Regulatory expression of MDP77 protein in the skeletal and cardiac muscles

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Received 23 July 2002; revised 28 August 2002; accepted 2 September 2002

First published online 12 September 2002

Edited by Amy McGough

Abstract The mdp77 gene was first cloned from the cDNA library of denervated chick muscles, while its role(s) in vivo was unknown. In the present study, using specific polyclonal antibodies against MDP77, we show that MDP77 was expressed specifically in the skeletal and cardiac muscle, and confirm its presence in the cytoplasm of the extrafusal muscle fibers. In mature muscles, MDP77 immunoreactivity was observed in a repetitive manner along the sarcomere. The onset of MDP77 expression occurred just after myotube formation both in vivo and in vitro. Furthermore, MDP77 was enriched in the intrafusal muscle fibers. Our findings suggest that MDP77 plays an important role(s) in the differentiation, maturation and function of both the skeletal and cardiac muscles.

Key words: Sciatic nerve; Denervation; Muscle spindle; Intrafusal muscle fiber; Cardiomyopathy; Myotube formation

1. Introduction

Nerve transection experiments have been used extensively for studying muscle atrophy, regeneration of peripheral nerves and muscles, reorganization of the neuromuscular junction and neuropathic pain [1–4]. The muscle-derived protein of molecular mass 77 kDa gene (mdp77) was identified and cloned from the cDNA library of denervated chick leg muscles [5]. Because there is no similarity to the already known proteins, the function of MDP77 in vivo remains unknown. The protein contains only a coiled-coil structure, like the myosin heavy chain (MHC), with a leucine zipper in the central region. Although the leucine zipper has been observed in numerous DNA binding proteins, there is no evidence to support the binding of the MDP77 protein to DNA or RNA. In the previous study, we have shown also that recombinant MDP77 in *Escherichia coli* had a neurite outgrowth activity for chick telencephalic neurons in vitro, though the mode of such activity is unknown [5]. There is, however, no evidence that the MDP77 protein is expressed in vivo.

In the present study, to elucidate the role of the intact MDP77 protein in vivo, we studied tissue distribution, cellular localization, onset of expression and change in expression in denervated muscles, using anti-MDP77 polyclonal antibodies.

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2. Materials and methods

2.1. Treatment of the experimental animals

In this study, we used embryonic and post-hatched chicks (white leghorn). All the animals were maintained according to the National Research Council's Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Animal Welfare, Care and Use Committee in AIST Kansai. The fertilized eggs were purchased from local farms, and incubated at 38°C in a humid chamber with rotation. The first day of the incubation was designated embryonic day (E) 0, and the day of hatching was designated post-hatched day (P) 0.

2.2. Antigen preparation, immunization, and antibody purification

The recombinant amino-terminal (Met(1)–Ser(149)) and carboxy-terminal (Ala(505)–Leu(634)) proteins of chick MDP77 (DDBJ accession number D89999) were prepared in *E. coli*. The obtained recombinant proteins were diluted at 1 mg/ml in Dulbecco's phosphate-buffered saline (PBS), and used for immunization by a standard method (Japan Lamb Co., Japan).

The produced rabbit anti-MDP77 antisera were purified by ligand affinity chromatography. One mg of recombinant protein was conjugated to 350 μg of CNBr-activated Sepharose 4B beads (Amersham Biosciences, USA). The antisera were loaded on the column and the IgGs bound to the beads were eluted with 0.1 M glycine buffer (pH 3.0). We used two kinds of purified anti-MDP77 antibodies (specifically labeled N6 and C9) for immunoblotting, immunoprecipitation and immunohistochemistry analyses.

2.3. Immunoblotting and immunoprecipitation

For immunoblotting, a variety of tissues or organs were homogenized in PBS containing proteinase inhibitors (1 μg/ml aprotinin, 5 μg/ml antipain, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride) on ice using glass–Teflon homogenizers or an electric homogenizer (Polytron; Kinematica, Switzerland). Twenty μg of homogenate per lane was electrophoresed on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and subsequently transferred to nitrocellulose membranes (Protran; Schleicher and Schuell, Germany) electrophoretically. After blocking with 5% skim milk in Tris-buffered saline containing 0.2% Tween 20 (TBST) for 6 h at room temperature or overnight at 4°C, the membrane was incubated with 0.1 μg/ml anti-MDP77 N6 or C9 polyclonal antibodies, and then peroxidase conjugate anti-rabbit IgG (Zymet Laboratories, USA) for 3 h at room temperature. Immunoreactive signals were detected by the chemiluminescent enhancement method (ECL plus; Amersham-Pharmacia Biotech, UK) and subsequently exposed to X-ray film (Super RX; Fuji Film, Japan).

For immunoprecipitation, extracts were obtained from the whole brain and the skeletal muscle homogenates of P9 chick by centrifugation at 75 000×*g*. One μg of N6 or C9 antibodies was added to 3 mg of these extracts each, and incubated at 4°C for 6 h with gentle rotation. Twenty μl of protein G Sepharose 4B (Amersham Biosciences) was added and the mixture was further incubated overnight at 4°C. As for the negative controls, extracts of the whole brain and skeletal muscle were incubated with protein G Sepharose 4B beads without antibodies. The beads were recovered and washed five times with 1 ml of TBST in each preparation. Then immunoblotting was performed using N6 and C9 antibodies, as described above.

0014-5793/02/$22.00
PII: S0014-5793(02)03386-0

2.4. Sciatic nerve transection

The procedure for sciatic nerve transection has been described in previous papers [2,5]. In brief, a P5 chick was anesthetized with halothane, and the lateral skin of the right leg was incised over a 2–3 mm length. The fascicle of the sciatic nerve was cut in the cleft of the adductor muscles without damaging the arteries. As a control, the skin of the left leg was incised and recovered without denervation in each operation. Chicks operated on were maintained in a humid chamber at 38°C. Four days following the operation, the chicks were killed and fixed with 4% paraformaldehyde, transcardially.

2.5. Immunohistochemistry

For the analysis of the distribution and localization of MDP77, we performed immunohistochemistry using the ABC method (ABC elite kit, Vector Laboratories, USA) as described in the previous paper [6]. Briefly, normal or denervated chicks were killed by hypothermia (for embryos) or administration of an overdose of halothane and sodium pentobarbital, perfused with saline, and fixed with freshly prepared 4% paraformaldehyde in PBS, transcardially. After post-fixation in the same fixative overnight at 4°C, the dissected tissues were embedded in OCT compound (Miles, USA) and frozen rapidly. Cryosections were cut 15 μm thick by a cryostat and mounted on 0.025% poly-L-lysine-coated slides. After washing with PBS and blocking with 5% skim milk in PBS containing 0.2% Tween 20, the slides were incubated with 0.1–0.25 μg/ml of the purified MDP77 N6 or C9 antibody overnight at 4°C. Subsequently, they were incubated with anti-rabbit IgG conjugated with biotin, incubated with streptavidin conjugated with horseradish peroxidase and, finally, visualized by 0.5 mg/ml of diaminobenzidine in Tris–HCl (pH 7.4). The slides were dehydrated with an ethanol series, cleared with xylene, and covered with coverslips using Enteran New (Merck, USA). The sections were observed under a microscope (BX60; Olympus, Japan), and photo images were taken with a CCD camera (Photometrics CoolSNAP cf; Roper Scientific, USA).

2.6. Primary culture of myoblasts

To obtain myogenic cells, legs of chick embryos on E10 were carefully dissected in cold PBS; removing the skin, adipose tissue, bones, blood vessels and nerves. The muscular tissue was cut and resuspended in Dulbecco's modified Eagle's medium (DMEM), and triturated using a pipette. The cell suspension was allowed to stand for 2 min, and the supernatant was transferred to a new tube. After centrifugation at 600 rpm, the cell pellet was recovered and resuspended in DMEM containing antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml gentamicin) and 10% fetal bovine serum. The isolated myoblasts were incubated on gelatin-coated six-well culture dishes or 35 mm diameter culture dishes in a humid CO_2 incubator. Twelve hours, 1 day, 2 days, 3 days, 4 days, and 5 days later, each dish was fixed on ice with cold 4% paraformaldehyde in PBS for 1 h. These samples were used for the immunohistochemistry in the same way as already mentioned.

3. Results

3.1. MDP77 was expressed only in the skeletal and cardiac muscles

To study tissue distribution, cytolocalization, onset of expression and change in expression of the intrinsic MDP77 proteins after sciatic nerve transection, we prepared two anti-MDP77 polyclonal antibodies (N6 and C9) against the amino-terminal protein (amino acid residues 1–149) and carboxy-terminal protein (505–634), respectively. These two antibodies were purified by ligand affinity chromatography for further studies. By immunoblotting, these two antibodies recognized dual bands at 115 kDa in the skeletal and cardiac muscles on P9 (Fig. 1A,B, arrows). The N6 antibody also recognized a non-specific band at 95 kDa (Fig. 1A, asterisk), but this band was not observed in the skeletal muscle. Because there is little similarity in the amino- and carboxy-termini of the chick and rat MDP77, these antibodies did not cross to the rat MDP77 from the skeletal muscle (Fig. 1A,B). In addition, no signal was observed in the immunoblotting using pre-immune antisera and without primary antibodies (data not shown). To elucidate the onset of expression of MDP77 in muscular systems, we performed immunoblotting using homogenates of the skeletal and cardiac muscles from embryonic to post-hatched stages. Expression of MDP77 was dramatically upregulated on E9 in skeletal and cardiac muscles, and its expression continued at a high level all the way through to the post-hatched stages (Fig. 1C). Next, immunoprecipitation was done to see if these antibodies could recognize native MDP77 as well as SDS-denatured MDP77. The 115-kDa bands were bound and recovered from the extract of skeletal muscle (Fig. 2, lanes M) by these two antibodies, but not from the extract of the forebrain (Fig. 2, lanes B). Moreover, the proteins pulled down by these two antibodies were each rec-

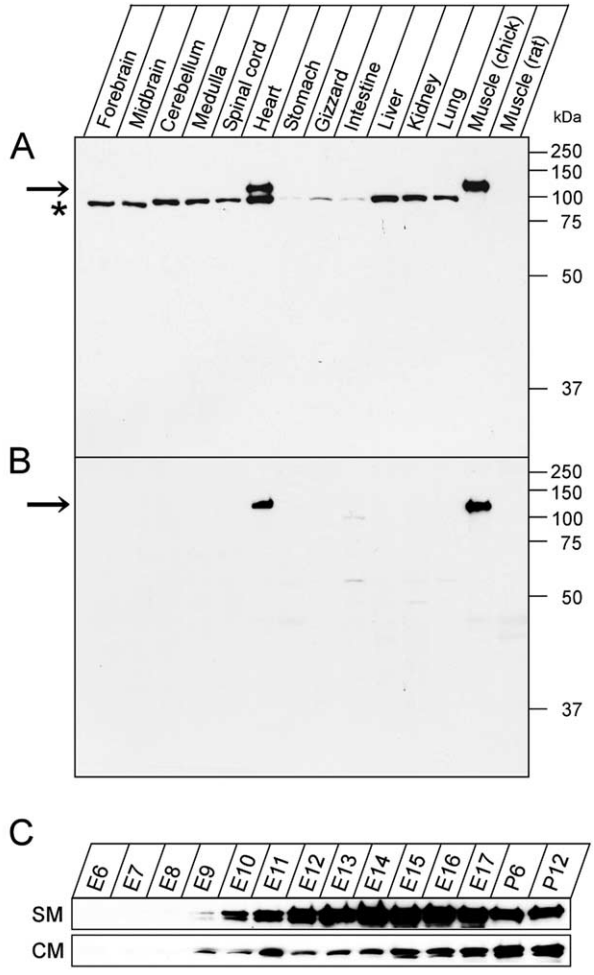

Fig. 1. Immunoblotting analysis of MDP77 in various tissues or organs. A: Immunoblotting with anti-MDP77 N6 antibody. MDP77 was detected in the skeletal and cardiac muscles at 115 kDa (arrow), and an asterisk indicates a 95-kDa unknown protein which was detectable only on SDS–PAGE under reduced condition. B: Immunoblotting with anti-MDP77 C9 antibody. Here, too, 115-kDa bands were only detected in the skeletal and cardiac muscles (arrow). C: A chronological analysis of MDP77 expression in skeletal muscle (SM) and cardiac muscle (CM). The onset of MDP77 expression was on E9, and the expression continued all the way through the post-hatched stages. Because it was difficult to discriminate skeletal muscle from embryonic legs on E6–E9, homogenates of whole embryo were used in SM.

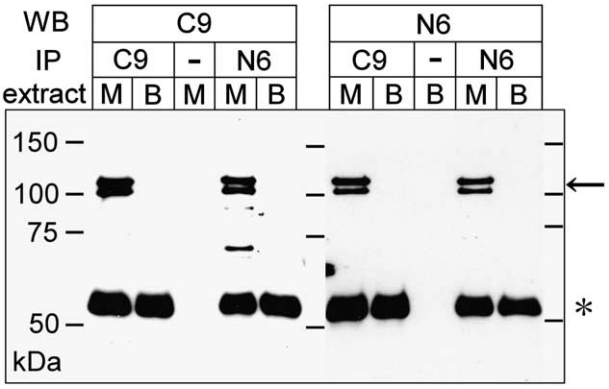

Fig. 2. Immunoprecipitation of MDP77. Extracts of skeletal muscle (M) and whole brain (B) on P9 were subjected to immunoprecipitation analysis, and the proteins bound to the antibodies were detected by anti-MDP77 C9 and N6 antibodies, respectively. Native MDP77 (arrow) in the extract of skeletal muscle (lanes M) was immunoprecipitated by each antibody and recognized with each antibody. The 95-kDa protein, which was detected in brain homogenates by immunoblotting using N6 antibody (Fig. 1A), was not observed in the case of brain extracts (lanes B). No immunoreactive signal was detected in negative control experiments without primary antibodies (−). WB: western blotting; IP: immunoprecipitation; N6: anti-MDP77 N6 antibody; C9: anti-MDP77 C9 antibody; asterisk: IgG(H).

ognized by both antibodies. No immunoreactive signal was detected in the lane without each antibody.

3.2. *MDP77 expression was upregulated after myotube formation in vivo*

Biochemical analysis revealed that MDP77 was expressed only in the muscular system except for the smooth muscles. To confirm the tissue distribution of the intact MDP77, we performed immunohistochemistry using anti-MDP77 specific polyclonal antibodies. Comparing the N6 and C9 antibodies in close sections under the same conditions, we found that the pattern of immunoreactivity was quite similar, but the signal using the N6 antibody was stronger than the one using C9 antibody (Fig. 3A,B). Thus, we have shown the results of the immunohistochemistry using the N6 antibody in the following study. We found that MDP77 was expressed only in the multinuclear-formed myotubes on E6 (Fig. 3C) and on E10 (Fig. 3D). There was a difference on the onset of MDP77 expression in each muscle. We could not observe MDP77 expression in the wing and leg on E6 (Fig. 3E). In the heart, MDP77 immunoreactivity was weaker than that in the skeletal muscles throughout the embryonic stages. The ventricles and atria were MDP77 immunoreactive (Fig. 3F). No signals were found in the artery, vena, aorta (including the tunica media which is the smooth muscle layer), connective tissue, cartilage, epidermis and other organs.

3.3. *MDP77 expression was upregulated after myotube formation in vitro*

Immunoblotting and immunohistochemistry revealed that

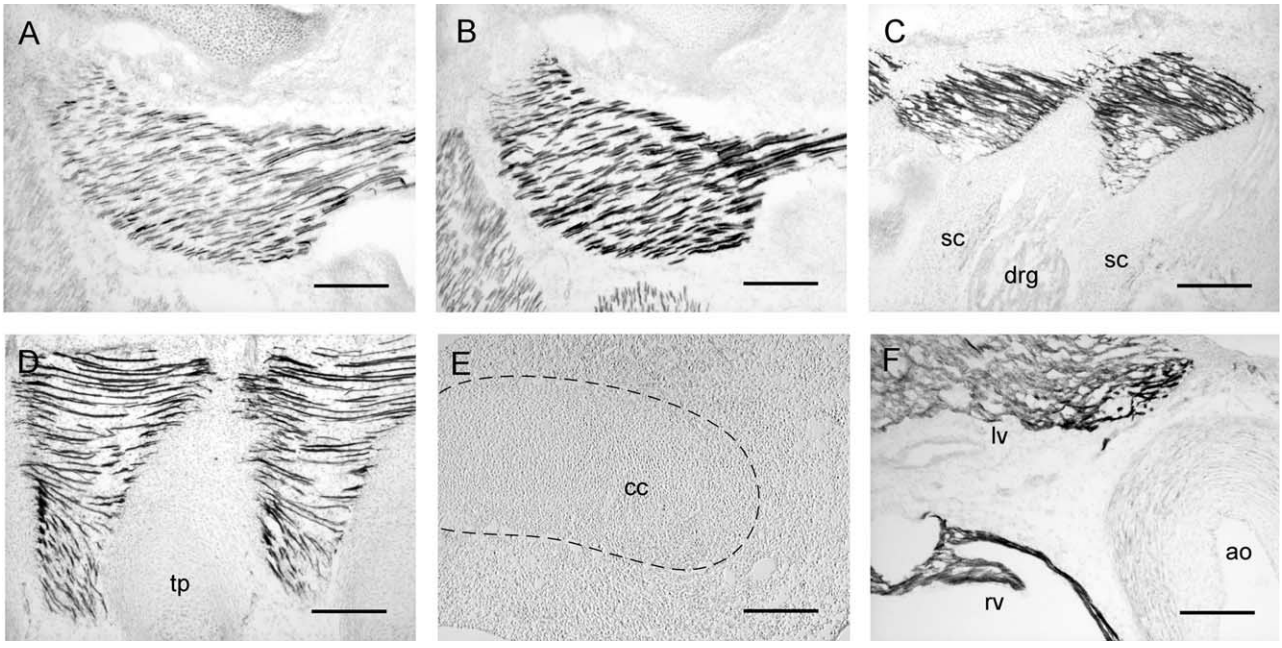

Fig. 3. Tissue distribution of MDP77 in chick embryos. The immunoreactivity of N6 and C9 antibodies was quite similar. However, the intensity of the signals with N6 antibody was stronger and there was less background than with C9 antibody using the same concentration (0.25 μg/ml). A: A cryosection at the elbow joint on E10. Fibrous myotubes were N6 immunoreactive. B: An adjacent section in A. As in the case of N6 antibody, C9 immunoreactivity was restricted in the muscle fibers. C: A parasagittal section in the trunk region on E6. Although trunk muscles were N6 immunoreactive, the sclerotomes (sc) and dorsal root ganglion (drg) were immunonegative. D: A parasagittal section in the trunk region on E10. The trunk muscle fibers were N6 immunoreactive, and the cartilaginous transverse process (tp) was immunonegative. E: A longitudinal section of E6 wing. In this stage, an aggregation of cartilaginous cells (cc, surrounded by a dotted line) was observed, but the myoblasts surrounding them were N6 immunonegative. To visualize the cells, the photograph was taken with a differential interference microscope. F: A coronal section of the heart on E10. The right (rv) and left (lv) ventricles are N6 immunopositive, but the aorta (ao) and the connective tissue were immunonegative. Bars = 200 μm in A–F.

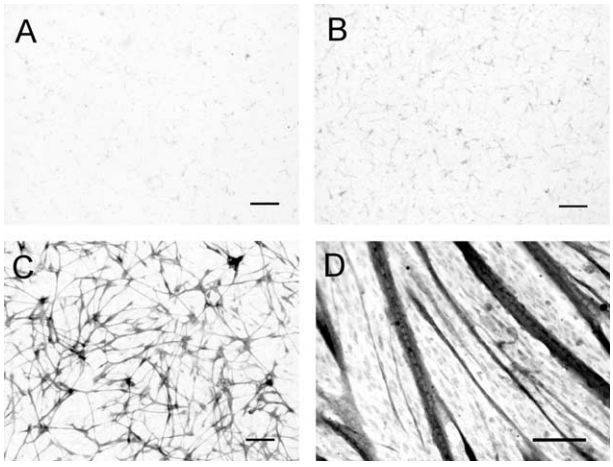

Fig. 4. The onset of MDP77 expression in vitro. Myoblasts from E10 leg muscles were harvested on gelatin-coated dishes and MDP77 expression was detected by immunohistochemistry using N6 antibody. A: Twelve hours in vitro; B: 1.0 day in vitro (DIV); C: 2.0 DIV; D: 5.0 DIV. The N6 immunoreactivity was clearly observed in the myotubes (C,D), but not in the unfused myoblasts (A,B). The localization of MDP77 was cytoplasmic and the nuclei were obviously immunonegative (D). Bars = 250 μm in A–C, 100 μm in D.

MDP77 was expressed only in the skeletal and cardiac muscles, and its expression was upregulated only after multinuclear myotube formation. To confirm this, we studied MDP77 expression using the primary culture system of immature muscle cells. Myoblasts prepared from E10 chick legs were triturated and cultured on gelatin-coated dishes. Twelve hours and 1 day later, the myoblasts were still single nuclear cells, and MDP77 immunoreactivity could not be found (Fig. 4A,B). Two days post-harvest later, the cells started fusing and formed the multinuclear myotubes, and a strong MDP77 immunoreactivity was observed in the myotubes (Fig. 4C). MDP77 expression continued in the later stages and was restricted in the cytoplasm (Fig. 4D).

3.4. *MDP77 expression and cellular distribution in the skeletal muscle of the post-hatched chick*

By immunoblotting analysis, we expected MDP77 to be expressed in the skeletal and cardiac muscles of the post-hatched chicks, as well as in the embryos. In mature muscle, the level of expression of MDP77 was moderate, and it was also restricted in the cytoplasm (Fig. 5A). Interestingly, at high magnification, MDP77 was represented in a periodical manner along the sarcomere (Fig. 5B). Furthermore, the intrafusal muscle fibers of the muscle spindles were MDP77 immunopositive (Fig. 5A,C,D). The MDP77 was clearly localized in the cytoplasm of the intrafusal muscle fibers except for the nucleus (Fig. 5C). In the transverse section, we found MDP77 in both the large and small fibers (Fig. 5D). Blood vessels, connective tissues (including fascial structures) and peripheral nerves in the muscular tissue were MDP77 immunonegative.

3.5. *MDP77 expression was upregulated in a subtype of muscle fibers after sciatic nerve transection*

To obtain data of MDP77 in denervated muscles, we performed transection of the sciatic nerve on P5, and studied MDP77 expression in the denervated muscles 4 days post-

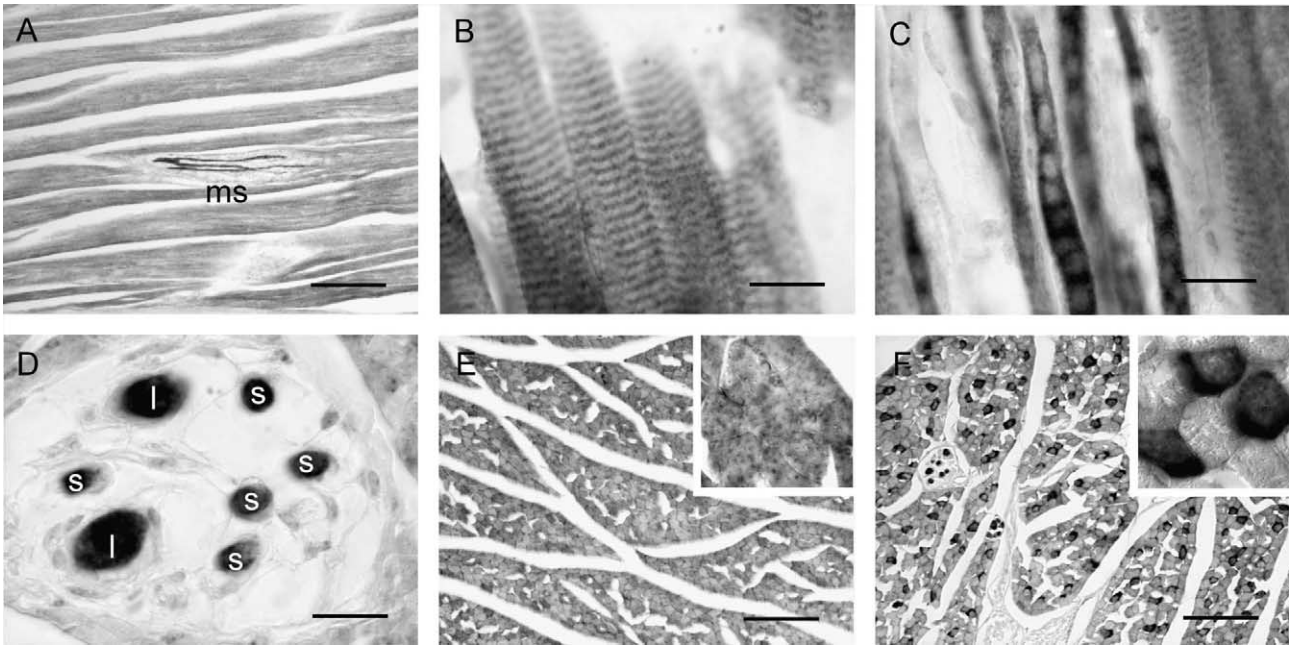

Fig. 5. Localization of MDP77 in the intrafusal and extrafusal muscle fibers in the post-hatched chick, and heterogeneous upregulation of MDP77 after sciatic nerve transection. A: A longitudinal section of the anterior tibial muscle on P9. Strong immunoreactivity was observed in the muscle spindle (ms) by immunohistochemistry using N6 antibody. B: In extrafusal muscle fibers, MDP77 was localized along the sarcomere, repetitively. C: In the equatorial region of intrafusal muscle fibers, N6 immunoreactivity was observed in the cytoplasm, but not in the nuclei. D: A transverse section of the extensor digitorum longus muscle. Two large (l) diameter and five small (s) diameter intrafusal muscle fibers were observed in the muscle spindle, and all were N6 immunoreactive. E,F: Transverse sections of the anterior tibial muscle 4 days after denervation on P5. N6 immunoreactivity was elevated in the subpopulation of denervated extrafusal muscle fibers (F) compared with a control (E). Differential cellular localization of MDP77 was observed between in denervated (inset in F) and sham-operated (inset in E) muscles. Bars = 200 μm in A; 20 μm in B–D; 100 μm in E,F.

operatively by immunohistochemistry. In a population of extrafusal muscle fibers (Fig. 5F), MDP77 expression was upregulated compared with the sham-operated control (Fig. 5E) within 4 days post-operatively. Whereas the cytolocalization of MDP77 in the sham-operated muscles (Fig. 5E and inset) was uniform, MDP77 was mainly localized beneath the plasmamembrane heterogeneously in the denervated muscle (Fig. 5F and inset).

4. Discussion

In the present study, we show that the MDP77 protein exists in the intact skeletal and cardiac muscles, and is localized in the cytoplasm in a repetitive manner. Through immunoblotting and immunohistochemistry analyses, the elevation of MDP77 expression was observed after multinuclear myotube formation in development and after sciatic nerve denervation. The most remarkable characteristic of MDP77 is its expression in the intrafusal muscle fibers of the muscle spindles. Muscle spindles are sensory receptors that detect muscle length and protect the muscles from laceration. Each spindle is comprised of four to nine intrafusal muscle fibers, inner and outer capsules, capillaries and axon terminals of the muscle sensory and fusimotor neurons. The structure, development, and regeneration of the muscle spindle in rodents [7,8], in birds [9], and in sheep [10] are well documented. One of the neurotrophic factors, neurotrophin-3, is essential for the development and differentiation of both the muscle sensory neuron and the intrafusal muscle fiber [11,12]. However, the molecular machinery of its innervation with the primary afferents is still unclear. One reason is that there is only a small number of muscle spindles, i.e. 17–35 per muscle mass [9]. Another reason is that it is difficult to discriminate the intrafusal muscle fibers from the extrafusal muscle fibers in biochemical and histological analyses. Some isoforms of the MHC are expressed in the intrafusal muscle fibers in rodents [13–16]. Previous studies have revealed that two calcium binding proteins, calbindin D-28k and calretinin, are expressed in the nuclear bag and chain fibers of rat intrafusal myotubes, respectively [17,18]. Because of the enrichment of the MDP77 protein in all types of intrafusal muscle fibers, MDP77 may be a useful histological marker for detecting the muscle spindles as well as these molecules.

In mature extrafusal muscle fibers, MDP77 immunoreactivity was observed in all the fibers, including the fast and slow muscle fibers. A repetitive pattern was shown along the sarcomere in the extrafusal muscle fibers and the polar region of the intrafusal muscle fiber, which was innervated by fusimotor neurons. The coiled-coil region of the MDP77 is similar to the rod region of the MHC. Hence, MDP77 may interact with some cytoskeletal elements.

The onset of MDP77 expression occurred just after multinuclear myotube formation in vivo and in vitro. After sciatic nerve transection, many events take place in the denervated muscles, i.e. dedifferentiation, reconstruction of cytoskeleton and activation of the muscle satellite cells for regenerating muscle fibers. Actually, a change in expression, during this process, of a large number of molecules has been reported [19–23]. In rodents, by de-efferentation, type 2B/2X MHC was reduced and embryonic MHC was strongly accumulated in small extrafusal muscle fibers assumed to be either newly formed or dedifferentiated type 2 fibers. In contrast, neonatal/adult fast MHC and embryonic/type 2B MHC were reduced, and type 2A MHC was upregulated in de-afferented intrafusal muscle fibers [23]. MDP77 is expressed in the developing muscle fibers. Interestingly, MDP77 immunoreactivity is also upregulated in a subtype of muscle fibers after sciatic nerve transection. It will be necessary to elucidate, in a future study, the chronological change of MDP77 expression in denervated extrafusal and intrafusal muscle fibers and just how MDP77 is controlled by muscle regulatory factors.

Both antibodies to the amino- and carboxy-terminal recombinants of MDP77 recognized 115-kDa bands. An immunoprecipitation study also revealed that these antibodies each recognized the same 115-kDa proteins. These results suggest that the immunoreactive 115-kDa bands are the MDP77 proteins. The apparent molecular mass of MDP77 on SDS–PAGE was larger than the deduced mass from the amino acid sequence of MDP77. The difference may be caused by the characteristics of MDP77 itself (pI = 4.75; Genetyx-Win program, Software Development Co., Japan) and post-translational modifications. Although the 95-kDa band was recognized only by the N6 antibody in the result of immunoblotting, we could not detect any immunoreactions in the immunoprecipitation and immunohistochemistry studies. These results suggest that the 95-kDa protein can form a structure resembling the amino-terminus of MDP77 only after SDS denaturation and the following renaturation.

Recently, a genomic analysis of mutant mice *In(10)17Rk* revealed that the mouse homologue of mdp77 was disrupted in the proximal breakpoint by a 50-Mb paracentric inversion on chromosome 10 [24]. Homozygous *In(10)17Rk* mice exhibit a *pygmy* phenotype due to the disruption of hmgi-c gene in the distal breakpoint. In *In(10)17Rk* mice, mdp77 disruption does not lead to a readily observed phenotype in addition to that of *pygmy*. As discussed in the paper [24], in humans, a similar sequence to chick and mouse mdp77 is mapped on 6q23.3 (GenBank accession number NT_025741.8) and dilated cardiomyopathies, CMD1F and CMD1J, are mapped on 6q23 and 6q23–24, respectively [25,26]. Although the role(s) of the MDP77 is still unclear, MDP77 may contribute to the etiology of some type of dilated cardiomyopathy. To manifest the essential role(s) of MDP77 in vivo, a detailed investigation of the phenotype of mdp77-disrupted mice must be done in further studies.

Acknowledgements: We thank Dr. Takashi Kawasaki for his helpful discussions and suggestions.

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