Keywords: Dystroglycan; Dystroglycanopathy; Fukutin-related protein; Glycosylation; Muscular dystrophy; Therapy

Introduction

Since the most recent workshop 2 years ago, significant progress has been made in key areas of dystroglycanopathy research. First, new genes have been identified as being involved in the glycosylation of α-dystroglycan (α-DG), bringing the total number of causative genes for dystroglycanopathy up to 18 and shedding new light on the pathway(s) underlying the functional glycosylation of α-DG. Second, the main glycan structure has been elucidated, and this represents a major leap forward. Third, studies of disease models of FKRP mutations have deepened our understanding of disease pathology and progression. Fourth, progress has been made toward developing experimental therapies for dystroglycanopathies. This includes further advances in AAV-mediated gene therapy for fukutin-related protein (FKRP)-associated muscular dystrophies; the application of oligonucleotide therapy for Fukuyama congenital muscular dystrophy; and an improved understanding of the therapeutic potential of modulating dystroglycan. Fifth, the potential efficacy of new and existing drugs, including glucocorticoid steroids, has been tested in animal models of relevant dystroglycanopathies. Sixth, great strides have been made in identifying tools that will be critical for future clinical trials: clinics for effective and accurate genetic diagnosis, a patient registry, biomarkers of disease and more appropriate outcome measures. Encouragingly, the workshop was attended by an unprecedented number of individuals from universities and independent companies with an interest in becoming involved in developing therapies for these diseases.

In summary, this workshop represented a new stage in dystroglycanopathy research, with the key genes in the pathway leading to the functional glycosylation of α-DG discovered, and their major functions understood. In the coming years, we expect to see the focus shift toward experimental therapies and clinic trials tailored specifically for dystroglycanopathies. Such developments will be particularly exciting for patients, parents, and their representative charities.

Background

Dystroglycanopathies are a subset of muscular dystrophies, and are caused by aberrant glycosylation of α-DG, which together with β-DG forms dystroglycan. This dimer is part of the dystrophin-glycoprotein complex (DGC), an entity that maintains stability of the muscle membrane by binding to the extracellular matrix (ECM), and the glycosylation of α-DG is crucial for this interaction. This “functional modification” of α-DG involves various putative pathways (ECM), and the glycosylation of α-DG is crucial for this interaction. This “functional modification” of α-DG involves various putative
been possible to establish an effective clinic management regimen or to develop an effective therapy. The development of experimental therapies for this subset of muscular dystrophies lags behind that for many other forms of this disease; indeed, no formal clinical trial has yet been conducted. One reason for this is the great variation in manifestation. This has hindered our understanding of disease progression, and constitutes a major hurdle for the evaluation of any therapeutic intervention. Nevertheless, over the last a few years significant progress towards developing therapies for these diseases has been made at both the clinical and pre-clinical levels, as presented in this workshop.

Summary of Sessions

Session A: Glycosylation

Chair: Dr. Kevin Campbell: The workshop began with a talk by Dr. Kevin Campbell regarding the pathway that leads to functional glycosylation of α-DG, the role of the LARGE enzyme in this process and the structures of the α-DG glycans. Recent publications by Dr. Campbell’s group have identified LARGE as a bifunctional glycosyltransferase with xylosyltransferase (Xyl-T) and glucuronyltransferase (GlcA-T) activities [1]. However, the ability of α-DG to bind to ECM proteins relies on the presence of a Xyl-GlcA disaccharide repeat. Also, the activity of LARGE requires the prior action of the xylose β-1,4-glucuronoltransferase B4GAT1 [2]. A glucuronic acid β-1,4-xyllose disaccharide synthesized by B4GAT1 has been proposed to function as an acceptor substrate for LARGE-mediated synthesis of the heteropolysaccharide chain, although the enzyme responsible for addition of the proposed first xylose remains to be identified. Although the structure of the α-DG glycan chain that mediates binding to proteins in the ECM is known, the functions of FKRP and fukutin remain to be discovered. In particular the relationship between LARGE and the two putative glycosyltrasferases is not clear. Earlier studies by Dr. Campbell’s group and others have shown that LARGE can bypass defects in α-DG glycosylation in fibroblasts from congenital muscular dystrophy patients, including those with mutations in FKRP and fukutin. Dr. Campbell has now further shown that this compensation relies on at least partial function of the mutant genes. This new finding provides a framework for fully understanding the pathway involved in α-DG glycosylation, which will be important for developing new therapies.

The functional glycosylation of α-DG is reduced or absent in dystrophic muscle of dystroglycanopathies however the presence of a small number of muscle fibers expressing high levels of functionally glycosylated α-DG (F-α-DG ) are present in the FKRP-P448Lneo-mutant mouse, referred to as revertant fibers. Dr. Qi Lu addressed the presence of these fibers and their relationship to regenerating fibers in LGEmyd and FKRP mutant mice. He demonstrated the restoration of F-α-DG is dependent on LARGE and partially functional FKRP. F-α-DG was noted in the regenerating fibers of the FKRP-P448Lneo-mouse but not in mature fibers. Therefore, factors other than FKRP play a significant role in the production of F-α-DG in regenerating fibers [3]. Dr. Lu’s group also examined the defects in FKRP on muscle regeneration, specifically on myogenic cell proliferation and fiber maturation, and concluded that muscle regeneration is not significantly impaired in the early stages of the disease muscles in dystroglycanopathy models [4]. The capacity of regeneration and other factors are likely important factors for considerable variation in levels of functional α-DG observed in diseased muscles in clinics.

Dr. Susan Brown expanded the scope of disease analysis by examining structural brain defects in mouse models for dystroglycanopathy, specifically lissencephaly. A comparison of three dystroglycanopathy mouse models at birth demonstrated a more severe brain phenotype in the FKRP0/FKRP0 compared to the LGEmyd and Pomgnt1null, suggesting a gene specific effect during cortical development in the dystroglycanopathies. Furthermore the severity of these defects was shown to be correlated with the mislocalisation of Cajal-Retzius cells which are instrumental in the development of the cortex. Overall the spectrum of defects that were observed recapitulates some aspects of the structural brain involvement seen in human patients.

Dr. Steve Winder previously showed that tyrosine phosphorylation of dystroglycan acts as a possible signal to promote the proteasomal degradation of the DGC. A knock-in mouse with a phenylalanine substitution at a key tyrosine phosphorylation site of dystroglycan, Y890, has no overt phenotype. However, the Y890F mice with mdx background (Dag1(Y890F)/Y890F) showed a significant improvement in muscular dystrophy and resistance to muscle damage following repeated eccentric contractions when compared with mdx mice. This is associated with reduction in DGC degradation. The results suggest that phosphorylation is an important pathway in the etiology of DMD and provides novel targets for therapeutic intervention [5]. Dr. Winder now reported his current study using tyrosine kinase and proteasome inhibitors directly in mdx mice and showed varying degrees of improvement in disease pathology, including a reduction in centrally nucleated fibers and circulating creatine kinase (CK) levels. Dr. Winder suggests that targeting this tyrosine phosphorylation could be explored as a therapeutic strategy for treating DMD and dystroglycanopathy.

Cardiomyopathy is one of the common outcomes of FKRP related muscular dystrophies. Defects in histology and cardiac function have also been demonstrated in FKRP mutant mice created in the McColl Lockwood Lab [6,7]. To address the question of how disease progression affects cardiac muscle and its function, Dr. Anthony Blaeser presented data with a one year longitudinal study of the FKRP-P448Lneo- mutant mice with ultrasound and histological examination of diaphragm and cardiac muscles. There is a marked increase in fibrosis with age in the diaphragm and the heart shows an increase in fibrosis but at low level. However, cardiac dysfunction is observed in the mutant animal as early as 6-8 weeks of age. Taken together the results offer new areas of focus for examining the effectiveness of treatments with regard to cardiac and respiratory function.

The final talk in Session A was given by Dr. Minoru Fukuda. Dr. Fukuda addressed the laminin-binding (LB) θ-glycan of α-DG and integrin α6 in prostate cancer (PC) cells. Dr. Fukuda reported that expression of LB O-glycan is down-regulated whereas integrin α6 is up-regulated in PC cells and the degree appears to be correlated to Gleason grading. Enhanced expression of LB and blocking the binding to α6 suppress tumor formation and metastasis of PC cells. Dr. Fukuda also presents evidence supporting the involvement of stromal derived factor-1 (SDF-1) and its receptor in the perineural invasion of PC cells. Human perineural cells secrete large amount of SDF-1 whereas expression of SDF-1 receptor (CXCR4) is increased in PC cells. Blocking the binding to the CXCR4 reduces the invasiveness of the PC cells. Dr. Fukuda suggests that LB O-glycan functions as a tumor suppressor and intervention of these pathways could constitute new approaches for reducing prostate cancer growth and invasion. The study of functional glycosylation of α-DG in non-muscle tissues is most interesting as it provides additional avenues for understanding the regulatory mechanisms, with which little is currently known, as well as possible targets of intervention for improving the expression of F-α-DG for dystroglycanopathies.
The use of non-invasive tools for disease identification is important. Dr. James Ervasti discussed his group’s effort to identify urinary biomarkers for muscular dystrophy pathogenesis. Previous reports suggest metabolic and signaling pathway alterations in dystrophic animals [8,9]. Dr. Ervasti hypothesizes that the reduced nitric oxide (NO)/cGMP signaling in dystrophin-deficient mice would lead to impaired energy production following mild exercise. A large scale metabolic screen of urine from dystrophin-deficient mdx mice identified possible biomarkers with regard to dysfunction in the Krebs cycle. He demonstrated the depletion of Krebs cycle metabolites in urine, skeletal and cardiac muscle of mdx mice suggesting the use of the urinary Krebs cycle metabolites as biomarkers for DMD. The levels of these metabolites may also provide possible mechanistic insights into skeletal muscle pathology in dystrophinopathies. Importantly, and relevant to dystroglycanopathies, urinary Krebs cycle metabolite biomarkers can be explored for monitoring disease progression and efficacy of therapeutic intervention as discussed in subsequent talks.

Session B: AAV therapy / drug discovery

Chair: Dr. Qi Long Lu: Dr. Tatiana Cohen began the second session with a presentation about the development of a xenograft mouse model and its use as a preclinical tool for therapeutic studies. The use of animal models is critical in developing disease therapies. However the transition from animal to human is not always clear. Treatments that may work in animal do not necessarily translate to human and vice versa. Dr. Cohen demonstrated the possibility of transplanting human biopsy and autopsy specimens into immunodeficient mice. These grafts were shown to become vascularized and innervated representing a functional muscle. She further showed xenografts from various muscular dystrophies, including facioscapulohumeral muscular dystrophy (FSHD), with a gene expression profile consistent with those in clinic. These human to mouse xenografts will be useful for evaluating therapies for dystroglycanopathies and other muscular dystrophies.

The use of antisense oligonucleotides (AON) has proven beneficial in treating various forms of muscular dystrophy, specifically DMD [10-12]. Fukuyama congenital muscular dystrophy (FCMD) is mainly caused by retrotransposon insertion of tandemly repeated sequences in the 3’ UTR region of the Fukutin gene. This insertion creates abnormal exonic splicing enhance and alters patterns of splicing, leading to non-functional transcripts. Results presented by Dr. Tatsushi Toda demonstrated successful rescue of fukutin mRNA expression initially with a cocktail of AONs targeting the predicted splicing enhanced sequences. This results in functional protein production and glycosylation of α-DG in FCMD patients’ cells and mouse model. Further testing by Dr. Toda identified a single AON showing effective recovery of α-DG glycosylation in FCMD myoblasts as well as in an FCMD mouse model. The possibility of moving to clinical trials with this treatment is currently being addressed. Dr. Motoki Kanagawa presented a new FCMD mouse model, a fukutin-Ki/dysferlin-deficient mouse, which can be used for testing the effectiveness of the AON treatment in vivo [13]. He also presented results of AAV gene therapy in fukutin conditional KO mice that more closely resembles that of FCMD patients. Treatment of these mice using AAV9-fukutin proved to be effective [14]. However AAV-LARGE was unable to rescue glycosylation of α-DG and/or ameliorate the pathology [15]. Dr. Kanagawa therefore concluded that LARGE overexpression may be used to compensate for reduced, but not for complete loss of function of Fukutin.

The use of mouse models for screening drugs and compounds in vivo is considered more relevant to clinic applications. This however becomes difficult for screening a multitude of drugs due to the number of animals necessary. The Kunkel lab therefore created zebrafish models in which FKRP knockdown was using morpholinos antisense oligomer and revealing a phenotype similar to that of dystroglycanopathies with a loss of α-DG glycosylation and shortened myofibers [16]. Dr. Matthew Alexander expanded on this by generating FKRPM mutant zebrafish using TALENs to introduce mutations into the FKRPM gene. He has also generated inducible FKRPM overexpressing transgenic fish lines for expressing human patient-specific FKRPM mutations on the FKRPM mutant background. These mutant zebrafish were then used to screen small compounds to identify any beneficial effects. Dr. Alexander reported the identification of several leading compounds able to correct muscle defects. The zebrafish model could therefore be a cost effective system for initial drug screening and secondary validation.

Despite wide-spread use of glucocorticoid steroids for muscular dystrophy, no study of the drugs has been conducted for dystroglycanopathies. Dr. Bo Wu presented her work examining the effectiveness of the steroids and bisphosphonates alone and in combination for efficacy in FKRPM mutant mice. She demonstrated an improvement in muscle histology together with immunosuppression with the steroid alone, but not with the bisphosphonate alone. The combination treatment produces the highest benefit index although immunosuppression remains. Unexpectedly, the treatment also improves functional glycosylation of α-DG. Dr. Wu concluded that the two drugs taken together could provide higher therapeutic value to FKRPM-related diseases.

Gene therapy is one of the most promising and fundamental treatments for muscular dystrophies. Dr. Xiao Xiao reviewed the progress in AAV gene therapy and reported the effectiveness of AAV therapy on an L276I-FKRP mutant mouse. Treatment of these mice with AAV9-FKRP showed a marked improvement of the dystrophic phenotype including prevention of cardiomyopathy and improvement of heart contractile function [17].

The overexpression of LARGE has been shown to compensate for the loss of F-α-DG in FKRPM mutant mice [18]. Dr. Charles Vannoy expanded on this study by examining the use of AAV mediated LARGE and FKRPM expression in FKRPM mutant mice. He demonstrated that both LARGE and FKRPM expression can restore functionally glycosylated α-DG in FKRPM-P448Lneo- mutants, resulting in amelioration of the dystrophic phenotype. However, FKRPM expression failed to correct the loss of F-α-DG in LARGEemyd mice. LARGE overexpression produced hyperglycosylated α-DG whereas FKRPM overexpression produced normal-sized α-DG in muscle, suggesting FKRPM gene therapy is more suitable to FKRPM-related dystroglycanopathies.

The first day was concluded with a talk by Dr. Jerry Mendell regarding clinical trials for muscular dystrophy with a focus on AAV gene therapy. His talk began with a recap of an earlier clinical trial of mini-dystrophin in DMD. The results of this study identified a potential for T-cell immunity to viral proteins and dystrophin transprotein as a consideration for future therapies [19]. Previous studies have also demonstrated positive results using α-sarcoglycan gene transfer in LGMD2D patients [20]. Currently, there are several AAV gene therapy clinical trials for muscular dystrophies. AAVrh7.4 α-sarcoglycan intramuscular trial demonstrated excellent gene expression in muscle in 5 of the 6 LGMD2D patients after 6 months. A new trial of AAVrh7.4 α-sarcoglycan delivered through the circulation has begun in two subjects and a third is ready at this time. One limitation with AAV mediated gene expression is the size of the gene that can be packaged. To get around this, an alternative strategy using two vectors and homologous recombination has been applied. This method is able to
achieve expression of the full-length dystrophin. This trial is ready to begin with FDA approval. Trials of AAV1-follistatin via intramuscular injection are also being conducted for DMD and BMD. Expression of Follistatin, an antagonist to myostatin, could build muscle size and improve its functions. Becker patients treated with AAV-follistatin have shown a significant improvement in the 6 minute walk test as well as a reduction in descending stair times. All the available results point to a promising outcome for AAV mediated gene therapy to muscular dystrophies. Dr. Mendell also presented new data of the exon skipping trials for Duchenne patients for skipping exon 51. These trials showed an increase in dystrophin-positive fibers as well as benefits to ambulation with no clinically significant adverse events over a 3 year period [11]. Plans are underway for trials targeting other dystrophin exons including 45 and 53.

Session C: Clinical management/endpoints

Chair: Dr. Amy Harper: The final session switched overall focus to clinical aspects of muscular dystrophy. Dr. Katherine Mathews presented four case studies of patients with muscular dystrophy caused by mutations in GMPPB. GMPPB has been shown to play a role in the early parts of the glycosylation pathway for α-DG and various mutations have been found in dystroglycanopathy patients [21]. Dr. Mathews presented clinical features of patients ranging from 3 to 32 years of age with a wide range of clinical severities. Although the pathology appears to be restricted to muscle and brain, there is no single phenotype specific for the patients, thus presenting the difficulties in clinical diagnosis and for clinical trial design. She also concludes that the heterogeneous nature of dystroglycanopathies requires different outcome measures for assessing effectiveness of treatments.

Dr. Anne Rutkowski’s presentation focused the discussion of appropriate outcome measures on those with cognitive impairment and a dystroglycanopathy. Current outcome measurement used in muscular dystrophy clinical trials includes the 6 minute walk test, forced vital capacity, MRI and quality of life measures. These outcome measures are not appropriate for those with mild to severe cognitive impairment with and without behavioral abnormalities. The majority of these individuals are neither capable of consistent voluntary efforts nor do they have fluent communication ability. To develop appropriate outcome measures for the cognitively impaired will require a thoughtfully designed natural history study that utilizes technology and non-effort dependent outcome measures. Limiting the participation of the cognitively impaired patients in future clinical trials is not ethical yet a significant financial investment will need to be made to develop the appropriate outcome measures to ensure participation of the cognitively impaired in clinical trials.

The clinical diagnosis of Dystroglycanopathies can be challenging due to phenotype variability. The brain and muscle can be involved to varying degrees. An identifiable gene variant may not manifest specific features and a wide range of severities can be seen. Dr. Amy Harper addressed some of these issues. The current number of genes involved in Dystroglycanopathies has grown over the past several years to 18. Despite recent advances, up to 50% of patients with Dystroglycanopathy remain genetically undiagnosed. Dr. Harper presented a case in which hypoglycosylation of α-DG in muscle was identified. However, all known genes involved in Dystroglycanopathies were normal. With the growing number of genes involved in Dystroglycanopathies, Dr. Harper suggests that next-generation whole exome sequencing may become a more cost effective and comprehensive approach to identifying candidate genes and cofactors for Dystroglycanopathies.

Dr. Madhuri Hegde presented her work on whole exome sequencing for the diagnosis of LGMD and CMD which are highly heterogeneous, genetically. Dr. Hegde presented data from 200 samples from LGMD and CMD patients in India. Using this approach she has been able to identify GNE Myopathy as the most common LGMD subtype in India with three common mutations. New genes are being discovered and assessed for their role in MD. Whole exome sequencing also provides a useful tool for identifying multiple mutations in single patients. This, in combination with functional data, could deepen our understanding of the contribution of multiple genes to the pathogenesis of disease.

The quality of life of muscular dystrophy patients is a topic of great discussion. Dr. Tina Duong presented her study assessing clinical outcome measures and various self-reported questionnaires for various LGMD and BMD patients, specifically Individualized Neuromuscular Quality of Life Questionnaire (INQoL) and ACTIVLM. By comparing the results of the questionnaires with patient perception it was demonstrated that there is a correlation between the two. INQoL and ACTIVLM proved to be useful for assessing perception of function and quality of life in LGMD patients. However, Dr. Duong suggests that these should be used as complimentary tools with regard to impairment and function based measures. Inability to differentiate between certain functions was also demonstrated by the questionnaires. With these results, Dr. Duong suggests a standardization of quality of life (QoL) tests for research. Longitudinal testing for both ACTIVLM and INQoL will also help to improve sensitivity.

The transition from basic research to clinical trials requires various methods and support. Dr. Claudia Mitchell introduced the support by the LGMD2I Fund and the various researches taking place to move to successful clinical trials. LGMD2I Fund currently supports several projects including the development of antibodies to a-DG, establishing dystroglycanopathy patient specific cell based assays for drug screening, biomarker discovery, and characterization and development of relevant animal models. With these models new therapies are currently being examined for translation to clinic. Dr. Mitchell concluded her talk by discussing the importance of a patient registry as well as a need for patient identification which will greatly facilitate clinical trials.

The workshop was concluded with a talk by Dr. Sebahattin Cirak describing the discovery and use of biomarkers for LGMD2I. Using samples from LGMD2I and CMD patients Dr. Cirak was able to identify various proteins with altered levels found in both LGMD2I and CMD compared to normal controls. Further examination of mass spectrometry data also showed biomarkers that were found in both LGMD2I and CMD. Many of the biomarkers were validated using ELISA. Biomarkers for LGMD2I were further assessed using NanoString technology for miRNA. From this data multiple miRNAs were identified as promising biomarker candidates, although further validation is still needed.

Workshop Organizers

The workshop was organized by Dr. Qi Long Lu, Dr. Anthony Blaeser, Dr. Kevin Campbell, Dr. Amy Harper, Caren Anderson and Melanie McDermid.

List of Speakers

(i) Matthew Alexander, Boston Children’s Hospital

(ii) Anthony Blaeser, Carolinas HealthCare System

(iii) Susan Brown, Royal Veterinary College, University of London
References


