CYSTIC FIBROSIS : LEAKAGE OF LYSOSOMAL ENZYMES AND OF ALKALINE PHOSPHATASE INTO THE EXTRACELLULAR SPACE

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Received June 16, 1977

SUMMARY

Nine lysosomal enzymes and alkaline phosphatase have been assayed with two different ultramicro techniques in the intra- and extracellular space of fibroblast cultures derived from the skin of cystic fibrosis patients, cystic fibrosis carriers, and normal controls, respectively. Evidence has been obtained for a multiple leakage of lysosomal enzymes and of alkaline phosphatase into the medium of fibroblast cultures from cystic fibrosis patients and carriers. The situation is comparable to a certain extent, to that observed in I-cell-disease (mucolipidosis II). This multiple leakage results in the decrease of intracellular activity of several lysosomal enzymes in cultures from cystic fibrosis patients and carriers and due to the coordinate regulation of the synthesis of the "leaky enzymes" in an overshooting of the intracellular alkaline phosphatase activity in cultures from cystic fibrosis patients. It also explains the retarded catabolism of certain molecules, such as the Tamm-Horsfall glycoprotein, in cystic fibrosis cells. It is speculated that the basic defect in cystic fibrosis leads to abnormal recognition sites on lysosomal enzymes and on alkaline phosphatase, and in consequence to the leakage of these enzymes into the extracellular space. The present findings allow one to develop methods for the pre- and postnatal diagnosis of cystic fibrosis with cell cultures, and for the detection of cystic fibrosis carriers with the peripheral blood.

Cystic fibrosis (CF) is the most frequent hereditary disease in people of Caucasian descent (1), and its social and economic implications are very important. Many laboratories are, therefore, searching for the primary defect in cystic fibrosis, and trying to develop diagnostic procedures as well as methods for the detection of CF-carriers.

The present paper demonstrates that cystic fibrosis is a type of mutation which affects lysosomes in a way which is somewhat reminiscent of I-cell-disease. The phenotype of cultured I-cell-fibroblasts is characterized by a multiple leakage of lysosomal enzymes into the extracellular space, i.e. the culture medium (2), and by a compensatory increase of the intracellular

ISSN 0006-291X

activity of non-leaky enzymes and of alkaline phosphatase (AP), respectively ; the latter phenomenon is due to a coordinate regulation, at the transcriptional level, of the replacement of these enzymes (3).

All experiments have been done with ultramicro techniques. They allow the expression of enzyme activities on a per-cell-basis instead of on a per-protein-basis. They are, therefore, particularly well suited to monitor very fine fluctuations of enzyme activities in cultured cells (4,5).

MATERIALS AND METHODS

Cell culture conditions (6), ultramicro methods to measure enzyme activities at the single cell level (4,5,6), individual enzyme assays based on the use of artificial fluorogenic methylumbelliferone-coupled substrates (8), the isolation of urinary Tamm-Horsfall glycoprotein (THP ; 6) and the principle of AP-induction with THP (6) have been described elsewhere.

For the present studies the AP-induction procedure has been modified and two different ultramicro techniques have been used.

Technique I . Culture medium : HAM F10 with 15% FCS. Fibroblasts were seeded into *Plastic Film Dishes (PFD's* ; 4,7) with an initial concentration of 1.5 x 10^5 cells per *PFD* .AP-induction : 24 hours after seeding, the fibroblast cultures were treated for 3 consecutive days with THP (100 µg/ml medium) ; for the last 2 days of induction 1 x 10^{-5} M isoproterenol and 1 x 10^{-3} M theophylline and for the last day 1 x 10^{-5} M isoproterenol were given in addition into the THP-containing medium. Then the cells were washed, lyophilized, isolated from the *PFD's* and enzyme activities were assayed in *Parafilm Micro Cuvettes (PMC's)* as described previously (3,4,5,6,8).

Technique II . Culture medium : HAM F10 with 15% FCS. Fibroblasts were grown to confluency in *PFD's* and then induced for 3 consecutive days with THP as described under Technique I. Then the induction-medium was replaced for 24 hours by HAM F10 with 0.1% heat-inactivated FCS to collect extracellular enzymes ; this medium was cleared by centrifugation (10,000 g for 5 minutes) and lyophilized to concentrate the collected extracellular proteins. The cells, remaining in the *PFD*, were washed, trypsinized and diluted with saline to a final concentration of 50 cells per 0.1 µl ; the cells were then disrupted by 3 cycles of freezing and thawing and the 10,000 g supernatant fraction of this extract was used for the enzyme assays. For the determination of enzyme activities in the extracellular space the lyophilized medium was dissolved in water in such a way that 0.1 µl of this solution had been conditioned by 50 cells.

Enzyme activities are expressed in 10^{-13} moles of substrate converted per cell per hour or as the percentage of the mean activities of controls.

RESULTS

Table 1 reviews the intracellular enzyme activity profiles in fibroblast cell strains from 5 patients with I-cell-disease as compared to 16 normal controls. The intracellular activities of β-galactosidase, hexosaminidase, α-galactosidase, α-mannosidase and β-glucuronidase are significantly (i.e.

Table 1 : Intracellular enzyme activity profiles, relative values, fibroblast cell strains from 5 patients with I-cell-disease and from 16 normal controls. Technique I, cf. methods.
Each determination was carried out in five-fold with 20 cells per incubation.

	Controls, n = 16 mean ± SD%	pat.1	pat.2	pat.3	pat.4	pat.5
β-galactosidase	100 ± 9.8% (4.3)*	8%	6%	6%	9%	6%
hexosaminidase	100 ±13.4% (28.5)	17%	19%	15%	25%	15%
α-galactosidase	100 ± 8.3% (0.5)	19%	22%	9%	45%	8%
α-mannosidase	100 ± 8.3% (0.6)	27%	29%	29%	30%	36%
β-glucuronidase	100 ± 9.1% (0.7)	31%	20%	8%	105%	15%
α-glucosidase	100 ± 7.0% (0.6)	218%	238%	157%	625%	153%
β-glucosidase	100 ± 6.9% (0.8)	168%	171%	174%	350%	184%
acid phosphatase	100 ± 8.5% (19.5)	199%	234%	217%	299%	195%
alkaline phosphatase	100 ±11.8% (0.5)	415%	465%	696%	2461%	335%

* The values in brackets in the control column indicate mean enzyme activities in 10^{-13} moles of substrate converted/cell/hour.

below -3 S.D.) decreased, with the exception of β-glucuronidase in patient 4 ; the intracellular activities of α-glucosidase, β-glucosidase, acid phosphatase and alkaline phosphatase, on the other hand, are significately (i.e. to over +3 S.D.) increased.

In comparison, Table 2 presents intracellular enzyme activity profiles in fibroblast cell strains from family 1 with cystic fibrosis (2 CF-homozygotes 2 CF-heterozygotes) as compared to 16 normal controls. In the CF-homozygotes β-galactosidase, hexosaminidase, α-mannosidase and acid phosphatase are clearly decreased below the -3 S.D. level, β-glucosidase is on the border of -3 to -2 S.D. ; α-galactosidase is only slightly decreased, while α-glucosidase and alkaline phosphatase are increased to or over the +3 S.D. level. The same trend is displayed by the obligate CF-heterozygotes. Induction with THP leads to a dramatic increase of the intracellular AP-activities in the CF-children

Table 2 : Intracellular enzyme activity profiles, relative values, fibroblast cell strains from family 1 with cystic fibrosis (2 CF-homozygous sibs, 2 CF-heterozygous parents) and from 16 normal controls. Technique I, cf. methods. Alkaline phosphatase without and with THP-induction (cf. methods). Each determination was carried out in five-fold with 20 cells per incubation.

	Controls, n = 16 mean ± SD%	sib 1 ♀	sib 2 ♂	father	mother
β-galactosidase	100 ± 9.8% (4.3)*	58%	63%	67%	69%
hexosaminidase	100 ±13.4% (28.5)	53%	58%	73%	79%
α-galactosidase	100 ± 8.3% (0.5)	80%	89%	81%	79%
α-mannosidase	100 ± 8.3% (0.6)	55%	65%	77%	75%
β-glucuronidase	100 ± 9.1% (0.7)	110%	120%	101%	107%
α-glucosidase	100 ± 7.0% (0.6)	165%	120%	91%	90%
β-glucosidase	100 ± 6.9% (0.8)	69%	89%	83%	87%
acid phosphatase	100 ± 8.5% (19.5)	50%	66%	87%	83%
alkaline phosphatase	100 ±11.8% (0.5)	210%	180%	105%	99%
alkaline phosphatase THP-induced	100 ±18.0% (0.7)	380%	430%	120%	130%

* The values in brackets in the control column indicate mean enzyme activities in 10^{-13} moles of substance converted/cell/hour.

but to only a very moderate increase in the heterozygous parents.

Fig. 1 reviews intra- and extracellular enzyme activity profiles, after THP-induction in fibroblast cell cultures from family 2 with cystic fibrosis (not identical with family in Table 2) as compared to 14 normal controls. In the CF- homozygotes the intracellular activities of acid phosphatase, α-fucosidase, α-mannosidase, α-galactosidase, β-galactosidase, hexosaminidase, α-glucosidase and β-glucosidase are below the -3 S.D. level ; the extracellular activities of the same enzymes are far above the +3 S.D. level ; the extra and intracellular alkaline phosphatase values are also very significantly increased. In the CF-heterozygotes the extracellular enzyme activities follow

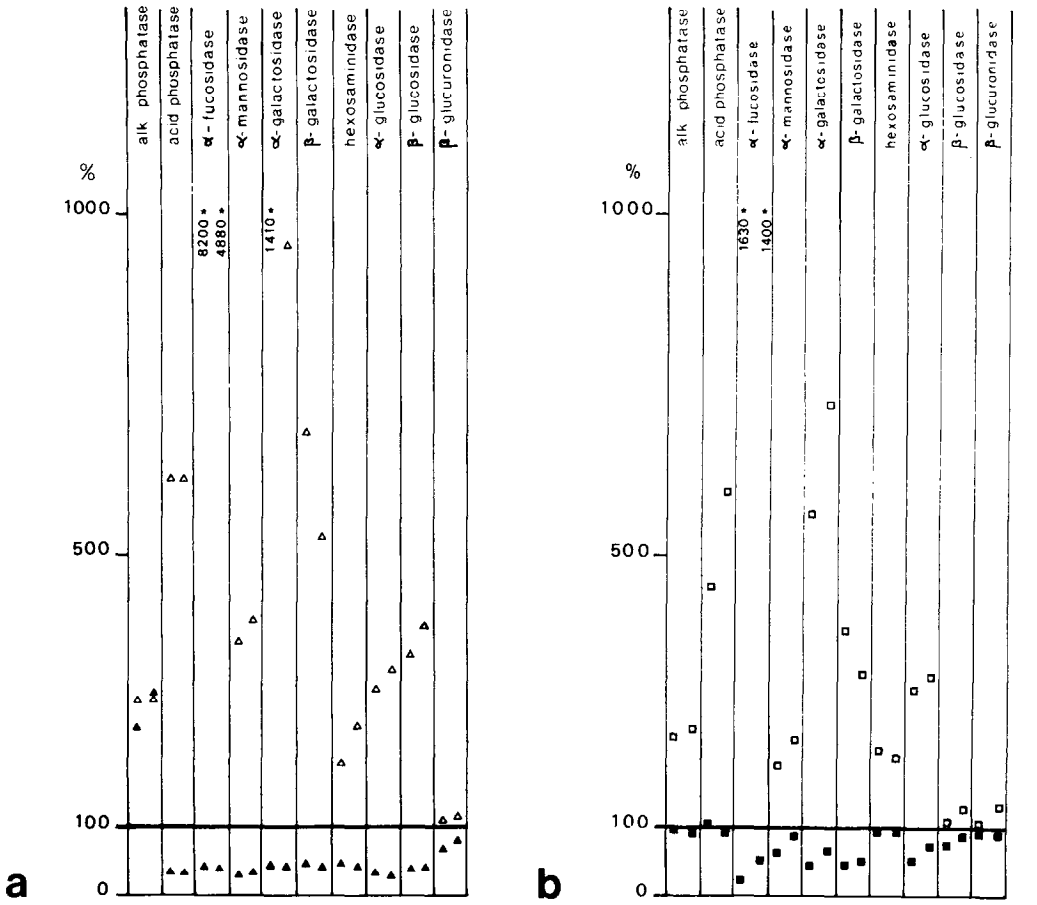

Fig.1. Intra- and extracellular enzymes activity profiles, relative values, fibroblast cell strains from family 2 with cystic fibrosis and from 14 normal controls, after THP-induction. Technique II, cf. methods (α-mannosidase, α-galactosidase, α-glucosidase, β-glucosidase were incubated at 25°C, the other enzymes at 37°C). Each point is the mean of 5 determinations and corresponds to 50 cells or the medium conditioned by 50 cells. The horizontal 100% bar corresponds to the mean of 14 normal control cell strains with the following standart deviations (intracellular/extracellular) : alkaline phosphatase (8.7/11.5) ; acid phosphatase (6.9/22.8) ; α-fucosidase (5.3/20.3) ; α-mannosidase (7.1/14.7) ; α-galactosidase (8.4/27.8) ; β-galactosidase (6.9/12.3) ; hexosaminidase (6.6/8.2) ; α-glucosidase (8.9/25.6) ; β-glucosidase (4.6/18.9) ; β-glucuronidase (7.1/14.3).
a. CF-children (♀, ♂) : ▲, intracellular ; △, extracellular
b. Parents (obligate heterozygotes) : ■, intracellular ; □, extracellular

the same trend, with the exception of β-glucosidase ; intracellularly a significant activity decrease is detectable only for α-fucosidase, α-mannosidase, α-galactosidase, β-galactosidase and α-glucosidase ; the intracellular activity

of alkaline phosphatase in particular is normal in heterozygotes.

DISCUSSION

This study confirms that the AP-induction with Tamm-Horsfall glycoprotein (6) permits one, as a rule, to discriminate with fibroblast cultures between CF-patients on the one hand, CF-carriers and normal controls on the other. To the present, 53 CF-homozygotes and 25 CF-heterozygotes have been treated with Tamm-Horsfall glycoprotein. 51 of the CF-homozygotes were THP-induceable ; 2 CF-homozygous sibs only, were not THP-induceable. The conclusion is that the vast majority of clinically diagnosed CF-cases are THP-induceable.

The present data indicate that in families where the index-patient is THP-induceable, a multiple leakage of lysosomal enzymes and of alkaline phosphatase into the extracellular space is displayed by all CF-homo- or heterozygous family members. So far 9 families with 14 CF-homozygotes and 15 CF-heterozygotes have been analyzed (data not shown) : all CF-homo- or heterozygotes in the families with a THP-induceable index-case display the multiple enzyme leakage. In the one exceptional family, where 2 sibs are not THP-induceable, none of the CF-homo- or heterozygotes show enzyme leakage ; in addition, the clinical picture of these CF-cases is very unusual. This rare type of cystic fibrosis, which is neither THP-induceable nor leaky for the studied enzymes, could be a biochemically completely different syndrome. Alternatively, it is possible that such types will be found leaky for other lysosomal enzymes and induceable by different substrates.

The multiple enzyme leakage results, with the exception of alkaline phosphatase (Table 2, Fig. 1), in a decrease of the corresponding intracellular enzyme activities. The coordinate regulation of the synthesis of lysosomal enzymes, of acid and of alkaline phosphatase (3,6,8) counteracts the leakage ; this results in an overshooting of the intracellular activity of alkaline phosphatase in CF-homozygotes (Fig. 1a). The same regulation takes place in CF-heterozygotes but here the stimulatory signal for the enzyme induction is weaker (3,6,8). This would explain the absence of an overshooting of intra-

cellular alkaline phosphatase activity and the tendency towards normalization of the intracellular activities of the other leaky enzymes in the CF--heterozygotes (Fig. 1b).

The primary defect in cystic fibrosis seems to lead to a loss of lysosomal enzymes and of acid and alkaline phosphatase into the extracellular compartment. This is reminiscent of I-cell-disease (2), though in I-cell-disease no data are available concerning a leakage of phosphatases. The difference between cystic fibrosis and I-cell-disease could, therefore, possibly be quantitative (lysosomal hydrolases) as well as qualitative (phosphatases).

It has been suggested (9) that the packaging of lysosomal hydrolases requires their secretion followed by specific recognition and uptake ; that is, the self-assembly of enzyme containing granules, such as primary lysosomes, needs receptors on the membrane or matrix of the granules on the one hand, specific recognition sites on the corresponding enzymes on the other hand.

One would expect to find two different types of mutations which could interfere with the normal self-assembly of digestive granules ; the one would affect the receptors in the granules, the other the recognition sites on the enzymes. I-cell-disease, and following the present study, cystic fibrosis would most likely qualify as recognition site mutants. This would explain why in these syndromes the heterozygotes as well as the homozygotes display the multiple enzyme leakage. In the Chediak-Higashi syndrome we found a multiple enzyme leakage in the homozygotes but not the heterozygotes (10) ; it seems likely that there we are dealing with a receptor mutant.

Enzyme recognition sites are probably relatively complex configurations. Different mutations could, therefore, interfere with different parts of the recognition site. This might explain the very high frequency of CF-carriers in the population (heterogeneity of mutants) and also the variability concerning the leakage of specific enzymes. α-glucosidase is, e.g., not leaky in family 1 (Table 2) but leaky in family 2 (Fig. 1) ; this has been observed previously (6).

This first evidence that the primary defect in cystic fibrosis is a "processing mutation" also allows one to design new diagnostic strategies. Preliminary studies indicate that the leaky enzymes are present in increased amounts in the blood of CF-heterozygotes and that it will become possible to screen populations for CF-carriers.

ACKNOWLEDGEMENTS

This study has been supported by grants from INSERM (ATP 36 76 68), DGRST (Nr. 75 70 004) and the Fondation de France.

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