ABNORMAL DISTRIBUTION OF α-L-FUCOSIDASE IN CYSTIC FIBROSIS: INCREASED ACTIVITY IN SKIN FIBROBLASTS

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SUMMARY α-L-Fucosidase activity is elevated in skin fibroblasts from cystic fibrosis patients when compared to controls. The activities of nine other acid hydrolases including neuraminidase are similar in cystic fibrosis and control fibroblasts. The relationship of these results to the recent finding of a decreased activity of α-L-fucosidase in the serum of cystic fibrosis patients is discussed. It is proposed that an abnormal distribution of α-L-fucosidase is involved in the pathogenesis of this disease.

CF[1] is one of the most common inherited diseases of the Caucasian race. Although it appears to have an autosomal recessive mode of inheritance, the precise biochemical lesion has not been identified (1). On the premise that a minor difference in glycoprotein structure could lead to the pathophysiology of CF, we have reopened the question of aberrant lysosomal enzymes by examining in particular, α-L-fucosidase (E.C.3.2.1.51) and neuraminidase (E.C.3.2.1.18). Both of these enzymes remove terminal monosaccharides from glycoproteins and neither has been examined sufficiently in CF (1). We recently found decreased activity of α-L-fucosidase in sera of CF individuals when compared to matched controls (2) and now report increased activity of this enzyme in CF skin fibroblasts.

METHODS Cell culture. Skin biopsies were obtained from eight individuals with CF and from six control individuals. Four of the latter were matched according to age, race, and sex of four CF individuals and the ages of all ranged from 1-20 years. The CF patients had Shwachman scores (3) from 40 to 90. Growth conditions have been described and no difference was observed between CF and control cells in rate of growth (4). Cells, seeded at $2 \times 10^4/cm^2$, were harvested in log phase ($2.3 \times 10^4/cm^2$) after 48 h. The remaining cultures were fed on Day 4 and growth continued for 3 days when the cells were harvested in confluent phase ($4.7 \times 10^4/cm^2$). In other experiments, the cells were harvested after 72 h in upper log phase of growth. The cells were examined between passages 6 and 14, and were negative for Mycoplasma.
Cell harvest. Cells were washed five times on the monolayer with Puck's saline solution and were harvested by treating with 0.25% trypsin (Flow Laboratories)

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[1]CF, cystic fibrosis; 4-MU, 4-methylumbelliferyl; PNP, p-nitrophenyl; PNS, p-nitrocatechol sulfate; MP-NeuAc, 2-(3'methoxyphenyl) neuraminic acid.

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for 5 min at 37°, followed by the addition of media. After removal from the monolayer, all steps prior to incubation were at 5°. The cells were further washed three times with 0.16 M NaCl. The cell count and cell viability were determined on a Biophysics Cytograph and the cells were at least 95% viable. In most cases, the CF and control cells were harvested on the same day and were within one passage for each reported experiment.

Preparation of enzyme extracts. The washed cells were immediately re-suspended at a concentration of 2.3×10^7 cells/ml of 0.1% Triton X-100 (Packard) and broken with 90 strokes of a tight-fitting B pestle in a Dounce homogenizer. The homogenate was centrifuged (630g/10 min) and appropriate dilutions were made from the supernatant solution for the respective assays. Proteins were determined on aliquots of each dilution (5). The supernatant solutions, containing 5-6 mg of protein per ml of homogenate, had approximately 80% of the total cell protein and 90% of the enzyme activity. Similar amounts of protein were present in CF and control cells (4).

Enzyme assays. All assay mixtures were in a final volume of 200µl and were incubated for 1 h at 37°. The enzyme activity was expressed as nmol of substrate hydrolyzed per mg of homogenate protein per h.

4-MU[1]-glycosides. The substrates used were 0.65 mM 4-MU-α-L-fucopyranoside, -β-D-galactoside, and -β-D-glucuronide (Research Products International). The reactions were in 50 mM citrate buffer, pH 5.8, pH 4.3, and 50 mM acetate buffer, pH 4.5, respectively. Two aliquots of homogenate, diluted with 0.1% Triton X-100 (25-50 µg of protein) were assayed for each value. The reactions were stopped by the addition of 1.8 ml of 125 mM glycine-carbonate buffer, pH 10, and read at 450nm after excitation at 360 nm (AMINCO SPF Spectrophotofluorometer).

PNP[1] and PNS[1] substrates. The incubation mixture contained 2mM substrate (Sigma), 50 µl of homogenate in two or three appropriate dilutions, and 0.1 M citrate buffer, pH 4.3 for all substrates with the exception of α-L-fucosidase which was assayed in 0.1 M citrate buffer, pH 5.0, and β-D-glucuronidase which was assayed in 0.1 acetate buffer, pH 4.5. The activity of α-L-fucosidase was stopped by the addition of 300 µl of 0.2 M sodium carbonate. The other reactions were stopped by the addition of 200 µl of 20% TCA and centrifuged (560g/15 min). The supernatant solution (200 µl) was treated with 300 µl of 0.5 M sodium carbonate. The released PNP was measured at 405 nm in the Zeiss Spectrophotometer, PM6. Sulfatase was assayed by a modification of the method of Antonowicz et al. (6) with 2 mM PNS, 50 mM acetate buffer, pH 4.5, and 50 µl of three dilutions of the homogenate (50-250 µg of protein).

MP-NeuAc[1]. For the neuraminidase assay, an aliquot of the homogenate was further centrifuged at 10,400g/20 min. The supernatant solution was assayed using 100 µl of three dilutions (80-300 µg of protein) in duplicate with 150 µg of MP-NeuAc (Boehringer Mannhein Biochemicals) and 50 mM phosphate buffer, pH 5.9, or 25 mM acetate buffer, pH 4.5. The reaction was stopped by the addition of alkali and, after development of the color, the released methoxyphenol was measured at 500 nm (7).

RESULTS α-L-Fucosidase activity. The activity of α-L-fucosidase was higher in fibroblast homogenates from seven CF individuals than in any of the controls using 4-MU-fucoside as substrate. The mean activity of the enzyme in all eight CF cell lines was 103 ± 29 nmol/mg of protein/h while that of the controls was 59 ± 13 (Fig. 1). Using PNP-fucoside as substrate, the mean activities were 36 ± 14 and 23 ± 10 nmol/mg of protein /h for 4 CF cell lines and 3 age, race, and sex matched controls, respectively. Clearly, the mean activity of α-L-fucosidase in the CF fibroblasts was higher than that of the controls using

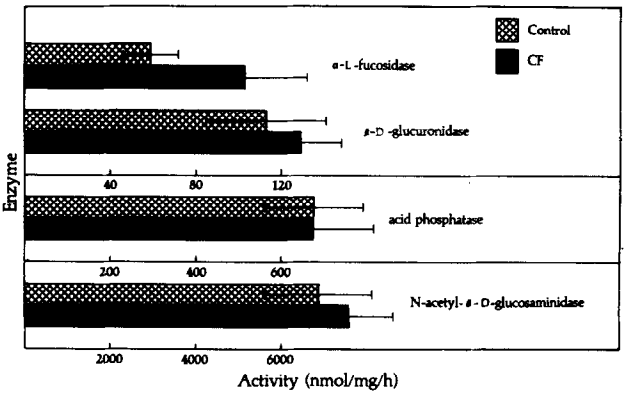

Fig. 1. Comparison of enzyme activities in CF and control skin fibroblasts. 4-MU-α-L-fucopyranoside and -β-D-glucuronide and PNP-phosphate and -N-acetyl-β-D-glucosaminide were used as substrates. The bars represent the mean ± S.D. (∓) of nmol of substrate hydrolyzed/mg/h by homogenates of 8 CF and 6 control skin fibroblast lines. The value for each of the fibroblast lines represented the average of several experiments in both logarithmic and confluent phase of growth. The Student's t-test was applied to the means using a two-tailed test: $2P < 0.005$ for the activities of α-L-fucosidase and for all other enzymes $2P > 0.2$.

either substrate, and was 175% and 158% of the controls with the 4-MU- and PNP-α-L-fucosides, respectively (Fig.2).

Using 4-MU-fucoside as substrate, no growth related differences in enzyme activity were observed in either cell type. The mean activities of the enzyme from logarithmically growing and confluent CF fibroblasts were 98 ± 35 and 109 ± 15 nmol/mg/h, respectively, and those of the controls were 57 ± 15 and 63 ± 13 nmol. For this reason the values were combined for Fig. 1.

Activity of other acid hydrolases. In contrast to the elevated activity of α-L-fucosidase in the CF fibroblasts, the activities of eight other lysosomal hydrolases were similar or lower than the controls (Fig. 1 and 2). Fig. 2a expresses the mean activities of the enzymes of four CF cell lines as percentage of the three matched controls. Using PNP-derivatives and PNS, 8 hydrolases which were assayed had 71-108% of the activity of the controls while α-L-fucosidase was elevated in the CF fibroblasts. When additional cell lines were examined for 3 of these enzymes, the activities remained similar for both cell types (Fig.

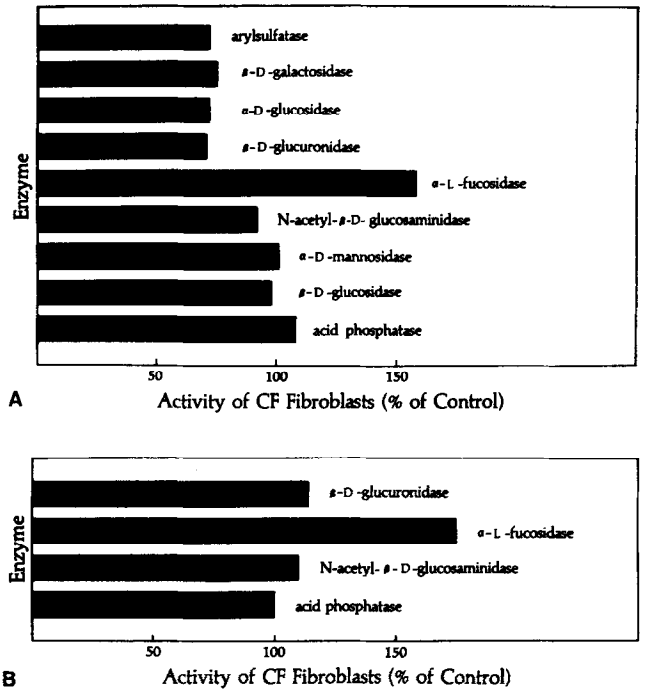

Fig. 2. Activity of lysosomal enzymes of CF fibroblasts expressed as percentage of the activity of control fibroblasts. A) Data from mean values of 4 CF and 3 matched control skin fibroblast lines using PNP- and PNS-substrates with cells harvested in the upper log phase of growth. B) Data from 8 CF and 6 control skin fibroblast lines using 4-MU-substrates for α-L-fucosidase and β-D-glucuronidase and PNP-substrates for the other two enzymes.

2b). The activity of β-D-galactosidase was also similar in both cell lines with PNP- and 4-MU-β-galactoside.

One control cell line showed that the activities of all the hydrolases were elevated above those from other control cultures. A repeat biopsy verified these results. In both cases, the cells grew in a non-fibroblast pattern with a slow generation time. The activity of α-L-fucosidase, although elevated, was not elevated in comparison to the other enzymes. These results are not included in Fig. 1 and 2.

Neuraminidase activity. Using MP-NeuAc as substrate, the activity of neuraminidase was similar in homogenates of CF and control fibroblasts. Both cell types in confluent growth hydrolyzed 140-170 nmol of substrate/mg protein/h in 25 mM

acetate buffer, pH 4.5. One third of this activity was found in 50 mM phosphate buffer, pH 5.9.

Parameters of α-L-fucosidase. Several parameters of the α-L-fucosidase activity of CF and control fibroblasts have been examined in at least one age, race, and sex matched pair. The enzyme from both cell types behaved in a comparable manner in the following experiments.

The activity of α-L-fucosidase toward natural substrates was examined using milk oligosaccharides labeled with tritium (8). A slow hydrolysis of fucose-linked α, 1→3 and α, 1→4 to GlcNAc was observed. In contrast, fucose-linked α, 1→2 to Gal was not hydrolyzed under similar conditions.

4-MU was released at a linear rate up to 150 min under the conditions of the assay. A broad pH maximum, pH 5 to pH 6, was observed. All detectable α-L-fucosidase activity was absorbed on a column of agarose-ε-amino-caproyl-fucosamine and eluted with 50 mM fucose (9). The enzyme was stable when the homogenate was held at 50° for 10 min, but partially inactivated at 55° for 15 min. In contrast, β-glucuronidase was stable under both conditions. Under more acidic conditions (50 mM citrate buffer, pH 4.8), α-L-fucosidase was partially inactivated at 50° for 10 min. Full activity was retained in homogenates stored at -20° for two weeks. Passages 6 through 14 maintained constant enzyme activity although prolonged passage in culture to passage 19 showed a 45% loss of activity when compared to passage 11.

DISCUSSION The finding of an elevated activity of α-L-fucosidase in CF fibroblasts when 9 other acid hydrolases have activities similar to control fibroblasts suggests an alteration in fucose metabolism in CF. Other observations support this hypothesis: i) a lowered activity of α-L-fucosidase in the sera of CF individuals when compared to age matched controls (2) and ii) the complex carbohydrates from the CF fibroblast cell surface were enriched in fucose when compared with the control fibroblasts (4). Additional support comes from studies by others. In 1959, Dische et al. (10) reported increased ratios of fucose to sialic acid in CF mucous secretions. Not all secretions showed this difference,

but many did (1). Differences have been shown in low molecular weight material labeled with radioactive fucose when CF and control fibroblast culture media were compared (11) and macromolecules containing increased amounts of fucose have been reported in CF serum (12).

This is the first report of neuraminidase activity in CF fibroblasts and, as found for 8 other acid hydrolases, the mean activity was comparable to the controls. There did appear to be a growth-related elevation of neuraminidase in both types of cells similar to that reported for hamster fibroblasts (7).

A number of studies have been reported which give the activity of α-L-fucosidase in skin fibroblasts from normal individuals (13-15). Using PNP-fucoside as substrate, Butterworth et al. (16) have examined fibroblasts from four patients with CF. They reported no difference from normal fibroblasts; however, their normals had a range of activity much greater than those reported here. Similar results were obtained with one patient (13). The activities were expressed per mg of homogenate protein and since the amount of protein depends on the method of cell breakage, centrifugation steps, and technical factors, comparisons between laboratories are difficult.

The relationship of α-L-fucosidase to the genetic lesion may be hard to define, although a number of possibilities can be examined. The parameters of α-L-fucosidase activity which we examined indicated no qualitative difference between the enzyme in CF and control fibroblasts. However, more detailed analyses are required since multiple enzyme forms (17) and inherited polymorphism in isozyme types (18) have been reported. Another possible explanation of the specific increase in α-L-fucosidase activity is an alteration in the secretion/recapture pathway. Studies of the recognition components of these receptor mediated processes show that minor alterations in carbohydrate moieties alter the intra- and extracellular distribution of glycoproteins (19-21), thus presenting an attractive hypothesis for the abnormal distribution of α-L-fucosidase in the serum and fibroblasts from CF individuals. Regardless of the mechanism, an al-

tered distribution of α-L-fucosidase is associated with CF and this may lead to the diverse manifestations of this disease.

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