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Proteomic analysis of dystrophin deficiency and associated changes in the aged *mdx-4cv* heart model of dystrophinopathy-related cardiomyopathy

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ABSTRACT

Cardiomyopathy is a serious complication in Duchenne muscular dystrophy, an X-linked neuromuscular disease of childhood that is triggered by primary abnormalities in the dystrophin gene. In order to directly correlate the deficiency in the membrane cytoskeletal protein dystrophin to secondary abnormalities in the dystrophic heart, this study has used label-free mass spectrometry to compare protein expression patterns in the aged *mdx-4cv* heart model of dystrophinopathy versus wild type heart. This report is the first successful identification of members of the cardiac dystrophin–glycoprotein complex by comparative whole tissue proteomics. The mass spectrometric analysis confirmed the loss of dystrophin and concomitant reduction of syntrophin and sarcoglycans in the dystrophin-deficient heart. Proteomic profiling of secondary changes identified distinct alterations in the basal lamina component laminin, the Ca^{2+}-binding protein sarcalumenin, the matricellular protein periostin, the proteoglycans asporin and lumican, the cardiac-specific myosin light chain kinase, heat shock proteins and a large number of mitochondrial and glycolytic enzymes. The proteomic findings indicate that the molecular pathogenesis of muscular dystrophy-associated cardiomyopathy is highly complex and involves impairments, modulations and/or adaptations of mitochondrial metabolism, glycolysis, protein chaperoning and ion homeostasis, as well as the maintenance of the contractile apparatus, the intracellular cytoskeleton and the extracellular matrisome.

Significance: The X-linked inherited disorder Duchenne muscular dystrophy is the most frequently inherited neuromuscular disease of childhood. Primary abnormalities in the dystrophin gene trigger progressive skeletal muscle wasting and impaired cardiorespiratory functions. In order to improve our general understanding of the molecular pathogenesis of muscular dystrophy-associated cardiomyopathy and to identify new marker candidates of cardiac changes in dystrophinopathy, we have carried out a comparative proteomic study of the *mdx-4cv* mouse model of Duchenne muscular dystrophy. The mass spectrometric profiling of whole heart preparations has identified the reduction in the dystrophin–glycoprotein complex and a large variety of secondary changes in the dystrophic heart. Cardiac proteins with a changed abundance were shown to be involved in fibre contraction, energy metabolism, cellular signalling, the cytoskeletal network, the extracellular matrix and the stress response. In the future, the newly identified cardiac proteins may be useful to improve predictive, diagnostic, prognostic or therapy-monitoring approaches in the field of muscular dystrophy and cardiomyopathy.

1. Introduction

Cardiorespiratory dysfunction is a common clinical manifestation in Duchenne muscular dystrophy [1–3], the most frequently inherited neuromuscular disorder of childhood [4]. Dystrophinopathies are caused by primary abnormalities in the dystrophin gene that result in the almost complete absence of the full-length dystrophin isoform Dp427 in muscle fibres [5]. Proximal skeletal muscle weakness is usually seen at 3 to 5 years of age in patients suffering from X-linked muscular dystrophy and the highly progressive pathogenesis causes a loss of ambulation by 12 years of age [6–8]. In most dystrophic children, the age of onset of abnormal systolic function varies considerably, but overt cardiomyopathy drastically increases age [9]. The majority of Duchenne patients show serious cardiovascular abnormalities during the second decade of life. Heart disease and skeletal muscle degeneration do not appear to correlate. Hence, molecular or cellular variations between different muscle types in relation to signalling, ion handling

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and bioenergetic pathways may contribute to the severe symptoms of cardiomyopathy [10–12]. The absence of the membrane cytoskeletal protein dystrophin is believed to destabilize cardiomyocytes and this loss of cellular integrity renders cardiac tissue more susceptible to stretch-induced fibre damage, surface membrane degradation, interstitial inflammation, necrosis, fatty tissue replacement and myofibrosis [13].

Many Duchenne patients die of respiratory impairments and cardiac dysfunction, making the elucidation of the molecular and cellular mechanisms involved in cardiorespiratory complications an important part of muscular dystrophy research [10]. The pre-symptomatic treatment of cardiovascular disease in dystrophic patients, using drugs such as inhibitors of angiotensin-converting enzyme, angiotensin-receptor blockers or corticoid receptor antagonists, may improve outcomes [9,14,15], but do not specifically target the underlying pathophysiological mechanisms. In this regard systematic profiling studies of the diseased heart may lead to the identification of novel therapeutic targets and this might result in the establishment of superior treatment options for cardiomyopathic patients in the long-term. Mass spectrometry-based proteomics has been widely applied in cardiology research [16], including a select number of studies on dystrophinopathy-related cardiomyopathy [12].

The proteomic characterization of the dystrophin–glycoprotein complex following immuno precipitation has identified new binding partners of cardiac Dp427 [17], indicating distinct differences of dystrophin complex formation between skeletal muscles and the heart [18]. Comparative proteomic profiling has been used to characterize 9-month old *mdx* hearts with the help of two-dimensional fluorescence difference in-gel electrophoresis [19,20]. In addition, a study focusing on intrinsic changes from 7-week old to 20-month old *mdx* hearts has determined the effects of aging on the dystrophic phenotype. During aging, dystrophin-deficient hearts undergo alterations in components of the cytoskeletal network, the basal lamina, iron binding processes, antibody response, the excitation–contraction–relaxation cycle and energy metabolism [21]. This is of biomedical importance, since the natural aging process itself is a major risk factor for cardiac disease and is generally characterized by increased fibrosis, atrial fibrillation, ventricular hypertrophy and decreased maximal contractile capacity [22]. The proteomic evaluation of age-related effects has established significant alterations in metabolic pathways, cellular signalling, the immune response, cellular support structures and the stress response in wild type rodent hearts [23–25].

Building on the findings from these proteomic studies, we have here used an alternative genetic model of X-linked muscular dystrophy, the *mdx-4cv* mouse. In contrast to the conventional *mdx* mouse that is characterized by a mutation in exon 23 of the dystrophin gene [26], the *mdx-4cv* model exhibits a mutation in exon 53 and shows a considerably lower number of dystrophin-positive revertant fibres [27–29]. This makes this mouse model very attractive for experimental treatment studies, such as testing the feasibility of exon-skipping therapy [30]. The analysis presented here is the first report that has identified members of the dystrophin–glycoprotein complex and simultaneously determined proteome-wide changes in the *mdx-4cv* heart model of dystrophinopathy-related cardiomyopathy using whole tissue proteomics. In order to study the advanced stages of cardiomyopathy related to Duchenne muscular dystrophy, the analysis of crude tissue extracts was carried out with the senescent and dystrophic cardiac *mdx-4cv* phenotype versus age-matched wild type hearts. Hence, this investigation has employed a highly suitable animal model of dystrophinopathy with a very low frequency of revertant fibres, and has also avoided any pre-fractionation steps that otherwise could potentially introduce analytical artefacts.

The proteomic study has established a significant decrease in a variety of mitochondrial proteins, the basal lamina component laminin and the luminal Ca^{2+}-binding protein sarcalumenin in the aged *mdx-4cv* heart. Importantly, the deficiency in dystrophin was shown to be associated with a considerable increase in the matricellular protein periostin and related extracellular matrix proteins, the protective anti-protease and anti-inflammatory factor anti-trypsin, and various heat shock proteins. Overall, the identified proteome-wide changes indicate dystrophinopathy-related changes in mitochondrial metabolism, the actomyosin apparatus, Ca^{2+}-handling, the extracellular matrix, the cytoskeletal network and the cellular stress response in the heart.

2. Materials and methods

2.1. Materials

Analytical grade reagents and materials used in the proteomic survey of wild type versus *mdx-4cv* cardiac preparations were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK). Ultrapure acrylamide stock solutions were purchased from National Diagnostics (Atlanta, GA, USA). Sequencing grade modified trypsin and Lys-C were from Promega (Madison, WI, USA). Invitrogen (Carlsbad, CA, USA) supplied Whatman nitrocellulose transfer membranes. The chemiluminescence substrate and protease inhibitors were obtained from Roche Diagnostics (Mannheim, Germany). Superfrost Plus positively-charged microscope slides were from Menzel Glaesser (Braunschweig, Germany). Primary antibodies were purchased from Abcam, Cambridge, UK (ab16048 to lamin LAM-B1; ab6588 to collagen isoform COL-VI; ab92721 to cardiac myosin light chain MLC-2; ab168348 to lumican; ab14734 to the voltage-dependent anion channel VDAC-1 and ab52488 to lactate dehydrogenase), Sigma Chemical Company, Dorset, UK (L-9393 to laminin), and Novus Biologicals, Cambridge, UK (NBP1-30042 to periostin; and NBP2-15492 to asporin). A polyclonal rabbit antibody to laminin fragment P1 was a generous gift from Dr. R. Timpl from the Max-Planck-Institute for Biochemistry, Martinsried, Germany. Normal goat serum and Cy3-conjugated antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). Chemicon International (Temecula, CA, USA) provided peroxidase-conjugated secondary antibodies. A variety of other general chemicals were of analytical grade and obtained from Sigma Chemical Company (Dorset, UK).

2.2. Dystrophic mdx-4cv mouse model of cardiomyopathy

Cardiomyopathy is a clinical feature of dystrophinopathies and this progressive cardiac dysfunction largely contributes to mortality in Duchenne patients [9]. Thus investigations into the pathobiochemistry of cardiac failure are clearly warranted. This study has used cardiac tissue isolated from 20-month old *mdx-4cv* mice, which represents an advanced stage of cardiac complications in dystrophinopathy. This genetic model of muscular dystrophy has been generated by chemical mutagenesis, using N-ethylnitrosourea to induce a C to T transition at position 7619 in exon 53 of the *Dmd* gene [27]. Thus in analogy to both the human disorder and the conventionally used *mdx* mouse, translation of the dystrophin protein is terminated prematurely [31]. This rodent model has advantages over the *mdx* mouse in that it displays 10-fold fewer dystrophin-positive fibres [28] and thus represents a more attractive model for the study of novel therapeutics [30]. For this proteomic study of the aged dystrophic heart, total tissue extracts of cardiac muscle from *mdx-4cv* mice and age-matched control C57BL6 mice were analysed. Fresh tissue samples were acquired from the Bioresource Unit of the University of Bonn, where the mice were kept under standard conditions according to German and Irish legislation on the use of animals in experimental research [32]. The animals were sacrificed by cervical dislocation. Fresh hearts were isolated immediately, quick-frozen in liquid nitrogen and stored at −80 °C prior to analysis [20].

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