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Letter to the Editor: Vitamin D Toxicity and Infantile Hypercalcemic Syndrome

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We appreciate Dr. Forbes' remarks and agree with him that the evidence of vitamin D toxicity or sensitivity during pregnancy leading to infantile hypercalcemic syndrome is not conclusive. Presently, we are conducting additional research to test the effect of maternal vitamin D ingestion on the fetus.

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ANOMALOUS α_2-MACROGLOBULIN-PROTEASE COMPLEXES IN CYSTIC FIBROSIS : DECREASED UPTAKE OF THE COMPLEXES BY FIBROBLASTS IN CULTURE

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SUMMARY

Immunochemical and functional properties of control and Cystic Fibrosis (CF) α_2Macroglobulin (α_2M) are compared. Crossed immunoelectrophoresis and Ouchterlony double diffusion revealed no qualitative differences between the two α_2M preparations.
Trypsin-esterase activity assayed with BAPNA as a substrate, in the presence of an excess STI, gave similar ratios between total and active α_2M. These α_2M-trypsin complexes were equally stable under various experimental conditions and maintained a constant STI non-inhibited esterase activity.
Normal and CF-α_2M-trypsin complexes were taken up by normal human fibroblasts to a similar extent during a four hour period.
The only significant difference was observed when the uptake of α_2M from untreated sera was examined. The uptake of α_2M from CF sera was always lower than from pooled control sera despite large variation. Mixing of control and CF serum did not affect the normal uptake and other serum components were taken up to the normal extent. Intracellular degradation of CF α_2M had a half life of 2.0 to 2.8 hours, which compares well to the normal half life of 2.2 hours. More work needs to be done on the nature of the interaction between α_2M and proteases before a reasonable explanation for the molecular nature of the abnormal behavior can be sought.

SPECULATION

An in vitro system is described which might be of value for the study of the molecular anomaly in the α_2M-protease complex of patients with CF. Moreover, our observations, if confirmed and extended, could represent a rapid, quantitative and reproducible assay for the detection of patients with CF. Optimalization of the assay might allow one to detect heterozygotes and possibly diagnose cystic fibrosis in amniotic fluid.

INTRODUCTION

We have recently demonstrated that α_2-Macroglobulin (α_2M) is taken-up selectively from the growth medium by fibroblasts in culture.
The uptake of α_2M is preceeded by the binding of the molecules to high affinity receptors at the cell surface, and results in rapid degradation (half life 2.2 hrs) of the molecules intracellularly. Moreover, comparison between the kinetic parameters of binding and uptake of purified 'native' α_2M and α_2M-protease complexes indicated that this cellular property was selective for the α_2M-protease complexes. Whereas the physiologic implications of this process remain obscure, we could demonstrate that certain human tumor-derived cell lines lacked the specific surface receptor (7,8,9).
Anomalies in the behavior of α_2M, purified from the plasma of patients with cystic fibrosis have been described (3,4,5,10). The reported anomalies pertained to the increased stability of α_2M-protease complexes as well as to the decreased affinity of proteases for this ± 750,000 MW serum glycoprotein.
The present report has examined immunochemical and functional properties of control and cystic fibrosis (CF) α_2M. No differences in the immunoelectrophoretic properties of α_2M was identified. After complexation to trypsin both CF and control α_2M-trypsin complexes remained stable.
Furthermore, the uptake by cultured fibroblasts of α_2M-trypsin complexes from control or CF sera was similar. The only significant difference with control α_2M was found in the variable but reduced uptake of naturally occurring α_2M-protease complexes by the cells. Possible explanations for this observation will be discussed.

MATERIALS

Blood samples were obtained from CF patients and their parents through the cooperation of the Belgian Cystic Fibrosis Foundation, and the Department of Pediatrics, Catholic University of Leuven. Only ambulant patients between 2 and 10 year of age with documented disease and increased sweat-chloride tests were included. Pooled control serum was obtained from the Red Cross Blood Centre, Leuven, Belgium.
The antibodies used in immunoelectrophoresis were purchased from Dakopatts, Denmark (11). Crystalline trypsin was from Sigma (12) and Soybean trypsin inhibitor was from Calbiochem (13). Enzyme substrates were obtained from Serva (14).

METHODS

Crossed and rocket immunoelectrophoresis were carried out as described (7,9). Enzymatic activity of trypsin in the presence of α_2M was determined as described by Ganrot (1). Purification of α_2M (more than 95%) was as described (8).

Cell culture
Diploid human fibroblast cultures were established from skin biopsies of healthy volunteers. The growth medium consisted of Dulbecco's Modified Eagle's medium (DME), containing 10% (v/v) heat-inactivated Newborn Calf Serum (NCS), 1 g/l $NaHCO_3$, 15 mM N-Tris (hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) and 15 mM N-2-hydroxyethyl piperazine N-2 ethane sulfonic acid (HEPES) buffered to pH 7.5 with 1N NaOH; no antibiotics were added.
Cell suspensions were obtained by trypsin as follows: after decanting the medium, the cell layers were washed once with 0.02% EDTA in Tris-buffered saline (2 mM Tris, pH 7.4) at 37° C. Crystalline trypsin (0.05%, 2x crystalline Sigma, in EDTA solution), was added to the cell layer and incubation was continued for 10 min at 37° C. Cold phosphate buffered saline (PBS) was added and the cell suspensions were centrifuged for 7 min at 160 g, 4° C.
The cell pellets were resuspended in cold PBS, centrifuged, decanted and processed. If necessary cell pellets were stored frozen at -20° C until used in immunoelectrophoresis.

Uptake of α_2M
Confluent cell layers were kept in 10% rabbit serum in DME for at least 48 hrs before the experiments. This is sufficient time to degrade all intracellular α_2M taken up from the newborn calf serum (7). After three washes with serum-free DME, the cell layers were incubated at 37° C in DME with the indicated concentration of serum. After 4 hrs the medium was removed, the cells washed and trypsinized and assayed for intracellular α_2M as described (7).

RESULTS

1. Immunochemical and functional characterization of α_2M
Serum or plasma from controls and CF patients was examined by immunoelectrophoresis (IE) using commercial rabbit-anti-human α_2Macroglobulin antisera. Quantitative results were obtained by rocket IE (RIE), while qualitative characteristics were examined by crossed IE (CIE). Representative patterns obtained with the latter technique for control- and CF- α_2M are shown in Fig. 1. No immunochemical differences between control or CF-α_2M could be detected by CIE or by Ouchterlony double diffusion. As expected (10) the levels of α_2M in CF-serum were always higher than in the control sera (Table 1).
Determination of the trypsin binding capacity of α_2M as described by Ganrot (1), is based on the ability of α_2M to protect trypsin against the inhibitory activity of the high molecular weight inhibitor of Soybean (STI). Enzyme activity under these circumstances is assayed with a low molecular weight substrate (Benzoyl-Arginyl-Para-Nitro Anilide, BAPNA). When the levels of α_2M in serum are compared to the trypsin-esterase activity after STI addition, a measure of the amount of active α_2M in the serum is obtained(6). The ratios of total to active α_2M for control and CF α_2M were comparable (Table I). Similar findings were obtained for partially purified α_2M from different sera.
The stability of α_2M-trypsin complexes was examined by the same technique: progressive dissociation of the complexes would indeed result in a gradual increase in the STI-inhibited esterase activity and decreased total esterase activity would be measured. A representative experiment on highly purified control α_2M is shown in Fig. 2. It is clear from these results that in the presence of an excess trypsin, a constant fraction of non-STI-inhibited esterase activity can be measured. Similar results were obtained with highly purified CF α_2M. The stability of the control α_2M-trypsin complexes was further examined by changing experimental conditions which might affect the activity of the trypsin. These included, prolonged incubation at 37° C (up to 5 hrs); large excess of trypsin and/or STI over α_2M; elimination of Ca^{2+} and addition of EDTA (10^{-2}M). None of these modifications had a marked effect on the non-STI-inhibited esterase activity. Experiments performed with Tosyl-Arginyl-Methyl Ester (TAME) as substrate gave essentially the same results. Examination by polyacrylamide gel electrophoresis under reducing (dithiothreitol) and denaturating (Sodium dodecyl sulphate) conditions, showed both control and CF α_2M to form subunits of 85.000 MW with concomittant disappearance of the band of 180.000 MW after complexation with trypsin (Results not shown).

2. Uptake of α_2M-trypsin complexes by normal fibroblasts in culture
Previous work has demonstrated that the mechanism of α_2M-uptake in normal skin fibroblasts in culture was selective for α_2M-protease complexes (7,8,9).
To further examine whether the α_2M of control and CF serum behaved 'normally', sera were treated with trypsin to obtain complete saturation of α_2M (7). The results indicated that the α_2M-trypsin complexes from CF serum, were taken up to a similar extent as the complexes from control sera (Fig. 3).

3. Uptake of naturally occurring α_2M-protease complexes by normal fibroblasts in culture
The results thusfar described, did not indicate an aberrant behavior of CF α_2M when complexed to trypsin. In a final attempt to demonstrate an anomaly in the α_2M of the CF patients the uptake of naturally occurring complexes of α_2M, present in untreated serum, was examined. Uptake of α_2M was found to be dose-dependent for both CF serum and for control serum.
However, for an equal α_2M content in the culture medium, a significant reduction (30%) in the amount of CF α_2M taken up by the cells was evident.
These observations were extended to a larger group of individual serum samples (Table 2). Without exception, the uptake of α_2M from CF sera was, although variable, lower than from pooled control serum, and lower than from individual age matched control sera. Also included in Table 2 are the results obtained with sera of obligate heterozygotes (parents of Vo.S.). The uptake of α_2M from these sera was between that of the affected child and the control values.

4. Interference with uptake and intracellular degradation
To exclude interference with the uptake of α_2M by serum components or factors, which might be present in the CF sera, uptake was also measured with mixtures of normal serum and CF sera, from which uptake was either intermediate (serum Vr.S.) or low (serum E.C.). Mixing of these sera with normal serum did not affect the uptake of normal α_2M (Table 2).
Quantitation of other serum components (albumin, α_1-anti-trypsin, and transferrin) also taken up by the cells from serum, showed that these components were taken up to the same extent from control and CF serum, indicating the effect to be specific for CF α_2M.
Another factor which might have contributed to the lower uptake of α_2M measured under the described conditions, is intracellular degradation. If CF α_2M would be degraded faster than control α_2M, which is degraded rapidly (half life 2.2 hrs), this would be reflected in our experiments by an apparent lower extent of uptake. Measurements of the intracellular degradation of CF α_2M showed it to be degraded with a half life between 2.0 and 2.8 hrs, which is very similar to the results obtained on normal α_2M.

DISCUSSION

The results of our investigations on the stability of α_2M upon complexation with trypsin, are in conflict with previously reported observations (3,4,5,10). Although extreme care was taken to reproduce the experiments under comparable

circumstances, no evidence for a lack of stability in the control α_2M-trypsin complexes could be obtained. Our results on the 'normal' uptake of these α_2M-trypsin complexes by fibroblasts, are consistent with a 'normal' behavior of these complexes under our experimental conditions. In contrast, the decreased uptake of naturally occurring α_2M-protease complexes in cystic fibrosis does suggest that an anomaly exists at the level of these complexes. Preliminary results rule-out that factors, present in the serum, might interfere with the normal clearance of the complexes. More extensive investigation of the latter possibility and of the potential effect of naturally occurring antibodies (2) is being undertaken. Much work needs also to be done in order to elucidate the exact nature of the interaction between α_2M and proteases, before a reasonable explanation for the molecular nature of this abnormal behavior can be sought. Our observations, if confirmed by others, might form the basis for a rapid, quantitative and reproducible assay for the detection of patients with CF and will be of potential value for the in vitro study of anomalies of the α_2M-protease complex.

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Table 1 : Total and active α_2M

	α_2M (mg%ml)	Trypsin activity after STI (units/ml)	Ratio α_2M/activity
Control serum	1.60	0.192	0.120
CF serum	3.92	0.442	0.112
Partially purified α_2M from :			
- control serum	1.32	0.162	0.123
- CF serum	2.05	0.218	0.106

Control serum was obtained by pooling sera from 100 healthy blood donors; CF serum consisted of a pool of three unrelated affected children. Total α_2M was determined by rocket immunoelectrophoresis with anti-human α_2M-antibodies as described (7). Active α_2M was determined as described by Ganrot (1). One unit of activity is defined as the amount of trypsin hydrolyzing 1 µmole of substrate (BAPNA) per minute at 25° C. The partially purified α_2M was obtained from the sera by gel filtration on Sephadex-G-200. α_2M comprised about 50% of the protein present in the fraction examined.

Table 2 : Uptake of α_2M by control fibroblasts in culture.

Serum	Uptake of α_2M	Relative (%)
Control serum (pool)	0.32 ± 0.03	100
Control serum (individual)		
V.K.	0.30	94
K.S.	0.26	81
V.R.C.	0.27	84
B.B.	0.33	103
CF serum : Vr.S.	0.15	47
E.C.	< 0.02	< 6
M.A.	0.25	78
M.L.	0.25	78
Vo.S.	0.12	37
B.W.	0.14	44
B.A.	< 0.02	< 6
L.G.	< 0.02	< 6
S.D.	0.06	19
Control serum + CF serum Vr.S.	0.37	116
Control serum + CF serum E.C.	0.35	109
Heterozygote serum (parents Vo.S.)		
Vo.C.	0.23	72
W.E.	0.24	75

Normal human skin fibroblasts were cultured in DME-10% rabbit serum for 48 hrs. Uptake was measured as described in the Methods Section.
The volume of serum added was adjusted to obtain a final α_2M concentration of 200 µg per ml medium. When control serum was combined with CF sera, the final α_2M concentration was 400 µg/ml. Uptake of α_2M is expressed as µg α_2M per 2 x 10^5 cells after 4 hours. For control serum a pool of hundred healthy donors was used and the mean with standard deviation of 5 experiments is given. Control serum (individual) denotes sera obtained from children, age matched to the CF patients. In the last column the results are expressed relative to the uptake with control serum.

Fig. 1 : Crossed immunoelectrophoresis of control and CF serum α_2M. Serum (15 µl) was applied in the well (bottom right) and electrophoretically separated in the first dimension (anode to the left) at 10 V/cm for 90 minutes at 5° C. In the second dimension, the immunogel contained rabbit-anti human α_2M-antibodies (Dakopatts, Denmark) (11) at 3 µl/cm^2.
Electrophoretic conditions were 4V/cm for 18 hours at 15° C.
(A) is control serum and (B) is cystic fibrosis serum (L.G.).
Concentration of α_2M in these sera were 162 and 506 mg% respectively.

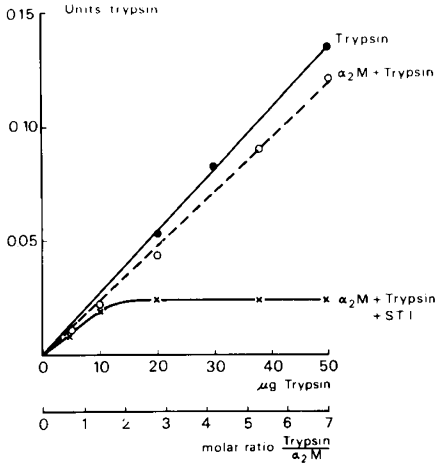

Fig. 2 : Activity of trypsin towards BAPNA in the presence of α_2M.
Trypsin activity was measured spectrophotometrically as described (1).
●—— ● : activity of trypsin alone
○--- ○ : activity of trypsin in the presence of 210 μg α_2M, assayed 30 min after mixing trypsin and α_2M
x——x : activity of trypsin in the presence of 210 μg α_2M and 50 μg Soybean trypsin inhibitor. STI was added 30 min after mixing trypsin and α_2M, and activity measurement was started 5 min later.
All incubations and assays were carried out at 25° C. The α_2M used was a highly purified preparation, obtained from control human plasma (8). Similar results were obtained with α_2M purified from CF serum.

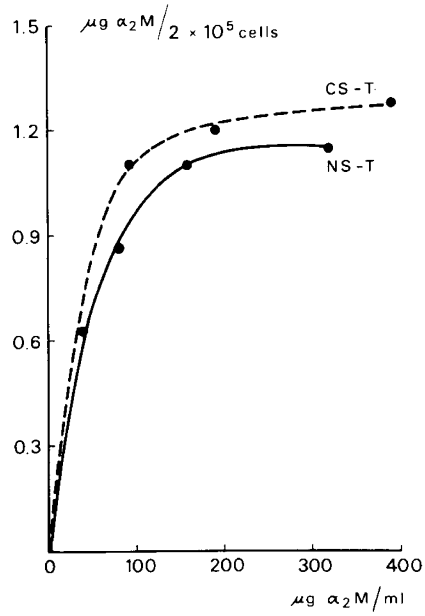

Fig. 3 : Uptake of α_2M by control fibroblasts in culture.
Skin fibroblast monolayers were cultured in DME-10% rabbit serum for 3 days. Control serum (NS-T) and CF serum (CS-T) treated with trypsin as described (7), was added to the medium to obtain a final concentration of α_2M in the culture medium as indicated.
Uptake of α_2M is expressed as μg α_2M per 2 x 10^5 cells, after 4 hours incubation at 37° C. All points are the mean of two determinations on separate cell layers.

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