cross-reacts poorly with fibrinogen or FPA-containing fragments (17–20). The tyrosinated analogues of Aα1-21 and Aα1-20 were each radioiodinated with ^{125}I by the chloramine-T method (26) to a sp act of 0.3–0.4 μCi/pmol. Specific binding of these tracers by antisera developed against the Aα14-21 and Aα1-20 conjugates, respectively, was then determined.

For assays using antisera developed against the Aα14-21 or Aα1-20 conjugates, dilutions of standard, test samples, antiserum, and tracer were made in 0.1 M NaCl buffered with 0.05 M Tris-HCl, pH 7.4 (TBS), containing 0.02% sodium azide and 0.1% normal sheep serum or 0.1% normal rabbit serum, respectively. Each assay tube contained 400 μl final vol consisting of the following, in order of addition: 250 μl of standard concentration of peptide to be measured or a dilution of test sample; 100 μl of antiserum (at a dilution sufficient to bind 30–35% of the total bindable counts); and 50 μl of tracer (~ 15,000 cpm/tube). After 18 h incubation at 4°C, tracer bound to antibody was precipitated using rabbit anti-sheep or goat anti-rabbit IgG (Sigma) in quantities sufficient to bind all of the sheep or rabbit IgG, respectively. In each case, the second antibody was diluted in TBS containing 20 mg/ml polyethylene glycol 8,000 (Fisher Scientific Co., Pittsburgh, PA). The tubes were then incubated for 30 min at 23°C, diluted with 2 ml of 0.9% NaCl, and centrifuged at 4,000 *g* for 20 min at 4°C. The supernatant fluid was aspirated and the pellets were counted for 1 min using a Clinigamma counter (LKB Instruments, Gaithersburg, MD).

Stability of Aα1-21 immunoreactivity. The stability of Aα1-21 immunoreactivity was determined in whole blood and in plasma. Using a 21-gauge butterfly needle, blood was collected from the antecubital veins of healthy volunteers into 10-ml vacutainer tubes (Becton Dickinson & Co., Mountain View, CA) prefilled with 1 ml of anticoagulant solution consisting of 1,000 kallikrein inhibitor units (KIU)/ml aprotinin, 1,400 U/ml heparin, and 0.1 mM MeO-Suc-Ala_2-Pro-ValCH_2Cl in Hepes-buffered saline, pH 7.4. After mixing with the anticoagulant, the blood was either used directly or cell-free plasma was prepared by centrifugation at 2,000 *g* for 15 min at 4°C. Synthetic Aα1-21 (20 pmol/ml) was then incubated in 2-ml aliquots of blood or plasma for 60 min at 37°C in the presence or absence of *o*-phenanthroline (final concentration, 10 mM). At intervals, 200-μl aliquots were removed, and fibrinogen was precipitated by the addition of 600 μl of chilled ethanol followed by centrifugation at 15,000 *g* for 5 min. The ethanol supernatants were then evaporated to dryness in a Speed-Vac concentrator (Savant Instruments, Inc., Hicksville, NY) and reconstituted to original volume with distilled water. The levels of Aα1-21 were then quantified using the three assays described above.

Effect of carboxypeptidases on Aα1-21 immunoreactivity in buffer. Synthetic Aα1-21, suspended in TBS at a concentration of 20 nM, was incubated for 60 min at 37°C in the presence or absence of carboxypeptidases A, B, or Y (at concentrations ranging from 0.01 to 10.0 U/ml). At intervals, 200-μl aliquots were removed, the reaction was terminated by the addition of *o*-phenanthroline (final concentration, 10 mM), and the levels of Aα1-21 were then quantified using the three assays described above.

HPLC analysis of carboxypeptidase-mediated proteolysis of Aα1-21. The time course of carboxypeptidase-mediated proteolysis of Aα1-21 also was monitored using reversed-phase HPLC. Aα1-21 was suspended in TBS at a concentration of 1.0 mg/ml. 1-ml aliquots were then incubated for 3 h at 37°C with carboxypeptidases A, B, or Y (at concentrations ranging from 0.01 to 10.0 U/ml). At intervals, 100-μl aliquots were removed, and the reaction was terminated by lowering the pH to 2.1 by the addition of 0.1% trifluoroacetic acid. After passage over Sep-Pak C18 cartridges (Waters Associates, Millipore Corp., Milford, MA), adsorbed peptides were eluted with 3 ml of 50% acetonitrile, evaporated to dryness in a Speed-Vac concentrator, and then reconstituted to original volume with 0.1% trifluoroacetic acid before HPLC analysis.

Analytical HPLC was performed using a liquid chromatograph (System Gold; Beckman Instruments Inc., Palo Alto, CA) equipped with two model 126 solvent delivery systems, and a model 506 automatic injector. Peptides were monitored using a model 167 variable wave length absorbance detector set at 214 nm. The column used for these studies was an Ultrasphere ODS C18 (4.6 mm i.d., Beckman Instruments). Solvents used in the chromatography were 0.1% trifluoroacetic acid (buffer A), and 0.1% trifluoroacetic acid containing 50% acetonitrile (buffer B). Using a flow rate of 1 ml/min, peptides were eluted from the column with a linear gradient constructed from buffers A and B, and extending from 30 to 70% buffer B over 30 min. 1-ml fractions were collected, evaporated to dryness in a Speed-Vac concentrator (Savant Instruments), and reconstituted with 500 μl of distilled water. The levels of Aα1-21 were then quantified using the three assays described above.

Clearance of Aα1-21 in marmosets. Synthetic human Aα1-21 was injected intravenously into four marmosets (*Callithrix jacchus jacchus*), and the disappearance of Aα1-21 immunoreactivity was then determined using the three assays described above. After anesthetizing

the animals with an intramuscular injection of ketamine (0.5 mg/kg), the femoral vein was exposed, and a 21-gauge catheter fitted with a two-way stopcock was inserted into the vein and held in place with a suture. Blood samples were taken before, and at intervals after, bolus intravenous injection of Aα1-21 (1 mg/kg), while patency of the catheter was maintained by infusing 0.5 ml of 0.9% NaCl containing 1 U/ml heparin after each blood sample was collected. At each time point, 900 μl of blood was collected into a plastic syringe, and immediately transferred into a polypropylene Eppendorf tube prefilled with 100 μl of anticoagulant solution consisting of aprotinin, heparin, and MeO-Suc-Ala_2-Pro-$ValCH_2Cl$ in the concentrations listed above. After mixing the blood with the anticoagulant, the red cells were sedimented by centrifugation at 15,000 *g* for 5 min, 300 μl of plasma was removed, and the fibrinogen was precipitated by the addition of 900 μl of chilled ethanol followed by centrifugation at 15,000 *g* for 5 min. The ethanol supernatants were evaporated to dryness in a Speed-vac concentrator (Savant Instruments), reconstituted to original volume with ethanol, and the levels of Aα1-21 were then quantified using the three assays described above.

Plasma peptide levels in subjects with known α_1-proteinase inhibitor (Pi) phenotype. Blood samples were collected from subjects with α_1-proteinase inhibitor deficiency of type PiZ, from their first-degree relatives, and from an additional control population with the normal (PiM) phenotype. The Pi type of each individual was determined by isoelectric focusing of dithioerythritol-treated serum on polyacrylamide gels at pH 4.2–4.9 (27, 28). Probands with α_1-proteinase inhibitor deficiency (type PiZ) were the first 50 subjects recruited for a genetic epidemiologic study aimed at investigating the variable expression of lung disease in this patient group, and their clinical features and means of ascertainment have been described in detail by Silverman et al. (29, 30). In addition, 71 first-degree relatives of these subjects also were investigated. Of these, 56 were heterozygotes (type PiMZ), while 15 had a normal phenotype (type PiM). A further control population of 8 healthy unrelated PiM individuals also was included. All individuals underwent pulmonary function testing using the previously described methods (29). In addition, smoking history was obtained from a modified version of the ATS-DLD-78 respiratory epidemiology questionnaire (31), and the serum concentrations of α_1-proteinase inhibitor were determined by particle-concentration fluorescence immunoassay as we have described (30, 32). This study was approved by the Institutional Review Board of the Jewish Hospital of St. Louis, MO.

After informed consent was obtained from each subject, blood was collected into 10-ml siliconized vacutainer tubes (Becton Dickinson) prefilled with 1 ml of anticoagulant solution consisting of 1,400 U/ml heparin, 1,000 KIU/ml aprotinin, and 0.1 mM MeO-Suc-Ala_2-Pro-$ValCH_2Cl$ in Hepes-buffered saline, pH 7.4. Extreme care was taken in blood collection and processing to ensure consistent and reliable assay results. After centrifugation at 1,700 *g* for 15 min at 4°C, 4 ml of plasma was carefully removed. Fibrinogen was precipitated by the addition of 12 ml of chilled ethanol followed by centrifugation at 4,000 *g* for 20 min at 4°C. The ethanol supernatants were evaporated to dryness in a Speed-vac concentrator (Savant Instruments) and reconstituted to original volume with distilled water. The levels of Aα1-21 were then quantified using the three assays described above. For α_1-proteinase inhibitor measurements, serum obtained from the same phlebotomy was stored at −90°C until analyzed.

Statistical methods. Since plasma peptides levels are log normally distributed, geometric means were calculated and all analyses were performed on log transformed data. The significance of differences was determined by analysis of variance or covariance and Tukey Studentized *t* tests using the SAS statistical package for the personal computer (SAS Statistical Institute, Cary, NC) on an IBM PS/2 Model 80 computer.

Results

Characterization of antisera. Three of four sheep injected with the Aα14-21 homologue produced antibodies. One antiserum, designated S-847, showed the highest titer, and was used in these studies. This antiserum is specific for Aα1-21 and does not cross-react with FPA or larger FPA-containing fragments (Table I). The carboxy-terminal Val residue of Aα1-21 is a critical element of the epitope for S-847 since the antiserum does not recognize FPA-containing fragments shorter than Aα1-21 (i.e., Aα1-20, Aα1-19, and Aα1-18).

All four rabbits immunized with Aα1-20 produced antibodies, and the antiserum with the highest titer, designated antiserum R20, was chosen for further study. This antiserum cross-reacts completely with Aα1-21, Aα1-20, and Aα1-19, but does not react with the larger FPA-containing fragments Aα1-23 and Aα1-27 (Table I). Although Aα1-18 also is recognized, R20 is virtually nonreactive with FPA or its homologues. These findings indicate that the antigenic determinant for R20 is at the carboxy-terminal region of the Aα1-20 fragment.

Comparison of plasma Aα1-21 levels measured with the three different assays. Using antiserum S-847, a radioimmunoassay specific for Aα1-21 was developed. The levels of Aα1-21 measured with this assay were then compared with TIFPA values in plasma samples collected from 30 subjects with a variety of clinical disorders. As illustrated in Fig. 1 *A*, the levels of Aα1-21 are considerably lower than the TIFPA values, and comparison of the results of the two assays using paired *t* tests demonstrates a highly significant difference between the two tests ($P < 0.0005$).

Antiserum R20 also was used for the development of a radioimmunoassay. In addition to measuring Aα1-21, this assay also recognizes smaller FPA-containing fragments (designated elastase-specific fibrinopeptides or ESF for reasons outlined below). In contrast to the results obtained with the specific Aα1-21 assay (Fig. 1 *A*), there is a good correlation between the TIFPA and ESF assays (Fig. 1 *B*). When the results

Table I. Relative Molar Concentrations of Analogues of the Aα-Chain of Fibrinogen Required for 50% Inhibition of Binding of ^{125}I-Tyrosyl-Aα1-21 or ^{125}I-Tyrosyl-Aα1-20 by Antisera S-847 and R20, Respectively

Analogue	Relative molar concentration	
	S-847	R20
FPA-containing analogues		
Aα1-27	ND	ND
Aα1-23	ND	ND
Aα1-21	1	1
Aα1-20	391	1
Aα1-19	5,282	0.5
Aα1-18	ND	4
Aα17-21	ND	3
FPA analogues		
Aα1-16 (FPA)	ND	1,858
Aα1-15	ND	ND
Aα1-14	ND	ND
Aα1-13	ND	ND
Aα1-12	ND	ND
Fibrinogen	ND	ND

The 50% inhibitory concentration of Aα1-21 (0.83±0.04 nM) or Aα1-20 (0.81±0.05 nM) was assigned an arbitrary value of unity. ND, no inhibition of binding with concentrations of 10^{-4} M.

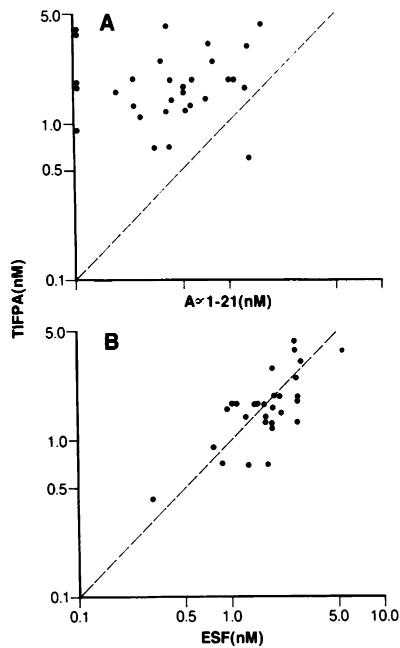

Figure 1. Comparison between the results of the TIFPA and Aα1-21 assays (*A*), and the TIFPA and ESF assays (*B*). Blood samples collected from 30 subjects (6 healthy individuals, 10 current smokers, and 14 patients with obstructive airways disease) were processed as described in Methods, and then assayed for TIFPA, Aα1-21, and ESF. The broken lines represent the lines of identity between the assays.

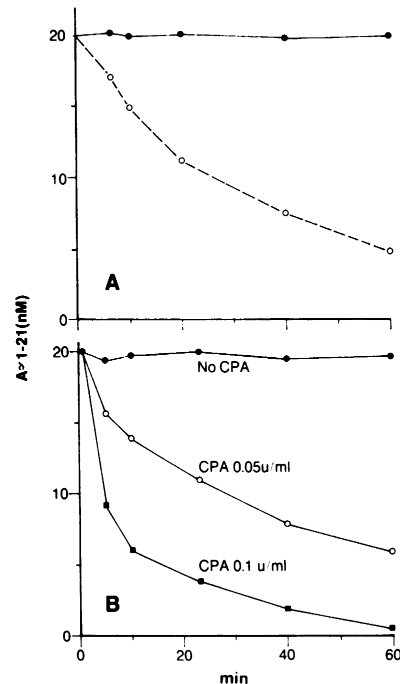

Figure 2. Stability of Aα1-21 immunoreactivity when measured with antiserum S-847. (*A*) Stability of Aα1-21 immunoreactivity in whole blood. Synthetic Aα1-21 was incubated in whole blood for 60 min at 37°C in the presence (●) or absence (○) of *o*-phenanthroline. At the times indicated, aliquots were removed, and the plasma was assayed for Aα1-21 using antiserum S-847 which is specific for intact Aα1-21. (*B*) Effect of carboxypeptidase A (CPA) on Aα1-21 immunoreactivity. Synthetic Aα1-21 was incubated in buffer for 60 min at 37°C in the absence or presence of CPA at the concentrations indicated. At the times indicated, aliquots were removed, and assayed for Aα1-21 using antiserum S-847.

obtained with the TIFPA and ESF assays are compared using paired *t* tests, the differences are not significant ($P = 0.23$).

Evidence that Aα1-21 is degraded to Aα1-20 and Aα1-19 by blood carboxypeptidase-like activity. To explore the reason for the discrepancy between the Aα1-21 and TIFPA values, the stability of Aα1-21 immunoreactivity in plasma and blood was determined. When measured as TIFPA or with antiserum R20, Aα1-21 immunoreactivity is stable in both blood and plasma (data not shown). In contrast, although Aα1-21 immunoreactivity is stable in plasma when measured with antiserum S-847 (data not shown), there is rapid and progressive loss of immunoreactivity when the peptide is incubated in whole blood (Fig. 2 *A*). The loss of immunoreactivity is blocked by the addition of *o*-phenanthroline suggesting that peptidase-mediated proteolysis of Aα1-21 produces smaller fragments that are not reactive with antiserum S-847. To test this possibility, Aα1-21 was incubated in buffer with carboxypeptidases A, B, or Y. As indicated in Fig. 2 *B*, incubation of Aα1-21 with carboxypeptidase A results in a loss of immunoreactivity similar to that which occurs in blood. Incubation of Aα1-21 with carboxypeptidases B or Y also results in a progressive decrease in its immunoreactivity (data not shown), thereby suggesting that all three carboxypeptidases are capable of degrading the peptide to fragments that do not react with antiserum S-847.

Carboxypeptidase-mediated proteolysis of Aα1-21 also was monitored using reversed-phase HPLC. Incubation with carboxypeptidase A results in rapid and progressive proteolysis of Aα1-21 first to Aα1-20, and then to Aα1-19 (Fig. 3). Aα1-19 appears to be the final product of this reaction since there is no further degradation of Aα1-19 in up to 3 h of digestion. Like carboxypeptidase A, carboxypeptidases B and Y also degrade Aα1-21 first to Aα1-20, and then to Aα1-19 (data not shown). Again, once converted to Aα1-19 the peptide does not undergo further proteolysis in up to 3 h incubation with either carboxypeptidase.

To determine whether degradation of the Aα1-21 fragment also occurs in vivo, marmosets were injected with human Aα1-21 and its disappearance was then monitored immunochemically using the TIFPA, Aα1-21, and ESF assays. More rapid

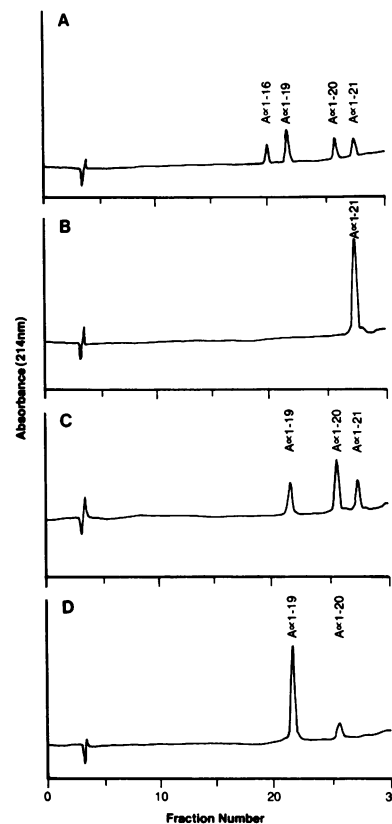

Figure 3. HPLC analysis of the time course of carboxypeptidase A mediated proteolysis of Aα1-21. (*A*) A mixture containing ~5 μg each of Aα1-21, Aα1-20, Aα1-19, and Aα1-16 (fibrinopeptide A) was injected onto the column. Using a flow rate of 1 ml/min, the peptides were then eluted as described in Methods. (*B*) Synthetic Aα1-21 (~ 20 μg) was injected onto the column using the same flow rate. (*C*) Synthetic Aα1-21 (1 mg/ml) was incubated with carboxypeptidase A (0.01 U/ml) for 15 min at 37°C. The reaction was terminated, and the carboxypeptidase A removed by lowering the pH to 2.1 by adding 0.1% trifluoroacetic acid, and by passage of the acidified sample over a Sep-Pak C18 cartridge. Approximately 20 μl of the digestion mixture was then injected onto the column using the same flow rate. (*D*) Synthetic Aα1-21 was incubated with carboxypeptide A for 45 min at 37°C. The reaction was terminated as described above, and 20 μl was then injected onto the column using the same flow rate.

disappearance of immunoreactivity occurs when the peptide is measured with the Aα1-21 assay than when it is quantified with either the TIFPA or the ESF assay. The rate of disappearance was then determined by linear regression analysis of the log transformed data, and the individual slope coefficients and their associated SEM for TIFPA, ESF, and Aα1-21 were calculated as -0.51 ± 0.03, -0.49 ± 0.01, and -0.78 ± 0.07 nM/min, respectively. Comparison of the three slopes indicates that those derived from the TIFPA and ESF data are not significantly different ($P = 0.75$) but both are significantly different from that derived from the Aα1-21 data ($P < 0.001$ for each comparison). These studies indicate that Aα1-21 is rapidly cleared in primates with a $t_{1/2}$ based on the results of the TIFPA, ESF, and Aα1-21 assay of 1.4, 1.5, and 0.8 min., respectively.

To explain the more rapid disappearance of Aα1-21 immunoreactivity, samples also were analyzed by reversed-phase HPLC using a gradient that separates intact Aα1-21 from its carboxypeptidase-derived degradation products. Fractions were collected and assayed for TIFPA, ESF, and Aα1-21. At 1 min, ~ 53% of the total immunoreactivity coelutes with Aα1-20, while 22% coelutes with Aα1-19. These findings indicate that 75% of the injected peptide is rapidly degraded. By 7.5 min, only 2% of the total immunoreactivity coelutes with Aα1-21, while 15% coelutes with Aα1-20 and the remainder coelutes with Aα1-19. Since unlike the TIFPA and ESF assays, only intact Aα1-21 is recognized in the Aα1-21 assay (Table I), these data explain the more rapid loss of Aα1-21 immunoreactivity when the peptide is quantified using the assay specific for Aα1-21. Further, these findings indicate that the sequence of degradation of Aα1-21 in vivo is identical to that produced in vitro by carboxypeptidases A, B, or Y.

ESF levels in subjects of known Pi phenotype. To examine the correlation between plasma ESF levels and Pi phenotype, a total of 128 subjects of known α_1-proteinase inhibitor phenotype was investigated (Fig. 4). The mean ESF level in PiZ individuals is significantly elevated over that in heterozygotes with the PiMZ phenotype (4.5 and 1.5 nM, respectively; $P < 0.01$). In addition, the mean ESF value in heterozygotes is significantly higher than that in subjects with the normal PiM phenotype (1.5 and 0.6 nM, respectively; $P < 0.01$). Thus, not only the severely deficient (type PiZ) subjects, but also heterozygotes for the deficiency (type PiMZ) have ESF values that are significantly higher than those in control (type PiM) subjects.

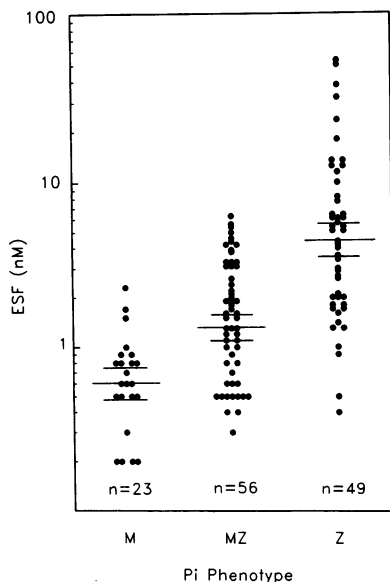

Figure 4. Correlation between plasma ESF levels and Pi phenotype. Blood samples collected from 128 subjects of known Pi phenotype were assayed for ESF. The horizontal bars represent the means, and the 95% confidence intervals.

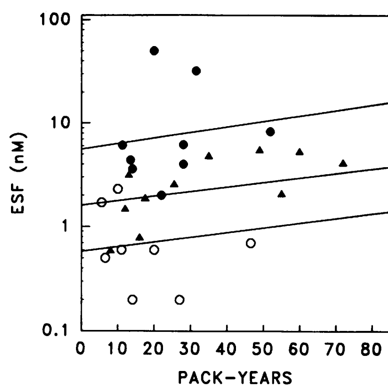

Figure 5. Analysis of covariance in which ESF levels in current smokers of each phenotypic group are plotted against pack-years of cigarette smoking. The ESF values in PiZ (●), PiMZ (△), and PiM (○) subjects are illustrated. The superimposed lines represent the best fit for each phenotypic group as determined by linear regression analysis but with the constraint of a common slope.

Since we have previously shown that cigarette smoking can influence ESF levels directly (21), part of the observed effect of Pi phenotype on ESF values may reflect differences in smoking history. To investigate this possibility in current smokers, analysis of covariance was performed to provide an estimate of the effect of Pi phenotype on plasma ESF levels which was then adjusted for differences in smoking history. The results of this analysis are illustrated in Fig. 5, in which the ESF values for each phenotypic group are plotted against pack-years of cigarette smoking. This model indicates a common trend of increasing ESF values with greater pack-years of smoking. In addition, however, there is a distinct separation ($P < 0.0001$) in the position (i.e., the intercept) of the relationship for each phenotype group. This vertical separation represents the influence of phenotype independent of the effect of cigarette smoking, and indicates a threefold increase in ESF levels in PiMZ subjects as compared to PiM individuals ($P = 0.02$), and a further fourfold elevation in ESF values in PiZ subjects as compared to PiMZ individuals ($P = 0.005$). Thus, this analysis demonstrates that the relationship between ESF levels and Pi phenotype that is illustrated in Fig. 4 is not the result of differences in smoking history but is caused by differences in the concentrations of α_1-antiproteinase inhibitor. This concept is further supported by the observation that the plasma ESF levels show a significant inverse relationship with the serum levels of α_1-proteinase inhibitor ($P < 0.001$), indicating increased HNE activity in those individuals deficient in the major regulator of the enzyme.

To test the concept that there is a link between unopposed HNE activity and lung dysfunction, plasma ESF values in 12 lifelong nonsmoking PiZ subjects were plotted as a function of the percent of predicted forced expiratory volume in 1 s (FEV_1%). The data on the 27 ex-smokers and 10 current smokers in the PiZ group were excluded from this analysis because cigarette smoking can directly elevate peptide levels (21), and can in itself lead to lung dysfunction (33). As shown in Fig. 6, plasma ESF levels are inversely related to FEV_1% ($P = 0.05$) indicating higher peptide values in PiZ individuals with impaired lung function.

HPLC characterization of peptides identified by the ESF assay. To characterize the peptides identified by the ESF assay

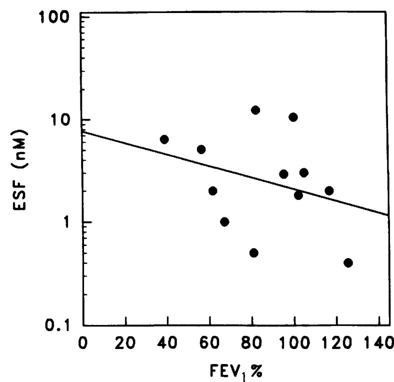

Figure 6. Correlation between plasma ESF values and FEV$_1$% in nonsmoking PiZ subjects as determined by linear regression analysis.

in the population described above, selected samples also were analyzed by HPLC using a gradient that separates Aα1-21 from its carboxypeptidase-derived degradation products (Fig. 7). Fractions were collected and assayed for TIFPA and ESF. Over 95% of the TIFPA or ESF immunoreactivity in the original samples was recovered in the HPLC fractions. With both assays, ~ 86% of the total immunoreactivity coeluted with Aα1-19, 12% with Aα1-20, and 2% with Aα1-21.

Discussion

In this study we have developed a direct assay for Aα1-21 and its degradation products (ESF). Using this test, we have confirmed our previous observations made with an indirect assay that peptide levels are measurable in normal individuals (16, 21), and are elevated in those deficient in α_1-proteinase inhibitor (16). These results support the concept that plasma peptide values can be used as an index of in vivo HNE activity. In addition, we have extended these observations by demonstrating that: (*a*) PiMZ heterozygotes have ESF values higher than those in PiM controls despite having only a 39% reduction in their mean serum concentration of α_1-proteinase inhibitor (30); (*b*) α_1-proteinase inhibitor deficiency and cigarette smoking have additive effects on HNE activity; and (*c*) in nonsmoking PiZ subjects there is an inverse correlation between plasma ESF levels and FEV$_1$% which provides direct support for the concept that unregulated HNE activity causes alveolar septal destruction.

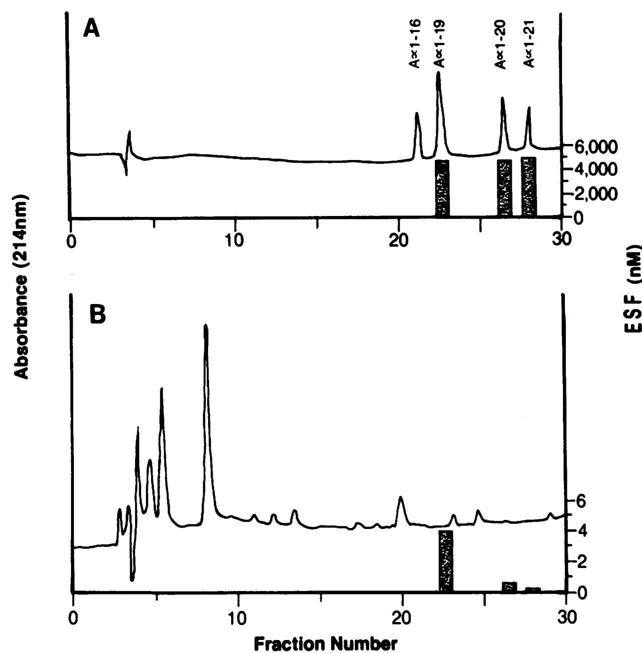

Figure 7. HPLC characterization of the peptides measured by the ESF assay in a representative patient sample. (*A*) A mixture containing ~ 5 μg each of Aα1-21, Aα1-20, Aα1-19, and Aα1-16 (fibrinopeptide A) was injected onto the column. Using a flow rate of 1 ml/min, the peptides were eluted as described in Methods. 1-ml fractions were then collected, evaporated to dryness, and assayed for ESF (*hatched bars*) using antiserum R20. (*B*) A patient sample was passed over a Sep-Pak C18 cartridge, and the adsorbed peptides were eluted as described in Methods. After reconstitution in 0.1% TFA, the sample was injected onto the column using the same flow rate. 1-ml fractions were collected, evaporated to dryness, and then assayed for ESF (*hatched bars*) using antiserum R20.

In developing a direct assay for Aα1-21 it proved necessary to determine the biologic fate of the peptide both in vitro and in vivo. Several lines of evidence indicate that Aα1-21 is degraded by blood carboxypeptidase-like activity. When quantified with R20, an antiserum that recognizes intact Aα1-21 fragment as well as Aα1-20 and Aα1-19 (Table I), peptide levels are not significantly different from those obtained with the TIFPA assay (Fig. 1 *B*). In contrast, the results obtained with antiserum S-847, which only measures intact Aα1-21 (Table I), are considerably lower than those obtained with the TIFPA assay (Fig. 1 *A*). This discrepancy reflects degradation of Aα1-21 into fragments that are not recognized by antiserum S-847 because the incubation of Aα1-21 in blood results in rapid and progressive loss of immunoreactivity which is blocked by the addition of *o*-phenanthroline (Fig. 2 *A*). Further, incubation of Aα1-21 with carboxypeptidases A, B, or Y results in a similar loss of immunoreactivity (Fig. 2 *B*), and HPLC analysis of the timecourses of Aα1-21 proteolysis indicates that these carboxypeptides progressively degrade Aα1-21 first to Aα1-20, and then to Aα1-19 (Fig. 3). The finding that all three carboxypeptidases produce the same pattern of Aα1-21 degradation can be explained on the basis of the specificities of these enzymes. Carboxypeptidase Y does not cleave a carboxy-terminal Arg residue, thus accounting for the stability of the Aα1-19 fragment (34). In contrast, carboxypeptidases A and B do not further degrade Aα1-19 because the Pro residue at position 18 makes the Arg residue at the carboxy-terminal of Aα1-19 an unfavorable site for further proteolysis (35, 36).

Two lines of evidence indicate that proteolysis of Aα1-21 by carboxypeptidase-like activity also occurs in vivo. First, clearance studies in nonhuman primates demonstrate that Aα1-21 immunoreactivity disappears almost twofold faster when the peptide is quantified with the specific assay for Aα1-21 than when it is measured using the ESF or TIFPA assay, and HPLC analysis of these samples indicates that this difference is due to the rapid conversion of the injected Aα1-21 to Aα1-20 and Aα1-19. Second, HPLC analysis of patient samples demonstrates that most of the ESF or TIFPA immunoreactivity coelutes with Aα1-19 and Aα1-20, while only a small fraction coelutes with intact Aα1-21 (Fig. 7). Although our previous HPLC studies suggested that TIFPA immunoreactivity coeluted with Aα1-21 (16, 21), the gradient used in those investigations does not adequately separate Aα1-21 from Aα1-20 and Aα1-19, thus accounting for this discrepancy.

Carboxypeptidase-mediated proteolysis of other fibrinopeptides has been well described. Both the thrombin-derived fragment fibrinopeptide B (37), and Bβ1-42 (38), a product of plas-

min action on fibrinogen, are rapidly degraded by carboxypeptidase-like activity. In the case of fibrinopeptide B, loss of the carboxy-terminal Arg residue does not affect the chemotactic activity of the peptide (39), suggesting that this process may not alter the biologic activity of these peptides.

Having developed and validated the direct assay for ESF both in vitro and in vivo, we used this test as an index of HNE activity in individuals of known phenotype for α_1-proteinase inhibitor (Fig. 4). The increased ESF levels in PiZ subjects with marked deficiency of the major regulator of HNE are not unexpected since these findings are consistent with our previous observations in this phenotypic group (16). Although there has been indirect evidence that unopposed HNE activity is responsible for the early onset of emphysema in PiZ individuals (1, 2, 4, 5), the inverse relationship between plasma ESF levels and FEV_1% in nonsmoking PiZ individuals (Fig. 6) provides direct support for the concept that unregulated HNE activity causes alveolar septal destruction in patients with α_1-proteinase inhibitor deficiency.

The presence of circulating ESF in PiM individuals with normal concentrations of α_1-proteinase inhibitor, and the higher values in PiMZ heterzygotes who have only a modest decrease in inhibitor levels, would not be predicted given the rapidity with which α_1-proteinase inhibitor complexes and inactivates free HNE (3, 40, 41). However, a plausible explanation for the measurable ESF levels in control subjects is provided by our recent studies of neutrophils migrating on a fibrinogen-coated surface in response to the chemoattractant FMLP. Upon stimulation, the neutrophils degrade extracellular fibrinogen and release Aα1-21 despite the presence of physiologic concentrations of macromolecular proteinase inhibitors (11). This phenomenon occurs because zones of close contact are formed between the neutrophils and the fibrinogen which prevent access of plasma antiproteinases to HNE released at the cell-substrate interface. Further investigations have demonstrated that the interaction between neutrophils and fibrinogen is mediated by the complement receptor type 3 (CR3; Cd11b/Cd18) on neutrophils which ligates fibrinogen through a discrete domain located on the carboxy-terminus of its gamma-chains (42).

The finding that ESF values in PiMZ heterozygotes are higher than those in PiM controls suggests that in addition to exclusion of antiproteinases from sites of cell contact with fibrinogen, there must be other mechanisms of HNE-mediated proteolysis that are critically dependent on the circulating levels of α_1-proteinase inhibitor. Support for this concept comes from the results of in vitro studies which demonstrate that the inactivation of HNE in the pericellular microenvironment of neutrophils and monocytes requires substantially higher concentrations of α_1-proteinase inhibitor than those needed to inactivate free enzyme (7–15). These findings have led to the suggestion that the proteolytic activity of HNE is confined to the vicinity of inflammatory cells. Inactivation of HNE at these sites requires more inhibitor both because the local concentration of enzyme is very high on or near the cell surface (43), and because cellular oxidants inactivate α_1-proteinase inhibitor (9). Based on this model, even a minor reduction in the serum concentration of α_1-proteinase inhibitor will result in increased HNE-mediated proteolysis.

Although cigarette smoking can directly influence plasma ESF levels (21), the correlation between peptide values and Pi phenotype is not the result of differences in smoking history. Analysis of covariance demonstrates highly significant differences for each phenotypic group that are independent of the pack-years of smoking (Fig. 5). Thus, this analysis supports the concept that cigarette smoking and α_1-proteinase deficiency have additive effects on HNE activity. Because subjects with α_1-proteinase inhibitor deficiency have ESF levels higher than those in normals, the additive effect of cigarette smoking is potentially more dangerous in these patients. These findings may explain why PiZ and some PiMZ subjects are at especially high risk for lung disease if they smoke (29, 30, 44–46).

There are many potential mechanisms by which cigarette smoking can increase HNE activity. Several lines of evidence indicate that cigarette smokers have increased neutrophil traffic through the lungs. Thus, cigarette smoking delays neutrophil transit through the pulmonary circulation (47), and increased numbers of neutrophils are recovered in the bronchial lavage fluid of smokers (48). Further, these migrating cells have the potential to produce more HNE-mediated damage because cigarette smoke promotes enzyme release from the neutrophils (49), and oxidatively inactivates α_1-proteinase inhibitor (50, 51). These findings may explain why ESF levels are higher in cigarette smokers (21), and why the effect of smoking on peptide levels may be greater in individuals deficient in α_1-proteinase inhibitor (Fig. 5). Thus, our present results directly support the concept that cigarette smoking and α_1-proteinase inhibitor deficiency interact to facilitate alveolar septal destruction.

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