Abnormal Distribution of Calcium-Handling Proteins: A Novel Distinctive Marker in Core Myopathies

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INTRODUCTION

Core myopathies (CM) are the most prevalent congenital myopathies (1). They manifest clinically with early-onset muscle weakness and are defined by characteristic histopathologic changes of the muscle fiber structure, termed “cores.” Core lesions are localized areas of mitochondrial depletion and sarcomere disorganization. Their presence in muscle fibers characterizes 2 congenital myopathies: central core disease (CCD) (2) and multi-minicore disease (MmD) (3).

Classically, CCD and MmD were considered independent entities. CCD was defined as an autosomal-dominant disorder characterized by central, well-delimited cores spanning the entire fiber length. Most CCD cases carry heterozygous mutations of the ryanodine receptor type 1 gene (RYR1) (4–6). In contrast, MmD is an autosomal-recessive disorder whose hallmarks are multiple, poorly defined cores that spread only a few sarcomeres in the longitudinal fiber axis. Four different clinical phenotypes have been distinguished in MmD (7, 8). The most prevalent one, termed “classical Mm,” is associated in most but not all cases with mutations in the selenoprotein N gene (SEPN1) (9–11).

Despite these classic differences between CCD and MmD, recent progress in the molecular characterization of CM has disclosed an unexpected overlap between both disorders. In RYR1-related CCD, the spectrum of morphologic manifestations can be highly pleomorphic, including multiple cores (5) or presence of only minicores (12, 13). Besides, homozygous or compound heterozygous RYR1 defects are responsible for 2 MmD forms (moderate MmD and MmD with ophthalmoplegia) (12, 14–16). In some of these cases, an age-related evolution of the morphologic lesions, from minicores to cores, has been observed (14, 17). Additionally, other known or unknown genes can be more rarely involved in CM (18); RYR1 and/or SEPN1 implication has been excluded in a number of patients, and mutations of the ACTA1 gene have been identified in 2 families with a dominant CM (19).

Therefore, establishing a precise diagnosis in CM is frequently challenging, particularly in young and/or sporadic cases. These difficulties are exacerbated by the lack of specific morphologic markers that could help selecting the most appropriate genetic test (20). The massive size of RYR1...
pyridine receptor) composed of 4 subunits (C1, C2, C3, and C4). It contains the voltage-sensitive Ca2+ channel DHPR (dihydropyridine receptor) which mediates Ca2+ release during excitation–contraction coupling (E-C coupling) and interacts with other proteins to form the triadic junction (Fig. 1). Yet, the pathophysiological consequences of RYR1 mutations remain controversial (22). Moreover, selenoprotein N (SelN) function is unknown (23). SelN contains a potential Ca2+ binding domain (EF-hand) and, like RYR1, is localized in the SR membrane and associated with core lesions. These common features allow hypothesizing a potential functional link between RyR1 and SelN.

To our knowledge, the distribution of the major proteins implicated in Ca2+ homeostasis and E-C coupling has not been ascertained in CM (except for sarcoplasmic reticulum Ca2+-ATPase SERCA [13]). This prompted us to study the immunolocalization of 6 of these proteins (RyR1, the dihydropyridine receptor DHPR-α1 subunit, SERCA isofoms 1 and 2, triadin, and calsequestrin) in skeletal muscles from genetically characterized CCD and MmD patients carrying RYR1 or SEPN1 mutations.

**MATERIALS AND METHODS**

**Patients**

Open diagnostic muscle biopsies from 12 ambulant patients with a clinical, histologic, and molecular diagnosis of SEPN1- or RYR1-related MmD or CCD were included in this study.

Histologic criteria in the 6 MmD cases included type I fiber predominance and relative hypotrophy as well as the presence of short cores in most type I and type II fibers. Five of these cases had a classical MmD phenotype (9): congenital weakness predominantly of axial muscles, scoliosis, and respiratory failure requiring nighttime ventilation. The sixth patient presented in infancy with the moderate form of MmD (pelvic girdle weakness, no ophthalmoplegia).

The 6 CCD patients showed long, well-delimited cores in 90% to 100% of type I fibers. All showed early-onset muscle weakness, mainly involving the pelvic girdle. One patient (CCD6) belongs to a previously reported family with recessive CCD (14) in which the affected siblings showed minicores in the muscle biopsies taken in childhood (no longer available) but long cores with rods in those taken in adulthood.

To validate our results, the 12 genetically characterized patients were compared with 6 CM patients (MmD7–MmD9, CCD7–CCD9) without SEPN1 or RYR1 mutations selected using the same clinical and morphologic criteria. SEPN1 mutations were excluded in the 6 MmD cases; RYR1 mutations were excluded in the 6 patients after screening of the whole RYR1 cDNA (in 5 cases) or the C-terminal encoding exons (in MmD7). Also, we excluded ACTA1 mutations in MmD8, CCD7, and CCD8 (the latter belonging to the same dominant family as CCD9).

**Linkage Analysis and Mutation Screening**

SEPN1 and RYR1 linkage studies were performed in the genetically informative families as described (9, 12). SEPN1 mutation screening was performed by direct sequencing as described (9). Because of its large size, the whole RYR1 coding sequence was screened when possible using complementary DNA (cDNA) obtained from a skeletal muscle biopsy (12). When muscle samples were unavailable, we screened RYR1 using genomic DNA and focused on the 15 3’-terminal exons containing most of the known CCD mutations (5). All samples were obtained after informed consent according to local ethics committees.

**Immunohistochemical and Ultrastructural Procedures**

Muscle samples were frozen immediately in isopentane cooled in liquid N2. Histologic and histochemical stainings were performed according to standard procedures using 10-μm cryosections (24). For immunohistochemistry, we used the following primary antibodies: polyclonal antibodies developed by one of the authors (IM) and directed...
against purified pig skeletal muscle RyR1 (anti-RyR, 1/500) (12) and the N-terminal domain of the rabbit triadin (anti-TNter, 1/20) (25, 26); commercial antibodies directed against purified alpha subunit of rabbit skeletal muscle DHPR (monoclonal anti-DHPRα1s; ABCAM, Cambridge, UK; 1:300), rabbit caldesmon (polyclonal anti-Calsq; Affinity BioReagents, Golden, CO; 1:200), and mouse SERCA 1 and 2 isoforms (monoclonal anti-SERCA1/2; Novocastra, Newcastle, UK; 1:100). Serial 7-μm cryosections were fixed with paraformaldehyde 4% in phosphate-buffered saline (PBS). After washing and incubation in PBS with 3% bovine serum albumin, labeling with primary antibodies was performed overnight at 4°C. Sections were then washed in PBS with Tween 0.1% and incubated 1 hour at room temperature with the following secondary antibodies: fluorescein isothiocyanate-conjugated (FITC) swine anti-rabbit (DAKO; 1:300); or CY3-anti-rabbit immunoglobulins (DAKO, Carpinteria, CA; 1:1000). Immunofluorescence was visualized with an Axioskop2 fluorescence microscope (Zeiss, Oberkochen, Germany) and recorded with a Photometrics Cool Snap fx camera (Roper Scientific, Tucson, AZ). For electron microscopy (EM) studies, muscle samples were fixed in glutaraldehyde, processed using standard procedures and observed using a Philips EM120 microscope.

RESULTS

Mutational Analysis

The mutations identified are shown in Table 1. The five patients with classical MmD carried SEPN1 mutations. Three of them were compound heterozygous for a missense mutation associated with either a splicing or a nonsense mutation (out-of-frame deletion or insertion). The 2 remaining patients were homozygous; MmD2 carried a common missense mutation, whereas in MmD3, an out-of-frame nucleotide insertion in exon 5 led to an absence of SelN protein verified by Western blot on fibroblast cultures (data not shown).

The 6 CCD patients and the moderate MmD case (MmD6) had RYR1 mutations. Five of them carried missense heterozygous changes in the C-terminal coding region of the gene (exons 100 and 102), a hot spot where most of the known CCD mutations cluster. The remaining 2 patients showed more uncommon genetic defects. Patient CCD5 had an in-frame heterozygous deletion of 3 bases in exon 91, predicting loss of one amino-acid residue. Patient CCD6 belonged to a consanguineous family with recessive CCD; she carried a homozygous missense change in exon 71, resulting in the replacement of a proline by serine (p.P3527S).

Histologic and Immunohistochemical Results

In all cases and with all the antibodies used, the preserved areas of the muscle fibers showed a labeling of the intermyofibrillar network indistinguishable from that observed in control muscle.

Multi-Minicore Disease Patients With SEPN1 Mutations

In these cases, both fiber types were well represented; type 1 fibers were slightly more numerous. Classic histotechnical changes (Fig. 2) showed minicores in a variable percentage of fibers, which was lowest (around 50%) in MmD3, maximum (100%) in MmD1 and MmD2, and intermediate in MmD4 and MmD5. However, these lesions were not visible by immunohistochemistry, because there was no focal modulation of immunolabeling with any of the antibodies analyzed. The expression patterns of all the Ca2+-related proteins studied were normal and identical to those observed in normal controls (Fig. 3; Table 2).

<table>
<thead>
<tr>
<th>Cases</th>
<th>Genes</th>
<th>Exons</th>
<th>Mutations</th>
<th>Pattern of Inheritance</th>
<th>Muscle Analyzed</th>
<th>Age at Muscle Biopsy</th>
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Mutations are designed according to the published reference sequences. Ho, homozygous; AD, autosomal-dominant; AR, autosomal-recessive.
Central Core Disease Patients With RYR1 Mutations

In all CCD samples, myosin ATPase reactions disclosed an extreme predominance of type I fibers (approximately 90–100%), all expressing SERCA2. Oxidative enzyme stainings revealed long cores, which were multiple and peripheral in all cases but one (Fig. 2D–H). Some cores were bordered by a dark rim, suggesting focal accumulation of SR (Fig. 2G–H). In 3 cases (CCD1, CCD3, and CCD6), cores were associated with rods. EM studies demonstrated different degrees of sarcomere disorganization, including structured cores (CCD3), unstructured cores (CCD2, CCD5), or coexistence of both (CCD1).

Independent of the core distribution and associated lesions, all samples displayed focal immunostaining abnormalities with all the antibodies. These abnormalities were consistent and restricted to the core lesions (Fig. 4). Strikingly, RyR1 immunolabeling was virtually absent from the cores, suggesting a focal depletion of this protein. In contrast, most cores were strongly immunolabeled for DHPRA, triadin, SERCA1/2 and, particularly, calsequestrin, indicating a focal accumulation of these proteins within and/or around the cores (Table 2).

Some cores were diffusely and homogeneously labeled with the 5 latter antibodies (“homogeneous pattern”), suggesting a focal accumulation of SR and triads.
devoid of RyR1. Conversely, other lesions showed a peripheral rim of immunocytochemical protein accumulation, which contrasted with a variable degree of labeling reduction in their center (“perilesional/halo pattern”) (Figs. 4, 5A). Both patterns of immunocytochemical protein accumulation could coexist in the same patient (Fig. 5B) or even in the same fiber, but never in the same core (Fig. 4). However, one of these patterns tended to predominate in a single patient. There was no correlation between the immunocytochemical pattern and the type of RYR1 mutation or the degree of sarcomere disorganization observed by EM (structured vs. unstructured cores, presence or absence of rods). No alternation in the distribution of SR or T-tubules within or around the cores was apparent on ultrastructural sections.

Interestingly, in 3 CCD patients (CCD1, CCD3, and CCD4), NADH-TR staining showed sparse fibers with only small, poorly defined cores, virtually identical to the minicores in MmD. All the labeling abnormalities described previously were retrieved in these RYR1-related minicores (Fig. 6A).

**Multi-Minicore Disease Patient With RYR1 Mutation**

Muscle from MmD6 showed short cores associated with centrally located nuclei and multiple foci of increased oxidative activity, staining dark blue with NADH-TR (Fig. 6B). The minicores showed a labeling pattern identical to that observed in CCD with RYR1 mutations (Fig. 6Ba–c, f, g, arrows). Noticeably, the dark oxidative-positive areas were often labeled for triadin and, occasionally, for DHPR $>_{1s}$ and RyR1, suggesting focal accumulation of these proteins outside the cores (Fig. 6Bf–h, asterisk).
Multi-Minicore Disease and Central Core Disease Patients Without SEPN1 or RYR1 Mutations

Labeling in these nonmutated cases was relatively variable, but none of them showed a pattern identical to either of those in SEPN1- or in RYR1-mutated patients (Fig. 7).

DISCUSSION

Recent progress in the molecular characterization of CM has shown that the classic diagnostic criteria based on core morphology alone are not sufficient for an exact diagnosis, stressing the necessity of novel markers. Previous studies demonstrated that all types of core lesions show increased immunoreactivity for several proteins such as filamin C, αB-crystallin, actin, dystrophin, gelsolin, desmin, α1-antichymotrypsin, β-amyloid precursor protein, β2-microglobulin, NCAM, and myotillin (10, 13, 20, 27–30). Filamin C and αB-crystallin have been considered the strongest immunohistochemical markers of core lesions (13, 20). However, the described immunohistochemical alterations are nonspecific; they are present in minicores, cores, and in other structural muscle defects such as target lesions (20, 28). Thus, they probably represent a secondary, stereotyped cellular response to sarcomere disorganization, nonindicative of the primary molecular defect.

In contrast, our study revealed a particular gene-related immunohistochemical pattern in CM. The distribution of Ca2+-handling proteins was normal in SEPN1-related MmD but was consistently altered in CCD and MmD with RYR1 mutations. Within or around the cores, we observed a virtual absence of RyR1 and a focal accumulation of calsequestrin, SERCA1/2, triadin, and DHPRα1s. This pattern was present in RYR1-related minicores as well as in cores; therefore, the immunohistochemical differences between RYR1- and SEPN1-related myopathies cannot be fully explained by the distinct core sizes but inform on the different molecular defects.

These distinctive immunohistochemical patterns represent a useful marker for differential diagnosis and may help choosing the appropriate genetic test for disease confirmation, especially in young CCD patients whose biopsies do not show the classic core structures. Cores showing the described immunolabeling abnormalities are not indicative of SEPN1 mutations, but advocate RYR1 screening. In our experience, RyR1 depletion and calsequestrin accumulation were the most conspicuous markers in RYR1-mutated cases. Therefore, we recommend the use of these 2 antibodies as a first immunohistochemical screening in CM. Calsequestrin accumulation within the cores is particularly noticeable as a result of the low level of labeling in normal fiber areas. In contrast, SERCA focal abnormalities are frequently inconspicuous as a result of the strong basal labeling for this protein in muscle fibers; actually, no modification in SERCA immunolabeling was observed in a previous study of 3 families with RYR1 mutations (13). Noticeably, in 5 CM cases without RYR1 or SEPN1 mutations, we observed dark oxidative-positive areas that were immunolabeled for calsequestrin, triadin, and DHPRα1s, whereas the cores showed

**FIGURE 5.** Perilesional versus homogeneous pattern in RYR1-mutated cases. (A) Serial transversal muscle sections from CCD1 (100×). NADH-TR (a), RyR1 (b), triadin (c), and DHPRα1s (d) immunolabeling. In the “perilesional/halo” lesions, triadin tended to be reduced in the core center (c), whereas DHPRα1s was often increased (d). This suggests that, at least in some cores, the junctional sarcoplasmic reticulum (SR) component of the triad (RyR1 and triadin) is preferentially depleted from the core center, whereas the tubular component (DHPRα1s) is present or accumulated. (B) Serial transversal muscle sections from patient CCD3 (40×); NADH-TR (e), myosin-ATPase pH 4.35 (f), SERCA2 (g), and SERCA1 (h). Those cores showing some degree of SR oxidative activity in their center (i.e. staining blue with NADH-TR; [e], arrow) tended to be homogeneously labeled ([g], arrow). In contrast, the cores whose center lacked NADH-TR staining tended to show a perilesional pattern ([e, g], arrowheads). Some fibers, which stained as type 1 with myosin-ATPase (f), expressed high levels of both SERCA2 and SERCA1 ([f, e], asterisk).
no labeling abnormality. Thus, identification of immunolabeled spots on a CM biopsy is not reliable in itself; comparison with serial NADH-TR or SDH-stained sections is necessary to verify whether they correspond to the cores. This could partly explain the RyR1 accumulation described in a previous *RYR1*-mutated case in which correlation with cores or serial sections are not illustrated (31).

Aside from its diagnostic applications, our results can provide some prospective indications about the pathophysiology of CM. It has been hypothesized that SelN could be implicated in skeletal muscle Ca$^{2+}$ handling and directly related with the SR Ca$^{2+}$-release structures (9). Our results do not support this hypothesis; on the contrary, they suggest that SelN is not directly involved in maintaining the structural integrity of the Ca$^{2+}$-release complex. Thus, different mechanisms could lead to core formation in muscles lacking normal SelN and in those carrying RyR1 defects.

Two models have been proposed to explain the functional consequences of *RYR1* mutations (22) based on data obtained using nonhuman and/or nonmuscular cells. According to the first model, RyR1 mutations would lead to a sustained calcium leak. The second model suggests that the mutated RyR1 would lose its functional connection with DHPR, leading to E-C uncoupling. Alternatively, protein accumulation in muscle fibers can be a nonspecific phenomenon. However, neither this possibility nor any of the 2 former pathophysiological models can explain the focal RyR1 immunocytochemical depletion that we observed in human

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**FIGURE 6.** Abnormal immunolabeling of minicores in central core disease (CCD) and multi-minicore disease (MmD) patients with *RYR1* mutations. (A) Serial transverse muscle sections from patient CCD3 (40×); NADH-TR (Aa) and immunolabeling for RyR1 (Ab). RyR1 reduction is observed both in the typical CCD cores and in the adjacent minicores or areas of uneven oxidative staining (arrows). (B) Serial longitudinal (a–c) and transverse muscle sections from MmD6. NADH-TR (Ba, d), DHPR915 (Bb), RyR1 (Bc, g, h), calsequestrin (Be, i), and triadin (Bf) immunolabeling. Minicores ([Bd], arrows) were short in length ([Ba–c], arrows) and showed RyR1 reduction ([Bc, g], arrows) and DHPR915 ([Bb], triadin ([Bf], arrows), and calsequestrin accumulation. Calsequestrin accumulation was restricted to the cores ([Bd, e, i], arrowheads); in contrast, triadin and RyR1 accumulation was occasionally observed in areas staining dark-blue with NADH-TR or in oxidatively normal areas adjacent to the cores ([Bd, f–h], asterisk). The muscle fiber boxed is in (Bd–f) and enlarged in (Bh, i). Original magnifications: (a–f) 25×; (g–i) 40×.
muscle expressing RYR1 missense mutations. Our results point to a focal accumulation of SR virtually depleted of RyR1 but not of the other Ca\textsuperscript{2+} handling proteins. This can be best understood according with a novel, hypothetical pathophysiological mechanism that we tentatively propose for core generation: some RYR1 mutations would lead to a relative and focal RyR1 depletion, thereby modifying the stoichiometry of proteins in the Ca\textsuperscript{2+}-release complex. This could cause initially a focal compensatory increase in SR and T-tubules; the absence of RyR1, and thus of a correct functional bridging between SR and T-tubules, would eventually provoke a loss of the normal SR distribution and its accumulation around the lesions, whereas T-tubules remain present within the cores. This evolving character of the cores is difficult to document and cannot be proven by this study, but would explain why 2 different patterns of protein accumulation ("homogeneous" and "perilesional") may coexist in the same biopsy.

So far, definition of cores has been based on focal mitochondria and sarcomere abnormalities. Our results suggest that, in cases with RYR1 mutations, SR and T-tubule abnormalities are a more distinctive change than the conventional EM defects in mitochondria and contractile apparatus. Based on the central role of SR and T-tubules in muscle cells, it is tempting to speculate that their pathologic modifications would lead to E-C coupling dysfunction, thereby causing muscle weakness. Along these lines, the mitochondrial and sarcomere modifications would represent secondary morphologic changes whose functional relevance remains to be proven.
In conclusion, our findings provide an appropriate tool to discriminate between RYR1- and SEPN1-related CM and suggest some novel pathophysiological indications. Further studies, including additional mutations and Western blot analysis, are needed to extend our results and gain a deeper insight into the generation of core lesions and muscle weakness in CM.

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