Cystine storage in cultured myotubes from patients with nephropathic cystinosis

Gregory S. HARPER,*† Isa BERNARDINI,* Orest HURKO,† Judith ZUURVELD† and William A. GAHL*

*Section on Human Biochemical Genetics, Human Genetics Branch, NICHD, NIH, Bethesda, MD 20892, and †Department of Neurology, Johns Hopkins Medical School, Baltimore, MD 21205, U.S.A.

Sorted muscle cells, cultured from a patient with nephropathic cystinosis, stored 100 times normal amounts of cystine. Subcellular fractionation and density-gradient centrifugation confirmed that the cystine was located in a lysosomal compartment. 2. Myoblasts from cystinotic patients in culture underwent fusion to myotubes in a normal fashion. 3. The free thiol cysteamine effectively depleted cystinotic-muscle cells of cystine. 4. Cultured myoblasts and myotubes offered a unique system for investigating the effects of lysosomal storage on differentiated cell functions.

INTRODUCTION

Nephropathic cystinosis, an autosomal recessive storage disorder, results from defective transport of cystine out of lysosomes (Gahl *et al.*, 1982*a,b*, 1983, 1984; Jonas *et al.*, 1982). Cystine forms crystals in corneal, conjunctival, renal, intestinal, lymph-node and other cells, and accumulates to 50 times normal concentrations in cultured fibroblasts, leucocytes and renal epithelial cells (Schneider *et al.*, 1968; Schneider & Schulman, 1983; Pellett *et al.*, 1984). Children with cystinosis have renal tubular and glomerular dysfunction ending in uraemia by 10 years of age (Gretz *et al.*, 1982). They also exhibit growth retardation, photophobia and hypothyroidism (Gahl, 1986), but mechanisms for such widespread organ damage have not yet been elucidated.

The brain and skeletal muscle are considered clinically uninvolved in cystinosis. Affected children have normal intelligence and neurological function, and their muscle appears free of pathology. The reason for this tissue sparing remains unknown, but may result from cell-specific resistance to cystine storage or variability in the extent of cystine storage among different tissues.

This issue can be addressed by studying the cystine contents of clinically uninvolved tissues. *Post mortem* brain specimens have normal cystine concentrations (Schulman & Bradley, 1973), and this may explain the normal central-nervous-system function in cystinosis. But muscle-biopsy specimens from cystinotic patients contain elevated cystine concentrations (Broyer *et al.*, 1981), which may reflect cystine storage by myotubes, fibroblasts, or other connective-tissue cells present in the biopsy sample. Only culture *in vitro* of a muscle biopsy with sorting of myoblasts from fibroblasts could reveal whether muscle cells themselves stored cystine.

We performed these experiments with sorted myotubes from cystinotic patients, to determine if clinically unaffected cells express the primary phenotype of cystinosis, i.e. cystine storage. We also studied whether these cells undergo normal myogenic fusion *in vitro*, and whether the cystine-depleting agent cysteamine, employed in the long-term therapy of children with cystinosis (Gahl *et al.*, 1986), depletes cystinotic-muscle

cells of cystine as effectively as it depletes other cells in culture (Thoene *et al.*, 1976; Pellett *et al.*, 1984). These investigations established cultured myotubes as a potential model system with which to study lysosomal storage disorders.

EXPERIMENTAL

Muscle tissue

Biopsy or *post mortem* specimens of muscle were minced and suspended in 400 μ l of 10 mM-*N*-ethylmaleimide (Pierce, Rockford, IL, U.S.A.) in 10 mM-sodium phosphate, pH 7.0, with a pestle and glass homogenizer. After extensive sonication, protein was precipitated with 4% (w/v) sulphosalicylic acid and assayed for protein as described by Lowry *et al.* (1951). The supernatant was assayed for cystine by the cystine-binding-protein method of Oshima *et al.* (1974).

Cell cultures

Skeletal-muscle tissue obtained from biopsies, after fully informed consent, was placed in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD, U.S.A.). The tissue was stored at 4 °C until dissociation was performed by repeated enzyme digestion and physical agitation, normally within 6 h of surgery (Wilson *et al.*, 1972; Yasin *et al.*, 1976, 1977). The dissociated mononuclear muscle cells were grown in air/CO₂ (9:1), in plastic culture plates with Dulbecco's modified Eagle's medium containing 10% (v/v) foetal-bovine serum (GIBCO, Grand Island, NY, U.S.A.), glutamine (10 mM), penicillin (100 units/ml), streptomycin (100 μ g/ml) and mycostatin (3 μ g/ml) (supplements obtained from the NIH media branch). To prevent fusion, cultures were repeatedly divided at 40% confluence with Dulbecco's phosphate-buffered saline (PBS: 144 mM-NaCl, 5.4 mM-KCl, 25 mM-glucose, 14 mM-sucrose, 5 mM-sodium phosphate, pH 7.3, 340 mosm) supplemented with 0.05% trypsin/0.02% EDTA (Whittaker M. A. Bioproducts, Walkersville, MD, U.S.A.), and replated at 1:2 dilution. Cells treated in this manner remained mononucleated for at least 15

† To whom reprint requests should be addressed.

passages. For myogenic fusion, cells were allowed to grow past 50% confluence in Dulbecco's modified Eagle's medium containing 2% (v/v) horse serum (Hyclone, Logan, UT, U.S.A.), glutamine and antibiotics. Cell fusion was normally complete within 3–5 days. Cultures were shown to be free of mycoplasma DNA (Gen-Probe, San Diego, CA, U.S.A.).

Sorting of myoblasts from fibroblasts

Cells grown in 100 mm-diam. culture dishes (typically 5×10^6 cells) were incubated in PBS/2 mM-EDTA at 37 °C, rinsed twice with PBS, and mechanically agitated to suspend them uniformly. After two washes with ice-cold PBS containing 10% heat-inactivated chicken serum (buffer A), the cells were resuspended in 0.4 ml of buffer A containing monoclonal antibody 5.1H11 (Walsh & Ritter, 1981). This antibody recognizes a surface antigen of human myoblasts and myotubes, but not fibroblasts. After 60 min on ice, the suspension was diluted to 50 ml with ice-cold buffer A and centrifuged; this was repeated three times to remove unbound antibody. The pellet was exposed to rhodamine-conjugated sheep anti-mouse antibody (Cappel, Westchester, PA, U.S.A.) for 60 min on ice, washed three times, resuspended in 5 ml of buffer A, and subjected to fluorescence-activated cell sorting ('FACS') in a Becton Dickinson FACS II apparatus equipped with rhodamine optics. Window settings for the separation were determined by using control muscle cultures which had not been treated with monoclonal antibody 5.1H11, and skin fibroblast cultures which had been exposed to both antibodies. Particular care was taken to minimize fibroblast contamination in the antigen-positive myoblast fractions. These fractions were replated and examined microscopically to verify the separation. Samples were also taken for tests of fusion competence, surface antigen staining and creatine kinase production, all measures of the efficacy of the sorting procedure (Hurko *et al.*, 1986).

Cysteamine treatment

Cystinotic myoblasts, grown to 60% confluence in 25 cm² plastic flasks, were fused into myotubes. At zero time, the growth medium was replaced with medium containing cysteamine (50–1000 μ M) (Sigma, St. Louis, MO, U.S.A.). The medium of control cultures (no cysteamine) was not changed. After 0, 10, 30 and 60 min

at 37 °C, duplicate cultures were rinsed twice with PBS, trypsin-treated, harvested and assayed for cystine and protein as described for muscle tissue.

Percoll-gradient separation of lysosomes

Cystinotic myotubes, grown in a 490 cm² rolling culture flask, were harvested, washed once in a 0.25 M-sucrose, and lysed in a nitrogen cavitation bomb (Kontes, Vineland, NJ, U.S.A.) at 207 kPa (30 lb/in²) for 10 min at 4 °C. A post-nuclear granular fraction was prepared by centrifugation at 17000 *g* for 10 min, resuspended in 0.25 M-sucrose/0.01 M-Hepes, pH 7.0, and applied to a self-generating 25% Percoll (Sigma) gradient prepared in this buffer, in a 38 ml centrifuge tube (Beckman; 25 mm $\times$ 89 mm), with a sample volume of 2 ml. The separation was performed in a vertical rotor (Vti 50) at 20000 rev./min for 1 h at 4 °C. The resulting gradients were fractionated in an ISCO tube fractionator, with Fluorinert (ISCO, Lincoln, NE, U.S.A.) as the displacing solution. The presence of lysosomes in the gradient fractions was determined by using β -hexosaminidase activity (Gahl *et al.*, 1982b) as a marker. The density profile was measured with marker beads (Pharmacia, Uppsala, Sweden).

RESULTS AND DISCUSSION

Cystine content of muscle tissue

Different portions of the same muscle biopsy contained vastly different amounts of cystine, whether determined on the basis of wet weight or protein content (Table 1). For all three specimens of cystinotic skeletal muscle, including those from two patients on long-term oral cysteamine, cystine contents were between 20 and 400 times the normal values, and ranged from 0.44 to 8.41 nmol of half-cystine/mg of protein (Table 1). A similar fold increase has been previously reported for cystinotic-muscle tissue (Broyer *et al.*, 1981).

Sorted cystinotic-muscle cells in culture

Muscle cells cultured from the biopsy of cystinosis patient 1 had endogenous cystine contents of 25.9, 35.5 and 44.4 nmol of half-cystine/mg of protein for three separate cultures. Normal control values are less than 0.35 nmol of half-cystine/mg of protein.

Table 1. Cystine content of muscle tissue from normal and cystinotic subjects

Multiple cystine assays were performed on different portions of the same biopsy specimen. Cystinotic patients 1 and 2 had received cysteamine for approx. 4 years at the time of biopsy.

Patient	Age (years)	Sex	Tissue source	Half-cystine (nmol/g wet wt.)	Half-cystine (nmol/mg of protein)
Normal	25	M	Biopsy	3.2 $\pm$ 0.7*	0.02 $\pm$ 0.005
Cystinotic 1	6	F	Biopsy	60	0.52
Cystinotic 2	7	F	Biopsy	80, 950	0.44, 7.07
Cystinotic 3	10	M	Autopsy	60, 270, 740	0.62, 2.21, 8.41

* Mean $\pm$ S.D. ($n = 5$), assayed by using the cystine-binding-protein method (Oshima *et al.*, 1974). A mean control value of 23 $\pm$ 3 nmol of half-cystine/g wet wt. ($n = 10$) was found by Broyer *et al.* (1981), by amino acid analysis. For the small amounts of cystine normally found in normal tissue samples, we have found the cystine-binding-protein method more reliable.

Fig. 1. Percoll-gradient fractionation of a granular fraction from cystinotic myotubes

A granular-fraction pellet was loaded on a Percoll self-generating density gradient. (a) Fractions were analysed for hexosaminidase activity (□) and cystine (■). (b) Fractions were analysed for protein (□) and density (----). The density profile was determined with density marker beads.

The cystinotic myoblasts grew normally in culture, doubling with normal frequency (24–36 h). The cells demonstrated the ability to undergo myogenic fusion in response to culture density and horse-serum supplementation. The fusion was complete within 7 days, as for normal cells.

Subcellular-fractionation experiments were performed to determine if cystine storage in the myotubes was within lysosomes, as for fibroblasts and leucocytes (Schneider *et al.*, 1968). The nuclear fraction contained 45% of the total cystine recovered, but was contaminated with the lysosomal marker β -hexosaminidase (40% of the total homogenate activity). The granular fraction contained 90 nmol of half-cystine (73 nmol/mg of protein), compared with only 37 nmol (12 nmol/mg of protein) in the cytosolic fraction. These fractions contained 44% and 17% of the total homogenate β -hexosaminidase activity respectively.

Percoll-gradient separation of the granular fractions was employed to verify the lysosomal location of the stored cystine. This technique gives separation of dense lysosomes from other, lighter, organelles (Rome *et al.*, 1979). For the myotube fractions, the distribution of

hexosaminidase suggested that most of the lysosomes band in the dense fraction (Fig. 1); approx. 50% of the total protein was separated from this peak. Most of the cystine sedimented with the hexosaminidase, whereas a minor proportion appeared in the top fractions of the gradient (4%) or spread through the light fractions of the gradient (11%). Other studies (G. S. Harper, unpublished work) suggest that for myotubes the light protein peak represents Golgi, mitochondria and plasma-membrane remnants.

Cysteamine depletion of cystinotic myotubes

Since intracellular accumulation of cystine occurred primarily within lysosomes, total cellular cystine content was used to measure the cystine-depleting efficacy of cysteamine in cystinotic myotubes. These cells were exposed to three concentrations of cysteamine for between 10 and 60 min at 37 °C (Fig. 2). After 60 min of exposure to cysteamine at concentrations of 250 and 1000 μ M, cystinotic myotubes were depleted of 60 and 86% of their intralysosomal cystine respectively. Cysteamine at a dose of 50 μ M was ineffective in depleting the cells of cystine.

Fig. 2. Cystine depletion of cystinotic myotubes by cysteamine

Cystinotic-myotube cultures were established in 25 cm² flasks. Cultures were exposed to medium containing 50 μ M-(○), 250 μ M-(△) or 1 mM-(□) cysteamine, for 0–60 min, and assayed for protein and cystine. Each point represents the mean of duplicate cultures.

From these investigations we conclude that: (a) mutant cells in culture expressed the basic biochemical defect of cystinosis, storing 100 times normal amounts of cystine in lysosomes; (b) intracellular cystine storage did not inhibit differentiation of myoblasts [a finding consistent with the apparently normal development and function of muscle in children with nephropathic cystinosis (Gahl, 1986)]; and (c) stored cystine was rapidly removed by cysteamine treatment ($t_{1/2}$ = 40 min at 250 μ M; Fig. 2). Cysteamine, which now serves as a drug of choice for the long-term treatment of nephropathic cystinosis, has been shown to retard renal glomerular deterioration and improve growth in affected children (Gahl *et al.*, 1986). Initially, its use was based on its depletion of cystine from cystinotic fibroblasts *in vitro* and leucocytes *in vivo* (Thoene *et al.*, 1976). Now these effects have been extended to cultured cystinotic renal cells (Pellett *et al.*, 1984), muscle cells (Fig. 2), and corneal cells (M. I. Kaiser-Kupfer & W. A. Gahl, unpublished work), which suggests the generalization that cysteamine can lower the cystine content of any cystinotic parenchymal cells to which the free thiol is adequately delivered.

We do not know why the muscle appears to be clinically spared in cystinosis, whereas other tissues deteriorate rapidly. The explanation probably does not lie in the amount of cystine stored in cystinotic muscle cells, which is 25–400 times normal. This range overlaps the fold increase reported for kidney (100–700 times normal), a severely affected tissue, as well as brain (2–70 times normal), a tissue considered to be unaffected in cystinosis (Schulman & Bradley, 1973). Rather, different tissues may have different susceptibilities to lysosomal cystine storage. For example, renal tubular cells may be

damaged in their reabsorptive function by cystine storage, whereas muscle cells can fuse normally in the presence of similar concentrations of lysosomal cystine. In addition, rates of cell turnover and the formation of cystine crystals in certain cells may influence whether a tissue is significantly affected by lysosomal cystine accumulation. Finally, differential tissue damage may be a temporal effect. Although it takes only months for renal tubules to be damaged in cystinosis, it takes 10 years for the destruction of renal glomeruli and thyroid cells, and it may take over 30 years for brain or muscle to manifest the adverse effects of long-standing cystine accumulation (Gahl, 1986).

The storage of cystine by cystinotic muscle cells emphasizes the similarities of lysosomal membrane function among different cell types. An operational cystine carrier system within the lysosomal membrane appears necessary to maintain cystine concentrations in the normal range in muscle cells, as for leucocytes and fibroblasts. Moreover, muscle cells apparently have a functional lysine-transport system in their lysosomal membranes, or else cysteamine might not deplete mutant cells of cystine. In fibroblasts, cysteamine reacts with cystine to form cysteine and cysteine–cysteamine mixed disulphide. The latter is recognized by a normal lysosomal lysine carrier in cystinotic cells (Pisoni *et al.*, 1985), and leaves the mutant lysosomes at a finite rate (Gahl *et al.*, 1985). The same sequence of events might be expected to occur in cystinotic muscle and kidney cells treated with cysteamine. Indeed, differences in cysteamine concentrations required to achieve equivalent extents of depletion may be related to differences among cell types in lysosomal lysine-carrier activity.

These investigations may represent the first time that cultured sorted muscle cells have been employed for the study of a lysosomal storage disorder. Although they have contributed substantially toward our understanding of cellular function in cystinosis, the full potential of the system is yet to be exploited. Different lysosomal disorders can be studied for the effect of storage on myoblast fusion and other functions, especially in diseases with clinical muscle involvement such as Type II glycogen-storage disease (Howell & Williams, 1983). It is even possible that fusion of normal myoblasts with myoblasts from lysosomal-disease patients would reveal whether individual lysosomal membranes fuse or remain distinct entities during this differentiation process.

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