PROSPECTS FOR PRENATAL DIAGNOSIS OF CYSTIC FIBROSIS : INDUCTION OF BIOCHEMICAL ABNORMALITIES IN FIBROBLASTS FROM PATIENTS WITH CYSTIC FIBROSIS BY A URINARY GLYCOPROTEIN

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SUMMARY

Alkaline phosphatase activity, assayed on a per cell basis with an ultra micro-method, can be increased up to 7-fold in fibroblast cultures derived from patients with cystic fibrosis by induction with a urinary glycoprotein. Fibroblasts from normal and heterozygous individuals are not significantly induced. There is a suggestion of heterogeneity among cases as reflected in varying baseline alkaline phosphatase and α-glucosidase levels.

Cystic fibrosis (CF) is the most common, severe autosomal recessive disease in populations of Caucasian descent. It should be eliminable by surveys of populations for heterozygous carriers followed by prenatal diagnosis. The observation that CF-fibroblasts often display metachromasia in culture (1) has suggested the possibility that cystic fibrosis might somehow qualify as a lysosomal storage disease. The purpose of the present communication is to demonstrate that this seems to be the case and that there are prospects for the prenatal diagnosis of cystic fibrosis through the induction of intracellular digestive tract enzymes by feeding cultured CF-fibroblasts with specific glycoproteins.

Simple ultra-microchemical methods have been described previously which permit the quantitative measurement of enzyme activities at the single cell level (2-3). The cells are cultivated in, and isolated from, the *Plastic Film Dish* (4). This allows one to select morphologically defined cells and to express the enzyme activities per cell instead of per total cell protein. This in turn facilitates the study of coordinate regulations of intracellular enzyme activities and allows the rapid prenatal diagnosis of metabolic errors in early pregnancy where the critical

separation of enzyme deficient, heterozygous and normal cells becomes very important (5).

Employing these microtechniques, one of us could recently describe the coordinate induction of enzymes involved in intracellular digestion (acid lysosomal hydrolases, acid and alkaline phosphatase). The retention of any type of nondigestible polymer induces the coordinate synthesis of intracellular digestive tract enzymes. The induction of alkaline phosphatase (AP) serves as a particularly good biochemical marker as the AP-activity of normal fibroblasts in culture is exceedingly low. Feeding a sphingomyelinase deficient Niemann-Pick-cell with sphingomyelin, for example, leads after a few hours to a dramatic increase in the intracellular AP-activity. This AP-induction can apparently serve to diagnose any type of lysosomal storage disease by feeding the cells in question with a "cocktail" containing the corresponding, nondigestible polymer (5).

Among other proteins, we choose the Tamm-Horsfall urinary protein (THP) as an inducer for CF-fibroblasts because it is an easily prepared human glycoprotein which had been thought to be abnormal in cystic fibrosis. Evidence from electron microscopy, sedimentation velocity measurements and viscometric studies had been interpreted as showing an altered monomer in the Tamm-Horsfall glycoproteins from patients with cystic fibrosis (6). However, thorough viscometric (7), light-scattering (8), and electron microscopical investigations (9) revealed no differences in Tamm-Horsfall glycoprotein related to cystic fibrosis.

MATERIALS AND METHODS

Tamm-Horsfall mucoprotein was prepared from the urine of healthy males by the method originally described (10). Briefly, the urine was diluted, the glycoprotein precipitated with 0.58 M NaCl, the precipitate washed with 0.58 M NaCl, the pellet dissolved in distilled water and extensively dialysed against the latter. Multiple batches were prepared, sometimes using higher salt concentrations to speed precipitation and both with and without chloroform (as a bactericidal agent) present. As the glycoprotein solution was too viscous for sterilization by filtration, part of the material was treated with 50 µg/ml kanamycin to avoid bacterial growth. An extinction coefficient of E_{280}(1%/1cm) of 10.7 was used to determine the concentration of the dissolved protein (11).

The fibroblasts from CF-patients, known heterozygotes (parents of cases) and normal controls were cultured in, and isolated from, *Plastic Film Dishes* (*PFD*'s). The *PFD*, which was originally developed by one of us (4), is comparable to a Petri dish with a very thin, absolutely flat, optically transparent plastic film bottom on which the cells are cultivated ; it is now commercially available from TECNOMARA-AG (Rieterstr. 59, 8059 Zürich, Switzerland).

The fibroblasts were cultured in Ham F10 medium and kept in a fully humidified atmosphere containing 5% CO_2 in air. After various times of incubation, with or without the THP present, the cultures were washed, shock-frozen in liquid nitrogen and lyophilized in conventional ways. Small plastic film leaflets, carrying 20 lyophilized fibroblasts, were then isolated under the stereomicroscope by free-hand dissection with a scalpel from the *PFD*-bottom and transferred into *Parafilm-Micro-Cuvettes* (*PMC*'s) for quantitative enzyme activity assays.

The *PMC*'s are very small, disposable enzyme reaction vessels. Immediately before use they are moulded in a parafilm strip. Each of them is filled by a constriction pipette with 0.3 µl of substrate and loaded with a plastic film leaflet carrying the 20 lyophilized fibroblasts to be assayed. The *PMC*'s are cold-sealed with a second parafilm strip and incubated submerged in a waterbath. The various enzymes have been assayed with artificial fluorogenic, methylumbelliferyl-coupled substrates. After the termination of the enzyme reaction, the sealing parafilm strip was peeled-off, the 0.3 µl of enzyme reaction mixture was washed out and diluted into 0.5 ml of alkaline buffer (carbonate buffer, 0.5 M, pH 10.7) and the now strongly fluorescent free methylumbelliferone was measured with a spectrophotofluorimeter (Perkin Elmer, MPF-4). The fluorogenic substrates were purchased from Koch-Light Laboratories and the following assay conditions were used : AP (4-methylumbelliferyl phosphate 5.5 mM in 2-amino-2-methylpropandiol-(1,3)-HCl buffer 0.2 M, pH 9.3 with 0.05% BSA ; incubation for 2 hrs at 37°C) ; α-glucosidase (4-methylumbelliferyl -α-D-glucopyranoside 2.3 mM in acetate buffer 0.125 M, pH 4.0 with 0.05% BSA ; incubation for 4 hrs at 37°C).

RESULTS

Baseline values. AP-activities in fibroblast strains from 8 CF-patients and from 4 CF-heterozygotes have repeatedly been tested for several years (12). AP-activities tended to be higher in CF-cell strains as compared to normal cell strains (CF strains up to 340% of controls) while heterozygous CF-cell strains had barely increased AP-activities. However, the values fluctuated considerably between different CF-cell strains and between different subcultures of the same CF-cell strain. More recently, and following a report of abnormal α-glucosidase levels in cystic fibrosis (13), a number of acid hydrolases were screened in these cell strains (α- and β-galactosidase, α- and β-glucosidase, β-glucuronidase, α-mannosidase, N-acetyl-α-D-glucosaminidase and acid phosphatase). Of these, α-glucosidase was elevated in strains from

Table 1

Induction of alkaline phosphatase by urinary glycoprotein in cultured skin fibroblasts from various patients with cystic fibrosis (100 µg/ml medium).

Patient	duration of induction	strain used as control	alkaline phosphatase activity 10^{-13} moles/hr/20 fibroblasts control	cystic fibrosis
1	48hrs	VOSa°	(5)11.8 ± 1.3*	(5)59.4 ± 7.6
1	72hrs	VOSd	(10) 5.1 ± 1.0	(10)29.9 ± 2.9
2	48hrs	KRUa	(10) 9.9 ± 1.5	(10)24.4 ± 2.6
2	72hrs	EVAb	(10) 7.8 ± 2.9	(10)23.3 ± 2.0
3	72hrs	EVAb	(10) 7.8 ± 2.9	(10)35.5 ± 9.9
4	72hrs	EVAa	(9) 6.0 ± 3.6	(10)32.8 ± 6.4
5	72hrs	EVAa	(9) 6.0 ± 3.6	(10)27.0 ± 4.9
6	72hrs	VOSd	(10) 5.1 ± 1.0	(10)33.6 ± 2.0
6	96hrs	VOSb	(10) 4.8 ± 1.9	(10)23.5 ± 6.1
7	48hrs	KRUa	(10) 9.9 ± 1.5	(10)24.7 ± 5.6
7	72hrs	VOSd	(10) 5.1 ± 1.0	(10)29.4 ±11.0
8	72hrs	EVAb	(10) 7.8 ± 2.9	(9)19.1 ± 2.5
9	72hrs	EVAb	(10) 7.8 ± 2.9	(10)32.9 ±10.0
10	48hrs	VOSb	(10) 4.8 ± 1.9	(10)24.9 ± 3.7
10	72hrs	VOSc	(10) 4.1 ± 1.4	(10)25.6 ± 3.7

° a,b,c, and d refers to successive passages of the used control cultures

* (number of determinations) mean ± S.D. All pairs of normal and cystic fibrosis were significantly different at $p < .01$ by Student's t-test.

several cases said to have unusually severe cystic fibrosis (up to 220% of controls) ; these same strains have normal baseline AP-activities (up to 140% of controls). Because of the fluctuation of the AP-baselines in different CF-strains and in various subcultures of the same CF-strain, THP-induced levels of alkaline phosphatase were compared to AP-levels in normal controls treated in an identical fashion.

Induction of alkaline phosphatase by Tamm-Horsfall glycoprotein.

Ten different CF-cell strains were induced with 100 µg of THP per ml culture medium for two to four days. As seen in table 1, all showed AP-levels significantly higher than the AP-levels in normal strains treated in the same way and at the same time. The induced levels were also significantly higher than the non-induced levels for each strain (data not shown).

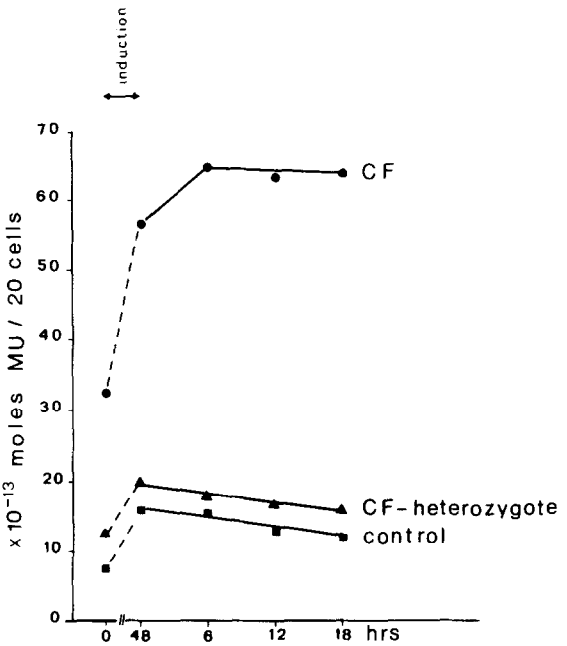

FIG. 1. Alkaline phosphate activity in a CF-patient, a CF- heterozygote, and a normal control. See text for details.

Since THP varies somewhat in its yield and properties depending, presumably, on physiological variations in urine, one CF-cell strain was induced with various preparations and concentrations of THP. Despite a variety of concentrations, minor variations in preservation and preservation methods, and ages of the THP preparation significant induction was found in every case (data not shown).

Wash out experiment. AP-activities were studied in fibroblasts from a CF-patient, a CF-heterozygote and a normal control before induction ; after two days of induction with 100 µg/ml THP ; and 6,12 and 18 hrs after the removal of the THP-inducer which had been fed for two days (fig.1). The AP-activity of the CF-homozygote was significantly increased even before induction, but rose to about 400% of the control after feeding for two days with 100 µg/ml THP. The normal control and the heterozygote

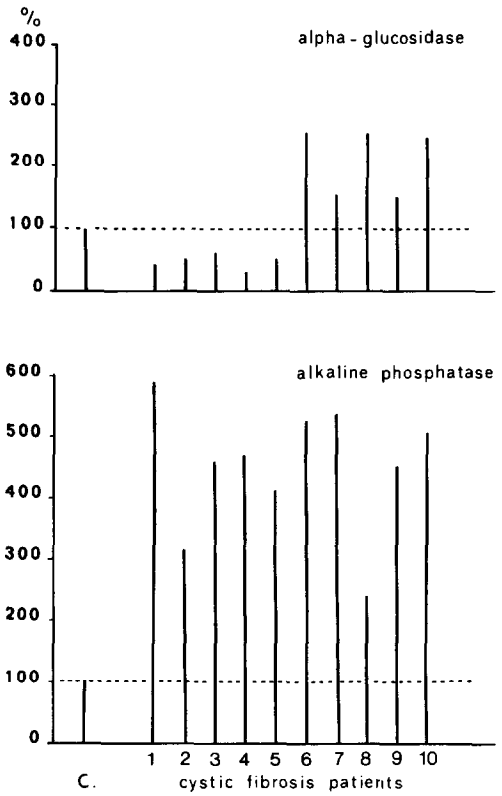

FIG. 2. α- Glucosidase and alkaline phosphatase activities during induction. See text for details.

react very little to feeding with THP. After the THP used for induction was removed from the medium, the AP-activity remained increased in CF-homozygotes, presumably because the internalized nondigestible THP continued to induce AP-synthesis. The activities in the normal control and the heterozygote, on the other hand, decreased towards the normal AP-baseline value, which will be reached about 24 hrs after removal of the inducer (data not included).

Comparison of alkaline phosphatase and α-glucosidase activities during induction. Fibroblast cultures of 5 cases with high spontaneous α-glucosidase activities (two of which were known to be clinically severe) and 5 CF-cases with low spontaneous α-glucosidase activities were induced

for four days with 200 µg/ml THP and tested for AP- and the α-glucosidase activities (fig.2). After four days of induction the AP-activities of all cases were clearly raised (up to about 500% of the control values), while the separation into two types on the basis of α-glucosidase levels persists.

DISCUSSION

From the present data we tentatively conclude that cystic fibrosis is probably caused by a block in the catabolism of specific glycoproteins. When challenged with an apparently non-catabolizable polymer, CF-fibroblasts behave like fibroblasts with a typical lysosomal storage disease, i.e. they display an increase in the synthesis of alkaline phosphatase. Cystic fibrosis is apparently heterogeneous from the clinical, the genetic and, following the present studies, the biochemical point of view.

The choice of alkaline phosphatase as an indicator of the coordinate induction of intracellular digestive tract enzymes has considerable advantages (5). The AP-activity of normal and CF- heterozygous fibroblasts is negligible and only slightly increased with induction, while CF-fibroblasts, (although showing somewhat higher, initial values) increase dramatically after feeding with THP. For these reasons normals and CF-heterozygotes can easily be separated from CF-homozygotes by AP-induction; to present, we have not encountered false positive or false negative cell strains. As the AP-assay is exceedingly sensitive, the diagnosis of cystic fibrosis could, in principle, be achieved with a few fibroblasts.

If fetal fibroblasts, obtained at amniocentesis, behave as skin fibroblasts (which has been the rule for other diseases), the induction of alkaline phosphatase by feeding with Tamm-Horsfall protein should provide an ideal and sensitive technique for the rapid prenatal diagnosis of cystic fibrosis in early pregnancy.

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