The Molecular Era of Myology

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Abstract. This review covers, in general terms, the salient features and impact of molecular myology under the following headings: its role in providing clues for the understanding of molecular etiology and pathogenesis of genetic myopathies, its contribution to the modernization and rationalization of the classification of muscle diseases, providing means of precise diagnosis and prevention of myopathies, development of radically new cell and gene therapies, and determination of future research directions. Myology appears to be among the medical disciplines that have benefited a great deal from molecular science. This remarkable progress will hopefully translate into effective treatment capabilities in the near future.

Key Words: Gene therapy; Genetic myopathies; Molecular myology; Muscular dystrophy gene mutation.

HISTORICAL PERSPECTIVES

Over the past two centuries, myology (i.e., the basic and clinical science of muscle and muscle disease) has passed through 3 major stages of development: the classical period, the modern stage, and the molecular era. The classical period spans the later parts of the nineteenth century and the earlier parts of the twentieth century. During this time, several major muscle diseases were clinically and pathologically characterized, including Duchenne muscular dystrophy (DMD), myotonic dystrophy, and facioscapulohumeral dystrophy, by master clinicians such as Charcot (1), Duchenne (2), Erb (3), Becker (4), Gower (5), Little (6), Meryon (7), and others. These accomplishments laid solid foundations for the momentous developments in the subsequent two periods.

The modern stage in the second half of the twentieth century is characterized by three major discoveries. First, it was observed that substantial elevation of the serum activity of creatine kinase indicates muscle damage or destruction (8). Then, the adaptation of modern histo-and cytochemical techniques to the study of muscle biopsies markedly improved the diagnostic accuracy and made possible the identification of new changes and structures (9). Examples of this are the demonstration of nemaline rods in nemaline myopathy (10, 11) and ragged red/blue muscle fibers in mitochondrial diseases (12). Thirdly, the advent of modern biochemical techniques permitted the identification of various enzyme defects/storages such as McArdle’s disease (13) and carnitine deficiency states (14).

The molecular era was made possible by the strikingly fast development of molecular biology and its application to muscle diseases. This permitted the identification of gene defects in many inherited diseases, leading to accurate and specific diagnosis. The best example of this is DMD and the discovery in the late 1980s of the gene at locus on Xp21 whose mutation causes the deficiency of an absolutely essential protein, dystrophin, in muscle fibers (15, 16). This was followed by an avalanche of discoveries revealing the molecular basis of dozens of hitherto mysterious muscle diseases. Parallel with the spectacular development of genomics in relation to muscle disease, histochemistry and immunoblotting also produced remarkable discoveries. For example, a number of sarcolemmal proteins were identified whose deficiency causes different forms of limb girdle dystrophy, including dystrophin (17), sarcoglycans (18), calpain (19), and caveolin (20) among others. Dramatic advances also occurred in immunopathology that had remarkable effects on the molecular diagnosis and treatment of nongenetic dysimmune muscle diseases (21).

The main purpose of this paper is to describe the salient features of the molecular era of myology. Of course, this does not mean a detailed account and molecular characterizations of all possible muscle diseases. There is a recent textbook that is devoted specifically to that purpose (22). In this paper, we shall highlight the key aspects of muscle diseases, which were made possible by the application of molecular techniques.

UNDERSTANDING THE CELLULAR AND MOLECULAR PATHOPHYSIOLOGY AND PATHOGENESIS OF GENETIC MYOPATHIES

In monogenetic diseases, three sequential sets of events lead to the clinical phenotype of a particular disease: the gene defect, the absence or abnormality of the corresponding protein, and dysfunction or damage, or even death, in certain cells and tissues in which the particular protein is normally expressed and plays a major physiological role. The application of molecular medicine in
genetic myopathies led to the discoveries that provided better diagnostic and therapeutic capabilities.

The Gene Defect

With regard to a given gene (and its transcript[s]) whose mutation causes a particular disease, the knowledge of several key features are important, including the physical map of the culprit gene, determination of whether it is nuclear or mitochondrial, and the splicing patterns of the primary transcript. Furthermore, knowledge of the following items has been helpful: chromosomal localization, size, exon number, constitutive promoter/enhancer elements, base sequence with notation of special domains and splice sites, phylogenetic conservation, expression characteristics (i.e. possible developmental regulation and control by specific cellular events), features of the pathogenetic mutations versus polymorphisms, and their genetic transmission patterns.

As far as splicing of the primary transcript is concerned, identification of splice sites, possible alternative splicing resulting in different isoforms, stability, and nuclear-cytoplasmic transfer of the fully processed mRNA are all important.

The type of mutation will determine if the relevant protein is absent or whether a full-length but functionally defective protein or truncated protein is formed, or whether the mutation affects one or more proteins.

The pedigree pattern may reveal if the genetic disease is sporadic due to a new mutation, or if the mutated gene has been transmitted from ancestors and perhaps there is a founder effect. The mutation can lead to dominant diseases with various degrees of penetration (i.e. limb girdle muscular dystrophy type 1 [23] or certain myotonias [24]), autosomal recessive diseases (i.e. merosin deficiency [25], limb girdle muscular dystrophy type 2 [23]), X-linked recessive diseases (i.e. dystrophinopathies [26] X-linked Emery-Dreifuss muscular dystrophy [27], and X-linked myotubular myopathy[28]), or maternal. The pedigree pattern is also useful in establishing whether imprinting is present. In dominant disease, a single mutated allele can exert a dominant negative effect, whereas in autosomal recessive disease, homozygosity or compound heterozygosity is present. Maternal transmission indicates a mitochondrial disease (29); however, a mitochondrial disease may be due to a nuclear gene mutation and Mendelian inheritance.

The Absence or Abnormality of the Corresponding Protein

According to the classical concepts of molecular genetics, a single gene mutation will result in the absence or dysfunction of the protein that the gene encodes. This is the “one gene one protein” concept (30). However, it is possible that a single gene defect gives rise to deficiency or deleterious alteration of more than one cellular protein, which can contribute the pathogenic effect. Examples of this “secondary effect” include the depletion of certain dystrophin-associated glycoproteins in dystrophin deficiency (31), the mutation of glycosylating enzymes affecting assembly of N-glycans (congential disorder of glycosylation type I), or N-glycan processing (congential disorders of glycosylation type II) (32).

There are two aspects of the affected protein that need to be established in each case: The nature and normal function of the affected molecule(s) and their subcellular localization. The combined information of the gene abnormality and the features of the affected protein(s) provide the best opportunity to identify a pathophysiological mechanism for a disease.

Understanding the cellular and molecular pathophysiology and pathogenesis of dysimmune myopathies includes two very important entities: autoimmune myasthenia gravis and the nongenetic myasthenic syndromes (33, 34) and inflammatory myopathies (21). Molecular myology has greatly contributed to acquiring important new knowledge pertaining to these entities. Answers have been sought and partly obtained regarding the following immunopathological questions (21): What are the primary offending antigens? Are they intrinsically or extrinsically derived molecules? If they are intrinsic, what transforms them to become deleterious? Are secondary antigens also operating? What are the antigen presenting cells and at what location and by what mechanisms do they operate? What are the principal cytokines and chemokines that serve as signal transmitters, adhesion molecules, and immuno-effector stimulants? What is the fundamental immunological process and how does it damage the target cells and molecules? Is continued action of the offending factors required or is the initial action merely an inductive process? What are the most effective means of eliminating or at least mitigating the pathogenic process for treatment?

Examples of the above items include the identification of the role of MuSK as an autoantigen in some cases of autoimmune myasthenia gravis (35), the classification of the various myasthenic syndromes by identifying the genetic versus dysimmune varieties (33, 34), and the use of immunomodulating agents as therapeutic means (36–39).

ESTABLISHMENT OF A RATIONAL CLASSIFICATION THAT WOULD ALSO FACILITATE THE EDUCATIONAL PROCESS IN THIS FIELD

In the pre-molecular era, the classification of muscle diseases was based on characteristic clinical and/or microscopic pathological features. For example, a disease with an early onset, x-linked recessive progressive proximal muscle weakness, large calves, and “dystrophic” microscopic pathology qualified for the category of “muscular dystrophy” (along with several other progressive genetic muscle diseases). Furthermore, episodes of...
profound hypotonic limb muscle weakness along with reduced serum potassium level would place such a disease in the category of “periodic paralyses.” In the molecular era, the basis of classification has changed and is still evolving. The basis for classification of genetic myopathies has become much broader and includes the following: mutational characteristics, affected proteins, microscopic features, the nature of the abnormal cellular process(es), principal organellar involvement, and distinctive clinical features. For a practical classification, two or more factors may be combined.

Three categories serve as a convenient basis for molecular classification:

1. Mutational Profile Plus Organellar Involvement

Primary Sarcolemmal Diseases Involving the Plasma Membrane or Basal Lamina: In this group we include dystrophinopathies (26), sarcoglycanopathies (18), merosin-deficient diseases (25), dysferlinopathies (40), and caveolin-related diseases (20).

Diseases with Primary Myonuclear Abnormalities: Examples in this category include emerinopathies (27), lamin A/C-related diseases (41), and myotubular-related centronuclear myopathy (28).

Diseases with Primary Involvement of Elements of Myofibrils or Cellular Cytoskeleton: Included in this group are actinopathies (42), core diseases (43), nemaline myopathies (44), plectin (45) and telethonin-related myopathies (46), myosin heavy chain type II a syndrome (47), and desminopathy (48, 49).

Diseases with Ion Channel or Ion Transporter Defects: Chloride/calcium/potassium/sodium channelopathies (myotonic or other periodic paralyses) (50), sarcoplasmic reticulum (SR) calcium release channel (ryanodine receptor) (51), and SR ATP-ase-related myopathy (Brody’s disease) (52, 53).

2. Nature of the Relevant Cellular Processes

Muscle Metabolism: Catabolic metabolism, including lysosomal disorders (e.g. lamp-2 deficiency [54], alpha glucosidase deficiency [55], and x-linked myopathy with excessive autophagy [56]) and nonlysosomal disorders (e.g. calpainopathy [57] and proteosomal disorders); carnitine and fatty acid metabolism (58); glycolytic pathways (59); and mitochondrial oxidative phosphorylation defects (60).

Neuromuscular Transmission: Congenital myasthenic syndromes (34) and ion channel disorders affecting voltage-regulated Na\(^+\) channel, or K\(^+\) and Ca\(^{2+}\) channels (50).

Glycosylation: Inclusion body myopathy with GNE deficiency (61, 62), muscle-eye-brain syndrome, and Fukuyama’s congenital muscular dystrophy (62, 63).

3. Special Complex Molecular Mechanisms

Trinucleotide (CTG) Repeat Expansion: In myotonin kinase gene (myotonic dystrophy, type 1) (64).

Trinucleotide (GCG) Repeat Expansion: In PABPN1 gene (oculopharyngeal muscular dystrophy) (65).

Tetrnucleotide (CCTG) Repeat Expansion: In the gene encoding zinc-finger protein ZNF9 (Myotonic dystrophy, type 2) (66).

Large Telomeric Deletion: On chromosome 4 in the D\(_{2}Z_{4}\) repeat zone (Fascioscapulohumeral muscular dystrophy) (67).

PROVIDING MEANS OF PRECISION DIAGNOSIS AND PREVENTION OF GENETIC MYOPATHIES

In the molecular era the diagnostic process of genetic or other myopathies must still start with obtaining a detailed history (including ascertainment of symptoms, pedigree, etc.) and performing a careful physical exam that is streamlined for characteristic signs of muscle disease. The next steps are electrophysiologic studies and microscopic study of muscle biopsies, using advanced histochemical analysis. Obtaining an appropriate data base by these two approaches is sufficient for establishing the diagnosis of a number of myopathies. However, here we emphasize instances where molecular testing is necessary. This includes mutational analysis and immunoblotting. For mutational analysis, one must focus on a highly suspected culprit gene. In cases of genetic myopathies where a certain type of mutation is predominant, conventional and cost-effective techniques with polymerase chain reaction (PCR) is the method of choice. In other instances, sequence analysis is necessary, which can be time consuming and expensive. Molecular analysis is performed either by commercial laboratories or molecular laboratories attached to clinical units. The cost-effective availability of a given mutational analysis in a genetic myopathy is facilitated by information available on Internet websites such as Gene Test (http://www.genetests.org). Some investigators give first preference to noninvasive molecular analysis vis a vis microscopic study of an invasive muscle biopsy (68), for example, in a case in which clinical and genetic history suggests DMD, but confirmation is necessary for differential diagnosis from Becker or other forms of muscular dystrophy. One school of thought advocates the demonstration of dystrophin deficiency by histochemistry/immunoblot on muscle biopsy. Another approach is to first perform mutational analysis by multiplex PCR of the dystrophin gene’s coding sequence and resort to muscle biopsy only if the former approach is not diagnostic (69). Another example for the absolute need for mutational analysis is carrier detection or prenatal diagnosis in DMD.
DEVELOPMENT OF SAFE AND RATIONAL MOLECULAR THERAPEUTICS

Based on the paradigm of three sequential events operating in the pathogenesis of genetic myopathies, therapeutic approaches may target any of those events.

GENE DEFECT AND/OR THE RESULTING PERTURBATION OF ONE OR MORE DOWNSTREAM GENETIC EVENTS

In this domain, various types of cell therapy or gene or genetic therapies are included.

Cell Therapy

Cell therapy consists of introduction of muscle progenitor cells into the treatable muscles that may have two possible roles. By fusing into the host’s muscle fibers, they may serve as agents for transferring normal genes into the host’s muscle fibers, or, by fusing with each other, they could give rise to new muscle fibers and serve as tissue replacement agents in situations when muscle fiber loss had occurred. Muscle progenitor cells for these purposes may be cultured myoblasts or stem cells deriving from either muscle or bone marrow (70) or other sources (71). The major problem with myoblast cell therapy for skeletal muscles is the rapid and massive cell death that occurs after injection into the host muscles (72). The precise molecular cause(s) of this event is not clear, but peculiar immediate immune reactions may be the culprit (73). Stem cells may actually avoid this fate after intramuscular or intravascular transfer. Cell therapy may only be expected to be practical if the transferred cells are introduced into the treatable muscles by a vascular route.

Gene Therapy

Gene therapy consists of the introduction of functional genetic material that counteracts the deleterious effects of the gene defect. Several forms of gene therapies are being explored in preclinical experiments for a few genetic muscle diseases (74). These methods include gene replacement and gene or transcript repair (75).

Gene Replacement: In gene replacement the coding sequence of a normal allele of the mutant gene is introduced into the nuclei of the treatable muscle fibers. Efficient and safe outcome of this procedure requires optimization of several items, including gene vectors (viral and nonviral), gene promoter/enhancer units, and the route of administration. Preclinical experiments in the mdx model have produced efficient, safe, longstanding and functional full-length dystrophin restoration in a large percentage of muscle fibers by using a drastically modified adenovirus vector and a powerful promoter/enhancer unit (76).

Gene Repair: The principal aim is removal of a primary stop codon from the primary transcript that gives rise to truncated and highly unstable protein and ultimately results in deficiency of that protein (77). In another application of gene repair, removal of one or more entire exons could transform an out-of-frame deletion to an in-frame-deletion that would eliminate the secondary stop codon downstream (78).

The most useful method of stop codon-bearing exon removal is corrupting the normal splicing pattern of the primary transcript by the use of specific antisense oligonucleotides. This approach has been used successfully in a dystrophin-deficient animal model of DMD (mdx mouse), where removal of exon 23, bearing a primary stop codon, gave rise to the production of normally localized but shortened dystrophin and mitigation of the dystrophic myopathology (78, 79). Another way of negating the deleterious effects of a primary stop codon is by producing a translational “readthrough” by corrupting the translational fidelity of ribosomes by aminoglycosides (80). By this method, the stop codon is ignored and full-length protein is formed. The efficiency of such a method is limited by the toxic effects of aminoglycosides. Therefore, the beneficial effects in the mdx model or in DMD/Becker muscular dystrophy (BMD) patients have been found to be quite variable (81, 82).

Another form of genetic therapy is upregulating a gene that encodes a functional analogue of the absent or defective protein. The best example of this approach is extrasynaptic upregulation of the level of utrophin in dystrophin deficiency (83). Utrophin is an efficient functional analogue of dystrophin, but it is normally expressed only in small quantities at the neuromuscular junction (84). The identification or development of a safe compound that can upregulate extrasynaptic utrophin could become the therapeutic agent of choice for dystrophin deficiency (85–87).

For dominant muscle diseases, such as myotonic dystrophy type 1 or oculopharyngeal dystrophy, where the mutant allele’s protein product has a dominant negative effect (even though the normal allele generate a normal transcript and protein), different gene therapeutic approaches should be used. The most imaginative of these is “gene silencing” or RNA interference by a short complimentary single-stranded RNA (RNAi), which prevents translation of the mutant transcript (88, 89).

Total or Partial Deficiency or Functional Abnormality of the Protein Product of the Mutant Gene or Secondary Protein Deficiencies

This also constitutes potential therapeutic targets. This therapeutic category includes replenishment of the absent protein (alpha glucosidase in Pompe’s disease) (90), custom-designed pharmacological agents that can correct the malfunction of a defective protein (quinidine for the correction of the mutant sodium ion channel function in a form of congenital myasthenia [slow channel syndrome])
(91), or neutralizing a toxic protein by generating specific intracellular antibodies to that protein (92).

**Disturbance of One or More Aspects of the Structure and/or Function of the Cells Affected by the Gene or Protein Defect**

This is also a potential therapeutic target. For example, by various myotrophic agents, such as IGF1, the resistance of muscle cells to the deleterious effects of a mutation could be enhanced (93). An excess of intracellular calcium that occurs in several genetic myopathies such as malignant hyperthermia or Brody’s disease is known to have deleterious effects on many cellular processes (94). This could be counteracted by calcium channel blockers or calcium scavengers (95). Relative stabilization of the plasmalemma by corticosteroids is thought to reduce the deleterious effect of dystrophin deficiency in DMD (96–101).

**FUTURE RESEARCH DIRECTIONS**

Despite the great depth and breadth of molecular discoveries pertaining to the theoretical and practical aspects of many myopathies, much remains to be explored. For example, in many genetic and nongenetic muscle diseases, the culprit gene and its protein product remains unknown. In some important diseases where the genetic defect has been identified, we still lack any understanding of the pathogenesis of muscle fiber damage. An example of this is fascioscapulohumeral dystrophy (67).

Some basic pathogenetic mechanisms that are possibly operating in myopathies or have potential therapeutic usefulness need to be explored in depth. These include the role of signaling systems, apoptosis, oxygen radical-induced damage, muscle cell development and differentiation, as well as related molecules, posttranslational processing of proteins, the interaction of nuclearly coded and mitochondrially-coded molecules, and perfecting gene-therapeutic methods.

**DEMONSTRATION OF THE VALUE OF MOLECULAR MYOLOGY BY THE EXAMPLE OF DMD**

Duchenne muscular dystrophy (DMD) is an excellent example of the value of molecular myology. DMD was the first triumph of the so-called reverse genetics in which revolutionary molecular techniques (subtraction hybridization) were used to discover the culprit gene and identify the mutations that do not permit the production of its protein product (dystrophin) (15, 16). The discovery of the dystrophin gene and linkage analysis have unleashed an avalanche of research that resulted in the discovery of the molecular background of dozens of other genetic muscle diseases (22).

**Research Related to Pathogenesis:** Even before the discovery of dystrophin, it was well established that the surface membrane of muscle fibers was “weak” and liable to break down, triggering segmental necrosis of muscle fibers (102). However, we now know that it is dystrophin that is responsible for mechanically reinforcing the plasma membrane by linking the intracellular to the extracellular cytoskeletons (15, 16). Muscle fibers are rendered relatively resistant to the mechanical strains and stresses that the normal contractile activity generates. In addition, the secondary marked decrease of several dystrophin-associated molecules also suggest that dystrophin deficiency also perturbs the extra-to-intracellular signaling function that are probably related to these molecules (103). The intracellular shift of nNOS and the depletion of dystrobrevin in dystrophin deficiency might also subvert the normal regulation of muscle blood flow (104).

**Research Related to Classification:** Before the molecular era of myology, DMD was classified in the large heterogeneous category of “muscular dystrophies.” In the era of molecular myology, DMD is now classified as an entity in the category of dystrophinopathies, along with BMD and other rarer phenotypes.

**Research Related to Diagnosis:** The various diagnostic options that prominently include mutational analysis by multiplex PCR and other techniques have already been discussed. The molecular analysis also improved the efficiency of carrier detection and prenatal diagnosis (105).

**Research Related to Therapeutics:** The usefulness of conventional treatment modalities is very limited. Even the use of corticosteroids only offers a reduction of the pace of downhill course and only in some patients (96–101). A great deal of expectation developed from cell and gene therapy, which has been outlined in the previous section. At present, most of the described methods are still in the preclinical stage. A limited phase I project in a few DMD/BMD patients using a plasmid-mediated dystrophin gene transfer into a single arm muscle has been initiated by the Association Francaise contre les Myopathies and Transgene Corp. (106). Other groups have undertaken limited short-term trials with gentamycin in DMD patients with a stop codon mutation (81). These trials yielded variable results in terms of dystrophin production in muscle. It appears that the nature of the stop codon and the flanking nucleotide sequence have a significant effect of the “readthrough” efficiency (80).

Among the greatest expectations is the prospect for the development of safe and cost-effective cell therapies using various types of stem cells, including the so called mesangiblasts that can populate muscle fibers after intraarterial injection and transdifferentiate into myogenic progenitor cells (71). Other preferred approaches include the finding of a nontoxic pharmacological agent whose chronic administration can upregulate the level of extrasynaptic utrophin to a level that is normally present in the postjunctional sarcolemma (107).

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