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Determination of α_1-proteinase Inhibitor Activity and Phenotypes in Patients with Emphysema

[Amfizemli Hastalarda α1-Proteinaz İnhibitörü Aktivitesi ve Fenotiplerinin Belirlenmesi]

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ABSTRACT

In our study, functionally active α_1-proteinase inhibitor (α_1-PI) levels and phenotypes were determined in sera of patients with emphysema and in that of healthy subjects. Patients were classified according to their smoking habits: Group I consisted of 7 nonsmoking (or limited history of smoking) patients. Group II consisted of heavily smoking 14 patients. Four patients having intermediate smoking habits were considered independently (Group III). Two of these patients were siblings with a history of familial emphysema. The Control group consisted of 28 healthy nonsmoking subjects. Active serum α_1-PI levels were assayed spectrophotometrically, by determining the extent of trypsin inhibition. α_1-PI phenotypes were determined by isoelectric focusing on polyacrylamide gels. Once the serum α_1-PI levels have been compared, the differences between Group I and Group II and the Control group were statistically insignificant ($p > 0.05$). Isoelectric focusing results showed that all but 4 patients had the M_1M_1 or M_1M_2 phenotype. (The siblings in the Group III had the ZZ phenotype and 2 patients in Group II had undefined phenotypes). Considering these results, we concluded that, with the exception the siblings expressing the ZZ variant, the development of emphysema in our patients was caused by an increase in elastase load rather than a deficiency in antielastase capacity. The increase in elastase load was most likely due to biological and chemical oxidants in the lungs.

Key Words: Emphysema, α_1-proteinase inhibitor, phenotypes, cigarette smoking, isoelectric focusing.

ÖZET

Çalışmamızda, amfizemli hastalarda ve sağlıklı kişilerde fonksiyonel olarak aktif serum α1-proteinaz inhibitörü (α_1-PI) düzeyleri ve fenotipleri belirlendi. Hastalar sigara içme alışkanlıklarına göre sınıflandırıldı: Grup I sigara içmeyen yada çok az sigara içen 7 hastadan oluştu. Grup II çok sigara içen 14 hastadan oluştu. Ayrıca orta derecede sigara içen dört hasta diğerlerinden bağımsız olarak göz önüne alındı. Bu hastalardan ikisi ailesel amfizem öyküsüne sahip ikiz kardeşlerdi. Kontrol grubu ise sigara içmeyen 28 sağlıklı bireyden oluştu. Aktif serum α_1-PI düzeyleri, spektrofotometrik olarak tripsin inhibisyonu üzerinden tayin edildi. α_1-PI fenotipleri ise poliakrilamid jelde izoelektrik odaklama yöntemi ile belirlendi. Serum α_1-PI düzeyleri karşılaştırıldığında; Grup I, Grup II ve kontrol grubu arasındaki farklar istatistiksel olarak önemsizdi ($p > 0.05$). İzoelektrik odaklama sonuçları 4 hasta dışında diğer tüm hastaların M_1M_1 yada M_1M_2 fenotipine sahip olduğunu gösterdi (Grup III deki ikiz kardeşler ZZ fenotipine sahipti; Grup II'deki iki hasta ise belirlenememiş fenotiplere sahiplerdi). Bu sonuçlar gözönüne alındığında; ZZ varyantına sahip ikizler dışında amfizem gelişiminin, antielastaz kapasitesindeki yetersizlikten ziyade elastaz yükündeki artış nedeniyle olduğu sonucuna varıldı. Elastaz yükündeki artış, büyük olasılıkla akciğerlerdeki biyolojik ve kimyasal oksidanlar nedeniyle idi.

Anahtar Kelimeler: Amfizem, alfa-1-proteinaz inhibitörü, sigara içimi, izoelektrik odaklama.

INTRODUCTION

Emphysema is an irreversible condition of the lung marked by progressive enlargement of the alveoli distal to the terminal bronchioles and destruction of alveolar walls (1,2). There are two major forms of the disease: Centriacinar and panacinar emphysema. Centriacinar emphysema develops in the central portion of the acinus in close proximity to the respiratory bronchioles and is predominantly associated with cigarette smoking. Less commonly observed panacinar emphysema involves air space enlargement throughout the acinus. Panacinar emphysema arises as a result of a deficiency in the hepatic synthesis and /or secretion of α_1- proteinase inhibitor (α_1-PI, also referred to as α_1-antitrypsin) (3).

Laurell and Ericksson (4) first described the relationship between inherited α_1-PI deficiency and the development of emphysema The current protease-antiprotease theory of the pathogenesis of emphysema claims that most human emphysema develops when the protective effect of α_1-PI is overwhelmed by the destructive effects of proteolytic enzymes, largely released by polymorphonuclear leukocytes (5-7). The inability of α_1-PI to cope with the proteolytic load may arise from a primary (genetic) shortage in the supply of inhibitor or from increased protease insult.

α_1-PI is able to inhibit a variety of serine proteinases, including neutrophil elastase, trypsin, chymotrypsin, cathepsin G, plasmin, thrombin, tissue kallikrein, factor Xa and plasminogen, but its major physiological target is neutrophil elastase, a powerful protease capable of cleaving a wide variety of extracellular matrix components, including elastin (8,9). α_1-PI is a quantitatively important component of the antielastase screen in human lung tissue, making up over 90 % of the antielastinolytic protection in the lower airways. Synthesized mainly in the liver, α_1-PI, is an acute phase protein and its plasma concentration increases in response to inflammation. In normal individuals α_1-PI serum levels are 20 to 48 μM (10).

α_1-PI is a 52 kDa glycoprotein with a single polypeptide chain of 394 amino acid residues, and carries three carbohydrate side chains linked to asparagine residues at positions 46, 83 and 247 (11). Variation in the carbohydrate composition of the molecule is the major cause of the observed multiple banding patterns on acid electrophoresis or isoelectric focusing (12). The protein is anionic at physiological pH with an isoelectric point of ~ 4.5. The reactive center of α_1-PI contains a methionine residue. This amino acid is susceptible to oxidation by a variety of oxidizing agents, resulting in a marked decrease in the association rate of the inhibitor with neutrophil elastase (13).

The gene responsible for the expression of α_1-PI is located on chromosome 14; the defect leading to α_1-PI deficiency is caused by a mutation on this gene. α_1-PI gene is very polymorphic with at least 75 alleles known (14). The most common type called as "M", accounts for 95 % of white, northern European genes. The classical deficient type, PI Z, results from a single substitution in the genetic code that replaces glutamic acid in position 342 with lysine. Subjects with PI ZZ have about 15 % of the normal quantity of α_1-PI in their plasma. Carriers of the deficiency PI MZ have about 60 % of normal levels. The S genotype, the most common of all non-M variants is found frequently in persons of European Spanish descent. It results from a point mutation that replaces the glutamic acid residue at position 264 with valine; the S type is not known to be associated with any disease (15-17).

Increased protease insult may lead to emphysema in persons with normal levels of α_1-PI, by depleting available inhibitor. Cigarette smoking is a specific risk factor in this context. Cigarette smoke contains irritants that cause recruitment of neutrophils and macrophages to the lung, resulting in increased proteinase burden. Neutrophils are capable of generating oxygen free radicals, and cigarette smoke itself contains oxidants that can inactivate the proteinase inhibitors and promote unrestricted proteolysis. Several studies showed that cigarette smoke not only increases the number of neutrophils or level of elastase present in the lung but also decreases the protective effects of α_1-PI by oxidative inactivation of this inhibitor [18, 21]. However, some studies suggested that smoking did not affect α_1-PI functional activity (22,23).

The purpose of this study was to investigate α_1-PI activity in emphysema patients, the possible relationship between cigarette smoking and inhibitor activity and the phenotypic occurrence of variants of α_1-PI in Turkish patients. The prevalence of α_1-PI variants varies between different geographic areas and different racial groups, making α_1-PI a useful molecular marker for the study of human population genetics. Although the population frequencies of α_1-PI alleles have already been studied in many countries (24-26), there are few studies about α_1-PI phenotypes in Turkey (27). This study offers supplementary phenotypic information derived from a short-term survey.

MATERIALS and METHODS

TPCK-treated bovine pancreatic trypsin and N-α-benzoyl-L-arginine ethyl ester were obtained from Sigma (USA). Remaining chemicals were purchased either from Sigma or Merck (Germany). Trypsin stock solutions (1 mg/ml; 43 μM) were prepared in 2 mM HCl and stored at 4 °C. Standard sera (for the α_1-PI phenotypes: M_1, M_1M_2, S, SZ, M_1S, M_2S, M_1Z, M_2Z and Z) were generously donated by Dr. J. P. Martin (Strasbourg, France), Dr. D. Cox (Toronto, Canada) and Dr. M.K Fagerhol (Oslo, Norway).

The study covered 25 patients diagnosed as emphysemic by clinical and radiological criteria (28,29) in the Department of Chest Diseases, Hacettepe University. Informed consent was obtained from all subjects. The patients were subgrouped according to smoking history.

Group I consisted of 7 patients (6 male, 1 female), aged 20-58 years (median, 32 years), nonsmoking or with a limited history of smoking (<8 package-years). (Package-year = no. of packages/day x years of smoking). Group II consisted of 14 patients (all male) aged 46-73 years (median, 60.5 years), with a history of heavy smoking (25-80 package-years; mean, 46 ±19). Four patients had intermediate smoking habits and two of them were siblings with a history of familial emphysema. These patients (age 28-41; smoking history, 15 <package years <25) were considered separately (Group III). Individualized information on the patients in the different groups is given Table 1.

Table I. Characteristics of patients with emphysema

Case no			Cigarette smoking habits			Serum	α_1-PI phenotype
	Age	Sex	Beginning age	Package-year	Quit smoking at	[α_1-PI], μM	
Group I							
1	25	M	-	-	-	20.5	M_1M_1
2	45	M	13	3	21	24.48	M_1M_1
3	33	M	26	7	still smoking	18.63	M_1M_1
4	32	M	-	-	-	17.74	M_1M_1
5	20	F	-	-	-	30.77	M_1M_1/M_1M_2**
6	22	M	19	3	still smoking	24.32	M_1M_1/M_1M_2**
7	58	M	-	-	-	8.87	M_1M_1/M_1M_2**
Group II							
1	48	M	23	25	still smoking	12.76	M_1M_2
2	46	M	21	25	still smoking	20.51	M_1M_1/M_1M_2**
3	49	M	24	25	still smoking	41.17	M_1M_1
4	72	M	47	25	still smoking	9.21	?*
5	65	M	17	72	still smoking	34.88	M_1M_1
6	73	M	16	55	71	23.25	M_1M_1
7	61	M	21	60	60	33.17	M_1M_2
8	53	M	13	40	still smoking	22	M_1M_1
9	70	M	20	50	still smoking	21	M_1M_1
10	61	M	21	40	still smoking	17.42	M_1M_1/M_1M_2**
11	62	M	19	43	still smoking	30.69	?*
12	60	M	20	40	still smoking	27.15	M_1M_1
13	49	M	17	80	still smoking	20.35	M_1M_1/M_1M_2**
14	46	M	16	62.5	41	18.17	M_1M_1/M_1M_2**
Group III							
1	35	M	14	20	34	0**	ZZ
2	41	M	20	25	40	0**	ZZ
3	31	F	16	15	still smoking	25.59	M_1M_1
4	28	M	13	15	still smoking	21	M_1M_1

*Phenotypes were not defined because of unavailability of reference standards.

** Patients that we could not determine exact subtypes.

The control group consisted of 28 nonsmoking volunteers (9 male, 19 female), aged 19-83 years (median, 28 years).

Collection and storage of blood samples. Venous blood samples were collected into nonheparinized tubes. Sera were collected, supplemented with 0.02 % sodium azide and divided into 2 parts. One part was stored at -20 °C for later use in α_1-PI variant analysis. The second part was dialyzed against 20 mM potassium phosphate buffer (pH 7.4), stored at 4°C and analyzed for α_1-PI activity.

Assay of trypsin activity. Trypsin activity was determined by using N-α-benzoyl-L-arginine ethyl ester (BAEE) as substrate (30). Trypsin (0.3- 1 µM was preincubated with 20 mM potassium phosphate buffer (pH 7.4) at 25 °C for two minutes. The assay was started by transferring 200 µl of the preincubation mixture to 1 ml of 0.5 mM BAEE in 10 mM Tris.HCI (pH 8.0). BAEE hydrolysis caused by trypsin was followed by monitoring the increase in absorbance at 253 nm. Activity measurements were carried out in duplicate and control trypsin activity was 0.745 ± 0.085 A /min -µM enzyme.

Assay of α_1-PI activity. α_1-PI activity was assayed by determining the extent of trypsin inhibition [30]. Diluted serum samples ((1/28.6, 1/25,1/20) were preincubated with 1 µM trypsin at 25° C in 20 mM potassium phosphate buffer (pH 7.4) for two minutes. Remaining trypsin activity in 200 µl preincubation mixture was determined as described above. α_1-PI levels in serum samples were calculated according to Equation I (f, dilution factor).

Equation I

$$[\alpha_1\text{-PI}],\ \mu M = (\Delta A/min^{Control} - (\Delta A/min^{Serum}) \div 0.74 \times f$$

α_1-PI phenotyping. Isoelectric focusing (IEF) was used for α_1-PI phenotyping. Before IEF, all serum samples were treated with dithioerythritol (DTE) to reduce any disulfide linkages at the single cysteine residue in α_1-PI. To this end, 10 µl of 200 mM DTE solution was added to 90 µl of serum and the mixture was incubated for 1 h at 37 °C.

IEF with carrier ampholytes was performed according to the instructions supplied with the Bio-Rad Model 111 mini IEF equipment, using flat bed polyacrylamide gels (0.4 mm thick). The gels (125×65×0.4 mm) were prepared by mixing 2 ml acrylamide stock solution (24.25 % (w/v) acrylamide and 0.75 % (w/v) N,N'-methylene-bis-acrylamide), 2 ml 25 % (v/v) glycerol, 0.5 ml of Pharmalyte solution (pH. 4.2-4.9) and 5.5 ml distilled water. After degasing for 5 min, 15 µl ammonium persulphate (10 %), 50 µl riboflavin-5'-phosphate (0.1 %) and 3 µl N,N'-tetrametylene-ethylenediamine (TEMED) were added. Polymerization was complete after 1 hour under fluorescent light. 1.5-3.5 µl of reduced serum samples were applied to the prepared gel.

Focusing was carried out under constant voltage conditions in a stepped fashion (100 V for 15 min, 200 V for 15 min, 450 V for 60 min). The gel was fixed in 4 % sulfosalicylic acid, 12.5 % trichloroacetic acid and 30 % methanol for 45 min and stained with 0.04 % Coomassie Brilliant Blue R-250 in Solution I (27 % isopropanol, 10 % acetic acid, % 0.5 $CuSO_4$) for 1 h. Destaining was carried out by washing with Solution I until the background was clear and further with Solution II (7 % acetic acid, 25 % isopropanol) for 1 h to remove the last traces of stain and $CuSO_4$.

Observed bands were compared with those of standard serum samples and genetically typed.

Statistical Evaluation. SPSS package program was used for statistical evaluation. Kruskal-Wallis one way variance analysis was used for comparison of the groups.

RESULTS and DISCUSSION

In this study we determined the α_1-PI levels and α_1-PI phenotypes of emphysema patients, diagnosed by clinical and radiological findings and classified into two groups based on their smoking habits. A healthy, nonsmoking Control group was also studied. The results of the patient and control groups were compared.

Table I shows the serum α_1-PI levels and α_1-PI phenotypes for all patients. Comparing the serum α_1-PI levels, the differences between Group I and II and the Control group were statistically insignificant. ($p > 0.05$). Mean serum α_1-PI levels of all three groups (Group I 20.76 ± 6.85 µM, Group II 23.70 ± 8.82 µM, Control group 21.46 ± 4.29 µM) were within normal limits (20-48 µM).

The IEF patterns of selected patients and of selected control subjects are shown in Figure 1 and Figure 2, respectively. Since serum α_1-PI levels were remarkably low in some of the patients and one subject in the Control group and almost undetectable in two patients in Group III, we thought that these subjects might have inadequate α_1-PI alleles. All of the patients in Group I, 12 of the 14 patients in Group II, 2 of the 4 patients in Group III and 27 of the 28 volunteers in the Control group had M_1M_1 or M_1M_2 phenotype. IEF patterns showed that the two patients (case 1 and 2) in Group III (the siblings) had the ZZ phenotype (Figure 3). The IEF profile of one volunteer (case 1) in the Control group was consistent with an M_1Z phenotype (Figure 2). We could not determine the phenotypes of the two patients (case 4 and 11) in Group II with our standard serum samples (Figure 1).

The main reason for emphysema is the impairment of elastase/antielastase balance in favor of elastase, incurred by an increase in elastase load and/or acquired or hereditary shortage in α_1-PI activity (5-7).

The predominance of the M allele in the emphysema patients in the present study and the fact that there was no meaningful difference between serum α_1-PI levels in the patient and control groups implicate an increase in elastase load due to biological and chemical oxidants in the lungs. (31). The early onset of emphysema in the nonsmoking (or light smoking) Group I patients suggests that physical and chemical insults other than those caused by smoking may have a deeper impact on the lungs. In

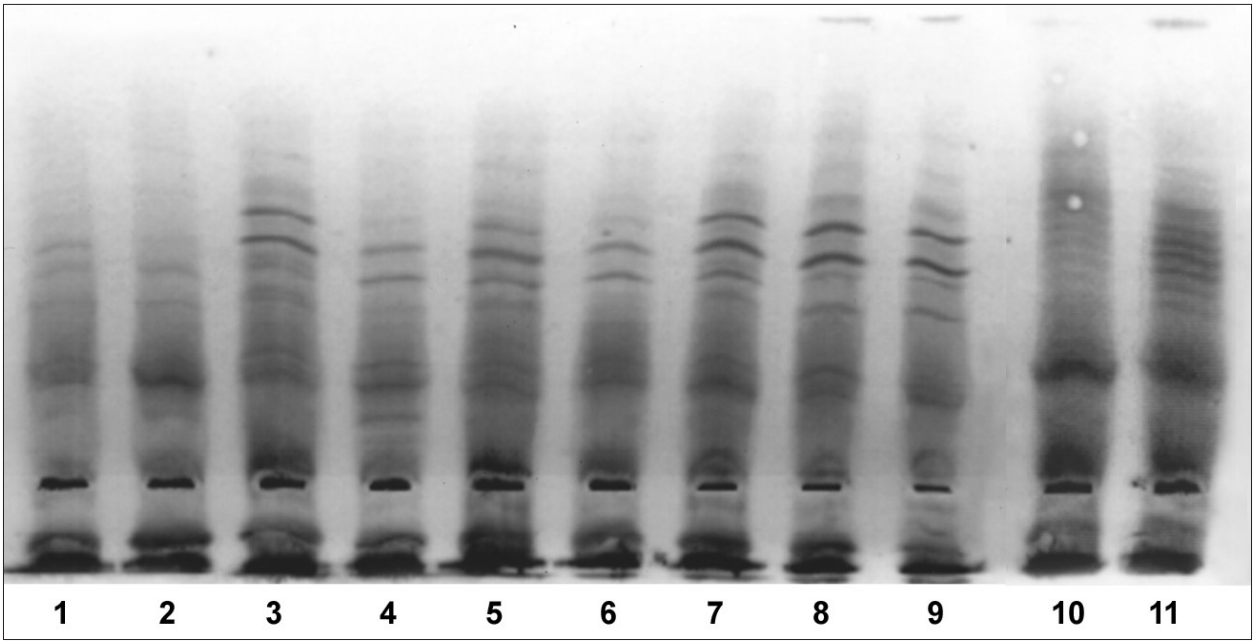

Figure 1 *The IEF patterns of standard serum samples and sera from selected patients in Group II.*
(1) *M_2Z* ***(2)*** *Z* ***(3)*** *M_1Z* ***(4)*** *SZ* ***(5)*** *M_2S* ***(6)*** *S* ***(7)*** *M_1S* ***(8)*** *M_1M_2* ***(9)*** *M_1* ***(10)*** *Grup II case 4* ***(11)*** *Grup II case 11 (anode at the top, cathode, at the bottom).*

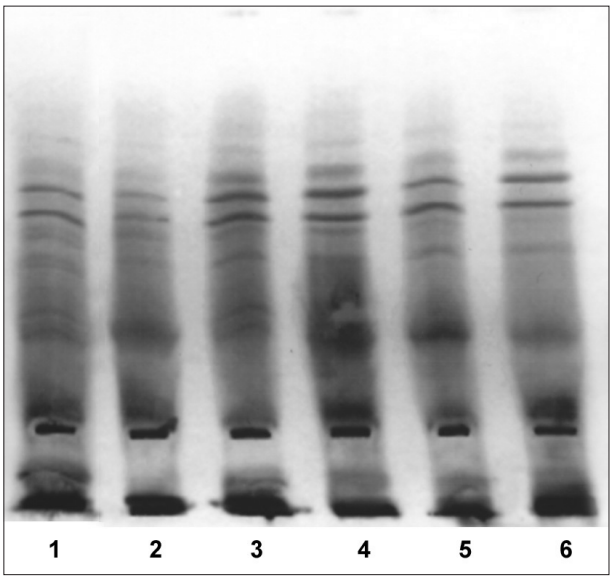

Figure 2 *The IEF patterns of selected control subjects*
(1) *M_1Z* ***(2)*** *Control group case1* ***(3)*** *M_1M_2* ***(4)*** *Control group case 25* ***(5)*** *M_1* ***(6)*** *Control group case 12 (anode at the top, cathode, at the bottom)*

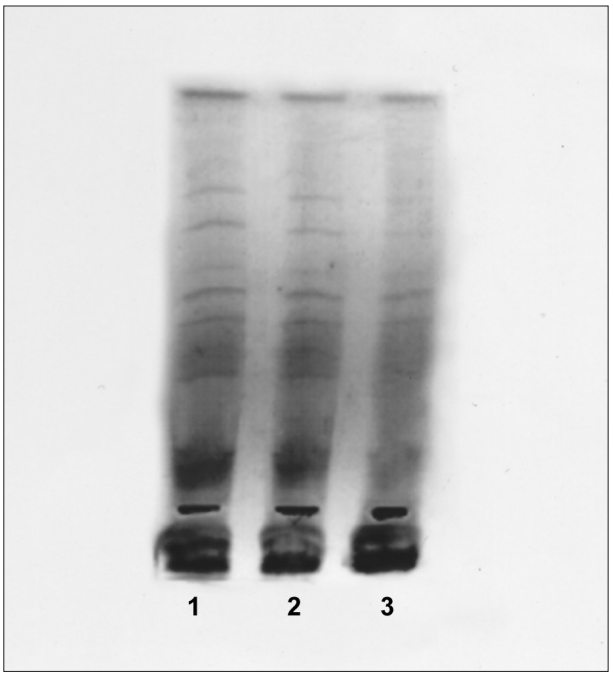

Figure 3 *The IEF patterns of two siblings in Group III*
(1) *Case 1* ***(2)*** *Case 2* ***(3)*** *Z*

our study cigarette smoking did not reduce the functional activity of serum α_1-PI. This finding contradicts a previous report on the impairment of the functional activity of serum α_1-PI with smoking (32) and is consistent with later studies on the subject (22,23,33). An extension of the study to include a larger number of patients and volunteers, a wider spectrum of hereditary and enviromental parameters; determination of elastase load in bronchoalveolar secretions should help to evaluate the various risk factors involved.

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