Muscle biopsy and cell cultures: potential diagnostic tools in hereditary skeletal muscle channelopathies

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One of the essential properties of the muscle membrane is its excitability, that is the ability to conduct electrical impulses across its membrane in response to an action potential and to propagate this electrical impulses along the muscle fiber itself. Electrical impulses travel throughout the nervous system by rapid shifts of the concentration of ions across the cell membranes. These shifts in ion channel concentration are conducted thorough ion-specific channels which, comprise a family of transmembrane glycoproteins that are made up of two or more subunits and use a variety of stimuli to trigger their opening or gating. Voltage-gated ion channels on the sarcolemma, are those selective for sodium, potassium, chloride or calcium ions which open by changes in voltage (usually depolarisations) across the sarcolemma.

When mutations arise on the genes encoding one of these skeletal muscle voltage-gated ion channels (usually occurring on the α-subunits of the ion channels), the resulting phenotype is that of a hereditary muscle channelopathy. In general, most mutations cause gain-of-function defects (Hanna et al. 1998, Davies NP et al 1999, Lehmann-Horn F 2002, Cannon SC 1997, 2001). Loss-of-function changes may also occur with disease-associated mutations in ion channels (Lehmann-Horn F 2002).

According to the channel involved there are sodium, calcium, chloride and potassium muscle channelopathies (Aguilar-Bryan L et al 1999; Ashcroft FM et al 1998; Barchi RL 1995). It is however worth noting that, decisive for the phenotype is the type of functional defect brought about by the mutations, rather than the channel affected. In fact, different phenotypes like hyperkalemic and hypokalemic periodic paralysis may be caused by the point mutations in different parts of the same gene. Similarly different genes can lead to similar phenotypes as is the case of some cases of cold-triggered myotonia associated with muscle weakness which may involve sodium or chloride channel gene mutations (Jurkatt-Rott K et
No matter which channel is involved, the hereditary muscle channelopathies share common characteristics: there is usually a dominant inheritance; the symptoms involved are typically episodic in nature, that is they are triggered by different internal and external factors; there is a general accepted rule that acetazolamide improves symptoms. These features lead to the general diagnosis of a channelopathy. The diagnostic criteria for each channelopathy have been set at the first European Consortium for the Periodic Paralysis. Since then, new channelopathies have been identified (Andersen’s syndrome) and many muscle disorders have been recognized as potential channelopathies. The muscular dystrophies in general have been considered so far as the result of the lack of structural proteins like dystrophin, which lacking, disrupt the sarcolemma and give rise to the dystrophic process and related weakness. The recent demonstration that chloride channel malfunctioning is involved in the myotonic phenomenon of the myotonic dystrophies types 1 and 2 (Charlet B et al. 2002; Mankodi et al. 2002a), indicates these disorders may also be considered as potential channelopathies and expands the field of the hereditary skeletal muscle channelopathies.

In this study we propose a diagnostic clinical and laboratory protocol to be applied as a screening in potential channelopathies. In particular we demonstrate that muscle biopsy and muscle cell cultures may be considered an important supportive tool for the correct identification of the channel involved. DNA-based diagnosis is now possible for many of the hereditary skeletal muscle channelopathies. This has obvious genetic counselling, prognostic and therapeutic implications. Collaborative efforts in the field of the muscle histopathology and molecular genetics have resulted in improved understanding of the disease mechanisms that underlie muscle channelopathies.

**Materials and Methods**

Fifty patients from 18 families fulfilling the general criteria for a potential hereditary muscle channelopathy according to the criteria described above were included in the study. Ten patients from 4 families with myotonic dystrophy type 1 (DM1) according to the criteria set at the International Myotonic Dystrophy Consortium (IDMC 2000) and 10 patients from 7 families with myotonic dystrophy type 2 (PROMM/DM2) according to the criteria designed at the European International Consortium.
Figure 2. Schematic representation of the muscle sodium channel (α-subunit) and associated mutations (filled circles).
Figure 3. Schematic representation of the chloride channel and associated mutations (filled circles).
were also included in the study.

After informed consent, all patients were subjected to the following: detailed family history to identify potential affected asymptomatic family members; neurologic examination including manual and quantitative muscle strength testing; electromyography with a standardized protocol to quantify myotonia; additional assessment of myotonia by subjective scales of severity, functional tests and quantitation of relaxation time after maximum voluntary contraction; muscle biopsy. Cell cultures from the muscle biopsy specimens obtained were prepared for research purposes.

**Patients**

**Potential calcium channelopathy:** twenty-patients with clinical and laboratory features of hypokalemic periodic paralysis or calcium channelopathy were studied (7 males, 13 females; age range: 20-48 years; mean age: 24.5±6.8) (Curtis BM et al 1984, Hosey MM et al 1988; Takahashi M et al 1987). An autosomal dominant transmission was recognized in 14 of 20 patients. Age at onset of symptoms was 11.8±3.4 and consisted of episodes of sudden weakness, affecting all 4 limbs, triggered by carbohydrate ingestion in 17 out of 20 patients. In the remaining 3 patients the episodes of weakness were triggered by prolonged exercise (football game, bicycle). In all cases the clinical diagnosis of hypokalemic periodic paralysis was made on the basis of low levels of serum potassium during an attack of weakness (below 3 mEq/L). The episodes, typically beginning in the morning on awakening, lasted 48-72 hours, exercise facilitating recovery. A common finding was that of triggering of symptoms also during episodes of emotional stress. During the interictal period 10 of 20 patients showed signs of a limb-girdle myopathy affecting the lower limbs more typically in the 4 range of the MRC scale. The remaining 10 patients had muscles of normal bulk and strength. Calcium channelopathies are determined by point mutations on the gene encoding for the voltage-gated skeletal L-type calcium channel protein on chromosome 1q (Ptacek LJ et al. 1994).

**Potential sodium channelopathy:** ten patients with the clinical diagnosis of sodium channelopathy were studied (7 males, 3 females; age range: 14-60...
years; mean age: 18.3±12.8) (Davies NP et al, 2000, Ebers GC 1991, Fontaine B et al 1990, Heinemann SH 1992, Lehmann-Horn F et al, 1987, McClatchey Al et al, 1992, Ptacek LJ et al, 1991, 1992, 1994; Rojas CV et al, 1991; Sansone et al, 1994; Stuhmer W et al, 1989). Of these 6 patients had episodes typically exacerbated by cold exposure. The episodes were characterized by paramyotonia and less often by episodes of weakness. The paramyotonic phenomenon was most pronounced in the facial district and in the hands compared to other body parts. The eyes were frequently affected. Repeated exercise worsened the contracture so that relaxation was increasingly impaired. The clinical diagnosis of paramyotonia was made in these patients. Four patients fulfilled the clinical criteria of a hyperkalemic periodic paralysis because serum potassium levels were above normal range during episodes of sudden weakness, typically triggered by exercise and fasting. Sodium channelopathies are determined by point mutations on the skeletal voltage-gated Na channel (Nav1.4) on chromosome 17q.

Potential chloride channelopathies: twenty patients fulfilling the diagnosis of myotonia congenita were studied (14 males, 6 females; age range: 30-58 years; mean age: 34.5±6.8) (Becker PE 1977, Koch MC et al, 1992, Fahlke C et al, 1997, Jentsch TJ 1994, Steinmeyer K et al, 1994, Wagner S et al, 1998; Wu FF et al, 2001). In 12 an autosomal dominant transmission was found. Symptom at onset was myotonia, typically present in the hands rather than on the face, exacerbated by cold. Repeated exercise resolved myotonia (warm-up phenomenon). In 4 patients there was associated proximal and distal weakness and in 2 patients myotonia was particularly painful. Dominant and recessive myotonia congenita are determined by point mutations on the skeletal voltage-gated Na channel gene on chromosome 17q.

Potential potassium channelopathies: four patients with episodes of sudden muscle weakness associated with severe cardiac arrhythmias and peculiar facial and skeletal abnormalities were classified as affected by Andersen’ syndrome (Andersen ED et al, 1971; Tawil et al, 1994; Sansone et al, 1997, Canun S et al, 1999, Plaster NM et al, 2001, Tristani-Firouzi M et al, 2002). Cell cultures were prepared as previously described (Meola G 1991) from muscle biopsies having myotonic dystrophy type 1 (5 males, 5 females; age range 19-45; mean age 43.2±9.1). Symptoms at onset were grip myotonia for 7 patients and distal hand weakness for 3 patients. Muscle weakness and atrophy were typically distal at onset. Multisystem involvement and specifically cardiac arrhythmias were present in 8 of 10 patients. Myotonic dystrophy type 1 is determined by the well-known large CTG repeat expansion (>50 repeats) on chromosome 19q.

Ten patients with autosomal dominant inheritance of predominantly proximal muscle weakness, cataracts and myotonia (4 males, 6 females; age range 29-65; mean age 48.6±5) with a normal size CTG expansion on the DMPK gene were diagnosed as having myotonic dystrophy type 2. None of the patients complained of symptoms attributable to cardiac involvement and no cardiac abnormalities were found on EKG. Myotonic dystrophy type 2 or proximal myotonic myopathy is determined by a CCTG expansion on chromosome 3q21.

Quantitation of muscle strength

Manual and isometric dynamometric muscle strength assessment: all subjects were tested manually for muscle strength using the 5-point MRC scale (Medical Research Council. 1976) and using an isometric dynamometer according to previously standardized protocols (Sansone et al. 2000).

Quantitation of myotonia: myotonia is quantified according to subjective self-assessment scales of severity, timed functional tests, relaxation time after maximum voluntary contraction and EMG recordings of relaxation time according to previously standardized protocols (Sansone et al 2000).

Muscle biopsy: muscle biopsy was performed on the right vastus lateralis or on the left biceps brachii under local anaesthesia with the consent of the patients. Cryostat sections (10 um thick) were processed for histochemical analysis as previously described (Dubowitz 1985). A battery of histological and histochemical reactions was performed (hematoxylin and eosin, modified Gomori trichrome, ATPase pH 9.4, 4.6 and 4.3, nicotinamide nucleotide dehydrogenase (NADH), succinic dehydrogenase (SDH), periodic acid Schiff (PAS), phosphorylase, acid phosphatase and oil red O.

Cell cultures

Cell cultures were prepared as previously described (Meola G 1991) from muscle biopsies
obtained from patients with congenital and adult forms of myotonic dystrophy types 1 and 2 to study the differentiation and replicative capacity of mutant DM1 and DM2 myoblasts in culture under different conditions.

**Results**

The results of the diagnostic protocol applied and in particular of the muscle biopsy results were confronted with the expected results solely based on the clinical diagnosis and with the results of genetic studies.

On the basis of clinical information we classified the patients as potential sodium, calcium, chloride or potassium channelopathies. We also classified patients with myotonic dystrophy of yet undetermined genetic background as potential type 1 and type 2 myotonic dystrophy.
After careful analysis of muscle biopsy we were able to outline the specific biopsy findings which characterize each channelopathy (Table 1).

In general, we demonstrate that sodium channelopathies are characterized by a normal morphology at trichrome Gomori and by subsarcolemmal areas, positively stained by NADH-TR (tubular aggregates) (Figures 6c and 6d). This helps in the differential diagnosis of hypokalemic periodic paralysis (HypoPP) types 1 (calcium channelopathy, chromosome 1) and types 2 (sodium channelopathy, chromosome 17) (Jurkat-Rott K et al 2000). In fact, HypoPP type 1, caused by a calcium gene mutation on chromosome 1q is typically associated with a vacuolar myopathy without tubular aggregates (Figures 6a and 6b). This vacuolar myopathy is represented by the presence of vacuoles in the middle of the fiber morphologically, or affecting only one type of fibers (type II) by histochemical staining. These are more typically present in the sodium channelopathies (Figures 6c and 6d) and in Andersen syndrome (Figure 7) (Sternberg D et al 2001).

Chloride channelopathies are also typically characterized by the absence (Figure 8c) or deficiency of type 2B fibers (Figure 8d) and this helps in the differential diagnosis with other myotonic syndromes, i.e. the myotonic dystrophies (Figures 9c and 9d).

Myotonic dystrophy type 1 is characterized by increased variability in fiber size, increased central nuclei, nuclear clumps and preferential type 1 atrophy (Figures 9a, 9b, 9c). Similar abnormalities but with no preferential type 1 atrophy have been described in myotonic dystrophy type 2 (Figure 9d). Our results confirm that myotonic dystrophy type 1 is characterized by preferential type 1 atrophy. We demonstrate that myotonic dystrophy type 2 is instead characterized by preferential type 2 atrophy (Figures 9c and 9d).

Conclusions

Our results demonstrate that although the diagnosis of the known and of the potential skeletal muscle channelopathies is ultimately a genetic one (Lehmann-Horn F et al 1995, 1999; Kleopa KA et al 2002), muscle biopsy may be a mandatory diagnostic tool in the correct identification of the chan-

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**Table 1. Summary of the clinical, muscle biopsy and genetic findings of our patients with calcium, sodium, potassium and chloride channelopathies and of our patients with myotonic dystrophy types 1 and 2 (potential chloride channelopathies).**

<table>
<thead>
<tr>
<th>CLINICAL FEATURES</th>
<th>MUSCLE BIOPSY FINDINGS</th>
<th>GENETIC RESULTS</th>
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<tbody>
<tr>
<td><strong>CALCIUM-CHANNELOPATHY</strong> (hypokalemic periodic paralysis type 1)</td>
<td>age at onset: 2nd decade triggers: CHO, exercise permanent limb-girdle myopathy no myotonia</td>
<td>vacuolar myopathy</td>
</tr>
<tr>
<td><strong>SODIUM CHANNELOPATHY</strong> (hypokalemic periodic paralysis hypokalemic periodic paralysis type 2)</td>
<td>age at onset: 1st decade triggers: fasting, rest after exercise myotonia: face &gt; hands &gt; limbs</td>
<td>tubular aggregates</td>
</tr>
<tr>
<td><strong>CHLORIDE CHANNELOPATHY</strong> (myotonia congenita)</td>
<td>age at onset: 1st decade triggers: cold temperature myotonia: face &gt; limbs &gt; face</td>
<td>type IIB fiber deficiency</td>
</tr>
<tr>
<td><strong>POTASSIUM CHANNELOPATHY</strong> (Andersen syndrome)</td>
<td>age at onset: 1st decade triggers: CHO, exercise no myotonia severe cardiac arrhythmias typical facial and skeletal features</td>
<td>tubular aggregates</td>
</tr>
<tr>
<td><strong>POTENTIAL CHANNELOPATHIES</strong></td>
<td></td>
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<tr>
<td>Myotonic dystrophy type 1</td>
<td>age at onset: birth-2nd predominantly distal muscle weakness myotonia: grip, percussion, tongue posterior lens indescent cataracts multisystem involvement</td>
<td>preferential type 1 atrophy</td>
</tr>
<tr>
<td>Myotonic dystrophy type 2</td>
<td>age at onset: 2nd-5th predominantly proximal muscle weakness myotonia: grip, percussion, tongue posterior lens indescent cataracts multisystem involvement</td>
<td>preferential type 2 atrophy</td>
</tr>
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Figure 8: a) Note hypertrophy of some fibers (EE x 40); b) Normal fiber type mosaicism of the biceps brachii at pH 9.4 ATPase (x 10); c) Note absence of type 2b fibers at pH 4.6 (x 10); d) Deficiency of type IIb fibers at pH 4.6 ATPase (x 40).

Figure 9. A) DM1 morphology (TG x 20): note fiber type variability, nuclear clumps; B) DM1: internal nuclei and nuclear clumps (*) (EE, x 20); C) Preferential type 1 fiber atrophy in myotonic dystrophy type 1 (ATPase 4.3, x 4); D) Preferential type 2 fiber atrophy in myotonic dystrophy type 2 (ATPase 9.4, x 10).
nel involved. This is particularly so when considering the time and cost of techniques such as linkage analysis, southern blot, PCR and mutational analysis especially for large size genes. In general the possibility to perform muscle biopsy rather than previous tests like potassium or glucose and insulin challenges used for the diagnosis of hypokalemic periodic paralysis is a great advantage because of the safety of the muscle biopsy procedure compared to the risk of secondary cardiac arrhythmias induced by variations in serum potassium levels. This applies particularly to the Andersen syndrome in which cardiac arrhythmias are a major concern and challenges should be avoided in any case.

Recognizing that hypokalemic periodic paralysis is more likely due to a sodium rather than a calcium channel on the basis of clinical and biopsy results has obvious clinical implications for the genetician who may direct time and money towards a more specific genetic analysis. It has also therapeutic implications because type 2 hypokalemic periodic paralysis is less likely to respond to acetazolamide or dichlorofenamide.

The importance of muscle biopsy studies in the channelopathies is also clearly demonstrated in patients with myotonia of unknown cause. This is especially true for uninformative families in which a dominant trait is difficult or impossible to determine. In fact, there may be patients with myotonic dystrophy type 2 or with myotonia congenita in whom myotonia may be the only clinical manifestation. In these patients the finding of preferential type 2 atrophy in the presence of normal distribution and size of type 2B fibers directs towards the diagnosis of myotonic dystrophy type 2 whereas the absence or deficiency of type 2B fibers is highly suggestive of myotonia congenita.

The results of our study also emphasize that muscle biopsy specimens from patients with myotonic dystrophy types 1 and 2 (Moxley et al, 2002, Mankodi et al, 2002) may be used to set up muscle cultures to investigate into the mechanisms involved in the pathogenesis of these disorders. Using muscle cell cultures it has been possible to recognize that myotonia in these disorders is determined by loss of the muscle-specific chloride channel due to misregulated alternative splicing (Charlet-B. N et al. 2002). For this reason the myotonic dystrophies have been recently considered potential channelopathies.

Muscle cell cultures may be considered as interesting models to study RNA processing and abnormal regulation of alternative splicing thus contributing to the understanding of the pathogenesis of DM1 and DM2 (Tapscott SJ et al, 2001). Previous studies have demonstrated that muscle cell cultures may be an in vitro model to study the effects of expanded CUG or CCUG RNAs on muscle and therefore to extrapolate these findings to other tissues. Recently investigators have shown that RNAs produced from mutant DM1 or DM2 alleles are retained in the nucleus in one or more discrete foci (Taneja KL, et al, 1995; Liquori CL et al, 2001). The expanded CUG and CCUG repeats retained in the nuclei and possibly additional components of the mutant DMPK and ZNF9 mRNAs inhibit myoblast differentiation and this may be investigated in vitro using this model (Khajavi M et al. 2001; Amack JD et al, 1999; Amack JD et al, 2001, Fardaei M et al, 2002). In addition to these observations are other previous studies showing that in vitro differentiation of congenital DM1 myoblasts is markedly impaired and that these cells undergo premature senescence (Furling D et al, 2001).

In conclusion, although the diagnosis of the channelopathies is ultimately a genetic one, muscle biopsy is an essential tool to direct the genetic approach towards the specific potential channel involved in the disease process. In addition, muscle cell cultures obtained from the biopsies of patients with myotonic dystrophy types 1 and 2 are very interesting models to study the possible toxic gain-of-function by the mutant RNA in the nuclear foci. Some manifestations of DM1 and DM2 like myotonia, cardiac arrhythmias, insulin resistance and cataracts like other aspects of the multisystem involvement of these disorders may result from transinterference with RNA processing. Understanding the exact mechanisms to overcome the toxicity of mutant RNA has obvious clinical and therapeutic implications.

The hereditary skeletal muscle channelopathies described here represent a small part of the rapidly expanding group of neurological channelopathies. The skeletal muscle channelopathies have served as paradigms for the understanding of other ion channel disorders, partly because of the availability of the tissues, which is not the case for the central nervous system channelopathies (Ptacek LJ 1997). Identifying the genetic locus of these diseases is important in the short-term for genetic...
counselling, but in the long-term is should lead to therapies, tailored to the particular dysfunctional channel.

This is an area yet to be explored and functional expression studies by cellular electrophysiology could result in improved mechanisms that underlie neurological channelopathies.

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