Myotonic Dystrophy Protein Kinase Is a Sarcoplasmic Reticulum Protein Specifically Localized in Type I Muscle Fibers without Colocalization of SERCA II ATPase

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Abstract

Myotonic dystrophy (DM) is the most prevalent muscular dystrophy involving multisystem disorder. Previously we showed that the gene product of DM: myotonic dystrophy protein kinase (DMPK or myotonin protein kinase) was mainly localized in the terminal cisternae of the sarcoplasmic reticulum (SR) by immunoelectron microscopy. We describe here that DMPK is specifically localized in muscle fibers with slow myosin heavy chain (MHC), but not with fast MHC by using double immunofluorescent labeling of anti-DMPK and slow or fast MHCs antibodies. Staining pattern of DMPK is observed in three different intensities (strong, moderate and weak) in type I muscle fibers with even intensity of slow MHC staining. In the high magnification of a type I muscle fiber, the cross-striated bands of DMPK are not co-localized with those of slow MHC nor SERCA II ATPase, which is well known to be localized in longitudinal SRs. This is consistent with our previous study that DMPK is localized in the terminal cisternae of SRs. Our present finding is quite important to investigate the function of DMPK and to know the pathophysiological basis of DM because it is well known that specific type I atrophy is commonly observed in DM muscles.

Key words: Myotonic Dystrophy (DM), Myotonic Dystrophy Protein Kinase (DMPK or myotonin protein kinase), myosin heavy chain, SERCA II ATPase, type I fiber.

Basic Appl. Myol. 7 (5): 311-316, 1997

Myotonic dystrophy (DM) is the most prevalent hereditary muscular dystrophy involving multisystem disorders [9]. The gene responsible for DM is localized on chromosome 19q13.3. The molecular defect of DM has been shown to be a (CTG)n multiple repeat situated in the 3' noncoding region of the myotonic dystrophy protein kinase (DMPK or myotonin protein kinase)[2, 3,8]. To investigate the localization and function of DMPK, we raised a polyclonal antibody against a synthetic peptide chosen within the deduced sequence of DMPK [18]. We already reported that DMPK was a membrane-bound protein localized mainly in the terminal cisternae of sarcoplasmic reticulum (SR) by immunoelectron microscopy [19].

Previous immunocytochemical analysis has suggested that DMPK is localized predominantly at sites of neuromuscular and myotendinous junctions in adult muscle [14, 20, 21]. We now investigate whether DMPK is localized in neuromuscular junction *in vivo* and *in vitro* by using our C-terminal polyclonal antibody against DMPK. It has been commonly accepted that DM muscle shows predominant type I atrophy. It is quite important to know the type-specific localization of DMPK in skeletal muscle. Regarding SR proteins, it is well known that ATP dependent calcium pumps in SR or endoplasmic reticulum (ER) are responsible for the maintenance of low cytoplasmic free Ca^{+} concentrations. The ATP pumps are encoded by a family of structurally related enzymes, termed the sarcoplasmic or endoplasmic reticulum (SERCA) ATPases. The SERCA I gene is exclusively expressed in type II (fast) skeletal muscle, but the SERCA II gene is subject to tissue dependent processing which is responsible for the generation of SERCA IIa muscle-specific isoform expressed in type I (slow) skeletal muscle, cardiac and smooth muscle and the SERCA IIb muscle-specific isoform expressed in all cell types. SERCA ATPases are known to be localized mainly in longitudinal SR. As it is important to know the

special localizations among SR proteins to know their functional roles in SRs, we also performed double immunofluorescent studies of DMPK and SERCA II ATPase in human skeletal muscle.

Materials and Methods

Innervated human muscle culture

Diagnostic muscle biopsies from five patients, whose biopsies muscles showed mainly neurogenic or nonspecific changes, were used. Aneural human muscle cultures were established according to an explant-re-explantation technique [1]. Aneural human muscle cells were cultured in 67% Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY), 23% medium 199 (Gibco), 10% fetal bovine serum (FBS), supplemented with 12.5 ng/ml human basic fibroblast growth factor (Pepro Tech, Rocky Hill, NJ), 10 ng/ml human epidermal growth factor (Pepro Tech), and 10 μg/ml insulin (Sigma Chemical Co., St. Louse, MO). The cultures were fed twice a week. For innervation experiments, explants of spinal cord with dorsal root ganglia from 13-day-old fetal rats (Sprague Dawley) were placed on top of the muscle cells in a monolayer as previously described [11, 13]. Innervated human muscle fibers were cultured in 90 % F14 (Kojin-Bio, Sakado, Saitama, Japan) and 10% FBS supplemented with 10 μg/ml insulin.

Immunocytochemical studies

We used an affinity-purified polyclonal antibody raised against human DMPK [19], monoclonal antibodies against human myosin, which recognize slow and fast types of myosin heavy chain (MHC) isoforms (a generous gift from Prof. Shimizu, Department of Neurology, Teikyo University), a monoclonal antibody against SERCA II ATPase (MA3-910, Affinity Bioagents,Inc., Neshanic Station, NJ), and fluorescein-isothiocyanate (FITC)-conjugated α-BGT (Molecular Probes, Inc., Eugene, OR) to identify acetylcholine receptors (AChRs).

Immunofluorescent study of double labeling of DMPK/α-BGTon innervated contracting human muscle fibers

For double labeling of DMPK/α-BGT, the innervated contracting human muscle fibers were fixed and incubated with anti-DMPK antibody for 60 minutes. After washing in PBS for 30 minutes, they were incubated with rhodamine-conjugated anti-rabbit IgG. Eventually they were incubated with FITC conjugated α-BGT.

Immunofluorescent studies of double labeling of DMPK/α-BGT, DMPK / slow or fast MHCsand DMPK / SERCA II ATPase on biopsied adult human skeletal muscle

Frozen muscle biopsy specimens showing only non-specific changes were sliced at 7 μm thickness in a cryostat and mounted on poly-L-lysine coated slide glasses and air-dried for 30 minutes. The cryosections were preincubated for 30 minutes with PBS containing 2% bovine serum albumin (BSA) and 5% goat serum (G solution). The sections described above were incubated with anti-DMPK antibody in G solution at 4°C overnight. After washing in PBS for 20 minutes, the sections were incubated with rhodamine-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA) in G solution at room temperature for 1 hour, washed in PBS. For double immunofluorescent labelings of DMPK/α-BGT, DMPK / slow or fast MHCs and DMPK / SERCA II ATPase, the tissue sections were incubated with FITC conjugated a-BGT, anti-slow or fast MHCs, or SERCA II ATPase antibodies at 4°C overnight, washed, and incubated with FITC labeled goat anti-mouse IgG (Cappel) at room temperature for one hour. For the immunocontrol staining, PBS or normal rabbit or mouse serum was used instead of the primary antibodies. After the application on coverslips, the sections were observed with a confocal laser scanning microscope (Carl-Zeiss, LSM 310).

Image analysis by confocal microscopy

For double immunofluorescent stained tissue sections or cultured cells, data from two channels were collected simultaneously, thus providing a precise colocalization, and individually analyzed to show the localization of each antigen. The data from one channel are indicated in green (FITC-fluorescence: excitation, 488 nm argon laser) and the data from the other channel are indicated in red (rhodamine-fluorescence: excitation, 543 nm HeNe laser). To examine the distribution of the two antigens, data from both channels were overlaid to produce a single image on which regions of colocalization are indicated in yellow. For tissue culture studies, phase-contrast micrographs were simultaneously taken with immunofluorescent pictures.

Results

Immunofluorescent double labeling studies of DMPK and a-BGT in vivo *and* in vitro

Fig. la & b clearly shows that DMPK is co-localized in a a-BGT binding site in adult human tissue and also appears in cross striation pattern in a longitudinal section of skeletal muscle. In human cultured muscle cells innervated with fetal rat spinal cord, immunofluorescent double labeling study of DMPK and a-BGT show that linear or varicose structure of DMPK (Fig. 1c) is colocalized in a cluster of ACHR (a-BGT binding site; Fig. 1d, e), where a nerve terminal is detected using phase-contrast microscopy (Fig. 1f).

Immunofluorescent double labeling studies of DMPK and slow orfast MHCs

Low power views of immunofluorescent double labeling of DMPK and slow or fast MHCs clearly show that DMPK staining is completely negative in type II muscle fibers (Fig. 2d-f) and DMPK is exclusively colocalized in type I muscle fibers (Fig. 2a-c). The intensity of DMPK in type

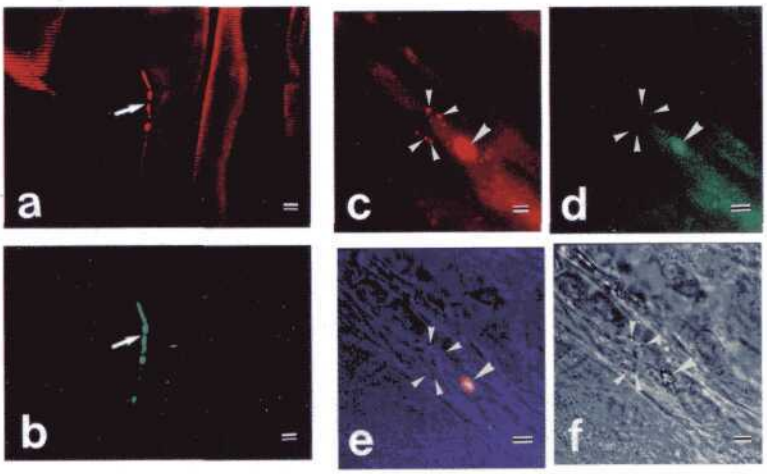

Figure 1. Double immunofluorescent labeling of DMPK (a, c) and α-BGT (b, d) on adult skeletal muscle (a, b) and innervated cultured human muscle cells (c-f). e: A simultaneous picture of double immunofluorescent pictures of DMPK /a-BGT and the phase-contrast picture (f) by confocal laser-scanning microscopy. In adult skeletal muscle, a cross-striated pattern of DMPK is observed in a longitudinal section (a) and varicose appearance of DMPK (arrow in a) is colocalized with the exact same appearance of a a-BGT binding site (arrow in b). The exact same co-localization of DMPK (c) and a-BGT binding site (d) are observed in the triple immunofluorescent and phase contrast picture (e), in which a nerve terminal is clearly detected (arrowheads in c-f). Co-culture was for 38 days. Bar, 10 μm.

I muscle fibers is variable [strong (arrows), moderate (arrowheads) and weak (double arrowheads) in Fig. 2a-f]. As immunofluorescent intensity of slow MHC in type I muscle fibers is equal, type I muscle fibers with strong, moderate or weak immunofluoresence of DMPK express orange, dark orange or green, respectively in the simultaneous double-immunofluorescent pictures (Fig. 2c). In high power views of a longitudinal section of human skeletal muscle, a binding pattern with doublet of DMPK exists between a cross-striated pattern of slow MHC and no colocalization of DMPK and slow MHC is observed (Fig. 2g-i).

Immunofluorescent double labeling studies of DMPK and SERCA II ATPase

Low power views of immunofluorescent double labeling of DMPK and SERCA II ATPase show that DMPK staining is exclusively colocalized in SERCAII ATPase-positive muscle fibers (Fig. 3a-c). As immunofluorescent intensity of SERCA II ATPase is almost equal like slow MHC, muscle fibers with strong, moderate or weak immunofluorescence of DMPK show orange, dark orange or green, respectively in the simultaneous double-immunofluorescent pictures of DMPK and SERCA II ATPase (Fig. 3c). In high power views, a banding pattern of DMPK exists between the banding pattern of SERCA II ATPase and no clear colocalization of DMPK and SERCA II ATPase is detected (Fig. 3d-f).

Discussions

Previously several authors reported that DMPK was localized at sites of neuromuscular and myotendinous junctions in adult tissues [14, 20, 21]. We confirmed this result by using our C-terminal antibody against DMPK. In our tissue culture studies, DMPK became developmentally localized in I band by innervation [19] and DMPK was colocalized in a cluster of AChRs in *in vitro* neuromuscular junctions. Abnormalities of the neuromuscular junction were previously reported in biopsied materials of extrafusal muscle fibers of patients with DM [4, 7, 15] and their intrafusal fibers [5]. We also reported that the nerve-muscle contacts on innervated DM muscle cells seemed less mature, as evidenced by impairment of the normal progression from multifocal to unifocal innervation and less well organized acetylcholinesterase patches, than equally long-term innervated cultured control muscle cells

[12]. Existence of apamin receptors in DM muscle [17] may cause repetitive discharges of DM muscle (appearance of myotonia) and remaining apamin receptor in DM muscle might be one of immature phenomenon of DM skeletal muscle by defective innervation. It is important to investigate the exact ultrastructural localization of DMPK

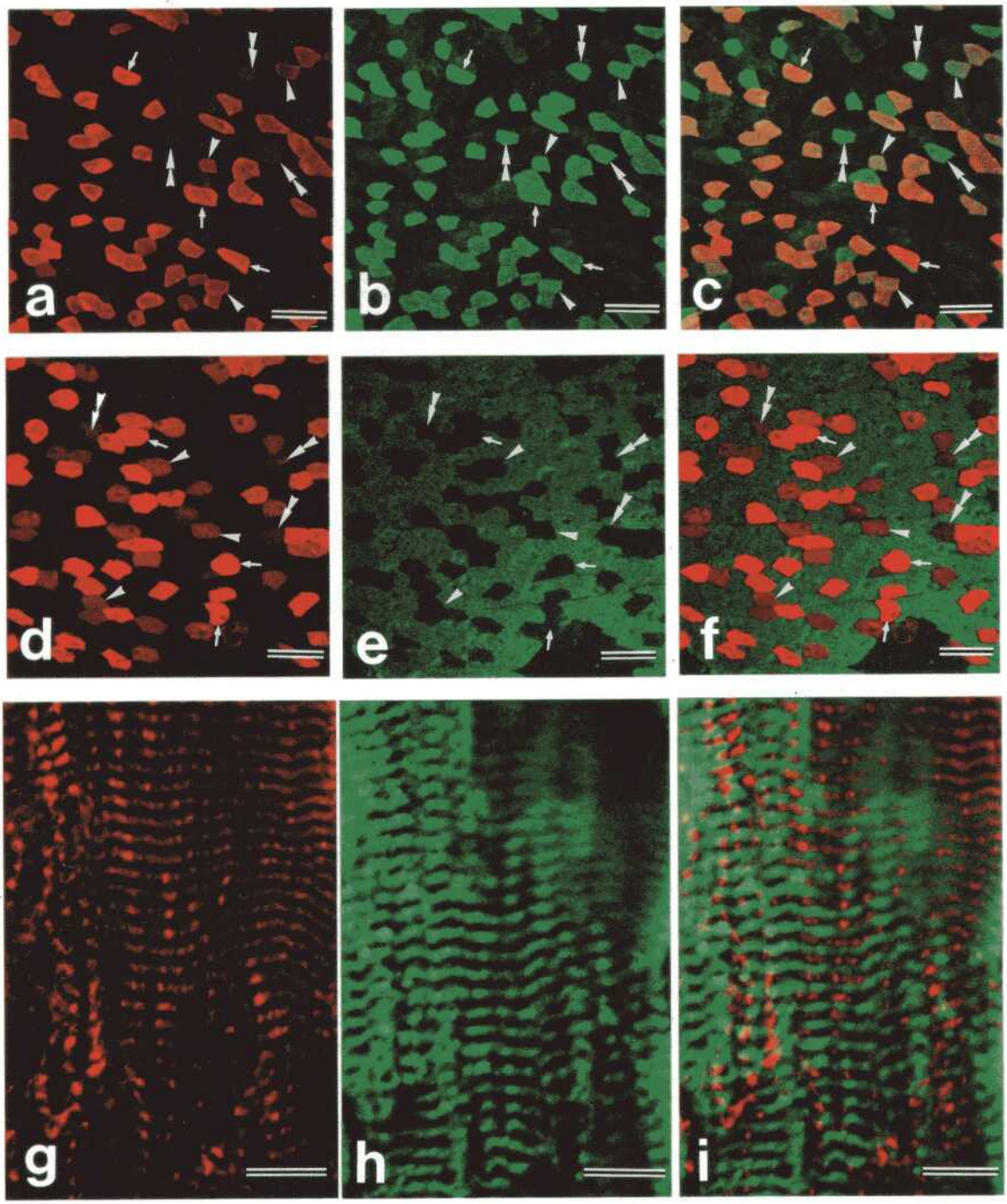

Figure 2. Double immunofluorescent labeling of DMPK (a, d, g) and slow (b, h) and fast (e) MHCs on adult skeletal muscle. c and f: Simultaneous double-immunofluorescent pictures of DMPK / slow and fast MHCs. Low power views (a-f) show that DMPK staining is completely negative in fast muscle fibers (d-f) and DMPK is exclusively colocalized in slow muscle fibers (a-c). The intensities of DMPK in slow muscle fibers are strong (arrows), moderate (arrowheads), and weak (double arrowheads). In high power views of a longitudinal section of human skeletal muscle, a binding pattern with doublet of DMPK is detected (g) between a cross-striated pattern of slow MHC (h) and no colocalization of DMPK and slow MHC is observed (i). Bar, 100 μm in a-f and 10 μm in g-i.

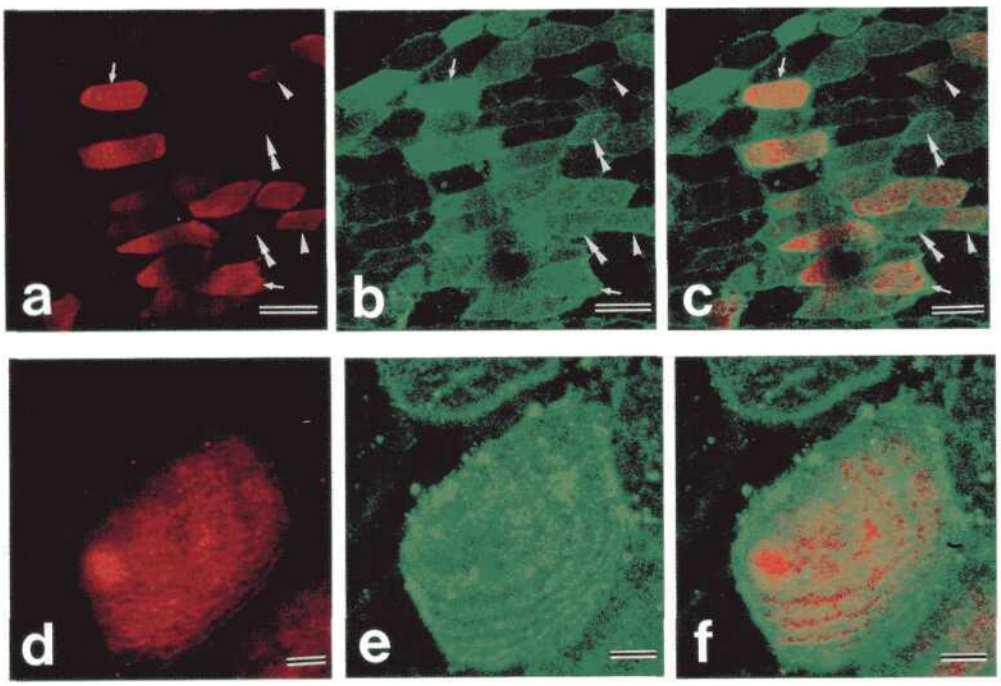

Figure 3. Double immunofluorescent labeling of DMPK (a, d) and SERCA II ATPase (b, e) on adult skeletal muscle. c and f: Simultaneous double immunofluorescent pictures of DMPK / SERCA II ATPase. Low power views (a-c) show that DMPK is colocalized in SERCA II ATPase-positive muscle fibers. The intensities of DMPK are strong (arrows), moderate (arrowheads), and weak (double arrowheads) in SERCA II ATPase-positive muscle fibers (a-c) similar to in slow muscle fibers in Fig. 2. In high power views, a binding pattern of DMPK (d) is detected between a cross-striated pattern of SERCA II ATPase (e) and no clear colocalization of DMPK and SERCA II ATPase is observed (f). Bar, 100 μm in a-c and 10 μm in d-f.

in neuromuscular junctions to know the functional role of DMPK in synaptic signal transmission and maturation of muscle fibers.

In the present studies DMPK expresses exclusively in type I muscle fibers. Dunne et al [6] also reported that a monoclonal antibody, which recognized the 64 kDa isoform of DMPK, reacted specifically in type I muscle fibers. This finding is quite important because it is well known that type I muscle atrophy is one of the significant finding of DM muscle pathology. We have now investigated the pathological changes of DMPK in DM skeletal muscles by confocal microscopy and immunoelectron microscopy (paper in preparation). DMPK might have some different physiological roles among subclasses of type I muscle fibers, because the type I muscle fibers with equal intensity of slow MHC have different intensities (strong, moderate and weak) of DMPK, which is localized in the terminal cisternae of SR, whereas the intensity of SERCA II ATPase, which is known to be localized in the longitudinal SR, is relatively equal in type I muscle fibers (Fig. 3b).

Recently it is reported that mice lacking the DMPK developed a late onset progressive myopathy [16] and in cultured myotubes derived from DMPK deficient mice calcium homeostasis was altered [10].

By investigating Ca homeostasis and other channel functions in triad systems, it might be possible to clarify the physiological role of DMPK and abnormal function of DMPK in DM skeletal muscle with the expansion of unstable trinucleotide repeats.

Acknowledgments

This study was supported in part by the grants from the Ministry of Health and Welfare to S. Ishiura and T. Kobayashi, and by the grand-in-aid for scientific research (07670702) from the Ministry of Education, Science, Sport and Culture to T. Kobayashi.

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References

[1] Askanas V, Engel WK: A new program for investigating adult human skeletal muscle grown aneurally in tissue culture. *Neurology* 1975; 25: 58-67.

[2] Aslanidis C, Jansen G, Amemiya C, Shutler G, Mahadevan M, Tsilfidis C, Chen C, Alleman J, Wormskamp NGM, Vooijs M, Buxton J, Johnson K, Smeets HJM, Lennon G, Carrana AV, Korneluk RG, Wieringa B, de Jong PJ: Cloning of the essential myotonic dystrophy region and mapping of the putative defect. *Nature* 1992; 355: 548-551.

[3] Buxon J, Shelbourne P, Davies J, Jones C, Van Tongeren T, Aslanidis C, de Jong P, Jansen G, Anvret M, Riley B, Williamson R, Johnson K: Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy. *Nature* 1992; 355: 547-548.

[4] Coers C, Telerman-Toppet N, Gerarad JM: Terminal innervation ratio in neuromuscular disease. *Arch Neurol* 1973; 29: 215-222.

[5] Daniel PM, Strich SJ: Abnormalities in the the muscle spindle in dystrophia myotonica. *Neurology* 1964; 14: 310-316.

[6] Dunne PW, Ma L, Casey D, Harati Y, Epstein HF: Localization of myotonic dystrophy protein kinase in skeletal muscle and its alteration with disease. *Cell Mot Cytoskelet* 1996; 33: 52-63.

[7] Engel WK, Brooke MH: Histochemistry of the myotonic disorders, in Kuhn (ed): *Progressive Muskeldystrophie, Myotonie, Myasthenie.* New York, Springer-Verlag, NY Inc., 1966, pp 203-222.

[8] Harley HG, Brook JD, Rundle SA, Crow S, Readon W, Buckler AJ, Harper PS, Housman DE, Shaw DJ: Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. *Nature* 1992; 355: 545-546.

[9] Harper PS, Rildel R: Myotonic dystrophy in Engel AG, Franzini-Armstrong C (ed): *Myology*. New York, McGraw-Hill, 1994, pp 1192-1219.

[10] Jansen G, Groenen P JTA, Bachner D, Jap P HK, Coerwinkel M, Oerlemans F, van den Broek W, Gohlsch B, Pette D, Plomp JJ, Molenaar PC, Nederholl MGJ, van Echteld CJA, Dekker M, Berns A, Mameister H, Wieringa B: Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. *Nature Genetics* 1996; 13: 316-324.

[11] Kobayashi T, Askanas V, Engel WK: Human muscle cultured in monolayer and cocultured with fetal rat spinal cord: importance of dorsal root ganglia for achieving successful functional innervation. *J Neurosci* 1987; 7: 3131-3141

[12] Kobayashi T, Askanas V, Saito K, Engel WK, Ishikawa K: Abnormalities of aneural and innervated cultured muscle fibers from patients with myotonic atrophy (dystrophy). *Arch Neurol* 1990; 47: 893-896.

[13] Kobayashi T, Ohno S, Park-Matsumoto YC, Kameda N, Baba T: Developmental studies of dystrophin and other cytoskeletal proteins in cultured muscle cells. *Microsc Res Techniq* 1995; 30: 437-457.

[14] Maeda M, Taft CS, Bush EW, Holder E, Bailey WM, Neville H, Perryman MB, Bies RD: Identification, tissue-specific expression, and subcellular localization of the 80- and 71-kDa forms of myotonic dystrophy kinase protein. *J Biol Chem* 1995; 270: 20246-20249.

[15] McDermot V: The histology of the neuromuscular junction in dystrophia myotonica. *Brain* 1961; 84: 75-84.

[16] Reddy S, Smith D BJ, Rich MM, Leferovich JM, Reilly P, Davis BM, Tran K, Raybum H, Bronson R, Cros D, Balice-Gordon RJ, Housman D: Mice lacking the myotonic dystrophy protein kinase develop a late onset progressive myopathy. *Nature Genetics* 1996; 13: 325-335.

[17] Renaud JF, Desnuelle C, Antornarchi HS, Huguss M, Serratrice G, Lazdunski M: Expression of apamin receptor in muscles of patients with myotonic muscular dystrophy. *Nature* 1986; 319: 281-287.

[18] Sasagawa N, Sorimachi H, Maruyama K, Arahata K, Ishiura S, Suzuki K: Expression of a novel human myotonin protein kinase (MTPK) cDNA clone which encodes a protein with a thymopoetin-like domain in COS cells. *FEBS Lett* 1994; 351: 22-26.

[19] Shimokawa M, Ishiura S, Kameda N, Yamamoto M, Sasagawa N, Saitoh N, Sorimachi H, Ueda H, Ohno S, Suzuki K, Kobayashi T: Novel isoform of myotonin protein kinase gene product of myotonic dystrophy is localized in the sarcoplasmic reticulum of skeletal muscle. *Am J Pathol* 1997; 150: 1285-1295.

[20] Tachi N, Kozuka N, Ohya K, Chiba S, Kikuchi K: Expression of myotonic dystrophy protein kinase in biopsied muscles. *JNeurolSci* 1995; 132: 61-64.

[21] van der Ven PFM, Jansen G, van Kuppevelt THSM, Perryman MB, Lupa M, Dunne PW, ter Laak HJ, Jap PHK, Veerkamp JH, Epstein HF, Wieringa B: Myotonic dystrophy kinase is a component of neuromuscular junctions. *Human Mol Genet* 1993; 2: 1889-1894.