Demonstration of Altered Acidic Hydrolases in Fibroblasts from Patients with Mucopolipidosis II by Lectin Titration

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Decreased binding by the lectins concanavalin A and wheat-germ agglutinin was found for a number of acidic hydrolases from skin fibroblasts of three unrelated patients with mucopolipidosis II. This decreased binding as compared with normal controls was demonstrated by titration of hydrolase activities with increasing amounts of immobilized lectins. Neuraminidase treatment slightly improved the binding of enzymes from mucopolipidosis-II patients, in contrast with the diminished binding found for hydrolases from control cell lines. The abnormality in binding by lectins of hydrolases of mucopolipidosis-II patients was observed for enzymes with various degrees of intracellular deficiency as well as for enzymes with normal intracellular activities. These findings suggest a generalized alteration of fibroblast acidic hydrolase molecules in mucopolipidosis II.

Mucopolipidosis II (I-cell disease) is a fatal neurodegenerative disorder which is clinically similar to the mucopolysaccharidoses and is inherited as an autosomal recessive condition (McKusick *et al.*, 1978). Skin fibroblasts from these patients have numerous membranous inclusion bodies, which appear to be lysosomes. Although the primary defect has not been described, several biochemical features of this disorder have been reported. In cultured skin fibroblasts from patients with mucopolipidosis II, there is a marked intracellular decrease in most lysosomal enzyme activities, with a concomitant increase of these activities in the culture media; similarly, plasma activities of most hydrolases are significantly elevated (Weisman *et al.*, 1974). Although no major specific substrate appears to be accumulating in these patients, a generalized increase of sialic acid along with a marked decrease in α -neuraminidase (EC 3.2.1.18) activity has been found in fibroblasts from mucopolipidosis-II patients (Thomas *et al.*, 1976). Early observations of a decreased capacity of several hydrolases of mucopolipidosis-II patients to be endocytosed by normal fibroblasts (Hickman & Neufeld, 1972), and altered electrophoretic mobilities of many hydrolases of mucopolipidosis-II patients (Champion & Shows, 1977), have suggested that the defect in mucopolipidosis II results in a generalized alteration of lysosomal hydrolases, perhaps through defective post-translational processing. Although treatment of hexosaminidase from fibroblasts of mucopolipidosis-II patients with neuraminidase did not alter its uptake by normal fibroblasts (Vladutiu & Ratazzi, 1978), the electrophoretic mobility of this enzyme appeared to return to normal. Finally, a somewhat lower

capacity of liver β -galactosidase to be bound by immobilized concanavalin A has also been reported (Miller, 1978), suggesting in conjunction with several of the above results that the defect in mucopolipidosis II may result in altered post-translational glycosylation of lysosomal hydrolases. In addition to these observations, evidence has been presented suggesting that the defect in mucopolipidosis II may also result in an altered membrane structure (Sly *et al.*, 1976).

In order to examine further the possibility that enzymes in mucopolipidosis II may be undergoing faulty glycosylation processes, we have utilized a sensitive system of titration to the binding of eight hydrolase activities, before and after neuraminidase treatment, to two immobilized lectins (concanavalin A and wheat-germ agglutinin) which are known to bind a large number of lysosomal enzymes through their carbohydrate moieties (Fiddler *et al.*, 1979).

Materials and Methods

Fibroblasts were derived, after obtaining informed consent, from skin biopsies of five normal controls, two patients with mannosidosis and three with mucopolipidosis II. The cells were cultivated and harvested as previously described (Ben-Yoseph *et al.*, 1977). Activity of lysosomal hydrolases towards synthetic 4-methylumbelliferyl substrates (Koch-Light, Colnbrook, Bucks., U.K.) were assayed as previously described (Fiddler *et al.*, 1979) with final concentration of 1 mM for 2-acetamido-2-deoxy- β -D-glucoside, β -D-galactoside and phosphate monoester, 2 mM for α -D-mannoside, α -L-fucoside, α -D-glucoside and β -D-glucoside and 5 mM for β -D-glucuronide.

Protein was measured by the method of Bradford (1976) by using the reagent kit supplied by Bio-Rad Laboratories (Richmond, CA, U.S.A.); bovine γ -globulin was used as protein standard.

Binding of fibroblast hydrolase activities by lectins was examined by using agarose-bound wheat-germ agglutinin, 7.0 mg/ml (Vector, Novato, CA, U.S.A.), and concanavalin A (Sigma Chemical Co., St. Louis, MO, U.S.A.) immobilized on Sepharose 4B (Pharmacia, Piscataway, NJ, U.S.A.) by the CNBr method (Porath *et al.*, 1967), 8.6 mg/ml. Fibroblast extracts were diluted with a 2 mg/ml solution of human serum albumin (Sigma) in order to compare the binding of equivalent amounts of enzymic activities. Diluted cell lysates were mixed with various amounts of immobilized lectin in 0.01 M- CaCl_2 /0.1 M- NaCl /0.05 M-Tris/HCl buffer, pH 7.0, in a final volume of 0.35 ml. After 1 h shaking at 37°C, the mixtures were filtered through Pasteur pipettes stoppered with glass wool and filtrates were assayed for unbound hydrolase activities.

Neuraminidase treatment was carried out by incubating 1 ml of fibroblast lysate with 20 μ l of a 1.5 units (1 unit = 1 μ mol hydrolysed/min)/ml solution of *Clostridium perfringens* neuraminidase (Worthington, Freehold, NJ, U.S.A.) in 0.05 M-sodium acetate buffer, pH 5.0, for 16 h at 25°C. Additional 20 μ l batches of neuraminidase were added after 4 and 14 h incubation. Control experiments demonstrated that neuraminidase hydrolysed sialic acid (Warren, 1959) from fetuin (Calbiochem, LaJolla, CA, U.S.A.) under these conditions. Neuraminidase and human serum albumin were free from proteinase and glycosidase activities and were not bound by either lectin.

Double immunodiffusion by using antiserum to GM_1 -ganglioside β -galactosidase (EC 3.2.1.23) (Ben-Yoseph *et al.*, 1977) was performed in agarose gel

(L'Industrie Biologique Française, Genevilliers, France; 10 mg/ml) in phosphate-buffered saline (0.14 M- NaCl /0.01 M-sodium phosphate buffer), pH 7.0.

Results

Fig. 1 illustrates that differences between fibroblast hydrolases from mucopolipidosis-II patients and normal subjects can be resolved when a constant enzymic activity is titrated by increasing amounts of immobilized concanavalin A; these differences progressively disappeared when quantities of concanavalin A were increased. For the three enzymes shown (β -N-acetylhexosaminidase, β -galactosidase and α -glucosidase), 12.5, 8.0 and 4.8 times more lectin respectively was required to remove 50% of the enzymic activity from fibroblast lysates of mucopolipidosis-II patients than from normal fibroblast lysates. The data shown represent the mean values for the replicates (two or three) for all cell lines examined; values ranged $\pm 5\%$ from these values. Treatment of the lysates with neuraminidase resulted in alterations of the titration curves for all three enzymes shown. More concanavalin A was required to bind these enzymes than untreated normal hydrolases and both untreated and treated hydrolases of mucopolipidosis-II patients. The titration curve of the normal fibroblast activities became similar to that of the enzymes of mucopolipidosis-II patients, which were only slightly affected by the neuraminidase treatment.

Fig. 2 illustrates the titration of β -N-acetylhexosaminidase by immobilized wheat-germ agglutinin. These results are similar to those in Fig. 1, although greater quantities of wheat-germ agglutinin were required to obtain the curve. Again, neuraminidase treatment resulted in a change of the normal

Fig. 1. Titration of glycosidase activities from fibroblasts of normal controls (●, ○) and patients with mucopolipidosis II (▲, △) before (●, ▲) and after (○, △) neuraminidase treatment by immobilized concanavalin A. Initial enzymic activities (nmol/h) in diluted lysates from normal subjects and mucopolipidosis-II patients: (a) β -N-acetylhexosaminidase, 559 and 622; (b) β -galactosidase, 46.3 and 52.5; (c) α -glucosidase, 78.7 and 84.1 respectively.

fibroblast activity to make it more closely resemble that of the untreated activity of fibroblasts from mucopolipidosis-II patients. With this lectin, however, neuraminidase treatment resulted in a curve for the

Fig. 2. Titration of β -N-acetylhexosaminidase activity from fibroblasts of normal controls ($\bullet$, $\circ$) and patients with mucopolipidosis II ($\blacktriangle$, $\triangle$) before ($\bullet$, $\blacktriangle$) and after ($\circ$, $\triangle$) neuraminidase treatment by immobilized wheat-germ agglutinin

Initial enzymic activity (nmol/h) in diluted lysates from normal subjects and mucopolipidosis-II patients: 559 and 622 respectively.

activity of mucopolipidosis-II patients which was closer to that of the untreated normal activity.

The same curves, as illustrated in Figs. 1 and 2, were obtained even if the initial activities were 10 times more diluted or concentrated.

Similar differences in the titration curves were observed for other acidic hydrolases. Table 1 presents a summary of the data obtained for eight hydrolase activities titrated with immobilized concanavalin A and five activities titrated with immobilized wheat-germ agglutinin. The intracellular values of these hydrolase activities in fibroblasts from patients with mucopolipidosis II ranged from 2.3% of control for α -fucosidase to 125% of control for acid phosphatase. However, for all eight activities marked differences were observed for the quantity of lectin required to bind 50% of the activity in fibroblast lysates of mucopolipidosis-II patients compared with normal cell lysates. For concanavalin A, the ratio of lectin (w/w) required to remove half of the activity from lysates of mucopolipidosis-II patients compared with normal lysates before neuraminidase treatment ranged from 3.0 for β -glucosidase to over 14 for acid phosphatase. Neuraminidase treatment markedly decreased all the ratios to below 1.0. Similar results were obtained from those intracellular hydrolases titrated with wheat-germ agglutinin. In addition, several hydrolases, including α -mannosidase from fibroblasts, obtained from two patients with mannosidosis provided results similar to these for normal fibroblasts.

Finally, examination of one of these hydrolases, β -galactosidase, from both untreated and neuraminidase-treated lysates of normal subjects and patients with mucopolipidosis II by double diffusion against an anti-(G_{M1} -ganglioside β -galactosidase) serum prepared against the normal human enzyme revealed no discernible antigenic differences between the

Table 1. Comparative binding of hydrolases from fibroblasts of normal subjects and patients with mucopolipidosis II with immobilized lectins

The ratio is μ g of lectin binding 50% of activity from lysates of mucopolipidosis-II patients/ μ g of lectin binding 50% of activity from lysates of normal controls. 'Pre' and 'Post' refer to neuraminidase treatment.

Enzyme activity	Intracellular activity in mucopolipidosis II (% of normal)	Ratio			
		Concanavalin A		Wheat-germ agglutinin	
		Pre	Post	Pre	Post
β -N-Acetylhexosaminidase (EC 3.2.1.30)	14.2	12.5	0.4	12.4	0.9
α -Mannosidase (EC 3.2.1.24)	16.5	12.6	0.8	3.1	0.9
β -Glucuronidase (EC 3.2.1.31)	20.1	5.8	0.6		
β -Galactosidase (EC 3.2.1.23)	4.3	8.0	0.7	6.5	0.9
α -Fucosidase (EC 3.2.1.51)	2.3	5.5	0.6		
β -Glucosidase (EC 3.2.1.21)	118.3	3.0	0.4		
Acid phosphatase (EC 3.1.3.2)	125.3	14	0.7	7	0.8
α -Glucosidase (EC 3.2.1.20)	70.1	4.8	0.8	13.7	0.1

β -galactosidases of normal subjects and mucopolipidosis-II patients.

Discussion

The technique of lectin titration enabled us to detect differences between fibroblasts of patients with mucopolipidosis II and control fibroblasts. Previous studies of the binding of β -galactosidase from liver of mucopolipidosis-II patients and normal subjects by column chromatography on Sepharose-bound concanavalin A revealed no differences in one case (Holmes *et al.*, 1975) and as much as 31–37% decrease in binding for another patient with mucopolipidosis II (Miller, 1978). We have observed 25–45% decrease in binding of all enzymes examined by both lectins. The specific binding of glycoproteins to concanavalin A and wheat-germ agglutinin is influenced by the presence and accessibility within the oligosaccharide chain of mannose or *N*-acetylglucosamine residues, respectively. The lower binding of lysosomal hydrolases from patients with mucopolipidosis II to concanavalin A and wheat-germ agglutinin indicates a possible change in the number, the availability or the orientation of carbohydrate residues in these enzyme molecules (Sharon & Lis, 1972; Goldstein & Hayes, 1978). The method of lectin titration appears to be an effective means of detecting differences between normal hydrolases and hydrolases of mucopolipidosis-II patients over a wide range of glycoprotein concentration. This point is emphasized by the experiments using fibroblasts from mannosidosis patients, which are known to contain mannose-rich glycopeptides, the concanavalin A-specific carbohydrate; no differences in hydrolase binding were observed from these three fibroblast lines compared with normal controls. This experiment, however, does not definitively rule out the possibility that other glycoproteins or glycolipids in cells of mucopolipidosis-II patients are altering the binding of the hydrolases. These changes may be consistent with many of the altered properties reported, such as 'recognition' markers (Hickman *et al.*, 1974), sialylation (Thomas *et al.*, 1976) and electrophoretic mobility (Champion & Shows, 1977). This alteration, however, does not seem to affect the activity of the enzyme protein. In previous studies of G_{M1} -ganglioside β -galactosidase (Ben-Yoseph *et al.*, 1977) and galactosylceramide β -galactosidase (EC 3.2.1.46) (Ben-Yoseph *et al.*, 1978) from fibroblasts of patients with mucopolipidosis II we have shown that the deficient intracellular activity is accompanied by a similar deficiency of antigenically cross-reacting material, and thus normal specific activity of cross-reacting material is retained by these enzymes in mucopolipidosis II.

After neuraminidase treatment the differences in binding to lectins of lysosomal hydrolases from

mucopolipidosis-II patients and normal subjects were much less remarkable than for untreated hydrolases. Deficiency of neuraminidase has been suggested as a defect in mucopolipidosis II (Strecker & Michalski, 1978), but the effect of the *Clostridium* enzyme could not be attributed to a simple 'correction' of this deficiency. This may be the result of differences in substrate specificities between human and the bacterial neuraminidases, but it should be noted that only slight differences were found in enzymes from mucopolipidosis-II patients, whereas major changes after neuraminidase treatment were seen in the normal enzymes. These observations suggest, however, that sialic acid residues have an important role in determining the conformation of carbohydrate moieties within the lysosomal enzymes, although no antigenic differences could be detected for G_{M1} -ganglioside β -galactosidase of mucopolipidosis-II patients and normal subjects before and after neuraminidase treatment.

The most consistent finding in fibroblasts of patients with mucopolipidosis II is a decrease in the intracellular activity of lysosomal hydrolases. The degree of deficiency varies with the enzyme, and the relationship of this variation to the molecular defect is not understood. We have studied the binding by lectins of three groups of lysosomal hydrolases with respect to their intracellular activity: A, β -*N*-acetylhexosaminidase, α -mannosidase and β -glucuronidase with lower activities (12–25% of normal); B, β -galactosidase and α -fucosidase with severely depressed activity (2–10% of normal); C, β -glucosidase, acid phosphatase and α -glucosidase with nearly normal activities. The abnormal binding by concanavalin A and wheat-germ agglutinin was found for all these enzyme groups before and after neuraminidase treatment. These findings suggest that a general alteration in carbohydrate composition of lysosomal hydrolases may be involved in the process of mucopolipidosis II.

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