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Functional Levels of α_2-Macroglobulin in Plasma in Relation to Emphysema

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Summary: In most studies, concentrations of α_2-macroglobulin are determined by immunological techniques. In this study, the amidolytic activity of porcine pancreatic elastase complexed with α_2-macroglobulin was measured using an elastase-specific substrate, succinyl-trialanyl-*p*-nitroanilide. The activities of plasmas from 47 emphysema cases were compared with 39 normal subjects. The age ranges of both groups were from 50 to 84 years. The mean activity of bound elastase in emphysema cases was 2.48 ± 0.03 kU/l of plasma. The mean for normal subjects was 1.48 ± 0.11 kU/l of plasma. The difference was very significant ($2P < 0.001$). All except 2 of the emphysema cases had smoked. The same results were obtained when only people who had smoked for 25 years or more were included in the analysis. All the plasma samples of people included in the study were assayed by an immunological method for absolute level (in g/l) of α_1-proteinase inhibitor. The levels of α_1-proteinase inhibitor for all the persons studied fell within the normal range for MM-phenotypes (2 to 4 g/l).

Introduction

Much work has been done on the elastase-inhibiting capacity of plasma. Attempts have been made to relate this to development of lung disease (1). The main elastase inhibitor in plasma is α_1-proteinase inhibitor. This inhibitor has been extensively studied and the relationship between α_1-proteinase inhibitor deficiency and development of emphysema, as originally suggested by *Laurell & Eriksson* (2), is well documented. However, since most emphysema cases have normal levels of α_1-proteinase inhibitor (3), there must be other factors responsible for the development of this disease, in addition to the variation in concentrations of α_1-proteinase inhibitor.

Smoking is one factor which is highly correlated to the development of emphysema (4, 5). However, not all smokers develop emphysema. Non-smokers with the ZZ phenotype for α_1-proteinase inhibitor, do not all develop the disease either. This leads to the conclusion that the disease is multifactorial.

A second elastase-binding factor, present in plasma, is α_2-macroglobulin. This has been classified as an elastase-inhibitor because it reduces the elastolytic activity of elastase against native elastin. However, it does not prevent attack on lower molecular weight substrates, such as succinyl-trialanyl-*p*-nitroanilide. Tropoelastin, a precursor of elastin, is also attacked by the complex of α_2-macroglobulin with elastase at about 19 times the rate of attack on native elastin (6), but according to *Keuppers* et al. (7), only fragments of relative molecular size $M_r < 10\,000$ are hydrolysed by the complex. The rate constants of the reactions are also dependent on the type of elastase under consideration. When porcine pancreatic elastase is complexed with human α_2-macroglobulin, the activity against succinyl-trialanyl-*p*-nitroanilide drops to 74% of that of the unbound porcine pancreatic elastase. On the other hand, human neutrophil elastase when bound to α_2-macroglobulin, is 15 times as active as the unbound neutrophil elastase (8).

A further consideration is the modulating effect of α_2-macroglobulin on the inhibition of elastase by α_1-proteinase inhibitor, when mixtures of α_2-macroglobulin and α_1-proteinase inhibitor react with porcine pancreatic elastase. We have used this property to

devise a method for measurement of the elastase-binding capacity of α_2-macroglobulin and α_1-proteinase inhibitor in plasma, using porcine pancreatic elastase (9).

The present communication shows that the elastase-binding capacity of α_2-macroglobulin is highly correlated to the development of emphysema.

Materials and Methods

Human α_1-proteinase inhibitor was obtained from Hoechst. Porcine pancreatic elastase was obtained from Boehringer, Mannheim. The substrate used for porcine pancreatic elastase was succinyl-trialanyl-*p*-nitroanilide from Hoechst. Tris(hydroxymethyl)aminomethane buffer and all other reagents were of analytical grade (Merck).

Subjects

Fresh human blood was drawn into a Venoject tube containing lithium heparin (Comopharm, Johannesburg). Patients and controls were in the age group 50 to 84 years. Forty-seven caucasian outpatients with severe chronic obstructive pulmonary disease and emphysema, as defined by criteria in the literature (10), and according to the physician's best diagnosis, were selected from the Respiratory Clinic of the J. G. Strydom Hospital in Johannesburg. The 39 controls were caucasian volunteers donating blood to the South African Blood Transfusion Service. All subjects had given informed consent for the taking of their blood for this study. Smoking history was recorded for all subjects.

Assay of activity of the complex between plasma α_2-macroglobulin and porcine pancreatic elastase

The theoretical aspects of this assay have been discussed in a previous paper and a comparison has been made with other published methods (9). Since the complex is active against succinyl-trialanyl-*p*-nitroanilide in the presence of excess α_1-proteinase inhibitor, the plasma was first allowed to react with excess porcine pancreatic elastase and then the residual unbound porcine pancreatic elastase was neutralized with α_1-proteinase inhibitor. For both reactions 99% of the binding is completed within 2 min (unpublished observations). Ten microliters of plasma were reacted at 37 °C for 5 min with 100 µg porcine pancreatic elastase in 3.5 ml of 0.1 mol/l Tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4. The excess porcine pancreatic elastase was then completely inhibited by addition of 250 µg of α_1-proteinase inhibitor in 100 µl of buffer as above. After binding of α_1-proteinase inhibitor at 37 °C for 5 min the assay was started by addition of 2 mg of succinyl-trialanyl-*p*-nitroanilide in 50 µl of buffer.

The temperature was maintained at 37 °C for a further 15 min and the hydrolysis then stopped by addition of 0.5 ml of 100 g/l citric acid solution. Absorbance was read at 410 nm. The activity of the porcine pancreatic elastase-plasma-α_2-macroglobulin complex was calculated in International Enzyme Units using the value for the molar lineic absorbance for *p*-nitroaniline of 960 m^2/mol.

Freezing and thawing does not affect elastase-binding capacity, but even when frozen, the capacity decreases slowly. This is in agreement with the work of *Gressner & Peltzer* (11) who found that it was possible to assay the trypsin-binding capacity of α_2-macroglobulin only up to 10 days if stored at 4 °C and up to 3 weeks if stored at −20 °C. To avoid any possible inactivation, this assay was always performed within 24 hours after blood collection.

The active site of porcine pancreatic elastase is not the same as the site to which α_2-macroglobulin binds. Therefore, one would expect that porcine pancreatic elastase which has lost elastolytic activity could still bind to α_2-macroglobulin. Hence, we used the same batch of porcine pancreatic elastase throughout for the assays and assayed the normals and patients in alternate batches of 9 to 12 to eliminate possible bias. Zero time blanks and elastase activity assays, without addition of plasma or α_1-proteinase inhibitor, were also performed each time. There was no loss of amidolytic activity of our batch of porcine pancreatic elastase over the period of the study. With the batch of porcine pancreatic elastase which we used, the amidolytic activity on succinyl-trialanyl-*p*-nitroanilide was determined in exactly the same way as the other assay described above, except that plasma and α_1-proteinase inhibitor were replaced with 110 µl water, and the incubation period with succinyl-trialanyl-*p*-nitroanilide before adding the citric acid was 2 min. In addition, the endogenous elastase activity was tested by running the whole assay without the addition of elastase.

Absolute assay of α_1-proteinase inhibitor

The absolute concentration of α_1-proteinase inhibitor was determined by an antibody precipitation, laser-nephelometric method (12). The monospecific antisera were obtained from Dako, Denmark. The standard used for α_1-proteinase inhibitor was 'Protein Standard, Plasma' from Behring, West Germany.

Effect of bronchodilators on binding activity of α_2-macroglobulin

In order to ascertain whether bronchodilators, which are often used by patients with emphysema and chronic obstructive lung disease, had any effect on the binding activity of α_2-macroglobulin, a study was performed on a volunteer. The binding activity of the subject's plasma was assayed before administration of two drugs and again after treatment. The regimen consisted of treatment 3 times a day with two puffs of fenoterol hydrobromide (Berotec®, Boehringer Ingelheim) over a period of 10 weeks. During the final week, 15 ml of Solphyllex® (theophylline 100 mg, etofylline 10 mg, diphenylpyraline HCl 8 mg, ammonium chloride 720 mg, Na-citrate 300 mg, ethanol (volume fraction 0.2), chloroform (volume fraction 0.002)) were taken 3 times daily after meals and also at bedtime.

Statistical evaluation

Four different criteria viz. sex, smoking, age, and state of health, were statistically evaluated using *Student*'s t-test for 2 different variables viz. absolute level of α_1-proteinase inhibitor and the measured elastase-binding capacity of α_2-macroglobulin in the plasma. The coefficient of variation of the method for repetitions on the same sample was 2.9%, while day to day variation on the same sample was 3.2%.

Results

Absolute α_1-proteinase inhibitor concentrations

There was no significant difference between healthy smokers and non-smokers or between those with emphysema, and healthy controls in the age group from

50 to 84 years. Females had slightly higher concentrations than males, which was significant in controls ($2P < 0.05$) but not in the patients ($2P < 0.6$). There was no correlation with age for the group from 50 to 84 years (linear correlation coefficient (r) = 0.108) (tab. 2).

Endogenous elastase activity

No residual endogenous amidolytic activity was present in any of the plasma samples.

Elastase-binding capacity of α_2-macroglobulin

Smoking made no difference to the binding activity. There was no correlation of binding activity with age in the age group from 50 to 84 years (r = 0.149 for controls and r = 0.065 for emphysema cases). There was no difference between males and females in the controls for the age group from 50 to 84 years ($2P > 0.6$) (tab. 1). The emphysema cases, however, showed a definite difference between the sexes (tab. 2), ($2P < 0.005$).

Comparison of emphysema cases with controls gave the results shown in figure 1. It is clear that elastase-binding capacity levels of the healthy subjects are much lower than those of emphysema patients. The calculated mean for 39 subjects who did not have emphysema (controls) was 1.48 (standard error = 0.08) kU/l plasma; that for the 47 emphysema patients was 2.48 kU/l plasma (standard error = 0.10). A *Student* t-test on the two groups gave t = 7.6059 with 84 degrees of freedom ($P < 0.001$). This is conclusive evidence that the two groups are different.

From figure 1 we can also conclude the following:

(i) None of the healthy smokers or ex-smokers has a porcine pancreatic elastase-binding capacity for the α_2-macroglobulin in their plasma, above 2.18 kU/l.

(ii) All except one of the healthy non-smokers had a porcine pancreatic elastase-binding capacity for the α_2-macroglobulin in their plasma, of less than 2.18 kU/l. The one with a level of 2.61 kU/l shows no sign of emphysema at age 61. He is known to have been exposed to a minimum level of side-stream smoke.

(iii) Most of the emphysema cases have a level of porcine pancreatic elastase-binding capacity for the α_2-macroglobulin in their plasmas, above 2.18 kU/l. There are, however, several cases with lower levels, one as low as 1.6 kU/l. All the emphysema patients had been heavy smokers for many years.

Tab. 1. Normal healthy volunteers from 50 to 84 years of age. Values of α_1-proteinase inhibitor and elastase-binding capacity of α_2-macroglobulin in plasma.

Parameter	n[a]	Groups	α_1-Proteinase inhibitor in plasma Protein (g/l)[b]	Elastase-binding capacity of α_2-macroglobulin in plasma kU/l[b,c]
Sex	28	Males	2.39 ± 0.16	1.44 ± 0.10
	11	Females	2.78 ± 0.15[d]	1.58 ± 0.13
Smoking	16	Smokers[e]	2.56 ± 0.14	1.33 ± 0.08
	11	Non-Smokers	2.34 ± 0.15	1.61 ± 0.14
Age	30	All	NC[f]	NC[f]

[a] Number of subjects used.
[b] Mean value in plasma ± standard error.
[c] kU/l = kilo-international units of elastase (amidolytic) activity per liter of plasma.
[d] Group difference significant at $2P < 0.05$.
[e] Ex-smokers excluded. Smokers 25 years or more compared with those who never smoked except for side-stream smoke.
[f] NC = no linear correlation.

Tab. 2. Emphysema patients from 50 to 84 years of age. Values of α_1-proteinase inhibitor and elastase-binding capacity of α_2-macroglobulin in plasma.

Parameter	n[a]	Groups	α_1-Proteinase inhibitor in plasma Protein (g/l)[b]	Elastase-binding capacity of α_2-macroglobulin in plasma kU/l[b,c]
Sex	29	Males	2.46 ± 0.10	2.25 ± 0.02
		Females	2.54 ± 0.09	2.85 ± 0.04
Smoking	37	Smokers[e]	2.46 ± 0.08	2.52 ± 0.12
	10	Ex- and non-Smokers	2.56 ± 0.13	2.33 ± 0.16
Age	47	All	NC[f]	NC[f]

[a] Number of subjects used.
[b] Mean value in plasma ± standard error.
[c] kU/l = kilo-international units of elastase (amidolytic) activity per liter of plasma.
[d] Group difference significant at $2P < 0.005$.
[e] Smoked for 25 years or more compared with those who stopped smoking before they had smoked for 25 years or never smoked at all except for side-stream smoke.
[f] NC = no linear correlation.

Effect of bronchodilators on elastase-binding capacity of α_2-macroglobulin

The regimen described had no significant effect on the elastase-binding capacity of α_2-macroglobulin in the volunteer (before treatment 2.61 kU/l of plasma, and after treatment 2.44 kU/l of plasma).

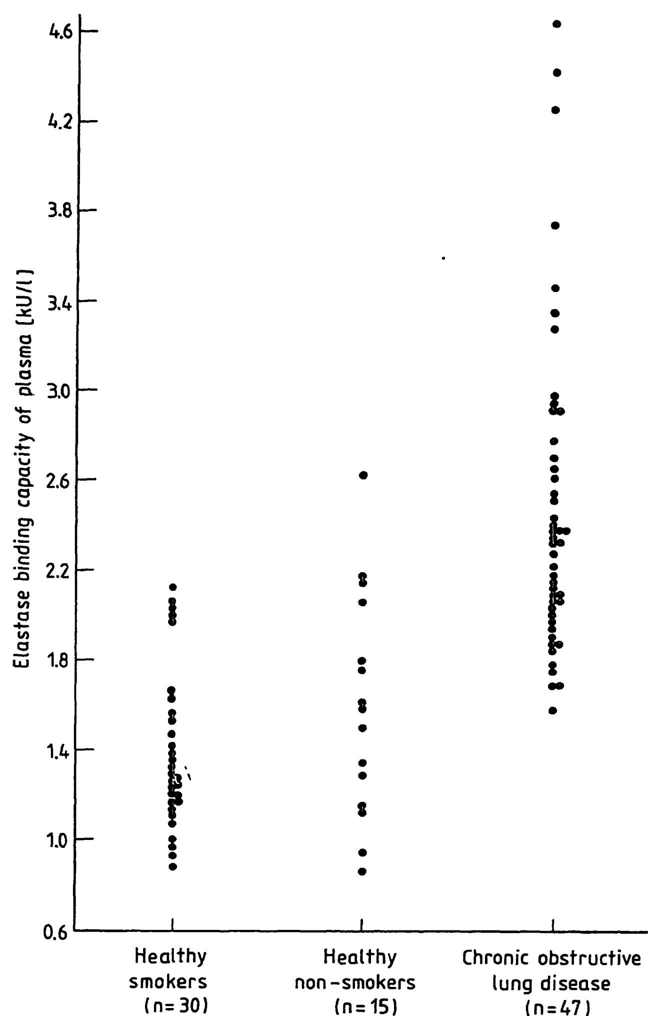

Fig. 1. Elastase-binding capacity of α_2-macroglobulin in plasma:
(a) 30 healthy smokers;
(b) 15 healthy non-smokers;
(c) 47 patients with chronic obstructive lung disease.
All three groups were from 50 to 84 years of age.

Discussion

Emphysema has a multifactorial aetiology. Smoking is strongly linked to the development of this disease. When cigarette smoke gets into the lung, it triggers a series of cellular and molecular events. The alveolar macrophages release a chemotactic factor which attracts polymorphonuclear leukocytes (13). Furthermore, this chemotactic factor acts on the neutrophils to release their granular contents, including elastase which is responsible for the destruction of elastin (14).

The main inhibitor of elastase is α_1-proteinase inhibitor. Since 1963, when *Laurell & Eriksson* (2) demonstrated the presence of different phenotypes of α_1-proteinase inhibitor, much attention has been devoted to the study of α_1-proteinase inhibitor deficiencies in relation to the incidence of emphysema. However, those studies have revealed that the majority of emphysema patients have normal plasma concentrations of α_1-proteinase inhibitor (15).

It has been suggested that cigarette smoke inactivates α_1-proteinase inhibitor in the lung, by oxidizing the critical methionyl residues. This mechanism was confirmed by the findings of *Carp* et al. (16). This oxidative inactivation is not necessarily permanent, as it can be reversed by the enzyme methionine sulphoxide-peptide reductase (17). At present there is considerable controversy as to whether or not smoking inactivates α_1-proteinase inhibitor. Recent studies were unable to show any difference in the functional activity of α_1-proteinase inhibitor from bronchoalveolar lavage, between smokers and non-smokers (18, 19). Also, smoking does not affect α_1-proteinase inhibitor activity in serum, as demonstrated by *Bridges* et al. (20).

Our results show that the plasma concentrations of α_1-proteinase inhibitor in all emphysema cases were in the normal range, and no difference was found between healthy controls and our patients ($P > 0.4$).

However the plasma concentrations of α_2-macroglobulin as expressed by elastase-binding capacity were significantly different in emphysema patients from those of healthy controls.

Pedersen & Franck (21), using the rocket immunoelectrophoresis technique for measuring α_2-macroglobulin plasma concentrations, also detected a significant difference between 20 patients with chronic airways obstruction and 20 age- and sex-matched controls. Using the same technique, however, other investigators found no difference between these two groups (22, 23, 24).

Since all except two of the emphysema cases were active smokers or were ex-smokers, and the two non-smokers had both been exposed to constant side-stream smoke from their spouses and work colleagues, a comparison was made between those who had smoked for at least 25 years in the two groups. Here the mean value of elastase-binding capacity of healthy long-term smokers ($n = 11$) was 1.33 kU/l of plasma while the mean value of elastase-binding capacity for the long-term smoking emphysema patients ($n = 39$) was 2.45 kU/l of plasma ($2P < 0.001$). From this we deduce that the difference in the concentrations is not due to smoking; some healthy non-smokers had even higher concentrations than the average of the healthy smokers. Since neither age nor smoking are the cause of high concentrations of elastase-binding capacity of α_2-macroglobulin in plasma, and because the emphysema cases have high values of this capacity, we postulate that a high elastase-binding capacity of α_2-macroglobulin in plasma, in conjunction with a

chronic lung challenge such as smoking, is a predisposing factor for the development of emphysema. A possible mechanism for this is that since the bound elastase is not inhibited by α_1-proteinase inhibitor, it can still cause proteolysis of elastin-precursors (6). This prevents the replacement of any elastin which is destroyed by the normal turnover mechanism in the body, resulting in a gradual depletion of elastin, which leads to emphysema. Tobacco smoke is known to inhibit ciliary action and it is possible that it also inhibits the phagocytosis of the complex between α_2-macroglobulin and neutrophil elastase to a sufficient extent to allow the complex to exert its proteolytic effect on elastin-precursors in the lung.

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