accumulations of phosphoserine-containing protein deposits in s-IBM vacuolated fibers. Western blots of muscle lysates demonstrated a 35 kD phosphoprotein. They concluded that the hyperexpression of 35kD protein may represent cytoskeletal by-products due to ERK activation and that the abundant expression of phosphoserine-containing protein in s-IBM implies that hyperphosphorylated myofibrillar proteins may be involved in the primary disease process.

4.3 ERK- or Elk-1–positive deposits are often perinuclear in s-IBM muscle fibers

With nuclear DNA staining, we found that ERK- or pElk-1–positive deposits were often detected on the external surface of the nuclei, although they were sometimes present also in the cytoplasm unrelated to the nuclear localization. There were sometimes overlaps of the positive deposits and nuclei. In rare fibers, protrusions of the positive deposits into nuclei were observed. A quantitative study of the relationship between ERK-positive deposits and nuclei in ERK-positive fibers showed that 78.2% of the nuclei were closely associated with the deposits; 3.2% of the nuclei had ERK-positive deposits occupying more than half of their area, and 75.0% of the nuclei were touched, penetrated, or partially covered by the deposits. The nuclear transcription factor pElk-1 displayed similar cytoplasmic aggregation and perinuclear localization. There was cytoplasmic and perinuclear inclusions of ERK in vacuolated fibers, but not of JNK or p38. JNK and p38, however, showed strong activity in regenerating fibers as ERK (Nakano et al 2001).
During muscle fiber differentiation, ERK is the last MAPK that becomes activated (Gredinger et al 1998, Zetser 1999). Therefore, the abnormality that causes ERK deposition may occur in the last phase of differentiation, when JNK and p38 activities have decreased.

4.4 Analysis of MKKs and MAP kinase phosphatases

4.4.1 MAP kinase kinases(MKKs)

ERK appeared to be up-regulated in vacuolated fibers in IBM and ERK is activated by MKK1/2 in the phosphorylation cascade triggered by extracellular stimuli (Fig. 3.). We therefore next tested MKK1/2 in s-IBM (Nakano et al 2003). Whereas in normal muscle fibers, weak immunoreactivity of MKK1/2 was observed, strong immunoreactivity of MKK1/2 was found in some of the regenerating or degenerating muscle fibers. In IBM, vacuolated fibers showed no or mild cytoplasmic immunoreactivity for MKK1/2, even fibers with ERK-positive inclusions. We then tested MKK3 and MKK4 to reject the possibility that other MKK might induce ERK in IBM, although MKK3 and MKK4 actually activate p38 MAPK or JNK, but not ERK (Fig. 3.) (Reffas and Schlegel 2000). Regenerating/degenerating fibers showed positive immunoreaction for these MKKs, vacuolated fibers in IBM were negative for MKK3 or MKK4.
Concerning to the increased MKKs in regenerating/degenerating fibers, growth factors promoting myogenesis (Groungs 1999) or cytokines locally produced or ischemic stresses in the affected tissue in inflammatory myopathies (Lundberg et al 1997) could induce them. As a proportion of vacuolated fibers also showed some positivity for MKK1/2, comparable myogenic factors or other extracellular signals might induce ERK cascade in vacuolated fibers in IBM. However, the intensity of the immunoreaction of MKK1/2 in vacuolated fibers was weaker than those regenerating/degenerating fibers in control specimens and the reaction did not form inclusions. The results exclude a possibility that a specific extracellular signal induces the increase of ERK protein.

4.4.2 MKPs

The study of MAP kinase phosphatases (MKPs), i.e., enzymes that deactivate MAPKs, was done with MKP-1, MKP-2 and MKP-3 (Nakano et al 2003). In MKP-1 analysis, some regenerating/degenerating fibers showed strong nuclear staining with moderate cytoplasmic positivity of MKP-1. In IBM, vacuolated fibers or some other structurally abnormal fibers contained inclusions that were strongly immunoreactive for MKP-1. The MKP-1-positive inclusions were colocalized with ERK in dual fluorescence study. Inclusions of MKP-2 with less conspicuous than MKP-1 were found in some vacuolated fibers. Although diffusely increased Immunoreactivity of MKP-3 was found in some regenerating fibers, MKP-3 was negative in vacuolated fibers.

MKP-1 expression increases during the early stage of myogenesis, and regulates ERK at the stage of muscle specific gene expression (Bennett and Tonks 1997, Shi et al 2010). The findings indicating that regenerating fibers showed increased expression of MKP-1 are consistent with the experimental results. In ERK phosphorylation cascade, MKP-1 serves as a negative regulator of ERK (Robinson and Cobb 1997). Moreover, MKPs make a tight complex with their substrates when catalyzed. Thus, it is highly probable that MKP-1 is induced to inactivate ERK in s-IBM vacuolated fibers.

4.5 Conclusion of MAPK cascades study: abnormal deposition of nuclear proteins involved in myogenesis

Nuclear migration of ERK is necessary for myogenic gene expression (Gredinger et al 1998). Based on the results of our MAPK cascade study, we hypothesize an inhibition of protein transport from the cytoplasm into the nucleus. In s-IBM muscle fibers, normal levels of activation of ERK phosphorylation cascade may proceed down to MKK1/2, the activations of which occur on the plasma membrane or in the cytoplasm, triggered by myogenic or other stimulation in s-IBM-vacuolated fibers. Moreover, frequent perinuclear accumulation of ERK protein in vacuolated fibers suggests that the nuclear translocation of ERK is inhibited. Due to aggregation of ERK, the ERK protein might accumulate in the cytoplasm and become unable to move across the nuclear envelope. Otherwise, due to impaired nuclear transmigration of ERK protein, it could deposit in the cytoplasm and perinuclear region. Activated ERK phosphorylates its nuclear substrates probably immediately after its synthesis and forms complexes in the cytoplasm. The abnormal activation of ERK could induce MKP-1. These enzyme-substrate complexes further congregate together in the cytoplasm. The protein complexes might grow to the "aggresomes" in the perinuclear region to process the aggregates with extralysosomal protein degradation system (Johnston et al 1999). Some of the components of aggresomes were indeed found in s-IBM muscle fibers (Ferrer et al 2005).

Nuclear transport of ERK is a mediated process. This process is required for the induction of many cellular responses, yet the molecular mechanisms that regulate ERK nuclear translocation are not fully understood (Lidke et al 2010). In s-IBM, presence of specific antibodies against the nucleus has been shown (Dalakas et al 1997). Sera from patients with s-IBM and other idiopathic inflammatory myopathies sometimes contain antibodies against nuclear enzymes and components (Brouwer et al 2001). Furthermore, several autoimmune-diseases are associated with autoantibodies against chaperone proteins as well as well-known anti-nuclear antibodies (Corrigall et al 2001). In experiment, injection of antibodies against a heat shock cognate protein 70 that assists nuclear transport results in cytoplasmic accumulation of several nuclear proteins in human cell cultures (Imamoto

et al 1992). Inhibition of carrier proteins or nuclear pore proteins involved in the nuclear transport results in the cytoplasmic and perinuclear accumulation of the cargo proteins (Görlich and Mattaj 1996). It is, therefore, suggested that a certain autoimmune mechanism could affect molecules involved in nuclear transport of ERK and induce cytoplasmic accumulation of ERK and its associated proteins in vacuolated fibers in IBM. Apart from autoimmune mechanism, nuclear envelope dysfunction could be aggravated by reactive oxygen species induced by inflammatory cytokines and by aging, as we will discuss them later.

Lack of proper nuclear migration of ERK inhibits MyoD expression (Gredinger et al 1998). Furthermore, forced induction of MKP-1 during myotube formation prevents myoblast fusion when the expression of the myosin heavy chain has occurred (Bennett and Tonks 1997). It is suggested that in s-IBM, there is an altered program of myogenesis due to abnormal aggregation of nuclear proteins that are associated with the differentiating process of muscle. The aggregation in turn may induce the protein degradation system, such as proteasmal pathways (Ferrer et al 2004) and autophagy (Kumamoto et al 2004), both of which are increased in s-IBM muscle fibers.

Our earlier report has shown CDK5-positive deposits in vacuolated fibers in s-IBM. A high proportion of the CDK5-positive deposits were perinuclear, as ERK. CDK5 co-localized with SMI-31 reactive deposits as ERK (Nakano et al 1999). CDK5, like ERK, belongs to the proline-directed kinases which can phosphorylate serine or threonine followed by proline sequences. CDK5 transiently appears in the nucleus during the terminal differentiation and promotes the process (Lazaro et al 1997). Thus, two protein kinases ERK and CDK5, both normally activated and translocated into the nucleus during the terminal phase of differentiation, accumulate in the cytoplasm and around the nuclei in s-IBM vacuolated fibers. We therefore hypothesize that the induction of ERK and CDK5 is part of the intrinsic program of muscle fiber differentiation, and one possibility is that the abnormally high concentration of these enzymes results from their aggregation in the cytoplasm and inability to enter the nucleus.

5. Nuclear abnormality in s-IBM: history

Several studies have shown distinct myonuclear alterations in s-IBM before establishing its nosology. In ultrastructural study, filamentous inclusions were sometimes detected in myonuclei as well as in the cytoplasm (Chou 1967). These inclusions in rare occasion appeared to be released from nuclei into the cytoplasm with breaks in the nuclear membrane. In s-IBM, but not in controls, myonuclei accumulate an unidentified a single stranded DNA-binding protein (Nalbantoglu et al 1994). Most of the sites of binding were myonuclei, whereas some were rimmed vacuoles. The figures suggest that rimmed vacuoles probably result from nuclear breakdown. Recent studies added valosin-containing protein (VCP) (Greenberg et al 2007b) and TDP-43 (Salajegheh et al 2009) to the list of proteins localized in both nuclear and rimmed vacuoles, supporting the hypothesis that nuclear breakdown results in rimmed vacuoles.

As our phosphoprotein study suggested the nuclear alterations in s-IBM vacuolated fibers, we thought to identify elemental components in rimmed vacuoles. If rimmed vacuoles originate from the nucleus, the basophilic components associated with them should be some components of the nucleus. Histones are representative of the basophilic substances, which prompted us to investigate histones in s-IBM.

While our preparation of papers with histone H1, another group showed existence of nuclear membrane protein emerin and lamin A/C within rimmed vacuoles (Greenberg et al 2006)

6. Immunolocalization study of histones in sporadic inclusion body myositis (s-IBM)

6.1 Histones and dynamic function of histone H1

In inactive chromatin, the DNA is combined to histones and forms nucleosomes. A nucleosome is an octamer of four pairs of the core histones H2A, H2B, H3 and H4. Double-stranded DNA twines around nucleosomes. Histone H1 binds to the linker DNA that connects the individual nucleosomes. Among histones, histone H1 shows dynamic behavior to regulate chromatin folding and gene expression, while core histones are integral components of chromatin fibers (Bustin et al 2005). The H1 binding to linker DNA is essential for the generation of the highly condensed chromatin structure and plays a pivotal role in gene regulation (Brown 2003). H1 is rich in arginine and lysine residues, which makes it highly basic (Woodcock et al 2006).

6.2 Histone H1, but not core histones (H2A, H2B, H3 and H4), are associated with rimmed vacuoles

Figure 4 displays the results in triple-fluorescence study of histone H1, emerin (a nuclear envelope protein associated with inner nuclear membrane) and DAPI (a marker of nuclear DNA). After the fluorescence studies, the same sections are stained with H&E as shown in the right column. The figures clearly show rimmed vacuole are products of nuclear degeneration. They reveal several modes of nuclear alterations. 1) H1-positive rings or deposits are associated with the nuclear membrane protein and DNA. Strong vacuolar H1-positive reaction colocalized with a DNA ring is detected inside or on emerin-positive reaction in a proportion of vacuoles. The figures suggest swelling or ballooning of nuclei with scarce nuclear matrix proteins. 2) H1-positive reaction that appeared to be leaking beyond emerin-positive lines is found in some other vacuoles. The cytoplasmic release of H1 is also observed in some morphologically intact nuclei (Nakano et al 2008). The H&E after the fluorescence study showed that H1 or emerin-positive products in vacuoles often appeared to correspond to the basophilic lines in H&E. A region of cytoplasmic H1-positive reaction usually corresponded to basophilic lakes around vacuoles or nuclei in H&E. The results indicated that release of H1 occurs even in an early phase of nuclear breakdown. In contrast, although regenerating fibers show increased H1 reactivity in their large and vesicular nuclei, H1-positive products fall within these nuclei. Calculation indicated that approximately 60% of vacuolated fibers contained H1-positive rings or other H1-positive remnants (Nakano et al 2008). Conversely, immunohistochemistry of histones H2A, H2B, H3 and H4 showed rare vacuoles harbored positive deposits. The comparative study with immunofluorescence and subsequent H&E staining suggested that histone H1 and other nuclear proteins comprise basophilic granules in rimmed vacuoles.

6.3 Cytoplasmic release of histone H1 suggests DNA double strand breaks

Cytoplasmic release of H1, but not other histones, has been observed in a type of apoptosis in an experimental study of cultured human cells: apoptosis induced by stimuli causing DNA double strand breaks such as X-ray irradiation, but not other apoptotic stimuli,

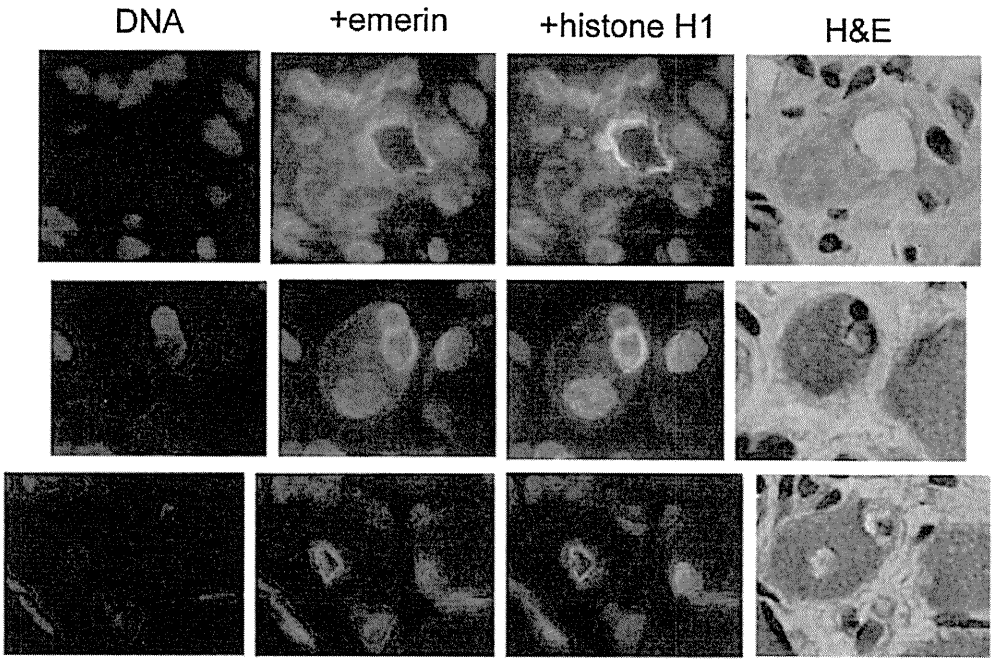

Fig. 4. Triple fluorescence studies in s-IBM vacuolated fibers. Blue: nuclear DNA; Green: emerin; Red: histone H1. Vacuoles usually appear to be more accentuated in H&E, probably due to dehydration process. The background green colour is purposely heightened to visualize muscle fibers.

releases histone H1 into the cytoplasm (Konishi et al 2003). Therefore, the cytoplasmic H1 release in s-IBM might indicate that some apoptotic stimuli causing DNA double strand breaks induce the s-IBM pathology. Apoptotic process exemplified by TUNEL revealed that it may scarcely operate in s-IBM muscle fibers (Hutchinson 1998). Nevertheless, several studies displayed some players of apoptosis in s-IBM muscle fibers (Behrens et al 1997, Li and Dalakas 2000b)

7. DNA double strand breaks (DSB) in s-IBM

7.1 The DNA damage responses

The primary structure of DNA is constantly exposed to cellular metabolites and extracellular DNA-damaging agents. These alterations can affect the cell to transcription of the genes. Other lesions induce potentially harmful mutations. Consequently, the DNA repair process must be constantly activated to respond to the damages in the DNA structure. Defects of the repair processes may cause genomic instability. To repair damage to one of the two paired molecules of DNA, there are many excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide complementary to that found in the undamaged DNA strand. The examples of these are base excision repair, nucleotide excision repair, and DNA mismatch repair.

DNA double strand breaks (DSB), in which both strands in the double helix are severed, are particularly serious to the cell because they can lead to genome rearrangements. DSB are produced by reactive oxygen species, ionizing radiation, chemicals that generate reactive oxygen species and replication error. DSB are also a normal result of V(D)J recombination and immunoglobulin class-switching process. DSB are repaired either by homologous recombination (HR) or nonhomologous end-joining (NHEJ) mechanism (O'Driscol and Jeggo 2006). HR plays only in replicating cells, while NHEJ functions in both cells in the cell cycle and those terminally differentiated. Mature muscle cells are terminally differentiated cells, that is, the cells withdraw from the cell replication cycle. Terminally differentiated cells do not possess a replication-associated DNA repair mechanism (HR mentioned above). This lack makes the terminally differentiated cells particularly sensitive to DNA damage (Lee and McKinnon 2007). In a muscle cell culture study, the exposure of differentiated myocytes to hydrogen peroxide, which induces reactive oxygen species, resulted in the accumulation of foci of DSB. It is exemplified by immunolocalization of phosphorylated histone H2AX (γ-H2AX) (Narciso et al 2007). The detection of γ-H2AX is a sensitive marker of DSB (Nakamura 2006). Histone H2AX that is a variant of histone H2A is rapidly phosphorylated at Ser 139 in the chromatin region surrounding a DSB (Kinner 2008). Immunocytochemical staining of γ-H2AX has been broadly applied to reveal DNA damage caused by cancer and other cellular stresses (Nakamura 2006, Kinner 2008).

DSB is different from the apoptotic DNA fragmentation that has been residually detected in the s-IBM muscles (Hutchinson 1998). In DSB, DNA breaks are induced directly and randomly by radiation or other genotoxic agents, whereas apoptotic DNA fragmentation occurs at a late stage of programmed cell death, when endonucleases sever DNA strands at regular lengths, making a ladder formation in Southern blotting.

DNA-PK is an enzyme involved in the initial step of the DSB repair process NHEJ, which does not require DNA replication, and therefore NHEJ is the major DNA repair mechanism in terminally differentiated cells (O'Driscoll and Jeggo 2006, Mahaney et al 2009). DNA-PK consists of a catalytic subunit (DNA-PKcs) and two regulatory subunits (Ku70 and Ku80). The binding of hetero-duplexes of Ku70 and Ku80 to DSB sites initiates the repair process (Mari et al 2006, Weterings and Chen 2007).

We immunolocalized γ-H2AX in s-IBM and we also tested DNA-PK to see whether the repair mechanism is defective or not (Nishii et al 2011). In the study, vacuolar peripheries often showed strong immunoreactivity to γ-H2AX and the three components of DNA-PK (DNA-PKcs, Ku70, and Ku80). The percentage of positive nuclei for γ-H2AX was significantly higher in vacuolated fibers than non-vacuolated fibers in s-IBM, or fibers in polymyosits suggesting that nuclear breakdown occurs along with the accumulation of DSB in muscle cells in s-IBM. Moreover, a triple fluorescence study of Ku70, emerin, and DNA suggested impaired nuclear incorporation of Ku70. Nuclear translocation of Ku proteins is important for DBS repair, and a deficiency in nuclear translocation caused hypersensitivity against X-ray irradiation due to the lack of DBS repair in a cell culture study (Okui et al 2002). Therefore, we hypothesized that defects in Ku70 nuclear import accelerate DSB formation in s-IBM.

Despite DSB was the highest in s-IBM vacuolated fibers, DSB was sometimes found to be increased in myonuclei without nuclear breakdown. Therefore, additional factors may be involved in the nuclear breakdown detected in s-IBM. We consider that a dysfunction of nuclear envelope may explain all the alterations in s-IBM: 1) nuclear fragility; 2) DNA double-strand breaks; and 3) impaired nuclear transport in s-IBM.

Impaired DSB results growth arrest, senescence, and apoptosis (Rossetto et al 2010). Our earlier examination showed aberrant expressions of proteins associated with myogenic differentiation. In s-IBM, the accumulation of DSB could result in arrest of muscle fiber maturation.

8. Possible mechanism of nuclear breakdown

Nuclear envelope dysfunction can cause both mechanical fragility of the nucleus and DNA damage. Lamins are proteins of nuclear intermediate filaments that comprise the lamina, the meshwork supporting inner nuclear membranes. Mutations in the genes that encode lamins and emerin cause Emery-Dreifuss muscular dystrophy and a number of different diseases collectively called laminopathies (Capell and Collins 2006). In several laminopathies, blebbing of the nuclei in cultured fibroblasts can be seen, and it is hypothesized that such mutations result in fragile and mechanically unstable nuclei (Goldman et al 2004). Indeed, emerin mutations can cause myopathy with rimmed vacuoles (Paradas et al 2005, Fidziańska et al 2004). Besides structural integrity, the lamina is also involved in various other processes, such as replication and gene transcription, which are intimately associated with DNA damage repair. Accordingly, impaired DNA repair has been found in several laminopathies. Fibroblasts possessing a laminopathy mutation show an excessive amount of un-repaired DNA damage, as exemplified by γ-H2AX immunohistochemistry (Liu et al 2005). 3) Furthermore, lamins are important in the spatial rearrangement of nuclear pore complexes and therefore nuclear protein transport. Nuclear protein import is reduced in cells expressing lamin A mutants (Busch et al 2009). We repeatedly detected figures suggestive of impaired nuclear import of proteins, as has been described in our phosphorylated protein study.

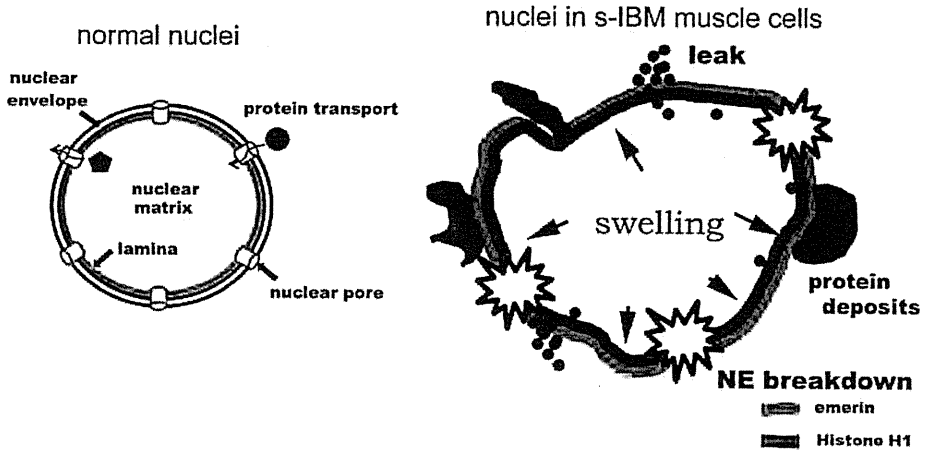

Fig. 5. Simplified schema of normal nuclei and nuclei in s-IBM muscle fibers. In s-IBM, nuclear proteins deposits occur in perinuclear regions due to inhibition of nuclear import, whereas histone H1 is released from nnclei. Finally, the nuclear envelopes break down to form rimmed vacuoles.

To summarize, dysfunctional lamins can explain the nuclear breakdown, accumulation of DSB, and impaired nuclear transport observed in s-IBM. As discussed in the section about perinuclear deposition of protein kinases, autoimmune mechanism could operate in the dysfunction of nuclear envelope. In addition, aging might increase the nuclear vulnerability and DNA damage. Nuclear pore complexes are not turned over in differentiated cells, and age-related alterations in nuclear pore complexes have been shown. Leaking of nuclear matrix proteins is dramatically accelerated during aging and that a subset of nucleoporins (components of nuclear pores) is oxidatively damaged in old cells (D'Angelo et al 2009). Moreover, several studies have indicated an age-dependent decline in DNA repair capacity (Gorbunova et al 2007). We suspect that these age-associated changes in nuclear envelope function and DNA repair mechanisms may predispose the muscles of the elderly to s-IBM pathology.

9. Similarity of s-IBM and DMRV/h-IBM

We found inclusions of a set of nucleus-oriented or nucleus-proper proteins in distal myopathy with rimmed vacuoles (DMRV)/hereditary inclusion body myopathy (h-IBM), a disorder in which the muscle biopsy displays rimmed vacuoles in muscle fibers as in s-IBM (Fig. 6) (Nakano et al 1999, 2001, 2003, 2008).

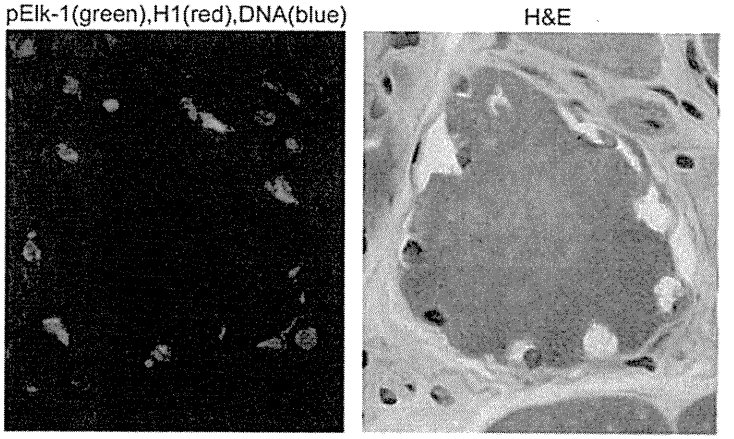

Fig. 6. Immunohistochemistry of pElk-1 and histone H1 in DMRV/h-IBM. Many pElk-1-positive deposits are seen in vacuoles .

Muscle pathology of DMRV/s-IBM shows rimmed vacuoles and tubulofilaments like s-IBM, but it lacks inflammation. The mutated gene for this autosomal recessive disease has been identified to be involved in glycosylation, named UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) (Eisenberg et al 2001, Nishino et al 2002). Remarkably, N-acetylglucosamine, the substrate of this enzyme is also a substrate of UDP-N-acetylglucosamine:polypeptide β-N-acetylglucosaminyl transferase (OGT), the enzyme that adds O-linked-β-N-acetylglucosamine (O-GlcNAc) to a protein. A thousand of proteins including transcription factors, cytoskeletal proteins, kinases and nuclear pore proteins are modified with O-GlcNAc (Zachara and Hart 2004). O-GlcNAc is a highly dynamic process

and acts as a modulator of protein function, in a manner analogous to protein phosphorylation. Moreover, there is a complex crosstalk between O-GlcNAc modification and phosphorylation. The two post-translational modifications often regulate in an opposite manner by competitive attachment to the same serine/threonine residue, but they sometimes function co-operatively by binding at different sites of the same molecule (Zeidan 2010). In DMRV/h-IBM as well as in s-IBM, abnormal expression of proteins concerning to phosphorylation could be related to perturbation of the O-GlcNAc modification of proteins.

10. Conclusion

We have examined myonuclear dysfunction s-IBM. Similar degenerative mechanism may exist in DMRV/h-IBM that shows almost identical pathology and nuclear breakdown concerning to muscle fiber degeneration (Nonaka et al 1998). To reveal how GNE enzyme dysfunction affects myonuclei in this disorder may contribute to unveil the etiology of s-IBM. In addition, myofibrillar myopathy is a genetic disorder in which mutations of several Z-line associated proteins have been identified. Muscle biopsy studies have found congophilic inclusions (Selcen and Engel 2010). Players involved in excessive protein processing have been detected in s-IBM and myofibrillar myopathy (Ferrer et al 2004, 2005). Moreover, the disorder sometimes accompanies rimmed vacuoles (Shinde et al 2008). The comparative study of s-IBM and myofibrillar myopathy may also be helpful.
Concerning to the relationship between inflammation and nuclear breakdown, some immunological mechanism could operate in nuclear envelope dysfunction. Otherwise, nuclear aging and decrease of DNA repair capacity due to aging could induce the nuclear degeneration.

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12. References

Askanas V, Engel WK, Alvarez RB. Light and electron microscopic localization of beta-amyloid protein in muscle biopsies of patients with inclusion-body myositis. Am J Pathol. 1992 Jul;141(1):31-6.

Askanas V, Engel WK, Bilak M, Alvarez RB, Selkoe DJ. Twisted tubulofilaments of inclusion body myositis muscle resemble paired helical filaments of Alzheimer brain and contain hyperphosphorylated tau. Am J Pathol 1994;144:177–187.

Askanas V, Engel WK. Inclusion-body myositis: muscle-fiber molecular pathology and possible pathogenic significance of its similarity to Alzheimer's and Parkinson's disease brains. Acta Neuropathol. 2008 Dec;116(6):583-95.

Banker B, Engel AG. Basic reactions of muscle. In: Engel AG, Franzini–Armstrong C, ed. Myology. 2nd ed. New York, NY: McGraw-Hill, 1994: 832–888.

Behrens L, Bender A, Johnson MA, Hohlfeld R. Cytotoxic mechanisms in inflammatory myopathies. Co-expression of Fas and protective Bcl-2 in muscle fibres and inflammatory cells. Brain. 1997 Jun;120 (Pt 6):929-38.

Bennett A, Tonks NK. Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. Science 1997;278:1288-1291.

Brouwer R, Hengstman GJ, Vree Egberts W, Ehrfeld H, Bozic B, Ghirardello A, Grøndal G, Hietarinta M, Isenberg D, Kalden JR, Lundberg I, Moutsopoulos H, Roux-Lombard P, Vencovsky J, Wikman A, Seelig HP, van Engelen BG, van Venrooij WJ. Autoantibody profiles in the sera of European patients with myositis. Ann Rheum Dis 2001;60:116-123.

Brown DT. Histone H1 and the dynamic regulation of chromatin function. Biochem Cell Biol 2003;81:221-227.

Busch A, Kiel T, Heupel WM, Wehnert M, Hübner S. Nuclear protein import is reduced in cells expressing *O*-GlcNAc nuclear envelopathy-causing lamin A mutants. Exp Cell Res 2009;315:2373-2385.

Bustin M, Catez F, Lim JH. The dynamics of histone H1 function in chromatin. Mol Cell 2005;17:617-620.

Capell BC, Collins FS. Human laminopathies: nuclei gone genetically awry. Nat Rev Genet 2006;7:940-952.

Carpenter S, Karpati G, Wolfe L. Virus like filaments and phospholipid accumulations in a case of chronic myopathy. J Neuropathol Exp Neurol. 1971 Jan;30(1):136-7.

Carpenter S, Karpati G, Heller I, Eisen A. Inclusion body myositis: a distinct variety of idiopathic inflammatory myopathy. Neurology. 1978 Jan;28(1):8-17.

Chou SM. Myxovirus-like structures in a case of human chronic polymyositis. Science. 1967 Dec 15;158(807):1453-5.

Corrigall VM, Bodman-Smith MD, Fife MS, Canas B, Myers LK, Wooley P, Soh C, Staines NA, Pappin DJ, Berlo SE, van Eden W, van Der Zee R, Lanchbury JS, Panayi GS. The human endoplasmic reticulum molecular chaperone BiP is an autoantigen for rheumatoid arthritis and prevents the induction of experimental arthritis. J Immunol 2001;166:1492-1498.

Dalakas M, Illa I, Gallardo E, Juarez C. Inclusion body myositis and paraproteinemia: Incidence and immunopathologic correlations. Ann Neurol 1997;41:100-104.

Dalakas MC. Sporadic inclusion body myositis--diagnosis, pathogenesis and therapeutic strategies. Nat Clin Pract Neurol 2006;2:437-447.

D'Angelo MA, Raices M, Panowski SH, Hetzer MW. Age-dependent deterioration of nuclear pore complexes causes a loss of nuclear integrity in postmitotic cells. Cell. 2009 Jan 23;136(2):284-95.

Dubiwitz V, Sewry CA. Metabolic myopathies I: Glycogenosis. Muscle biopsy. 3rd ed., Sanders/Elsevier 2007: pp 453-468

Eisenberg I, Avidan N, Potikha T, Hochner H, Chen M, Olender T, Barash M, Shemesh M, Sadeh M, Grabov-Nardini G, Shmilevich I, Friedmann A, Karpati G, Bradley WG, Baumbach L, Lancet D, Asher EB, Beckmann JS, Argov Z, Mitrani-Rosenbaum S. The UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase gene is mutated in recessive hereditary inclusion body myopathy. Nat Genet. 2001 Sep;29(1):83-7.

Ferrer I, Carmona M, Blanco R, Moreno D, Torrejón-Escribano B, Olivé M. Involvement of clusterin and the aggresome in abnormal protein deposits in myofibrillar myopathies and inclusion body myositis. Brain Pathol. 2005;15(2):101-8.

Ferrer I, Martín B, Castaño JG, Lucas JJ, Moreno D, Olivé M. Proteasomal expression, induction of immunoproteasome subunits, and local MHC class I presentation in myofibrillar myopathy and inclusion body myositis. J Neuropathol Exp Neurol. 2004 May;63(5):484-98.

Fidziańska A, Rowińska-Marcińska K, Hausmanowa-Petrusewicz I. Coexistence of X-linked recessive Emery-Dreifuss muscular dystrophy with inclusion body myositis-like morphology. Acta Neuropathol 2004;107:197-203.

Force T, Bonventre JV. Growth factors and mitogen-activated protein kinases. Hypertension 1998; 31: 152–161.

Goldman RD, Shumaker DK, Erdos MR, et al. Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. Proc Natl Acad Sci U S A 2004;101:8963-8968.

Gorbunova V, Seluanov A, Mao Z, Hine C. Changes in DNA repair during aging. Nucleic Acids Res 2007;35:7466-7474.

Görlich D, Mattaj IW. Nucleocytoplasmic transport. Science 1996; 271: 1513–1518.

Gredinger E, Gerber AN, Tamir Y, Tapscott SJ, Bengal E. Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. J Biol Chem 1998;273:10436-10444.

Greenberg SA, Pinkus JL, Amato AA. Nuclear membrane proteins are present within rimmed vacuoles in inclusion-body myositis. Muscle Nerve 2006;34:406-416.

Greenberg SA, Pinkus GS, Amato AA, Pinkus JL. Myeloid dendritic cells in inclusion-body myositis and polymyositis. Muscle Nerve. 2007a Jan;35(1):17-23.

Greenberg SA, Watts GD, Kimonis VE, Amato AA, Pinkus JL. Nuclear localization of valosin-containing protein in normal muscle and muscle affected by inclusion-body myositis. Muscle Nerve. 2007b Oct;36(4):447-54.

Greenberg SA. How citation distortions create unfounded authority: analysis of a citation network. BMJ. 2009 Jul 20;339:b2680.

Griggs RC, Askanas V, DiMauro S, Engel A, Karpati G, Mendell JR, Rowland LP. Inclusion body myositis and myopathies. Ann Neurol 1995;38:705–713.

Grounds M. Muscle regeneration: molecular aspects and therapeutic implications. Curr Opin Neurol 1999;12:353-543.

Hartlerode AJ, Scully R. Mechanisms of double-strand break repair in somatic mammalian cells. Biochem J. 2009 Sep 25;423(2):157-68.

Hill C, Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 1995; 80: 199–211.

Hunter T, Karin M. The regulation of transcription by phosphorylation. Cell.1992 Aug 7;70(3):375-87.

Hutchinson DO. Inclusion body myositis: abnormal protein accumulation does not trigger apoptosis. Neurology. 1998 Dec;51(6):1742-5.

Imamoto N, Matsuoka Y, Kurihara T, Kohno K, Miyagi M, Sakiyama F, Okada Y,Tsunasawa S, Yoneda Y. Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. J Cell Biol 1992; 119: 1047-1061

Johnston JA, Ward CL, Kopito RR. Aggresomes: a cellular response to misfolded proteins. J Cell Biol 1998; 143:1883-1898.

Keyse S. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr Opin Cell Biol 2000;12:186-192.

Kinner A, Wu W, Staudt C, Iliakis G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res 2008;36:5678-5694.

Konishi A, Shimizu S, Hirota J, Takao T, Fan Y, Matsuoka Y, Zhang L, Yoneda Y, Fujii Y, Skoultchi AI, Tsujimoto Y. Involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks. Cell 2003;114:673-688

Kumamoto T, Ueyama H, Tsumura H, Toyoshima I, Tsuda T. Expression of lysosome-related proteins and genes in the skeletal muscles of inclusion body myositis. Acta Neuropathol. 2004 Jan;107(1):59-65.

Kyriakis J, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81:807-869.

Lazaro J, Kitzmann M, Poul MA, Vandromme M, Lamb NJ, Fernandez A. Cyclin dependent kinase 5, cdk5, is a positive regulator of myogenesis in mouse C2 cells. J Cell Sci 1997; 110: 1251–1260.

Lee Y, McKinnon PJ. Responding to DNA double strand breaks in the nervous system. Neuroscience 2007;145:1365-1374.

Li J, Johnson SE. ERK2 is required for efficient terminal differentiation of skeletal myoblasts. Biochem Biophys Res Commun. 2006 Jul 14;345(4):1425-33.

Li M, Dalakas MC. The muscle mitogen-activated protein kinase is altered in sporadic inclusion body myositis. Neurology. 2000a Apr 25;54(8):1665-70.

Li M, Dalakas MC. Expression of human IAP-like protein in skeletal muscle: a possible explanation for the rare incidence of muscle fiber apoptosis in T-cell mediated inflammatory myopathies. J Neuroimmunol. 2000b Jul 1;106(1-2):1-5.

Lidke DS, Huang F, Post JN, Rieger B, Wilsbacher J, Thomas JL, Pouysségur J, Jovin TM, Lenormand P. ERK nuclear translocation is dimerization-independent but controlled by the rate of phosphorylation. J Biol Chem. 2010 Jan 29;285(5):3092-102.

Liu B, Wang J, Chan KM, Tjia WM, Deng W, Guan X, Huang JD, Li KM, Chau PY, Chen DJ, Pei D, Pendas AM, Cadiñanos J, López-Otín C, Tse HF, Hutchison C, Chen J, Cao Y, Cheah KS, Tryggvason K, Zhou Z. Genomic instability in laminopathy-based premature aging. Nat Med 2005;11:780-785.

Lluís F, Perdiguero E, Nebreda AR, Muñoz-Cánoves P. Regulation of skeletal muscle gene expression by p38 MAP kinases. Trends Cell Biol. 2006 Jan;16(1):36-44.

Lotz BP, Engel AG, Nishino H, Stevens JC, Litchy WJ. Inclusion body myositis. Observations in 40 patients. Brain. 1989 Jun;112 (Pt 3):727-47."

Lundberg I, Ulfgren A, Nyberg P,Andersson U, Klarreskog L. Cytokine production in muscle tissue of patients with idiopathic inflammatory myopathies. Arth Rheum 1997;40:865-874.

Lünemann JD, Schmidt J, Schmid D, Barthel K, Wrede A, Dalakas MC, Münz C. Beta-amyloid is a substrate of autophagy in sporadic inclusion body myositis. Ann Neurol. 2007 May;61(5):476-83.

Mahaney BL, Meek K, Lees-Miller SP. Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. Biochem J 2009;417:639-650.

Maurage CA, Bussière T, Sergeant N, Ghesteem A, Figarella-Branger D, Ruchoux MM, Pellissier JF, Delacourte A. Tau aggregates are abnormally phosphorylated in inclusion body myositis and have an immunoelectrophoretic profile distinct from other tauopathies. Neuropathol Appl Neurobiol. 2004 Dec;30(6):624-34.

Mari PO, Florea BI, Persengiev SP, Verkaik NS, Brüggenwirth HT, Modesti M, Giglia-Mari G, Bezstarosti K, Demmers JA, Luider TM, Houtsmuller AB, van Gent DC. Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. Proc Natl Acad Sci U S A 2006;103:18597-18602.

Mendell JR, Sahenk Z, Gales T, Paul L. Amyloid filaments in inclusion body myositis. Novel findings provide insight into nature of filaments. Arch Neurol. 1991 Dec;48(12):1229-34.

Mirabella M, Alvarez RB, Bilak M, Engel WK, Askanas V. Difference in expression of phosphorylated tau epitopes between sporadic inclusion-body myositis and

hereditary inclusion-body myopathies. J Neuropathol Exp Neurol. 1996 Jul;55(7):774-86.

Nakamura A, Sedelnikova OA, Redon C, Pilch DR, Sinogeeva NI, Shroff R, Lichten M, Bonner WM. Techniques for gamma-H2AX detection. Methods Enzymol 2006;409:236-250.

Nakano S, Akiguchi I, Nakamura S, Satoi H, Kawashima S, Kimura J. Aberrant expression of cyclin-dependent kinase 5 in inclusion body myositis. Neurology 1999; 53: 1671–1676.

Nakano S, Kawashima S, Satoi H, et al. Active form of ERK2 is a major phosphorylated protein in vacuolated fibers in sporadic inclusion body myositis. Muscle & Nerve 1998; S130. Abstract.

Nakano S, Shinde A, Ito H, Ito H, Kusaka H. MAP kinase phosphatase-1 is induced in abnormal fibers in inclusion body myositis. Neurology. 2003 Aug 12;61(3): 322-6.

Nakano S, Shinde A, Kawashima S, Nakamura S, Akiguchi I, Kimura J. Inclusion body myositis:expression of extracellular signal-regulated kinase and its substrate. Neurology. 2001 Jan 9;56(1):87-93.

Nakano S, Shinde A, Fujita K, Ito H, Kusaka H. Histone H1 is released from myonuclei and present in rimmed vacuoles with DNA in inclusion body myositis. Neuromuscul Disord 2008;18:27-33.

Nalbantoglu J, Karpati G, Carpenter S. Conspicuous accumulation of a single-stranded DNA binding protein in skeletal muscle fibers in inclusion body myositis. Am J Pathol 1994;144:874-882.

Narciso L, Fortini P, Pajalunga D, et al. Terminally differentiated muscle cells are defective in base excision DNA repair and hypersensitive to oxygen injury. Proc Natl Acad Sci U S A 2007;104:17010-17015.

Needham M, Mastaglia FL. Sporadic inclusion body myositis: a continuing puzzle. Neuromuscul Disord 2008;18:6-16.

Nishii M, Nakano S, Nakamura S, Wate R, Shinde A, Kaneko S, Kusaka H. Myonuclear breakdown in sporadic inclusion body myositis is accompanied by DNA double strand breaks. Neuromuscul Disord. 2011 May;21(5):345-52.

Nishino I, Noguchi S, Murayama K, et al. Distal myopathy with rimmed vacuoles is allelic to hereditary inclusion body myopathy. Neurology 2002;59:1689-1693.

Nonaka I, Murakami N, Suzuki Y, Kawai M. Distal myopathy with rimmed vacuoles. Neuromuscul Disord 1998; 8: 333–337.

Nukina N, Kosik KS, Selkoe DJ. Recognition of Alzheimer paired helical filaments by monoclonal neurofilament antibodies is due to crossreaction with tau protein. Proc Natl Acad Sci USA 1987; 84: 3415–3419.

O'Driscoll M, Jeggo PA. The role of double-strand break repair - insights from human genetics. Nat Rev Genet 2006;7:45-54.

Okui T, Endoh D, Kon Y, Hayashi M. Deficiency in nuclear accumulation of G22p1 and Xrcc5 proteins in hyper-radiosensitive Long-Evans Cinnamon (LEC) rat cells after X irradiation. Radiat Res 2002;157:553-561.

Paradas C, Márquez C, Gallardo E, et al. X-linked Emery-Dreifuss muscular dystrophy and vacuoles: an immunohistochemical characterization. Muscle Nerve 2005;32:61-65.

Reffas S, Schlegel W. Compartment-specific regulation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) by ERK-dependent and non-ERK-dependent inductions of MAPK

phosphatase (MKP)-3 and MKP-1 in differentiating P19 cells. Biochem J 2000;352:701-708.

Robinson M, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997;9:180-186.

Rossetto D, Truman AW, Kron SJ, Côté J. Epigenetic modifications in double-strand break DNA damage signaling and repair. Clin Cancer Res. 2010 Sep 15;16(18): 4543-52.

Salajegheh M, Pinkus JL, Taylor JP, et al. Sarcoplasmic redistribution of nuclear TDP-43 in inclusion body myositis. Muscle Nerve 2009a;40:19-31.

Salajegheh M, Pinkus JL, Nazareno R, Amato AA, Parker KC, Greenberg SA. Nature of "Tau" immunoreactivity in normal myonuclei and inclusion body myositis. Muscle Nerve. 2009b Oct;40(4):520-8.

Schillace R Scott JD. Organization of kinases, phosphatases, and receptor signaling complexes. J Clin Invest 2000;103:767-772.

Schmidt J, Barthel K, Wrede A, Salajegheh M, Bähr M, Dalakas MC. Interrelation of inflammation and APP in sIBM: IL-1 beta induces accumulation of beta-amyloid in skeletal muscle. Brain. 2008 May;131(Pt 5):1228-40.

Selcen D, Engel AG. Myofibrillar Myopathy. 2005 Jan 28 [updated 2010 Jul 27]. In: Pagon RA, Bird TD, Dolan CR, Stephens K, editors. GeneReviews [Internet]. Seattle (WA): University of Washington, Seattle; 1993-. Available from http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=mfm

Sherriff FE, Joachim CL, Squier MV, Esiri MM. Ubiquitinated inclusions in inclusion-body myositis patients are immunoreactive for cathepsin D but not beta-amyloid. Neurosci Lett. 1995 Jul 14;194(1-2):37-40

Shi H, Scheffler JM, Pleitner JM, Zeng C, Park S, Hannon KM, Grant AL, Gerrard DE. Modulation of skeletal muscle fiber type by mitogen-activated protein kinase signaling. FASEB J. 2008 Aug;22(8):2990-3000.S

Shinde A, Nakano S, Sugawara M, Toyoshima I, Ito H, Tanaka K, Kusaka H. Expression of caveolar components in primary desminopathy. Neuromuscul Disord.2008 Mar;18(3):215-9.

Soulez M, Rouviere CG, Chafey P, et al. Growth and differentiation of C2 myogenic cells are dependent on serum response factor. Mol Cell Biol 1996; 11: 6065–6074.

Treisman R. Ternary complex factors: growth factor regulated transcriptional activators. Curr Opin Genet Dev 1994; 4: 96–101.

Weterings E, Chen DJ. DNA-dependent protein kinase in nonhomologous end joining: a lock with multiple keys? J Cell Biol 2007;179:183-186.

Woodcock CL, Skoultchi AI, Fan Y. Role of linker histone in chromatin structure and function: H1 stoichiometry and nucleosome repeat length. Chromosome Res 2006;14:17-25.

Zachara NE, Hart GW. O-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. Biochim Biophys Acta. 2004 Jul 6;1673(1-2):13-28.

Zeidan Q, Hart GW. The intersections between O-GlcNAcylation and phosphorylation: implications for multiple signaling pathways. J Cell Sci. 2010 Jan 1;123(Pt 1):13-22.

Zetser A, Gredinger E, Bengal E. p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. J Biol Chem 1999; 274: 5193–5200.

A Congenital Muscular Dystrophy with Mitochondrial Structural Abnormalities Caused by Defective De Novo Phosphatidylcholine Biosynthesis

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Congenital muscular dystrophy is a heterogeneous group of inherited muscle diseases characterized clinically by muscle weakness and hypotonia in early infancy. A number of genes harboring causative mutations have been identified, but several cases of congenital muscular dystrophy remain molecularly unresolved. We examined 15 individuals with a congenital muscular dystrophy characterized by early-onset muscle wasting, mental retardation, and peculiar enlarged mitochondria that are prevalent toward the periphery of the fibers but are sparse in the center on muscle biopsy, and we have identified homozygous or compound heterozygous mutations in the gene encoding choline kinase beta (*CHKB*). This is the first enzymatic step in a biosynthetic pathway for phosphatidylcholine, the most abundant phospholipid in eukaryotes. In muscle of three affected individuals with nonsense mutations, choline kinase activities were undetectable, and phosphatidylcholine levels were decreased. We identified the human disease caused by disruption of a phospholipid de novo biosynthetic pathway, demonstrating the pivotal role of phosphatidylcholine in muscle and brain.

A spontaneous mutant mouse with a neonatal-onset autosomal-recessive rostral-to-caudal muscular dystrophy (*rmd* mouse) due to a loss-of-function mutation in choline kinase beta (*Chkb*) was identified in 2006.[1] Interestingly, *rmd* mice exhibit a unique mitochondrial morphology in muscle fibers, which show enlarged mitochondria at the periphery of the fiber but none at the center (Figure S1). These features are similar to those seen in a congenital muscular dystrophy (CMD) that we previously reported in four Japanese individuals.[2] We therefore screened 15 genetically undiagnosed cases of CMD with fairly homogenous clinical features (Table 1) for mutations in choline kinase beta (*CHKB*); we included the four cases from in our previous study in these 15 cases. Features included peculiar mitochondrial changes in muscle as well as motor delay followed by the appearance of severe mental retardation and microcephaly without structural brain abnormalities (Figure 1 and Table 1).

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent. The study was approved by the Ethical Committee of the National Center of Neurology and Psychiatry. All mouse protocols were approved by the Ethical Review Committee on the Care and Use of Rodents in the National Institute of Neuroscience, National Center of Neurology and Psychiatry. For muscle pathology, samples of skeletal muscle were obtained from biceps brachii or quadriceps femoris in humans and from quadriceps femoris muscle in 8-week-old *rmd* mice. Muscles were frozen and sectioned at a thickness of 10 μm according to standard procedures, and a battery of routine histochemical stains, including hematoxylin and eosin (H&E), modified Gomori trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), and Oil Red O, were analyzed. For electron microscopic analysis, muscles were fixed as previously described,[3] and ultra-thin sections were observed at 120kV or 80kV. All affected individuals exhibited nonspecific dystrophic features (Figure 1A). However, in mGT, NADH-TR, SDH, and COX staining, prominent mitochondria at the periphery as well as central areas devoid of mitochondria were seen (Figures 1B and 1C). Oil Red O staining was unremarkable (data not shown). Electron microscopy confirmed enlarged mitochondria (Figure 1D).

We directly sequenced all exons and their flanking intronic regions in *CHKB* (MIM 612395, NM_005198.4, GenBank Gene ID 1120) in genomic DNA extracted from individuals' peripheral lymphocytes. All 15 individuals in three different populations (Japanese, Turkish, and British) had homozygous or compound heterozygous mutations in

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Table 1. Summary of Clinical and Laboratory Features

			Phenotypic Findings									Muscle Pathology					Mutations				
Individual	Sex	Origin	Age at Last Follow-Up	Floppy at Birth	Walk Alone	Serum Creatine Kinase (IU/liter)	Head Circumference (percentile)	Mental Retardation	Seizure	Cardiomyopathy	Skin Change	Age at Muscle Biopsy	Necrotic Fiber	Regenerative Fiber	Endomysial Fibrosis	Mitochondrial Enlargement	Status	cDNA	Consequence	Exon	Literature ref. on phenotype
1	F	Japanese	died at 13 yr	+	2 yr 6 mo	370	ND	+	-	+	-	7 yr3 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
2	M	Japanese	died at 23 yr	+	1 yr 9 mo	190–2676	25–50	+	+	+	-	1 yr 2 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
3	F	Japanese	28 yr	+	1 yr 6 mo	502	ND	+	+	+	-	8 yr	+	+	+	+	het	c.116C>A	p.Ser39X	1	2
																	het	c.458dup	p.Leu153PhefsX57	3	2
4	M	Japanese	22 yr	+	2 yr 6 mo	230	3–10	+	+	-	-	4 yr 11 mo	+	+	+	+	het	c.116C>A	p.Ser39X	1	
																	het	c.458dup	p.Leu153PhefsX57	3	
5	M	Turkish	7 yr	-	2 yr 6 mo	843	<3	+	-	-	+	6 yr	±	+	+	+	homo	c.611_612insC	p.Thr205AsnfsX5	5	
6[a]	M	Turkish	died at 2 yr 6 mo	+	no	258	<3	+	-	+	-	1 yr 3 mo	±	±	+	+	homo	c.922C>T	p.Gln308X	8	
7	F	Turkish	2 yr	-	no	368	3-10	+	-	-[b]	-	9 mo	-	±	+	+	homo	c.847G>A	p.Glu283Lys	8	
8	M	Turkish	13 yr	ND	2 yr	1122	ND	+	-	-	-	12 yr 10 mo	±	±	+	+	homo	c.1130 G>T	p.Arg377Leu	11	
9	F	Turkish	17 yr	+	3 yr	2669	<3	+	-	ND	-	17 yr	±	±	+	+	homo	c.554_562del	p.Pro185_Trp187del	4	
10	F	Turkish	16 yr	+	3 yr	1103	<3	+	-	-[c]	+	3 yr	-	±	+	+	homo	c.677+1G>A	ND	5	
11	F	Turkish	3 yr 3 mo	+	no	497	10-25	+	-	ND	-	3 yr	±	-	+	+	homo	c.677+1G>A	ND	5	
12	F	Turkish	5 yr	-	3 yr 6 mo	467	25–50	+	-	-[d]	+	4 yr 6 mo	±	+	+	+	homo	c.677+1G>A	ND	5	
13	M	Turkish	3 yr 6 mo	+	no	428	<3	+	-	+	+	3 yr	+	+	+	+	homo	c.1031+1G>A	aberrant splicing	9	
14	F	Turkish	6 yr 4 mo	-	1 yr 3 mo	1606	3–10	+	-	+	-	4 yr	+	+	+	+	homo	c.1031+1G>A	ND	9	
15	M	British	died at 8 yr	-	3 yr 4 mo	607–1715	<3	+	-	+	+	2 yr 2 mo	+	-	+	+	homo	c.852_859del	p.Trp284X	8	

Detailed clinical information for individual 1 to 4 was previously described (2). Eleven CHKB mutations were identified in 15 affected individuals. All exhibited generalized muscle hypotonia and weakness from early infancy. Ambulation was delayed, and gait in those who achieved walking was limited. In addition, all displayed marked mental retardation, and most never acquired meaningful language. Microcephaly with head circumferences at or below the 3rd to 10th percentile was observed in most cases. Cranial magnetic resonance imaging showed no developmental brain defects. Six individuals had dilated cardiomyopathy, and two had cardiac anomaly. Individuals 1, 2, 6, and 15 died from cardiomyopathy at ages 13 yr, 23 yr, 2 yr 6 mo, and 8 yr, respectively. No one had respiratory insufficiency. Ichthyosiform skin changes were frequent. All showed mildly to moderately elevated serum creatine kinase (CK) levels. Individuals 7 and 9 also had homozygous single-nucleotide substitutions, c.902C>T (p.Thr301Ile) and c.983A>G (p.Gln328Arg), respectively. CHK activities of recombinant CHK-β proteins with p.Thr301Ile and p.Gln328Arg were only mildly decreased (Figure S2), suggesting these are likely to be neutral polymorphisms or only mildly hypomorphic mutations. Individuals 10, 11, and 12, who have same c.677+1G>A mutation, and individuals 13 and 14, who have same c.1031+1G>A mutation, are not siblings. Abbreviations are as follows: ND, not determined; p, percentile; F, female; and M, male.

[a] An affected sibling had ichthyosis and died at age 6 years with cardiomyopathy.
[b] Patent ductus arteriosus.
[c] Atrial septal defect.
[d] Mitral valve prolapse.

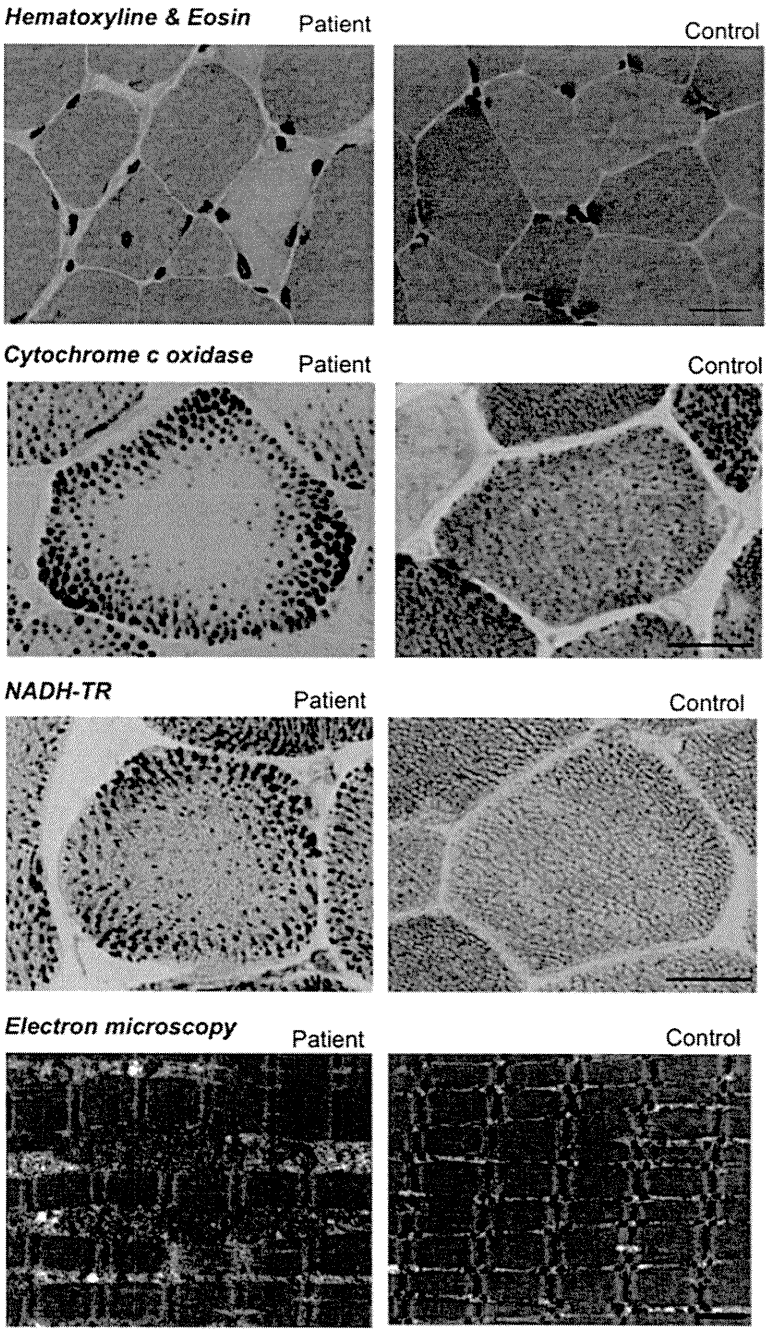

Figure 1. Muscle Pathology of the Affected Individuals

Cross-sections of muscle fiber from a human control and individual 4.

(A) On H&E staining, nonspecific dystrophic features with necrotic and regenerating fibers, internalized nuclei, and endomysial fibrosis are seen. The scale bar represents 25 μm.

(B) On cytochrome c oxidase staining, enlarged mitochondria at the periphery and central areas devoid of mitochondria were seen. The scale bar represents 20 μm.

(C) On NADH-TR staining, the intermyofibrillar network was preserved even in the central areas that are devoid of mitochondria, suggesting the presence of myofibrils and only absence of mitochondria. The scale bar represents 20 μm.

(D) Electron microscopy confirmed enlarged mitochondria. The scale bar represents 1 μm.

CHKB (Table 1). Among a total of 11 mutations identified, six were nonsense, two were missense, one was a 3 amino acid deletion, and two were splice-site mutations. The six nonsense mutations, c.116C>A (p.Ser39X), c.458dup (p.Leu153PhefsX57), c.611_612insC (p.Thr205AsnfsX5), c.810T>A (p.Tyr270X), c.852_859del (p.Trp284X), and c.922C>T (p.Gln308X), were predicted to truncate the protein and eliminate highly conserved domains of CHK.[4,5] Individuals 1 and 2 (unrelated, Japanese) had the same homozygous nonsense mutation of c.810T>A (p.Tyr270X). Individual 2's mother, who was healthy, had the heterozygous c.810T>A (p.Tyr270X) mutation. Unfortunately, a DNA sample from the father of individual 2 was not available. DNA samples from other family members of individual 1 and 2 were not available. Individuals 3 and 4 (siblings, Japanese) had the same compound heterozygous mutation c.116C>A (p.Ser39X) and c.458dup (p.Leu153PhefsX57). Both parents were healthy, and the father was heterozygous for mutation c.116C>A (p.Ser39X), whereas the mother was heterozygous for mutation c. 458dup (p.Leu153-PhefsX57), thus confirming a recessive inheritance pattern. These mutations cosegregated with the disease phenotype in all family members tested.

We therefore measured CHK activity in biopsied muscle. For all biochemical analyses, because of the limiting amounts of remaining tissue, biopsied muscle samples were available only from individuals 2, 3, and 4. Biopsied muscle samples from these three individuals were homogenized in 3 volumes of 20 mM Tris-HCl (pH 7.5), 154 mM KCl, and 1 mM phenylmethanesulfonyl fluoride with a sonicator (MISONIX), and supernatant fractions (105,000 × g, 60 min) were prepared and analyzed for CHK activity as previously described.[6] Similar to muscles of *rmd* mice,[1] muscles from individuals 2, 3, and 4, who carried homozygous or compound heterozygous nonsense mutations, did not have any detectable CHK activity (Figure 2A). Individuals 7, 8, and 9 had homozygous missense mutations c.847G>A (p.Glu283Lys) and c.1130 G>T (p.Arg377Leu) and a homozygous 3 amino acid deletion, c.554_562 del (p.Pro185_Trp187del), respectively.We screened 210 control chromosomes for the identified missense mutations and small in-frame deletion by direct sequencing or single-strand conformation polymorphism (SSCP) analysis. SSCP was performed with Gene Gel Excel (GE Healthcare) as previously described.[7] These missense mutations and this small in-frame deletion were not identified in control

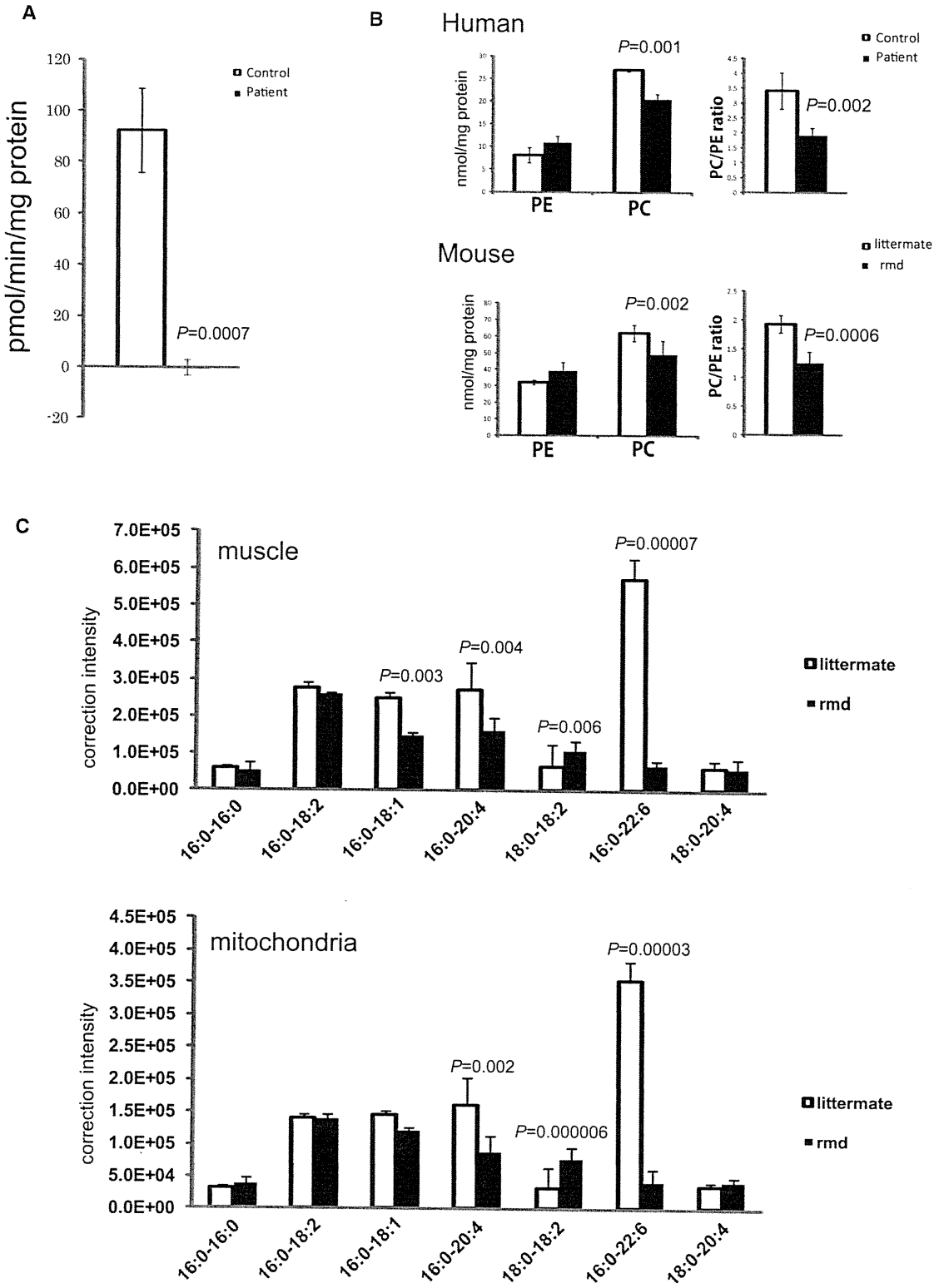

Figure 2. Choline Kinase Activity and Phospholipid Analyses

(A) In muscle tissue from individuals 2, 3, and 4, CHK activity cannot be detected (n = 3). Data represent the mean of three individuals. (B) PC and PE content in frozen biopsied muscle tissues from individuals 2, 3, and 4 and hindlimb muscles from 8-week-old *rmd* mice (n = 4) and control littermates (n = 5) were analyzed by thin-layer chromatography followed by phosphorus analysis. PC and the PC/PE ratio are significantly decreased in affected individuals and *rmd* mice (n = 3 for humans, n = 4 for *rmd* mice, n = 5 for littermates). (C) Fatty acid composition of PC molecular species in muscles and isolated mitochondria from hindlimb muscles of *rmd* mice are determined by electrospray ionization mass spectrometry (ESI-MS). We observed that 34:1-PC (16:0-18:1), 36:4-PC (16:0-20:4), and 38:6-PC (16:0-22:6) species are significantly decreased, whereas 36:2-PC (18:0-18:2) is increased in *rmd* muscle. Similarly, in isolated mitochondria from hindlimb muscle, 36:4-PC (16:0-20:4) and 38:6-PC (16:0-22:6) species are decreased, whereas 36:2-PC (18:0-18:2) is increased.

chromosomes. To elucidate the pathogenesis of these substitutions, we measured CHK activity in recombinant proteins with mutations. We cloned the open reading frame of *CHKB* into pGEM-T easy (Promega), then subcloned it into pET15b (Novagen) to make His-tagged CHK-β.[8] Each mutation was induced by site-directed mutagenesis.[7] Plasmids were transformed into *Escherichia coli* strain BL21 (DE3) and inoculated at 20°C to an OD_{600} of approximately 0.5, and the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside induced expression. The His-tagged CHK-β proteins were subjected to affinity purification on a nickel column (GE Healthcare) and eluted with 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 300 mM imidazol, and 1 mM phenylmethanesulfonyl fluoride, and 25 ng protein was analyzed for CHK activity. CHK activity of recombinant proteins with these mutations decreased to less than 30% of wild-type CHK activity, suggesting that these mutations are causative in these individuals (Figure S2). For individual 13, who had a mutation at the splice site of the exon-intron border after exon 9 (c.1031+1G>A), we also analyzed cDNA sequences. Exons 4 through 10 were amplified from the first-strand cDNAs, and direct sequencing followed. cDNA analysis of *CHKB* in skeletal muscle from individual 13 showed four splicing variants, all of which remove consensus domains for *CHKB* (Figure S3). This suggests the same loss-of-function mechanism in humans and *rmd* mice.

Because phosphorylation of choline by CHK is the first enzymatic step for phosphatidylcholine (PC) biosynthesis,[9] we anticipated that PC content should be altered in affected individuals' muscles. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and total phospholipid amounts were measured in biopsied muscles from individuals 2, 3, and 4 and in leg muscles from 8-week-old *rmd* mice by either one-dimensional or two-dimensional thin-layer chromatography (TLC) followed by phosphorus analysis.[10,11] As expected, PC levels decreased in affected individuals' skeletal muscle (Figure 2B), as they did in *rmd* mice (Figure 2B and Sher et al.[1]), suggesting that the CMDs due to *CHKB* mutations in humans and *rmd* mice are not only pathologically but also pathomechanistically similar.

PC is present in all tissues and accounts for around 50% of phospholipids in biological membranes in eukaryotes. Selective tissue involvement can be explained by the different tissue distribution of CHK isoforms. There are two CHK isoforms: CHK-α and CHK-β, encoded by distinct genes, *CHKA* (MIM 118491) and *CHKB*, respectively. They are known to form both homodimers and heterodimers, with differential tissue distribution.[12] In mice, disruption of *Chka* causes embryonic lethality,[13] suggesting the importance of CHK-α in embryonic development. In skeletal muscles from *rmd* mice, CHK activity is absent, and PC levels are decreased.[1] In other tissues, however, CHK activity is only mildly decreased, PC levels are not altered, and no obvious pathological change is seen.[1] CHK activity in skeletal muscle from individuals 2, 3, and 4 is barely detectable, and PC levels are significantly decreased, suggesting that CHK-β is the major isoform in human skeletal muscle. In support of this notion, CHK-α was not detected in human muscle (Figure S4). These results suggest that muscular dystrophy in affected individuals and *rmd* mice is caused by a defect in muscle PC biosynthesis. In addition, in *rmd* mice, hindlimb muscles are more significantly affected than forelimb muscles.[1] This is most likely explained by the fact that CHK activity is detected, though decreased, in forelimb muscles in *rmd* mice as a result of the continued post-natal expression of *Chka*.[14] This indicates that the severity of muscle involvement is determined by the degree of deficiency of CHK activity.

Generally, phospholipids have saturated or monounsaturated fatty acids at the *sn-1* position and polyunsaturated fatty acids at the *sn-2* position of glycerol backbone.[15] It has been shown that phospholipids have tissue-specific fatty acid composition.[15] For example, heart PC and muscle PC mainly contain docosahexaenoic acid (22:6) (Nakanishi et al.[15] and Figure 2C), but liver PC includes various fatty acids.[15] NanoESI-MS analyses of PC molecular species in muscle and isolated mitochondria were performed with a 4000Q TRAP (AB SCIEX, Foster City, CA, USA) and a chip-based ionization source, TriVersa NanoMate (Advion BioSystems, Ithaca, NY, USA).[16] Quadriceps femoris (hindlimb) and Triceps (forelimb) muscle from affected *rmd* mice and littermate controls were frozen with liquid nitrogen, and total lipid was extracted by the Bligh and Dyer method.[10] The ion spray voltage was set at −1.25kV, gas pressure at 0.3 pound per square inch (psi), and flow rates at 200 nl/min. The scan range was set at m/z 400~1200, declustering potential at −100V, collision energies at −35~−45V, and resolutions at Q1 and Q3 "unit." The mobile phase composition was chloroform: methanol (1/2) containing 5 mM ammonium formate and was normalized to the muscle weight. The total lipids were directly subjected by flow injection, and selectivity was analyzed by neutral loss scanning of the polar head

In muscle and isolated mitochondria, the 38:6-PC molecular species is profoundly decreased (n = 6 for muscle, n = 5 for isolated mitochondria).

Mitochondria from skeletal muscles of whole hindlimbs of *rmd* mice were isolated by the differential centrifugation method. Fresh muscle was minced and homogenized with a motor-driven Teflon pestle homogenizer with ice-cold mitochondrial isolation buffer (10 mM Tris-HCl [pH 7.2], 320 mM sucrose, 1mM EDTA, 1mM DTT, 1 mM PMSF, 1 mg/ml BSA, and protease inhibitor cocktail [Roche]) and centrifuged at 1,500 × *g* for 5 min. The supernatant fraction was centrifuged at 15,000 × *g* for 20 min, the pellet was resuspended in mitochondrial isolation buffer, and the centrifugation/resuspension was repeated twice more.

All data are presented as means ± standard deviation (SD). Means were compared by analysis with a two-tailed t test via R software version 2.11.0.

group for PC in negative-ion mode.[17] Interestingly, there was a 10-fold decrease (9.8%) in the 16:0-22:6-PC levels versus the control in *rmd* hindlimb muscle and also in muscle mitochondria (Figure 2C), indicating the importance of the PC de novo synthesis pathway for maintaining not only PC levels but also fatty acid composition of PC molecular species. Similarly, in forelimb muscle 16:0-22:6 PC levels were also decreased in comparison to the control, but to a milder extent (18.2%), suggesting an association between severity of muscle damage and fatty acid composition alteration of PC (data not shown). In *rmd* mice, it has been shown that muscle PC can be delivered from plasma lipoprotein,[18] suggesting that non-decreased PC molecular species might be derived from the plasma, whereas 16:0-22:6 PC might be synthesized only in muscle (and possibly in brain). However, confirmation of this requires further studies.

Individuals with *CHKB* mutations have severe mental retardation in addition to the muscular dystrophy. Interestingly, polymorphisms near the *CHKB* locus and decreased CHKB expression have been associated with narcolepsy with cataplexy, suggesting a link between CHK-β activity and the maintenance of normal brain function in humans.[19] Furthermore, brain damage in pneumococcal infection has been attributed to the inhibition of de novo PC synthesis, suggesting the importance of PC synthesis for the brain.[20] Our data provide evidence that altered phospholipid biosynthesis is a causative agent for a human congenital muscular dystrophy, and further studies will elucidate the detailed molecular mechanisms of the disease in both muscle and brain.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
R software version 2.11.0, http://www.r-project.org/

References

1. Sher, R.B., Aoyama, C., Huebsch, K.A., Ji, S., Kerner, J., Yang, Y., Frankel, W.N., Hoppel, C.L., Wood, P.A., Vance, D.E., and Cox, G.A. (2006). A rostrocaudal muscular dystrophy caused by a defect in choline kinase beta, the first enzyme in phosphatidylcholine biosynthesis. J. Biol. Chem. *281*, 4938–4948.
2. Nishino, I., Kobayashi, O., Goto, Y., Kurihara, M., Kumagai, K., Fujita, T., Hashimoto, K., Horai, S., and Nonaka, I. (1998). A new congenital muscular dystrophy with mitochondrial structural abnormalities. Muscle Nerve *21*, 40–47.
3. Hayashi, Y.K., Matsuda, C., Ogawa, M., Goto, K., Tominaga, K., Mitsuhashi, S., Park, Y.E., Nonaka, I., Hino-Fukuyo, N., Haginoya, K., et al. (2009). Human PTRF mutations cause secondary deficiency of caveolins resulting in muscular dystrophy with generalized lipodystrophy. J. Clin. Invest. *119*, 2623–2633.
4. Liao, H., Aoyama, C., Ishidate, K., and Teraoka, H. (2006). Deletion and alanine mutation analyses for the formation of active homo- or hetero-dimer complexes of mouse choline kinase-α and -β. Biochim. Biophys. Acta *1761*, 111–120.
5. Aoyama, C., Yamazaki, N., Terada, H., and Ishidate, K. (2000). Structure and characterization of the genes for murine choline/ethanolamine kinase isozymes alpha and beta. J. Lipid Res. *41*, 452–464.
6. Ishidate, K., and Nakazawa, Y. (1992). Choline/ethanolamine kinase from rat kidney. Methods Enzymol. *209*, 121–134.
7. Matsumoto, H., Hayashi, Y.K., Kim, D.S., Ogawa, M., Murakami, T., Noguchi, S., Nonaka, I., Nakazawa, T., Matsuo, T., Futagami, S., et al. (2005). Congenital muscular dystrophy with glycosylation defects of α-dystroglycan in Japan. Neuromuscul. Disord. *15*, 342–348.
8. Mitsuhashi, H., Futai, E., Sasagawa, N., Hayashi, Y., Nishino, I., and Ishiura, S. (2008). Csk-homologous kinase interacts with SHPS-1 and enhances neurite outgrowth of PC12 cells. J. Neurochem. *105*, 101–112.
9. Aoyama, C., Liao, H., and Ishidate, K. (2004). Structure and function of choline kinase isoforms in mammalian cells. Prog. Lipid Res. *43*, 266–281.
10. Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37*, 911–917.
11. Rouser, G., Fkeischer, S., and Yamamoto, A. (1970). Two dimensional then layer chromatographic separation of polar