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Filamin C accumulation is a strong but nonspecific immunohistochemical marker of core formation in muscle

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Abstract

Filamin C is the muscle isoform of a group of large actin-crosslinking proteins. On the one hand, filamin C is associated with the Z-disk of the myofibrillar apparatus and binds to myotilin; on the other hand, it interacts with the sarcoglycan complex at the sarcolemma. Filamin C may be involved in reorganizing the cytoskeleton in response to signalling events and in muscle it may, in addition, fulfill structural functions at the Z-disk. An examination of biopsies from patients with multi-minicore myopathy, central core myopathy and neurogenic target fibers with core-like target formations (TF) revealed strong reactivity of all the cores and target formations with two different anti-filamin C antibodies. In all three conditions, the immunoreactivity in the cores for filamin C was considerably stronger than that for desmin. Only for αB-crystallin were comparable levels of immunoreactivity detected. There was no difference in intensity for filamin C between the three pathological conditions. Thus, filamin C along with αB-crystallin is a strong and robust, but nonspecific marker of core formation. The reason why filamin C accumulates in cores is unclear at present, but we postulate that it may be critically involved in the chain of events eventually leading to myofibrillar degeneration.

Keywords: Cores; Filamin C; αB-crystallin; Congenital myopathies; Multi-minicore disease; Central core disease; Target formations

1. Introduction

Cores in a muscle biopsy refer to distinct lesions that are best seen in stains for the oxidative enzymes NADH and SDH [1]. In these histochemical reactions, cores can be recognized as round zones lacking in enzyme activity as they are depleted of mitochondria and glycogen. They may be located centrally or eccentrically, as single or multiple lesions [2,3]. Cores are the pathological hallmark in two forms of congenital myopathies, central core disease (CCD) [4] and multi-minicore disease (MmD) [5,6]. Core-like lesions are also frequently encountered in denervated muscle, in which affected fibers are referred to as target fibers, and the core-like lesions as target formations (TF) [7,8]. A number of experimental situations may also result in the formation of cores, in particular, tenotomy combined with neurotomy [9]. Although the cores and target lesions in these diverse conditions do show recognizable pathological differences, these lesions also have important features in common. The most characteristic pathological feature is a change in Z-disk alignment

Abbreviations: CCD, central core disease; MmD, multi-minicore disease; TF, target formations.

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leading to Z-disk streaming followed by Z-disk disruption. This process may ultimately lead to focal myofibrillar degeneration resulting in unstructured cores. The various core lesions differ with respect to their position within the fiber, number per fiber, longitudinal extent of the lesion, and the formation of zones delineating the lesion. Immunohistochemical investigations in cores have revealed abnormal immunoreactivity in particular for the intermediate filament desmin [10,11], but also of a number of other proteins including increased immunoreactivity for actin, α-actinin, gelsolin, dystrophin, α1 antichymotrypsin, β-amyloid precursor protein, β-microglobulin, and NCAM [11]. More recently, increased immunoreactivity for αB-crystallin was also noted in cores and target fibers [12].

Filamins are a group of actin-binding proteins that are highly conserved at least between avians and humans, and a homologous protein is even found in *Dictyostelium* [13]. Only very recently, the leading laboratories in the field have agreed on a common nomenclature, which we have adopted in this work. Common to all filamins is an amino-terminal actin-binding domain that is followed by a long carboxy-terminal region comprised of a series of immunoglobulin-like domains [14]. The almost ubiquitously distributed isoform filamin A (previously called filamin 1, α-filamin or ABP-280) has been studied most extensively and a plethora of interacting proteins have been identified. These include β-integrins [15–17], caveolin 1 [18], furin [19], presenilin [20] and SEK-1 [21] (see also the review by van der Flier and Sonnenberg [14]). Correspondingly complex are the functions implied for filamin A that range from the organization of stress fibers [22] or contractile units in smooth muscle cells [23] to roles in neuronal migration [24] and the control of hemostasis [25].

Recent studies have also begun to define specific interactions of filamin C, the filamin isoform that is expressed mainly in striated muscle [26] (previously called filamin 2 or γ-filamin). At the sarcolemma, filamin C interacts with γ- and δ-sarcoglycan (mutated in autosomal recessive muscular dystrophy) [27], while at the myofibril, it binds to myotilin (mutated in autosomal-dominant muscular dystrophy) [28] as well as to FATZ/myozenin/calsarcin [29,30]. It seems therefore that filamin C is capable of shuttling between the submembraneous cytoskeleton and the contractile apparatus. Alterations in the subcellular distribution of filamin C observed in various muscular dystrophies corroborate such a concept [27]. These findings prompted us to examine filamin C histochemical staining patterns in other muscular diseases. We show here that filamin C along with αB-crystallin is the strongest immunohistochemical marker thus far for the lesions characteristic for central core disease, multi-minicore disease and target formations. Thus, although filamin C accumulation is not specific for a particular type of the cores investigated, it could indicate a common pathway for the formation of these lesions. In addition, filamin antibody stains may serve as a good marker for core formation in general.

2. Material and methods

A total of seven biopsies with a histological [3] and clinical diagnosis of multi-minicore disease (MmD) [31,32], six biopsies with a histological and clinical diagnosis of central core disease (CCD) [33], and five biopsies with the histological features of target fibers/target formations (TF) [7] and a compatible clinical history and examination were included.

Muscle biopsies were snap-frozen in isopentane cooled in liquid N_2 and stored at -80 °C until further use. Routine histological examinations and histochemical reactions were performed according to standard procedures [1].

The following primary antibodies were used: (1) polyclonal rabbit antibody FLN2 against a peptide sequence specific for filamin C as described by Thompson et al. [27] (1:500); (2) monoclonal anti-filamin C antibody (mAb) RR90 as described by van der Ven et al. [28] (1:10); (3) a rabbit polyclonal antibody specific for α-actinin 2 as described by Chan et al. [34] (1:1000); (4) a rabbit polyclonal anti-telethonin antibody as described by Mues et al. [35] (1:100); (5) monoclonal antibodies against αB-crystallin (1:1000) and (6) desmin (1:200) were purchased from Novocastra (Newcastle upon Tyne, UK); and (7) against α-actin from DAKO (Denmark) (1:50); 8. the FATZ/myozenin/calsarcin antibody has been described by Takada et al.

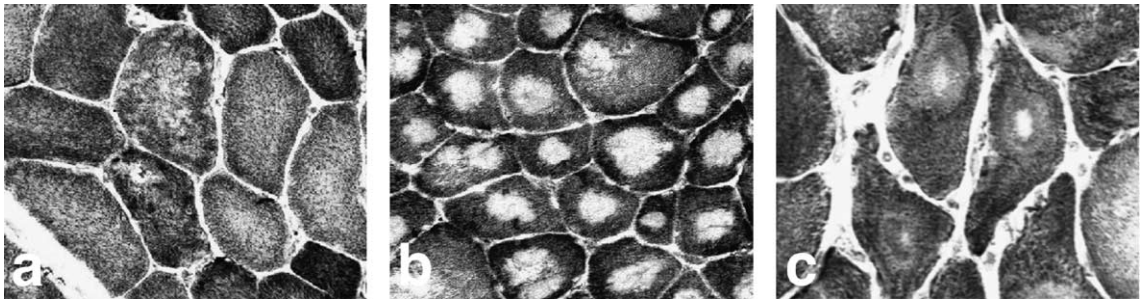

Fig. 1. Appearance of the three lesions in NADH-TR histochemical stains. (a) Multi-minicore disease (MmD), (b) central core disease (CCD), (c) target formations (TF). Note the multiple lesions with somewhat indistinct borders in MmD, large centrally located defects with sharp borders in CCD, and zoned lesions in TF. (Magnifications 40 ×).

[29] (1:1500). Indirect immunohistochemical analysis on 6–9 μm frozen sections was performed as described by Bönnemann et al. [36] and Schröder et al. [37]. Appropriate secondary antibodies were coupled to Cy3, fluorescein isothiocyanate or Texas red and were applied according to the recommendations of the manufacturers (Southern Biotechnology Associates, USA; Jackson Immunoresearch Laboratories, USA).

Slides were examined using a Zeiss Axiophot fluorescence microscope with epi-illumination and acquired with a Hamatsu CCD camera or with a Nikon Eclipse E 800 microscope.

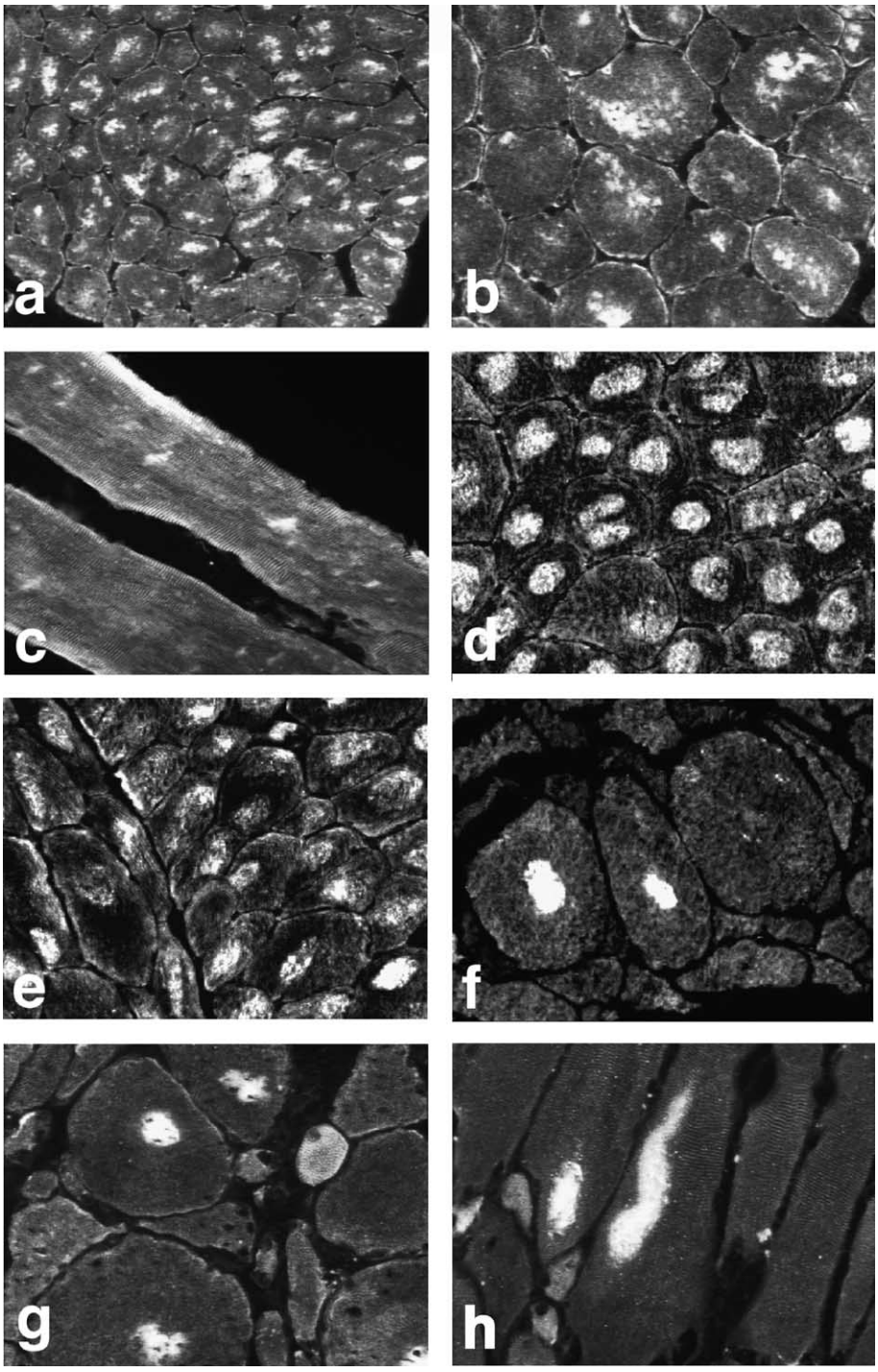

Fig. 2. Immunoreactivity for filamin C within the three types of cores, generating similar results with both antibodies used (FLN2, polyclonal; RR90, monoclonal). (a–c) MmD/FLN2; (d, e) CCD/RR90; (f) TF/RR90; (g, h) TF/FLN2. Note the sharp border of accumulations in all three types of cores. In MmD, the short longitudinal extent of the lesions is reflected by the filamin C accumulations (c), whereas the extent is intermediate in TF (h). In some CC fibers, note the less reactive zone aroud a central zone of intense immunoreactivity (d, e). (Magnifications (b), (c), (g), (h) = 40 ×; (a), (d–f) = 20 ×).

Immunoelectronmicroscopy was performed according to earlier studies [38]. The primary antibody used was the polyclonal antibody FLN2 [27] at a concentration of 1:200, the secondary antibody was coupled to 10 nm immunogold particles and used at a concentration of 1:20.

3. Results

3.1. Immunohistochemistry

The diagnosis (MmD, CCD, TF) were confirmed on routine stains. In particular, the NADH-TR histochemical stain showed the core morphology typical for the three conditions (Fig. 1). Next, immunofluorescence microscopy was performed on sections from the same biopsies. Since a hallmark of these diseases is an altered Z-disk morphology, we mainly focused on thin filament-associated proteins, but also included the intermediate filament desmin and αB-crystallin in our analysis since accumulations in cores had been previously demonstrated for these proteins [12]. Most strikingly, the immunostains demonstrated accumulations of material intensely immunoreactive for filamin C in the cores in all three types of biopsies (Fig. 2). These accumulations consistently showed a sharp border in all three pathological conditions, and there was no apparent internal structure to the accumulations. In less than 40% of CC fibers there was decreased immunoreactivity around the intensely reactive core (Fig. 2d, e). In TFs, the filamin C accumulations were always found in the inner core, while in some fibers, there appeared to be a slightly less reactive zone around the core. In longitudinal sections, the intense immunoreactivity followed the longitudinal extent of the core lesions, i.e. it was short in the case of MmD, followed the extent of the fiber in CCD, and was of intermediate length in TFs. The results were identical for the two anti-filamin C antibodies used (FLN2, polyclonal, Fig. 2a, b, c, g, h; RR90, monoclonal, Fig. 2d, e, f).

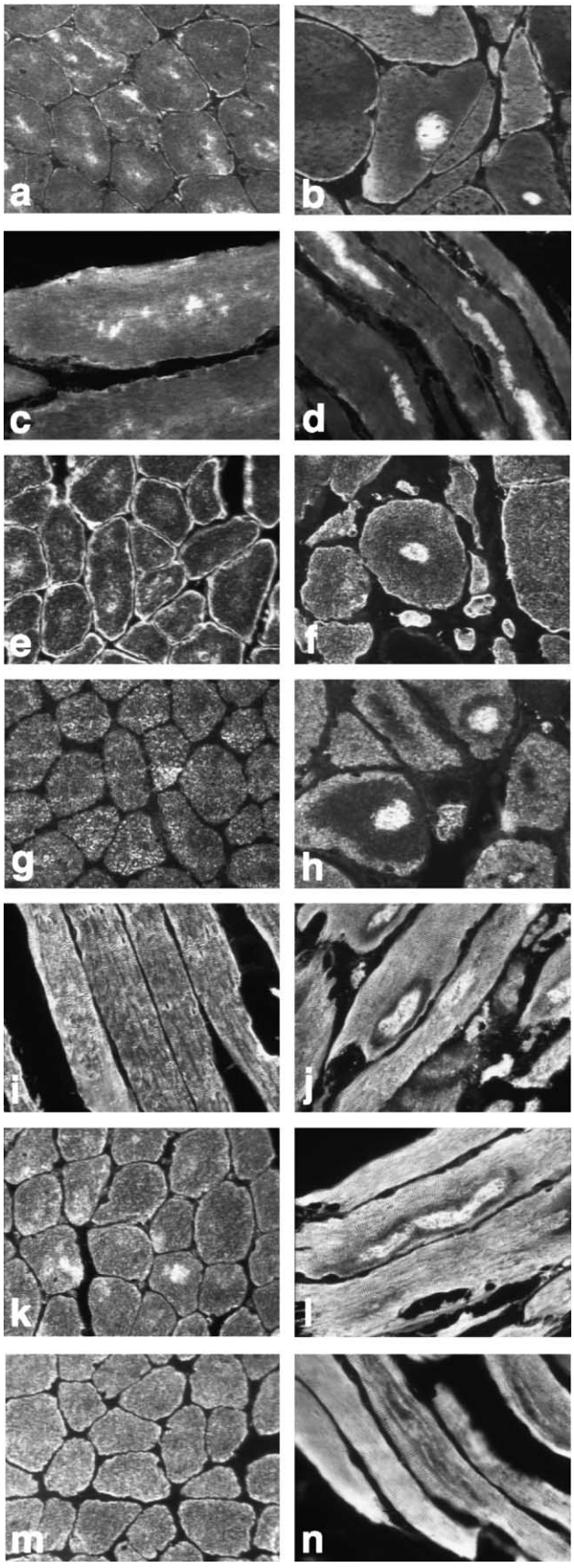

Fig. 3. Immunoreactivity for a number of additional antigens in MmD (left column) and in TF (right column): αB-crystallin (a–d); desmin (e, f); actin (g, h); α-actinin 2 (i, j); FATZ/myozenin/calsarcin (k, l); telethonin (m, n). Note the intense immunolabelling seen for the αB-crystallin in both types of cores (a–d). Note the less distinct border for desmin in MmD (e), whereas a dense accumulation of desmin with a less reactive zone around it was seen in some but not in all TFs (f). Actin immunoreactivity was not noticeably abnormal in MmD (g) but displayed striking accumulations within a zone of severely reduced immunoreactivity in TF (h). α-Actinin 2 in MmD only showed Z-disk disarray (i), whereas in TF the picture was similar to that seen for actin (j). FATZ/myozenin/calsarcin showed some increased immunoreactivity with indistinct borders in the core lesions of MmD (k) and again inTF appeared very similar to actin and α-actinin 2 with dense central accumulations within a halo of decreased immunoreactivity (l). Telethonin did not label cores in MmD (m), whereas in TF three zones could be recognized: a center of decreased immunoreactivity, a ring/border of increased immunoreactivity surrounded by a halo of decreased immunoreactivity (n). (Magnifications 40 ×).

Immunolabelling was of comparable intensity using the anti-αB-crystallin antibody (Fig. 3a, b, c, d). However, a direct quantification of intensities is difficult because αB-crystallin is a much smaller molecule; therefore, the stoichiometry of epitopes recognized by the antibody may be expected to be much higher in the lesion. Additional immunolabelling for desmin, actin, α-actinin, FATZ/myozenin/calsarcin and telethonin were performed in four cases of MmD and three biopsies with TF. Desmin immunoreactivity in MmD was moderately increased within the core zones with less distinct borders compared to filamin C and αB-crystallin (Fig. 3e), whereas in TF, there was desmin accumulation in some but not all of the target fibers (Fig. 3f). Actin appeared normal in MmD (Fig. 3g) but showed striking accumulations within a rim of severely reduced immunoreactivity in TF (Fig. 3h). For α-actinin 2, we observed irregularities in MmD mostly corresponding to the Z-disk disarray (Fig. 3i), whereas in TF, there were strong central accumulations with a halo of decreased immunoreactivity (Fig. 3j). Immunolabelling for the small filamin C-binding protein FATZ/myozenin/calsarcin was also increased in the core lesions of MmD; however, with less distinct borders and less intensity compared to both filamin C and αB-crystallin (Fig. 3k). In the case of TF, FATZ/myozenin/calsarcin stains appeared identical to the findings for α-actinin 2 (Fig. 3l). Quite in contrast, telethonin did not accumulate in the cores in MmD (Fig. 3m), whereas in TF, three zones with distinct immunoreactivities were revealed: a center of decreased immunoreactivity, followed by a ring/border of increased immunoreactivity, which in turn was surrounded by a halo of decreased immunoreactivity (Fig. 3n).

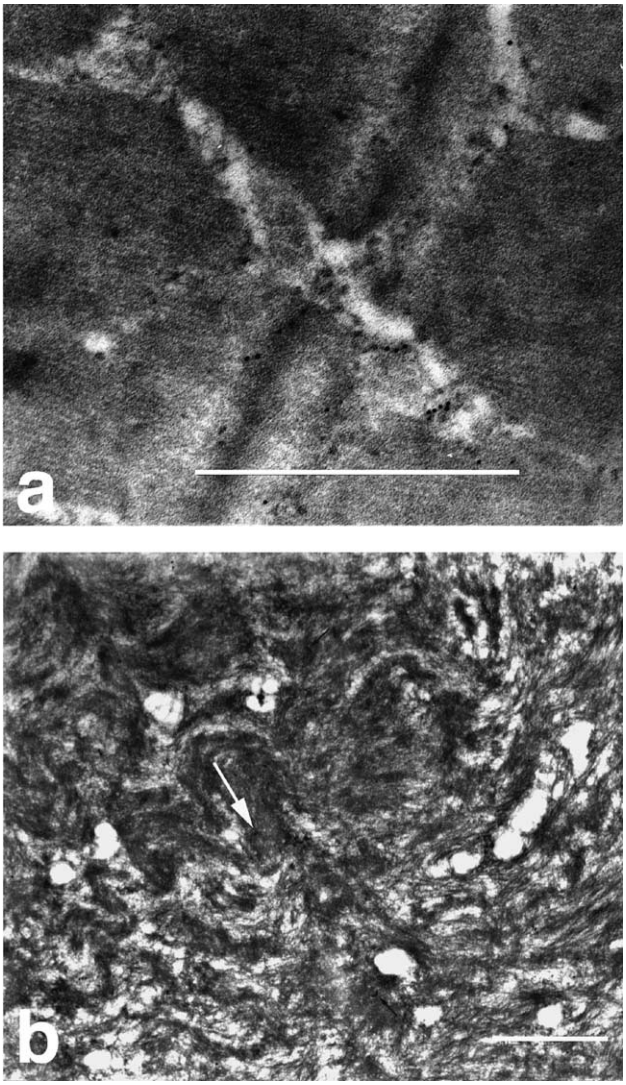

Fig. 4. Electron microscopical immunogold localization of filamin in core lesions. (a) Normal localization of filamin C at the Z-disk periphery (Magnification × 106.000 before reduction). (b) Localization of filamin C in a core formation of a TF in neurogenic atrophy (Magnification × 40.000 before reduction), revealing disrupted sarcomeres. Labelling is indicated by 10 nm gold grains (arrow) using the poyclonal anti-filamin C antibody FLN2 (bars = 1 μm).

3.2. Immunoelectronmicroscopy

Immunoelectronmicroscopy using the polyclonal antibody FLN2 [27] was performed on normal muscles and on biopsies with MmD or with TF. As has been reported before [27,39], in normal muscles, most of the grains localized to the edge of the Z-disk (Fig. 4a) with a minor portion also at the sarcolemma (not shown). In the cores (not shown) and in the target formations (Fig. 4b), accumulations of immunogold particles were observed within the entire lesion without predilection for either center or perimeter.

4. Discussion

In this study, we have investigated the immunohistochemical properties of multi-minicore disease (MmD), central core disease (CCD) and target formations (TF) with an emphasis on thin filament-associated proteins. Most interestingly, we find that filamin C along with αB-crystallin is a strong and consistent immunohistochemical marker of core and target formations irrespective of the primary cause of the lesion. The accumulation of filamin C was identical in appearance using two independently raised antibodies specific for distinct portions of the molecule [27,28]. This rendered it extremely unlikely that the observed immunoreactivity might represent a crossreaction with some other protein.

Although filamin C accumulation is not specific for a particular type of core lesion, our findings may point towards a common mechanism underlying the formation of these lesions. Common to all core lesions is an abnormality of Z-disk morphology that ranges from Z-disk streaming to disintegration of the Z-disk or a total dissolution of the myofibrillar apparatus. Filamin C is found at the Z-disk, particularly at its edge [27,39], where it is involved in thin filament organization. It is also involved in the very

early stages of Z-disk formation [39]. In addition, filamin C could also play a role in the linkage between the Z-disk and the submembraneous actin cytoskeleton and associated transmembrane complexes [27]. A disturbance of one or all of these links could thus be an important but inconspicuous early step in the formation of cores. MmD probably is genetically heterogeneous [31,32]. We have sequenced the filamin C gene in two patients with MmD and were not able to find causative mutations (TGT, CGB, LMK, data not shown). Mutations in the ryanodine receptor gene have been associated with familial and sporadic cases of CCD [40,41]. Therefore, it appears interesting to investigate the possibility of a direct interaction between filamin C and the ryanodine receptor.

In this study, we also find strong accumulation of the small heat-shock protein αB-crystallin within the core lesions. This molecule accumulates in the cores together with another of the small heat-shock proteins, heat-shock protein 27 (hsp 27) [42]. Small heat-shock proteins/αB-crystallin proteins have been found to accumulate together with cytoskeletal proteins in a number of situations. Examples include Alexander disease due to GFAP mutations [43], over-expression of GFAP in mice [44] as well as lesions in MS [45]. Mutations in αB-crystallin itself cause desmin-positive inclusions in muscle [46]. Small heat-shock proteins/αB-crystallin are probably recruited into these accumulations to help bring the proteins in the precipitates back into their native configuration [47]. Thus, αB-crystallin accumulation in muscle has been noted in a number of conditions in which focal destruction of muscle cytoarchitecture occurs. Examples include ragged red fibers [48], so-called desmin-related myopathies [46,47,49], and inclusion body myositis [12]. αB-crystallin has chaperone roles for actin and desmin as well as other proteins [50,51]. However, the molecular mechanisms of HSP induction, regulation and their role in muscle function are not completely understood. For filamin C, we have also observed nonspecific precipitations in the case of ragged red fibers and in severe glycogen storage (not shown). Thus, like desmin and many other proteins, filamin C may also be recruited into cytoarchitectonic lesions nonspecifically.

Interestingly, the examination of several other Z-disk-associated proteins revealed that not all the components of the Z-disk accumulate within the core lesions indiscriminately. Due to limited biopsy material, these additional stainings could not be performed in a systematic fashion in CCD. However, even with the more limited examples available, these findings were consistent. A comparison of relative intensities of immunoreactivity revealed that the levels of α-actinin 2 (Fig. 3i), telethonin (Fig. 3m) and actin (Fig. 3g) in the cores are barely elevated in comparison to αB-crystallin and filamin C. Even FATZ/myozenin/calsarcin, a ligand of filamin C, was only slightly accumulated (Fig. 3k). However, the picture was strikingly different in TF: dense accumulations in the center of the lesion were surrounded by a halo of greatly decreased immunoreactivity for actin, α-actinin 2 and FATZ/myozenin/calsarcin (Fig. 3h, j, l). In the case of telethonin in TF, there was an additional center of very low immunoreactivity within the accumulation (Fig. 3n). Thus, these distinct patterns of accumulation of Z-disk components underline histopathological differences already apparent in histochemical reactions (Fig. 1) and indicate differences in the final steps of pathogenesis leading to these different types of cores.

From the diagnostic point of view, the strong immunoreactivity for filamin C and αB-crystallin described here allows for the unambiguous and easy recognition of core lesions in muscle specimen. This could be of great help, in particular, for the diagnosis of MmD in which condition the lesions in oxidative stains may be spurious and electron microscopic examination is not always available as it is far more complicated and time consuming.

Note added in proof:

Confirming genetic heterogeneity of multi-minicore disease, mutations in the ryonidine receptor (Ferreiro et al. Ann Neurol 2002;51:750–759) as well as in SEPN1 (Ferreiro et al. Am J Hum Genet 2002;71:739–749) have now been identified in two forms of the disorder.

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