The Ultrastructure of Skeletal and Smooth Muscle in Experimental Protein Malnutrition in Rats Fed a Low Protein Diet

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Summary. Light microscopy of the pectoralis muscle of rats on a low protein diet did not show such morphological alterations as atrophy, degeneration, or sarcoplasmic edema, but electron microscopy occasionally demonstrated ultrastructural changes only in the sarcomeres of myofibrils. In the affected sarcomeres, the Z-line was disrupted and often showed a jagged structure. The Z-substance with electron opacity was frequently present flowing along the long axis of myofibrils, here referred to as the streaming of Z-lines. In addition, regular striations formed by the reciprocal arrangement of thick and thin filaments disappeared from the affected sarcomeres, though these filaments were still discernible. Two or more consecutive sarcomeres in a single myofibril were occasionally involved in these changes. A further two or more neighboring sarcomeres at the same level of myofibrils were affected transversely by these structural alterations. On the other hand, the ultrastructure of the intestinal smooth muscle was not affected by protein deficiency. The study suggests that the ultrastructural damage induced by a low protein diet is attributed to the activation of endogenous protease by the excess leaking of Ca^{2+} into the cytosol as a result of lipid peroxidation of cell membrane by raised free radicals, owing to the depletion of glutathione production by protein deficiency. It also suggests that the smooth muscle cells differ in their susceptibility to protein deficiency from the skeletal muscle cells.

The skeletal muscle is known to be a large reserve of protein that constantly undergoes synthesis or degradation of protein in its normal state (YOUNG, 1974). The rate of this protein metabolism, however, is impaired and morphological changes are induced when subjected to protein malnutrition (CHAUHAN et al., 1965; HANSEN-SMITH et al., 1979a, b). In our

previous study, we described ultrastructural changes in the myocardial cells induced by protein deficiency in rats fed a low protein diet (OUMI and YAMAMOTO, 1999). Recently malnutrition, particularly protein deficiency, has become a serious problem in many areas of the world. Although biochemical and light microscopic studies on the skeletal and smooth muscles suffering protein-energy malnutrition have been reported (VINCENT and RADEMECKER, 1959; CHAUHAN et al., 1965; HANSEN-SMITH et al., 1979a, b), information on the ultrastructure of skeletal and smooth muscle cells during protein malnutrition has been very scanty, except for a recent report on the voluntary muscle in childhood malnutrition by BROOKS (1995).

The aim of the present study was to demonstrate ultrastructural changes in both skeletal and smooth muscles as induced by a low protein diet, and so obtain insight into the factors responsible for muscle damage in protein malnutrition.

MATERIALS AND METHODS

Twenty wistar strain male rats immediately after weaning were used in the present study. Ten were fed a low protein diet for 2 weeks, and the others a control diet. Tap water was also offered *ad libitum* to rats of both groups. The control diet was prepared according to the composition of AIN-93G, containing casein 20 g/100 g (20% protein), soybean oil 7.0 g/100 g, cornstarch 39.7 g/100 g, dextrin 13.2 g/100 g, sucrose 10.0 g/100 g, cellulose 5.0 g/100 g, mineral mix 3.5/100 g, vitamin mix 1.0 g/100 g, L-cystine 0.3 g/100 g, and choline bitartrate 0.25 g/100 g (REEVES et al.,

Fig. 1. Regularly arranged bandings of myofibrils in the pectoralis muscle of a rat fed a control diet. $\times 35,000$

1993). The low protein diet was identical in composition to the control diet except for additionally containing casein 5.0 g/100 g (5% protein) and corn-starch 54.7 g/100 g. Rats were weighed and their dietary consumption was recorded daily. For pair feeding, ten rats were offered control diet amounts restricted to the intake of the low protein diet group.

The rats were anaesthetized with ether, and parts of the pectoralis major muscle and the jejunum were removed from individual rats. Immediately after removal the materials were cut into small pieces and then fixed in half-Karnovsky's fixative (1.6% para-formaldehyde and 1.7% glutaraldehyde buffered with 0.1 M phosphate, pH 7.4) for 2 h followed by postfixation in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4 for 1 h. After dehydration in a graded ethanol series, the materials were embedded in epoxy resin. Thick sections were made with glass knives on a Porter-Blum microtome, stained with toluidine blue, and then examined by light microscopy. Thin sections were cut with a diamond knife on a Reichert Ultracut microtome, stained with uranyl acetate and lead, and examined in a JEOL 1200 EX electron microscope.

RESULTS

The body weight gain of the control group was 90.9 ± 5.6 g, that of the low protein group 46.6 ± 15.8 g, and the pair-fed group was 90.1 ± 3.2 g. Thus, there was a great difference in body weight gain between the control group and the low protein group, but not such a significant difference between the control group and the pair-fed group.

Pectoralis muscle

The pectoralis muscle in the control group consisted of well-organized striated muscle cells. Myofibrils of individual cells showed a regular pattern of striation, i. e., the A-band, I-band, H-band, M-line, and Z-line were each clearly identified in respective myofibrils. The T-tubules were present at the A-I junction of the myofibrils. A flattened sarcoplasmic reticulum was located in the extremely scanty sarcoplasm between two adjacent myofibrils (Fig. 1).

In the pectoralis muscle of the rats fed a low protein diet, light microscopy did not show such

Fig. 2. Myofibrils of the pectoralis muscle from a rat fed a low protein diet for 2 weeks. One discrete sarcomere is involved in alteration. Two adjacent Z-lines of the affected sarcomere show the streaming of the Z-substance flowing along the long axis of the myofibril. Randomly arranged myofilaments are discernible. $\times 24,000$

morphological alterations as atrophy, degeneration, or sarcoplasmic edema of muscle cells. On the other hand, electron microscopy occasionally revealed some ultrastructural changes in some sarcomeres of myofibrils. In the affected sarcomeres the Z-line was disrupted and often showed a jagged appearance (Figs. 2, 3). The Z-substance with electron opacity in the affected sarcomeres was frequently present flowing along the long axis of myofibril, here referred to as the streaming of the Z-line (Figs. 2, 3). Sometimes the streaming of the Z-substance extended to the adjacent Z-line over the affected sarcomere (Figs. 2, 3). Such regular striations of the sarcomeres as observed in the control muscle disappeared, thick and thin filaments were still discernible, but their reciprocal arrangement was disturbed (Figs. 2, 3). Thick filaments seemed to locate at the central region of affected sarcomeres, but some thin filaments appeared to attach to the jagged Z-line and/or Z-substance (Figs. 2, 3). However, the structural relationship between the streaming Z-substance and myofilaments could not be observed clearly. The sarcomeres just adjacent to the affected ones in the myofibril often

took an arching shape in appearance as a whole, corresponding to the shape of the affected Z-line. The A-band consisting of thick and thin filaments was relatively well preserved, but the I-band often disappeared (Figs. 2, 3). Such myofibrillar fracturing of the I-band region as observed in the myocardial cells of the rats fed a low protein diet was not seen in the pectoralis muscle. These structural changes were observed not only in a single sarcomere (Fig. 2), but also in two or three consecutive sarcomeres in a single myofibril. In some muscle cells, two or more neighboring sarcomeres at the same level of myofibrils were involved in these changes (Fig. 3). The sarcoplasmic reticulum surrounding the affected sarcomeres was almost normal in appearance, though with a tendency to be a little dilated (Fig. 3). The T-tubules disappeared from the affected areas of myofibrils (Figs. 2, 3). Mitochondria did not show any structural alterations,

In the pair-fed rats, no structural changes in the pectoralis muscle cells were observed by either light or electron microscopy.

Fig. 3. Another typical change in myofibrils of the pectoralis muscle induced by a low protein diet. Three adjacent sarcomeres from different myofibrils are involved in the irregular streaming of Z-lines at the same level of myofibrils. Disarranged myofilaments are discernible in the affected sarcomeres. $\times 24,000$

Intestinal smooth muscle

None of the detectable alterations of structure was recognizable in the intestinal smooth muscle cells of the rats fed a low protein diet for two weeks by either light or electron microscopy (Fig. 4).

DISCUSSION

The present study demonstrated for the first time that ultrastructural changes are induced in the skeletal muscle of rats on a low protein diet (containing 5% protein) in contrast to a control diet (20% protein). Since there was no difference in weight gain between the control group and the pair-fed group, but considerable difference between the control group and the low protein group, it seems apparent that protein deficiency is responsible for inducing these ultrastructural changes in skeletal muscle as observed in the cardiac muscle (OUMI and YAMAMOTO, 1999).

Morphological changes in skeletal muscles during

protein malnutrition have been reported mainly in the children with kwashiorkor by light microscopy (VINCENT and RADEMECKER, 1959; MONTGOMERY, 1962; HANSEN-SMITH et al., 1979a, b). Only one report of an animal experiment has been available on rhesus monkeys receiving a negligible amount of protein in their diet (CHAUHAN et al., 1965). All of these light microscopic studies described such structural features common to skeletal muscles affected with protein malnutrition as atrophy, degeneration, and sarco-plasmic edema of muscle fibers. The degree and severity of structural damage varied by individual case, probably depending on the stage of protein malnutrition. In the pectoralis muscles examined in the present study, however, we could not confirm such morphological changes as described above by light microscopy. It seems that the pectoralis muscle examined in our study was probably at an earlier stage of protein deficiency.

There have been very few papers dealing with the ultrastructure of the skeletal muscle affected with protein malnutrition. BROOKS (1995) recently reported on the ultrastructure of the skeletal muscle (M.

Fig. 4. Smooth muscle cells from the jejunum of rat fed a low protein diet. No ultrastructural changes are recognizable. $\times 12,000$

vastus lateralis and M. rectus abdominis) in children with protein malnutrition. He described variable depletions and the focal absence of myofibrils, with a disorganized Z-line in the most severe case. In addition, he also observed in some other specimens such effects as sarcomere disorganization, mitochondrial swelling, glycogen depletion, sarcoplasmic edema, and focal contractions of sarcomeres. He noted that he was dealing with a highly selected group in which muscle damage was likely to be more extreme than in cases which would eventually recover. In the present study, however, the pectoralis muscle was probably at earlier stages of protein deficiency and did not show such ultrastructural changes as observed by BROOKS (1995), except for the disorganized Z-line and the sarcomere disorganization. He described neither the streaming of the Z-line nor the myofibrillar disorganization in detail. He particularly noticed the variable depletion and focal absence of myofibrils and sarcoplasmic edema in the severely malnourished infants who died after agonal stress. As he mentioned, only one point in an ongoing process was recorded in his paper. Unfortunately, he did not discuss the pathogenesis of the ultrastructural changes observed in malnourished children.

Although it is not known what factors are involved in the disorganization of sarcomeres and the Z-line streaming of myofibrils induced by a low protein diet, it is conceivable that these changes are closely associated with the metabolic disorder of proteins in the muscle cells. The myofibrils of skeletal muscle cells contain two types of giant protein filaments in addition to contractile myofilaments, i. e., connectin (titin) (MARUYAMA et al., 1977; WANG et al., 1979; HOROWITS and PODOLSKY, 1987; TRINICK, 1994) and nebulin (HOROWITS et al., 1986; WANG and WRIGHT, 1988; PIEROBON-BORMIOLI et al., 1989). Connectin filament is known to project from the M-line, in close association with thick filaments in the A-band and extending into the I-band, to attach to the Z-line (HOROWITS and PODOLSKY, 1987; WANG and WRIGHT, 1988). In contrast, nebulin filament, which is absent in myocardial cells (WANG and WRIGHT, 1988), constitutes a set of intimate association with actin filaments in skeletal muscle (WANG and WRIGHT, 1988; KRUGER et al., 1991; TRINICK, 1994). Both connectin and nebulin are thought to act as protein ruler to regulate the assembly of myosin and actin filaments precisely (TRINICK, 1994). Desmin further serves to link two Z-lines of neighboring myofibrils (CAMPBELL

et al., 1979; O'SHEA et al., 1979; TOKUYASU et al., 1983). Judging from the properties of these cytoskeletal proteins, it is thought that the disintegration of these cytoskeletal filaments is responsible for the sarcomere disorganization. Since it has been shown that activated calpain removes α -actinin connecting between actin filaments and the Z-line (REDDY et al., 1975; GOLL et al., 1991), and further degrades connectin, nebulin, and desmin (LAZARIDES, 1980; OUALI, 1990; THOMPSON et al., 1993; TAYLOR et al., 1995), the activation of calpain—which is an endogenous protease in the muscle cells—appears to be one of the key factors for inducing the ultrastructural alterations with a low protein diet. As calpain is activated by Ca^{2+} (REDDY et al., 1975; HATHAWAY et al., 1982; GOLL et al., 1991; THOMPSON et al., 1993; TAYLOR et al., 1995), elevations of Ca^{2+} concentration in the cytosol must be required for the activation of calpain. Furthermore, Ca^{2+} is known to induce the weakening of the Z-line by a mechanism different from the removal of Z-lines by calpain (HATTORI and TAKAHASHI, 1982). Thus, elevations of Ca^{2+} concentration in the cytosol appear to be responsible for induction of the Z-line streaming and the disorganization of sarcomeres.

In dietary protein deficiency, free radicals are an important factor for causing injuries because of the diminished production of glutathione that is a potent antioxidant (REED and FARISS, 1984; JACKSON, 1986; GOLDEN and RAMDATH, 1987). The raised formation of free radicals may result in an increase of membrane lipid peroxidation, followed by an increase of membrane permeability to ions. Thus excess Ca^{2+} is leaked into the cytosol and then activates calpain. This interpretation might be supported by the fact that the Z-line streaming and disorganization of sarcomeres of myofibrils are induced in the human skeletal muscle by long-term eccentric exercise (FRIDÉN et al., 1981, 1983; ARMSTRONG et al., 1983; FRIDÉN, 1984; BELCASTRO et al., 1988; BELCASTRO, 1993), in which a raised formation of oxygen free radicals followed by lipid peroxidation of the cell membrane is responsible for muscle damage (SJÖDIN et al., 1990).

Since Ca^{2+} appears to activate both nonlysosomal and lysosomal protease in muscles (ZEMAN et al., 1985), the possibility cannot be excluded that lysosomal protease activated by raised Ca^{2+} is associated with myofibrillar damage induced by a low protein diet.

It is known that the smooth muscle also contains the Ca^{2+} -activated protease, calpain (HATHAWAY et al., 1982). However, no ultrastructural changes detectable under the electron microscope were induced in the intestinal smooth muscle cells by a low protein

diet. The reason for this is not clear at present, but the possibility is suggested that susceptibility to free oxygen radicals and/or the proteolytic action of calpain in the smooth muscle cells differs from those of skeletal muscle cells. Further studies are required in this connection.

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