Application of magnetic chromatography to the isolation of lysosomes from fibroblasts of patients with lysosomal storage disorders

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Abstract A method for the purification of lysosomes from fibroblasts has been developed which uses endocytosis of superparamagnetic colloidal iron dextran particles followed by separation of the iron-containing lysosomes in a magnetic field. This permitted isolation of lysosomes from fibroblasts from patients with infantile sialic acid storage disorder and other lysosomal storage diseases in which a shift in lysosomal density induced by the storage material prevents purification by centrifugation in a Percoll gradient. The magnetic lysosomes isolated from these cells are very similar to those from normal cells as judged by lysosomal marker enzyme activity and 2D-PAGE analysis of the enriched proteins.

Key words: Lysosome; Purification; Iron dextran; Infantile sialic acid storage disorder; Lysosomal storage disease; Two-dimensional polyacrylamide gel electrophoresis

1. Introduction

It is very important for proteome research and the diagnosis of specific protein defects to be able to purify organelles from cultured cells to obtain preparations of organelle-associated proteins. These proteins can then be identified by searching databases, checked for aberrations or, if still unidentified, sequenced and cloned. In the case of the lysosome there are several inherited disorders where the specific protein defect has not been identified. This is especially true for lysosomal membrane proteins about which very little is known apart from their enzymic or transport functions.

Lysosomes can normally be isolated from many different types of cells by centrifugation in density gradients. In the case of infantile sialic acid storage disorder (ISSD) though, it has been observed by others [1,2] and ourselves that the density of the lysosomal compartment is decreased by the stored material so that purification by gradient centrifugation is impossible. The same is true for cells from patients with α-mannosidosis (unpublished data), Batten's disease [3], Farber disease [4] and presumably a number of other lysosomal storage diseases. Therefore we sought a method which would allow purification of lysosomes which is independent of their buoyant density and their morphology, both of which are likely to be altered in lysosomal storage diseases. Even if gradient centrifugation could be modified to produce an enrichment of the lysosomes with an altered density, the contaminating proteins co-purified under the new conditions would be different from those obtained under standard conditions and would further complicate the identification of specific lysosomal proteins by 2D-PAGE. Rodriguez-Paris et al. [5] have used endocytosis of iron dextran (FeDex) followed by magnetic chromatography to isolate phagolysosomes from *Dictyostelium*. In this paper we show that lysosomes with decreased density due to the accumulation of storage material can be isolated efficiently from cultured fibroblasts by magnetic chromatography after endocytosis of FeDex.

2. Material and methods

2.1. Chemicals

Protease inhibitors were from Boehringer Mannheim except for benzyloxycarbonyl-L-phenylalanine-L-phenylalanine-diazomethyl ketone (ZFF) which was generously provided by Heidrun Kirschke (Halle-Wittenberg, Germany). Millex-GV Filters (0.22 μm) were from Millipore S.A. (Molsheim, France). Nutrient mixture Ham's F-10, trypsin EDTA, Dulbecco's PBS and penicillin/streptomycin (5000 IU/ml, 5 mg/ml) solutions were from Gibco BRL/Life Technologies (Paisley, UK). FCS was from Imperial Laboratories (Andover, UK). 4-Methylumbelliferyl substrates were purchased from Melford Laboratories Ltd. (Ipswich, UK). MiniMACS separation columns were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Immobiline dry strips were from Pharmacia Biotech (St. Albans, UK). All other chemicals were from Sigma (Poole, UK).

2.2. Cell lines and cell culture

Human skin fibroblasts were derived from two normal individuals, three patients with ISSD, one patient with nephropathic cystinosis and one with late-infantile neuronal ceroid lipofuscinosis (variant CLN6). Cells were cultured in Ham's F-10 medium with 10% FCS and 1% PenStrep in a 5% CO_2 atmosphere.

2.3. Preparation of colloidal iron dextran particles

Superparamagnetic particles were prepared as described by Rodriguez-Paris et al. [5]: 10 ml of 1.2 M $FeCl_2$ and 10 ml of 1.8 M $FeCl_3$ were mixed in a 500 ml beaker. The solution was constantly agitated (no magnetic stirrer) while 10 ml of 30% NH_3 was added. Agitation continued until the suspension was freely flowing again. Next, 100 ml of 5% NH_3 was added and the beaker placed on top of a magnet until the precipitate gathered at the bottom. With the magnet still in place the supernatant was decanted. After removal of the magnet the sediment was resuspended in 100 ml H_2O and then collected again on the magnet. This washing step was repeated once more. The final sediment was resuspended in 80 ml of 0.3 M HCl and stirred with a magnetic stirring bar for 30 min. After this time 4 g of dextran T40 was added and stirring continued for at least 30 min. This suspension was dialyzed for 2 days at 4°C against 8 l of deionized water with three changes of the dialysis bath. Dialysis can be monitored by checking the pH of the dialysis bath prior to each change. Large aggregates were removed by centrifugation for 10 min at 15000 rpm (Sorvall SS-34). The supernatant was removed, sterile filtered at 0.22 μm to yield FeDex particles. This solution could be stored at 4°C for up to 2 months and had a dry weight of 53 mg/ml.

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; FeDex, colloidal iron dextran; HSA, human serum albumin; ISSD, infantile sialic acid storage disorder; PNS, postnuclear supernatant

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2.4. Loading of fibroblasts in culture with FeDex

The FeDex suspension (13.5 ml) was mixed with 20 ml of culture medium and evaporated to a total of 20 ml in a Speed Vac evaporator. The FeDex-containing medium was immediately sterile filtered at 0.22 μm and added to a confluent layer of fibroblasts in a 175 cm^2 flask. Loading was carried out for 9 h or the time indicated at 37°C after which the cells were washed once with PBS and left with fresh medium overnight.

2.5. Harvesting of cells

The cells were detached in 3 ml of trypsin-EDTA solution for 15–20 min at 37°C, after which 5 ml of MEM medium containing 10% FCS was added and the suspension transferred to a 15 ml conical tube. The flask was rinsed with 6 ml of Dulbecco's PBS (0.1 g $CaCl_2$/l) which was also added to the suspension. The cells were centrifuged for 10 min at 600×*g* at 4°C. From this point onwards all steps were performed in an ice/water bath or in the cold room. The supernatant was removed and the cells were washed once with 6 ml of 250 mM sucrose in 4 mM imidazole/HCl buffer, pH 7.4 (buffer A).

The cell pellets were brought to a volume of 600 μl with buffer A* (buffer A containing 5 mM iodoacetamide, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml ZFF and 500 μg/ml Pefabloc [4-(2-aminoethyl)-benzenesulfonyl fluoride]). Cells were broken up in four 150 μl aliquots by 8 strokes in a '0.1 ml' Potter-Elvehjem homogenizer. The homogenizer was rinsed with 150 μl of buffer A* which was added to the homogenate. The homogenate was centrifuged at 600×*g* for 5 min and the postnuclear supernatant (PNS) removed.

The Ca^{2+}/Mg^{2+}/EDTA content of the above solutions resulted in optimal recovery of enriched lysosomes. A higher Ca^{2+} concentration will reduce the recovery in the PNS while EDTA will prevent binding of vesicles to the magnetic column.

2.6. Magnetic affinity chromatography

MiniMACS columns, which have been developed for cell separation by magnetic chromatography, were equilibrated with buffer A. The PNS was split into three aliquots (200 μl) which were applied to three columns in parallel with the magnet attached and unbound material was collected by gravity. The column was washed with 1.5 ml of TBS (150 mM NaCl; 5 mM Tris-HCl pH 7.4) before the bound material was eluted in two successive steps. First 100 μl of hypotonic buffer B (5 mM Tris-HCl pH 7.4 with the same protease inhibitor concentrations as in buffer A*) were added and held on the column for 15 min to lyse the lysosomes osmotically. The soluble lysosomal proteins were eluted with another 100 μl of buffer B whilst the lysosomal 'ghosts' were retained on the column by the FeDex. These two eluates were combined to form the luminal protein fraction (fraction 1). The lysosomal membrane proteins were eluted by removal of the magnet and addition of 100 μl of buffer B containing 0.5% (v/v) Triton X-100. After incubation for 15 min another 100 μl aliquot of the same solution was applied. These two eluates were combined to form the lysosomal membrane fraction (fraction 2). All samples were frozen as 200 μl aliquots in liquid nitrogen and stored at −70°C.

2.7. Enzyme assays

β-Hexosaminidase [6], β-galactosidase [7] and α-fucosidase [8] were assayed as described previously. β-Glucosidase was assayed as follows. The sample was diluted in 2 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.1% HSA. To 50 μl of this was added 50 μl of the following substrate solution: 10 mM 4-methylumbelliferyl-β-D-glucopyranoside; 50 mM sodium taurocholate; 0.2%(v/v) Triton X-100; 0.1% HSA; 40 mM $CaCl_2$ in McIlvaine's citric acid-sodium phosphate buffer, pH 5.4. After incubation at 37°C for 60 min the reaction was stopped with 1 ml of 0.25 M glycine/NaOH, pH 10.4. The released 4-methylumbelliferone was measured at 450 nm using an excitation wavelength of 365 nm. Citrate synthase was determined as published [9]. Protein was determined with the Bio-Rad Protein assay (Bio-Rad, Munich, Germany) using bovine IgG as a standard protein.

2.8. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The separation of proteins by 2D-PAGE was carried out as previously reported [10] with the following changes. Immobiline dry strips with a pH range of 4–7 and a length of 180 mm were used for the first dimension. Focusing was carried out at 24°C for approximately 70 kVh at a maximum voltage of 3500 V. The gels were silver-stained following the protocol of Jungblut and Seifert [11].

3. Results

Confluent fibroblasts from normal controls and patients with lysosomal storage disease were loaded with FeDex for

Table 1
Enzyme activities in lysosomes purified with FeDex ($n = 4$)

Fraction	Specific activity (nmol/min/mg)	Enrichment relative to homogenate	Recovery % of homogenate
β-Hexosaminidase			
Homogenate	210 ± 27	1.0	100
PNS	198 ± 64	0.93 ± 0.18	52 ± 13
Fraction 1	2494 ± 758	12 ± 4	4.8 ± 1.6
Fraction 2	1902 ± 784	8.9 ± 2.5	19 ± 4
β-Glucosidase			
Homogenate	6.5 ± 0.5	1.0	100
PNS	5.7 ± 1.5	0.87 ± 0.15	48 ± 11
Fraction 1	17 ± 4	2.7 ± 0.9	1.0 ± 0.2
Fraction 2	86 ± 37	13 ± 5	27 ± 6
α-Fucosidase			
Homogenate	0.87 ± 0.51	1.0	100
PNS	0.75 ± 0.34	0.93 ± 0.19	52 ± 14
Fraction 1	30 ± 22	31 ± 8	12 ± 2
Fraction 2	4.1 ± 2.0	5.0 ± 1.2	11 ± 2
β-Galactosidase			
Homogenate	16 ± 1	1.0	100
PNS	15 ± 3	0.91 ± 0.15	51 ± 11
Fraction 1	577 ± 128	36 ± 6	14 ± 3
Fraction 2	95 ± 31	5.9 ± 1.7	12 ± 2
Citrate synthase			
Homogenate	136 ± 28	1.0	100
PNS	117 ± 38	0.85 ± 0.13	48 ± 9
Fraction 1	(none detected)	< 0.5	< 0.2
Fraction 2	47 ± 25	0.33 ± 0.14	0.74 ± 0.34

Lysosomes were isolated from two normal fibroblast lines by uptake of FeDex followed by magnetic chromatography using the two-step elution protocol. Fraction 1 is the material eluted under hypotonic conditions with the magnet still switched on. Fraction 2 is material eluted with a detergent-containing eluent with the magnet removed. Enzyme activities and protein content were assayed in the homogenate, PNS and the two fractions. Citrate synthase activities in fraction 1 were below the detection limit of the assay. Values are means ± S.D.

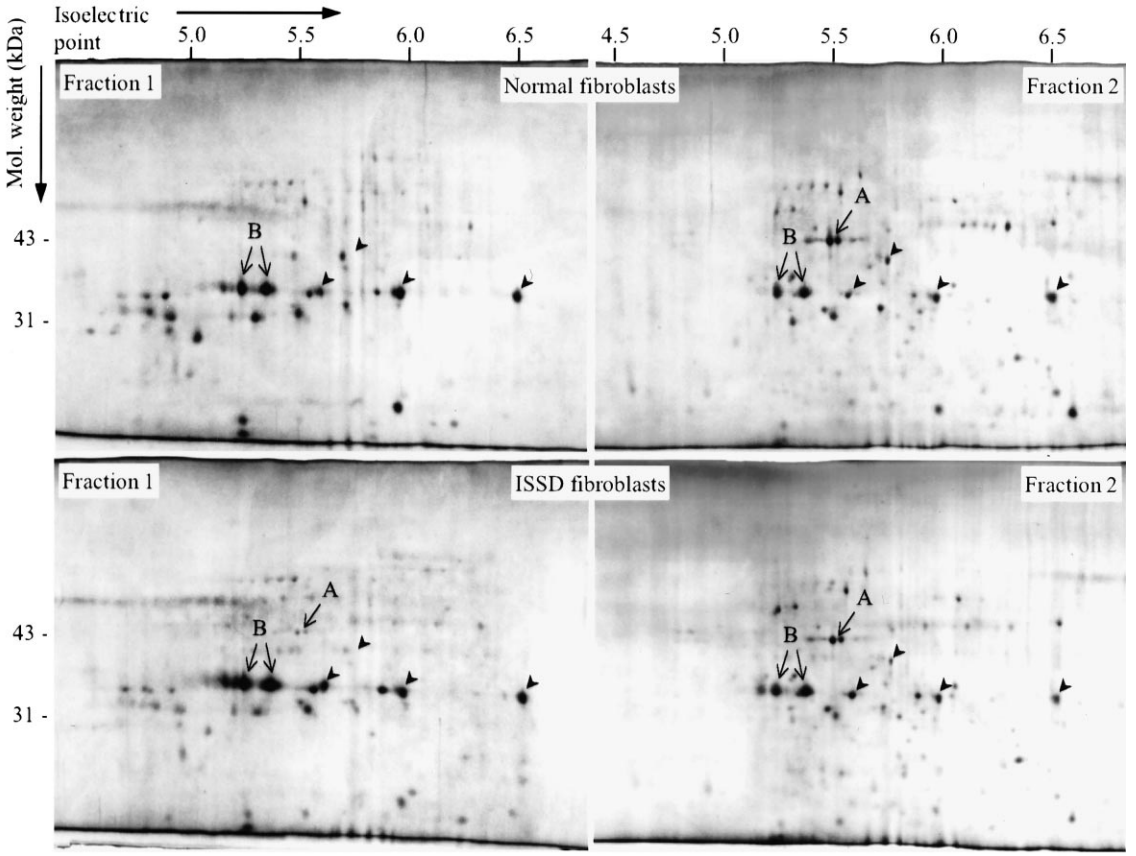

Fig. 1. 2D-PAGE with silver staining of lysosomal preparations from normal fibroblasts (top panels) and fibroblasts from patients with ISSD (bottom panels). Fractions 1 and 2 were prepared as described in the legend to Table 1. Spots A have been identified as actin. Spots B are thought to be cathepsin D. Arrowheads point to proteins previously shown to be lysosomal proteins.

different times and then incubated with fresh normal medium overnight. The appearance of the cells was monitored by light microscopy and no morphological changes were found for up to 12 h contact with the FeDex. A loading time of 9 h was adopted for all subsequent preparations. The cells were harvested, homogenized and the PNS applied to the MiniMACS column. It was observed that after removing the magnet from the column, the recovery of presumably intact lysosomes by elution with isotonic buffer was only minimal and that the application of hypotonic or detergent-containing buffers was necessary to obtain reasonable yields. Glombitza et al. [12] had seen similar results.

Therefore a two-step elution protocol was devised: release of soluble luminal proteins by lysis of bound lysosomes with hypotonic buffer (fraction 1) followed by elution of bound material by detergent-containing buffer after removal of the magnet (fraction 2). The results for the duplicate analysis of two normal fibroblast lines are given in Table 1. β-Hexosaminidase, α-fucosidase and β-galactosidase, which are soluble luminal enzymes, were eluted in both of the fractions but the enrichment was always much greater in fraction 1. In contrast, β-glucosidase, which is a membrane-associated enzyme, was found almost exclusively in the second fraction. Mitochondrial contamination as assessed by citrate synthase activity was not detected in fraction 1 and less than 1% was recovered in fraction 2.

This shows that the procedure efficiently separates lysosomes from mitochondria and that the luminal and membrane proteins of the lysosome can be selectively eluted from the magnetic column.

This technique was then applied to the PNS of fibroblasts from patients with ISSD and other lysosomal storage disorders (Table 2). Allowing for the variation in activities within each fibroblast type, there is no apparent difference in the efficiency of the purification of lysosomes between the control lines and any of the storage fibroblast lines, indicating that the endogenous storage material does not affect the separation.

To illustrate further the similarity of the lysosomal preparations from normal cells and those from a storage disease, the enriched fractions 1 and 2 from control and ISSD lines were analyzed by 2D-PAGE (Fig. 1). From our previous experience the group of spots designated A are very likely to be cathepsin D. The protein designated B was confirmed to be actin, which is known to interact with and bind to lysosomes [13–15]. The spots marked by arrowheads had also been identified previously as lysosomal [10]. Three were present in both fractions albeit relatively more abundantly in the luminal fraction while the largest (~40 kDa) was found exclusively in fraction 2, suggesting it was membrane-associated.

4. Discussion

Our results show that lysosomes from fibroblasts from patients with a range of lysosomal storage diseases, in which the accumulation of storage material has altered the density of the lysosomes and prevents their separation from other organelles

Table 2
Recovery and enrichment of marker enzymes in lysosomes prepared from PNS from different fibroblast lines

	Enrichment relative to PNS		Total recovery[a] (% of PNS)
	Fraction 1	Fraction 2	
Normal ($n=4$)			
β-Hex	13 ± 5	9.5 ± 1.0	46 ± 4
β-Gluc	3.2 ± 1.4	15 ± 3	59 ± 2
ISSD ($n=3$)			
β-Hex	14 ± 4	6.0 ± 1.0	22 ± 5
β-Gluc	1.4 ± 1.2	16 ± 6	32 ± 7
Cystinosis ($n=1$)			
β-Hex	13	7.0	37
β-Gluc	1.2	16	44
NCL6 ($n=1$)			
β-Hex	10.5	7.9	43
β-Gluc	0.9	19	58

Lysosomal fractions from two normal fibroblast lines (same experiment as Table 1), three ISSD lines and one line each of patients with nephropathic cystinosis and late infantile neuronal ceroid lipofuscinosis (CLN6) were prepared as described in the legend to Table 1. The PNS and the two eluate fractions were assayed for β-hexosaminidase (β-Hex), β-glucosidase (β-Gluc) and protein content.
[a]Ratio of the sum of total enzyme activities in fractions 1 and 2 over the total activity in the PNS. For normal controls and ISSD cells values are means ± S.D.

by density centrifugation, can be isolated by magnetic chromatography. The simplicity of the protocol also makes it convenient for the isolation of lysosomes from normal fibroblasts. It is possible that the method might not be applicable to fibroblasts from some storage diseases because the lysosomes cannot tolerate the additional load of FeDex. Subconfluent ISSD cells were seen to show some damage morphologically after 12 h of loading. With sensitive fibroblasts the pulse time can be decreased to 3 h with a decrease in recovery of lysosomes of only approximately 5%.

It is assumed that the method relies on the non-specific uptake of FeDex (37 mg iron dextran per ml medium) by fluid phase endocytosis and its transfer by the default endocytotic pathway to highly acidified secondary lysosomes, in a similar manner to dextran alone [16]. In the original work on *Dictyostelium* phagolysosomes by Rodriguez-Paris et al. [5] a much lower concentration of FeDex (2 mg/ml) and only 15 min exposure time to the FeDex-containing medium could be used because of the high pinocytotic activity of this slime mold. Glombitza et al. [12] used an endocytosis time of 24 h and unknown FeDex concentration for the isolation of lysosomes from keratinocytes. Becich and Baenziger [17] used FeDex (0.2 mg/ml) covalently conjugated with Gal-BSA or asialofetuin to isolate endosomes and lysosomes from HepG2 cells by exploiting the asialoglycoprotein receptor expressed on this cell type. They only monitored acid β-galactosidase as a lysosomal marker and found a recovery of 8–9% of the activity relative to the PNS with a 9-fold enrichment. In contrast, in the present study recoveries relative to the PNS of 29% in the first fraction and 25% in the second were obtained with enrichments of 40- and 6-fold, respectively. For fibroblasts, the resulting purity of the lysosomes is similar to that achieved by Percoll density gradient centrifugation [18]. Generally, the maximum achievable enrichment of lysosomes will depend on the abundance of lysosomes in the starting material.

Using the two-step elution protocol soluble lysosomal enzymes were eluted in both fractions but the enrichment was always greater in fraction 1, in which less than 1% of the membrane-associated β-glucosidase was recovered. Association with the colloidal gel may be responsible for the soluble lysosomal enzymes found in fraction 2.

The 2D-PAGE patterns of lysosomal proteins were very similar in the corresponding eluted fractions for normal and ISSD cells. The storage material did not lead to major secondary changes in the molecular weight and isoelectric point of proteins.

This method allows lysosomes from patients with a wide range of lysosomal storage diseases to be purified and subjected to biochemical analysis. One application is the comparison of the protein patterns obtained by 2D-PAGE for lysosomes from controls and patients to identify underlying protein defects. This procedure has already revealed candidate proteins for the lysosomal sialic acid transporter, that is defective in ISSD, indicating the value of the procedure.

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