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DECREASED MYOTONIN-PROTEIN KINASE IN THE SKELETAL AND CARDIAC MUSCLES IN MYOTONIC DYSTROPHY

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SUMMARY: To investigate the role of myotonin-protein kinase (MT-PK) in the pathophysiology of myotonic dystrophy (DM), we developed specific antibodies against synthetic MT-PK peptides. The antibody identified a 53kDa protein in skeletal muscle and recognized decreases in the amount of the protein in both adult and congenital DM patients, compared with amounts in controls and in patients with other muscle diseases. In cardiac muscle, this antibody identified a 62kDa protein, and in brain, both the 53 and 62kDa proteins were detected. These results suggest the presence of tissue-specific isoforms of MT-PK.

Myotonic dystrophy (DM), inherited as an autosomal dominant trait, affects multiple organ systems. DM patients present variable clinical symptoms with myotonia, muscle weakness, cardiac conduction defects, cataracts, mental dysfunction, testicular and ovarian atrophy, and diabetes(1). The genetic basis of the disease is now known to include the mutational expansion of an unstable $[CTG]_n$ repeat in the 3'untranslated portion of the MT-PK gene at chromosome 19q13.3. The MT-PK gene encodes a protein with putative serine/threonine protein kinase(2-9), and the protein has kinase activity(10,11). The size of the $[CTG]_n$ repeat expansion may increase in succeeding generations, and this is paralleled

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by increasing clinical severity (anticipation) (4,12-15). There is also a congenital form of DM. However, there are conflicting results whether the repeat expansion produces increased or decreased MT-PK mRNA and protein levels.

In this study, we raised polyclonal antibodies against the synthetic MT-PK peptides deduced from the MT-PK cDNA to identify the MT-PK protein levels in muscle and brain tissues obtained from patients with DM, and other muscle diseases.

MATERIALS AND METHODS

Development of MT-PK antibodies. Two peptide fragments, DSTAETYGKIVHYKEH (peptide DM1) and EAEARNRDLEAHVRQ(peptide DM2) were synthesized, using standard procedures, and conjugated with KLH(keyhole lympet hemocyanin). Peptide DM1 corresponds to part of the amino acid sequence of human exons 7 and 8 within the serine/threonine protein kinase domain, but downstream of the putative kinase site involved in the regulation of kinase activity. Peptide DM2 corresponds to part of the amino acid sequence of human exon 12 within the α-helical coiled domain. Neither peptide is involved in alternatively spliced regions(8)(Fig.1) and neither has homology to other known protein kinases. Polyclonal antibodies were raised in New Zealand white rabbits by usual

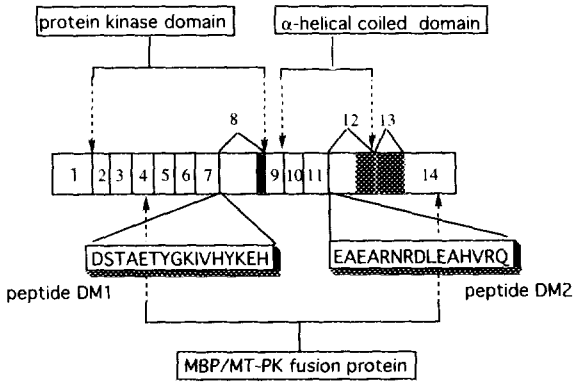

Fig.1. Localization of the synthetic peptides used as antigen in human MT-PK cDNA(Accession No. M87312). Numbers indicate exons and shaded boxes represent alternative spliced regions. The positions of the two synthetic peptides used for the development of the DM-PK antibodies and the truncated cDNA region used for the production of the MBP/MT-PK fusion protein are shown.

method. The antibodies were affinity-purified from the sera, using the peptide antigens(Prot On™ kit, Multiple Peptide System Co., U.S.A.).

Production of a MT-PK fusion protein. Truncated human MT-PK cDNA(Fig.1) was cloned into the MBP expression vector pMAL-p2 as indicated by the manufacturer's protocol (New England Biolabs,Inc.). The MBP/MT-PK fusion protein was produced in E.coli, and this was followed by affinity purification for MBP through a column containing amylose.

Immunoblotting. Tissue samples were weighed, solublized in 20 volumes of SDS sample buffer(2% SDS, 0.125M Tris -HCl buffer, pH6.5, 5% 2-mercaptoethanol, 50% glycerol), and boiled for 5 min. After centrifugation, samples were loaded onto 10% SDS polyacrylamide gel for electrophoresis, after which the proteins were electroblotted onto a nitrocellulose membrane and incubated with the affinity-purified MT-PK antisera. The blots were then incubated with biotinilated secondary antibodies and developed in substrate solution, using an ABC kit (Vector Lab.). For the quantitative analysis of MT-PK, the myosin heavy chain content of each sample solution was pre-measured by densitometric analysis of CBB-stained 6% SDS polyacrylamide gel. Immunoblotting was done under conditions in which each sample solution had equal myosin heavy chain content, and the optical density of the MT-PK bands was analyzed by densitometer.

RESULTS

The MBP/MT-PK fusion protein was used to characterize the specificity of the antibodies, both antibodies reacted with this protein, as expected (Fig.2).

Normal human skeletal muscle showed immunoreactive protein against the anti-DM2 antibody as a distinct 53kDa band, localized mainly in the soluble/cytoplasmic fraction (Fig.3). The titer of the anti-DM1 antibody, however, was not sufficient to detect the same 53kDa band in human muscle, although both antibodies detected the MBP/MT-PK fusion protein and the full length MT-PK expressed in the Cos cells (data not shown).

In normal human cardiac muscle, a 62kDa band was detected, not the 53kDa band of skeletal muscle, while, in brain, both the 53 and 62kDa bands were detected(Fig.4). The level of MT-PK in brain was higher than that in skeletal and cardiac muscles(Fig.4). In two autopsy patients with

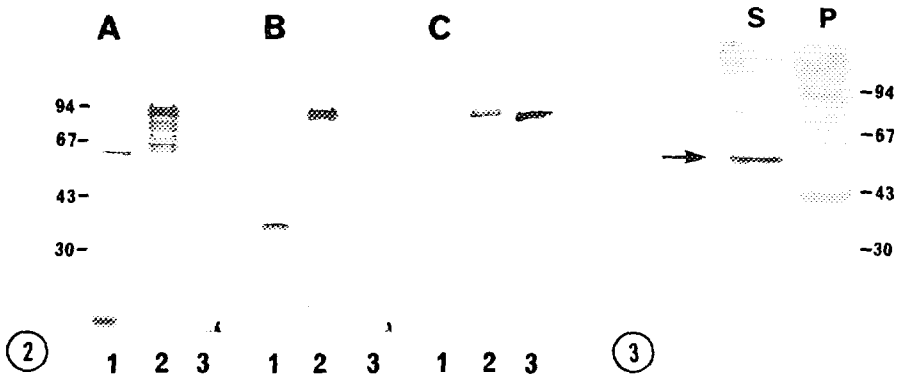

Fig.2. Characterization of anti-MT-PK antibodies on immunoblot of MBP/MT-PK fusion protein. MBP/MT-PK fusion proteins, purified and digested with V8 protease but not separated (lane 1), purified but not digested (lane 2), and MBP/U2AF(U2 auxiliary factor(23)) fusion protein, purified but not separated (as control, lane 3) were analyzed by immunoblot with anti-peptide DM1(panel A), anti-peptide DM2(panel B), and anti-MBP(panel C) antibodies. Molecular weight markers are phosphorylase b(94kDa), albumin(67kDa), ovalbumin(43kDa), and carbonic anhydrase(30kDa).

Fig.3. Immunoblot of protein detected with antibody to peptide DM2. Soluble/cytoplasmic fraction(lane S) and particulate fraction(lane P) of control human skeletal muscle were analyzed by immunoblot with anti-peptide DM2. Arrow shows the position of the 53kDa band, molecular weight markers, as in Fig.2.

adult DM, the 53kDa MT-PK protein of skeletal muscle and the 62kDa MT-PK protein of cardiac muscle were dramatically decreased compared with the control autopsy samples. These autopsy samples had been flash frozen within 2 hours after the patients died. No changes were recognized in the amount of brain MT-PK proteins (Fig.4).

Quantitative analysis revealed differences in the relative abundance of MT-PK protein in skeletal muscle among normal controls(100%), DM patients(36.0±36.9%), and patients with other muscle diseases (77.2±37.1%). In both adult and congenital type DM, MT-PK protein levels were either decreased or unchanged, but showed a significant difference from those in other muscle diseases [Duchenne muscular dystrophy(n=3), polymyositis(n=3), dermatomyositis(n=3) and other muscle diseases(n=3)] (Table1).

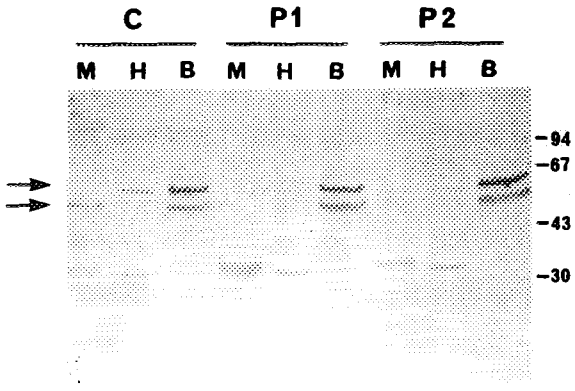

Fig.4. Immunoblot analysis of autopsy tissues of control and DM patients. Autopsy tissue, skeletal muscle (lanes M), cardiac muscle (lanes H) and brain (lanes B) of human control (C), DM patient 1 (P1) and DM patient 2 (P2) were analyzed by immunoblot with anti-peptide DM2. Arrows show positions of the 53 and 62kDa proteins, molecular weight markers, as in Fig.2.

DISCUSSION

Since the identification of the DM gene, several studies have focused on MT-PK mRNA levels in the muscles of control and DM patients. Some of these studies have reported reduction of MT-PK expression(16-19), while

Table 1 Comparison of relative cellular abundance of MT-PK protein in skeletal muscles of patients with DM and patients with other muscle diseases. Quantification of the MT-PK protein was performed by immunoblot and by measurement of the optical density of MT-PK bands, as described in MATERIALS AND METHODS. Relative abundance is expressed as a percentage of the amount in the control muscle, values, means ± S.D.

Type of patient	Number of patient	MT-PK protein level* (% of control)	
DM**	14	36.0 ± 36.9	p=0.0117#
Other muscle diseases***	12	77.2 ± 37.1	

* Control amount, 100%.
** 7 patients with adult DM and 7 with congenital DM.
*** This includes Duchenne muscular dystrophy, inflammatory myopathies, and patients with non-specific muscle weakness.
# Mann-Whitney's U test.

others have reported elevation(20). Reports of the localization of MT-PK protein have also differed(17,21,22). Therefore, we developed polyclonal antibodies specific for MT-PK protein to elucidate the expression of MT-PK protein in DM.

The polyclonal anti-DM2 antibody (specificity proven using the MBP/MT-PK fusion protein, Fig.2) detected the 53kDa protein in skeletal muscle, the 62kDa protein in cardiac muscle and both 53 and 62kDa proteins in the control brains. These results suggest that MT-PK protein has at least two isoforms, one being the skeletal muscle type and the other, cardiac muscle type; both type isoforms exist in brain. In brain, it has been reported that Ca^{2+}/calmodulin-stimulated protein kinase was expressed at high levels and that the antibody raised against the peptide fragment in the kinase domain of MT-PK cross-reacted to this protein(21). Since the peptide fragment DM2 used to raise the antibody here is not in the kinase domain of MT-PK and has no homology to any other known protein kinase, we conclude that the anti-DM2 antibody detected a 62kDa protein other than Ca^{2+}/calmodulin-stimulated protein kinase.

Decreased levels of both skeletal muscle type and cardiac muscle type MT-PK proteins were detected in DM patients. Since cardiac involvement is one of the common symptoms of DM(1), the decreased level of cardiac muscle type MT-PK protein in DM-patients is expected. However, in brain, levels of MT-PK mRNA have been reported to be low compared with levels in skeletal and cardiac muscles (3). In our study, the MT-PK protein level in adult brain was significantly higher than that in skeletal and cardiac muscles, and there was no detectable difference between the DM and other samples. We cannot explain this finding, but our observations may support the clinical evidence that few adult patients with DM show abnormal mental function, while those with the congenital form of DM exhibit mental retardation frequently. However, we cannot totally exclude the possibility that brain tissue contains a protein related to MT-PK that was recognized by our antibodies.

Although decreased level of MT-PK protein has been reported(17), no relevant methods was used. Further, antibodies they used have not been fully characterized yet. Since the quantitation of MT-PK is dependent on the amount of muscle tissue loaded, we have determined the amount of myosin heavy chain content as a reference. The abundance of MT-PK protein showed reduced levels in the muscles of adult and congenital DM patient compared with levels in control adult muscles. The relative abundance of MT-PK protein was estimated at below 50% in 12 of 14 DM patients, although 2 congenital type patients showed almost the same level as the control. We also found significant differences between the MT-PK protein levels in the muscles of DM patients (36% of control) and in the muscles of patients with other muscle diseases (77% of control) ($p=0.0117$). These results suggest that a decreased amount of MT-PK protein is pathognomonic in myotonic dystrophy.

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