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Formation of gelsolin amyloid fibrils in the rough endoplasmic reticulum of skeletal muscle in the gelsolin mouse model of inclusion body myositis. Comparative analysis to human sporadic inclusion body myositis

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Abstract

Sporadic inclusion body myositis has a significant impact on life of elderly. Despite some similarities to other myopathies with established genetic defects little is known about mechanisms of its development and no effective treatment is available. Therefore there is a need for animal models, which would faithfully reconstitute important aspects of this human disease. We recently expressed mutant form of human gelsolin in mice under control of muscle specific promoter. This induced myopathic changes reminiscent to human inclusion body myositis. In this study we use immunogold labeling to further characterize this model. We are able to demonstrate a presence of gelsolin amyloid deposits within the rough endoplasmic reticulum. We further compare this mouse model to human sporadic inclusion body myositis.

Keywords

gelsolin; amyloid; endoplasmic reticulum; IBM

Introduction

Sporadic inclusion body myositis (s-IBM) is the most common progressive muscle disease of elderly, predominantly affecting men over 50 years of age (1, 2). It progressively compromises muscles of hand and thigh, resulting in severe functional disabilities (3), and

can eventually progress to dysphagia and respiratory insufficiency. Histologic evaluation reveals varying degrees of lymphocytic inflammatory infiltrates. Myofibers demonstrate severe hypertrophy admixed with angular grouped atrophy. Hallmark of the disease is rimmed vacuoles, which by electron microscopy are rimmed by degenerative organelles, proliferations of smooth endoplasmic reticulum and are associated with myeloid dense bodies. Immunohistochemical characterization revealed a plethora of proteins involved in generation of various types of amyloid (4). In particular, the rimmed vacuoles might contain A β peptide derived from 751 kDa splicing isoform of Amyloid precursor protein (APP), as well as some of the proteases, involved in its generation: *BACE1* and *BACE2*(5), although these findings have not yet received a universal recognition (6). Additional features frequently detected in IBM are cytoplasmic filamentous and intramitochondrial paracrystalline inclusions. Filamentous inclusions are reported to have a paired helical ultrastructure, reminiscent of hyperphosphorylated tau seen in neurofibrillary tangles of Alzheimers's disease and in other brain tauopathies. These "tubulofilaments" are composed of 7–10 nm individual filaments and have a periodicity of 41–55 nm. Their combined external diameters were measured as 15–21 nm (7, 8). Filaments are stainable by some antibodies to tau and are apparently ubiquitinated (8, 9). Rimmed vacuoles are heterogeneous in molecular composition and are not specific to s-IBM. They have recently been described in a genetically heterogeneous group of diseases called distal myopathies (10). However, the presence of AD-related components is detected more frequently in s-IBM (3). Uncertainty concerning the pathogenesis of s-IBM correlates with a lack of effective treatment options (11).

In one respect s-IBM is a disease of amyloid accumulation, albeit intra- rather than extra-cellular. Therefore general mechanisms governing amyloid formation, trafficking, degradation and mechanisms of toxicity are relevant to s-IBM. One of naturally occurring amyloid diseases is a familial amyloid polyneuropathy type IV (12). The disease manifests in cranial and peripheral neuropathy, corneal lattice dystrophy, cutis laxa and is caused by *G654A* or, rarely, *G654T* gelsolin mutation (13), with subsequent systemic deposition of mutant—Asp187Asn or Asp187Tyr gelsolin, respectively (14, 15). We recently expressed a pathogenic human D187N gelsolin construct in mice under control of creatine kinase promoter (16). This mutant plasma gelsolin has no role in muscle cells as it is a secreted form of intracellular gelsolin. The role of plasma gelsolin is not entirely clear but is thought to act as an extracellular actin scavenger. The rationale for the expression of the mutant gelsolin in muscle was that although plasma gelsolin is ubiquitously expressed throughout the body, it has been shown that skeletal muscle, constituting ~30% of body's bulk is the main contributor to gelsolin's plasma levels. Therefore, we hypothesized that an abundant secretion of mutant gelsolin would be able to reconstitute various features of Finnish Familial Amyloidosis. In this model we have demonstrated that homozygous D187N (+/+) mice by 12 months showed myopathic changes similar to one detected in human s-IBM. It featured not only myofiber atrophy, but also vacuolar changes and congophilic cytoplasmic inclusions, immunohistochemically positive for gelsolin. Interestingly, although only human D187N gelsolin transgene was expressed by myofibers, immunohistochemical detection also revealed the presence of wild type derived beta amyloid (16) and overexpression of APP. The origin of intracellular congophilic deposits of gelsolin and their role in vacuolar

degeneration of myofibers remains to be established. Amyloidogenic 5 and more prevalent 8 kDa fragments of gelsolin are generated from 68kDa C-terminal fragment (C68) in extracellular space, presumably by activity of metalloproteases (MMP-14 and others), after abnormality folded protein is aberrantly cleaved in trans-Golgi network by furin protease (17). Therefore the mechanisms and sites of intracellular deposits are not entirely clear. In this study we perform a more comprehensive immunohistochemical analysis of gelsolin localization in affected muscle of transgenic mice. Additionally we compare ultrastructural findings of mouse model to that of patients with s-IBM.

Materials and Methods

Animal tissue preparation and analysis

Muscle specimens (vastus lateralis, cranial tibial) from 18 months old mice were excised fresh immediately following humane euthanasia and fixed in 2.5% solution of glutaraldehyde (GA) in phosphate buffered saline, pH 7.4 for 4 hours. Subsequently the tissue was cut in 1 mm³ fragments, postfixed in 1% aqueous osmium tetroxide for 1 hour, dehydration in graded alcohol and acetone and embedded in Araldite resin. Thick sections (1μ) were stained with methylene blue for light microscopic examination. Areas of interest were selected and thin sections (65 nm) were counterstained with uranyl acetate (UA) and lead citrate before examination in a JEOL 100CX electron microscope at 80 kV. Digital imaging and size measurement was done by using Image Capture Engine Software Version 5.42.538 by Microfire AMT 542 Camera by Optronics (East Muskogee, OK 74403 USA). For immunoelectron microscopy thin sections were collected on formvar-carbon coated nickel grids. After a brief wash in 0.05M Tris buffer pH 7.2, the samples were blocked in 10% fetal calf serum (FCS) in Tris for 40 min at room temperature. In order to improve binding of antibody to gelsolin fibrils we used various protocols, which alternate resin etching by 4% Sodium metaperiodate (SMP) or 1% periodic acid (PA), antigen retrieval from aldehyde cross-linking by heat treatment in Sodium citrate (SC) buffer for 10 min, and loosening of fibrils with full strength (95%) formic acid (FA) for 5 min at room temperature (rt) (see results and table #1). Subsequently to a final wash in 0.05M Glycine-Tris-5% FCS buffer the grids were incubated for 2 h in primary antibody in 0.05M Glycine-Tris-5% FCS buffer at rt. For the labeling we used polyclonal rabbit antibodies raised against GST 8 kDa gelsolin fragment (18). These antibodies do not recognize full length protein. Subsequently to the primary antibodies the grids were washed in 0.05M Glycine-Tris-5% FCS for 10 min, followed by 6nm gold conjugated goat anti-rabbit antibody for 1h at rt, followed by a wash in 0.05M Glycine-Tris-5% FCS, then distilled water and counterstaining with uranyl acetate and lead citrate.

Human tissue preparation and analysis

For comparison with animal model of IBM we analyzed 20 cases of s-IBM collected during routine patient care at Cedars-Sinai Medical Center in 2006–2013. This retrospective study was approved by the Institutional Review Board. Biopsies were diagnosed as s-IBM based on established clinico-pathologic diagnostic criteria (3, 19, 20) using both histochemical studies and electron microscopy. Tissue preparation was similar to the animal study with

minor variations. We used 2% GA for 12–18 hours and employed additional *en block* UA staining in cacodylate buffer.

Results

As we have previously reported (16), 18 months old mice showed a striking accumulation of amyloid-like substance in endomysial, but not in perimysial compartment with entrapment of myofibers and distortion of their contours (Figure 1). Nerve bundles were also predominantly spared and showed no loss of myelin or axonal degeneration. Each section showed a large fraction of myofibers containing vacuoles with basophilic rimming. Thin sections showed bundles of amyloid fibrils intimately juxtaposed to bundles of collagen (Figure 2). Ultrastructurally, the fibrils measure ~ 7 nm in diameter. The rimmed vacuoles showed abundance of myeloid dense bodies (MDB) (Figure 3). There were numerous, tightly packed stacks of rough endoplasmic reticulum (RER), with interconnected cisternae (Figure 4). Some of the mitochondria in the proximity to the rimmed vacuoles show enlargement, but no paracrystalline inclusions were found. Next we searched for intracellular localization of amyloid fibrils in myocytes. Although we find some deposits of thin amyloid-like filaments free in the cytoplasm (Figure 3), these were not particularly numerous or abundant. Since the generation of amyloid in gelsolin model ought to be extracellular and its deposits commence at a perivascular space (16) we expect to find intracellular amyloid fibrils within the endosomal compartments. In fact, one hypothesis for the origin of MDB posits their derivation from endosomes. However, electron microscopic examination finds no evidence of endosomal localization of amyloid. Although non-membrane bound amyloid (asterisks on Figure 3) can be potentially derived from endosomes, which could be potentially lysed by toxic effects of amyloid, yet we find no appreciable intra-endosomal deposits of amyloid to confirm such a possibility. Additionally, Golgi complexes were not abundant and no identifiable amyloid could be discerned in Trans Golgi Network (TGN). In contrast, we observe abundant, thick and often electron dense deposits of amyloid like substance in distended organelles with a shape, distribution and direct connections (arrow on Figure 5) with RER. Direct measurements of RER-associated fibrils reveal a diameter of 9nm, which is larger than that found in the deposits of amyloid in the extracellular matrix.

We next set to determine whether RER-associated fibrils indeed contain gelsolin as defined by antibody staining. For this study we used 2.5% GA/1% osmium fixed and Araldite resin embedded tissue. GA/osmium fixation is optimal for preservation of conventional appearing morphology. Nevertheless, GA fixation extensively cross-links proteins, thus limiting access of antibodies. Osmium cleaves peptide bonds and might alter antigen to make it unrecognizable to abs. Hydrophobic and non-porous plastic also limits an access of antibodies, whereas unbound aldehyde groups react with antibodies and neutralize them. However, the sheer abundance of antigen in our animal model helps to maximize labeling efficiency. In addition, several protocols were gradually introduced to partially overcome the above limiting factors. In particular, one can etch the surface of plastic to expose more antigen (21), use heat retrieval with acidic or basic buffers $\pm$ EDTA in order to refold proteins to original confirmation, recognizable by antibodies (22). Incubation with amino-

acids, such as glycine can block free aldehyde groups of residual GA. Treatment of amyloid protein aggregates with 95% formic acid can loosen them up to maximize labeling.

In order to reliably localize gelsolin derived amyloid in Araldite sections at the ultrastructural level we performed a series of experiments in which combinations of ancillary techniques were used to improve labeling efficiency. We used FAF (Familial amyloidosis Finnish type) is a primary rabbit polyclonal antibodies. We have previously demonstrated that these antibodies recognize amyloid form of gelsolin, but do not bind to a full length protein or C68 peptide. The outline of experiments is listed in the Table 1.

We found that placement of tissue into and removal from 95% formic acid produces physico-chemical diffusion gradients with violent physical disruption of formvar-carbon coating. Given our successful labeling in other protocols we did not attempt to modify procedure and abandoned this protocol. No appreciable labeling was detected without etching step with sodium metaperiodate. We did not attempt to do immunolabeling without heat retrieval step. Addition of periodic acid after the etching step and heat retrieval did not improve the density of labeling. From our experiments we can conclude that etching of plastic followed by citrate buffer heat retrieval can provide sufficiently high specific density of labeling with our antigen-antibody pair. No significant non-specific labeling was observed and omission of primary antibody produced no labeling.

FAF antibodies disclosed intense staining of the extracellular compartment (Figure 6A), with appropriate sparing of collagen fibers. Within the myofibers we detected dense labeling of membrane bound compartments, with associated recognizable ribosomes and in a shape identical to distended RER observed on routine EM (Figure 6B).

Thus from our combined study we can conclude that intracellular deposits of amyloid gelsolin is predominantly found in the RER of myocytes in 18 months mice.

Next we compared electron microscopic findings of gelsolin model to human s-IBM. Demographic data on our cohort of 20 patients is shown in Table 2.

All of the cases demonstrated rimmed vacuoles, and contained myeloid dense bodies by TEM. Additionally, in 90% of cases we observed filamentous cytoplasmic inclusions whereas intramitochondrial paracrystalline inclusions were detected in 30% of cases (Figure 7). Many of the rimmed vacuoles had associated arrays of patterned smooth endoplasmic reticulum. However, we did not find a case with dilated endoplasmic reticulum with amyloid fibrils or endosome associated membrane bound amyloid deposits.

In conclusion, we find that gelsolin model of s-IBM produces formation of myeloid dense bodies akin to one seen in human s-IBM. However, we find multiple differences.

These include the presence of amyloid fibrils in RER vs cytosol, failure to generate intramitochondrial paracrystalline inclusion of human disease and also different morphology of sarcoplasmic reticulum aggregates.

Discussion

Human s-IBM is a disease of elderly, likely to be related to of cargo degradation by proteasome (23), and failing endoplasmic reticulum stress and unfolded protein response (24). We have previously shown that mutant gelsolin expressed in skeletal muscle leads to morphologic changes reminiscent of s-IBM, and produces muscle weakness (16). In this study we demonstrate that the gelsolin mice model has some differences from the human s-IBM, in particular accumulation of amyloid fibrils in RER.

It is unclear how amyloid fibrils can be produced in the RER, given a well established mandatory roles of TGN-associated furin and extracellular matrix associated MMP14 during the multi-step gelsolin amyloid processing pathway. One can propose that retention of misfolded gelsolin in RER triggers alternative proteolytic pathways, perhaps proteasome derived. In this scenario the myeloid dense bodies, whose cellular origin in IBM still remains to be firmly established, could be derived from ER. This could explain their laminated structure and extremely large membrane surface.

In this study we could not confirm our initial hypothesis, that skeletal muscle can absorb amyloid from the extracellular milieu, leading to engorgement of endosomes, their cytolysis and subsequent degenerative changes in the cytoplasm. Rather it seems like degenerative cytoplasmic changes might be initiated through the ER-associated unfolded protein response. Additional studies are needed to verify this hypothesis.

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Figure 1. Methylene blue stained 1 μ thick Araldite sections of 18 months old homozygous D187N (+/+) mouse. Note heavy deposits of amorphous endomysial substance (*) with relative sparing of nerves and vessels. Numerous rimmed vacuoles are indicated with arrows. Original magnification x 400.

Figure 2. Low (A) and high (B) magnification of extracellular amyloid deposits (*) in 18 months old homozygous D187N (+/+) mouse. Original magnifications: x 6,000 (A) and x 55,000 (B).

Figure 3. Ultrastructure of myeloid dense bodies (MDB) and cytosolic amyloid deposits (*) in 18 months old homozygous D187N (+/+) mouse. Original magnifications: x 20,000 (A), 18,000 (C), x 12,000 (B, D).

Figure 4. Proliferations and stacking of endoplasmic reticulum in 18 months old homozygous D187N (+/+) mouse. Original magnifications: x 25,000 (A–D).

Figure 5. Appearance of intracellular membrane bound amyloid deposits in 18 months old homozygous D187N (+/+) mouse. Connection to ribosome-studded RER is denoted with arrow on B. Original magnification x 30, 000.

Figure 6.
6 nm immunogold labeling of gelsolin amyloid using FAF antibodies in extracellular (A) and intracellular compartment (B). Original magnification x 110, 000.

Figure 7. Features of human s-IBM: myeloid dense bodies (A, B), filaments (asterisk on C) and mitochondrial paracrystalline inclusions (arrows on D). Original magnification x 2,000 (A, B), x 55,000, x 6,500 (D)

Table 1

Pre-treatment steps applied to Araldite resin embedded tissue before application of primary antibodies.

Step 1 (10min)	Step 2 (10min)	Step 3 (5min)	Step 4 (5min)	Labeling efficiency
1	SC heat retrieval	-	-	Negligible
4% SMP ²	SC heat retrieval	-	-	Good ³⁼⁴
4% SMP ³	SC heat retrieval	1% PA	-	Good ³⁼⁴
4% SMP ⁴	-	FA	-	Formvar failure
4% SMP ⁵	SC heat retrieval	1% PA	FA	Formvar failure

SMP: sodium metaperiodate, SC: citric acid, PA: periodic acid, FA: formic acid.

Table 2

Case #	Age	Gender	Filamentous inclusions	Intramitochondrial paracrystalline inclusions
1	64	M	No	No
2	68	M	Yes	Yes
3	64	M	Yes	No
4	79	M	Yes	Yes
5	74	F	Yes	No
6	77	F	Yes	No
7	65	F	Yes	No
8	80	M	Yes	No
9	75	M	No	No
10	70	M	Yes	No
11	56	F	Yes	No
12	78	M	Yes	No
13	71	M	Yes	No
14	72	M	Yes	Yes
15	66	M	Yes	No
16	69	M	Yes	Yes
17	71	M	Yes	Yes
18	64	M	Yes	Yes
19	63	F	Yes	No
20	77	M	Yes	No