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Receptor-Mediated Uptake of Acid α-Glucosidase Corrects Lysosomal Glycogen Storage in Cultured Skeletal Muscle

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ABSTRACT. Attempts at treatment of glycogenosis type II and other lysosomal storage disorders by enzyme replacement have been reported. Parenteral enzyme administration has been ineffectual. Treatment by bone marrow transplantation is currently under investigation. We have used cultured skeletal muscle cells from a patient with infantile glycogenosis type II to study fundamental aspects of enzyme replacement therapy. Efficient uptake of acid α-glucosidase was achieved by using the mannose-6-phosphate receptor on the cell surface as a target for an enzyme precursor with phosphorylated high-mannose types carbohydrate chains purified from human urine. We found that the enzyme was channeled to the lysosomes and converted to mature acid α-glucosidase. Glycogen storage was reversed. The results are discussed in relation to treatment of glycogenosis type II. (*Pediatr Res* 24: 90–94, 1988)

Abbreviation

IGF, insulin-like growth factor

Pompe's disease or glycogenosis type II is an autosomal recessive disorder (1, 2). Deficiency of acid α-glucosidase is the primary defect, leading to lysosomal accumulation of glycogen (3). Cardiomegaly, hypotonia, and moderate hepatomegaly are characteristic of the rapidly progressive infantile form of the disease. Death occurs in the first or second year of life as a result of cardiorespiratory failure (4). In late onset forms of the disease (juvenile and adult variants) impairment of skeletal muscle function is usually the only symptom (5). Patients more than 60 yr old have been described (6).

In the past, enzyme replacement has been attempted in various lysosomal storage disorders by parenteral administration of purified lysosomal enzymes (7, 8). Infantile Pompe patients received acid α-glucosidase purified from *Aspergillus niger* (9, 10) or human placenta (4). None of these attempts was clinically successful. At present, this form of therapy has been abandoned, and treatment of lysosomal storage disorders by bone marrow transplantation is currently under investigation (11–16). Some beneficial effects have been reported in the mucopolysaccharidoses. However, the few attempts at treatment of glycogenosis type II with bone marrow transplantation have not been successful (15, 16). No increase of acid α-glucosidase activity was found in muscle tissue. Elevated enzyme levels were only measured in blood cells, which in fact reflected the presence of donor cells.

The lack of an efficient treatment for lysosomal storage diseases in general and glycogenosis type II in particular has stimulated us to investigate fundamental aspects of receptor-mediated enzyme replacement therapy.

During the last decade much has been learned about the role of receptors as signal transducers, and as mediators of selective transport of macromolecular compounds. The mannose-6-phosphate receptor was recognized by its function in endocytosis of high-uptake forms of lysosomal enzymes in cultured fibroblasts (17, 18). Later it became evident that the receptor is predominantly localized intracellularly, and is mainly involved in selective transport of lysosomal enzymes from the Golgi complex to lysosomes (19). Various cell types appear to contain the receptor (20), but a systematic study of the expression of the mannose-6-phosphate receptor on the plasma membrane of different cell types has not been performed.

In a previous study, we have shown that mannose-6-phosphate receptors are present at the cell surface of myotubes and mediate efficient uptake of lysosomal enzymes containing carbohydrate chains with mannose-6-phosphate residues (21). However, the most important question remained unanswered: does exogenously supplied enzyme reach the glycogen storage vacuoles of skeletal muscle cells and does it degrade the accumulated glycogen. To answer this question cultured skeletal muscle cells from an infantile glycogenosis type II patient were used as a model system, and supplied with a high-uptake precursor of acid α-glucosidase purified from human urine.

METHODS

Cell culture procedures. Muscle cell cultures were obtained from an infantile glycogenosis type II patient by dissociation of a biopsy from the quadriceps muscle (0.1 g wet weight, Fig. 1) as described by Yasin *et al.* (22). Primary cultures were preplated once to select against fibroblasts (23). Dulbecco's modification of Eagle's medium supplemented with fetal calf serum (20%), chicken embryo extract (2%), and antibiotics were used as growth medium. An atmosphere of 10% CO_2 and 90% air was maintained. Experiments were performed in 24-well plastic tissue culture plates. At a density of 10^4 cells/cm^2 myoblast fusion was stimulated by a change of medium. The fusion medium consisted of Dulbecco's modification of Eagle's medium supplemented with horse serum (2%), in an atmosphere of 5% CO_2 and 95% air. In the next 4 days more than 90% of the mononuclear cells fused to form myotubes.

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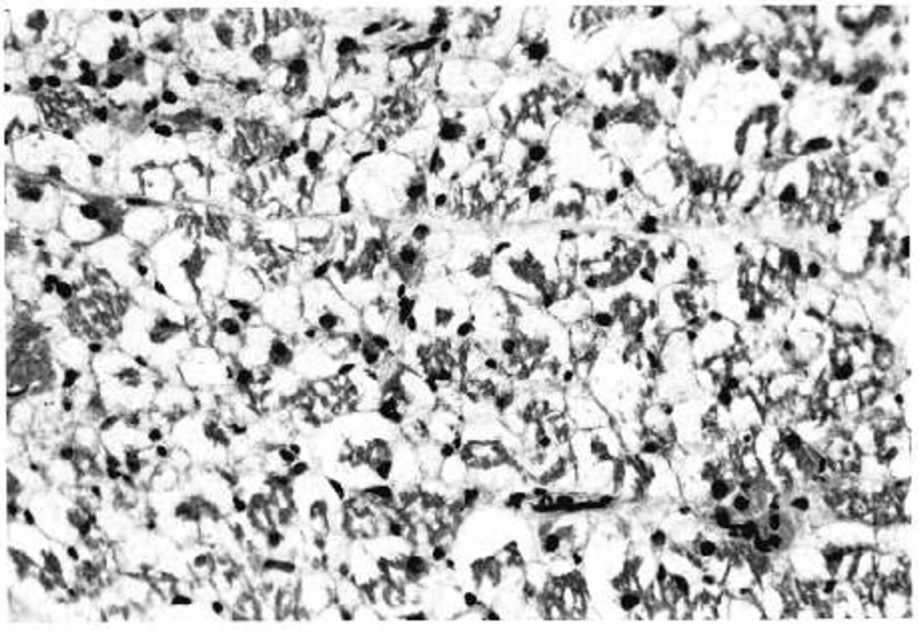

Fig. 1. Light micrograph of muscle tissue derived from the severely damaged quadriceps of the patient at 9 months of age (hematoxylin and eosin, × 630).

A high-uptake 110 kDa precursor of acid α-glucosidase (sp. act. 180.2 μmol 4-methylumbelliferone/mg protein/h) was purified from human urine as described by Oude Elferink *et al.* (24). Myotubes were incubated with enzyme in a final concentration of 1 μmol 4-methylumbelliferone/500 μl/h during 16 h as described previously (21).

Miscellaneous. Acid α-glucosidase activity was measured with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside or with glycogen (25, 26). Glycogen content of tissues, cells, and lysosomes was determined as described by Koster *et al.* (27). A crude lysosomal fraction was prepared by homogenizing muscle cells in a Potter-Elvejhem homogenizer at 1300 rpm in 250 mM mannitol with 10 mM EDTA and 20 mM imidazol, pH 7.4. Nuclei and intact cells were removed by centrifugation at 100 × *g*. Lysosomes were subsequently spun down from the supernatant at 1000 × *g* and washed once with homogenization buffer. Protein concentrations were determined according to Lowry *et al.* (28) and creatine kinase levels were measured as described previously (23). All assays were carried out in duplicate.

Immunocytochemistry was performed according to Van Dongen *et al.* (29), using mouse monoclonal antibodies (43G8) (30) raised against acid α-glucosidase and rabbit polyclonal antibodies against β-hexosaminidase (31). Immune complexes were visualized with goat anti-mouse IgG conjugated to a green fluorescent dye and goat anti-rabbit IgG conjugated to a red fluorescent dye, respectively.

Glycogen was demonstrated by transmission electron microscopy following the procedure described by De Bruijn (32).

Table 1. *Acid α-glucosidase activity**

Cells/tissue	Substrate: Glycogen	Substrate: 4-methylumbelliferyl-α-D-glucopyranoside
Leukocytes		
Patient	10	
Control range (n = 10)	70–220	
Muscle		
Patient	1.1	0.54
Control range (n = 10)	70–350	7–40
Cultured fibroblasts		
Patient		1.1
Control range (n = 10)		40–150
Cultured muscle cells		
Patient		0.83
Control range (n = 5)		50–120
Patient after enzyme uptake		150–180

* Activities are expressed as nmol glucose or methylumbelliferone/h/mg protein.

RESULTS

The clinical diagnosis of infantile glycogenosis type II was confirmed by deficiency of acid α-glucosidase activity in leukocytes, cultured skin fibroblasts, and muscle tissue. The same enzyme deficiency was demonstrated in cultured skeletal muscle cells derived from the patient (Table 1). Using immunocytochemistry, enzymatically inactive acid α-glucosidase was shown to be absent (Fig. 2*A*). However, when human urine acid α-glucosidase was applied to the deficient cells, a bright intracellular labeling pattern was observed after 16 h (Fig. 2*B*). The distribution of the fluorescent spots suggested compartmentalization in the lysosomes. Indeed, when a double labeling was performed for acid α-glucosidase (Fig. 2*C*) and β-hexosaminidase (as lysosomal marker) (Fig. 2*D*), exactly the same localization was obtained.

Subsequently, the effect of endocytosed acid α-glucosidase on the cellular glycogen content was determined. The experiments were performed with muscle cell cultures consisting of more than 90% myotubes. The high creatine kinase activity of the cells (1010–1024 mU/mg protein) was indicative of their advanced stage of differentiation. Uptake of enzyme, measured after 16 h, had been very efficient. Intracellular activity above the control range was achieved (Table 1; Fig. 3, day 1). The half-life of endocytosed acid α-glucosidase varied between 6 and 9 days in different experiments. The effect on the glycogen content of the

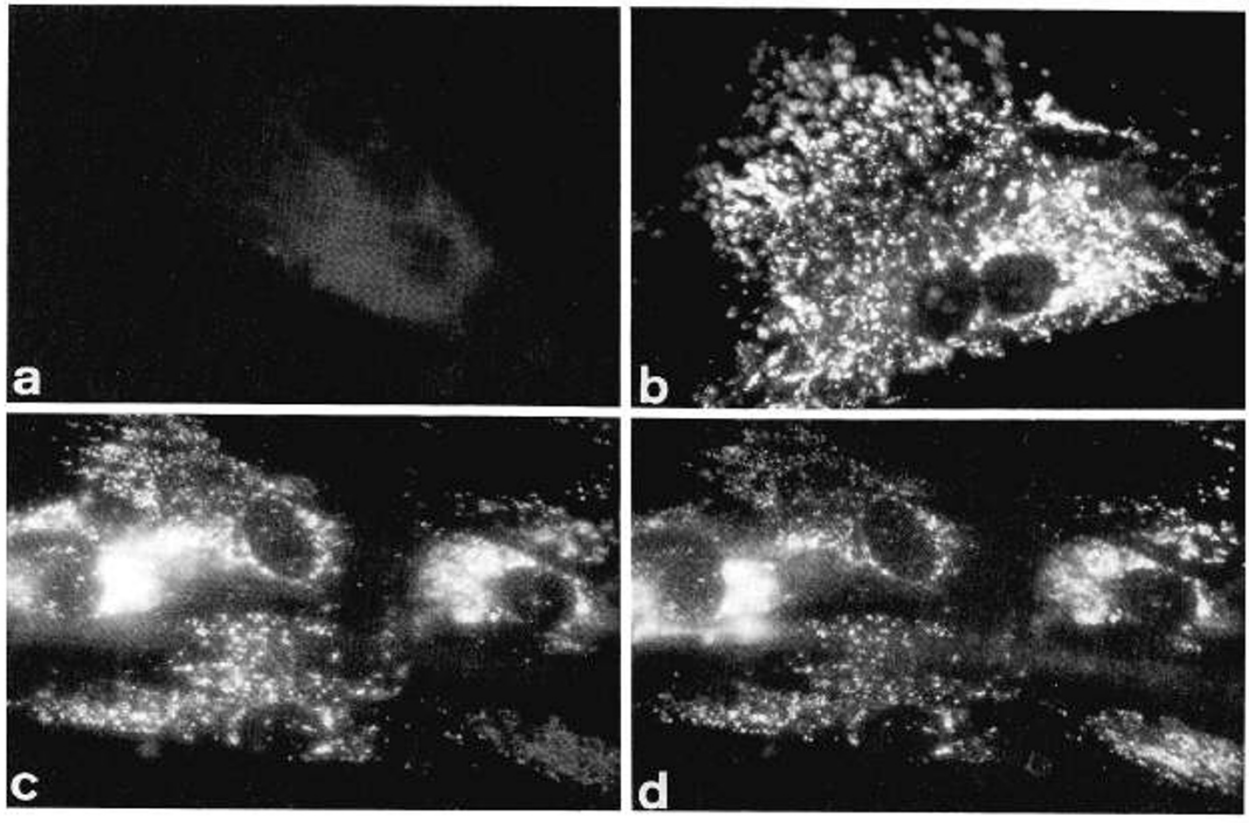

Fig. 2. Immunocytochemical demonstration of acid α-glucosidase of cultured muscle cells from the patient: *A*, multinuclear muscle cell before enzyme addition. *b*, binuclear cell 16 h after enzyme addition. *c*, intracellular localization of acid α-glucosidase after endocytosis. *d*, localization of β-hexosaminidase (as lysosomal marker) in the same cells as in *c*.

cells was most pronounced 6 days after enzyme addition, when the amount of cellular glycogen was approximately 33% reduced (see Fig. 3 for a representative experiment). In four independent experiments the reduction of cellular glycogen varied from 32–35%. In control cells no degradation of glycogen was measured after acid α-glucosidase addition.

Clearance of glycogen was examined by electron microscopy. A striking difference was observed between treated and untreated cultures inspected 6 days after enzyme addition (Fig. 4). Abundant accumulation of glycogen was seen in lysosomes of cells that did not obtain enzyme. In contrast, treated myotubes could not be distinguished from normal. Significant changes in the amount of cytoplasmic glycogen were not observed.

To further substantiate these observations, the distribution of glycogen over cytoplasm and lysosomes was quantitated. In control cells 18–24% of the total cellular glycogen was found in the lysosomal fraction, whereas in cultured muscle cells from the patient the lysosomal glycogen fraction was between 45 and 52% in various assays. Six days after enzyme addition the lysosomal glycogen content of cells from the patient had returned to control values.

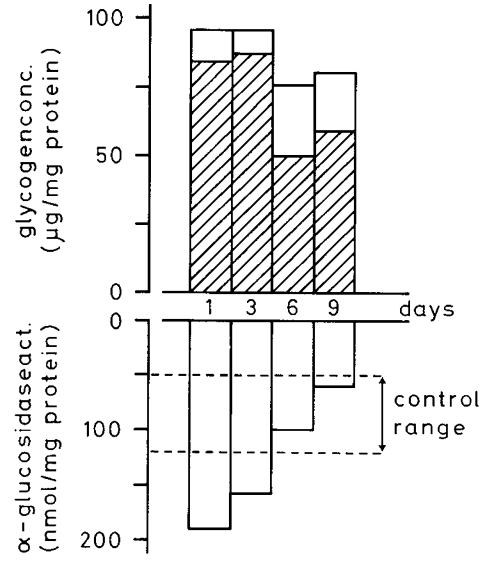

Fig. 3. Acid α-glucosidase activity of cultured myotubes after uptake of human urine enzyme (*open bars downward*). Cellular glycogen content without (*open bars upward*) and after enzyme addition (*hatched bars*). Assays of enzyme activity and glycogen content were carried out in duplicate wells. The average values of the duplicate experiments are indicated. The variation between duplicates was less than 4%.

DISCUSSION

Receptors with different specificities are essential mediators in a variety of cellular and biochemical processes. Two distinct receptors with specificity for the mannose-6-phosphate recognition marker of lysosomal enzymes have been characterized. A 215-kDa cation independent as well as a 46-kDa cation dependent mannose-6-phosphate receptors seem involved in intracellular transport of lysosomal enzymes (33–35). Endocytosis of exogenous lysosomal enzymes seems mainly ascribed to the 215-kDa receptor (36). Recently, it was shown that the latter receptor is identical to the IGF II receptor (37). The exact physiological function of the mannose-6-phosphate receptor as IGF II receptor is not yet understood. IGF II seems to function primarily in fetal and early neonatal development.

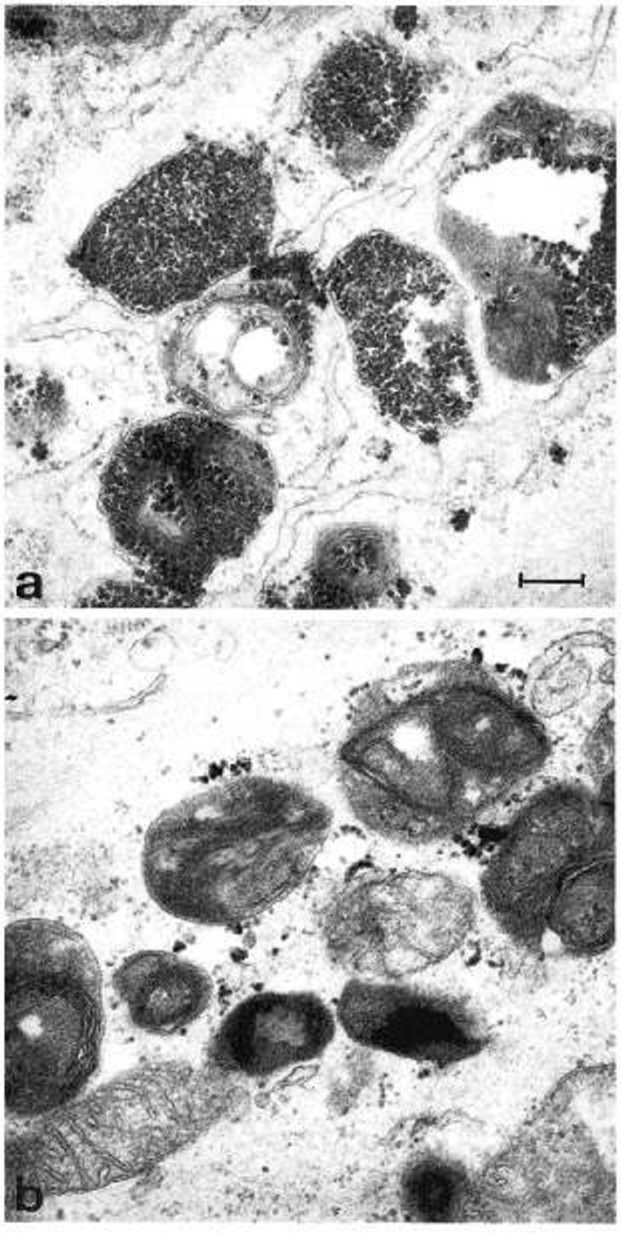

Fig. 4. Transmission electron micrographs: *A*, cultured muscle cells from the patient; *B*, a duplicate culture 6 days after treatment with human urine acid α-glucosidase. *Bar*, 2.5 μm.

Mannose-6-phosphate receptors are transiently present on the plasma membrane of a variety of cell types (17–19, 33, 38). We have previously shown that mannose-6-phosphate receptors on the surface of skeletal muscle cells can be used as targets for acid α-glucosidase species with a high mannose-6-phosphate content (21). The precursor of acid α-glucosidase, which is rather abundant in urine, has this desired characteristic (24, 39), and was used in our study as the enzyme source. After uptake by muscle cells the precursor was converted to fully active mature enzyme. This is concluded from the fact that endocytosed acid α-glucosidase can be visualized with monoclonal antibody 43G8, that only recognizes mature enzyme, and not the administered precursor (30, 40). In addition, the immunocytochemical labeling pattern demonstrates that all lysosomes are reached, and not a selected subset. Uptake of enzyme appeared very efficient. Activities above the control range were reached. Nine days after correction, the acid α-glucosidase activity was still at the lower limit of the control range. Clearance of glycogen from lysosomes, as assessed by transmission electron microscopy and quantitative assays, demonstrates that exogenously supplied acid α-glucosidase can functionally replace deficient endogenous enzyme. Our results suggest that the reported degradation of glycogen in cultured bovine muscle cells after enzyme addition also occurs in lysosomes (41). Thus, receptor-mediated enzyme replacement therapy for glycogenosis type II may be feasible, if high uptake forms of acid α-glucosidase would gain sufficient access to affected muscle tissue.

In the past, results obtained with administration of enzyme to patients with lysosomal storage disorders, and to animals, have been disappointing (7–10). Enzymes administered parenterally appeared to be captured predominantly by the Kupffer cells of the liver (42), and the initial enthusiasm about the applicability of enzyme replacement therapy subsided. However, with the present knowledge about low and high uptake forms of lysosomal enzymes and the occurrence of cell type-specific receptors (8, 17–19, 21, 33–39) it has to be concluded that in these trials the optimal conditions for enzyme replacement therapy were not used. In retrospect, the wrong species of acid α-glucosidase were administered in attempts to correct glycogenosis type II (4, 9, 10). Acid α-glucosidase purified from human placenta does not contain mannose-6-phosphate residues and is very poorly taken up by cultured muscle cells (21, 43). In contrast, mannose-6-phosphate-containing enzyme from urine is rapidly endocytosed by muscle cells. By supplying high uptake forms of acid α-glucosidase muscle cells may compete with macrophages for enzyme capture (38).

At present, the possibilities of bone marrow transplantation and cell and tissue transplantation are being explored (11–16, 44–47). The effectiveness of these procedures depends on transfer of donor enzyme to the affected organs and cell types. Our data also predict that these latter forms of therapy will be more efficient when the transplanted cells supply high-uptake forms of acid α-glucosidase to deficient muscle.

We have shown that cultured skeletal muscle from patients with glycogenosis type II is a suitable model system for fundamental studies on enzyme supplementation. Studies on animal models are in progress to test the *in vivo* feasibility of enzyme replacement therapy by infusion of high uptake forms of acid α-glucosidase. An important question will be whether the enzyme can cross the capillary wall to reach its ultimate goal. Suggestive evidence exists that various macromolecules are actively transported across endothelial cells via plasmalemmal vesicles (48–50).

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