DECREASED FORMATION OF α_2-MACROGLOBULIN-PROTEASE COMPLEXES IN PLASMA OF PATIENTS WITH CYSTIC FIBROSIS

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SUMMARY

Alpha$_2$-macroglobulin from patients with cystic fibrosis is shown to have reduced binding with papain, trypsin, and thrombin. The obligate heterozygotes for cystic fibrosis revealed intermediate values between the controls and the patients. Since papain and trypsin are not plasma endopeptidases, it becomes evident that the absence of α_2-macroglobulin-protease complex in cystic fibrosis is due to a molecular defect within the macroglobulin.

INTRODUCTION

The basic defect in cystic fibrosis (CF), the most common significant autosomal recessive disorder affecting Caucasians, remains as yet unknown [1]. Previous work in our laboratory has documented the deficiency of a proteolytic activity with arginine esterase specificity in saliva [2] and plasma [3, 4] of patients with CF. On the basis of these observations, Rao and Nadler [2] suggested that the various "factors" present in CF resulted from a deficiency of this proteolytic activity. Wilson and Fudenberg [5] provided additional evidence for this hypothesis when they demonstrated a deficiency of an α_2-macroglobulin (α_2M) fragment in plasma of patients with CF and obligate heterozygotes when compared to controls. They suggested that an abnormality

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Abbreviations: CF: cystic fibrosis disease
α_2M: α_2-macroglobulin
PBS: phosphate buffered saline, pH 7.0

in the binding affinity of the α_2M for plasma proteases may account for the presence of the CF factors. Recently, we have documented [6] the absence of an α_2M-protease complex in plasma of patients with CF. In order to determine if a deficiency of α_2M fragment [5] and the absence of α_2M-protease complex in CF [6] is secondary to either an abnormal human serum protease or a molecular defect within the α_2M, the following study was performed.

Since α_2M binds numerous endopeptidases both with thiol or serine groups in their binding sites [7], the binding of papain, bovine trypsin, and thrombin to normal and CF α_2M was determined. The studies demonstrate a decreased binding of these endopeptidases to α_2M from patients with CF and the obligate heterozygotes when compared to controls.

MATERIALS AND METHODS

Papain (lot 36A779) and bovine trypsin (lot 36A797) were obtained from Worthington Biochemical Corporation (Freehold, New Jersey); bovine thrombin (lot 1146-8310) and p-hydroxymercuribenzoate were from Sigma Chemical Company (St. Louis, Missouri); dithiothreitol was from Calbiochem (San Diego, California); agarose was from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin); and $Na^{125}I$ was from New England Nuclear (Boston, Massachusetts). Rabbit antiserum to human α_2M and protein standard serum B were purchased from Behring Diagnostics (Somerville, New Jersey). All other reagents were reagent grade.

Blood was collected using plastic equipment. Contact with glass surfaces was avoided in order to prevent activation of proteolytic proenzymes. The blood samples were brought to 0.38% sodium citrate and the plasma was separated. Plasma samples were obtained from 10 age-matched controls, 15 cystic fibrosis patients, and 13 obligate heterozygotes for CF.

The quantitative determination of α_2M concentration was performed by single radial immunodiffusion according to Mancini et al. [8]. The anti-α_2M antiserum final concentration in the agarose was 2%. The plasma samples applied for determination were diluted v/v with phosphate buffered saline (PBS) and 6 µl were applied in duplicate. Four serial dilutions of serum with a known α_2M concentration (protein standard serum B) were used for the standard curve. The diffusion was completed at 38° C.

Iodination of trypsin, papain, and thrombin with ^{125}I was performed by the chloramine T procedure [9]. The enzyme concentrations were determined spectrophotometrically assuming a specific extinction coefficient at 280 nm of $E^{1\%}_{1\,cm} = 13.5$ for trypsin [10], $E^{1\%}_{1\,cm} = 24.6$ for papain [11], and $E^{1\%}_{1\,cm} = 19.5$ for thrombin [12]. The radiolabeled enzymes had 19×10^6 cpm/mg papain, 28×10^6 cpm/mg trypsin, and 27×10^6 cpm/mg thrombin. Trypsin solutions were kept at pH 3.0 and brought to pH 7.0 immediately prior to use in experiments.

Binding of Proteases to α_2M: Alpha$_2$M concentration was determined in plasma of control (1.6 mg/ml), patient with CF (2.1 mg/ml), and obligate heterozygote (2.4 mg/ml). Each sample was diluted with PBS to a final α_2M concentration of 1 mg/ml. To 100 µl aliquots, increasing concentrations of papain were added (100 µl of the enzyme in the presence of 3 mM dithiothreitol and 2 mM EDTA) and incubated for 30 minutes at 38° C. To prevent the digestion of IgG antibodies by papain excess, the enzyme was inhibited by adding 0.6 ml of 2 mM p-hydroxymercuribenzoate in PBS. In order to precipitate selectively the α_2M-papain complexes, 200 µl of anti-α_2M antiserum were added, incubated at 38° C for 60 minutes and overnight at 4° C. The immuneprecipitates were separated by centrifugation, washed x 3 with 1 ml cold PBS and dissolved in 1 ml 0.1 M NaOH. The bound papain in the immuneprecipitate was determined by cpm in the dissolved immuneprecipitate.

Similar experiments were performed using ^{125}I-trypsin. In these experiments, the α_2M concentrations of the plasma samples were 2.1 mg/ml in the control, 1.7 mg/ml in the CF, and 1.9 mg/ml in the heterozygote. The samples were diluted with PBS to a final α_2M concentration of 1 mg/ml. The procedure was followed as with papain only neither activation nor inhibition of the enzyme was indicated and the volume was brought to 0.8 ml with PBS before adding the anti-α_2M antiserum. In these experiments, the ^{125}I-labeled enzymes were diluted 1:1 with "cold" enzyme.

In an attempt to determine the binding ratio using a simpler procedure for a larger group of samples, the following experiment was performed. To 50 µl of plasma samples, 100 µg of ^{125}I-trypsin in an equal volume were added and incubated for 30 minutes at 38° C. Duplicate aliquots (6 µl) were applied for single radial immunodiffusion against anti-α_2M antiserum. The α_2M concentration was determined by calculating the ring area as compared to a standard curve of serial dilutions of control human plasma with a known α_2M concentration. After the diffusion was completed, the plates were washed exhaustively in PBS, the rings were punched out and dissolved in 1 ml of hot 1 M NaOH. The bound trypsin was determined according to the cpm in the dissolved immuneprecipitate. The binding of ^{125}I-trypsin to 1 mg of α_2M was calculated from the ratio of cpm in the ring to the α_2M concentration. The same procedure was used to determine the binding ratio of ^{125}I-thrombin to α_2M.

RESULTS

The binding of increasing concentrations of ^{125}I-papain to 100 µg α_2M in plasma from controls, patients with CF, and obligate heterozygotes are shown in Figure 1. The CF plasma samples revealed decreased binding of the enzyme in the α_2M immuneprecipitate when compared to the control. The binding in the heterozygote showed intermediate values. The experiment with ^{125}I-labeled bovine trypsin demonstrated similar findings and the results are shown in Figure 2.

The binding of ^{125}I-trypsin to 1 mg of α_2M, determined by the modified single radial immunodiffusion procedure, in plasma samples of

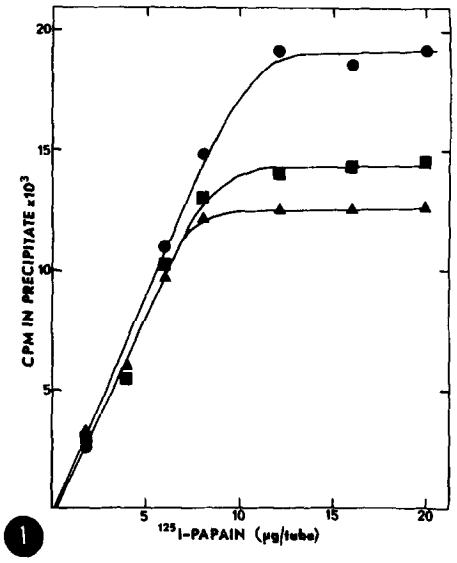

Figure 1: ^{125}I-Papain (9,500 cpm/µg) in the immuneprecipitate of 100 µg α_2-macroglobulin from a patient with cystic fibrosis (▲----▲), an obligate heterozygote (■----■), and a control (●----●) with anti-α_2-macroglobulin antiserum.

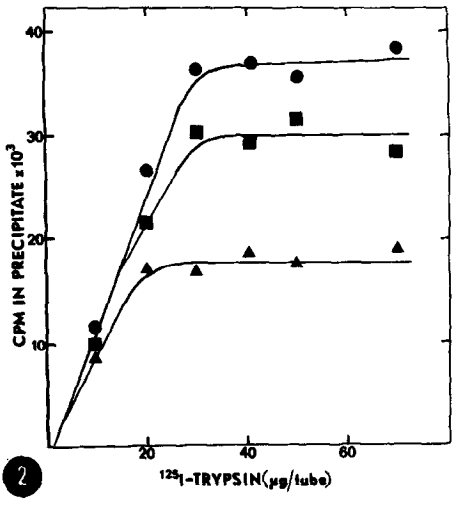

Figure 2: ^{125}I-Trypsin (14,000 cpm/µg) in the immuneprecipitate of 100 µg α_2-macroglobulin from a patient with cystic fibrosis (▲----▲), an obligate heterozygote (■----■), and a control (●----●) with anti-α_2-macroglobulin antiserum.

10 controls, 15 patients with CF, and 13 obligate heterozygotes is shown in Figure 3. The trypsin bound to α_2M, immunologically quantitated, was significantly reduced in the CF samples ($t = 7.2$; $p < 0.01$). The obligate heterozygotes represented an intermediate group with individual samples overlapping both the normal and the CF groups. The mean value for the obligate heterozygotes was significantly lower than the normal mean value ($t = 3.6$; $p < 0.01$) and significantly higher than the mean value of the CF group ($t = 2.9$; $p < 0.01$).

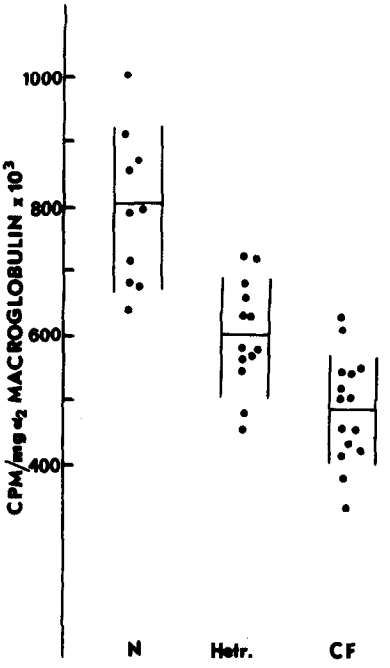

Figure 3: ^{125}I-Trypsin (28,000 cpm/µg) bound to 1 mg of α_2-macroglobulin of patients with cystic fibrosis (CF), obligate heterozygotes (Hetr) and controls (N).

The binding of ^{125}I-thrombin to 1 mg of α_2M is shown in the following table.

Table I

Binding of ^{125}I-Thrombin (27,000 cpm/µg) to 1 mg of α_2-Macroglobulin

	Number of Samples	cpm x 10^3	
		Mean Value	Range
Normal	5	834	610-1172
Cystic fibrosis	8	306	183- 514
Cystic fibrosis heterozygotes	6	656	262- 845

DISCUSSION

The present study, using an immunological methodology, demonstrates decreased binding of α_2M to papain, bovine trypsin, and bovine thrombin in plasma of patients with CF and obligate heterozygotes. Since papain and trypsin are two endopeptidases which are not serum proteases, it is apparent that the molecular defect leading to the deficiency of α_2M fragment [5] and the α_2M-protease complex [6] in CF must, therefore, be due to a defect within the α_2M. The observation that both a thiol peptidase (papain) and two serine peptidases (trypsin and thrombin) revealed decreased binding suggests that similar abnormalities would be shown with other endopeptidases that are bound to α_2M.

Since α_2M binds only peptidases in their proteolytic active form, it has been postulated that the enzyme cleaves a peptide bond in a sensitive region of the macroglobulin causing a conformational change in the α_2M that traps the enzyme irreversibly [7, 13]. The α_2M-protease complex retains proteolytic activity towards only low molecular weight substrates [7, 13], therefore, its absence might well explain the appearance of various CF "factors" of a polypeptide nature [1]. The biological role of the α_2M-protease complex is not clear. It has been suggested that it may be of importance for the development of local inflammatory responses and the breakdown of biologically active polypeptides [13]. Although our sample size was small, the observation that the obligate heterozygotes revealed intermediate values of α_2M binding suggests that the abnormal α_2M is, in fact, an inherited metabolic defect in CF. The molecular defect in CF α_2M could be caused by a lower affinity to proteases, the release of the protease from the CF α_2M-protease complex that in normals is irreversibly bound [7, 13], or that binding sites in part of the CF α_2M are blocked.

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