Autophagy in Natural History and After ERT in Glycogenosis Type II

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Abstract We studied the role of autophagy in a series of 10 infantile-, juvenile-, and adult-onset GSDII patients and investigated autophagy blockade in successive biopsies of adult cases during disease natural history. We also correlated the autophagosome accumulation and efficiency of enzyme replacement therapy (ERT) in four treated cases (two infantile and two juvenile-adult onsets).

The autophagic flux was monitored by measuring the amount of p62-positive protein aggregates and compared, together with fibre vacuolisation, to fibre atrophy.

A blocked autophagic flux resulted in p62 accumulation, increased vacuolisation, and progressive atrophy of muscle fibres in biopsies collected from patients during natural history. On the contrary, in the GSDII cases early treated with ERT, the autophagic flux improved and muscle fibre atrophy, fibre vacuolisation, and acid phosphatase activity decreased.

The functionality of the autophagy-lysosome system is essential in GSDII muscle, which is characterised by the presence of swollen glycogen-filled lysosomes and autophagic build-up. Defining the role of autophagy and its relationship with muscle loss is critical for understanding the disease pathogenesis, for developing new therapies, and for improving ERT efficacy in GSDII.

Introduction

Glycogenosis type II (GSDII, MIM# 232300) is an autosomal-recessive disorder caused by the deficiency of the lysosomal enzyme acid α -glucosidase (GAA), which catalyses the hydrolysis of α -1,4 and α -1,6 links of glycogen (Angelini and Engel 1973; Hirschhorn and Reuser 2001; van der Ploeg and Reuser 2008). The enzyme deficiency leads to a spectrum of clinical phenotypes ranging from an infantile and rapidly fatal form (Pompe disease) to a slowly progressive late-onset form (Laforet et al. 2000). The slow progression of the disease and the variable organ involvement complicate the prognosis and the efficacy of therapy of adult GSDII cases. Untreated adult GSDII patients have a poor quality of life (Hagemans et al. 2004).

The first successful trials with enzyme replacement therapy (ERT) were done on selected infantile Pompe patients, because of the severity of the disease and the rather homogeneous natural course (Kishnani et al. 2007). Recombinant human α -glucosidase (rhGAA) treatment was demonstrated to be effective in reducing left ventricular mass and improving survival in infantile patients. In adults, treatment efficacy is variable (Angelini et al. 2012). We aim to describe morphological changes and autophagy biomarkers in muscle biopsies from both infantile and adult forms of GSDII.

Material and Methods

Patients

We selected ten patients (three Pompe, seven adult onset) with molecular diagnosis of GSDII (Table 1), who underwent clinical examination, muscle biopsy, measurement of

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Table 1 Clinical and genetic data of GSDII patients

Pt. No.	Biop. No.	Phenotype	ERT duration	Onset	Age at biopsy	Disease duration	Ventilator use	GAA gene mutations
1	8475	Infantile onset	—	3 months	7 months	4 months	—	c.2015G>A, p.R672Q, homozygote
2	8484 8946	Infantile onset	— 18 months	3 months	5 months 2 years	2 months 21 months	—	c.1564C>G, p.P522A, homozygote
3	8668	Infantile onset	17 months	2 weeks	18 months	18 months	—	c.1933G>A, p.D645N, homozygote
4	8557 8868	Juvenile onset	— 6 months	18 years	18 years 19 years	0 1 years	—	c.546+1G>T; n.d.
5	4512 7786	Adult onset	—	40 years	56 years 65 years	16 years 25 years	—	c.-32-13T>G; c.307T>C, p.C103G
6	5638 7774	Adult onset	—	34 years	54 years 60 years	20 years 26 years	50 years	c.-32-13T>G; c.2219delTG, p.V740fsX55
7	5639 7797	Adult onset	—	41 years	42 years 48 years	1 years 7 years	—	c.-32-13T>G; c.546+1G>T
8	6373	Adult onset	—	27 years	28 years	1 years	—	c.-32-13T>G; c.2066_2070duplAGCCG, p.A691fsX6
9	6374	Adult onset	—	32 years	32 years	0	—	c.-32-13T>G; c.2066_2070duplAGCCG, p.A691fsX6
10	7340 9045	Adult onset	— 36 months	50 years	59 years 64 years	9 years 14 years	59 years	c.-32-13T>G; c.2530_2541del, p.A844_L847del

GAA activity in muscle or lymphocytes, and identification of mutations in the *GAA* gene (Nascimbeni et al. 2008). Of the ten patients studied (Table 1), four cases (two Pompe, two adult onset) underwent ERT treatment before the only (patient 3) or a second muscle biopsy was obtained (patients 2, 4, 10), and three untreated adult-onset cases (patients 5, 6, 7) underwent two biopsies during the natural course of the disease.

Muscle Morphology, Immunohistochemistry, and Immunoblot

Muscle biopsy sections were stained using a panel of ten different routine stains (including the lysosomal acid phosphatase), to analyse fibre size and the degree of vacuolisation. We studied muscle autophagy markers and atrophy at different times of the disease progression. For immunohistochemical analysis, muscle sections were incubated with primary antibodies against caveolin-3, p62, LC3, according to methods previously reported (Nascimbeni et al. 2008, 2012). The percentage of vacuolated fibres and p62-positive fibres was defined as those presenting either diffuse or scattered intracytoplasmic vacuoles or staining, respectively. Fibre cross-sectional area (CSA) was measured in at least 200 fibres in each biopsy using ImageJ software.

Results

Patients in Natural History (Table 1)

Patient 1. This child was born from consanguineous parents and has a first-degree cousin also affected. At 3 months, he presented delayed psychomotor development, high CK (1,260 U/L), axial and limb hypotonia, and poor motility. At age 7 months, he underwent muscle biopsy and molecular diagnosis was obtained. *Patient 8.* This 28-year-old man was the brother of case 9. He had stepping gait and proximal weakness. CK was 3,318 U/L and muscle CT scan showed atrophy in paraspinal and gluteus muscles. Spirometry showed moderate respiratory insufficiency. Muscle GAA activity was 15%. *Patient 9.* This 32-year-old woman had myalgia and elevated CK (825 U/L). Muscle biopsy showed a vacuolar myopathy and GAA activity was 17%. Spirometry revealed slight respiratory insufficiency, and muscle CT showed moderate atrophy of gluteus.

Patients in ERT Treatment (Table 1)

Patient 2. This female child at 5 months had severe hypotonia, growth retardation, hepatomegaly, and cardiomegaly. Echocardiography showed cardiac hypertrophy and

wall motion abnormalities. CK was 575 U/L. Muscle biopsy showed massive vacuolisation (Fig. 1), and GAA gene mutations were identified. She was ERT treated (20 mg/kg every 3 weeks) only after 8 months. At age 2 years, following *aspiration* pneumonia, she was not able to sit unassisted. A second muscle biopsy at age 2 years showed marked vacuolisation and increased fibrosis (Fig. 2). This patient was a nonresponder to ERT treatment.

Patient 3. This female child was born at 41 g.w. by caesarean section due to foetal bradycardia. Echocardiography showed bilateral ventricular hypertrophy. CK was elevated. EMG showed myotonic discharges. High levels of urinary Glc4 were found. GAA activity in lymphocytes was reduced. Mild hypotonia and macroglossia were noted. ERT (20 mg/kg every 3 weeks) was started at 20 days of life. Gradual improvement in left ventricular hypertrophy was observed. At 18th month follow-up, a muscle biopsy showed relatively mild myopathic changes with 10% of vacuolated fibres. At 2 years, echocardiography was normal as well as psychomotor development, except for a mild speech delay.

Patient 4. This 17-year-old boy complained of generalised asthenia and cramps after intense exercise, but was virtually asymptomatic (except for MRC in iliopsoas 4/5). CK was 1,012 U/L. Spirometry showed FVC = 91%, FEV1 = 83%, and pO₂ = 93%. Echocardiography showed mild left ventricular and septal hypertrophy, and ejection fraction was 49.7 %. Muscle biopsy showed glycogen storage with 50% vacuolated fibres (Fig. 1). Muscle GAA was 20%. He had a second biopsy after 6 months of ERT treatment, showing decreased vacuoles and acid phosphatase (Fig. 1). The patient improved after ERT, presenting less fatigue and increased 6-minute walking test. He is a good ERT responder.

Patient 5. This 40-year-old woman complained of progressive difficulty in climbing stairs, walking, and rising from the floor. At 43 year, she walked with a cane and had marked weakness of iliopsoas and quadriceps muscles and hypotrophy of thighs. ECG was normal. Spirometry showed a slightly restrictive pattern with FVC = 79%. Muscle CT scan showed hypertrophy of gluteus, quadriceps, and posterior thigh muscles. Muscle biopsy at age 56 showed 1% of vacuolated fibres (Fig. 2). Muscle GAA activity was 5%. A second biopsy at age 65 was done, and then, ERT treatment was started. Her 6-minute walking test improved from 80 to 160 m. FVC was 1.8 and 1.6 L after 6 months (Angelini et al. 2009). After 4 years of ERT, she still presented a stable condition.

Patient 6. This woman had difficulty walking, climbing stairs, and rising from the floor since 34 years of age. Since 50 years, she had progressive respiratory insufficiency and FVC decreased 13% every year. She then used overnight oxygen and had dyspnea at rest. At age 54, a first muscle biopsy showed 20% vacuolated fibres (Fig. 2). Muscle GAA activity was 7%.

Muscle CT scan showed marked proximal atrophy in posterior thigh muscles. At age 60, a second biopsy was done. Then, ERT was started. At age 64, she had lordotic gait, was able to climb stairs and to rise from a chair, but could not lift legs. MRC on deltoid was 3/5, on triceps 4/5, external shoulder rotators 3/5, and iliopsoas and quadriceps 3/5. Her 6-minute walking test was 120 m before ERT, 160 m. after 6 months ERT, and returned to 110 m after 12 months. FVC was initially 0.5 L and remained stable after 12 months ERT (Angelini et al. 2009).

Patient 7. This 43-year-old woman, since 7 years complained of weakness, could rise from the floor only using two hands and climb stairs only using the rail. A first muscle biopsy showed 3% of vacuolated fibres (Fig. 2). Muscle GAA activity was 12%. A second muscle biopsy was done at age 48 before ERT was started. She had an allergic reaction during ERT treatment, consisting of erythematous thoracic rash, and had to discontinue ERT.

Patient 10. This 59-year-old woman had onset at age 50, with weight loss and muscle weakness. She needed ventilator since 59 years of age, when she underwent the first muscle biopsy that showed 5% of fibres with vacuoles. Muscle GAA activity was virtually absent. At age 64 years, a second muscle biopsy was done after 36 months of ERT treatment, showing decreased vacuoles and acid phosphatase reaction (Fig. 1). ERT resulted in stabilisation of motor functions, and only nocturnal ventilator support was required (Vianello et al. 2013).

Disease Progression and Autophagy Impairment

Infantile and late-onset patients have different levels of autophagic flux and accumulation of p62-positive protein aggregates. Infantile patients show impaired autophagy, whereas late-onset patients display a correlation between autophagic block and muscle atrophy with disease progression (Fig. 2). The comparison of three adult patients who underwent two biopsies during natural history (Table 1, Fig. 2) showed a different degree of autophagy impairment as revealed by accumulation of p62 protein aggregates. Cross-sectional area (CSA) decreased in all patients in the second biopsy. Patient 7 showed a time-dependent increase of abnormalities: in the first biopsy, she had an almost normal fibre morphology, and in the second biopsy, there was a 60% decrease in CSA, which correlated strikingly with the increase in p62-positive aggregate and vacuolated fibres (from 2 to 40%) (Fig. 2).

Early ERT Treatment Restores Autophagy

One infantile patient (patient 2) and two adult patients (patient 4, 10) were ERT treated for 16, 6, and 36 months, respectively, and after treatment, a second biopsy was taken.

Fig. 1 Comparison of pre-ERT (**a**, **c**, **e**, **g**) and post-ERT muscle biopsies (**b**, **d**, **f**, **h**) stained for acid phosphatase (**a–d**) and

immunolabelled for p62 protein aggregates (**e–h**) in adult early-treated patient 4 (**a**, **b**, **g**, **h**), in late-onset patient 10 (**c**, **d**), and in

The juvenile patient 4 showed a low level of mature GAA that increased after ERT. ERT treatment in this patient greatly reduced the number of p62-positive and vacuolated fibres from 60 to 34% and from 44 to 35%, respectively (Fig. 1), which remained atrophic. MRC of iliopsoas muscle increased from 4 to 5 and his 6-minute walking test also improved. The adult patient 10, after ERT, had less degree of fibre vacuolisation and acid phosphatase reaction (Fig. 1). Consistently, with morphology, therapy in adult patients resulted in less fatigue and better exercise performance.

The infant patient 2 started therapy at 8 months old, an age probably too late for successful recombinant GAA uptake. Indeed, the second biopsy suggested a persistent autophagic impairment despite ERT treatment: p62-positive aggregate fibres increased from 70 to 98%, while vacuolated fibres were 94% before ERT and 98% after ERT (Fig. 1). This patient had no detectable GAA mature forms and instead elevated levels of the inactive 110 kDa precursor (Nascimbeni et al. 2012). ERT induced the appearance of mature 76 kDa GAA even if at very low level. The p62-positive aggregate accumulation and number of vacuolated fibres were increased, possibly contributing to the poor ERT response.

Another early-treated infantile patient (patient 3), who underwent only one biopsy after 18 months of ERT, showed normal CSA value and only few fibres with p62 aggregates (6%) or vacuoles (3%). Also in this case, we can conclude that early treatment, prior to massive autophagy-build-up onset, is effective in restoring the autophagy flux.

Discussion

Autophagy is a highly conserved homeostatic process for lysosome-mediated degradation of cytoplasmic components, including damaged mitochondria and toxic protein aggregates. In infantile Pompe patients, the enormous build-up of glycogen-filled lysosomes appears to cause the muscle damage (Raben et al. 2007). Recent studies showed that autophagy impairment contributes to both disease progression and fibre atrophy (Nascimbeni et al. 2012). A residually functional autophagic flux is important for an efficient ERT delivery in muscle lysosomes, since mature GAA forms were found in our ERT-responsive juvenile case. The maturation steps of GAA, from the synthesis of the immature protein in the endoplasmic

reticulum to the final cleaved active protein in lysosomes, are complex and require a functional system of vesicle trafficking.

A limited number of ERT trials and observational studies have been published on late-onset patients (van der Ploeg 2010; Strothotte et al. 2010; Angelini et al. 2012; Regnery et al. 2012). An observational study (Deroma et al. 2014) in eight juvenile GSDII cases reported marked decrease of CK after ERT and stabilisation of disease course. According to treating clinicians, not all patients respond to therapy to the same extent and in the same time frame (Angelini et al. 2012; Strothotte et al. 2010; Regnery et al. 2012). A number of prognostic factors were observed or suspected, including patient gender, age, body composition, genotype, disease duration, and clinical conditions. Good clinical condition and short disease duration seem to be the most important predictors of good response (Angelini et al. 2012; Regnery et al. 2012; Deroma et al. 2014). The majority of infantile patients in whom ERT was started before the age of 6 months and before the need of ventilator support improved and showed longer ventilator-independent survival, reduced cardiac mass, and acquisition of motor skills. Infants who started treatment before the occurrence of extensive muscle damage had better motor outcomes than patients who began treatment at more advanced stages. The same parameters seem to apply to early-treated juvenile cases, where often the main feature is hyperCKemia. At the same time, ventilatory-dependent patients fail to achieve independence from ventilation. Effectiveness of ERT seems to be more limited in advanced cases, even if clinical stabilisation in motor and respiratory function was observed in some advanced cases (Regnery et al. 2012).

The muscle structure is more severely affected in the infantile form, whereas the degree of vacuolisation is variable in late-onset patients. A further difference between infantile and late-onset form is vacuolar compartmentalisation by membranes with sarcolemmal proteins, as shown by caveolin-3 staining (Nascimbeni et al. 2008); this additional feature could be important in determining response to ERT. An important pathological feature is failure of autophagosomal turnover and massive autophagic build-up in fibres. This contributes to myofiber atrophy that increases during natural history in late-onset GSDII (Nascimbeni et al. 2012).

Our data suggest that functional autophagy might protect myofiber from disease progression and muscle atrophy in

Fig. 1 (continued) late-treated infantile patient 2 (e, f). The early ERT treatment resulted in a reduction of fibre vacuolisation and lysosomal acid phosphatase reaction, suggesting a lower degree of lysosomal impairment. When started early, before autophagy block onset, ERT is

efficient and restores the autophagy block, as evidenced by a reduced number of p62-positive fibres in patient 4, but not in patient 2. Bar = 40 μ m

Fig. 2 H&E staining (a, c, e) shows a different degree of muscle fibre vacuolisation in different forms of GSDII. The majority of fibres are vacuolated in the infantile patient 2 (a), whereas vacuolated fibres correspond to atrophic fibres in patient 6 (c). Muscle biopsy shows mild myopathic changes, consisting of central nuclei and atrophic fibres in patient 5 (e). The immunohistochemical analysis of p62-positive aggregates (b, d, f) showed that the majority of fibres present p62 aggregates in infantile patient 2 (b) and that most of p62-positive fibres are atrophic and vacuolated in adult patients 6 (d) and 5 (f). The comparison of fibre cross-sectional areas between p62-positive and negative fibres and between vacuolated and non-vacuolated fibres in the infantile-onset patient 2 (g) and adult-onset patients 5 (h), 6 (i), and 7 (j) showed that vacuolated/engulfed fibres display autophagy-related atrophy, suggesting a key role of impaired autophagy and subsequent autophagosomes accumulation in myofibrillar disorganisation and alteration of endocytic trafficking

late-onset patients. The problem of autophagy and fibre atrophy remains to be addressed. Non-pharmacological therapies and drug therapies promoting cellular clearance (or preventing autophagic build-up) might be a future strategy in GSDII management.

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Synopsis

Muscle fibre atrophy and autophagy are linked in the progression of glycogenosis type 2; ERT might reverse such features.

Compliance with Ethics Guidelines

Conflict of Interest

Dr. Corrado Angelini has received compensation from Genzyme European Registry.

Dr. Anna C. Nascimbeni declares that she has no conflict of interest.

Dr. Marina Fanin declares that she has no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (University of Padova, Italy) and with the Helsinki Declaration of 1975 and revised in 2000.

Informed consent was obtained from all patients (or their parents) for being included in the study.

Contribution of Individual Authors

Drs. Angelini, Nascimbeni, and Fanin have contributed pertinent aspects of the planning, conduct, and reporting of the work described in the article.

References

- Angelini C, Engel AG (1973) Subcellular distribution of acid and neutral alpha-glucosidases in normal, acid maltase deficient, and myophosphorylase deficient human skeletal muscle. *Arch Biochem Biophys* 156:350–355
- Angelini C, Semplicini C, Tonin P, Filosto M, Pegoraro E, Soraru G, Fanin M (2009) Progress in enzyme replacement therapy in glycogen storage disease type II. *Ther Adv Neurol Disord* 2(3):143–153
- Angelini C, Semplicini C, Ravaglia S et al (2012) Observational clinical study in juvenile-adult glycogenosis type 2 patients undergoing enzyme replacement treatment for up to 4 years. *J Neurol* 259:952–958
- Deroma L, Guerra M, Sechi A et al (2014) Enzyme replacement therapy in juvenile glycogenosis type II: a longitudinal study. *Eur J Pediatr* 173(6):805–813
- Hagemans MLC, Janssens ACJW, Winkel LPF et al (2004) Late-onset Pompe disease primarily affects quality of life in physical health domains. *Neurology* 63:1688–1692
- Hirschhorn R, Reuser AJ (2001) Glycogen storage disease type II: acid alpha glucosidase (acid maltase) deficiency. In: Scriver BA, Sly W, Valle D (eds) *The metabolic and molecular bases of inherited disease*. McGraw-Hill, New York, pp 3389–3420
- Kishnani PS, Corzo D, Nicolino M et al (2007) Recombinant human acid [alpha]-glucosidase: major clinical benefits in infantile-onset Pompe disease. *Neurology* 68:99–109
- Laforet P, Nicolino M, Eymard PB et al (2000) Juvenile and adult onset acid maltase deficiency in France: genotype-phenotype correlation. *Neurology* 55:1122–1128
- Nascimbeni AC, Fanin M, Tasca E, Angelini C (2008) Molecular pathology and enzyme processing in various phenotypes of acid maltase deficiency. *Neurology* 70:617–626
- Nascimbeni AC, Fanin M, Masiero E, Angelini C, Sandri M (2012) The role of autophagy in the pathogenesis of glycogen storage disease type II (GSDII). *Cell Death Differ* 19:1698–1708
- Raben N, Takikita S, Pittis MG et al (2007) Deconstructing Pompe disease by analyzing single muscle fibers: to see a world in a grain of sand. *Autophagy* 3:546–552
- Regnery C, Kornblum C, Hanisch F et al (2012) 36 Months observational clinical study of 38 adult Pompe disease patients under alglucosidase alpha enzyme replacement therapy. *J Inher Metab Dis* 35:837–845
- Strothotte S, Strigl-Pill N, Grunert B et al (2010) Enzyme replacement therapy with alglucosidase alpha in 44 patients with late onset glycogen storage disease type 2: 12-month results of an observational clinical trial. *J Neurol* 257:91–97
- van der Ploeg AT (2010) Where do we stand in enzyme replacement therapy in Pompe's disease? *Neuromusc Disord* 20:773–774
- van der Ploeg AT, Reuser AJ (2008) Pompe's disease. *Lancet* 372:1342–1353
- Vianello A, Semplicini C, Paladini L et al (2013) Enzyme replacement therapy improve respiratory outcomes in patients with late-onset type II glycogenosis and high ventilator dependency. *Lung* 191:537–544