CYSTIC FIBROSIS: DECREASED THERMOSTABILITY OF α-MANNOSIDASE IN CRUDE EXTRACELLULAR FLUIDS OF PATIENTS AND CARRIERS

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1. Introduction

Cystic fibrosis (CF) is the most common autosomal recessive genetic disease in people of Caucasian descent. It affects about 1 in 2000 live-born children and the frequency of carriers, i.e., CF-heterozygotes, has been estimated to be about 1 in 20 in the general population [1]. Affected children suffer from chronic pulmonary obstruction, variable degrees of exocrine pancreatic dysfunction and increased levels of sweat electrolytes [2]. The sweat test [3] is so far the only generally accepted tool for the laboratory diagnosis of cystic fibrosis: it presupposes, however, the presence of the patient in the laboratory, is cumbersome and only reliable if handled by very experienced technicians [4]. Neonatal identification of CF-patients through new-born screening, which would allow one to apply early therapy and to possibly improve the prognosis of pulmonary obstruction has been tried [5] with limited success and, therefore, has been partly abandoned. Approaches to prenatal diagnosis of cystic fibrosis with amniotic fluid have been proposed, but so far remained in a purely experimental stage [6–8]. Not only have reliable diagnostic tools been lacking, but the basic metabolic defect of this most important genetic disease remained itself elusive [2].

We could, previously, demonstrate that the genetic defect of cystic fibrosis affects the lysosomal system [6], and that it leads to a multiple leakage of lysosomal hydrolases into the extracellular space with a concomitant multiple partial intracellular deficiency of the corresponding hydrolases [9]. To explain these observations we formulated the hypothesis that the basic genetic defect in cystic fibrosis affects enzyme recognition markers on lysosomal hydrolases [9–11] which serve to anchor these hydrolases onto specific lysosomal membrane receptors [12]. These enzyme recognition markers are thought to be phosphorylated oligosaccharides [13] fixed onto the enzymatic proteins by a secondary processing during their passage through the cavities of the endoplasmic reticulum.

If lysosomal enzymes carry abnormal recognition markers, it was reasonable to test if the altered enzymatic glycoproteins would display abnormal thermostabilities.

Now we wish to demonstrate that an abnormal thermostability has, indeed, been found in crude extracellular fluids for α-mannosidase, the first of the 'leaky' [9] enzymes analyzed. More extensive studies involving several of the 'leaky' hydrolases are under way and they indicate that the observed thermolabilities can be exploited to develop the urgently needed simple diagnostic tools for cystic fibrosis.

2. Materials

Growth medium: HAM F10 (GIBCO), supplemented with 15% FCS (GIBCO). Corning tissue culture flasks: 75 cm^2. 4-Methylumbelliferyl-α-D-mannopyranoside (Koch-Light Laboratories, Colnbrook, Bucks, U.K.). Spectrophotofluorimeter Perkin-Elmer MPF-4 (Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, U.K.).

3. Methods

Fibroblast cell cultures were established in conventional ways from skin biopsies of normal controls, CF-patients and CF-heterozygotes (carriers), and kept for three days at 37°C in growth medium in confluency. After washing, the growth medium was replaced for two days with collection medium (2.2 ml HAM F10, supplemented with 5% FCS which had been previously heat-inactivated for 3 h at 56°C). The collection medium was then cleared by 2 min centrifugation at 5000 × *g*. Aliquots of this medium were distributed into Eppendorf polypropylene tubes (1.5 ml, without cover), frozen and stored in liquid nitrogen until experimental use. Thawed aliquots must be immediately used and can not be refrozen.

Venous blood was drawn into plastic tubes containing 25 I.U. heparin Roche per ml blood, put into ice water and processed as soon as possible, but at the latest in one hour. The blood tubes were first centrifuged for 5 min at 400 × *g* and subsequently for 10 min at 3000 × *g* in a refrigerated swing-out centrifuge. Plasma aliquots were then distributed into Eppendorf tubes, frozen in liquid nitrogen and processed as described for collection medium.

A new technique has been developed for the sequential heat inactivation and enzyme assay of very small samples of collection medium or of plasma in disposable Eppendorf tubes. The important point is to protect the small incubation volumes against evaporation by covering them with non-fluorescent Uvasol paraffin oil (Merck). After each pipetting, the oil is forced to cover the upper surface of the incubation sample by centrifugation of the incubation vessel (Eppendorf propylene tube) in the Runne-Zentrifuge (model 104-A, Heidelberg).

Collection media or plasma were thawed in ambient air and kept in ice-water until heat inactivation. To 10 μl of plasma (prediluted 1 : 10 with 0.9% NaCl) or collection medium in Eppendorf tubes were added 25 μl of Uvasol oil. Samples were centrifuged 20 sec at 6000 × *g*, heated for 0–240 min at 41°C in a water-bath, and placed 5 min on ice. 10 μl of 10 mM 4-methylumbelliferyl-α-D-mannopyranoside were added, samples centrifuged as above, and incubated at 30°C for 90 or 180 min, respectively. Reactions were stopped by placing tubes in ice-water for 5 min. 1 ml of 0.5 M carbonate buffer (pH 10.7) was added

flattens off and behaves like the normal control during the second part of heat inactivation. Extrapolation of the last 2 h CF-heterozygote values to zero time seems to indicate that culture media and plasma from heterozygotes are composed of two different types of α-mannosidase molecules: about 50% of the molecules seem to be thermolabile (as in the CF-homozygote), about 50% thermostable (as in the normal control). As will be discussed, this observation must, however, be interpreted with great caution.

A mixture of equivalent amounts of cell culture medium from a normal control with that from a CF-homozygote resulted in heat-inactivation kinetics which were almost identical to the one described for the medium of CF-heterozygotes (data not shown). The heat-inactivation curve of CF-homozygotes in blood plasma, as compared to the one in culture medium, seems to flatten off slightly during the last hour of inactivation. This might indicate the presence of relatively higher levels of 'residual thermostable' α-mannosidase in blood plasma as compared to cell culture medium.

4. Results

Figure 1a displays heat-inactivation curves of human α-mannosidase in fibroblast culture medium from one normal individual, one obligate CF-heterozygote and one CF-homozygote, exposed for various lengths of time to 41°C and assayed at pH 5.4.

Figure 1b displays heat-inactivation curves of human α-mannosidase in blood plasma of one normal individual, one obligate CF-heterozygote and one CF-homozygote, exposed for various lengths of time to 41°C and assayed at pH 5.4. Different individuals have been used for heat-inactivation studies with cell culture medium and blood plasma, respectively.

For corresponding genotypes the kinetics of heat inactivation of α-mannosidase at 41°C seems to be almost identical in culture medium or blood plasma. While the normal control shows hardly any loss of activity, the CF-heterozygote as well as the CF-homozygote are inactivated to about the same degree during the first 40 min of incubation; thereafter the heat-inactivation curve of CF-heterozygotes rapidly enzymes [9–11]. The fact that abnormal thermolabilities are observable as well in culture media as in

plasma of CF-patients and carriers underlines that we are not dealing with a cell culture artefact.

In order to define the molecular defect in cystic fibrosis, it will be necessary to study if the observed 'thermolability' is due to the action of proteinases, or if it is due to abnormally stable inhibitors, or if the α-mannosidase will remain thermolabile in the purified stage. At present, we can only speculate on why the inactivation of the α-mannosidase fraction in CF-homozygotes is not considerably faster than observed.

To exploit the present observations for the development of diagnostic tools, it will have to be shown that the apparent thermolability of α-mannosidase or other lysosomal hydrolases in crude extracellular fluids is a general characteristic of CF-patients and carriers. We are presently studying heat inactivation kinetics of a large number of controls, carriers and CF-patients, in culture medium as well as in blood plasma. The results of this study have, so far, been very encouraging and follow the expected pattern.

It seems, therefore, likely that based on present observations simple methods will be developed which allow one to carry out post- and prenatal diagnosis, neonatal screening and carrier detection in cystic fibrosis.

5. Discussion

Three technical points must be strictly followed in order to successfully repeat the present study.

First, blood plasma and collection media must be rapidly prepared and processed immediately. Some of the hydrolases studied seem to be thermolabile, even in normal individuals, and plasma or media aliquots can, therefore, not be repeatedly thawed and frozen.

Second, evaporation of the small incubation volumes must be avoided by covering with non-fluorescent oil in order to detect the real kinetics of heat-inactivation.

Third, blood plasma instead of serum must be used. During coagulation non-leaky thermostable hydrolases are released from thrombocytes; the level of thermostable hydrolases is, therefore, increased in serum as compared to plasma and distorts the thermo-

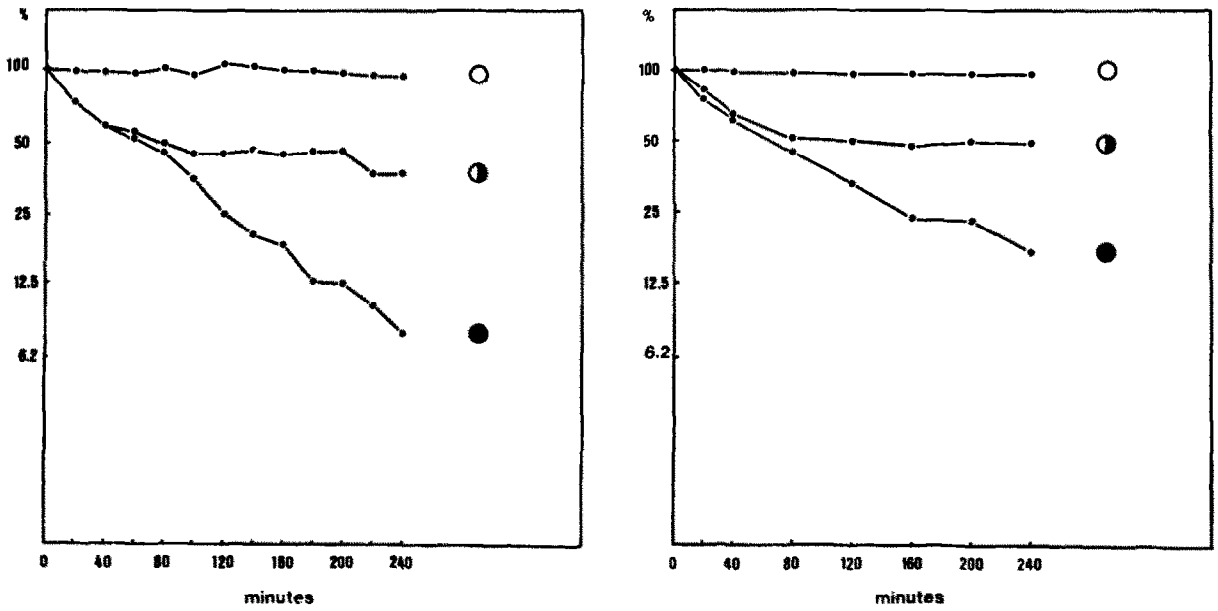

Fig.1(a) Heat-inactivation at 41°C of human α-mannosidase in medium of fibroblast cell cultures. ○ normal control; ◐ CF-heterozygote; ● CF-homozygote. (b) Heat-inactivation at 41°C of human α-mannosidase in blood plasma. ○ normal control; ◐ CF-heterozygote; ● CF-homozygote.

inactivation kinetics of thermolabile enzymes.

The previous observation that several lysosomal hydrolases leak into the medium of cell cultures from CF-patients and CF-heterozygotes [9], and the present demonstration that at least one of these 'leaky' enzymes – α-mannosidase – is abnormally thermolabile in crude extracellular fluids, supports the theory that the genetic defect of cystic fibrosis leads to abnormal recognition markers on lysosomal and samples centrifuged as above. Fluorescence reading was performed with the spectrofluorimeter, excitation wave length 360, emission 448.

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