Molecular Biology of Muscular Dystrophy

International Workshop between Japan and France

held at Toranomon Pastoral, Tokyo

on 17-18 January , 1996

Sponsored by : Science and Technology Agency (STA)
: Japan International Science and Technology Exchange Center (JISTEC)
: Ministry of Health and Welfare

National Institute of Neuroscience
National Center of Neurology and Psychiatry(NCNP)

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Proceeding of International Workshop betwenn Japan and France

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Preface

This book represents the proceeding of international workshop between Japan and France entitled "Molecular Biology of Muscular Dystrophy" held in Tokyo, 17-18 January, 1996. The antecedent of the workshop dates back to 1995, when Robert G.Whalen and Hideo Sugita organized a small workshop in Paris. Progress in the field of muscular dystrophy has clearly been facilitated by the international cooperation, exchange of ideas and generous sharing of biological reagents. As such, we would like to acknowledge our indebtedness to Drs. Whalen and Sugita for setting these conferences in motion. All of us look forward to the next meeting tentatively scheduled for France in the Autumn of 1997.

Progress in the field of muscular dystrophy over the past several years has been impressive, and the contribution of Japan and France was becoming bigger and bigger. Especially, the cloning of the sarcoglycan genes and the subsequent identification of the mutations in some of the patients of limb-girdle muscular dystrophy has been done by Japanese and French researchers. This finding was high-lighted in this meeting. After identification of the causative gene of muscular dystrophy, most important step is to verify underlying mechanism of muscle necrosis, nevertheless the cause of muscle necrosis was deficiency of dystrophin, sarcoglycan, or laminin molecule.

One more important subject in this field is to develop gene therapy for muscular dystrophy. In life-threatened disease such as Duchenne muscular dystrophy, there is no more hopeful treatment than gene therapy. In this meeting, some of the challenging approach using cell-mediated transfer as well as using direct gene transfer, has been proposed. Progress next few year should tell the advantage and disadvantage of gene therapy.

We gratefully acknowledge Science and Technology Agency(STA) and Japan International Science and Technology Exchange Center(JISTEC) for their sponsorship of this meeting.

Eijiro OZAWA
Organizer

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The localization of dystrophin and its associated proteins on the sarcolemma of mammalian striated muscle cells

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Abstract

In confocal laser microscopy combined with indirect immuno-fluorescence with a monoclonal anti-dystrophin antibody, dystrophin was seen to be localized along the sarcolemma in a nonuniform and even discontinuous pattern in normal guinea pig skeletal and cardiac muscle cells. Sections cut tangential to the sarcolemma showed a lattice-like image of longitudinal and transverse striations. The transverse striations were often regularly spaced resembling the sarcomere pattern. An intense immuno-fluorescence staining was seen at the myotendinous junctions in skeletal muscle cells and at the intercalated discs in cardiac muscle cells. This staining pattern suggests that dystrophin may also be localized in the adherens junctions where myofibrils attach. When muscle cells were stained with both anti-dystrophin and anti-β-dystroglycan, the staining pattern was completely identical suggesting that both proteins are colocalized along the sarcolemma. In contrast, the staining of vinculin was not well superimposed on that of dystrophin.

To clarify the exact relationship between the sarcolemma and dystrophin and other related proteins, crude sarcolemmal vesicles were prepared from rabbit and rat skeletal muscles according to the methods described by Ohlendieck et al.(1991). When such crude sarcolemmal vesicles were examined by negative staining and thin section electron microscopy, they were found to be heterogeneous in population varying in size and shape. Among them, large vesicles of irregular shape contained fine filamentous structures and vesicular fragments. Such filamentous structures formed networks which were closely associated with the inner surface of the vesicle membrane. Interestingly, the

filamentous networks were restricted to the membrane domains where particulate structures projected from the outer surface. Gold-labeled wheat germ agglutinin (WGA) selectively bound to such large vesicles. With some other findings, the large vesicles were considered to be of sarcolemmal origin. When the crude sarcolemmal vesicles were mounted on a grid and immunologically stained with anti-dystrophin antibody after brief Triton treatment, immunogold particles were found only on the filamentous networks beneath the vesicle membranes. Replica electron microscopy showed characteristic granular structures on most of large vesicles. They seemed to correspond to the particulate structures projecting from the outer surface of vesicles as seen in thin sections. Exogenous laminin could be reconstituted on the sarcolemmal vesicles in close association with such particulate structures, suggesting that they may represent dystroglycan complexes.

Dystrophin: localization and regulatory regions for expression

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Introduction

Although the gene that, when defective, results in Duchenne muscular dystrophy was isolated and called dystrophin gene[1-2)], mechanism of muscle degradation in this condition is still obscure, because of the unknown physiological or mechanical function of the gene transcript. To determine the localization and functional significance of dystrophin, we studied various tissues from almost entire body of control and mdx mice, and control rats, using polyclonal antibodies against dystrophin. We also studied human muscle biopsy specimens.

It is known that the muscle type promoter is involved in expression of the dystrophin gene in skeletal, cardiac and smooth muscle as well as in glial cells. It is conceivable that the muscle type dystrophin promoter contains the different regulatory regions for skeletal, cardiac and smooth muscle in vivo, but the detail is not well known. With this background we started to investigate the mechanism of developmental and tissue-specific regulation of the muscle type dystrophin gene, especially promoter region in mice, using transgenic mice.

Materials and methods

Immunohistochemical study

All the tissues from mice and rats, including the cerebrum, cerebellum, spinal cord, eye, tongue, nose, heart, lung, kidney, digestive organs, liver, spleen, muscle, skin and blood vessels were obtained immediately after anesthetic death. Indirect immunofluorescence and ABC method were used to stain cryostat sections on gelatinized coverslips. We used several kinds of dystrophin antibodies(Fig. 1).

Transgenic mice

We generated transgenic mice carrying the 900bp genomic fragment from the muscle type dystrophin promoter region, fused to the coding region of the bacterial *lacZ* gene(Fig. 2). Inserts from pBlue dys promoter-*lacZ* were excised by Xhol-BamHl digestion, purified with Gene Clean(Bio 101), and micro-injectedinto 400 fertilized mouse eggs collected after mating(Fig. 3).

Results

Immunohistochemical study

We observed a immunohistochemical dystrophin reaction in synaptic regions such as neuromuscular junctions, equatorial region of intrafusal fibers, myo-tendinous junctions, outer plexiform layer of the retina, the taste buds, papillary layer of the dermis, tactile nerve endings in the skin and neurons in the brain, as well as on the surface membrane of skeletal, cardiac and smooth muscle fibers. Then we analyzed mRNAs from the retina of mice with the use of the reverse transcription and polymerase chain reaction (RT-PCR) method. The 5' sequences, corresponding to the first exon, of dystrophin transcripts (DT) in the retina was mainly the brain type(Fig. 4), whereas in the 3' region of DT that corresponds to the C-terminal domain of dystrophin, some additional RT-PCR products were detected.

Base sequences in three of them showed homology to these for previously reported human brain type dystrophin isoforms(Fig. 5).

Transgenic mice

Nineteen independent transgenic founder mice were identified, in six of which the expression of *lacZ* was detected in the right heart. Fourteen of the nineteen transgenic lines produced offspring. Various tissues of transgenic adult mice were stained with X-gal to detect *lacZ* expression. Six of the fourteen transgenic mouse lines showed *lacZ* expression only in the right heart(Fig. 6). The reporter gene expression was detected first in the presumptive right atria and ventricular myocardium of the embryos at 8.5 days post coitum.

We compared the promoter sequence to that of the previously reported the human sequence of 850bp region upstream from the cap site[3] and reported the mouse sequence of 400bp upstream from the cap site[4]. The homology between the human and mouse 140bp regions upstream from the initiation codon reaches the 93%. Upstream from this point, the mouse and human sequences look more divergent (66.8% homology). Non-specific region, ATA and GC box, and muscle specific regions, CArG box, are conserved in human and mouse.

Discussion

We observe rather strong immunohistochemical reactivity in synaptic regions[5-9] as well as on the skeletal, cardiac and smooth muscle fiber membrane. It is well known that these synaptic regions, which showed immunohistochemical dystrophin reaction, contain abundant synapses and/or non-myelinated nerve endings, as a common histological characteristics. With this background we consider that the localization of dystrophin in the synaptic regions suggests its physiological function in the conduction system rather than a mechanical one. In 1993, Pillers et al.[10] reported abnormal electroretinogram in Duchenne and Becker muscular dystrophy patients, that is markedly reduced amplitude of the b-wave in the dark-adapted state. The results suggested that the dystrophin has some function to keep normal function in the retina. Recently, D'Sauza et al.[11] finally reported a novel isoform of dystrophin (Dp260) present in the mouse retina and that is required for normal function in the retina. So, we still don't know the full length brain type dystrophin has function or not.

But, anyway it is not likely that dystrophin plays a critical role in the conduction system, because there has been no report of apparent retinal abnormalities in DMD patients. Dystrophin may play a delicate role, acting as

only a trigger propagating contraction within the cell. Dysfunctioning of the conduction system due to a defect in dystrophin is still an important factor in the muscle degradation in DMD. As for the vascular system, no smooth muscle cells receive nerve endings, which are usually several microns away from the cells. This suggests that the vascular system may be one of the most vulnerable tissues to a defect in dystrophin although it play a minor role in the conduction system due to anatomical characteristics[12]. I would like to emphasize that we should not ignore the localization and function of dystrophin on the smooth muscle layer, especially on the vascular system. As for the transgenic mice, by using the E.coli *lac Z* reporter gene instead of the CAT assay, it has been possible to follow visually the temporal and spatial expression of dystrophin both during embryonic and postnatal mouse development. The *lacZ* gene has advantage of visualizing expression in specific organs.

We found that the 900bp promoter region of mouse muscle type dystrophin was active only in the right heart, i.e. not in skeletal smooth or left heart muscle. On the other hand, Muntoni et al.[13] reported family cases with only severe cardiomyopathy, i.e. no clinical muscle symptoms, due to possible deletion the muscle type dystrophin promoter, but the cardiac-specific region of the dystrophin promoter has not been identified to date. Our data in this study may be convenient to explain the cases reported by Muntoni et al.

These results suggest that several delicately divided elements for dystrophin expression exist in muscle tissues, such as left heart, skeletal and smooth muscle cell.

Fig. 1 **Antidystrophin antibodies**

Affinity-purified, region-specific dystrophin antibodies used in this study. kD=kDa

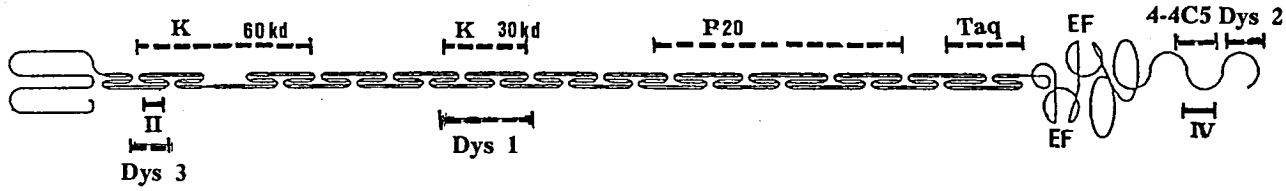
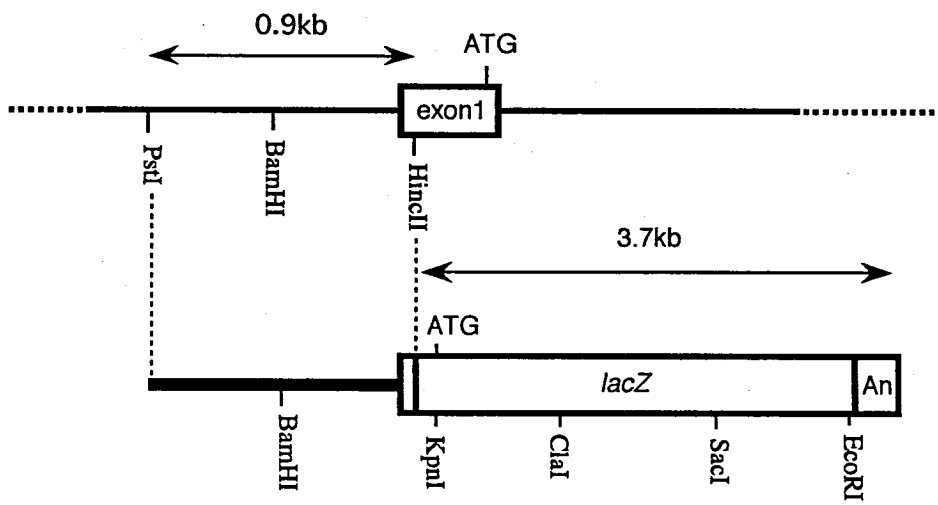


Fig. 2

Mouse Dystrophin Promoter-*lacZ* Constract



Structure of dystrophin muscle type promoter lacZ transgene.
900bp PstI-HincII fragment derived from Mouse dystrophin promoter region was fused to the coding region of the E.coli, lacZ gene.

Fig. 3

Scheme of the procedure for producing transgenic mice