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Quantification of muscle pathology in infantile Pompe disease

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Abstract

The effects of enzyme replacement therapy (ERT) in infantile Pompe disease are variable, necessitating the identification of biomarkers to assess the severity of disease and response to ERT. The aims of this study were to investigate whether quantification of muscle pathology in infantile Pompe disease prior to and during ERT is feasible at the light microscope, and to develop a score that summarizes the degree of muscle pathology in a comprehensive manner from PAS-stained resin sections alone. We, therefore, determined glycogen load, extent of muscle fibre disruption, and amount of autophagic vacuoles in resin-embedded muscle biopsy specimens from 11 infantile Pompe patients and 2 with early childhood phenotype by quantitative methods, correlated the findings with ultrastructural analyses, compared PAS-stained resin sections with conventional PAS-stained cryosections, and related the quantified degree of muscle damage from infantile patients to the effects of ERT. Comparison of electron and light microscopic findings demonstrated that important alterations of skeletal muscle morphology can also be depicted by examining PAS stained resin sections. Infantile patients with good response to ERT had lower muscle pathology score values prior to and during ERT than those with moderate and poor response, but the number of tissue samples available for evaluation was limited. These findings suggest that quantification of muscle pathology by analysing PAS stained resin sections is in principle feasible and useful to monitor disease progression and therapy response. These results have to be validated by investigating a larger group of patients.

Keywords: Pompe disease; Glycogen storage disease type II; Enzyme replacement therapy; Lysosomal storage disease; Acid maltase deficiency; Autophagy

1. Introduction

In Pompe disease (glycogen storage disease type 2) deficiency of the lysosomal enzyme acid α-glucosidase (GAA) leads to pathological glycogen accumulation in various tissues of the body, especially in muscles [1]. Based on age at manifestation and residual enzyme activity, different types can be distinguished. The infantile type is characterized by GAA activities <1% in cultured fibroblasts and muscle tissue, and usually presents with hypertrophic cardiomyopathy and skeletal muscle weakness within the first six months of life [1]. Most patients die within the first year of life without achieving any motor milestone such as turning, sitting or walking. The childhood type manifests as progressive skeletal myopathy without cardiac involvement from birth till school-age; while the juvenile and adult types set in later [1,2].

In infantile Pompe disease enzyme replacement therapy (ERT) with recombinant human GAA (rh-GAA) improves survival, cardiac and respiratory function, and motor development, but the success of ERT is variable [3–7]. While some subjects achieve independent ambulation, others show only minor gain of motor functions [8,9]. Factors known to negatively influence outcome are late start of ERT, complete absence of any native enzyme, referred to as negative cross immunological material (CRIM) status, and

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high rhGAA antibody titers [10–12]. However, the mechanisms why some patients respond better than others are not fully understood. Therefore, additional biomarkers assessing severity of disease and response to therapy are warranted [13].

Several studies [14–17] have shown that muscle biopsy analysis is principally feasible to monitor changes in skeletal muscle pathology in infantile Pompe patients, and that glycogen clearance and ultrastructural damage correlate with clinical outcome. However, the application of this technique in the diagnostic routine is limited as the analysis by electron microscopy is cumbersome and time consuming. In addition, glycogen accumulation and other pathological alterations such as extent of myofibrillar structure disruption or amount of non-glycogen-filled vacuoles have so far not been routinely assessed in detail.

The aims of this study were to investigate whether detailed assessment and quantification of muscle pathology is feasible at light microscopy, and to develop a score, that summarizes the degree of muscle pathology in a standardized, reproducible, and comprehensive manner from PAS-stained resin sections alone. A further goal was to explore whether quantitatively assessing muscle damage by this technique is principally useful in monitoring disease progression or response to ERT. We, therefore, determined glycogen load, extent of muscle fibre disruption, and amount of autophagic vacuoles in resin-embedded muscle biopsy specimens from a smaller cohort of infantile Pompe patients, correlated the findings with ultrastructural analyses, compared PAS-stained resin sections with conventional PAS-stained cryosections, and related the quantified degree of muscle damage to the effects of ERT.

2. Patients and methods

2.1. Ethical statement

This work was approved by the ethical committee of the University of Giessen, and written informed consent was obtained from all parents of the patients.

2.2. Patients

15 muscle biopsies from 11 children with infantile Pompe disease confirmed by significantly reduced GAA activities in fibroblasts or lymphocytes, and by detection of GAA mutations in both alleles were retrospectively analysed (Table 1). Age at biopsy ranged from 1.5 to 136 months. Four patients had two biopsies (P1, P2, P3, P11). Eight tissue samples were obtained prior to start (P1a, P2a, P3a, P5, P6, P7, P9, P11) and 7 were taken during ERT (P1b, P2b, P3b, P4, P8, P10, P11b). All Pompe patients were treated with 20 mg/kg rh-GAA every other week. The clinical effects of ERT on motor function were classified as good response (R1) if subjects achieved independent walking, as moderate (R2) if they reached sitting without support or as poor (R3) if they did not gain any motor milestone. In addition, we analysed 4 biopsies from 2 patients with early-childhood onset Pompe disease (P12a, P12b, P12c, P13). 6 children (range 5–114 months) with non-specific muscular hypotonia and/or motor developmental delay, and normal GAA activity served as further controls (P14-P19).

2.3. Muscle biopsies

Open muscle biopsies were taken (Table 1) and processed according to standard procedures [18]. Biopsies were performed either to establish diagnosis or during surgical interventions such as port catheter implantation or gastric tube insertion. Small biopsies were fixed with 6% glutaraldehyde/0.4M phosphate buffered saline (PBS) and were processed with a Leica EM TP tissue processor. From resin embedded tissue 1–2 µm thin sections were stained with 2% p-phenylenediamine (PPD) and a Periodic acid-Schiff (PAS). In brief, for PAS staining, the sections were first stained with a methylene blue-azur II solution (Richardson's solution), treated with 5% periodic acid and incubated with Schiff's reagent [15]. For electron microscopy the ultrathin sections were contrasted with 3% lead citrate-3H2O with a Leica EM AC20 (ultrostain kit II) and were examined with a Zeiss EM 109 transmission electron microscope. 6 µm cryosections were stained with H&E and PAS according to the standard procedure [18].

2.4. Visualisation of muscle pathology by ultrastructural analysis and PAS stained resin sections

First, electron microscopic sections were evaluated in order to explore which morphological alterations were present in the patient cohort. Next, we evaluated PAS stained resin sections and analysed which abnormalities identified by electron microscopy could also be visualized at the light microscopic level.

2.5. Muscle pathology in PAS stained resin sections and cryosections

To investigate whether PAS staining of resin sections is better suited to visualize muscle fibre damage and glycogen load than PAS stained cryosections, we compared such stainings from two biopsies, one with severe and one with mild pathology.

2.6. Quantification of muscle pathology

2.6.1. Pompe muscle pathology score (PMPS)

PAS stained resin sections were analysed with an Olympus BX 51 microscope (Olympus, Hamburg, Germany) equipped with a motorized stage (Ludl Electronic Products, Hawthorne (NY), USA), a digital camera and a computer with the stereology software newCAST (Visiopharm, Horsholm, Denmark). The sections were delineated using a mask tool at a magnification of ×1.25. Fields of view for morphometric analysis were generated in a systematic uniform random way automatically by the software. In general, 100% of the sample area was investigated at a magnification of x400. To determine the fractions of the different grades of muscle fibre pathology (0–5) a point grid consisting of 9×9 crosses was projected on each field of view, where a point is the intersection of the vertical and the horizontal line of a cross. The points hitting a specific muscle pathology grade were counted and the fraction of each grade was calculated for every section [19].