CURRENT RESEARCH IN MUSCULAR DYSTROPHY, JAPAN

(CLINICAL RESEARCHES)

The Proceedings of the
Annual Meeting of Muscular
Dystrophy Research Group,
1980, Tokyo

PREFACE

Following the last Proceedings of the Annual Meeting of Muscular Dystrophy Research Group, 1978, 1979, "Current Research in Muscular Dystrophy, 1980" is published.

This volume contains not only the abstracts of the papers presented at the Annual Meeting of the Clinical Research Group of Muscular Dystrophy in 1980, but also some of the research works performed during past three years.

Meanwhile, a real progress has been made in the fields of the genetic, clinical, morphological and metabolic aspects of the disease and this may lead to the understanding of the fundamental defects. Still, we have to emphasize that these and other discoveries, while they are significantly advanced and certainly very encouraging, are by no means final.

Lastly, I as the chairman of this Research Group, would like to express my sincere appreciation to the Ministry of Health and Welfare, National Center for Nervous, Mental and Muscular Disorders, and Muscular Dystrophy Association of Japan for persistent financial support to this Research Group for three years.

My thanks are also due to Dr. H. Sugita, the co-editor, who has made it possible to publish this report.

Kazuo Miyoshi

March, 1981

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EARLY MORPHOLOGICAL CHANGES IN DYSTROPHIC CHICKEN MUSCLES

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We previously reported[1] that early morphological changes appeared in PLD muscles at 1 week after hatching and these changes became more prominent at 3 weeks after hatching. This time, we studied the caveolae on the plasma membrane surface of the superficial pectoral muscles at various stages after hatching in control and dystrophic chickens.

Materials and methods

We used the superficial pectoral muscles, white fibers, of control (line 412) and dystrophic chickens (line 413) at 1 day, 1 week and 3 weeks after hatching. For the freeze-fracture EM study, the muscle specimens were routinely fixed in 2.5% glutaraldehyde and cut into 1-mm cubes following fixation. Then, the blocks were immersed in 25% glycerol. These specimens were frozen by immersion in liquid nitrogen. They were fractured and shadowed with platinum and carbon in a freeze-fracture device operated at $-115°C$ and beneath 1×10^{-6} Torr. Replicas were cleaned in bleach and washed with distilled water and examined with an electron microscope. 200-micron square areas representing a total sampling area were counted in order to determine average caveolar density.

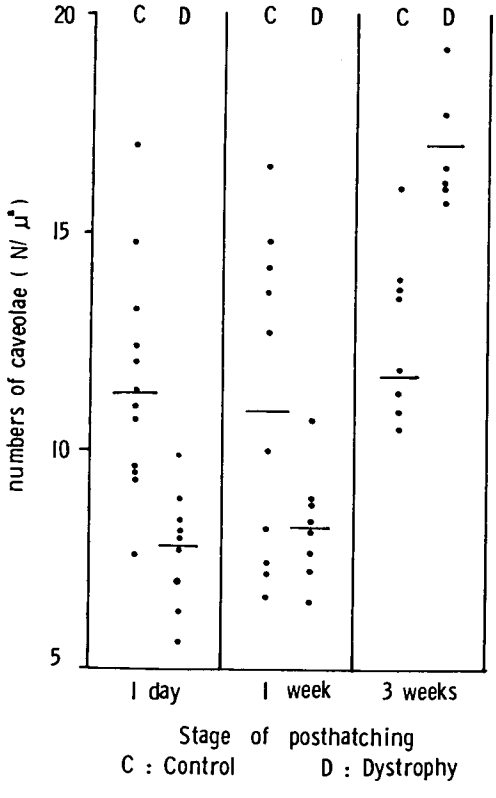

Fig. 1 CHANGES OF CAVEOLAR DENSITY
(total sampling areas: 200 μm^2)

Results (Fig. 1)

At 1 day and 1 week after hatching, there was a difference in caveolar density between control and dystrophic muscles. The distribution, shape and size of caveolae were more irregular in dystrophic chickens (Fig. 2).There were no remarkable differences in caveolar density between 1 day and 1 week after hatching. However, the caveolar distribution showed more random arrangement in dystrophic muscles. At 3 weeks after hatching, there was an increase of caveolar density in dystrophic muscles as compared with control muscles and the caveolar distribution was more irregular and random in dystrophic muscles. In control muscles, the caveolae were arranged in a regular pattern. There were no remarkable changes in caveolar density of control muscles at 1 day, 1 week or 3 weeks after hatching.

Discussion

The caveolae are considered to be either pinocytotic vesicles or T-system openings. There were no obvious changes in dystrophic

muscles at 1 day after hatching in our previous study, while the changes of caveolar density, distribution and size were already seen at 1 day after hatching in dystrophic muscles. This study of caveolae is considered to be more useful for detecting the early morphological changes of the dystrophic process.

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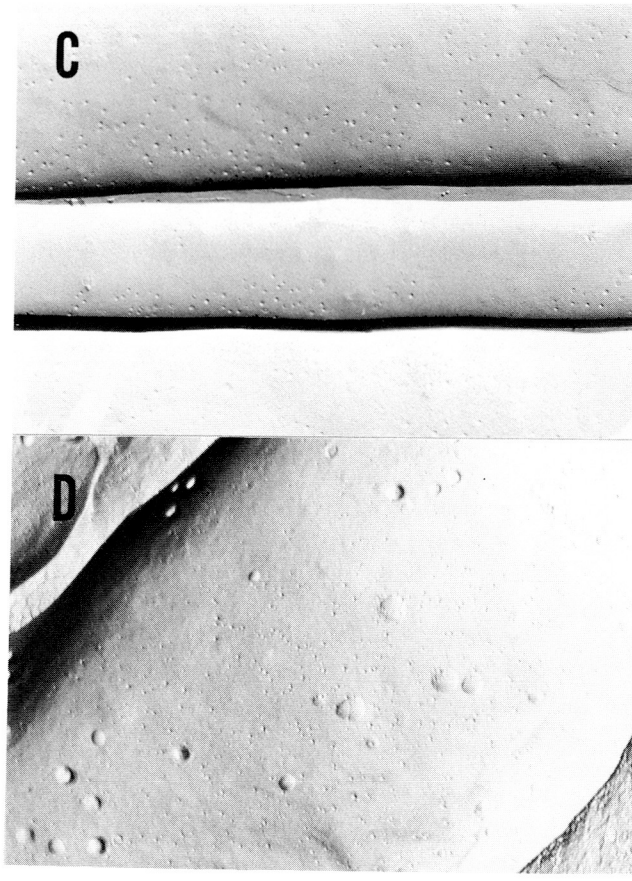

Fig. 2
(C) P face of muscle plasmalemma from a 1 day old normal chicken. The caveolar distribution has a regular pattern. × 21.000
(D) E face of muscle plasmalemma from a 1 day old dystrophic chicken. The caveolar distribution is more random and the distribution and size are more variable than in control chickens. × 40.000

MORPHOMETRIC INVESTIGATION OF DEVELOPING MUSCLES OF DYSTROPHIC CHICKEN WITH SPECIAL REFERENCE TO THE POSTERIOR LATISSIMUS DORSI MUSCLE

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The posterior latissimus dorsi muscle (PLD) is a well-known affected muscle in the dystrophic chicken of line 413, it is in striking contrast to the healthy appearance of the anterior latissimus dorsi muscle (ALD), and has various beneficial points for experimental procedures. This investigation was designed to measure the number and diameter of muscle fibers in order to gain some basic morphological data for PLD in comparison with those for ALD, superficial pectoralis (PS), biceps brachii (BB) and gastrocnemius muscle.

Materials and methods

The PLD and ALD muscles were removed as a whole from dystrophics at 3, 5, 8 and 16 weeks after hatching and small strips of the PS, BB and gastrocnemius muscles were obtained from dystrophics at 5, 12, 19, 26 and 33 days after hatching, and also from age-matched controls. In order to calculate the total number of fibers in PLD and ALD, the images of each muscle trunk were reconstructed from many pictures of paraffin cross sections after fixing in paraformaldehyde solution. The diameters of about 500 muscle fibers were measured at random on magnified microphotographs of each case. For the PS, BB and gastrocnemius muscles, cryostat sections were used for measurement of fiber diameters. The data were evaluated by statistical analysis.

Results

Table 1 shows the mean value and standard deviation of the total number of PLD muscle fibers in each group. The total number at 3 weeks after hatching was about 9,800 in dystrophics, corresponding to 2.5 times the value for ALD muscle fibers (about 3,700), but showed no significant difference from the controls. The total number of fibers had a decreasing tendency in the following stages of dystrophics and had diminished definitely at 16 weeks. On the other hand, the total numbers of fibers in the dystrophic ALD muscles were 3,772.0 ± 712.5, 3,410.3 ± 506.8 and 3,356.0 ± 140.6 at 3, 5 and 8 weeks of age, respectively, showing no significant difference from controls.

The mean muscle diameters of PLD increased gradually in developing stages without a significant difference between dystrophics and controls until 8 weeks of age, and the mean value was about 42 μm at 16 weeks in dystrophics, being higher than that of controls (about 30 μm) ($P < 0.01$). The mean muscle diameters of PS and BB muscles showed higher values in dystrophics than controls after 12 days of age ($P < 0.05$). The distribution of muscle fiber diameters was compared using megahistograms of the diameters of each stage. Large fibers were observed at 3 weeks in PLD of dystrophics, small fibers were seen additionally at 5 and 8 weeks, and many hypertrophic fibers of over 60 μm in diameter appeared at 16 weeks. No significant change was observed in the distribution of fiber diameters in the ALD muscle. On the other hand, many fibers became larger in diameter than controls after 12 days of age in PS and BB muscles of dystrophics (Imaoka).

Table 1. Total number of muscle fibers in PLD

Age (weeks)	Control	Dystrophic	P
3	11,797.0 ± 1,098.7 (n=3)	9,825.0 ± 1,984.1 (n=3)	NS
5	10,496.7 ± 616.9 (n=3)	8,042.0 ± 6,112.4 (n=3)	NS
8	9,483.3 ± 2,736.9 (n=3)	6,194.0 ± 1,186.6 (n=3)	NS
16	12,742.8 ± 1,965.4 (n=9)	7,164.8 ± 2,465.8 (n=6)	<0.005

NS : not significant

Summary

Morphometric results obtained for the PLD muscle in developing dystrophic chickens can be summarized as follows.

1. The total number of muscle fibers exhibited no significant decrease at 3 weeks after hatching in comparison with the controls, being about 2.5 times higher than the value for the ALD muscle, and decreased significantly at 16 weeks.

2. The mean value of fiber diameters was increased at 16 weeks, but not different significantly from controls until 8 weeks. The fiber diameters showed wide variation after 3 weeks of age compared with controls.

3. In the PLD muscle the increase in mean fiber diameter and the variation in diameter occurred later than in the PS and BB muscles. The PLD muscle in developing stages was supposed to be only mildly damaged in comparison with the PS and BB muscles.

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INTRACYTOPLASMIC VACUOLATION OF αW FIBERS IN CHICKEN MUSCULAR DYSTROPHY — PROBABLE EARLY PATHOLOGICAL EVENT INITIATES MASSIVE FIBER NECROSIS —

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The main pathological changes in dystrophic chicken include muscle fiber necrosis with an active regenerating process which is also a characteristic finding in human muscular dystrophy. However, the evidence of preferential damage to the white muscle, intracytoplasmic vacuolation, a lack of opaque fibers overloaded by calcium ions in dystrophic chicken lead us to assume that the pathophysiology in fiber necrosis might be different between human and chicken muscular dystrophy. In addition to the degenerative changes, a delay in fiber type transformation from the immature αR to the mature αW fiber in the dystrophic white muscle has been shown by histochemical examination (1, 2). The intracytoplasmic vacuoles localized mainly in the αW fibers have been thought to play an important role in developing fiber necrosis (3). To elucidate the pathogenesis of fiber necrosis in dystrophic white muscle, an electron microscopic study was performed to correlate histochemical fiber type with vacuolar formation.

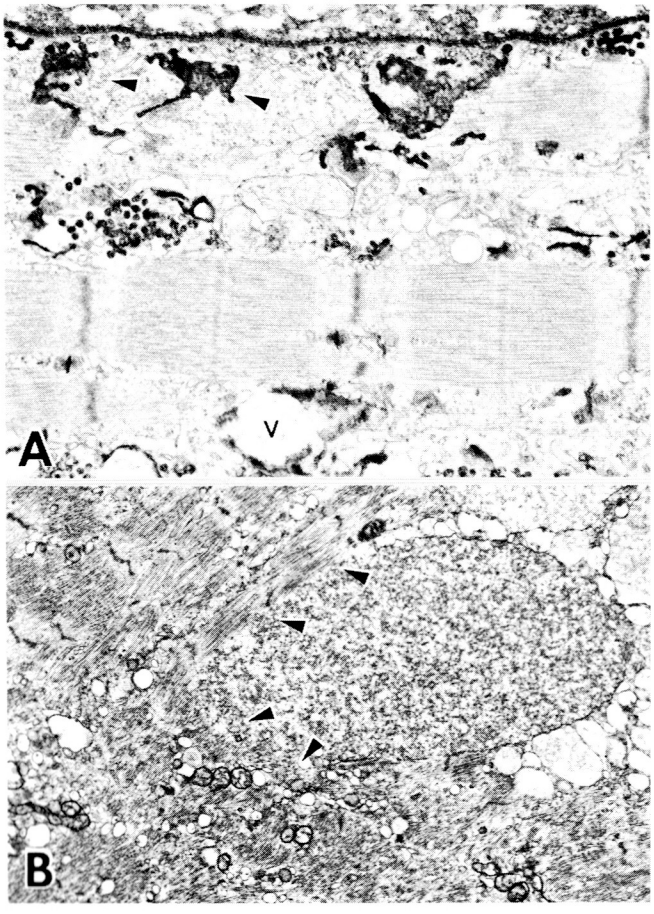

Fig. 1A: Proliferative caveolae with a honeycomb structure forming a large vacuole (arrow head) and a vacuolated T-tubule (v) in an αW fiber from dystrophic PLD. Lanthanum nitrate staining, × 17,000.
1B: Myofibrillar degeneration and Z-band loss in the vicinity of a large vacuole with a focal membrane defect (arrow heads). × 15,300.

Materials and methods

To examine the electron microscopic characteristics of each fiber type, the pectoralis superficialis (PS) and posterior latissimus dorsi (PLD) muscles were dissected from both non-dystrophic (line 412) and dystrophic (line 413) chickens at 2-4 months after hatching. The muscles were immediately immersed in relaxing solution at pH 6.8 containing EGTA, ATP, TRIS and maleate. A teased single fiber was divided into two segments in the solution under a dissecting microscope; one for histochemical examination to identify the muscle fiber type and the other for electron microscopic examination. Small pieces of the specimens obtained from dystrophic and non-dystrophic white muscles were fixed in cacodylate buffered 2.8% glutaraldehyde solution for 2-4 hours. After rinsing in the same buffer solution, they were post fixed in s-collidine buffered 1.3% OsO_4

containing lanthanum nitrate (Revel and Karnovsky).

Results

In the αW fibers from the PS muscle, the transverse (T) tubules ran at the level of the Z-line which measured 500-600A in width. Whereas the T-tubules were located at A-I junction in both αR and αW fibers from the PLD muscle. Since the Z-line in αR fibers measured 800-1000A and that in αW fibers 600-800A, two subtypes of the muscle fibers in PLD were distinguishable by electron microscopy.

When lanthanum nitrate was used for electron microscopic examination, the T-tubules were clearly demonstrated. The T-tubules in the dystrophic αW fibers appeared to be proliferated forming a honeycomb to conglomerated structure (Fig. 1A). The small intracytoplasmic vacuoles were stained by lanthanum nitrate suggesting that they originated from the proliferated T-system. A connection between the vacuoles and T-tubules or caveolae was also demonstrated by lanthanum nitrate staining.

In the vicinity of the vacuoles with a partial defective membrane, focal myofibrillar degeneration, Z-streaming, loss of Z-line and vacuolated organelles were recognized (Fig. 1B). On the other hand, the T-system and myofibrils were well preserved in the αR fibers in which the mitochondria were enlarged and remarkably increased in number.

Discussion

The present study again confirmed preferential damage to white muscle in dystrophic chicken (1, 2). Even in the white muscle, only the αW fiber undergoes vacuolar change in association with focal myofibrillar degeneration. Therefore the degenerative events seem to be expressed once the muscle fiber has acquired the histochemical characteristics of an αW fiber. The sarcotubular system proliferation may be the earliest pathological manifestation in the dystrophic process in chicken. The proliferated T-tubules and caveolae subsequently form large vacuoles by their fusion and/or expansion of unknown causes (3).

Since the T-tubules are opened to the extracellular space, the vacuoles probably contain high concentrations of calcium ions. A focal defect of the vacuolar membrane, as shown in Fig. 1B, may permit calcium influx into the sarcoplasm which activates calcium dependent proteases such as calcium activated neutral protease (CANP). Digestion of the Z-band by the protease initiates myofibrillar degeneration followed by massive fiber necrosis with phagocytic invasion. From the present results, it can be concluded that the proliferative event in the sarcotubular system is a prelude to the necrotic process in dystrophic chicken. Understanding the mechanism of the proliferative process may help to solve the pathophysiology in fiber necrosis in chicken muscular dystrophy.

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A RADIOAUTOGRAPHIC STUDY OF THE ACTIVATED SATELLITE CELL IN DYSTROPHIC CHICKEN MUSCLE

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Satellite cells which were first reported by Mauro[1] were increased in number in myopathies. They undergo mitosis and are incorporated into muscle fibers and become muscle nuclei.[2] Thus satellite cells are considered to be the source of muscle fibers when the fibers are injured or show degeneration.

In the pectoralis muscle of the dystrophic chicken, many satellite cells are observed. The purpose of this study was to distinguish the activated satellite cell from the quiescent satellite cell by means of radioautography using ^{3}H-thymidine and to perform ultrastructural observation of the activated satellite cell.

Materials and methods

4 dystrophic chickens (line 413) and 4 control chickens (line 412) of either sex aged one day and 21 days after hatching were used. They received 4 times every 6 hr intraperitoneal injections of doses of 22-67 u Ci g body weight of ^{3}H-thymidine. The superficial pectoralis muscle was fixed by immersion in 3% glutaraldehyde for 20 min, postfixed in O_sO_4, dehydrated in alcohol and propyleneoxide, and embedded in epon-araldite. 1 um sections and ultrathin (gold-silver interference color) sections were treated with SAKURA NR M2 or SAKURA NR H2 by the dipping or wire-loop method.[3] The specimens were exposed for 4 to 6 weeks in the refrigerator and developed by the LIGOP method.[4] For light radioautography the specimens were stained with toluidine blue and for electron microscopic radioautography they were stained with lead citrate.

In light radioautography, we estimated the number of activated satellite cells by the following labeling index.

$$\frac{\text{numbers of labeled nuclei}}{\text{whole numbers of nuclei}} \times 100$$

Using an oil lens, 100 different visual fields were counted.

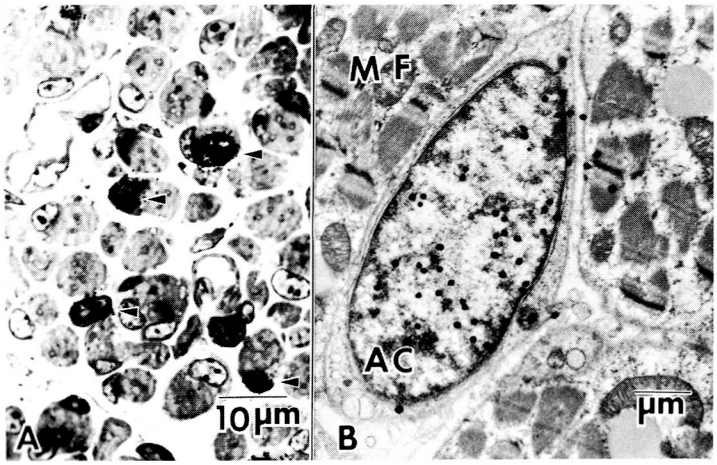

Fig. 1 A: Light microscope radioautogram of the pectoralis muscle one day after hatching. The nuclei (arrow head) are overlaid with silver grains.
B: Electron microscope radioautogram; the satellite cell nuclei (AC) are overlaid with silver grains. MF; muscle fiber.

Results and comments

A light microscope radioautograph shows labeled nuclei readily (Fig. 1. A). One day after hatching, the labeling index of control chickens is 4.59, and that of dystrophic chickens is 4.19. 21 days after hatching, that of control chickens is 3.86, and that of dystrophic chickens is 5.68. At this stage, the labeling index of dystrophic chickens was higher than that of the control chickens.

On electron microscope radioautography, the label was found

only in satellite cells (Fig. 1, B), none of the true muscle nuclei were labeled.

The labeled nuclei of satellite cells (activated satellite cells) had a round, oval or irregular form. Chromatin in the nuclei of the activated satellite cells showed variation from much to little. The contents of the activated satellite cells also showed variation. In some activated satellite cells, the plasma membrane of the satellite cell and that of the muscle fiber showed the "foot" structure. This suggested that the satellite cell is incorporated into the muscle fiber.

The label is also found on the nuclei of the satellite cells undergoing mitosis.

The cytoarchitectural characteristics could not be used to differentiate the activated satellite cells from the quiescent satellite cells in the early stage.

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TROPHIC EFFECTS OF SYMPATHETIC GANGLIA ON NORMAL AND DYSTROPHIC CHICK SKELETAL MUSCLES IN TISSUE CULTURE (2) — ACETYLCHOLINESTERASE AND CATECHOLAMINE STAINING —

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We have reported that the muscles from dystrophic chick degenerated earlier than those from normal chick in single cultures. In co-cultures of the muscles and the sympathetic ganglia, the muscles, either the normal or the dystrophic ones, were maintained for a longer time than those in single cultures. In co-cultures of them, a bridge was formed with abundant muscle and sympathetic nerve fibers between both explants. Some of the nerve fibers terminated on the muscle fibers where the acetylcholinesterase activity positively appeared (1). The present study aimed to clarify the relationship between cholinergic and adrenergic fibers in co-cultures of various combinations of the muscles and the sympathetic ganglia using histochemical techniques.

Materials and methods

The pectoral muscles from normal and dystrophic chick embryos of 11 days old and the cervical sympathetic ganglia from those of 13 days old were used as materials. Fragments of the muscles and the ganglia were explanted alone or together with special separation of about 2-3 mm on collagen coated cover slips. Combinations of the tissues were as follows: A, normal muscle (NM); B, dystrophic muscle

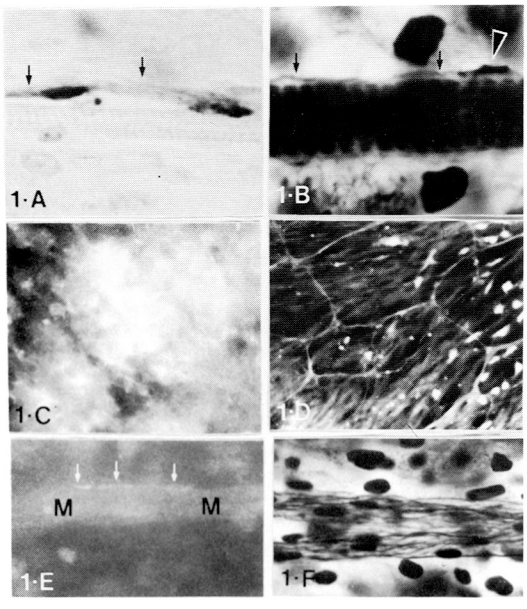

Fig. 1-A: Co-culture of the normal muscle (for 7 days) and the normal sympathetic ganglion (for 5 days). AChE activity appears on the sarcolemma, forming crescents (arrows show sympathetic nerve fibers) AChE staining by Nakamura's rubeanic acid technique. × 490.

B: Co-culture of the normal muscle (for 23 days) and the dystrophic sympathetic ganglion (for 21 days). A sympathetic nerve fiber runs along the muscle fiber and terminates at the positive site of AChE activity on the sarcolemma. Bodian's silver impregnation after AChE staining. × 1200.

C: Single culture of the dystrophic sympathetic ganglion for 13 days. The cytoplasm of sympathetic neurons in the explant shows bright fluorescence of CA. The glyoxylic acid technique. × 120.

D: Single culture of the dystrophic sympathetic ganglion for 13 days. Sympathetic nerve fibers radiating from the explant show bright fluorescence of CA. The glyoxylic acid technique. × 60.

E: Co-culture of the dystrophic muscle (for 17 days) and the normal sympathetic ganglion (for 15 days). A sympathetic nerve fiber running parallel with the muscle fibers (M) shows fluorescence of CA (arrows). The glyoxylic acid technique. × 260.

F: Co-culture of the dystrophic muscle (for 30 days) and the normal sympathetic ganglion (for 28 days). Sympathetic nerve fibers run forming a dense bundle between both explants. Bodian's silver impregnation. × 210.

(DM); C, normal sympathetic ganglion (NS); D, dystrophic sympathetic ganglion (DS); E, NM and NS; F, NM and DS; G, DM and NS; H, DM and DS. Cultures were maintained in a humidified air-tight chamber which was gassed with 3% CO_2 and 97% air. The fluid medium was changed three times a week. It consisted of 80% Eagle's MEM with Hanks' salts, 5% chick embryo juice, 15% horse serum and 200 u/ml of penicillin. For demonstration of acetylcholinesterase (AChE) activity, the modified Karnovsky-Roots' and Nakamura's rubeanic acid techniques were used. For demonstration of catecholamine (CA), liquid formaldehyde and glyoxylic acid techniques were used.

Results and discussion

(AchE staining)

In any combination of the muscles and the sympathetic ganglia, positive sites of AchE activity appeared in the form of dots or filaments along the sarcolemma of the muscle fibers in the bridge formed between both explants after one to two weeks of culture. This could be demonstrated more in detail by use of an enhancement medium containing rubeanic acid as compared with use of the modified Karnovsky-Root's technique. In this method, the positive sites appeared in the form of crescents (Fig. 1-A) or semicircles.

After three to four weeks of culture, they appeared in the muscle explant and further on the muscle fibers radiating from the explant. Among the combinations in co-cultures, no prominent difference was observed in the form and number of the positive sites of AchE activity.

(Bodian's silver impregnation after AchE staining)

Sympathetic nerve fibers ran along or parallel with the muscle fibers and some of them terminated at the positive sites of AchE activity on the sarcolemma (Fig. 1-B).

(CA staining)

With the liquid formaldehyde method, the cytoplasm of both large neurons in the explant and small neurons migrating out from the explant were shown to contain fluorescent granules of CA in cultures of the sympathetic ganglia. But fluorescence was not induced in the sympathetic nerve fibers. The glyoxylic acid technique of Furness and Costa yielded good results as compared with the liquid formaldehyde method. With this method, not only the cytoplasm of both the large and small neurons, but also sympathetic nerve fibers radiating from the explant exhibited bright fluorescence of CA (Fig. 1-C, D).

In co-cultures of the muscles and the sympathetic ganglia, however, only a few fibers showed fluorescence of CA along the muscle fibers in the bridge between both explants (Fig. 1-E), although many sympathetic nerve fibers were shown to exist on Bodian's silver impregnation (Fig. 1-F).

Patterson et al. showed that the presence of certain nonneuronal cells or conditioned medium of them had a profound effect on the type of transmitter chosen by the sympathetic neurons (2, 3). From this point, it is suggested that the sympathetic adrenergic nerve fibers changed into the cholinergic ones in co-cultures of the skeletal muscles and the sympathetic ganglia.

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THE MOVEMENT OF "FILOPODIA" TO NORMAL AND DYSTROPHIC CHICK MUSCLES

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It has been suggested that there are membrane abnormalities in the muscles of those with progressive muscular dystrophy. This theory is based on many observations such as an increase in serum CPK, depolarization of the resting membrane potential, decreased duration of the outward potassium current during muscle excitation, and a decrease in the number of intra-membrane particles. On the other hand, filopodia – fibrous processes projecting from the growth cone of a dissociated neuron – are now assumed to be able to discriminate objects by mere touch.[1,2] Therefore, in this study, an attempt was made to find out when these membrane abnormalities are recognized by the filopodia of spinal motoneurons.

We analysed dynamically the movement of filopodia and growth cones of normal spinal motoneurons in four different groups: (1) 5-day cultured normal muscle, (2) 5-day cultured dystrophic muscle, (3) more than 2-week cultured normal muscle, and (4) more than 2-week cultured dystrophic muscle. The superficial pectoral muscle was excised from 11-12 day old embryos (stage 37-38) of normal and dystrophic chickens. Muscle tissue was dissociated by 0.25% trypsin, suspended in the fluid medium for muscle (Eagle's MEM: extract of the chick embryo: calf serum = 8:1:1), and plated on collagen-coated dishes. These dishes were incubated for three hours at 37°C, in an atmosphere of 5% CO_2 and 95% air saturated with water vapour. Then, the dishes were rinsed with a Ca^{2+}–and Mg^{2+}–free solution, and treated with 0.05% trypsin for 15 minutes at room temperature. Myogenic cells removed from the bottom of the dishes by these procedures were again plated on collagen-coated dishes, which were incubated under the same conditions described above. Cells still floating after the two-hour incubation were used for the selective culture of the muscle. The cells were seeded in other collagen-coated dishes at a concentration of about 10,000 cells per 1 ml of culture medium, and cultured for 5 days to 3 weeks. The cell density was low so that an overgrowth of muscle would not interfere with the observation of filopodia. As for the culture of motoneurons, according to the method used by Masuko et al. (1979)[3], the ventral half of the spinal cord was obtained from normal 60-66 hour old chick embryos (stage 17). Cord fragments were cultured at 37°C for 24 hours in a plugged tube, which was rotated at 70 rpm in a gyratory shaker and contained a fluid medium for nerves (Eagle's MEM: extract of the chick embryo: calf serum = 8:0.5:1.5) with 10 μM of cytosine arabinoside. These fragments were then dissociated by 0.25% trypsin, and the density of neurons was set at 60,000 to 80,000 cells per 1 ml of culture medium. The suspension of neurons was seeded directly into dishes containing the muscle culture, and incubated for 2 hours under the same conditions as previously described. The fluid medium containing much debris was then replaced by a completely new one, after observing the establishment of neurons on the bottom of the dishes. The movement of filopodia was then recorded by phase-contrast cinematography for three hours per neuron. After that, some of the dishes were cultured for one to three weeks for further observation of morphological changes of motoneurons, whereas the others were fixed in FEA solution (5% formalin, 5% acetic acid, 90% alcohol) and stained by a silver impregnation technique to confirm the presence of motoneurons.

1) The movement of filopodia to the normal muscle. In four cases out of each of the two groups of five 5-day cultured muscles and five of the more than 2-week cultured muscles, the growth cone making contact with the muscle surface had a tendency to immobilize itself and to cease projecting filopodia in an hour. However, in one case of each group, the growth cone and its filopodia moved away from the muscle in about an hour after their initial contact with the muscle. 2) The movement of filopodia to the dystrophic muscle. In three cases out of the five 5-day cultured dystrophic muscles, the growth

cone was immobilized on the muscle surface by the same process as that in the four cases of the normal muscle. In two cases, however, the growth cone did not cease moving, and the filopodia continued palpating the muscle surface for more than an hour after initially touching the muscle. On the other hand, in four cases out of five of the more than 2-week cultured dystrophic muscles, the growth cone, once having touched the muscle, moved away from it. In one case, the growth cone of a motoneuron attached itself firmly to the dystrophic muscle and its detachment was not observed within three hours.

Although it is certain that there are other kinds of well-differentiated neurons besides motoneurons in the posterior horn, as well as at the junction of the anterior and the posterior horns of the spinal cord[4,5,6], which are technically hard to eliminate completely, the obtained suspension of neurons is believed to be composed of about 90% motoneurons and less than 10% contaminants (personal communication from Dr. Shimada). It is also true that the movement of filopodia shows diverse varieties, and the 3-hour observation may not be enough to assert that the filopodia of a certain motoneuron stop moving. Consequently, in this study, we paid attention to the overall rate of occurrence of the removal of filopodia, as well as trying to make as many observations as possible. From these observations it may be possible to say that the more severe the muscular degeneration is, the more likely it is to observe the removal of the growth cone and filopodia from the muscle. Supposing that the filopodia are the "probing chemical touch system"[7] and that they are able to recognize the surface structure of muscle, it may be postulated that the frequency of occurrence of the removal of filopodia reflects abnormalities of the surface structure (the basement membrane or the plasma membrane) of dystrophic muscle. However, since our present observations were not numerous enough to draw any definite conclusions, it is absolutely necessary to examine more cases in the future.

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STUDY ON NEUTRAL LIPID IN THE MUSCLE OF DYSTROPHIC CHICKEN

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It has long been recognized that there is an excessive amount of neutral lipids in the muscle in muscular dystrophy. However, the true mechanism of the accumulation has not been clarified. In the present work, the amount of neutral lipids in the muscle and the fatty acid compositions were compared between dystrophic and control chickens.

The amount of triglyceride was 1.94 ± 0.93 mg/100 mg wet weight in the dystrophic muscle compared to 0.24 ± 0.13 mg/100 mg w.w. in the control. Diglyceride was also increased in dystrophy, but monoglyceride could not be determined because of the small amount.

As shown in the figure, the fatty acid compositions of triglyceride differed between the dystrophic and control muscles in that there were decreases in C16 palmitic acid and C18:1 oleic acid, but increases in C18 stearic acid and C18:2 linoleic acid in the dystrophic chicken. On the other hand, no significant change was seen in the fatty acid composition of diglyceride. These decreases or increases of fatty acid subfractions of triglyceride were compared to those of subcutaneous adipose tissue. The fatty acid compositions were similar in the dystrophic and control birds, and no significant difference was seen. It was found that the above mentioned changes in C16, C18, C18:1 and C18:2 in the muscle of dystrophic chicken grossly resembled those of the adipose tissue in both dystrophic and control birds. These facts suggest that the accumulation of neutral lipids in the dystrophic muscle is not due to a primary defect in intracellular lipid metabolism but secondary to loss of muscle fibers of an obscure origin.

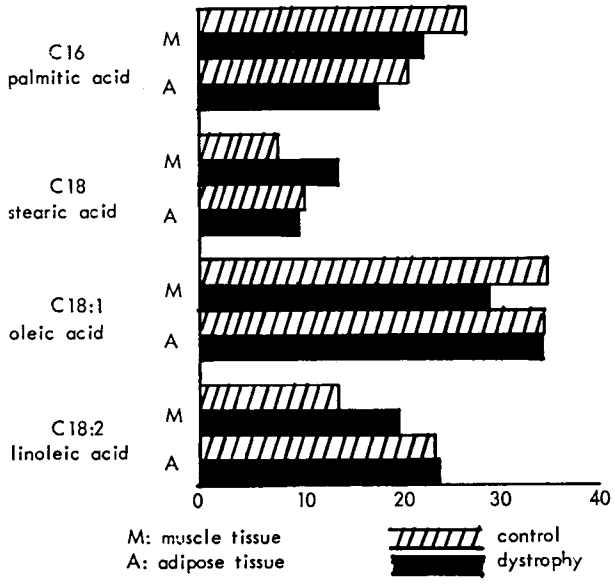

Fig. Fatty acid composition of Triglyceride

GANGLIOSIDES IN DYSTROPHIC CHICKEN MUSCLE

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Introduction

Abnormalities of muscular gangliosides have been reported in denervated muscles, the muscle of dystrophic mouse and the muscle of experimental rabbit muscular dystrophy. Recently gangliosides have been found to be located exclusively in the cell membrane and to play an important role in such reactions as receptor interaction, cellular adhesion and cellular recognition. We have analyzed the structures of the muscular gangliosides in chickens and compared the gangliosides of dystrophic chicken and control chicken.

Materials and Methods

1. Determination of structures of main gangliosides in chicken skeletal muscle

Total lipids were extracted from 30kg of muscle of white leghorn chickens with chloroform-methanol (1:2 and 1:1, by volume) at room temperature. The extracts were applied to a DEAE-Sephadex column. The neutral lipids were eluted with methanol and then gangliosides were recovered by elution with 0.2 M sodium acetate in methanol. The ganglioside fractions were further separated and purified by thin layer chromatography (TLC). Each purified ganglioside was analyzed for sugar by gas liquid chromatography (GLC).

2. Ganglioside composition of skeletal muscle of dystrophic chicken

Gangliosides were extracted from 3g of superior pectoral muscles obtained from 10 dystrophic chickens (line 413) and 10 control chickens (line 412), and analyzed by thin layer chromatography with two solvent systems (Ando, et al.[1]).

Results and Discussion

5 gangliosides, A, B, C, D and E, were isolated. A has the same Rf value as GM_3, and B that of GD_3. The other gangliosides showed unique movement on TLC, and the Rf values of these gangliosides do not coincide with any known gangliosides. Each ganglioside appeared as a doublet.

The sugar compositions of the purified gangliosides were determined by gas chromatography. Gangliosides A, B and C contain glucose, galactose and N-acetylneuraminic acid. The molar ratio of glucose, galactose and N-acetylneuraminic acid is 1:1:1 in A, 1:1:2 in B, and 1:1:3 in C, and D contains glucose, galactose, N-acetylglucosamine, N-acetylneuraminic acid, in the molar ratio of 1:3:2:1. Ganglioside E was recovered in a too small amount to be analyzed. Gangliosides A, B and C were hydrolyzed with Clostridium perfringens neuraminidase, and the hydralyzates were examined by TLC. All gangliosides were converted to lactosylceramide. These results indicated that all gangliosides have lactosylceramide as the core structure. To determine the sugar linkages in these gangliosides, permethylation studies were performed. Gas liquid chromatography of partially methylated neuraminic acid showed that from ganglioside A, there was only one peak, and from ganglioside B, there were two peaks. The ratio of the first to second peak was 1:1, and from ganglioside C, there were two peaks, the ratio of the first to second peak was 1:2. Each peak was examined with a mass spectrometer. The first peak was determined to be 1, 2, 4, 7, 8, 9-O-methyl-N-acetylneuraminic acid, and the second peak was determined to be 1, 2, 4, 7, 9-O-methyl-8-OTMS-N-acetylneuraminic acid. From these findings, the structures of gangliosides A, B and C were determined to be NANA-Gal-Glc-Cer, NANA-NANA-Gal-Glc-Cer and

NANA-NANA-NANA-Gal-Glc-Cer, respectively. A was GM_3, and B was GD_3. C was GT_3 and was a very unique ganglioside. This ganglioside had been found only in cod fish brain[2], and so here is first demonstrated in muscle. Ganglioside D contains N-acetylneuraminic acid, galactose, N-acetylglucosamine and glucose in the molar ratio of 1:3:2:1. The sugar linkages in ganglioside D have not been determined, but considering the sugar composition and the movement on TLC, the structure of ganglioside D was considered to be the same as NANA-Gal-GlcNA-Gal-GlcNA-Gal-Glc-Cer, which was one of three monosialongangliosides isolated from chicken muscles very recently by Chien and Hogan[3]. The structure of ganglioside E was not determined.

The ganglioside patterns of the dystrophic chicken muscles and control chicken muscles were examined with both the alkaline and the neutral solvent systems. The 5 gangliosides, A, B, C, D and E, migrated, and moreover, two additional gangliosides were found. Ganglioside F co-migrated with sialylparagloboside which was isolated from human red cell membrane. The structure of this ganglioside from chicken muscles was determined to be NANA-Gal-GlcNA-Gal-Glc-Cer by Chien and Hogan[3] and the structure was the same as sialyl paragloboside in human red cell membrane. G was a novel ganglioside and its structure was partially determined to be NANA-Gal-GalNA-Gal-Gal-Glc-Cer by Chien & Hogan[3]. Each ganglioside content was measured by scanning these 7 spots on two different plates with a densitometer. In dystrophic chicken muscles, total gangliosides increased 3.6 times. Especially, GM_3, ganglioside D, was highly increased. Ganglioside F, GD_3 and GT_3 also increased. E and G were not changed (Table).

Table Ganglioside composition of normal and dystrophic chicken muscles

Ganglioside	Control (N = 10)	Dystrophy (N = 10)	Dystrophy/Control
	μg NANA/g protein		
Total	13.5 ± 4.2	48.7 ± 22.5	3.6
A (GM_3)	5.5 ± 1.2	23.1 ± 8.1	4.2
B (GD_3)	2.0 ± 1.1	5.3 ± 3.0	2.7
C (GT_3)	0.3 ± 0.2*	0.6 ± 0.4*	2.0
D	3.7 ± 2.3	16.8 ± 13.6	4.5
E	0.5 ± 0.4	0.6 ± 0.4	1.2
F	0.5 ± 0.2	1.7 ± 0.9	3.4
G	1.1 ± 0.6	0.7 ± 0.8	0.6

*$P < 0.025$

The increased amounts of these gangliosides could be due to two possible causes. First is an increase of membrane components in dystrophic muscle tissue containing an increase of the sarcotubular system in necrotic muscle fibers. The second is increased biosynthesis or decreased catabolism of these gangliosides in dystrophic muscles. At this time, the metabolic pathways of these gangliosides are not clarified, so it is difficult to discuss the disturbance of ganglioside metabolism in dystrophic muscles. Further studies are needed to clarify the problem.

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GENETIC ANALYSIS OF AMYOTROPIC DISEASES — DUCHENNE TYPE MUSCULAR DYSTROPHY —

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For the analysis of X-genetic linkage between the loci for Duchenne type muscular dystrophy, Xg^a blood groups, and colour blindness, a study will be made using as a proband of Duchenne type muscular dystrophy (DMD) Kagoshima data (about 48 families) and Tokushima data (about 40 families) and tests of linkage and estimation of crossover values will be conducted. This year a study was made on 10 families in the Kagoshima data. The results of this study are given in the following table.

Table 1. Results of Xg^a blood group and colour blindness tests in DMD during 1980

S \ r	1	2	3	Number of sibships	Number of sibs	Number of DMD	Test completed: Xg^a blood groups	Test completed: colour blindness	Father: Xg^a blood groups	Father: colour blindness	Mother: Xg^a blood groups	Mother: colour blindness
1	5			5	5	5	3 (60.0)	3	2 (40.0)	2	2 (40.0)	2
2	12	5		17	34	22	17 (50.0)	18 (52.9)	4 (23.5)	4	5 (31.3)	5
3	11	3		14	42	17	17 (40.5)	17	3 (23.1)	3	3 (21.4)	3
4	4	2		6	24	8	3 (12.5)	3	Test completed			
5	2	1		3	15	4	2 (13.3)	2				
6	1		1	2	12	4	2 (16.7)	2				
7	1			1	7	1						
Total				48	139 (Male: 85)	61	44 (31.7)	45 (32.4)	9 (19.1)	9	10 (21.3)	10

S: Number of affected persons in each sibship. r: Number of DMD ( ): %

As all the data were collected by single ascertainment, the segregation ratio (P) is estimated from the following equation

$$P = \frac{R - N}{T - N}$$

where R is the total number of DMD cases, N the number of sibships, and T the total number of children included in the sibships. Using the foregoint equation the segregation ratio was estimated to be as follows:

$$P = 0.351 \pm 0.0785$$

The difference between this estimated value and the expected value (0.5) is 1.9 times larger than the error, which is not statistically significant at the 5% level. Thus, DMD does not conform with the theory of X-linked recessive inheritance. It is, however, a fact that the segregation ratio is lower than the expected value, but here gene selection may be involved.

At any rate, for the testing of X-linked inheritance, it is a prerequisite that the genetic mechanism of this entity be elucidated. In the year following after completion of the study on the cases yet to be examined, it is necessary that a review be made regarding this point.

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FACIOSCAPULOHUMERAL SYNDROME; THE NON-HEREDITARY VARIETY

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The facioscapulohumeral (FSH) type of muscular dystrophy was first recognized by Landouzy and Dejerine nearly a century ago. Because the FSH distribution of muscle wasting is not limited to muscular dystrophy, the term "FSH syndrome" is used to include myopathic and neuropathic diseases. Three cases with characteristics distinct from the usual FSH syndrome were described.

Case 1 – 27-year-old female. At 18 months of age, she had a febrile illness with a temperature of nearly 40°C. One month later, paralysis of the child's mouth and unclear speech were detected. She was unable to run as fast as other children. At age 4, the retrospective diagnosis was polio. At age 27, she was a tall woman with myopathic facies. Muscle atrophy was apparent in the shoulder, hip and peroneal regions. Scapula alata was prominent. Serum CPK was 237 (normal $<$ 100). EMG showed a great decrease in the number of motor units and fibrillation potentials. Family history was negative.

Case 2 – 15-year-old female. At the age of 15 months,discovery of horseshoe kidney led to a 4-hour operation under general anesthesia. After leaving the hospital, she had difficulty picking herself up from a sitting position. She had no facial expression. At age 4, the FSH type of muscle wasting was observed. Muscle biopsy of the right tibialis anterior showed no particular changes. At age 15, muscle wasting was observed in the scapuloperoneal region with slight weakness of the orbicularis oculi. Serum CPK was 29. EMG findings of denervation in the gastrocnemius and tibialis anterior suggested a neurogenic process. Family history was negative.

Case 3 – 49-year-old female. At the age of 5 years and 1 month, she developed high fever, nausea and vomiting. Two months later, her mother noticed that the child dragged her legs and her back was not straight. She could not stand up without using her hands and could not smile. A doctor told the parents that the child had had "infantile paralysis". At age 49, her clinical features were those of an advanced FSH syndrome. Serum CPK was 40. EMG revealed no denervation potentials. Family history was negative.

The onset and progression of the most FSH syndrome are insidious and may occur anywhere from childhood to late adulthood. Tyler and Stephen's analysis of the dystrophic FSH syndrome showed that the onset was from 7 to 55. In our analysis of the neurogenic FSH syndrome, the earliest onset was 7 years. The cases cited in the present study had onsets of 15 months, 18 months and 5 years, respectively. The onset was subacute and initial diagnoses were "polio" in all the cases. Generally, there was no significant progression or slight partial recovery in muscle weakness except for 2 episodes with the sudden appearance of new symptoms in Case 1.

In our patients symptoms were first obvious after an acute febrile illness or surgical operation. Occasionally, we have seen ambulant patients with neuromuscular disorders lose a lot of strength after a few days in bed due to a febrile illness. The lack of family history in all our cases will rule out the possibility that their diseases are premature manifestation of the usual FSH syndrome.

Hanson and Rowland described 3 unrelated patients who were originally thought to have Möbius syndrome because of facial weakness in infancy, but were later diagnosed to have FSH muscular dystrophy. Characteristics of their cases were: (1) early onset of the disease, (2) relatively slow progression, (3) asymmetry and (4) lack of family history. Our cases have similar characteristics to theirs, but were not congenital.

A search of the literature revealed similar patients. In 1914, Dejerine described a 17-year-old boy with scapulohumeral muscle wasting, whose disease appeared at 2½ years of age after pulmonary infec-

tion of unknown origin. His condition was stationary after age 6. A 16-year-old boy with the neurogenic FSH syndrome developed his disease after febrile illness (possibly whooping cough) at age 3 (Kanbayashi et al). These cases also lacked a family history.

Polymyositis rarely represents FSH muscle wasting. In our cases no findings of polymyositis were detected. However, it cannot be ruled out that inflammation might have played a role in the pathogenesis of the disease at the initial stages.

Although little attention has been paid to such cases, recognition of the presence of this variety is of importance in prognostication and in genetic counseling.

FAMILIAL OCULOCRANIOSKELETAL NEUROMUSCULAR DISEASE WITH ABNORMAL MUSCLE MITOCHONDRIA – A FOLLOW UP STUDY

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There have been many case reports of progressive external ophthalmoplegia associated with numerous neurological signs and symptoms and abnormality of mitochondria. The pathogenesis and nature of this syndrome are still controversial. Recently we studied one such case which had been already reported by Tamura et al in 1974 under the title of familial oculocranioskeletal neuromuscular disease with abnormal muscle mitochondria. The purpose of this report is to describe the clinical course of this patient over the period of the last 7 years and to discuss the problems in the classification and relationship of similar disorders.

A 34-year-old woman was seen in September 1980 with the chief complaint of ophthalmoplegia. She was the product of an uncomplicated pregnancy and delivery. Her parents were first cousins. She had been slow in physical and mental activities since school days. Bilateral blephaloptosis started at 18 years of age. Then ophthalmoplegia, nasal voice, dysphagia, hearing loss, generalized muscle atrophy and weakness successively developed. At 27 years of age she was evaluated by Tamura et al[1]. She was not followed over the next seven years, while her symptoms were slowly progressive. In 1980 she was referred to us by an otorhinolaryngologist whom she visited for her hearing loss. Four out of 6 siblings including herself were affected by the same disease to various degrees.[1]

The physical findings and the laboratory data are summarized in Table 1 in comparison to those of Tamura et al. The characteristic features were mild mental deficiency, marked external ophthalmoplegia with ptosis, moderate facial and bulbar palsy, neurogenic hearing loss, generalized muscle wasting and weakness predominantly in the nuchal and proximal muscles. Deep tendon reflexes were generally hypoactive. There was no pigmentary degeneration of the retina nor cerebellar signs. No myotonia nor sensory loss were noted. The gait was almost normal, though mild Gowers' sign was observed.

The cerebrospinal fluid showed a mild increase of protein content (50 mg/dl). There was no conduction defect on ECG. The EEG demonstrated 5 to 6 Hz generalized theta slowings. Needle EMG revealed different findings in each muscle tested, as shown in Table 1. "Myopathic changes" include short duration, low voltage NMU and full interference pattern. "Neurogenic changes" include high voltage, polyphasic NMU as well as giant action potentials, and decreased number of NMU.

The muscle biopsy from the lt. quadriceps muscle disclosed mild variations in the size of the muscle fibers, most of which were angular fibers. There were also muscle fibers showing granular changes. Central nuclei were observed in 37% of the fibers. There was a moderate increase of connective tissue with cell infiltration. Modified Gomori-trichrome staining demonstrated a few ragged red fibers in the area of granular changes shown by the H.E. staining. DPNH staining revealed dark stained areas at the subsarcolemma of the ragged red fibers. Moth-eaten fibers were also observed. Electron microscopic examination revealed abnormally increased mitochondria, glycogen and lipid in the subsarcolemma. However, the increased mitochondria were almost normal in shape and size. No paracrystalline inclusions were observed in the mitochondria.

CT scanning demonstrated diffuse symmetric low density in the white matter.

Discussion

As shown in Table 1, most of the clinical manifestations of this patient were complete by 27 years of age, and further progression of her disease was slow over the next 7 years. There were only a few new findings such as apparent mental disturbance, abnormal EEG and diffuse low density of the white matter

Table 1 A comparison of the clinical features and laboratory data of Tamura et al, 1973, and of the authors, 1980.

	TAMURA (1973)	AUTHORS (1980)
Age (years)	27	34
Body weight (kg)	34	29
Ptosis & ophthalmoplegia	marked	marked
Muscle weakness & atrophy		
facial	moderate	moderate
bulbar	moderate	moderate
nuchal	moderate	moderate
proximal	moderate	moderate
distal	mild	mild
Mental function	normal	full scale IQ 65 (WAIS)
Hearing loss (neurogenic)	mild	Rt. 20 dB, Lt. 30 dB. (1000 Hz)
Optic fundi	normal	normal
DTRs	decreased	decreased
Cerebellar signs	none	none
Sensory loss	none	none
ECG	normal	normal
EEG	normal	5 to 6 Hz diffuse theta slowings admixed.
CSF protein	75 mg/dl	50 mg/dl
Serum CPK, LDH	normal	normal
NCV	not done	Rt. facial; mildly slow Rt. median, ulnar & peroneal; normal
EMG		
myopathic	Rt. biceps	Rt. sternocleidomastoid, Rt. deltoid & Lt. biceps
mixed	------	Rt. triceps & Rt. thenar
neurogenic	------	Rt. quadriceps
Muscle biopsy		
site	Lt. biceps	Lt. quadriceps
variations in the size of muscle fibers	mild	mild
central nuclei	not increased	increased
granular changes	present	present
connective tissue	increased	increased
angular fibers	absent	present
ragged red fibers	present	less remarkable
mitochondrial changes		
number	increased	increased
size	enlarged	almost normal
paracrystalline inclusion	present	absent
CT scan	not done	diffuse low density of the white matter

in the CT scan. There were also some different findings in EMG and muscle biopsy. It is not certain whether these differences reflect the chronological changes or topographic variations.

This is a unique familial disease whose clinical manifestations and laboratory findings including mitochondrial abnormalities mimic those of Kearns-Sayre syndrome which has been considered as a sporadic disease.[2] Since the original report of this case by Tamura et al, there have been some reports[3),4)] of similar familial diseases whose spectrum of symptoms and mode of inheritance are not completely identical in each case. The nomenclature and the relationship between each case as well as Kearns-Sayre syndrome are still controversial. At present solely seminologic discussion without etiologic

consideration would be of limited value. Further etiologic investigations are required to resolve these problems.

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COMPUTERIZED TOMOGRAPHIC FINDINGS OF THE CENTRAL NERVOUS SYSTEM IN MYOTONIC DYSTROPHY

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Introduction

Patients with myotonic dystrophy (MD) often show mental impairment, hypersomnia, abnormality of smooth pursuit movement, light-near dissociation and inclusion body of the thalamus, that suggest central nervous system (CNS) involvement in this disease. We examined CNS of MD by computerized tomography (CT).

Materials and methods

A control group (C) and an MD-patient group were used for this study. The C group consisted of 16 males and 15 females who didn't have any organic diseases. The MD group consisted of 10 males and a female. The examinations were carried with CT-scanners, EMI 1010 (160 × 160 matrix) and DELTA 50 FS (256 × 256 matrix). The slice widths were 10 mm and 8 mm, respectively. The angle of CT was 15° over the canthomeatal line. Measurements were made on the x-ray films using a translucent millimeter rule. In each CT, the following dimensions were measured: A. the maximum transverse inner diameter of the skull, B. the maximum width of cella media, C. the maximum distance between the tips of the anterior horns, D. the maximum width of the third ventricle and E. the maximum width of the interhemispheric fissure. From the absolute values of the above data, (1) the cella media index (B/A), (2) the anterior horn index (C/A) and (3) the third ventricle index (D/A) were derived.

Results

Patients with MD generally showed dilatation of the ventricular system on CT. The cella media indices of the fifth and sixth decades were significantly greater than those of the C groups of corresponding ages (Table 1). Likewise, the anterior horn indices were greater in patients of the fourth and sixth decades than age-matched controls. The third ventricle indices in MD were greater in all age groups and the differences between MD and C groups were most prominent. On the other hand, the interhemispheric fissures were only slightly larger, though not significantly, in MD groups, probably because of the difficulty in measurements.

Table 1 Indices of the ventricular system on CT

Age		30 – 39 Y.O.		40 – 49 Y.O.		50 – 59 Y.O.	
Sex		Male	Female	Male	Female	Male	Female
(1)	MD	24.3 ± 3.4		30.8 ± 4.6*	25.6	29.4 ± 3.8*	
	C	18.8 ± 3.8	20.7 ± 1.6	21.5 ± 2.7	22.6 ± 2.4	21.1 ± 2.2	25.4 ± 3.7
(2)	MD	30.9 ± 3.0*		30.6 ± 4.7	28.2	31.4 ± 2.3*	
	C	25.5 ± 2.3	21.3 ± 6.8	25.9 ± 3.0	26.6 ± 1.1	26.5 ± 1.2	25.8 ± 3.1
(3)	MD	4.8 ± 1.7*		5.7 ± 2.4*	3.9	6.6 ± 1.7*	
	C	2.5 ± 0.5	2.4 ± 0.1	2.6 ± 0.5	2.3 ± 0.9	3.1 ± 1.0	3.2 ± 1.1

M ± SE % * significant ($p < 0.05$)

Discussion

The present study demonstrates the greater third ventricle indices in MD patients than those of controls. The enlargement of the third ventricle is especially remarkable. Patients with MD are known to have hypertrophic changes of the skull due to possible abnormal secretion of growth hormone. In our studies, however, the maximum transverse inner diameter of the skull in MD was not significantly different from that in the C group. Then we used this diameter to calculate various indices which were also easy to measure. Pneumoencephalographic studies in MD revealed the dilatation of the third ventricle and the body of the lateral ventricle. These observations are compatible with our CT findings. The enlargement of the third ventricle seems particularly important, since patients with MD often show many neuroendocrinological abnormalities which can be ascribed to the dysfunction of the diencephalon. It is possible that the dilatation of the third ventricle is of significance, specifically for MD. However, CT findings change with age and various states. It is important, therefore, to study the correlation between the dilatation of the ventricular system and duration of the disease or neuroendocrinological findings.

Conclusion

Patients with MD showed enlargement of the ventricular system, especially the third ventricle on CT, suggesting the organic change of the NCS.

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AN UNUSUAL CASE WITH FACIO-SCAPULO-HUMERAL MUSCULAR ATROPHY

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Introduction

Progressive muscular atrophy was regarded as a neurogenic or myogenic disorder and it has been known that neurogenic atrophy is remarkable in the distal portions of extremities, while myogenic atrophy is predominant in proximal portions. However, the relationship between neurogenic and myogenic atrophy has been discussed on the basis of the age of onset, muscle involvements, progression and genetics, and many cases have been studied by researchers in order to come to an agreement[1,2].

As for cases with muscular atrophy involving the face, shoulder and humerus, they have been subdivided into three groups, i.e. facio-scapulo-thigh type, facio-scapulo-peroneal type and scapulo-peroneal type, and furthermore cases of each group were also different.

An unusual case with facio-scapulo-humeral muscular atrophy is presented here.

Case report

A 30-year-old man was completely normal in childhood. At the age of 15, he could not lift a heavy object above his head and became gradually unable to elevate his arms. At the age of 17, he became unable to run fast and had difficulty in dorso-flexion of his ankle joints and climbing steps. At the age of 29, he fell frequently and had difficulty in extending his left hand, and then showed facial muscle atrophy.

His past history was unremarkable. His parents were not consanguineous. His one elder brother complained of gait disturbance. General physical examination was normal. Neurological examination revealed only facial muscular weakness in the cranial nerve territory and he could not close his eye-lids or whistle. Evaluation of motor function revealed weakness of the deltoids, biceps, triceps, right forearm extensors, hip flexors and adductors, quadriceps and tibial extensors. Muscular atrophy was remarkable in the proximal portions of the extremities except for tibial atrophy. Fasciculation, sensory disturbance, ataxia, involuntary movements and joint contractures were not observed. All deep tendon reflexes were decreased. He had a waddling gait, had to get up from the floor with the help of his hands and could not stand on his toes.

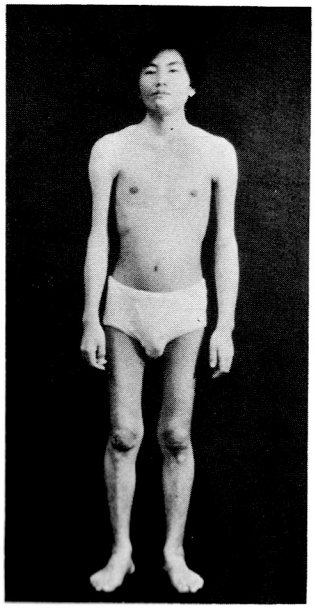

Fig. 30 years old, male

Laboratory examinations showed a normal hemogram and urinalysis. The erythrocyte sedimentation rate was 4 mm/hr. Latex agglutinations for rheumatoid arthritis and the antinuclear antibody test were negative and liver function, serum electrolytes and endocrinological studies were all normal. Serum CPK was 154 u. (normal < 70) and aldolase was 3.1 u. (normal < 2.2). Daily urinary excretion of creatine was 610 mg/day. Chest, cervical and lumbal X-rays and ECG were normal.

Electromyography of the biceps, triceps, forearm extensors, quadriceps, gastrocnemius and tibial anterior revealed no fibrillations at rest, and large action potentials and full interference patterns with partially low amplitude at maximum voluntary contraction.

Frozen sections of biopsied specimens from the right gastrocnemius were used for examinations including H.E. staining, modified trichrome

staining and SDH and ATPase (pH 9.4) staining. H.E. staining showed almost a normal histogram and only sporadically floccular change. ATPase and modified trichrome staining were also normal.

Comment

The characteristic features of our case were as follows: (1) onset at 15 years-old, (2) slow progression, (3) slight elevations of serum enzyme levels, (4) unusual muscle involvements as described above, (5) electromyographic findings not compatible with typical neurogenic or myogenic changes, and (6) almost normal histological findings with the so called "floccular change".

Benign muscular atrophy involving the face, shoulder and humerus has been reported as facio-scapulo-humeral muscular dystrophy, Kugelberg-Welander disease (K-W disease), chronic polymyositis, scapulo-peroneal atrophy and Emery-Dreifuss disease[3]. Among these diseases, chronic polymyositis would show skin rash, muscle pain, increased erythrocyte sedimentation rate and positive latex agglutination for rheumatoid antibody. Emery-Dreifuss disease would show joint contractures and disorders of the cardial conduction system, however, these findings could not be found in our case.

His characteristics, especially findings (2), (3), (5) and (6) described above lead us to include him within the category of K-W disease, although his muscle involvement is apart from the classical form of K-W disease which simulates limb-girdle muscular dystrophy.

Emery[4] categorized K-W disease as one of the subgroups of chronic spinal muscular atrophy, while Furukawa[5] tentatively divided K-W disease into 4 subgroups on the forms of the muscle involvements in muscular dystrophy.

As to muscle atrophy of the facio-scapulo-humeral type of this origin, the various forms have been subsequently reported as more divided subtypes with or without muscle involvement of face, of thigh, or of lower leg. Our case showed muscle atrophy in proximal portions of the upper extremities at first, then in the lower legs, thighs, face and forearms in that order. Considering these points, facio-scapulo-humeral muscular atrophy may have to be regarded as one category.

Therefore, it is necessary that we study more cases over longer terms in order to determine which facio-scapulo-humeral atrophy should be lumped into one category or divided into more subgroups.

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CENTRONUCLEAR MYOPATHY
A DISTAL FORM WITH JUVENILE ONSET AND NO OCULAR INVOLVEMENT

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Since the first report of myotubular myopathy by Spiro and his colleagues in 1966[1], more than 50 patients with myotubular or centronuclear myopathy have been reported. The clinical manifestations of this myopathy are quite variable[2], commonly consisting of ptosis and weakness of extraocular, facial, palatal and masticatory muscles. In this communication, we report the second case characterized by juvenile onset, distal atrophy and absence of ocular involvement.

A twenty-seven year old man started to experience difficulty in dorsiflexion of the left ankle at age 12, and noticed difficulty in climbing a staircase at age 16 and in a chinning exercise at age 20. He never experienced double vision or difficulty in swallowing. Physical examination showed normal cranial nerves, moderate atrophy of bilateral forearms and legs with slight contracture of the left ankle joint. On manual muscle testing, generalized weakness (4) and distinct distal weakness (3) were disclosed with hypoactive deep tendon reflexes. Neither cerebellar sign nor sensory abnormality was present. Laboratory studies showed normal CK (43 IU), slightly elevated serum creatine (1.0 mg/dl), creatinuria (145 mg/day) and decreased urine creatinine (417 mg/day). Electromyography showed fibrillation voltage in the left anterior tibial muscle and myopathic patterns in the other muscles. Biopsy was performed on the right anterior tibial muscle. H&E and Gomori's trichrome stainings disclosed central nuclei, myotubular structure, variable size of muscle fibers and fatty degeneration (Fig. 1-a). ATPase stainings at pH 9.4, 4.6 and 4.3 and NADH reaction revealed small fibers with central nuclei, consisted of both type I and type II fibers (Fig. 1-b). An electron microscopic study showed no abnormalities in mitochondria except for their deformity, without a tendency of perinuclear accumulation. Moxley et al

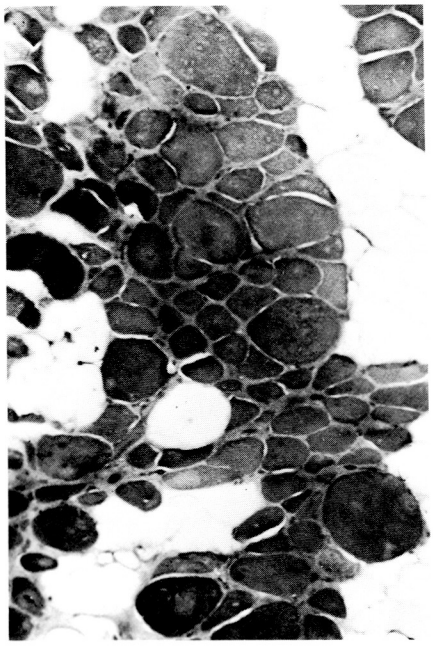
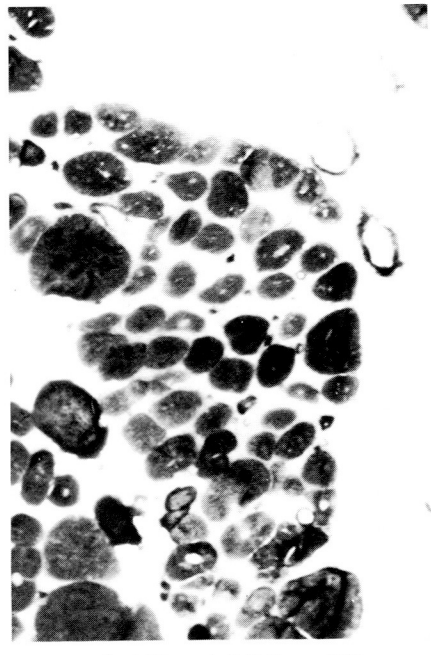


Fig. 1 a: Trichrome stain × 200



b: ATPase (pH 9.4) × 200

reported in 1978 a case with a similar clinical picture, but they noticed granular material and mitochondria around the nucleus[3]. As to the pathogenesis, functional denervation during the myogenesis is postulated.

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A CASE OF ATYPICAL MYOPATHY WITH CONTRACTURES OF JOINTS AND CARDIAC INVOLVEMENT

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A case of atypical myopathy with cardiac conduction abnormalities, myocardial disease and tricuspid regurgitation (TR) was reported, a 27-year-old female.

She showed unsteady gait at the age of three. At age five, it was noticed she had muscle wasting of the extremities. When she was 15 years old, wasting of the facial muscles developed. At age 22, she was admitted to a hospital because of palpitation and general edema. At this time, a chest X-ray disclosed marked cardiomegaly and creatine phosphokinase (CK) was 38U (0-25). She was referred to our clinic for further evaluation of muscular weakness and cardiac disease at age 24, on May 26, 1977, An Adams-Stokes attack occured and a permanent pacemaker was inserted, at age 26. At the age of 27, she died of heart failure.

No consanguinity was found in her parents, both of whom had marked cardiomegaly; her mother had essential hypertension and her father ischemic heart disease disclosed by ECG. CK for her mother was 27U (0-25). They had neither muscular weakness nor EMG abnormalities. Her two brothers were healthy.

Neurological examination at age 24 revealed moderate to severe wasting and weakness of the muscles of the limb girdles and four extremities, predominantly in the proximal muscles, mild wasting of facial muscles, and contractures of neck, elbow, hip and knee joints (Fig. 1). There was no hypertrophy of the calves. All deep tendon reflexes were absent. Pathological reflexes and sensory disturbance were not observed. CK was 127 IU (30-230). EMG showed short duration and low amplitude potentials. Biopsy of the biceps brachii muscle revealed moderate non-specific myopathic change, derangement of the interfibrillar network on NADH staining and type I predominance on ATPase staining, suggesting myopathy. The heredity, clinical course, distribution of muscular atrophy and CK value are not compatible with any type of congenital myopathy, arthrogryposis multiplex congenita, myotonic muscular dystrophy, Duchenne, facioscapulohumeral or limb-girdle muscular dystrophy, or cardiopathic muscular dystrophy (Norris et al.)[1]. Our case resembles malignant limb-girdle muscular dystrophy described by Miyoshi et al.[2]. However, the CK value, facial wasting and heart disease are not compatible with this type.

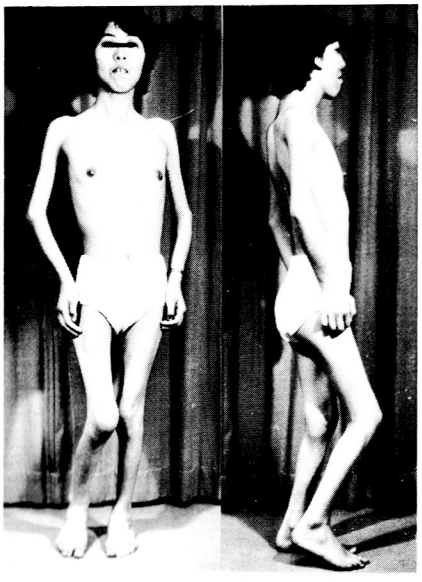

Fig. 1 Photographs of patient showing contractures of elbow, hip and knee joints, and wasting of facial muscles, limb-girdles and extremities.

Cardiac examination revealed right-sided heart failure, tricuspid regurgitant murmur and globular cardiomegaly (CTR 63%). ECG showed atrial flutter, complete atrioventricular block, idioventricular rhythm with QRS patterns of complete left bundle branch block and ventricular ex-

trasystoles. Right heart catheterization and ventriculography disclosed isolated TR. Right ventricular endomyocardial biopsy revealed hypertrophy and degeneration of myocytes, derangement of muscle bundles, interstitial fibrosis and endomyocardial thickening, and electron microscopic examination disclosed sparsity of myofibrils, widening of intercalated discs, increase of glycogen deposition, mitochondrial change, and dilatation and increase of the sarcotubular system, suggesting myocardial disease. Thus, her cardiac disease was regarded as a secondary myocardial disease accompanied by atypical myopathy. Several cases of degenerative neuromuscular disease with mitral regurgitation have been reported[3], but isolated TR is extremely rare.

Clinical characteristics of our case resemble Emery-Dreifuss muscular dystrophy[4,5], which consists of the following components: (1) X-linked recessive inheritance, (2) contractures at elbows, ankles and neck appearing early in childhood, (3) the distribution of weakness, affecting arm muscles more severely than scapular muscles in the upper limbs, in the lower limbs distal muscles are affected earlier than proximal muscles, (4) very slowly progressive course, and (5) cardiac conduction abnormalities appear universally in affected individuals leading ultimately to persistent atrial arrest. This syndrome is regarded as a form of X-linked muscular dystrophy, although histological and EMG examinations reveal either myopathic or neurogenic patterns or mixtures of the two. Our case differs from it in the following points: (1) she was a sporadic case and not Turner's syndrome, (2) contractures at hip and knee joints were prominent and ankle joints were intact, and (3) complication of TR. Our case is considered atypical myopathy with contractures of joints and cardiac involvement, not being Emery-Dreifuss syndrome, although the possibility that either her mother is a carrier or her father has an incomplete form of this syndrome can not be ruled out.

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MUSCLE PATHOLOGY OF DISTAL MYOPATHY

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Introduction

Distal myopathy was established as a distinct entity by Welander in 1951. There followed reports of the disease varying in heredity, age of onset and its clinical course, indicating the clinical heterogeneity of this disorder. We studied distal myopathy to see whether there are any common features or not from the viewpoint of muscle pathology.

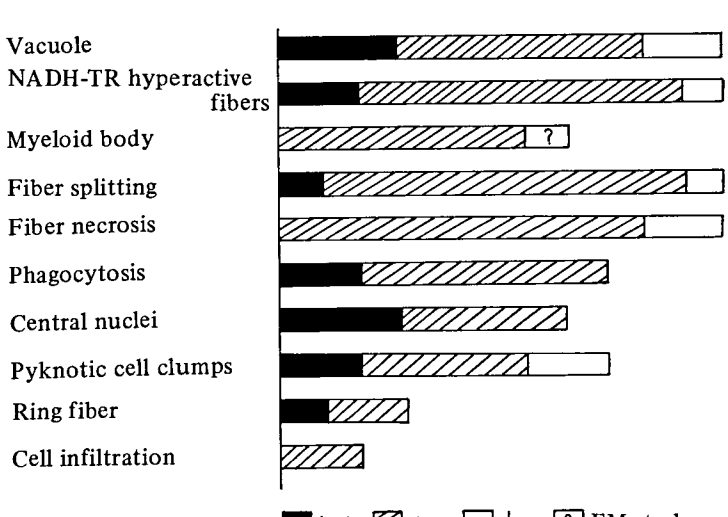
Materials and methods

Thirteen muscles were obtained from nine patients with clinical diagnosis of distal myopathy. They were from 23 to 37 years old. Two of them were male and seven were female. Only one of the cases had a family history of autosomal recessive inheritance. The sites of biopsy were M. biceps brachii, M. quadriceps femoris or M. gastrocnemius. In 4 cases, muscles were obtained from two different sites.

One part of each biopsied muscle was freshly frozen and studied histologically and histochemically. The other part was fixed in formalin and in glutaraldehyde, and studied by light microscopy and electron microscopy.

Results

All the muscles examined showed essentially identical changes, although they varied in degree of changes. In severer cases, almost all muscle fibers were replaced by fat tissues. The distal muscles were more severely affected than proximal muscles when different muscles were examined in the same patient. Nonspecific dystrophic changes observed in all the muscles included marked variation in fiber size, fiber splitting and necrotic fibers. Central nuclei, pyknotic cell clumps and phagocytosis of muscle



	CASES(%)	MUSCLES(%)
Vacuole	9/9 (100)	13/13 (100)
NADH-TR hyperactive fibers	9 (100)	13 (100)
Myeloid body	6 (67)	9 (69)
Fiber splitting	9 (100)	11 (85)
Fiber necrosis	9 (100)	10 (77)
Phagocytosis	8 (89)	10 (77)
Central nuclei	7 (78)	9 (69)
Pyknotic cell clumps	8 (89)	11 (85)
Ring fiber	3 (33)	3 (23)
Cell infiltration	2 (22)	2 (15)

Fig. Muscle pathology of distal myopathy

fibers were present in most cases. Phagocytosed muscle fibers showed acid phosphatase activity. Ring fibers were present in three cases, and mild cell infiltration was observed in two cases, which did not respond to steroid therapy.

The characteristic changes in our cases were the presence of vacuoles and NADH-TR hyperactive fibers. Vacuoles varied in size and were situated either centrally or peripherally. They were negative for PAS and Oil Red O stain. The rim of vacuoles was stained basophilic with H&E, and red with modified Gomori trichrome. The vacuoles were acid phosphatase positive. They were present in either type 1 or type 2 fibers. On electron microscopy, these vacuoles were or were not bound by a single membrane. They contained myeloid bodies, degraded mitochondria or other membranous structures, degraded fibrous bodies and other cytoplasmic degraded products. Some myeloid bodies were present independently of vacuoles.

The other characteristic change was NADH-TR hyperactive fibers resembling small angulated fibers. However, they differed from those appearing on denervation in the respect that most of them contained vacuoles. Also, many of these fibers seemed to be derived from the neighboring fibers by splitting.

Discussion and conclusion

Routine histopathological studies in both sporadic and inherited cases of distal myopathy revealed that the common muscle pathology was dystrophic changes. Vacuoles were present in some cases, but were absent in others. For example, Welander described the presence of vacuoles in some cases in 1951, but Edström failed to find vacuoles in his histological and histochemical studies of Welander type distal myopathy in Sweden, and he concluded that the characteristic muscle pathology was type 1 atrophy resembling myotonic dystrophy. The Japanese, autosomal recessive distal myopathy described by Miyoshi also has no vacuoles.

In contrast, our cases were characterized by vacuoles and NADH-TR hyperactive fibers. The same histochemical and ultrastructural changes of distal myopathy were described by Markesbery and Sunohara. These vacuoles were characterized by acid phosphatase activity and by the content of degraded substances revealed by electron microscopic studies, and thought to be autophagic vacuoles. NADH-TR hyperactive fibers were also thought to be exocytosed degradation products from their fiber splitting nature. These degenerating processes of distal myopathy should be differentiated from the other types of muscular dystrophy.

In conclusion, it is our opinion that there are two types of distal myopathy in view of the muscle pathology, i.e., with or without vacuoles. However, no distinct correlation has sofar been found between the different muscle pathology and clinical heterogeneity of the disease.

DISTAL MYOPATHY AND LYSOSOMES

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The lysosome-system plays an important role in the protein catabolism or metabolic process in the cytoplasm of the cell. On the other hand, this system is suspected to participate in cell injury or focal cytoplasmic degradation in cells, including muscle fibers (de Duve and Wattiaux, 1966[1]; Pitt, 1975[2]).

The purpose of this study was to investigate the participation of the lysosome-system in the muscle fibers of various myopathies, mainly of distal myopathy, in view of "degradation".

Materials and methods

17 muscle specimens from patients with muscular dystrophies were studied. They included 7 of the Duchenne type, 2 of the Becker type, 3 of the facioscapulohumeral (FSH) type, 3 of distal myopathy, and 2 of myotonic dystrophy (MD). Each patient showed almost typical clinical features, respectively. One of the 3 cases with distal myopathy was sporadic, but the other 2 were familial, and their parents were first cousins and three of their five siblings had a similar neuromuscular disease. In all 3 cases, muscle weakness and wasting started in the legs with subsequent involvement of arms at around 23 to 30 years of age.

Biopsies were performed under local anesthesia on the rectus femoris or on the biceps brachii. Specimens were frozen in isopentane coolled in dry-ice. Transverse cryostat sections were processed for routine staining (Dubowitz and Brooke, 1973[3]). For electron microscopical observation, tissue slices were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), postfixed in 1% OsO_4 solution in 0.1 M phosphate buffer, dehydrated in graded alcohol, and embedded in epon-araldite. Ultrathin sections were stained with uranyl acetate and lead citrate. Also, acid phosphatase (ACPase) preparations, a marker enzyme of lysosome, were stained (Barka and Anderson, 1962[4]).

Results

Histopathological findings in the muscles of the Duchenne, Becker and FSH types of muscular dystrophy and MD showed characteristic findings of each disease, as reported by many authors.

In distal myopathy, light microscopy showed mild or moderate variation of the fiber size, and a number of small angulated fibers sporadically. There was a moderate increase of centrally placed nuclei in the muscle fibers. Simple necrosis and fiber splitting were often shown. There was no evidence suggesting an inflammatory reaction. Macrophages were scarcely recognized. The most striking finding for the muscle fibers of all three cases was rimmed vacuoles (Dubowitz and Brooke, 1973[3]; Fukuhara et al., 1980[5]), which were located in the center or periphery of many small or normal-sized fibers. These vacuoles were ringed by material that was intensely basophilic in H. E. preparations and red in Gomori trichrome. The ATPase and DPNH preparations showed that both fiber types involved vacuoles, but they were more frequent in type I than type II fibers. The fibers with vacuoles were atrophic and stained darkly by DPNH occasionally.

ACPase preparations showed almost negative staining inside of the vacuoles. A positive ACPase reaction was seen in the margin of some vacuoles, but rarely. Under an electron microscope in these vacuoles could be seen membranous lamellar structures of various sizes and shapes and various heterogenous materials, which were enclosed by a limiting membrane.

Frequencies of rimmed vacuoles and ACPase positive fibers in each muscle studied are given in Table 1.

In the Duchenne, Becker and FSH types of muscular dystrophy, rimmed vacuoles were not found,

Table 1 Frequency (%) of rimmed vacuoles and acid phosphatase (ACPase) positive fibers

Materials	No. of cases	Rimmed Vacuoles mean ± SD (min.-max.) (%)	ACPase positive fibers mean ± SD (min.-max.) (%)
Progressive muscular dystrophy (except for distal myopathy)	12	0	0.01 ± 0.14 (0-0.3)
Duchenne type	7	0	0.13 ± 0.16 (0-0.3)
Becker type	2	0	0
FSH type	3	0	0.08 ± 0.15 (0-0.3)
Distal myopathy	3	5.10 ± 8.05 (0.4-14.4)	15.60 ± 7.00 (8.5-22.5)
Myotonic dystrophy	2	0	4.65 ± 0.49 (4.6-5.0)

and ACPase positive fibers were seen in only 0.1% of the fibers. Although ACPase activity was slightly, present and was recognized in the perisarcolemmal regions. Of course, marked activity was shown in the cytoplasm of numerous macrophages. MD also showed no rimmed vacuoles, but showed ACPase positive fibers in 4.65 ± 0.43%. ACPase activity was almost all found in the degenerating fibers, particularly around the internal nuclei or in the macrophages invading the muscle fibers. In distal myopathy rimmed vacuoles were seen in 5.10 ± 8.05% and muscle fibers with ACPase positive granules were noted in 15.60 ± 7.00%. ACPase preparations showed positive granules inside of the almost normal-looking fibers as well as in the fibers with rimmed vacuoles.

Discussion

In the present study, numerous rimmed vacuoles were found in distal myopathy. ACPase activity was rarely found in the margins of the rimmed vacuoles, but electron microscopical studies showed such vacuoles which were presumed to be autophagic in nature. Therefore, rimmed vacuoles were presumed to be autophagic vacuoles or secondary lysosome, without or with ACPase activity (de Duve and Wattiaux, 1966[1]). On the other hand, numerous muscle fibers in our cases had ACPase positive granules. ACPase is one of the rather specific lysosomal enzymes and works as a good marker for lysosomal activity (Essner and Novikoff, 1961[6]; Holt and Hicks, 1961[7]). In addition to numerous autophagic vacuoles and the increased activity of ACPase, few macrophages were found in distal myopathy. In other muscular dystrophies rimmed vacuoles were seldom seen except in oculopharyngeal muscular dystrophy (Dubowitz and Brooke, 1973[3]) and ACPase was also not increased except in the necrotic fibers invaded by macrophages.

Conclusions

The degenerative process in distal myopathy appeared to progress mainly through the lysosomal system and might differ from other muscular dystrophies.

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TUBULOMEMBRANOUS MYOPATHY

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Recent histochemical and ultrastructural studies on skeletal muscle revealed many neuromuscular diseases as clinico-pathological entities. We have reported a new progressive myopathy with tubulomembranous inclusions[1]. The origin and nature of the inclusions are obscure, but it would be worthwhile to report the morphological features of the skeletal muscle.

Case history

A boy, born in 1961, was admitted to Niigata University Hospital for disturbance of gait at the age of 16. His parents were first cousins once removed. He was the slowest runner at school, but his parents did not regard him as abnormal because they also had poor exercise abilities. Since the age of 15, he ran less easily than before, walked with a waddling gait, and got tired very easily. Mild weakness and wasting were noted in the proximal limb muscles and facial muscles. Laboratory data were normal except for slightly increased serum CPK. Needle EMG showed myopathic features in several muscles. Nerve conduction velocities were normal. Leukocyte lysosomal enzyme analysis revealed normal levels of arylsulfatase A and B, α- and β-galactosidase, and N-acetyl-β-D-glucosaminidase. Ultrastructural studies on peripheral blood lymphocytes and skin also revealed no abnormal inclusions. A trial of prednisolone had no significant effect on muscle strength. Further progression of the muscle wasting was noted in the lower extremities after that time. He walked with a marked waddling gait and needed support. A marked increase of the lumbar lordosis and a prominent Gowers' sign were noted. Serum CPK was 1236 mU/ml (normal: below 100).

Morphological studies

A muscle biopsy was performed on the left quadriceps femoris. Light microscopical observations showed moderate variation of fiber diameters, extensive fiber-splitting and central nuclei with mild endomysial fibrosis. Numerous rimmed vacuoles[2,3] were seen. In semi-thin epoxy sections stained with toluidine blue, intrasarcoplasmic inclusions stained faintly, measuring 2.5 μm by 10 μm at the greatest diameter, were noted in 40-50 % of fibers of which the architecture was otherwise normal. PAS preparations did not show any abnormal accumulation of glycogen. The most remarkable findings under an electron miscroscope were numerous abnormal inclusions consisting of laminated tubulomembranous structures (TMS) (Fig. 1). The lamellae were regularly spaced with a periodicity of 8.5-9 nm. These laminated TMS were sometimes curved a little and in places they appeared as concentric lamellae according to the plane of sectioning. The second type of inclusion consisted of curvifilamentous materials abutting on to the type I inclusions. The diameter of each filament

Fig. 1 Tubulomembranous structures × 18,000

was 3-6 nm. The 3rd type of inclusion was moderately electron-dense granular material surrounded by a single unit membrane which sometimes included lamellar structures identical with those in the first type of inclusions. Glycogen granules aggregating within a membrane was also observed although rarely.

Discussion

The pattern of the muscle involvement in this patient was similar to limb girdle dystrophy, except for the facial involvement and rapid progression of muscular wasting. The TMS in the present patient somewhat resembles the inclusions of Fabry disease,[4] but clinical and biochemical data were different. The periodicity and form of parallel membranes of the inclusions were also different.[4] Type II inclusions in this patient vaguely resembled curvilineal profiles in ceroid lipofuscinosis, but his clinical picture was different. Type III inclusions have some similarities to the "dense granulofilamentous masses" in the dominantly inherited myopathy described by Fardeau et al.[5] Therefore, ultrastructural findings of the muscle biopsy specimen in the present case did not conform with any of the neuromuscular diseases hitherto described.

In conclusion, we suspect that this patient suffers from a hitherto unrecognized progressive muscular disease and the descriptive term "tubulomembranous myopathy" is proposed.

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DEBRANCHER DEFICIENCY MYOPATHY: THREE ADULTS INCLUDING TWO SIBLINGS

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A 34 year old female (case 1), her 27 year old brother (case 2) and a 32 year old female (case 3) had slowly progressive muscle weakness and atrophy which began in the third decade of life. The weakness was greater distally than proximally, but sternomastoid and thigh adductor muscles were also severely involved. Parents of the affected siblings were double cousins. There was no consanguinity in the other family.

Liver function test results were normal and no hepatomegaly was found even on a technetium scintigram. Serum CPK levels were slightly elevated.

The abnormal accumulation of glycogen was observed in subsarcolemmal areas in PAS stained frozen sections of biopsied muscles. On electron microscopic observation, many glycogen containing vacuoles were found in the muscle.

Biochemical analyses of muscle were done in cases 2 and 3 (Table 1): (1) Glycogen content was slightly increased. (2) Debranching enzyme activity for phosphorylase limit dextrin was reduced. This became more marked when α-glucosidase was inhibited with cadmium or antibodies to yeast α-gluco-

Table 1 Results of Biochemical Anayses of Biopsied Muscle

		Case 2	Control	Case 3	Control
Glycogen content		23.86	10.63	14.70	7.30 mg/g.w.w. of muscle
Phosphorylase	Total	0.30	0.47	0.46	0.70 μM/mg protein/min.
	A	0.08	0.17	0.30	0.40 μM/mg protein/min.
	B	0.22	0.30	0.16	0.30 μM/mg protein/min.
Debranching enzyme activity					
(1) with phosphorylase limit dextrin as substrate					
(a) with inhibitor for α-glucosidase					
anti-yeast α-glucosidase serum		0.5	3.0	–	– μM/mg protein/min.
cadmium		0.6	3.2	0.09	5.13 μM/mg protein/min.
(b) without inhibitor for α-glucosidase		3.6	6.6	0.18	6.60 μM/mg protein/min.
(2) incorporation of ^{14}C-glucose into glycogen		253.8	583.8	–	– U/mg protein/min.
β-Amylolysis		11.0	34.3	–	– % digestion
α-Glucosidase					
with glycogen as substrate					
pH 4.0		0.08	0.03	0.07	0.03 μM/mg protein/min.
pH 7.0		0.11	0.10	0.11	0.09 μM/mg protein/min.
Acid phosphatase		0.21	0.12	2.9	0.1 μM/mg protein/min.

Table 2 Debrancher Activity in Erythrocytes

Case 2	Case 3	Control
0.36	2.29	2.45 units/gHb/min.

sidase. Incorporation of ^{14}C-glucose into glycogen was also reduced (case 2). (3) Glycogen had short outer chains on β-amylolysis (case 2). (4) α-Glucosidase activity was increased at pH 4.0 and normal at pH 7.0. (5) Acid phosphatase activity was increased.

Biochemical analyses of erythrocytes were also done in cases 2 and 3 (Table 2): Incorporation of ^{14}C-glucose into glycogen was reduced in case 2 but normal in case 3.

Before DiMauro et al[1] reported 5 patients and we[2] reported these 3 patients, only 3 patients with debrancher deficiency myopathy had been reported[3,4]. In our work, debrancher activity was measured by two different assay methods. One is based on reversal of the physiological reaction, with incorporation of labeled glucose into glycogen. The other method is based on the phosphorolytic degradation of PLD, which depends on the presence of debrancher. The debrancher activity was found to be reduced by both methods in case 2, this patient had amylo-1, 6-glucosidase deficiency myopathy. In case 3, the debrancher activity was definitely reduced with the former method in muscle, but was normal with the latter method in erythrocytes. This result suggests that case 3 may belong to subgroup III D of Van Hoof and Hers[5]. In these patients, the transferase activity is missing while the amylo-1, 6-glucosidase function of the enzyme is preserved[5]. Patient 3 in DiMauro's study[1] was also supposed to belong to this subgroup.

Our thanks are due to Dr. A.G. Engel and Dr. S. DiMauro for their kind comments.

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TRANSPLANTATION OF HUMAN DYSTROPHIC MUSCLE TO NUDE MICE: A MORPHOLOGIC STUDY

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It is unknown whether the regeneration of human dystrophic muscles is normal, although this is a very important subject. Some investigators have suggested that the muscle regeneration is ineffective in this genetic disorder (1, 2). However, why the muscle regeneration is ineffective is unknown. Therefore, I conducted an experiment on human dystrophic muscle transplantation into nude mice and compared these results morphologically with those of normal human muscle transplantation into nude mice (3).

Biopsied muscles used in this study were obtained from 7 patients with Duchenne muscular dystrophy (DMD) and five histochemically normal muscles were obtained from orthopedic patients without any neuromuscular diseases. Small pieces of muscles were inserted into the subcutaneous space of the back of nude mice and the incisions were closed by cotton sutures. The mice were sacrificed 1, 2, 3, 4 weeks after transplantation. Each transplant was cut into small pieces, fixed, dehydrated and embedded in plastic, and processed for light and electron microscopy. The results of normal human muscle transplantation have already been reported elsewhere (3). In this report, I would like to present the results of DMD muscle transplantation up to 2 weeks.

Light microscopically, after 1 week of transplantation, regenerating myoblasts and multinucleated myotubes were seen, especially at the periphery of the transplant (Figure). After 2 weeks, the growth of myotubes was observed.

Electron microscopically, after 1 week, myoblasts and multinucleated myotubes were observed within the basal lamina of the degenerating muscle fibers of DMD and these cells were usually closely aligned within the basal lamina, wrapping the cell debris of degenerating muscle fibers. Moreover, these cells were not always associated with the degenerating muscle fibers, and were also present within the basal lamina of the interstitial space, being packed compactly with each other with the intertwined cell processes. The basal lamina of these fibers was duplicated and partly pleated. The myoblasts and myotubes contained randomly distributed actin and myosin filaments, Z-line material, intermediate sized filaments, microtubules and abundant ribosomes and polysomes which are also present along nascent myofilaments. In addition, clusters of glycogen granules with and/or without membrane materials and lipid droplets were observed. After 2 weeks, electron microscopic examination of the regenerating myotubes in the transplanted DMD muscles revealed the partial cross striation of the

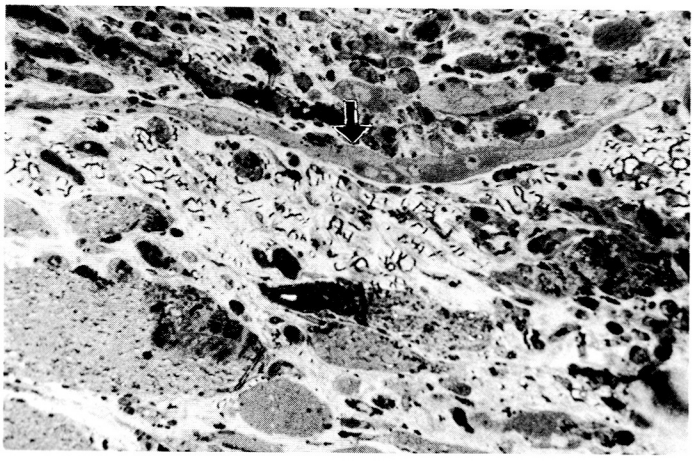

Fig. The light micrograph shows degenerating muscle fiber and a regenerating myotube (arrow) after 1 week of transplantation in a DMD transplant. The degenerating muscle fiber usually occupies the center of the transplant and shows the granular cytoplasmic feature; whereas the regenerating myotube which usually occupies the periphery of the transplant is thinner, and contains a fine cytoplasmic matrix and several euchromatic nuclei with prominent nucleoli. (Toluidine blue × 500)

myofilaments, leptomeres in the subsarcolemmal and perinuclear regions and tangle formation of T-tubules in addition to the findings noted in myotubes of DMD transplants after 1 week.

The plasma membrane of the regenerating myotubes was continuous and its disruption, such as reported in DMD muscle plasma membrane (4), was not observed in this study. No conspicuous differences of the morphology of the regenerating myotubes between control and DMD muscle transplants were qualitatively observed up to 2 weeks. A further study up to 4 weeks after transplantation will be completed soon.

Schotland et al. (5) have reported the alteration of the intramembranous structure of the DMD muscle plasma membrane utilizing the freeze fracture technique. So a freeze fracture study of the regenerating muscle fibers on long term transplantation may provide further information about the pathogenesis of human muscular dystrophy.

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A MORPHOLOGICAL STUDY OF EXPERIMENTAL MITOCHONDRIAL MYOPATHY WITH 2, 4-DINITROPHENOL

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"Mitochondrial myopathy" may be defined as a muscle disease in which the mitochondrial changes (aggregation, enlargement, cristal alterations, paracrystalline inclusions) are the major pathological feature, but its pathogenesis has not yet been clarified. In some cases of mitochondrial myopathy the loose coupling of oxidative phosphorylation of mitochondria was demonstrated. These mitochondrial changes have been demonstrated in rat skeletal muscles which were kept under the ischemic condition or following intra-arterial infusion of 2, 4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation.[1,2,3)]

We demonstrated the appearance of morphological changes similar to mitochondrial myopathies by intra-arterial infusion of DNP, and by intra-muscular injection of DNP which has not yet been tried. We confirmed that the latter produced the same morphological changes of the muscles as in the case of intra-arterial infusion.

Materials and methods

Male Sprague-Dawley rats weighing 250-300 g were used. The rats were anesthetized by i.p. injection of Nembutal, and the abdomen was opened. The iliac artery on one side was ligated, and the aortic artery was cannulated with a small-bore polyethylene catheter. Fifteen mg of DNP was introduced through the intracatheter over a period of 3-5 min. The muscle biopsies from bilateral femoral muscles were taken 15 min, 30 min, 45 min and 60 min after the injection of DNP. The biopsied muscle on the side of which the iliac artery was not ligated was the intra-arterially infused group, and the muscle specimen on the opposite side was the control group. In the third group the rats were anesthetized by i.p. injection of Nembutal, and fifteen mg of DNP was injected intramusculary. The muscle specimens were obtained from the femoral muscles 15 min, 45 min and 60 min after the injection of DNP. Each specimen was divided into two parts. One of the parts was immediately frozen in isopentane, and about 10 μm thick transverse cryostat sections were prepared and serial sections were stained with hematoxylin and eosin, modified Gomori trichrome and NADH tetrazolium reductase. The second part was fixed in OsO_4 following fixation in 2.5% glutaraldehyde and embedded in Epon 812. Ultrathin sections were viewed under a JEM 100B electron microscope.

Results

The H.E. stain showed no remarkable changes in each group. From 15 min after the intra-arterial infusion of DNP the modified Gomori trichrome stain showed distinct ragged red fibers (RRF), being most marked in the biopsied muscles of 30 min after the infusion. In the third group the entire body musculature became rigid between 45 and 60 min after intramuscular injection of DNP, and the rats died in a few minutes. Marked RRF were demonstrated in the necropsied muscles immediately after death.

Only one of the control groups showed distinct RRF. This could be caused by the effect of the ischemia and/or infusion of DNP.

The percentage of RRF each time was estimated as follows. Fifteen min after i.a. infusion showed 10-17% (mean 12.1%); 30 min, 8-45% (26.9%); 45 min, 18-35% (27.0%); and 60 min, 21%, respectively. In the control group only one sample, in which distinct RRF were seen, showed high values, 9% of RRF at fifteen min after injection, 19% at 30 min, and 16% at 45 min, while the others showed low values,

0-10% (4.4%).

NADH tetrazolium reductase reactions showed high activities in the fibers which were seen as RRF with modified Gomori trichrome methods, and these activities were most marked in the subsarcolemmal areas. These fibers were considered to be type 1 fibers. There were no high activities except for in one sample which showed RRF in the control group.

An electron microscopic study revealed no mitochondrial abnormalities in the control muscles. In the muscles infused with DNP intraarterially, marked marked aggregation of mitochondria was seen in the subsarcolemmal space and intermyofibrillar space, and enlargement of the mitochondria and cristal alteration were seen, but paracrystalline inclusions were not demonstrated. In the muscles injected with DNP intramuscularly, the same changes of the mitochondria were noted as in the intra-arterially injected group.

Discussion

Although many patients with mitochondrial myopathies have been reported, the pathogenesis has not yet been clarified. To study the pathogenesis of this disease, Melmed et al[2] and Sahgal et al [3] experimentally reproduced RRF and mitochondrial changes similar to mitochondrial myopathies by intra-arterial infusion of DNP. In the first place we also infused DNP intra-arterially, and from fifteen min after infusion RRF were seen and the peak was seen at thirty min after infusion.

Because of the technical difficulties of intra-arterial infusion and of keeping the rat alive during the long period the repetitive administration of DNP is almost impossible, therefore the chronic effect could not be studied. The i.p. injection of DNP failed to reproduce the mitochondrial changes. For these reasons we tried intramuscular injection of DNP, and confirmed the appearance of mitochondrial changes as in intra-arterial infusion.

From the clinical aspect, the experimental models of mitochondrial myopathies which were reported in the past were not identical to human myopathies. More appropriate models have to be made in future experiments. We would like to make experimental models which are identical to human myopathies in both symptoms and morphological changes by repetitive intramuscular administration of small doses of DNP.

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MYOPATHY WITH MITOCHONDRIAL INCLUSION BODIES — CLINICAL AND ELECTRONMICROSCOPIC STUDIES —

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Introduction

Abnormalities of skeletal muscle mitochondria, including paracrystal line-like inclusions, have been reported in a great number of various muscle disorders with usually obscure aetiology[1]. It is well known that prominent abnormal mitochondria are present in patients with ocular myopathy or Kearn-Sayre syndrome. Such abnormal mitochondria are found not only in skeletal muscles but also in other organs. In this paper, mitochondrial inclusion bodies in three cases with ocular myopathy, a case with humeroperoneal muscular atrophy, and a case with quadriceps myopathy are reported.

Case report

Case 1 (Ocular myopathy). The patient was a 48-year-old female who had been healthy until about 33 years of age, when she started to notice right blephaloptosis. The symptom progressed gradually, and at the age of 46 left-side ptosis appeared, too. Physical examination revealed bilateral ophthalmoplegia, and EMG showed slight myopathic change. CPK level was 29 I.U.

Case 2 (Ocular myopathy + slightly generalized myopathy). The patient was a 46-year-old female who had been healthy until about 30 years of age, when she started to notice bilateral blephaloptosis after the third delivery. Since she had an operation for a goiter at the age of 33, the symptom progressed. Physical examinations revealed bilateral ophthalmoplegia, and slight weakness of face, neck and shoulder muscles. EMG showed a myogenic change and EEG indicated a dysrhythmic basic pattern. CPK level was 170 I.U., and slight elevation of TTT, ZTT and CCF indicated chronic hepatitis. Thyroid function and BMR were normal.

Case 3 (Kearns-Sayre syndrome). The patient was a 39-year-old male whose eye-lids were slightly ptotic since childhood. After adolescence bilateral blephaloptosis progressed rapidly and at the age of 30, he began to lose his visual acuity slightly. Physical examinations revealed bilateral ophthalmoplegia, and slight weakness and atrophy of neck and distal extremities. The cerebellar sign was questionable. Laboratory examinations revealed dysrhythmia in EEG, myogenic change in EMG, elevation of CSF protein (101 mg/dl), calcification of the basal ganglia and cortical atrophy in CT, slight mental retardation (WAIS 75), slight deafness, CPK 45 I.U., and slight elevation of lipids. Liver biopsy indicated fatty degeneration.

Case 4 (Humeroperoneal muscular atrophy). The patient, a 48-year-old male, was slender from childhood and unskillful in chinning exercises and running. At the age of 27, A-V block was detected by ECG, and he has complained of palpitation and dyspnea for the past 10 years. From about the same time he noticed weakness of the extremities. He received an operation for a pacemaker at the age of 48. Physical examinations revealed weakness and atrophy of humeroperoneal muscle, and contracture of cervical vertebrae and spine. Laboratory examinations revealed a complete A-V block in EEG, myogenic and slightly neurogenic changes in EMG, and CPK was 54 I.U.

Case 5 (Quadriceps myopathy). The patient was a 31-year-old male who had had difficulty in running fast since childhood. At the age of 18, he had difficulty in going up and down stairs and jumping, and he started to notice atrophy of the thigh. Physical examinations showed localized atrophy and weakness in the thigh. Laboratory findings showed myogenic change in EMG, CPK 490 I.U., and slight elevation of GOT, GPT and LDH. CRP, RA test and LE test had negative results.

Histochemical study

The deltoid muscle in Cases 2 and 3 showed ragged-red fibers (20-30%) and type 2 atrophy. The frontal muscle and external ocular muscle of Cases 1 and 2 revealed scattered muscle fiber atrophy and an increase of collagen fibers. As the biceps in Case 4 revealed collagen fibers only, we reexamined the pectoralis major and found scattered ragged-red fibers and type I fiber atrophy. The quadriceps in Case 5 revealed variability in fiber size, scattered atrophic fibers, type I fiber atrophy and ragged-red fibers, but cell infiltration was not recognized.

Ultrastructural study

In all cases, we recognized aggregated and modified mitochondria, and paracrystalline inclusions in mitochondria. The abnormalities in mitochondria were more severe in Cases 1, 2 and 3 than in Cases 4 and 5. In Case 3, we found paracrystalline inclusions in the liver mitochondria, too. We examined the fine structure of the paracrystalline inclusions with various projections, and we concluded that this inclusion body had a box-like shape containing filamentous subunits in three dimensions (Fig.)

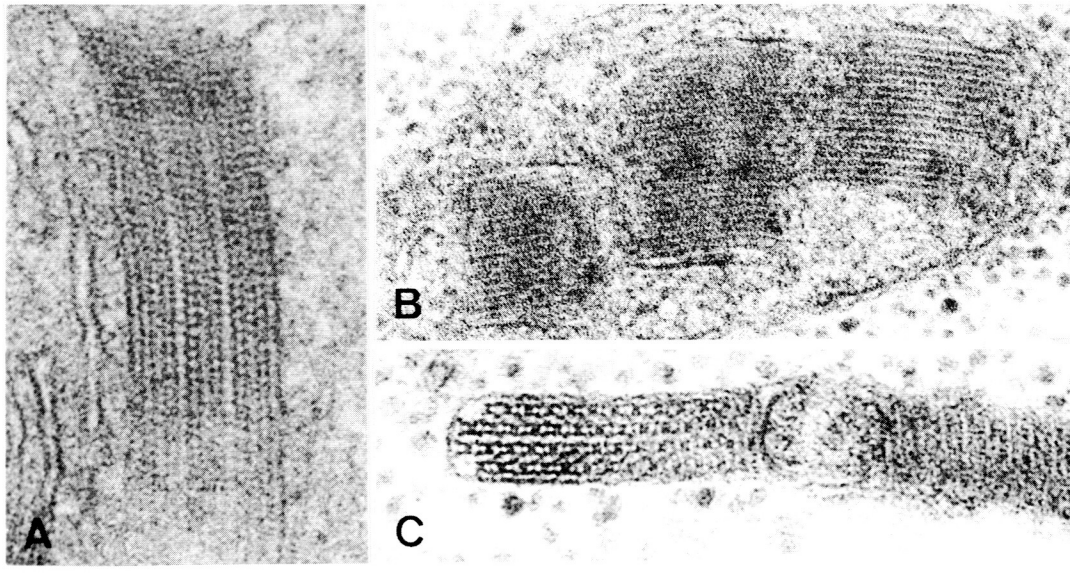

Fig. Electron micrographs show a portion of a paracrystalline intramitochondrial inclusion with a variety of projections with presumed side (A), top (B) and vertical-cut (C) sections.
A: × 160,000, B: × 120,000, C: × 150,000

Discussion

In addition to the ocular myopathy, Cases 2 and 3 showed slight myopathic changes in extremities. Case 3, so-called Kearns-Sayre syndrome, had involvement of other organs, liver, central nervous system and retina. Case 4 is almost the same category as humeroperoneal muscular atrophy or scapulo-peroneal dystrophy, or Emery-Dreifus syndrome, which involved neurogenic and myogenic changes and revealed autosomal recessive inheritance. Case 5 is so-called quadriceps myopathy, which is thought to be a syndrome, such as polymyositis, muscular dystrophy and neurogenic atrophy. Case 5 was treated with predonisolone, 40 mg daily. After two weeks treatment there was marked improvement in his CPK level. Therefore, this patient was diagnosed as polymyositis syndrome.

Abnormal mitochondria containing paracrystalline inclusions are found in various disorders of muscle, especially in ocular myopathy. But little is known about their chemical abnormalities. As

DiMauro[2] mentioned, many more "mitochondrial myopathies" (in a restrictive biochemical sense) will be recognized as systematic, biochemical studies of mitochondrial function are carried out.

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STUDY ON DISUSE ATROPHY

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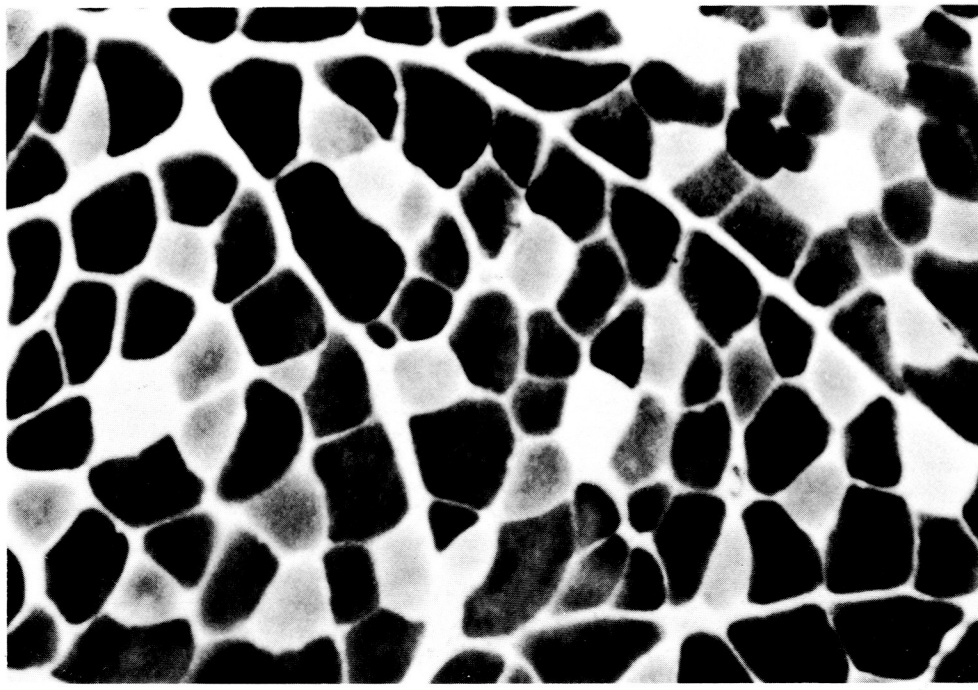
In order to investigate the mechanism of disuse atrophy, a cast immobilization experiment was performed on adult albino rabbits. Immobilization was performed by three different methods: the knee was flexed and the ankle was dorsi-flexed in group 1, the knee was flexed and the ankle was planter-flexed in group 2, and the knee was extended and the ankle was dorsi-flexed in group 3.

In group 1, the anterior tibial muscle showed atrophy of type II A fibers 4 weeks after the start of immobilization, and subclassification of type II was difficult in the 8th week. In this group in the gastrocnemius muscle, both type I and type II A fibers were atrophied. The change of diameter of type I fibers was most marked in group I and least significant in group 3.

This tendency was absolutely parallel to the grade of stretch of the muscle. On the other hand, atrophy of type II A fibers was observed in all groups regardless of the grade of stretch. Therefore it was considered that the true effect of disuse was seen in type II A fibers most significantly, and the decrease in diameter of type I fibers was probably due to loss of proper muscular tonus.

The most severe histological change was seen in the anterior tibial muscle in group 3 which was significantly relaxed. Two weeks after the start of immobilization, the muscle began to lose its normal colour and elasticity showing tumor-like swelling within the muscle.

There were many atrophied fibers with central areas of which the intensity of staining with SDH or routine myosin ATPase was either too dark or too pale compared to that of usual areas. Also central nuclei were frequently seen in these fibers. Further investigation is necessary for classification. Contradicting some previous studies in the literature, the ratios of the number of fibers of each histochemical type did not show any significant change after continuous immobilization. In the anterior tibial muscle, the ratios of types I, II A and II B were 7.9, 34.7 and 57.4%, respectively, in the control rabbit, and were nearly equal 4 weeks after the start.



Change of fiber size after 4 weeks immobilization in the knee-flexed, ankle-dorsi-flexed position. Diameter of intermittent (type II A) fibers was decreased.
Routine myosin ATPase. x 100.

—HISTOCHEMICAL STUDY ON LIPID METABOLISM IN MOTOR NEURONE DISEASE —

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In the previous paper, which reported the clinical and laboratory findings in two cases of Kennedy's disease found in two different families, it was pointed out that abundant deposition of droplets stained by oil red 0 in muscle fibers, fatty degeneration of the liver and hypertropic cardiomyopathy were commonly seen. In one case, electron microscopic examination of biopsied heart muscle revealed deposition of lipid bodies in cardiocytes.

An increase of plasma triglyceride level with increased pre-B-lipoprotein fraction was also noted in both cases. Then a quantitative study on the deposition of fat granules in muscle fibers in Kennedy's disease was performed.

Materials and Methods

Muscle specimens were obtained from quadriceps femoris muscle in two cases with Kennedy's disease (cases 1 and 2) as well as two cases with typical amyotrophic lateral sclerosis (cases 3 and 4). Serial cryostat sections were submitted to myosin ATPase staining at pH9.4 and oil red 0 staining. Then deposition of fat granules stained in each type 1 fiber and type 2 fiber was determined. For quantitative determination, the line intercept method reported by Lucas and Seber[1] was employed. Photographic prints (1120 magnification) of each fiber were made and the number of deposited granules in 40 fibers each of type 1 and type 2 selected at random was calculated.

Lipid coverage per unit (= C) of each fiber was estimated by the line intercept technique as follows.

$$C = \frac{1}{4}\ \pi \overline{W}\ \frac{Ml + Ms}{Ll + Ls}$$

Ml, Ms = number of droplets on long, short diameter
Ll, Ls = length of long, short diameter
$\overline{W}$ = average droplet size for the group

In addition, the fiber type histogram was also studied.

Results

1) The fiber type histogram showed a marked increase in the number of hypertrophic fibers of more than 100 micr. in muscles obtained from cases 1 and 2.

2) In case 1, percent lipid coverage was 5 to 15 percent (average 10.76%) in type 1 fibers and about 5 percent (average 5.12%) in type 2 fibers.

In case 2, percent lipid coverage in type 1 fibers was 3 to 5 percent (average 3.99%) in many fibers, though in the same fibers it reached 10 percent. In type 2 fibers, the findings were almost the same (average 4.64%) as in type 1 fibers.

In both cases 1 and 2, there was no difference in percent lipid coverage between hypertrophic fibers and non-hypertrophic fibers of both types 1 and 2.

In muscles obtained from patients with ALS, percent lipid coverage was less than 2 percent (average 1.35 and 2.04% in type 1 fibers, and 0.67 and 1.69% in type 2 fibers) in many fibers of both types.

Discussion

The distribution and quantity in muscle fibers of neuromuscular disorders have rarely been reported. The incidence of lipid droplets in human skeletal muscle in various abnormal states was examined by Harriman and Reed.[2] They examined 13 cases of motor neuron disease and found moderate deposition of fat in 10 cases. However, they did not do a quantitative study. We found more abundant deposition of fat droplets in both fibers of type 1 and 2 in Kennedy's disease as compared with the deposition in ALS.

While the percent lipid coverage seen in muscles from our patients with Kennedy's disease was lower than that in neutral lipid storage disease demonstrated by McKeran et al.[3], this finding suggests that there may be a metabolic disturbance of lipid as the basis of Kennedy's disease, which may also cause cardiomyopathy, fatty liver and hyperlipidemia.

However, it is known that the amount of lipid in muscle is influenced by age, sex and nutritional state. Further histochemical studies on the deposition of fat in muscle fibers are desired.

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—HISTOLOGICAL STUDIES ON MUSCLE IN A CASE WITH SCHWARTZ JAMPEL SYNDROME —

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Twenty six cases of Schwartz Jampel syndrome characterized by blepharophimosis, short stature and abnormalities of bones and joints have been reported. However, only a few histochemical studies on muscle have been performed. We studied biopsied muscle histochemically in a case with the syndrome and found type 1 fiber predominance.

Case record

A seven-year-old boy was admitted in August, 1978, to Osaka Rosai hospital because of abnormality in gait and other body actions. His parents were first cousins, and his maternal grandfather and grandmother were also consanguineous. He was born at full term, 3,100 grams body weight, after an uncomplicated pregnancy. He began to walk at the 14th month, and since age 4 progressive clumsiness in gait and other body actions has been noted in addition to shortness of stature.

Findings on admission: His height was 103.4cm (−3.36SD), while skeletal muscles were well developed and intelligence was normal. His face was pinched with blepharophimosis and he looked sad. He had marked lumbar lordosis with slight scoliosis and pigeon-breast. His gait was stiff and clumsy like a marionette. Grip myotonia, unaffected by cold, was demonstrated. Percussion myotonia was not detected. Muscles were stiff and firm on palpation while there was no weakness. The firmness of muscle decreased only slightly during general anesthesia being unaffected by warmth or cold. Deep tendon reflexes were almost absent, and Babinski's toe sign was not elicited. Other physical findings were negative.

Laboratory findings: Routine laboratory examinations revealed no abnormality except a slight increase of serum CPK and IgA deficiency, less than 10mg/dl. Urinary amino acid analysis revealed no abnormality. The results of endocrinological and chromosome studies were all normal. Radiographic studies revealed maldevelopment of the facial skeleton, and deformities of skull, chest, spine and acetabulum.

Electrophysiological studies: EMG findings during voluntary contraction were normal, while a myotonic response was evoked by insertion of a needle electrode. Motor nerve conduction velocities in ulnar and tibial nerves were within normal range.

Muscle biopsy studies: Biopsy of the right quadriceps femoris muscle was performed.

1. Light microscopic studies revealed the following.

Only a few fibers with slight non-specific myogenic changes were found in HE stained sections. Diameters of muscle fibers determined with frozen tissue stained for ATP-ase at pH9.4 varied from 10 to 60 micr. with averages of 30 for type 1 fibers and 31.8 for type 2 fibers. However, there was marked type 1 fiber predominance, 74% of all fibers. Almost all end-plates demonstrated by cholinesterase staining showed elongation.

2. Electron microscopic studies revealed swelling, destruction and vacuolization of the mitochondria, and partial disappearance of Z-lines. However, on the whole, the myofibrillar architecture was well preserved.

Comment:

The present case was diagnosed as Schwartz Jampel syndrome from the clinical findings. The muscle

stiffness was generalized, continuous and not decreased by movement. So the stiffness was more similar to that in the syndrome of continuous muscle-fiber activity according to Isaacs,[1] or neuromyotonia than that in congenital myotonia and myotonic dystrophy.

Noteworthy in nine cases of Schwartz Jampel syndrome in the literature was the spontaneous continuous electrical activity, resembling electrophysiological findings in the syndrome of continuous muscle-fiber activity or neuromyotonia.

In our studies, type 1 fiber predominance was clearly demonstrated. The pathogenesis of this finding is not known. However, it is interesting to point out the speculated correlation between type 1 predominance and continuous muscle activity in this syndrome.

Salmons et al[2] and Romanul et al[3] reported that they could change the muscle fiber type not only by cross innervation but also by continuous electrical stimulation with a particular pattern using rabbits. According to them, when a nerve to a "fast" muscle was continuously stimulated at a slow rate, the "fast" muscle fibers converted into "slow" fibers histochemically and physiologically within 4–8 weeks.

This interesting experiment shows that the electrophysiological state of muscle plays a role in maintaining the fiber type. Thus it is suspected that the type 1 fiber predominance in our case may be caused by continuous stiffness on the basis of the clumsy movement.

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SINGLE FIBER EMG OF PROGRESSIVE MUSCULAR DYSTROPHIES

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In order to investigate electrophysiological characteristics, the authors compared single fiber electromyographic findings of muscular dystrophies with those of other neuromuscular disorders. For various single fiber electromyographic parameters, we studied the mean interspike interval (MISI) and the velocity recovery function (VRF) which reflects variability of the interpotential interval (IPI) of two muscle fibers belonging to the same motor unit.

Method and Materials

We measured the mean interspike interval and the jitter in the voluntarily contracted extensor digitorum communis (EDC) muscle in patients with various types of progressive muscular dystrophies and other neuromuscular disorders using a single fiber electrode (Medelec SF-25).

The mean interspike interval (MISI) is the mean interval of early action potentials of muscle fibers belonging to the same motor unit. In each patient, MISI was measured as the mean value of more than five MISIs from each EDC muscle using a superimposed method.

The jitter was measured with a signal processor (Sanei 7T08) and calculated as the mean consecutive difference. For voluntarily irregular innervation rates, the action potentials were recorded with a data recorder (TEAC R-410). In order to examine the relationship between the interpotential interval (IPI) and the preceding interdischarge interval (IDI), the signal processor plotted the IPI in a horizontal line and the preceding IDI in a vertical line of one to two hundred potential pairs of the same muscle fibers (Figure 1).

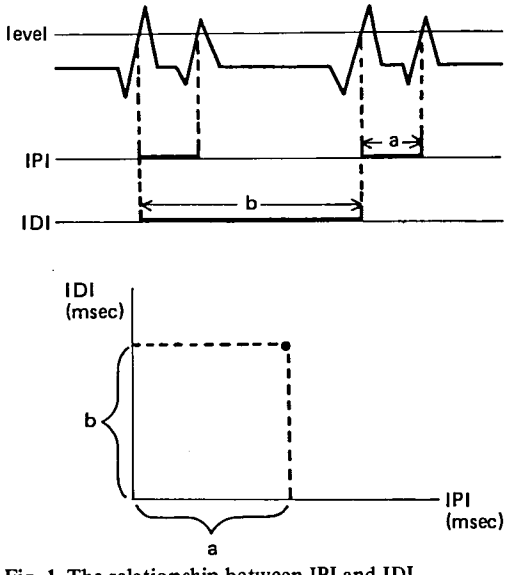

Fig. 1 The relationship between IPI and IDI. The interpotential interval (IPI) and the preceding interdischarge interval (IDI) were measures as 'a', and 'b' in this figure which were defined as intervals of each crossing point using a level technique. Each interval was mapped in the manner in the lower figure. For each potential pair, one to two hundred points were plotted.

Results

In patients with progressive muscular dystrophies, the mean value of the MISIs was significantly higher than that of control subjects. In patients with other myopathic disorders and chronic polyneuropathy, the mean value of the MISIs tended to be higher than that of control subjects.

In potential pairs from normal EDC muscle, the IPI only slightly, or did not fluctuate with irregular innervation rates. In potential pairs from patients with progressive muscular disorders and other myopathic disorders, the long IPI, more than 5 msec, tended to be shorter during strong voluntary contractions than during weak contractions. Among three action potentials belonging to the same motor unit, the IPI of a potential pair with a longer IPI was more remarkably influenced by the irregular innervation rates than that with a shorter IPI. On rare occasions, the IPI with a short interval, less than 1 msec, tended to be longer during strong voluntary contrac-

tions. The IPI of a certain double discharge tended to be longer during strong contractions and the double discharge disappeared over a certain innervation rate.

In myasthenia gravis and peripheral nervous disorders, the most potential pairs with abnormal jitter did not show an apparent relationship between the IPI and the IDI. In patients with Guillain-Barré syndrome and Hirayama disease, the long IPI also tended to become short during strong voluntary contractions.

Discussion

The tendency of long MISI of multi-action potentials belonging to the same motor unit involves some factors such as variance of the length of nerve twigs, ectopic motor end-plates and muscle fiber diameters or muscle propagation velocities. In muscular disorders, the variance of muscle fiber diameters, which is a characteristic feature of morphological changes in progressive muscular dystrophies, may reflect the tendency of long MISI. But the single fiber technique could not define the relationship of each factor with the MISI.

Concerning the relationship between the IPI and the IDI, the degree of the velocity recovery function (VRF)[1] of each muscle fiber participates in the fluctuation of the IPI. As the muscle fiber propagation velocities of later components tended to be slower than those of earlier components[2] and the IPI tended to become long during strong contractions, the muscle fibers with slow propagation velocities must have more pronounced VRF than those with fast propagation velocities. This tendency was observed in both myopathic muscle fibers and neurogenic muscle fibers[3]. We concluded that the muscle fibers with slow propagation velocities showed pronounced VRF due to changes of muscle fiber membrane characters in primary myopathic disorders and secondary muscle fiber changes in neurogenic disorders.

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EXPERIMENTAL MYOTONIA IN HUMAN EXTERNAL INTERCOSTAL MUSCLES

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In order to apply the methods of Excitation-Contraction (E-C) Uncoupling developed by the authors and others[1] for mammalian muscles to human skeletal muscles, experiments were performed on human external intercostal muscles obtained during thoracotomies. The purpose of this study was as follows: (1) to record the resting membrane potentials of normal human external intercostal muscles, (2) to apply E-C uncoupling to human skeletal muscle specimens for the purpose of making intracellular recordings during muscle activities, and to observe the morphological changes of the human muscles after E-C Uncoupling by electron microscopy, and (3) to induce myotonia in the human muscle specimens in vitro by exposure to anthracene-9-carboxylic acid[2].

Methods

Experiments were performed on human external intercostal muscle specimens which were obtained from two cases of thoracotomies under general anesthesia: one for lung cancer, and the other for traumatic tracheal stenosis. These two patients did not have any muscle disorders. Great care was taken not to injure the muscle fibers, and intact muscle specimens from tendon to tendon were used.

Excitation and contraction was uncoupled by exposing the human external intercostal muscle specimens to 400 mM glycerol in a liter of mammalian Ringer's solution for 60 minutes, and then washing solution for 20 minutes. The latter contained 5 mM calcium and 5 mM magnesium in mammalian Ringer's solution for better maintenance of the resting membrane potentials.[3] The muscle specimens were then exposed to 5 mg/L dantrolene sodium solution for another 20 minutes.

Myotonia was induced in the human muscle specimens by adding anthracene-9-carboxylic acid to the muscle chamber at the concentration of 10^{-4} M/L.[4] During the experiments the muscle specimens were constantly oxygenated with a 95% O_2 and 5% CO_2 mixture that passed through the muscle chamber.

Resting membrane potentials, myotonic bursts, were recorded intracellularly by conventional techniques using glass microelectrodes filled with 3 M KCl.

Results

(1) The mean resting membrane potentials of the normal human external intercostal muscle specimens were -84.4 ± 2.9 (S.D.) mV, and -81.2 ± 2.9 (S.D.) mV. These were the results for two specimens in the oxygenated Tyrode Solution.

(2) Myotonia was induced in the human external intercostal muscle specimens by anthracene-9-carboxylic acid without reduction of the resting membrane potentials: -83.1 ± 1.6 (S.D.) mV, and -81.5 ± 2.2 (S.D.) mV.

(3) Myotonia was successfully induced after E-C Uncoupling as well, and myotonic bursts were recorded intracellularly without movement induced artifacts. During myotonic bursts there was 9 to 11 mV initial depolarization before each action potential started.

(4) Electron microscopic observation of semithin (0.2 μm) sections of the human external intercostal muscle specimens before and after subjecting to the present method of E-C Uncoupling revealed that vesicles were produced near the triad after E-C Uncoupling. The myofibril, myofilament, and the

Z-band were all intact. High power (x 72,000) observation of the vesicles suggested the continuation of the vesicles to the t-tubules. Further investigations are required to confirm whether the terminal cistern or the t-tubules are disrupted in association with the production of the vesicles near the triad. Some external tracer would be required to clarify this point.

Discussion

In order to make the intracellular recordings for the skeletal muscle fibers during repetitive activity Eisenberg and others developed a technique of E-C Uncoupling for frog skeletal muscle. The authors and others developed a method of E-C Uncoupling which is applicable to mammalian muscle using a hyperosmolar glycerol solution, washing solution, and dantrolene sodium solution. The present investigation revealed that the latter technique could be successfully applied to human external intercostal muscle specimens in vitro. Myotonia was induced in the human muscle specimens by anthracene-9-carboxylic acid and the intracellular recordings were made during myotonic bursts with the help of E-C Uncoupling as described above. Electron microscopic observation of the human external intercostal muscle specimens before and after subjecting to the present method of E-C Uncoupling revealed the formation of vesicles near the triad without producing any changes of the myofibril, myofilament, or Z-band. With this technique, prolonged intracellular recordings with human skeletal muscle specimens during activities are possible.

Since the present experiments involved myotonia induced by anthracene-9-carboxylic acid in normal human muscle specimens, further correlation of human myotonic disorders to the results of the present experiments has to be performed.

Acknowledgement

Professor Shukuro Araki kindly reviewed the manuscript.

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CONTRACTILE AND CHEMOSENSITIVE PROPERTIES OF MUSCLE TREATED WITH CALCIUM IONOPHORE (A23187)

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Muscle membrane abnormalities have been suggested for the pathogenesis of human muscular dystrophy. Several of the theories depend on an abnormal influx of calcium. An excessive intracellular calcium concentration may account for the increased protein degradation, and through enhancement of Ca^{++}-activated neutral proteases or ATP depletion, lead to muscle necrosis and weakness. Calcium ionophore, A23187, which raises the intracellular calcium concentration, is a useful tool for studying this calcium-related theory of muscle cell necrosis. In the present investigation, contractile properties of A23187-treated muscle were studied and compared with those which we previously obtained for human dystrophic muscle.

Methods

Diaphragms (63.7–70.8 mg wet weight) were dissected from young adult male rats of the Wistar strain. Control muscle was equilibrated for 60 minutes at 37°C in mammalian Ringer solution containing ethanol (10 μl/ml), gassed with 5% CO_2 in oxygen. Test muscle was immersed for 60 minutes at 37°C in Ringer solution which contained ethanol-dissolved A23187 (10 μg/ml) and was aerated with O_2 + CO_2 (95:5). Each muscle was then placed in the oxygenated solution in a chamber containing platinum plate electrodes for massive stimulation, and was connected to a strain gauge and carrier amplifier for isometric recording. Direct stimulation was assured by curarization of the muscle (d-TC, 6 μg/ml). Using twitch (Pt), tetanus (Po), and their derivatives, contractile analyses were done on the basis of the concept of the "active state", which was subdivided into the time component ($T_{1/2R}$; $T_{dPt/dt}$), and the intensity component (d^2Pt/dt^2; dPo/dt).

Results and Discussion

A23187-treated muscle showed prolongation of $T_{dPt/dt}$ (time from onset to point of maximum velocity of twitch development) and $T_{1/2R}$ (time from onset to the half-relaxation level of twitch). The former, the duration of the active state, was measured as a factor expressing the amount of calcium liberated from the sarcoplasmic reticulum and also the rate of the reaction between calcium and troponin. The latter factor, the decay of the active state, reflects the removal of calcium from troponin and the uptake of calcium by the sarcoplasmic reticulum, and may also be related to a relationship between calcium-binding by troponin and ATP-binding by myosin. Abnormalities of these indices which relate to subcellular events were also found in dystrophic muscle. Post-tetanic prolongation of the relaxation time of twitch ($T_{1/2R}$) was also abnormally increased in both A23187-treated muscle and dystrophic muscle. The maximum acceleration of twitch development (d^2Pt/dt^2; active state intensity of shortening) was measured to express the amount of calcium released from the sarcoplasmic reticulum at the initiation of excitation-contraction coupling. This was normal in A23187-treated muscle, while it was reduced in dystrophic muscle. The maximum tetanic force (Po; active state intensity of load-bearing), attributed to the number and intrinsic strength of the actin-myosin cross-bridges, was decreased in both A23187-treated muscle and dystrophic muscle. Also decreased in both muscles was the maximum velocity of tetanus development (dPo/dt) which relates to the force-velocity relation of the contractile material if not contaminated by abnormal series-elastic components. The twitch/tetanus ratio (Pt/Po) was markedly increased reflecting greater reduction of the tetanic force and the protracted time course of the active state, while it was decreased in dystrophic muscle.

Sensitivity to epinephrine of muscle contraction, mediating through beta-receptors in the cAMP-dependent or cAMP-independent pathway, was previously found by us to be useful for estimation of membrane/submembrane calcium-related abnormalities in dystrophic muscle and in experimentally denervated muscle. Immersion of the rat diaphragms in a solution containing isoproterenol (2 μg/ml) caused the twitch to increase with prolongation of the active state duration and decay. The same change in twitch as in the control after isoproterenol treatment was seen in A23187-treated muscle, while dystrophic muscle responded in a manner different from the control.

Conclusion

The treatment of skeletal muscle with A23187 induced marked changes in tetanus parameters that mainly relate to abnormalities in contractile proteins and part of the activation process. Twitch parameters including its response to beta-adrenergic stimulants were not remarkably altered by A23187 except parameters expressing the time course of the active state. Our previous study demonstrated that, compared with changes in tetanus, abnormalities of twitch, particularly in the acceleration of twitch development and response of twitch to beta-adrenergic stimulants, tend to appear in an early stage of muscle dystrophy. The twitch/tetanus ratio was increased in the A23187-treated muscle, while it was decreased in dystrophic muscle. Therefore, the A23187-treated muscle is not wholly similar to dystrophic muscle.

PHOSPHATIDYL INOSITOL METABOLISM IN DUCHENNE ERYTHROCYTE MEMBRANES

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It still remains obscure whether erythrocyte membranes are primarily involved in Duchenne muscular dystrophy, despite the fairly large number of biochemical and morphological abnormalities of the membranes already reported.[1] Dise et al reported more pronounced facilitation of K efflux by calcium ionophore A23187 in Duchenne erythrocytes than in controls.[2] Increased fragility and echinocytosis in Duchenne erythrocytes were also reported. Phosphatidyl inositol metabolism is coupled with physiological responses of cell membranes.[3] Allan and Michell reported that microvesiculation was accompanied by activation of polyphosphoinositide phosphodiesterase with the formation of 1,2-diacylglycerol and phosphatidate.[4] These reports prompted us to study phosphatidyl inositol metabolism in Duchenne erythrocytes, including the effect of calcium ions.

Methods

Blood specimens were taken into heparinized syringes from patients with Duchenne dystrophy and controls including normal subjects and patients with spinal progressive muscular atrophy. Erythrocytes were separated by the use of dextran, followed by washing twice with 0.9% NaCl, hemolysis and washing with several kinds of hypotonic buffers. The following conditions gave the maximum incorporation of ^{3}H-inositol into the membranes: 20 mM Tris-HCl buffer (pH7.5), 0.5 mM ATP, 0.25 mM CTP, 20 mM $MgCl_2$, 0.05 mM ^{3}H-inositol, 100 μl erythrocyte membranes (around 0.1 mg protein) in a total volume of 200 μl, incubated at 37°C for 30 min. The reaction was terminated with 5% TCA, followed by extraction of phospholipids with a mixture of chloroform, methanol and conc. HCl (200:100:1, v/v) and partitioning with 0.1 N HCl. The bottom layer was subjected to thin-layer chromatography or radioactivity measurement.

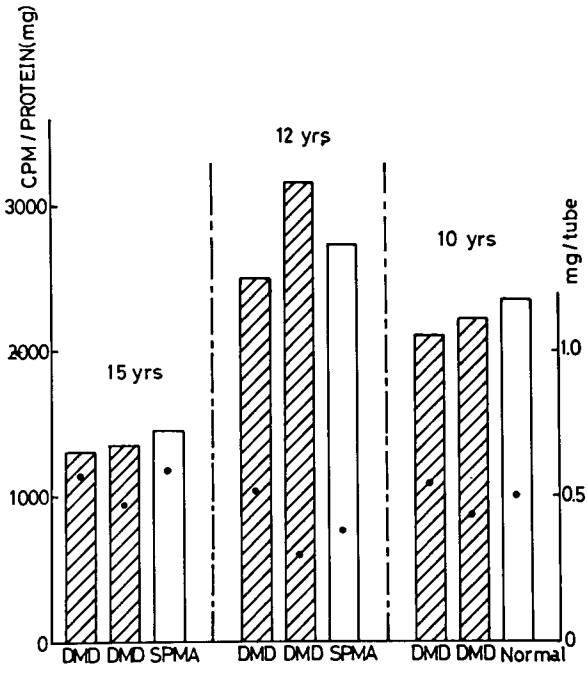

Fig. 1 Incorporation of ^{3}H-inositol into erythrocyte membranes lysed in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol.

Results:

Calcium ions markedly reduced the incorporation of ^{3}H-inositol into the membranes. EGTA prevented this inhibition, suggesting a Ca-specific inhibition. No essential difference, however, was seen between the patients and controls. A thin-layer chromatogram on Silica Gel H disclosed almost complete incorporation of ^{3}H-inositol into diphosphoinositide under these conditions. The highest incorporation of ^{3}H-inositol was achieved by the use of membranes, prepared with 10 mM Tris-HCl, pH 7.5, includ-

ing 1 mM EDTA and 1 mM dithiothreitol. The ^{3}H-inositol incorporation into erythrocyte membranes from the patients and controls simultaneously prepared with this buffer showed a significant difference as shown in Fig. 1.

Simultaneously measured adenylate cyclase activities of the membrane preparations also failed to reveal any difference between the two groups and no activation by epinephrine was demonstrated at all, unlike the results reported by Wacholtz et al.[5)]

These results did not support systemic involvement of erythrocyte membranes in Duchenne muscular dystrophy.

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CONCANAVALIN A BINDING ON THE ERYTHROCYTES IN DUCHENNE MUSCULAR DYSTROPHY

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As was previously reported, red blood cells (RBCs) from genetically dystrophic chickens show a 20 to 40% reduction of Con A binding sites compared to normal controls.[1] Using the same techniques, we have investigated the Con A binding on RBCs from Duchenne muscular dystrophy (DMD) patients.

RBCs were obtained from six DMD patients, of 8 to 23 years of age, and six normal controls, of 17 to 19 years of age. Unfixed RBCs (1×10^6/ml) were incubated with ferritin conjugated concanavalin A (Fer-Con A; 30–60 μg/ml of Con A) at 37°C for 30 min. RBCs labeled with Fer-Con A were washed with phosphate buffered saline (PBS; pH 7.2) and suspended at a concentration of 50 volume %. A small volume of this solution was dropped onto the surface of distilled water. Some of these RBCs were ruptured at the air-water interface. The RBC membranes spread flat by surface tension, were picked up on a grid with a supporting film, washed with distilled water, and processed for transmission electron microscopy. As controls, RBCs were incubated with free ferritin, or with Fer-Con A in the presence of 0.2 M α-methyl-D-mannoside. In both cases, no ferritin was found on the cell surface.

RBC membranes prepared by lysis at the air-water interface showed the topological distribution of Fer-Con A on the membrane surface and showed strikingly the difference between normal and DMD patients. Numerous ferritin particles, singly or in small clusters, were tagged onto the surface of RBCs obtained from normal controls. While, in the case of DMD patients, the number of ferritin particles on the membrane was less. On the electron microscopic photographs (enlarged, x 60,000), four regular squares of 1,000 nm side length were chosen randomly, and were subdivided into 100 nm x 100 nm unit squares. The numbers of particles per unit square in each case were 1.07 ± 1.45, 1.18 ± 1.70, 1.29 ± 1.95, 1.30 ± 1.68, 1.47 ± 1.68 and 1.63 ± 1.68 for DMD, and 1.48 ± 2.07, 1.75 ± 2.52, 2.11 ± 2.69, 3.32 ± 3.27, 4.04 ± 3.28 and 4.15 ± 3.68 for the controls. The difference between them was significant at $p < 0.05$ level.

In order to assess the distribution pattern of ferritin particles, Irimura et al[2] introduced, as a parameter of clustering, the ratio $\sigma(k)/\sigma_{random}(k)$. $\sigma_{random}(k)$ is the standard deviation of the number of particles contained in unit squares with a side length of 250 x kÅ randomly distributed which is obtained theoretically from a table of random numbers. According to Irimura et al, the value of $\sigma_{random}(k)$ is a function of variable k and of the total number of particles (TN = the number of particles in 1,296 unit squares), as shown by the equation:

$$\sigma_{random}(k) = 0.029\ TN/k \quad (k < 6)$$

$\sigma(k)$ is the standard deviation of the number of ferritin particles in unit squares with a side length of 250 x kÅ, counted on electron micrographs. $\sigma(4)/\sigma_{random}(4)$ ratios for DMD were less than those of normal controls as shown in Fig. 1 ($p < 0.01$).

The evidence presented here indicates that RBC membranes from DMD patients have significantly fewer receptors for Con A, as shown in the previous report for dystrophic chicken RBCs. It has been suggested that band 3 proteins and/or other glycoproteins make up the intramembrane particles (IMPs)

revealed by freeze-fracture techniques.[3] Band 3 proteins are said to be major binding sites for Con A in human RBCs. Therefore, the results of our present study seem to be consistent with the description of Wakayama et al[4] with regards to DMD-RBC membranes and IMPs. It is also suggested that Con A receptors may participate in the modulation of membrane fluidity, and consequently, may contribute to the regulation of membrane enzymes.[5] Taking all these reports into consideration, we may find some plausible explanations for the functional abnormalities in the membrane of dystrophic RBCs.

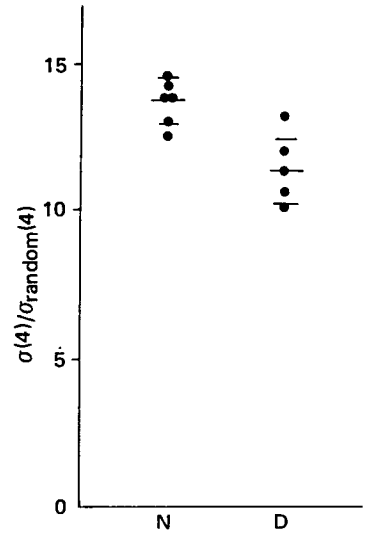

Fig. 1

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CALCIUM FLUX OF ERYTHROCYTES IN DUCHENNE MUSCULAR DYSTROPHY

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The calcium flux of erythrocytes from patients with Duchenne muscular dystrophy (DMD) was measured. Passive influx was determined using erythrocytes incubated with lanthanum chloride as a specific inhibitor for calcium-activated magnesium-dependent ATPase. There was no significant difference in calcium influx in cytosol and in membrane fractions between DMD and control age-matched boys. Influx of calcium was also determined using ATP-depleted erythrocytes incubated without the inhibitor. Again there was no significant difference between DMD and controls. Efflux of calcium was measured using erythrocytes loaded with calcium ionophore A23187. The calcium efflux speed was related to intracellular calcium concentration and it reached a plateau at about 1 mM concentration. There was no significant difference in calcium efflux rate as a function of intracellular calcium concentration between DMD and controls.

Refefence

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Ca AND ATP CONTENTS OF ERYTHROCYTES IN DUCHENNE MUSCULAR DYSTROPHY

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Recently, high calcium accumulation in muscle cells of patients with Duchenne Muscular Dystrophy (DMD) has been reported. The present study deals with the mechanism of this phenomenon.

Last year, we reported that there was no appreciable difference in the occurrence of deformed erythrocytes between DMD and controls when the erythrocytes were in plasma or were stored in a buffer solution containing dextrose without Ca^{2+}. On the other hand, when the erythrocytes from DMD and controls were stored in Phosphate Buffered Saline containing Ca^{2+} (PBS (+)) with no dextrose, a large number of echinocytes in DMD erythrocytes occurred. It is generally accepted that the echinocytes occur by either calcium accumulation or by ATP depletion in the erythrocytes.

The present study attempted to clarify the cause of the occurrence of the echinocytes in DMD erythrocytes by using a buffer solution containing Ca^{2+} with dextrose for preventing the ATP depletion. Also calcium and ATP contents of DMD erythrocytes were compared with those of controls.

Subjects and Methods

The buffer solution used in this study was Phosphate Buffered Saline containing Ca^{2+} and Mg^{2+}, with 5 mM-dextrose added (PBSD (+), pH 7.4 281 mOsm/1.) Heparinized venous blood was obtained from DMD patients and age-matched healthy boys as controls. Erythrocytes separated from plasma together with the buffy coat were washed three times with PBSD (+). After each washing, the buffy coat was removed. Then, they were suspended in PBSD (+), in 10–15% hematocrit proportions, and stored at 4°C for 72 hours, being stirred occasionally. The stored erythrocytes were centrifuged and then were adjusted in PBSD (+) to 45% hematocrit proportions, and were subjected to calcium and ATP level measurements. The ATP in the fresh erythrocytes just after washing three times with PBSD (+) was also measured.

Ca determination: Erythrocyte calcium was determined by ejecting the calcium in the external solution (Trapped Plasma) that remains in the spaces between the packed cells. The Trapped Plasma was measured with Indocyanine green (ICG: gift from Daiichi Seiyaku Co., Ltd.). The samples for trapped plasma measurement were prepared by centrifugation at the same time as for the sample for Ca determination. Calcium was measured by atomic absorption spectrophotometry. Samples for Ca determination, both the packed cells and the external solution were treated with HNO_3-H_2SO_4.

ATP determination: ATP in erythrocytes extracted with 0.6N perchloric acid, stored at 4°C, was measured within 24 hours by the enzyme assay method (ATP-test; Boehringer)

Hemoglobin (Hb) determination: Hb was measured by the azide-methemoglobin method (Hemokit – S: Nihonshoji) to evaluate the ATP content relative to Hb. All solutions used were prepared just before use.

Results and Discussion

Erythrocyte calcium: The calcium content of the erythrocytes from seven DMD patients (average 19.3 years old) was 2.5 times higher than that of seven healthy boys (average 19.7 years old). It was confirmed that the ATP of the erythrocytes was not depleted under the present conditions. The high calcium content in DMD erythrocytes as compared with controls, is surmised to be caused by the accumulation of calcium in the erythrocytes and by the easy occurrence of Ca influx. Because Ca^{2+}-ATPase activity for the Ca efflux pump of DMD erythrocytes stored at 4°C might be low since Ca^{2+}-

ATPase activity of the erythrocytes stored in the cold is inhibited or low. These proposals are also supported by other reports[1] that confirmed higher Ca^{2+}-ATPase activity of DMD erythrocytes at 37°C.

Accordingly, the occurrence of a large number of echinocytes in DMD erythrocytes when they are stored in PBS (+) solution containing Ca^{2+} without dextrose would be due to accumulation of calcium in the DMD erythrocytes based on depletion of ATP.

Erythrocytes ATP: The ATP content of erythrocytes from ten DMD patients (average 16.4 years old), after storing at 4°C for 72 hours and just after washing, was 12–15% higher than that of fourteen healthy boys (average 16.8 years old). This result coincides with the results reported by Danon[2]. One of the reasons for the high ATP level in DMD erythrocytes might be the high calcium content of DMD erythrocytes, because the ATP in the erythrocytes controls Ca binding to membranes.

Conclusions

1) Ca content of DMD erythrocytes stored in PBSD (+) at 4°C for 72 hours was 2.5 times higher than that of controls.

2) ATP contents of DMD erythrocytes after storing in PBSD (+) at 4°C for 72 hours and just after washing three times were 12–15% higher than those of controls.

3) The occurrence of a large number of echinocytes in DMD erythrocytes in PBS (+) solution would be due to the accumulation of Ca in the erythrocytes based on ATP depletion in cells.

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ATPase AND ADENYLATE KINASE OF ERYTHROCYTE MEMBRANES IN HUMAN MYOTONIC DYSTROPHY

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Various abnormalities of erythrocytes (or erythrocyte membrane) have been reported in human myotonic dystrophy. They included (Na + K)-ATPase, (Ca + Mg)-ATPase, cation exchange, protein kinase, adenine uptake to ATP and ADP, electron spin resonance pattern, membrane deformability and erythrocyte shape. However, there are contradictory reports on these reported abnormalities. Therefore, we re-examined ATPase activity of erythrocyte membranes in human myotonic dystrophy. Adenylate kinase was also examined, because most of the reported abnormalities were related directly to adenine nucleotides.

Patients with myotonic dystrophy (MD) and age-matched controls (patients with lung tuberculosis) were in the same institution for more than one month. Blood from the subjects and controls was taken into heparinized tubes on the same day at almost the same time. Thereafter, preparation of erythrocyte membranes and assay of enzymes were simultaneously done for MD and control patients. After separation of plasma and buffy coat, erythrocytes were washed three times with 8.5% NaCl and the buffy coat was removed at each washing. The erythrocytes were hemolyzed with 5 mM Tris-HCl (pH 7.4) and membranes were washed four times with the same solution by centrifugation at 25,000g for 20 min. The white membranes were finally resuspended in the same solution and stored at -35°C until use. ATPase activities were assayed by using a coupled enzymatic method. The assay mixture for "total ATPase" consisted of 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 15 mM KCl, 1 mM ATP, 1 mM PEP, 0.25 mM NADH, pyruvate kinase, LDH and erythrocyte membranes (300 to 400 μg). The ouabain concentration was 0.1 mM, when it was used. After equilibration at 37°C, the activity was followed by recording the absorbance at 340 nm. Adenylate kinase activity in the direction ATP + AMP → 2 ADP was determined by adding 1 mM AMP to the assay mixture of ATPase including 0.1 mM ouabain, and adenylate kinase activity was calculated by subtraction of the ATPase activity from the activity with AMP. Adenylate kinase activity in the direction 2 ADP → ATP + AMP was also assayed in a reaction mixture containing 100 mM tris-acetate, pH 7.0, 10 mM Mg-acetate, 20 mM glucose, 0.6 mM NADP, 1 1 mM ADP, hexokinase, G-6-PDH and membranes, and the activity at 37°C was determined by recording at 340 nm. The same method was used for the assay of adenylate kinase activity in plasma.

The enzyme activities of erythrocyte membranes are shown in the Table, and no difference of the activities between MD and control patients was observed. Adenylate kinase activity of the erythrocyte membranes was much higher than the ATPase activity in the present study. An apparent increase of adenylate kinase of plasma was noted in two out of five patients with MD, but the mean activity of the enzyme was not significantly different between MD and control patients.

Table Erythrocyte membrane ATPase and adenylate kinase activity in myotonic dystrophy and controls.

	Control (n = 6)	Myotonic (n = 5)
Total ATPase	9.9 ± 2.6	9.4 ± 2.3
(Na + K)-ATPase	2.8 ± 0.8	2.7 ± 0.7
Adenylate kinase		
ATP + AMP → 2 ADP	39.1 ± 8.8	41.1 ± 16.0
2 ADP → ATP + AMP	26.1 ± 4.7	28.2 ± 9.1

Enzyme activities; mμ moles min/mg. membrane protein (at 37°C)

Our results for ouabain sensitive ATPase of erythrocyte membranes of MD contradicted

those of the previous reports. There are also contradictions among the reported abnormalities. Brown et al.[1] reported that (Na + K)-ATPase from two patients with MD was stimulated by 0.1 mM ouabain, and the results of Dobosz[2] showed less inhibition of the enzyme by 0.1 mM ouabain than that of normal erythrocyte membranes. Mishra et al,[3] who used a similar assay method for ATPase activity to that in the present study, reported that 1 mM ouabain inhibited the enzyme from MD greater than that from normal controls. The exact cause(s) of the dicrepancy among the reports is not known. Slight differences in preparation of erythrocyte membranes may have caused inconsistency of the properties of the membranes or inconsistent results among the reported abnormalities. In the present study, the patients with MD and controls were in the same institution, and samples from patients with MD and controls were obtained and processed simultaneously.

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ERYTHROCYTE ACETYLCHOLINESTERASE IN MYOTONIC DYSTROPHY

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We studied the erythrocyte membrane in myotonic dystrophy (MyD) with acetylcholinesterase (AchE). Patients with MyD and controls (patients with mild pulmonary tuberculosis) were in the same institution for more than 6 months and were matched by age and sex. Experiments 1 (study on hemolysates) and 2 (study on ghosts) were done on separate days but for each experiment samples from patients and controls were obtained at almost the same time on the same day and processed simultaneously.

Hemolysates were prepared almost identically to as before[1]. In Experiment 2, the erythrocytes were washed three times with isotonic saline after the plasma and buffy coat were removed carefully. The erythrocytes were hemolyzed with 10 volumes of 10 mM tris-HCl, pH 7.4, and the membranes were sedimented at 20,000 g for 20 min. The membranes were washed 5 times with the same buffer and collected by the same centrifugation. The AchE activity was measured by the method of Ellman et al[2] slightly modified. The activation energy, transition temperature and Hill coefficient for the inhibition of the enzyme activity by fluoride were obtained graphically as described previously[1].

There was no difference between MyD patients and controls in the enzyme activity in hemolysates or in ghosts within the temperature range studied (7–14°C in Experiment 1, and 25–41°C in Experiment 2). No significant difference was noted between MyD patients and controls in the transition temperature (Tc) or in the activation energy either above or below Tc in Experiment 1. In Experiment 2, the activation energy did not differ between MyD patients and controls. The Hill coefficient for the inhibition by fluoride was approximately 1 in MyD patients and controls at 13, 25 and 37°C. The fluoride dose for 50% inhibition of the enzyme activity was different between different temperatures but there was no difference between MyD patients and controls.

The activation energy, transition temperature and Hill coefficient for the inhibition of AchE activity by fluoride were considered to reflect properties of the membrane. Thus, the present results suggest that there is no gross abnormality in AchE or its environment in the erythrocyte membrane in MyD.

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CONGENITAL MYOPATHY AND ABNORMAL TRYPTOPHAN METABOLISM

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We previously found and reported abnormal findings of the tryptophan-kynurenine pathway in patients with nemaline myopathy and related myopathies. Here we describe abnormal metabolism of the pathway in two cases with so-called arthrogryposis multiplex congenita (AMC) from two pedigrees, who were newly found by us, and furthermore summarize the results obtained to date.

Materials and Methods

Two cases with so-called AMC, their parents and the grandmother from 2 pedigrees were included in the study. Tryptophan metabolism was investigated by the method reported previously. Briefly, after the loading of L-tryptophan at a dose of 100 mg/kg (5g, in subjects weighing more than 50 kg), 24-hour urinary excretion of kynurenic acid (KA) and xanthurenic acid (XA) was assayed by the Satoh-Price method.

Results

1. Cases: Subjects were 2 cases from 2 pedigrees; case 1 (M. T., 17-yr-old male) and case 2 (Y. U., 14-yr-old female). Their parents were apparently healthy and were not consanguineous.

Cardinal manifestations in both cases were ankylosis of multiple joints at birth or postnatally, marked scoliosis, dislocation of hip joints, high-arched palate and generalized muscular atrophy.

Serum creatine kinase (CK) activity was normal in both. Histological examinations of biopsied muscle revealed marked type 2 fiber predominance as well as diffuse, mild atrophy of muscle fibers in the gastrocnemius muscle of case 1, and variation of fiber size, marked type 1 fiber predominance and multicore-like findings in the latissimus dorsi muscle of case 2.

2. Tryptophan metabolism: Urinary excretion of KA and XA before the loading was normal in both, less than 0.5 μmoles/kg/day. After the loading, however, 24-hr urinary excretions of both KA and XA were abnormally increased, i.e., case 1, KA 18.7, XA 2.4; case 2, KA 11.7, XA 2.7 (normal values; KA 2.0–5.0, XA 0.7–2.0 μmoles/kg/day). The increase was not corrected by the administration of vitamin B6. Increased excretion of KA and normal excretion of XA were observed in the mother of case 1 and the maternal grandmother of case 2. Fathers gave normal values.

Discussion

Abnormal findings observed in the two patients with AMC led to the presumption that a deficiency or decreased activity of 3-HA-oxygenase in the tryptophan-kynurenine pathway is the basis of the disorder. Increased excretion of KA observed in the mother was supposedly attributable to expression of the same basic abnormality as in the patients.

With the same method, we found and reported various, different abnormalities of tryptophan metabolism in patients with nemaline myopathy, in patients with congenital fiber type disproportion, in one case with unclassified myopathy with type 1 fiber atrophy and in patients with Hartnup-like disease.

Four patients from 4 pedigrees with nemaline myopathy showed markedly increased excretion of KA and normal XA excretion after the loading of tryptophan. Similar abnormalities were found also in their mothers and the maternal grandmother of one case. We presumed the presence of a deficiency or decreased activity of kynurenine-3-hydroxylase of the tryptophan-kynurenine pathway. X-linked domi-

nant inheritance of the disease as well as this abnormal metabolism was also suggested.

Both of the two cases in a family (the proband and his father) affected with congenital fiber type disproportion showed a marked increase of KA and a mild increase of XA, which was not corrected by the administration of vitamin B6. A deficiency or decreased activity of 3-hydroxyanthranilate oxygenase of the pathway was indicated in this disorder.

The patient with unclassified myopathy with type 1 fiber atrophy gave remarkably high values of KA and XA, especially of the latter. The remarkable increase of XA excretion was reduced by the administration of a high dose of PALP (300 mg/day). Similar findings were also found in the daughter. Vitamin B6 dependency of kynureninase was suggested in this disorder.

Urinary excretions of both KA and XA after the loading of tryptophan were abnormally low in two brothers affected by Hartnup-like disease. Despite the similarity of clinical manifestations with Hartnup-like disease, both patients demonstrated no aminoaciduria and normal absorption and urinary excretion of L-tryptophan. Similar findings were observed in the apparently healthy parents. A deficiency or decreased activity of formamidase and an autosomal recessive pattern of inheritance were suggested in this disease.

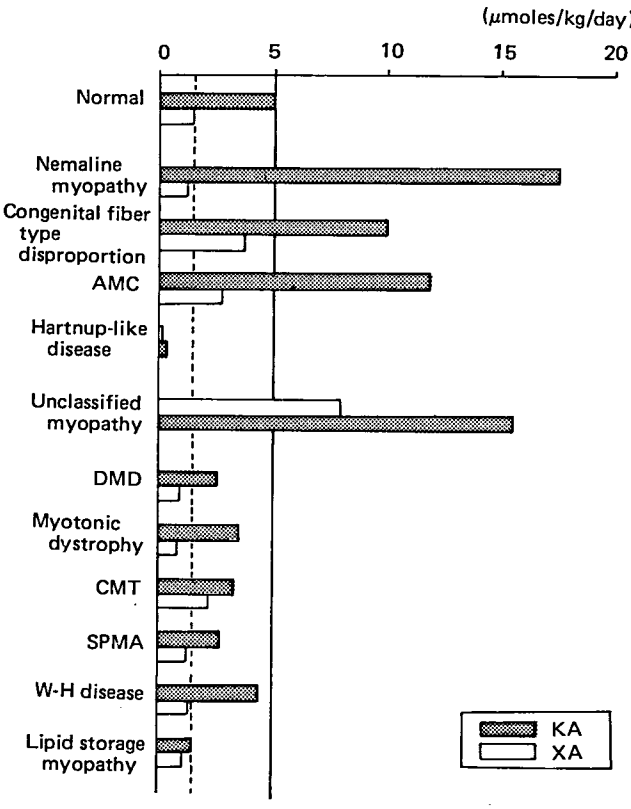

Fig. The Urinary Excretion of KA and XA in Neuromuscular Disorders (after loading of L-tryptophan at a dose of 100 mg/kg)

No abnormal findings of the metabolism were found in DMD, myotonic dystrophy, CMT, W-H disease, SPMA etc.

In this report two cases with AMC were newly included.

Summary

Disorders with abnormal findings in the tryptophan-kynurenine pathway were restricted to rare, so-called congenital myopathies. Abnormal metabolism of the pathway might be related to the etiology of these disorders.

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SOME PROPERTIES OF CREATINASE IN HUMAN SKELETAL MUSCLE

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Recently, the presence of creatinase, which catalyzes the hydrolysis of creatine to sarcosine and urea, in human skeletal muscle was reported[1]. It was also reported that the kinetic behavior of creatinase was altered in skeletal muscle of patients with Duchenne muscular dystrophy[2].

The present study was undertaken to confirm the presence of creatinase and to examine a few properties of the enzyme.

Materials and Methods

Human skeletal muscle was obtained from non-myopathy patients at autopsy. Muscle was homogenized in physiological saline with a Polytron and the homogenate was centrifuged at 18,000 x g for 30 min. The supernatant was then subjected to gel filtration on a Sephadex G-150 column (2.5 x 50cm).

Creatinase activity was assayed by creatine consumption: the enzyme reaction was carried out at 37°C for 30 min and the remaining creatine was determined by the diacetyl α-naphthol method.

Results

Fig. 1 shows the elution profile of creatinase activity on a Sephadex G-150 column. Two peaks of creatinase activity were observed; one at a molecular weight of 50,000 and the other at 25,000.

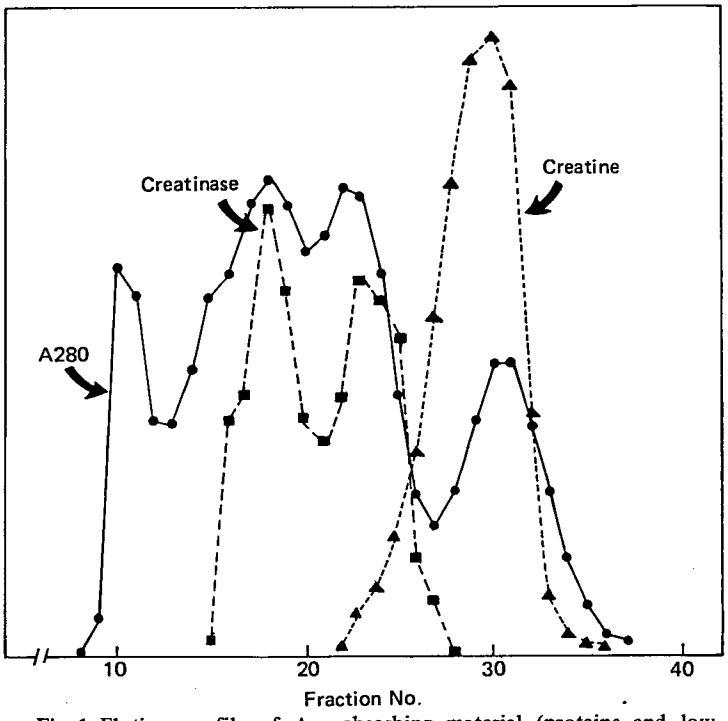

Fig. 1 Elution profile of A_{280}-absorbing material (proteins and low-molecular-weight substances), creatinase activity and creatine on Sephadex G-150 column (2.5 x 50cm) filtration. Eight ml fractions were collected.

Each eluate fraction was treated with trichloroacetic acid (final 5%), centrifuged, and the concentration of creatine in the supernatant was also determined. Creatine was found to be eluted in the low-molecular-weight fraction, not associated with creatinase activities.

Discussion

Our results confirm the presence of creatinase activity in human skeletal muscle reported by Miyoshi and his coworkers[1),2)], but differ from their results in that we detected two peaks of creatinase activity on Sephadex G-150 gel filtration. Whether or not the peak at M.W. 50,000 represents a dimer of the M.W. 25,000 species remains to be elucidated.

The gel filtration study also indicates that creatine in muscle is apparently not associated with

creatinase or any other binding-proteins but exists in a free state.

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ABNORMAL METABOLISM OF CREATINE IN SKELETAL MUSCLES OF MUSCULAR DYSTROPHIES

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In previous papers, we reported the existence of creatinase activity in human skeletal muscles and its abnormal kinetic behavior for creatine in DMD muscles.

In this paper, we present results of further experiments. However, we would like to make a correction in this paper, what we have hitherto called merely creatinase we now call a creatinase-like activity in a broad sense including creatinase. The reasons are that our previous results were obtained with crude materials and that stoichiometrical measurements were difficult. The true nature of this activity will be disclosed after further experiments have confirmed it.

Here we describe the results showing that this activity in skeletal muscles of fetuses and of patients with another type of muscular dystrophy, autosomal recessive distal muscular dystrophy (ARDMD), which was previously reported by us in Japan, differs from that in adult skeletal muscles.

Materials and Methods

Subjects were (1) healthy adults or non-myopathy patients, (2) human fetuses (19th and 32nd week of gestation) and a 5-month-old infant, and (3) patients with autosomal recessive distal muscular dystrophy (ARDMD). Skeletal muscles obtained by autopsy in (1) and (2) and by biopsy in (3) were used for the experiments.

Skeletal muscles stored at $-60^{\circ}C$ were homogenized and centrifuged to obtain supernatants. The active fraction obtained by gel filtration of each supernatant was used as the material.

Assuming the existence of creatine degradative activity, the decrease of creatine was assayed. Namely, in a reaction mixture of the material and creatine, the amount of creatine was measured by the diacetyl α-naphthol method before and after the reaction and the difference was calculated.

Results

Results for skeletal muscles of human fetuses and an infant.

In muscles of fetuses at the 32nd week, the peak activity was found in the same fraction on gel filtration as in healthy adults. The molecular weight of the active substance was the same as that of healthy adults, about 50,000.

The effect of increasing the concentration of the substrate creatine on the reaction velocity was studied using the fraction collected. A hyperbolic curve was obtained in fetal and infantile muscles as in muscles of healthy adults. A linear curve was obtained in either muscle on double reciprocal plotting of this curve (Lineweaver-Burk plot). However, the Km value of skeletal muscles of a 19th-week fetus was 3.8×10^{-4} M, compared with the value of 8.0×10^{-5} M in muscles of healthy adults. A 32nd-week fetus and the 5-month-old infant showed intermediate values.

Thus, the presence of a different type of this activity in fetal muscles, which might be called the fetal type, was suggested.

Results for skeletal muscles of patients with ARDMD.

In skeletal muscles of patients with ARDMD, the peak activity was found in the fractions corres-

ponding to a molecular weight of about 50,000 as in those of healthy adults and DMD patients.

However, kinetic analysis of the activity against the increasing creatine concentration gave a sigmoidal curve in ARDMD muscles as in DMD muscles. The concentration of creatine giving the half value of Vmax was 3.6×10^{-4}M in DMD, and 1.0×10^{-3}M in ARDMD.

Vmax measured in DMD and ARDMD muscles with the same protein content was 2.7 times and more than 4 times that of healthy adults, respectively.

Summary

It was found that the creatinase-like activity in human skeletal muscles varied among adults, fetuses, and types of muscular dystrophies, DMD and ARDMD, in its kinetic behavior.

The altered characteristics of the creatinase-like activity found in skeletal muscles of patients with muscular dystrophies might be intimately related with the abnormal creatine metabolism and, furthermore, with the etiology of the disease.

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ENZYMATIC STUDIES ON THE MECHANISM OF CREATINURIA IN RATS WITH EXPERIMENTAL THYROTOXIC MYOPATHY

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Introduction

Thyrotoxic myopathy is common in middle-aged male patients with hyperthyroidism. The weakness and wasting of the proximal muscles of the extremities are recognized prior to the signs of thyrotoxicosis and disappear with the treatment for hyperthyroidism. There is no change of serum creatine kinase in spite of the increased urinary excretion of creatine.

In this work, we tried to clarify enzymatically the mechanism of creatinuria on thyrotoxic myopathy with the use of animal studies.

Materials and Methods

(1) Materials: Male albino rats of the Wister strain weighing 110–130g, male albino rabbits weighing 3 kg, and mice of the C_{57} BL/6J strain weighing 30–40g were used. All animals were sacrified by decapitation and tissue was removed as quickly as possible in each animal. The human tissues were obtained by autopsy of a patient with osteosarcoma who had died 10 hours before.

(2) Food for animals: Food was given to an individual animal ad libitum. The following basal diet was used; casein (20.0%), dextrin (55.9%), sucrose (15.0%), cellulose (1.0%), vitamin mixture (1.0%), salt mixture (4.0%), choline chloride (0.1%), oil (2.0%) and water.

(3) Injection of triiodothyronine (T_3): T_3 (from Sigma Co. Ltd) was dissolved in saline with 0.02% NaOH to 50 gamma/ml. This solution was subcutaneously injected into each rat at the rate of 50 gamma/100g body weight. Saline containing 0.02% NaOH without T_3 was given to the control rats.

(4) Enzyme preparation: The tissues were homogenized with a Potter homogenizer with 10 volumes of 50 mM Tris-HCl buffer (pH 7.4) for the assay of creatine kinase (CPK), with 10 volumes of 100 mM phosphate buffer with 1 mM of EDTA and 0.15% of deoxycholate (pH 7.4) for glycine amidinotransferase (GAT), with 20 volumes of distilled water for arginase, and with 10 volumes of distilled water for creatine. Each homogenate was centrifuged at 8000×g for 10 minutes. The supernatants were used as the materials for the assay. All procedures described above were performed at 4° C.

(5) Estimation of enzyme activities and creatine: CPK, GAT and creatine were determined by the methods of Noda[1], Walker[2] and Folin[3], respectively. The activity of arginase was measured according to the method of Schimke[4] except that 1/80M glycine-NaOH buffer (pH 9.4) was used in the assay.

Results and Discussion

(1) The relationship between the contents of creatine and the activities of GAT in tissues

In the brain, heart, liver, pancreas, kidney and skeletal muscle of rats, the contents of creatine and the activities of GAT were determined. The tissues in which the contents of creatine were high showed low GAT activity. They were also found to be high in CPK activity. In addition, these characteristics of the distributions of these three substances in the tissues were also demonstrated in the tissues of rabbit, mouse and human.

The tissues for the synthesis are different from those for the storage of creatine. Therefore an

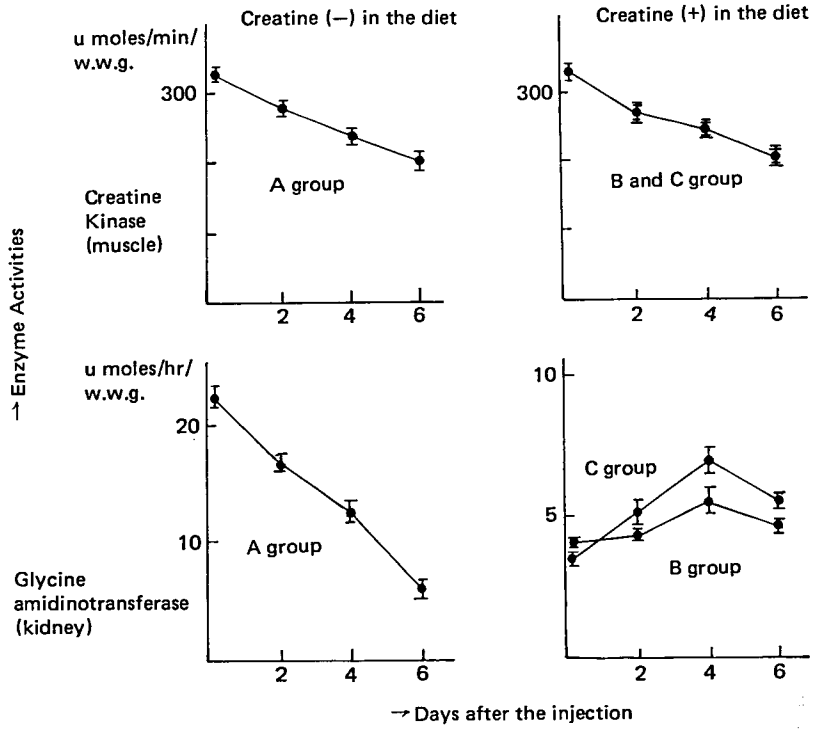

Fig. 1 Effects of T_3 on the activities of both the creatine kinase of skeletal muscle and the glycine amidinotransferase of kidney.

Male rats were divided into three groups. The rats of A group were fed on the basal diet, B group on the basal diet including 0.25% of creatine, and C group on the basal diet including 0.5% of creatine for about one week. Afterwards fifty gamma of T_3 per 100g body weight was subcutaneously injected into the rats of each group every day with the diet described above in each. Each circle shows the average of 3–6 animals.

imbalance between synthesis and storage, as a result of the metabolic abnormality in each tissue or of hormonal disturbance, is most probably the reason for the creatinuria.

(2) Physiological changes in the activities of kidney GAT

Food for rats was changed from the basal diet to a 0.5% creatine added basal diet, and then to a creatine free basal diet. As a result, after creatine was added to the diet, the activity of kidney GAT gradually decreased. On the contrary, after creatine was removed from the diet, the level increased again. These findings indicate that, when the body is "full" of creatine, the activity of this enzyme – the rate limiting enzyme in creatine biosynthesis – is inhibited by the creatine itself.

Male rats continued on the basal diet grew to 110g body weight. Afterwards, only water was given to these animals for four days. The result was that the activity of kidney GAT remarkably decreased. Over these four days, the body weight of the rats decreased to 70g. There was the expected large amounts of muscle wasting. However, an increase of creatine could not be detected in their urine. Creatine biosynthesis is supposed to be inhibited by the creatine driven from the muscle.

(3) The activities of muscle CPK and kidney GAT in rats with experimental thyrotoxic myopathy

The conditioned rats were divided into three groups. The rats of A group were fed on the basal diet, B group on the basal diet including 0.25% of creatine, and C group on the basal diet including 0.5% of creatine for about one week until the body weight reached 110–150g. Fifty gamma of T_3 per 100g body weight was injected into the rats of each group every day.

As a result, the activities of muscle CPK decreased day by day to 87% after 2 days, 85% after 4

days, and 60% after 6 days of the injections. These decreases of muscle CPK were independent of creatine contents in the diet and induced only by the injection of T_3.

On the other hand, the activities of kidney GAT in rats fed on the basal diet decreased day by day to 78% after 2 days, 58% after 4 days, and 26% after 6 days of the injections. On the contrary, in rats fed on tha basal diet including 0.25% of creatine, the levels increased to 112% after 2 days, 143% after 4 days, and 120% after 6 days. Furthermore, in rats fed on the basal diet including 0.5% of creatine, the levels increased also to 150% after 2 days, 200 %after 4 days, and 160% after 6 days of the injections.

With the injections of T_3, the activity of kidney GAT decreased. That may be due to feed back inhibition by the creatine driven from muscle as that of fasting rat is supposed so. If so, with creatine in the diet, the lower levels of this enzyme have to be expected with creatine from both muscles and diet. However, an unreasonable response occurred.

Conclusion

(1) Tissues for synthesis of creatine were different from those for storage. An imbalance between synthesis and storage of creatine is most probably the reason for creatinuria.

(2) The activity of kidney GAT was reduced easily by creatine in the diet, or with wasting muscle in the fasting state. However, with the injections of T_3, in spite of the decrease of muscle CPK activity, an inverse (decreasing and increasing) response occurred between the levels of kidney GAT of the rats fed on the diet without and with creatine. Creatine in the diet has to overflow through thyrotoxic myopathy into the urine.

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EXERTIONAL RHABDOMYOLYSIS AND MYOGLOBINURIA: A CASE REPORT AND METABOLIC STUDY

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Introduction

Skeletal muscle utilizes carbohydrate and lipid as energy sources and disturbances of their metabolism might cause the acute destruction of muscles. We describe in this report a case of acute exertional rhabdomyolysis and myoglobinuria, and biochemical studies on lipid and carbohydrate metabolism in the patient.

Case report

The patient was a twenty year old male. He had suffered from swelling, tenderness, and weakness of muscles after strenuous exercise for fifteen years, and had recovered spontaneously after at least one hour of rest. Muscle groups which were used to excess were involved. He had experienced several episodes of pigmenturia since thirteen years old. These symptoms were induced by short strenuous exercise rather than that of long duration and moderate strength. There were no relations between their occurrence and temperature, moisture, contents of food, and fasting. The so-called second wind phenomenon was once noted during marathon. There was no history of a similar illness in any member of the family. The past history was uncontributary. He had not been exposed to any toxins and had taken no medications.

Physical examination revealed a well-developed and well-nourished male. No muscle tenderness, weakness, atrophy or hypertrophy was demonstrated by neurological examination.

Laboratory data including blood cell counts, serum electrolytes, blood urea nitrogen, creatinine, GOT, GPT, bilirubin, plasma non-esterified fatty acid (NEFA), triglyceride (TG), and thyroid hormones were all normal. Glucose tolerance test results were normal. But even at rest, the values for serum creatine phosphokinase (CPK) and aldolase were slightly elevated. Electromyography showed the normal pattern.

A biopsy specimen of the quadriceps femoris muscle was reported as showing a small number of scattered muscle fibers undergoing degeneration and minimal myopathic changes. Glycogen and lipid droplets were not accumulated in the muscles. Electron microscopically muscle mitochondria had normal structures.

An exercise test was performed by climbing up stairs until swelling, tenderness, and weakness appeared in muscles of the lower extremities. The values of serum CPK and aldolase rose markedly to 15,420 IU/l and 49.1 U, respectively. Twenty minutes after the exercise, he noticed extremely dark urine, in which myoglobin was demonstrated with a spectrophotometer.

Methods

An ischemic forearm exercise test was performed and venous blood lactate was measured. Biopsy samples of muscles were obtained under procaine anesthesia and stored in liquid nitrogen immediately after they were excised. Muscle enzyme activities of the glycolytic pathway including phosphorylase, phosphoglucomutase, phosphohexose isomerase, and phosphofructokinase were determined by the standard assay methods, using the supernatant fraction of 10,000 x g centrifugation of homogenized muscles.

A seventy-two hour fasting test was performed and plasma NEFA, TG, serum CPK, and urine

ketone bodies were measured. Five normal twenty year old male volunteers served as the control group.

The fatty acid composition of plasma NEFA was analysed as follows. Total lipids were extracted from two ml of lyophilized plasma with chloroform-methanol (2:1, v/v). NEFA was separated by thin layer chromatography using a development system of n-hexane-ethyl ether-acetic acid (90:10:1, v/v/v), converted to methyl esters with 2.5% methanol-HCl and analysed by gas liquid chromatography.

Carnitine palmityltransferase (CPT) was assayed by two different methods: the hydroxamate method (Crabtree and Newsholme, 1972) and the isotope exchange method (Norum, 1964). Muscle carnitine content was assayed by the enzymatic method using carnitine acetyltransferase.

Results

The ischemic forearm exercise test produced no muscle cramps. The venous lactate value rose four-fold promptly after exercise.

In the assay of the glycolytic enzymes, the activities of phosphorylase and phosphoglucomutase were shown to be normal and those of phosphohexose isomerase and phosphofructokinase were slightly higher than those of normal controls.

During the fasting study, myoglobinuria was not induced. Plasma TG rose normally and urine ketone production was normal, but the elevation of plasma NEFA was lower than that of the control group. Palmitic and stearic acid in plasma NEFA were slightly higher in the patient. In the control group, oleic acid was increased after fasting, but in the patient its increase was smaller.

The activity of CPT assayed by two different methods was shown to be normal. Muscle carnitine content was also in the normal range.

Comments

Myoglobinuria is a relatively rare syndrome, which is known to be caused by various conditions[1)2)3)]. Because abnormal muscle energy metabolism could exist in this case, metabolic studies were undertaken to investigate the two main ATP-producing systems of muscles in exercise: one was the anaerobic glycolytic pathway which plays an important role in the initial stage of exercise, and the other was the aerobic one utilizing plasma free fatty acid as an energy source.

The clinical symptoms of this case suggested myoglobinuria due to defects in the anaerobic glycolytic pathway. However, the biopsied muscle revealed no accumulation of glycogen. Venous lactate was produced promptly under ischemic exercise. The activities of muscle glycolytic enzymes studied were shown to be normal, so then well-known diseases such as myophosphorylase deficiency (McArdle) and phosphofructokinase deficiency (Tarui) and lactic dehydrogenase M-type subunit deficiency, which was recently found in a patient with myoglobinuria, could all be ruled out.

In order to be utilized as an energy source in muscle, NEFA must first react with CoA, and the acyl CoA must then be transported into the mitochondria with the aid of carnitine-CPT systems located at the inside and outside surfaces of the mitochondrial inner menbrane.

In this case, long-chain saturated fatty acids $C_{16:0}$ and $C_{18:0}$, the substrates of CPT, rose normally after fasting. The activity of CPT was shown to be normal. Muscle carnitine content was also normal.

The above results revealed that this case did not correspond to any myoglobinuric syndrome in which the underlying metabolic disturbance was demonstrated in terms of enzyme defects, and suggested that this might be a new, not yet known type of myoglobinuria. The underlying pathophysiological mechanism remains to be elucidated.

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TURNOVER OF HUMAN MYOGLOBIN AS STUDIED BY RADIOIMMUNOASSAY

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To elucidate the processes of metabolism of myoglobin (Mb) in humans, we studied half-lives ($T_{1/2}$) of serum Mb in various clinical cases with hypermyoglobinemia and also investigated the $T_{1/2}$ in healthy volunteers and patients with nephropathy, hepatopathy or myopathy after intravenous loading of Mb.

Materials and Methods

Subjects were clinical cases with acute or transient hypermyoglobinemia such as malignant hyperthermia, acute myocardial infarction, LDH M-subunit deficiency (Hamamatsu Medical College, Nishimura et al) and sports (baseball, long distance running). Serum Mb concentrations were measured serially and $T_{1/2}$ were determined in these cases. On the other hand, human Mb prepared by us was administered intravenously (i.v.) as a single dose (10 mg/60 kg) to healthy volunteers, patients with renal insufficiency, cirrhosis of the liver, Duchenne type muscular dystrophy (DMD), myotonic dystrophy and neurogenic muscular atrophy. Then serum Mb concentration and $T_{1/2}$ of these cases were determined. Urinary Mb was also assayed. Mb was measured by a radioimmunoassay method developed by us (sensitivity, 1 ng/ml).

Results

1) $T_{1/2}$ of serum Mb in clinical cases: $T_{1/2}$ of serum Mb were 5 h to 9 h in malignant hyperthermia, 4 h to 9 h 30 min in acute myocardial infarction, 4 h 10 min in LDH M-subunit deficiency, 4 h 20 min to 13 h 30 min in baseball, and 4 h 40 min to 5 h 30 min in long distance running. Among them, the shortest $T_{1/2}$ were 4 h to 4 h 10 min, and was close to the $T_{1/2}$ of human Mb in serum, probably because Mb was released from the muscle once and during a short time.

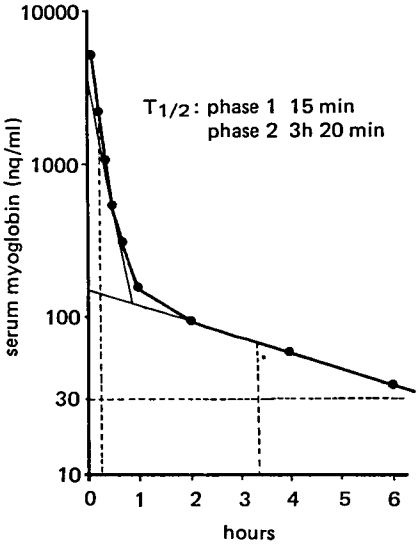

Fig. Disappearance curve of serum myoglobin in a healthy volunteer (M.I.)

2) Correlation of initial concentration of serum Mb (maximal value) with $T_{1/2}$ in clinical cases: Cases with remarkable hypermyoglobinemia such as malignant hyperthermia ($1 \times 10^4 - 1 \times 10^5$ ng/ml) and those with relatively mild myoglobinemia such as sports ($5 \times 10 - 1 \times 10^3$ ng/ml) did not differ in $T_{1/2}$ values.

3) $T_{1/2}$ as determined by i.v. loading of Mb in healthy individuals and some clinical cases: $T_{1/2}$ of serum Mb in two healthy volunteers were 3 h 20 min (Fig.) and 3 h 30 min. $T_{1/2}$ of some cases with renal insufficiency were prolonged to 5 h. $T_{1/2}$ was prolonged to 5 h in one case with cirrhosis of the liver. Two cases with DMD gave remarkably prolonged values; 6 h 10 min and 8 h 20 min. $T_{1/2}$ were 3 h 55 min and 3 h 10 min in two cases with myotonic dystrophy, and 3 h 50 min and 3 h 40 min in two cases with neurogenic muscular atrophy.

4) Urinary excretion of Mb after i.v. loading of Mb: Urinary excretion of Mb was not observed in most cases after i.v. loading of 10 mg/60 kg of Mb.

Discussion and Summary

With the recent development of a radioimmunoassay for human Mb in Japan[1] and abroad,[2] reports of serum and urinary Mb values in myocardial, neuromuscular and renal disorders have increased.

Previous reports on Mb turnover include studies in dogs by Amako et al[3] and those in humans with i.v. injection of whale Mb by Koskelo et al.[4] Results of Mb turnover in humans with human Mb were reported only by Sylvén[5] in 1978. He injected ^{125}I-Mb intravenously into healthy individuals and determined $T_{1/2}$, which was reportedly 3 h 20 min in phase 2. He supposed that Mb was metabolized in the kidney, based on the result that labelled Mb was not demonstrated in the urine. However, catabolism of Mb in organs other than the kidney remained unclear.

We investigated $T_{1/2}$ of serum Mb in various clinical cases with hypermyoglobinemia and Mb turnover after i.v. loading of Mb in various disorders. The results can be summarized as follows; 1) $T_{1/2}$ of serum Mb in some clinical cases with hypermyoglobinemia were close to that of healthy individuals as determined with a single i.v. injection of Mb, while in most cases they were prolonged. The prolongation was probably due to continuous efflux of Mb from the muscle and some other factors. 2) $T_{1/2}$ in renal insufficiency and liver disorders as determined by Mb loading was prolonged, which suggested the role of the kidney and liver in Mb metabolism. $T_{1/2}$ in DMD was supposed to be prolonged mainly because of continuous leakage of Mb from the muscle.

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GOT ISOZYMES IN MUSCLES OF PATIENTS WITH OPHTHALMOPLEGIA PLUS SYNDROME AND EXPERIMENTAL MITOCHONDRIAL MYOPATHY

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It has been reported that morphological abnormality of mitochondria is involved in muscles of patients with the ophthalmoplegia plus syndrome[1]. However, little is known about the mitochondrial enzymes in muscles of patients with the ophthalmoplegia plus syndrome. In the present study, we determined the activities of GOT isozymes located in the mitochondrial matrix (m-GOT) and cytoplasm (s-GOT) in muscles of two patients with the ophthalmoplegia plus syndrome and of rats with experimental mitochondrial myopathy.

Materials and Methods

Muscle specimens were obtained from deltoid muscles of the following two patients with the ophthalmoplegia plus syndrome.

Case 1 was a 24 year old female with ophthalmoplegia, ptosis and muscle weakness of facial, pharyngeal, neck and limb muscles. Short stature, retinal degeneration, mild EEG abnormality and auditory dysfunction were observed. Ragged red fibers and abnormal mitochondria with paracrystalline inclusions were revealed on histological examination of the muscle.

Case 2 was a 24 year old female with weakness of extraocular, facial and limb muscles. Retinal degeneration and mild EEG abnormality were observed. Histological examination of a muscle specimen did not show ragged red fibers.

Muscle specimens obtained from 7 patients with other neuromuscular diseases were used for the control (polymyositis, 4; amyotrophic lateral sclerosis, 1; FSH form muscular dystrophy, 1; LG form muscular dystrophy, 1).

Production of experimental mitochondrial myopathy

1) DNP (2'4'-dinitrophenol) method[2]: Male Wistar rats weighing about 150 gm were anesthetized by i.p. injection of pentobarbital. The right iliac artery was cannulated and 5ml of DNP solution (3 mg/ml, neutralized with NaOH) was injected intra-arterially. The right gastrocnemius muscle was removed and frozen 30 min after DNP injection.

2) Ischemia method[3]: Right hind legs of male Wistar rats were made ischemic by touniquets and 24 hours later right gastrocnemius muscles were removed and frozen.

Determination of GOT isozymes[4]

Muscles were homogenized in 20 vol. of phosphate buffered saline (pH 7.4) after 4 cycles of freezing at $-20^{\circ}C$ and thawing at room temperature. The homogenates were centrifuged at 10,000 x g for 15 min at $4^{\circ}C$. The supernatants were used for the assay of total GOT activity. The activity of m-GOT was determined after s-GOT was removed by absorption of s-GOT with sheep red blood cells coated with anti-s-GOT antibody. The solution for the GOT assay contained 10 mM Asp, 3.3 mM 2-oxoglutaric acid, 0.1 mM NADH, 0.1 M Tris buffer, pH 7.4, and 3 μg/ml of MDH. The final volume of the reaction mixture was 1 ml and the reaction was carried out at $37^{\circ}C$. Activity was calculated by following the reduction at OD 340 nm.

Results

1) Fig. 1 shows the activities of s-GOT and m-GOT (A) and ratio of m-GOT in the total GOT activity (%m-GOT) (B) in the muscles of patients with the ophthalmoplegia plus syndrome and other neuromuscular diseases. There is no difference in the activities of s-GOT and m-GOT. However, %m-GOT was decreased in the muscles of the patients with the ophthalmoplegia plus syndrome.

2) Fig. 2 shows the activities of GOT isozymes and %m-GOT in the muscles of the control rats and rats with experimental mitochondrial myopathies. In both experimental mitochondrial myopathies, the decrease of m-GOT was more marked than that of s-GOT. The decrease in %m-GOT was slight but significant in mitochondrial myopathies.

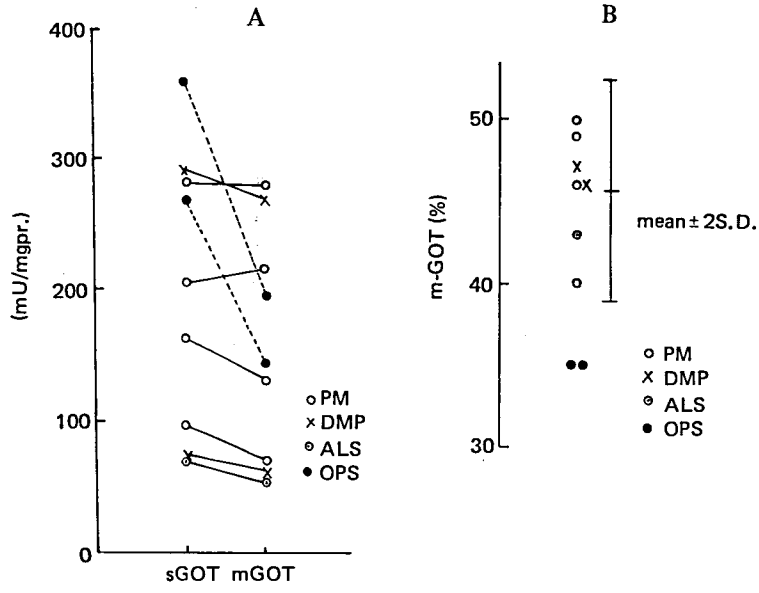

Fig. 1 The activities of GOT isozymes in muscles of patients with ophthalmoplegia plus syndrome (OPS) and other neuromuscular diseases (A) and the percentage of m-GOT in the total GOT activity (%m-GOT). (PM, polymyositis; ALS, amyotrophic lateral sclerosis; DMP, progressive muscular dystrophy)

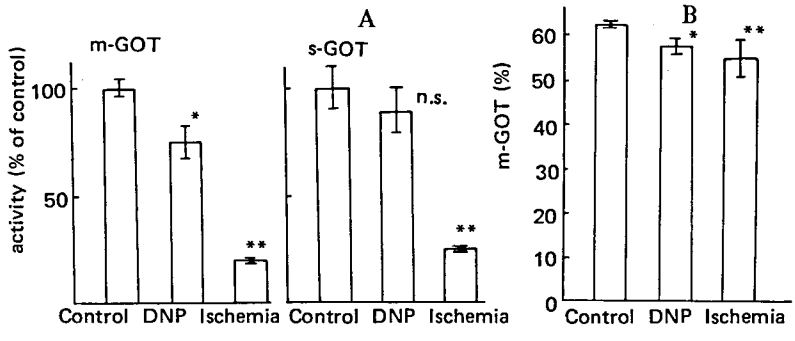

Fig. 2 The activities of GOT isozymes (A) and %m-GOT (B) in the muscles of rats with experimental mitochondrial myopathies. Each histogram shows the mean ± SD for 5 samples. (* $P < 0.05$, ** $P < 0.01$)

Discussion

Berenberg et al[1] studied the biochemical properties of mitochondria obtained from three patients with the opthalmoplegia plus syndrome. They reported that in the mitochondria of one of the three patients, respiration and DNP stimulated ATPase activity were decreased. However, in the other two patients, biochemical abnormality was not revealed. And yet now common biochemical abnormalities of muscles of patients with the ophthalmoplegia plus syndrome are not known.

GOT isozymes were located in the mitochondrial matrix and cytoplasm, and some changes in mitochondria may induce changes in the activities of GOT isozymes. As revealed in this work, %m-GOT was decreased in the muscles of two patients with the ophthalmoplegia plus syndrome. Because the same phenomenon was observed in the muscles of experimental mitochondrial myopathy, it is suggested that the decreased %m-GOT reflects the damage to mitochondria.

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ANALYSIS OF THE CONTRACTILE PROTEIN OF A SINGLE HUMAN MUSCLE FIBER

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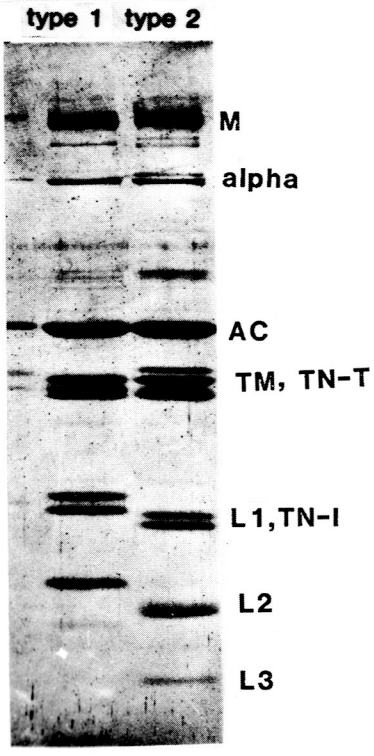
Single muscle fibers were prepared from biopsy specimens of Duchenne muscular dystrophy (DMD) and other diseases. The contractile protein of single fibers was analyzed and compared to the physiological property. Our main interest was to study the intermediate fiber (type x), which is abundant in DMD muscle[1]. The muscle fiber of experimental myopathy was also studied.

Results and discussion

(1) The protein composition of single muscle fibers was studied by one dimensional (1D) or two dimensional (2D) gel electrophoresis (Fig. 1). In 1D electrophoresis, the protein composition was different between type 1 and type 2 fibers, especially in the myosin light chain (MLC) pattern. The troponin complex and tropomyosin might also differ between them, but exact differences could not be confirmed with a 1D gel only. 2D electrophoresis demonstrated that every muscle fiber contained two tropomyosin subunits, alpha and beta chains. The type 1 fiber had two MLC. The molecular weight of MLC 1s was 27,000 and that of MLC 2s was 19,000. The type 2 fiber had MLC 1f, MLC 2f and MLC 3f, and the molecular weight of each component was 26,000, 18,500 and 16,000, respectively.



Legend to figure 1
The contractile protein of single type 1 and type 2 muscle fibers was analyzed by SDS-gel electrophoresis. M, myosin heavy chain; alpha, alpha actinin; AC, actin; TM, TN-T, tropomyosin alpha and beta subunits and troponin T; L1, TN-I, myosin light chain (MLC) 1 and troponin I; L2, MLC 2; L3, MLC 3.

(2) In DMD, type 1 fibers exhibited the same MLC pattern as control type 1, and the MLC pattern of type 2 was same as that of control type 2. The MLC of type X fibers was equal either to control type 1 or control type 2. Thus, differentiation seemed complete as far as MLC was concerned.

(3) Experimental muscular atrophy was produced by immobilizing the ankle joint of rats. Single muscle cells from the atrophic soleus exhibited two types of MLC. These muscle fibers are supposed to be half way in the transformation from type 1 to type 2.

(4) Hypothyroidism was induced in rats by surgical thyroidectomy. No change in the MLC composition was found in the soleus or EDL muscle, although the time course of twitch was prolonged by thyroidectomy.

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STUDIES ON THE STRUCTURAL PROTEINS IN EXPERIMENTAL SEROTONIN MYOPATHY

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Introduction

Many kinds of myopathy have been reported but the modes of destruction of the structural proteins in the various myopathies are scarcely known.

We[1] previously confirmed pathologically the production of experimental serotonin myopathy in rats. Therefore, we studied the mode of muscle destruction in experimental serotonin myopathy using dodecyl sulfate polyacrylamide gel electrophoresis.

Materials and Methods

Production of serotonin myopathy

Experimental serotonin myopathy was produced in male Wistar rats weighing about 100g. Serotonin in isotonic saline was injected intraperitoneally once a week and injections were repeated 24 times.

Preparation of protein sample

Muscle from the hind limb was homogenized in 9 volumes of 0.1 M NaCl containing 5 mM $NaHCO_3$ with a Waring Blender. The homogenate was then centrifuged at 10,000 x g for 20 minutes and the residue was resuspended in the same solution as above. This procedure was repeated 3 or 4 times. The residue thus obtained was used as the protein sample to be subjected to electrophoresis.

Gel electrophoresis

The method for the electrophoresis using dodecyl sulfate polyacrylamide gel was essentially the same as that of Fairbanks et al (1971)[2]. The protein sample was dissolved in a solution containing 10 mM Tris-HCl buffer, pH 8.0, 1% SDS, 1% β-mercaptoethanol, 1 mM EDTA, 8M urea and 10% sucrose, and heated for 20 minutes at 80°C. The concentration of the protein sample was 1–2 mg/ml. Approximately 30 μg of protein was applied to the gel. The concentration of the acrylamide was 5.6%. Electrophoresis was performed at a constant current of 4mA per gel. The running time under these conditions was about 4 hr.

Table 1 Comparison of the Percentages of Bands

		Results (Densitometer)	
Band	MW x 10^3	Control (%)	Serotonin myopathy (%)
1	185	22.96	26.54
2	155	4.44	2.56
3	120	14.07	11.13
4	95	2.96	0.85
5	68	1.48	4.96
6	50	2.96	
7	38	10.37	8.93
8	25	13.33	18.83
9	15	7.4	6.33
10			
11		20.03	20.87
12			

Results

Pattern of normal skeletal muscle

Electrophoresis of normal skeletal muscle gave a pattern in which 13 well resolved bands – arbitarily labeled 1-13 – are predominant. The percentage of each band was calculated with a dinsitometer (Table 1). Molecular weight estimates for bands 1-13 were obtained with markers (Table 1).

Pattern of serotonin myopathy

Obvious changes of pattern compared to normal muscle were observed in bands 1-9 of which the molecular weights ranged from 185,000 to 15,000. The changes were as follows (Table 1).

(1) The width as well as tearing of band 1, and increases in bands 1 and 8 were observed.

(2) Decreases in bands 2, 3, 4, 7, 9, respectively, were also seen. The most remarkable decrease was shown in band 4 of molecular weight 95,000.

(3) Fusion of band 5 with 6 was observed but the percentage of fused bands was substantially equal to normal.

Discussion

The electrophoretic pattern of normal skeletal muscle obtained under our conditions probably revealed structural proteins and many other components of muscle because 13 bands were clearly distinguished and their molecular weights ranged from 185,000 to less than 15,000. In conclusion, two points became clear from this rough analysis. One of the most remarkable changes in structural proteins of serotonin myopathy is the decrease in α-actinin. Another important finding is the width as well as tearing and increase of the heavy chain of the myosin band.

It is reported[3] that the most characteristic changes in the structural proteins in patients with Duchenne muscular dystrophy at a relatively early stage of the disease are the decreases in α-actinin and troponin components while the degradation of the heavy chain of myosin is observed at a later stage.

The decrease in α-actinin and degradation of the heavy chain of myosin in serotonin myopathy suggest analysis of the mode of muscle destruction in various myopathies.

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CALCIUM-DEPENDENT DEGRADATION IN MUSCLE PROTEIN —EFFECT OF Ca IONOPHORE (A23187) AND A CANP INHIBITOR (E-64-C) —

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Many data have accumulated suggesting that calcium ions (Ca^{2+}) are involved in the degradation of muscle structural proteins.

In Duchenne dystrophy, there is the idea that the increased Ca^{2+} due to an excessive influx of extracellular Ca^{2+} through a certain defective plasma membrane may stimulate calcium activated neutral protease (CANP) and initiate the degradation of the structural proteins[1].

To test this hypothesis, we experimentally increased the intracellular Ca^{2+} concentration of excised intact rat muscles by using Ca ionophore, A23187, *in vitro* and observed the degeneration of muscle fibers morphologically and biochemically. The effect of E-64-c, a potent inhibitor of CANP, was also investigated.

Two experimental media were prepared, one was Krebs-Ringer solution (1 mM Ca^{2+}) with cycloheximide and A23187 (solution A), and the other had 1 mM EGTA instead of Ca^{2+} (solution B). The excised rat soleus and extensor digitorum longus (EDL) muscles were kept in the above solutions, one in solution A and the contralateral one in solution B, and incubated for 3 hours at 37°C.

During incubation, the amount of protein liberated from the muscle was determined. After incubation, a part of the muscle was used for an electron-microscopic study and the rest for an electrophoretic study.

The proteins released from the muscle into the media were more abundant when incubated in solution A than in solution B, 23% more in EDL and 63% in soleus. As the released protein measured by creatine phosphokinase was almost equal, the relative increase in liberated protein in solution A compared with solution B was supposed to mainly originate from the solubilization of the degenerated structural proteins.

Slab gel electrophoresis of myofibrillar proteins of both EDL and soleus muscles which were incubated in the solution containing Ca^{2+} showed a greatly reduced band of α-actinin, more prominent in DEL than soleus.

In contrast, the incubation of both EDL and soleus in the presence of EGTA resulted in no detectable change in α-actinin.

Electron microscopy showed almost selective degradation and disappearance of the Z-band with relative preservation of other structures. The mitochondria were swollen and disintegrated.

On the other hand, the muscles incubated in the presence of EGTA showed well preserved myofibrils and mitochondria.

To show that the disappearance of α-actinin from the Z-band was not a direct cause of excessive intracellular Ca^{2+}, we tested the effect of E-64-c on the degradation of the Z-band.

Addition of E-64-c to solution A resulted in a remarkable suppression of the release of α-actinin from the muscle, especially in the case of EDL. A similar tendency was reported by Kameyama and Etlinger[2] using another inhibitor, leupeptin, in rat soleus.

It became clear from our *in vitro* incubation experiment that incubation of the intact muscle with Ca^{2+} and Ca ionophore caused the selective degradation of the Z-band and preferential liberation of α-actinin from the Z-band. This result is essentially the same as that reported by Uchino and Chou[3].

As the loss of α-actinin from the Z-band was suppressed by an inhibitor of CANP, E-64-c, the degradation of the Z-band is supposed to be caused not by a direct effect of increased Ca^{2+}, but by a

proteolytic action of CANP, activated by an excessive influx of extracellular Ca^{2+}.

Therefore, this experiment further supports the hypothesis that CANP may play a crucial role in the initial step of the degradation of the structural proteins in Duchenne dystrophy.

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DEGRADATION OF MYOFIBRILLAR PROTEINS BY CATHEPSIN H

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It is known that lysosomal proteases play an important role in the turnover of myofibrillar proteins. But little is known about which lysosomal proteases degrade which myofibrillar proteins and in what manner except for cathepsins B, D and L[1)2)3)]. In this work, the mode of degradation of myofibrillar protein by cathepsin H was investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Only troponin-T was digested by cathepsin H, troponin-I and troponin-C were not. Figure 1 shows the degradation pattern of troponin with cathepsin H. Troponin-T showed two degraded bands, having molecular weights of 32,000 and 13,000, respectively. Cathepsin H could not degrade other myofibrillar proteins such as myosin, actin, tropomyosin or α-actinin. Degraded products from troponin-T on cathepsin H treatment have similar molecular weights to those with cathepsin B treatment. The following experiment was done to examine the differences of the cleaving point of cathepsin B and cathepsin H. The hydrolyzing activity was determined fluorometrically as the amounts of amino acids or peptides released into the supernatant from the digested protein. Cathepsin H was 100 times less sensitive to E-64 than cathepsin B. Figure 2 shows the time course of troponin digestion with the following protease systems; cathepsin H only, cathepsin B only, cathepsin H after cathepsin B digestion or cathepsin B plus cathepsin H. Cathepsin H digested troponin after pretreatment with cathepsin B neither faster nor slower than treatment with cathepsin H only. This might suggest that cathepsin H cleaved a different point of troponin to cathepsin B. And cathepsin H might not have a stronger ability for the product of troponin with cathepsin B than native

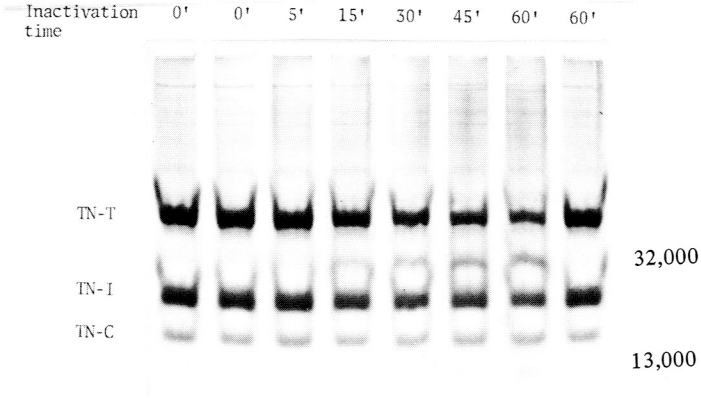

Fig. 1 Degradation patterns of troponin on cathepsin H treatment

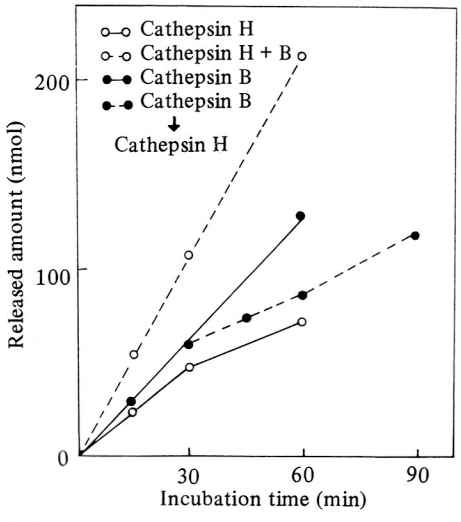

Fig. 2 Released amount of amino acids or peptides from troponin on cathepsin treatment

troponin. Cathepsin B and H act on troponin in a non-involutional but additional manner. Cathepsin H and cathepsin B may attack in an independent manner. The Table presents a comparison of susceptibilities of myofibrillar proteins to cathepsin B, cathepsin H and mast cell serine protease[2) 4)].

Troponin-T was degraded by all of these proteases. Myosin, tropomyosin and troponin-I were degraded by cathepsin B and mast cell serine protease. α-Actinin and troponin-C were not degraded. Some kind of cooperation among these proteases may occur in the degradation of these myofibrillar proteins.

Table Susceptibilities of myofibrillar proteins to proteases

	Cathepsin B	Cathepsin H	Mast Cell Serine Protease
Myosin H chain	+++	–	+++
L chain	–	–	–
Actin	–	–	+
α-Actinin	–	–	–
Troponin TN-T	++	++	+++
TN-I	+	–	+
TN-C	–	–	–
Tropomyosin	+	±	++
M-Protein			–

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IMMUNOGLOBULIN AND THE IMMUNE COMPLEX IN FUKUYAMA TYPE CONGENITAL MUSCULAR DYSTROPHY

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Whilst the genetic patterns indicated autosomal recessive transmission of Fukuyama type congenital muscular dystrophy, pathological findings such as meningeal infection, micropolygyria, and anomalies of the cerebral cortical layers suggested the etiological significance of intrauterine infection. In a few reports, immunoglobulins were shown to be elevated in this disease.

In 14 patients with the disease aged from 6 months to 16 years, we estimated serum gammaglobulins G, A and M, monoclonal bands of immunoglobulin in the CSF, as well as circulating immune complexes with the following results;

1) In all 14 cases, the amounts of Ig G, A and M were within age-specific normal limits with a few exceptions in relatively old cases. In all cases, Ig M was normal.

2) Isoelectrofocusing electrophoresis of the CSF of 2 patients examined showed negative results for the oligoclonal band of immunoglobulin.

3) The circulating immune complexes were slightly elevated in 5 of the 14 cases examined.

We were unable to disclose immunological evidence of the prenatal infection, which may be etiologically related with this disease.

ISOLATION OF NERVE GROWTH FACTOR FROM HUMAN PLACENTAL TISSUE

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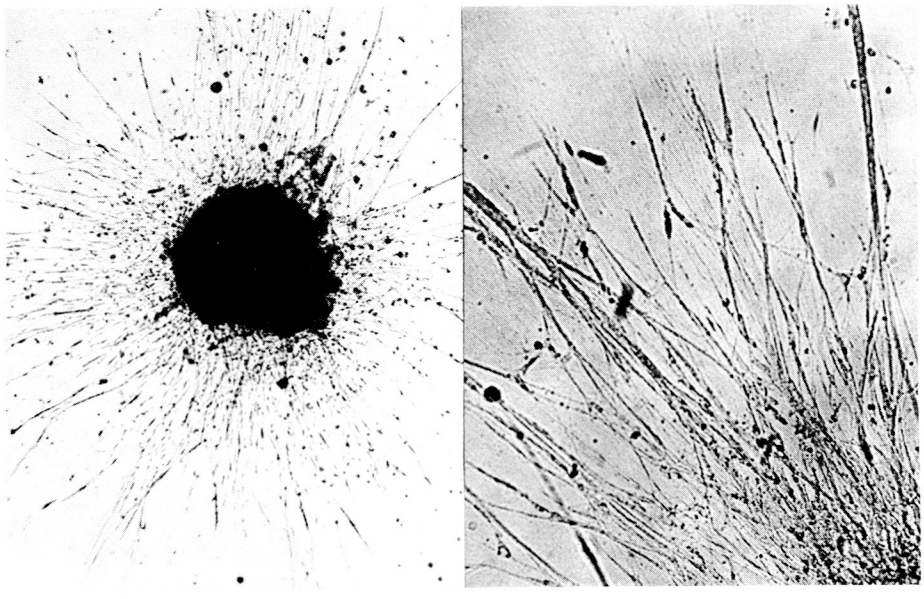
A number of recent papers[1-3] have suggested that there might be a relationship between altered levels of nerve growth factor (NGF) activity in human tissues and neurologic disorders that have a known defective genetic component. At present, the NGF levels in human tissues are usually measured and standardized by using mouse NGF and its antibodies, yielding many contradictory results. The availability of purified human NGF will allow the standardization and unequivocal quantitative measurements of NGF levels in human tissues and fluids in a number of neuropathies.

This paper deals with the isolation of human NGF from human placental tissue according to the method described by Goldstein et al.[4] with some modifications.

Human term placental tissue was homogenized in cold saline (20% w/v) at 20,000 rpm for 2 min. The homogenate was adjusted to pH 13.0 with 10 N NaOH, re-homogenized at 20,000 rpm for 30 sec, and centrifuged at 30,000 × g for 10 min. The resulting supernatant was adjusted to pH 3.8 with glacial acetic acid, allowed to stand for 5 min at 0°C, and centrifuged at 30,000 × g for 30 min.

The supernatant was then adjusted to pH 5.0 with 4 N NaOH, centrifuged at 30,000 × g for 50 min, and the supernatant was dialyzed against distilled water, centrifuged at 20,000 × g for 10 min, and applied to a column of CM-52 cellulose. The combined active CM-52 fractions were re-chromatographed on a column of CM-52 cellulose. The active fractions were pooled, concentrated by pressure dialysis

INDUCTION OF NERVE FIBER OUTGROWTH BY HUMAN PLACENTA NERVE GROWTH FACTOR FROM THE DORSAL ROOT GANGLIA OF CHICK EMBRYO



40× magnification 100× magnification

Fig. 1 The dorsal root ganglia were cultured at 37°C for 3 days in the presence of human nerve growth factor (7 ng/ml).

and gel-filtered on a Sephacryl S-300 column. The yield of purified material was about 10 mg from one human placental.

Gel filtration using Sephacryl S-300 gave a molecular weight of about 160,000. The purified NGF was tested by immunodiffusion against antiserum to purified mouse NGF, and thereby compared with mouse NGF. The interaction of the precipitin lines shows that there are antibodies that recognize determinants common to both human and mouse NGFs, but that the mouse antigen has determinants not present in human NGF (spur formation). Purified NGF gave a single band on polyacrylamide disc gel electrophoresis at pH 7.5. But, as shown on sodium dodecyl sulfate disc gel electrophoresis, purified high-molecular weight NGF showed several bands. Thus, high-molecular weight NGF was subjected to preparative disc gel electrophoresis at pH 2.3. The 280 nm absorption profile of the column fractions revealed plural bonds, one of which showed specific biological activity for NGF. The NGF was maximally active at approximate 7 ng/ml with the tissue culture method using chick embryo dorsal root ganglia as shown in Fig. 1.

Isoelectric focusing using a gradient between pH 3.5 and pH 10.0 gave a pI value corresponding to about 5.0. Sodium dodecyl sulfate disc gel electrophoresis of the NGF obtained gave a molecular weight of about 65,000. This NGF differs from other NGFs including mouse β-NGF and snake NGFs in having a high molecular weight and may correspond to the high molecular weight NGF isolated from mouse submandibular gland by Young et al.[5)]

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LEVEL OF NERVE GROWTH FACTOR-LIKE IMMUNOREACTIVITY IN HUMAN SERUM

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Previously, we reported that, as measured by the tissue culture method, the level of nerve growth factor (NGF) in the submaxillary glands of male heterozygous dystrophic mice exceeded that of male homozygous mice by an order of more than two[1], and also as measured by radioimmunoassay, the level of NGF-like immunoreactivity in the muscle of homozygous dystrophic mice was about half that of heterozygous mice[2]. These results suggested that a dysfunction of the nerves to the skeletal muscles resulted in the development of the disease.

In connection with this, we tried to examine the level of NGF-like immunoreactivity in sera of patients with dystrophy using the solid-phase radioimmunoassay method. First, we examined the assay procedures for the level of NGF-like immunoreactivity in human serum. The results revealed that there were at least two NGF-like molecules in human serum, and that most of the NGF in the human serum existed in a complex form associated with serum components of high molecular weight[3]. Unlike the NGF, this complex possessed no biological or immunological activity.

(a) Presence of Serum Components which Bind NGF. In a competitive radioimmunoassay, any decrease in radioactivity in the bound antibodies is interpreted as showing the presence of NGF. We examined the NGF level in human serum with a two-antibody radioimmunoassay system, in which the ^{125}I-β-NGF bound to its anti-rabbit antibody was precipitated by anti-goat rabbit IgG antibody. The level of NGF-like immunoreactivity in human serum measured by this method was found to be 30—500 ng/ml. But human sera showed only a weak response in the nerve fiber outgrowth test. After gel filtration of human serum on a Sepharose CL-6B column, each fraction was measured by the two-antibody radioimmunoassay system. Remarkable binding inhibition of ^{125}I-β-NGF was observed in the eluate of the macroglobulin fraction, but this fraction showed no NGF activity whatsoever. Hence, if the ^{125}I-β-NGF was bound to the serum components, its binding to the antibodies would be prevented, and the result would be an overestimation of the NGF in the serum. We therefore tested whether NGF-binding components were present in the sera. ^{125}I-β-NGF was gel filtered in the presence or absence of human sera on a column of Sepharose CL-6B (Fig. 1). A considerable shift of the peak was observed, suggesting that NGF indeed binds to two large molecules in the serum. However, only one component was found to interfere with the antigen-antibody reaction. The component eluted in the second fraction did not show competition in the radioimmunoassay. These results suggest that the serum components, which migrated as macroglobulin, bound competitively to ^{125}I-β-NGF, contributing to the apparently high levels of NGF-like immunoreactivity found in human serum. Fabricant et al.[4] have also reported the same results for a NGF radioimmunoassay. Therefore, at the beginning of the assay, it is necessary to ensure the absence of these components in the often contaminated sera so that reliable values of NGF in sera or other tissues can be obtained.

(b) Distribution of NGF-like Immunoreactivity in Human Serum under Neutral Conditions. To avoid the interaction between NGF-like immunoreactivity and macromolecules in human serum, we tried to use a solid-phase radioimmunoassay system for human sera. The advantages of this method are that mixing of serum and ^{125}I-β-NGF samples is avoided and the competitive binding components, when bound to endogenous NGF, form a complex that dissociates under alkaline conditions.

(c) Distribution of NGF-like Immunoreactivity in Human Serum under Alkaline Conditions. On gel filtration of human serum on a Sephadex G-150 column under alkaline conditions, two peaks with NGF-like immunoreactivity appeared; one was almost at the position observed at neutral pH, and the

other was a new peak eluted approximately at the column volume. With the tissue culture method using chick embryo dorsal root ganglia, the first peak gave only a weak response, but the second peak produced distinct outgrowth of nerve fibers. These results suggested that there were at least two NGF-like molecules in human serum, and that most of the NGF existed in a complex form associated with serum components of high molecular weight. Unlike the NGF, however, this complex possessed no biological or immunological activity. The other NGF-like molecules obtained with gel filtration on Sephadex G-150 had molecular weights of 30,000 to 50,000. These findings indicated that the NGF-like activities in human serum were distributed in these categories. Actually, a large number of NGF-like molecules seemed to be eluted in a complex form with no biological activity. The serum components, i.e., ^{125}I-β-NGF-binding components, that migrated as macroglobulin might be carrier proteins which participate in the regulation of the biological function of NGF in human serum.

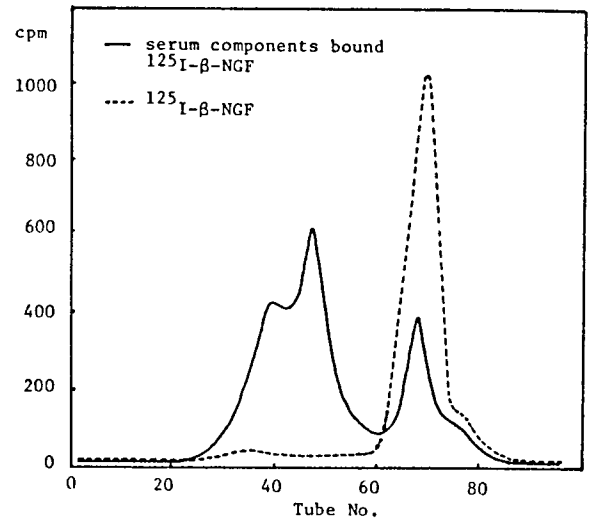

Fig. 1 Elution patterns of ^{125}I-β-NGF in the presence or absence of human serum on a column of Sepharose CL-6B. Normal human serum (320μl) was mixed with ^{125}I-β-NGF (12,000 cpm) and incubated for several hours at room temperature. The inclubation mixture was then applied to a column of Sepharose CL-6B (105 × 1.0cm) equilibrated with 0.05M phosphate buffer, pH 6.8, and eluted with the same buffer. 400μl of 0.05M phosphate buffer, pH 6.8, containing 0.5% bovine serum albumin and ^{125}I-β-NGF (10,000 cpm) was applied to the same column equilibrated with 0.05 M phosphate buffer, pH6.8. Fractions of 1.2 ml each were collected. The solid line indicates the distribution of radioactivity in the presence of serum; the dashed line, in the absence of serum.

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