RAPID COMMUNICATION

Abnormal Increases of Lysosomal Cysteinine Proteinases in Rimmed Vacuoles in the Skeletal Muscle

K. II, MD, K. HIZAWA, MD, I. NONAKA, H. SUGITA, MD, E. KOMINAMI, MD, and N. KATUNUMA, MD

From the First Department of Pathology and Institute for Enzyme Research, School of Medicine, The University of Tokushima, Tokushima; and the Division of Ultrastructural Research and Division of Neuromuscular Research, National Center for Nervous, Mental and Muscular Disorders, Tokyo, Japan

Skeletal muscle obtained from a patient with distal myopathy with rimmed vacuole formation (DMRV) was examined by a new direct labeled antibody enzyme method of immunohistochemistry. Abnormal increases of cathepsins B and H, capable of degrading the myofibrillar proteins, were demonstrated to be localized at the site of vacuoles and other intramyofibral portions and not to be associated with concomitant increases of their endogenous inhibitors. Autodigestion by these intramyofibral lysosomal proteinases may be of major importance in focal destruction of myofibers and formation of vacuoles, because no myofiber necrosis with invasion of macrophages, overt myositis, and neuropathy was seen in the muscle. These findings provide the first reasonable explanation of myofibral breakdown and atrophy in DMRV, and should be helpful in further studies on the mechanism of myofibral breakdown in various vacuolar myopathies and other myopathies of so far unknown pathogenesis. (Am J Pathol 1986, 122:193–198)

IN MANY myopathies such as progressive muscular dystrophy and polymyositis, the main pathologic event is myofiber necrosis. Macrophages then invade the necrotic myofibers as scavengers, and the proteinases that they produce digest the denatured myofibral components.[1] This event is usually followed by a repair process.

In contrast, autophagy without a repair process has been recognized in Type II glycogenosis.[2,3] In this autophagic process, lysosomes are thought to be derived from the Golgi apparatus in the myofibers themselves. The intracytoplasmic vacuoles rimmed by basophilic granular materials, called "rimmed vacuoles,"[4,5] which have been observed in some myopathies such as a distal myopathy with rimmed vacuole formation (DMRV), may be lesions caused by autophagy. Rimmed vacuole formation seems to be a key to myofiber atrophy and loss in DMRV because no apparent myofiber necrosis with myophagocytosis, no infiltration of macrophages and inflammatory cells, and no other major pathologic abnormalities are seen in DMRV. Histochemically, rimmed vacuoles are known to have high acid phosphatase and nonspecific esterase activities, which suggests their relation to lysosomes. Some lysosomal proteinases have been suggested to participate in vacuole formation,[6] and attempts have been made by Katunuma et al to prove this.

In the present study, we applied a new enzyme-immunohistochemical method to the skeletal muscle from a patient with DMRV to determine whether cathepsins B and H and their endogenous inhibitors are involved in myofiber degeneration and breakdown in rimmed vacuoles.

Supported in part by grants from the Ministry of Education, Science and Culture of Japan, from the National Center of Nervous, Mental and Muscular Disorders (NCNMMD) of the Ministry of Health and Welfare of Japan, and from the Tokyo Biochemical Research Foundation.

Accepted for publication December 9, 1985.

Address reprint requests to Kunio Ii, MD, The First Department of Pathology, School of Medicine, The University of Tokushima, Kuramoto Cho 3, 18-15, Tokushima Shi, Tokushima 770, Japan.

Materials and Methods

Materials

Biopsy specimens of the left biceps brachii, a mildly affected muscle from a patient (27-year-old female)[7,8] with DMRV who had severe bilateral muscular atrophy of the distal parts of the extremities (Figure 1a), and normal human skeletal muscle (control) were used. Normal human skin and liver obtained at atuopsy were also used as positive controls of reactions for cystatins α and β, respectively.

Methods

Biochemically, cathepsins B and H and cystatin-β were purified from normal human liver, and cystatin-α was purified from normal human skin obtained at autopsy by reported methods,[9-13] with minor modifications for human materials (to be published). Fab′ fragments of anti-cathepsin B, cathepsin H, cystatin-α, and cystatin-β antibodies (rabbit IgGs), respectively, were obtained by digestion of each antibody with pepsin, and Fab′-horseradish peroxidase (HRP) conjugates (10 μg/ml) were prepared by the maleimide method of Ishikawa et al.[14]

Serial sections (7 μ thick) of frozen skeletal muscles of the patient and controls were placed side by side in pairs on eight slides. One slide was stained with hematoxylin and eosin (H&E), one with modified Gomori's trichrome, and four (one slide each for each kind of conjugate) immunohistochemically with the conjugates by the direct labeled antibody enzyme method reported previously.[1,15,16] Two slides were processed in the same way but were incubated with phosphate-buffered saline (PBS; 0.1 M phosphate buffer, pH 7.4, containing 0.85% NaCl) and with normal rabbit serum instead of the conjugates. As positive controls, a section of normal skin was stained with anti-cystatin-α Fab′-HRP conjugate and a section of normal liver with anti-cystatin-β Fab′-HRP conjugate. All sections were counterstained briefly with hematoxylin, dehydrated, cleared, and mounted.[1,15,16] Positive reactions were seen as brown discoloration of oxidized DAB (3.3′-diaminobenzidine tetrahydrochloride).

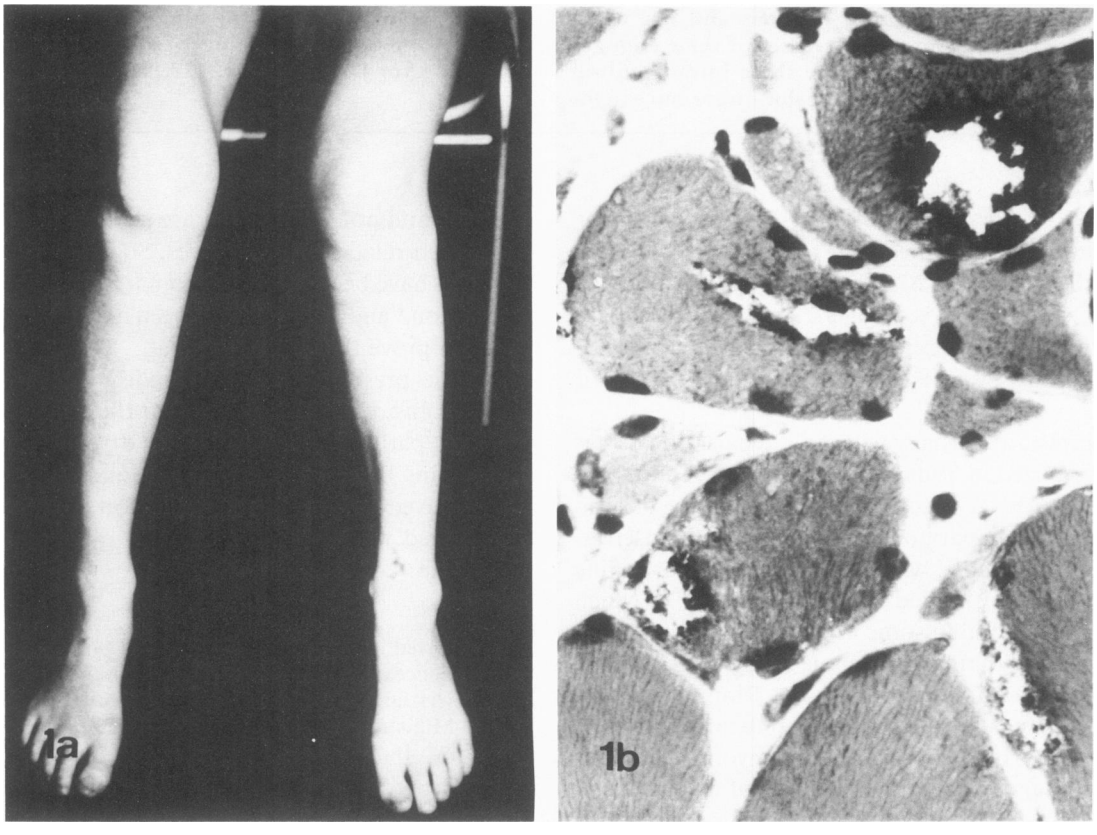
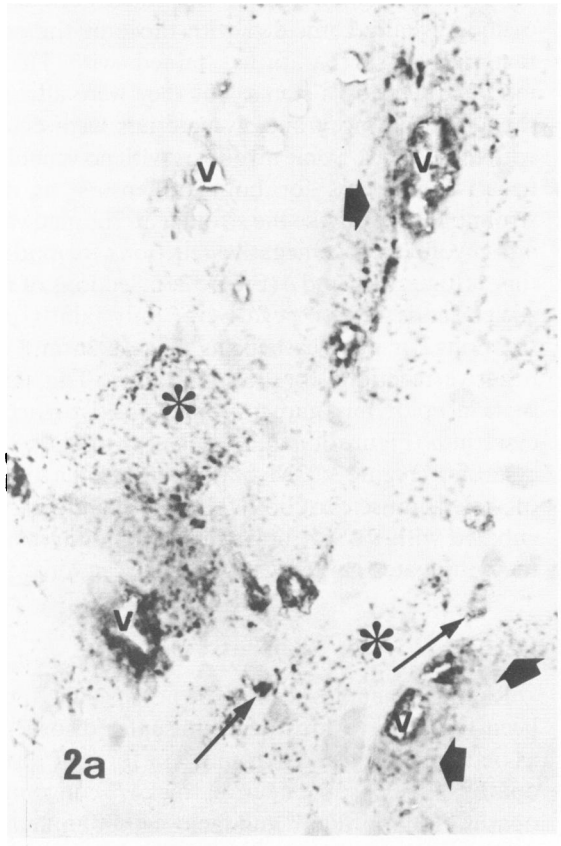
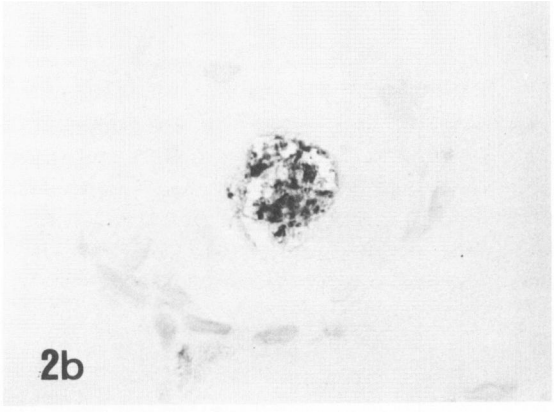
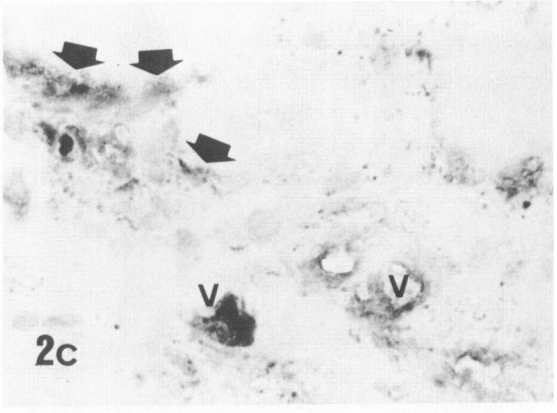
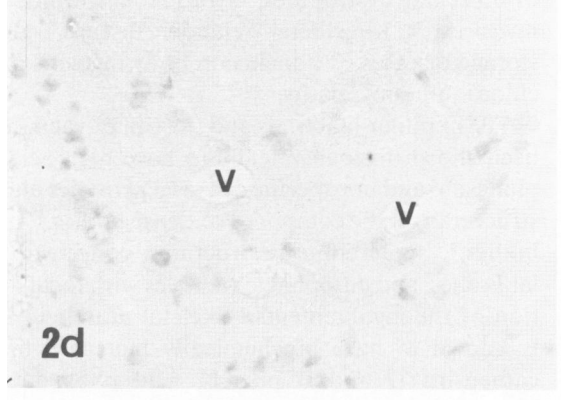


Figure 1a—Marked muscular atrophy of the distal parts of the lower extremities, especially the tibialis anterior muscles, of the patient. **b**—Typical rimmed vacuoles of various sizes formed in the central, peripheral, and subsarcolemmal portions of myofibers. No apparent myofiber necrosis with myophagocytosis or infiltration of macrophages and inflammatory cells is seen. Note granular materials in and around the vacuoles, in subsarcolemmal regions and in intermyofibrillar spaces. (H&E, × 600)









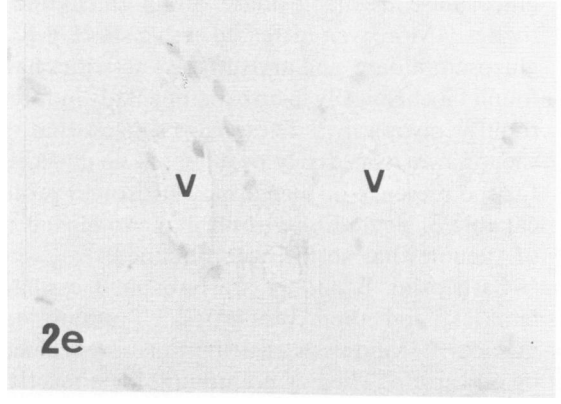


Figure 2a—Strong positive reactions for cathepsin B in almost all rimmed vacuoles (*V*) of various sizes, predominantly in the periphery and rim, subsarcolemmal regions (*thick arrows*), perinuclear portions (*thin arrowheads*), and intermyofibrillar spaces (*asterisks*) are seen mostly as fine granules or clusters of granules. **b** and **c**—Strong positive reactions for cathepsin H within and in the rim of a large vacuole (**b**) and smaller ones (**c**) and in subsarcolemmal regions (**c**, *thick arrows*). All positive reactions for cathepsins are seen within the myofibers, not in the stroma. **d** and **e**—Absence of stainings for cystatin-α (**d**) or β (**e**) in rimmed vacuoles (*V*), other intramyofibral portions, and the stroma. (Immunohistochemical stains: **a**, **b**, and **c**, × 660; **d** and **e**, × 330)

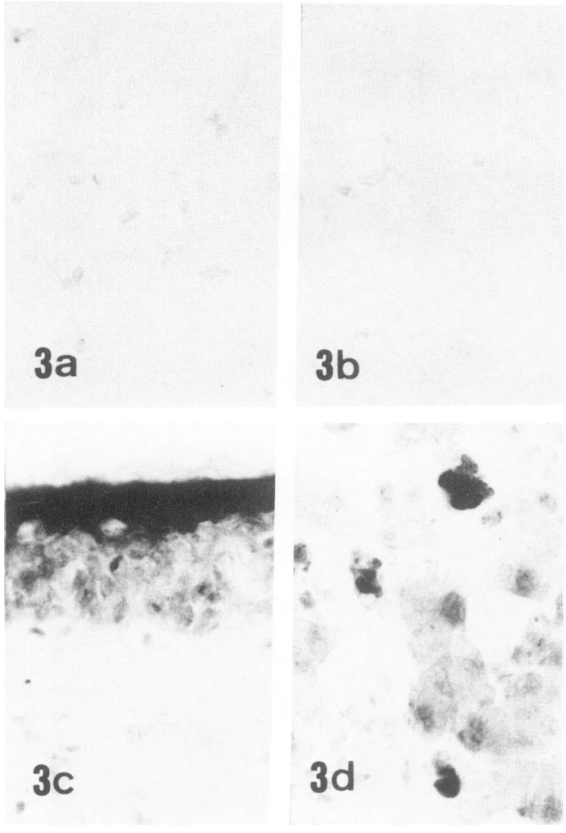


Figure 3—Cathepsins B and H and cystatins α and β in normal human tissues (controls). Negative or only faintly positive stainings for cathepsins B (**a**) and H (**b**) in skeletal muscle and strongly positive reactions for cystatin-α in the skin (**c**, superficial epidermis) and for cystatin-β in the liver (**d**, Kupffer cells). (Immunohistochemical stains: **a**, **b**, and **c**, × 330; **d**, × 660)

Results

Histological examination showed great variation in fiber size. Numerous vacuoles rimmed by granular materials staining basophilically with H&E stain and reddish purple with modified Gomori's stain were observed, especially in atrophic myofibers (Figure 1b). Some portions of myofibers contained only granular materials with no visible vacuoles in the subsarcolemmal, perinuclear, and possibly intermyofibrillar regions. No necrotic myofibers, myophagocytosis or infiltration of macrophages and inflammatory cells were observed (Figure 1b).

Immunohistochemically, almost all rimmed vacuoles gave strongly positive reactions for both cathepsins B and H, frequently predominantly in the periphery and rim (Figure 2a–c). Positive reactions for both cathepsins B and H were also seen in subsarcolemmal, perinuclear, and possibly intermyofibrillar regions of some atrophic and nonatrophic myofibers where no vacuoles were visible (Figure 2a and c), and seemed to represent early-stage lesions. Their distributions and locations usually coincided with those of the granular materials in preparations stained with H&E and modified Gomori's stains, but they were also seen in regions where no granular materials were detectable with these stains. Some myofibers with no vacuoles gave negative reactions for both cathepsins, as did the stroma. However, like the stroma, all rimmed vacuoles and myofibers gave negative reactions for both cystatins α (Figure 2d) and β (Figure 2e). Sections of normal skeletal muscle gave negative or only faintly positive reactions for both cathepsins (Figure 3a and b) and negative reactions for both cystatins. The skin (superficial epidermis) gave a strongly positive reaction for cystatin-α (Figure 3c), and the liver (Kupffer cells), for cystatin-β (Figure 3d). Myofibers and stroma in sections of skeletal muscles of both the patient and controls incubated with PBS or normal rabbit serum instead of the conjugates gave negative DAB reactions.

Discussion

Rimmed vacuoles seem to be nonspecific; they have been found in various neuromuscular disorders such as oculopharyngeal dystrophy,[4,5] a form of distal myopathy,[5,7,8,17–21] some neuropathies,[5,20] congenital myopathy,[5] limb-girdle[5,20] and facioscapulohumeral types of muscular dystrophies, myotonic dystrophy,[20] polymyositis,[5,20] Kugelberg–Welander disease,[5] glycogen storage diseases,[2,3,22] inclusion body myositis,[23,24] and chloroquine myopathy.[2,25–27]

The granular materials and the rim of vacuoles have been shown histochemically to have high acid phosphatase[5,7] and nonspecific esterase[7] activities and ultrastructurally to be composed of dense bodies,[17] myeloid bodies,[7,17] membranous structures, concentric lamellar bodies, and autophagic vacuoles with focal destruction of the myofilaments.[7] Skeletal muscle in DMRV is known to have biochemically increased levels of cathepsins D and B plus L, acid phosphatase, α-glucosidase, α-galactosidase, and β-gluculonidase activities.[21] Moreover, increased cathepsin H, β-N-acetylglucosaminidase, and arylsulfatase activities have been found biochemically in an experimentally induced chloroquine myopathy.[27] These facts suggest that rimmed vacuoles are related to lysosomes, but no direct evidence for the presence or increase of lysosomal proteinases capable of degrading myofibrillar proteins at the site of vacuoles has so far been obtained.

Cathepsins B and H are lysosomal cysteine proteinases; and their properties,[10,28] amino acid sequences,[28,29] and tissue distributions[12] were determined by our group. There is no immunologic reactivity be-

tween the two enzymes.[28] Furthermore, it has been proven by our group that cathepsin B degrades the myofibrillar proteins, myosin, troponin T and I, tropomyosin with susceptibilities in this order, whereas cathepsin H degrades only troponin T.[28,30,31]

Two types of endogenous cysteine proteinase inhibitor (cystatin-α, epidermal type, and cystatin-β, liver type) were also purified; and their properties,[11,28] amino acid sequences,[32,33] tissue distributions, and absence of immunologic cross-reactivity[13] were reported by our group. The contents of these cathepsins and inhibitors, especially cathepsin H and cystatin-α, in normal skeletal muscle were much lower than those in organs such as the kidney and liver,[12,13] which were confirmed immunohistochemically also in the present study.

In dystrophic hamsters, which are a model of human myopathy showing apparent myofiber necrosis with myophagocytosis and macrophageal infiltration, we demonstrated immunohistochemically that the marked increase of cathepsin B plus L and the concomitant increases of their endogenous inhibitors in the skeletal muscle were due to these activities in macrophages invading and surrounding the necrotic myofibers.[1] In the present case, unlike the dystrophic hamsters, there was no apparent myofiber necrosis with macrophageal infiltration, or evidence of overt myositis and neurogenic muscular atrophy, and abnormal increases of cathepsins B and H (with no concomitant increases of their endogenous inhibitors) were demonstrated at the site of rimmed vacuoles and in some other regions within the myofibers. This suggests that autodigestion, including active myofibrillar breakdown, by these intramyofibral lysosomal proteinases is a major event, causing vacuole formation, atrophy, and loss of myofibers.

Considering the wide variety of diseases in which rimmed vacuole formation has been found, the causes and mechanisms of activation of these proteinases seem to be heterogeneous. These may be as follows: 1) abnormality of some myofibral constituents including the myofilaments, causing activation of lysosomal proteolytic functions as "abnormal proteins"; 2) abnormality of the lysosomes themselves in the steps of maturation, segregation, organization, and regression; 3) abnormality of lysosomal enzymes themselves, including abnormal gene expression of cysteine proteinases in transcriptional or translational steps; and/or 4) abnormality of regulation of lysosomal proteinases, including abnormality of their inhibitors. In any case, the new findings presented here suggesting that autodigestion by intramyofibral lysosomal proteinases is important in myofibral breakdown provide a reasonable explanation for the destruction of myofilaments at the site of rimmed vacuoles. These findings provide an important clue to the mechanism of breakdown, atrophy, and loss of myofibers in various myopathies in which rimmed vacuoles are formed and in other myopathies.

References

1. Kominami E, Bando Y, Ii K, Hizawa K, Katunuma N: Increases in cathepsins B and L and thiol proteinase inhibitors in muscle of dystrophic hamsters. Their localization in invading phagocytes. J Biochem (Tokyo) 1984, 96:1941-1948
2. Engel AG: Vacuolar myopathies: Multiple etiologies and sequential structural studies, The Striated Muscle. Edited by CM Pearson, FK Mostofi. International Academy of Pathology Monograph. Baltimore, Williams & Wilkins, 1973, pp 301-341
3. Schotland DL: Ultrastructure of muscle in glycogen storage diseases,[2] pp 410-426
4. Dubowitz V, Brooke MH: Ocular and oculopharyngeal dystrophies, Muscle Biopsy: A Modern Approach. London, W. B. Saunders, 1973, pp 231-241
5. Fukuhara N, Kumamoto T, Tsubaki T: Rimmed vacuoles. Acta Neuropathol (Berl) 1980, 51:229-235
6. Katunuma N, Kominami E: Cytosolic protein degradation by autolysosomes. Neurol Med 1984, 20:599-605 (in Japanese, with English title)
7. Nonaka I, Sunohara N, Ishiura S, Satoyoshi E: Familial distal myopathy with rimmed vacuole and lamellar (myeloid) body formation. J Neurol Sci 1981, 51:141-155
8. Nonaka I, Sunohara N, Satoyoshi E, Terasawa K, Yonemoto K: Autosomal recessive distal muscular dystrophy: A comparative study with distal myopathy with rimmed vacuole formation. Ann Neurol 1985, 17:51-59
9. Kirschke H, Langner J, Wiederanders B, Ansorge S, Bohley P, Hanson H: Cathepsin H: An endoaminopeptidase from rat liver lysosomes. Acta Biol Med Germ 1977, 36:185-199
10. Towatari T, Kawabata Y, Katunuma N: Crystalization and properties of cathepsin B from rat liver. Eur J Biochem 1979, 102:279-289
11. Kominami E, Wakamatsu N, Katunuma N: Purification and characterization of thiol proteinase inhibitor from rat liver. J Biol Chem 1982, 257:14648-14652
12. Kominami E, Tsukahara T, Bando Y, Katunuma N: Distribution of cathepsins B and H in rat tissues and peripheral blood cells. J Biochem (Tokyo) 1985, 98:87-93
13. Kominami E, Bando Y, Wakamatsu N, Katunuma N: Different tissue distributions of two types of thiol proteinase inhibitors from rat liver and epidermis. J Biochem (Toyko) 1984, 96:1437-1442
14. Ishikawa E, Imagawa M, Hashida S, Yoshitake S, Hamaguchi Y, Ueno T: Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. J Immunoassay 1983, 4:209-327
15. Ii K, Hizawa K, Kominami E, Bando Y, Katunuma N: Different immunolocalizations of cathepsins B, H and L in the liver. J Histochem Cytochem 1985, 33:1173-1175
16. Ii K: A labeled antibody enzyme method (maleimide method by Ishikawa) of immunohistochemistry: The technical procedure for immunohistochemical staining. Jpn J Clin Chem 1985, 14:74-80 (in Japanese, with English title)
17. Markesberry WR, Griggs RC, Herr B: Distal myopathy: Electron microscopic and histochemical studies. Neurology 1977, 27:727-735

18. Cullen MF, Mastaglia FL: Pathological reactions of skeletal muscle. Vacuolar degeneration, Skeletal Muscle Pathology. Edited by F. L. Mastaglia, J Walton. Edinburgh, Churchill Livingstone, 1982, pp 105–106
19. Fukuhara N: Vacuolar degeneration in the skeletal muscle. Neurol Med 1984, 20:613–621 (in Japanese, with English title)
20. Tanabe H, Uchigata M, Kojima S, Matsui T, Isozaki E, Shiosawa R, Nozawa T, Yamamoto A, Matsubara S: [A comparative study of "distal myopathy with rimmed vacuoles" with "slowly progressive neurogenic muscular atrophy group with rimmed vacuoles."], Annual Report of Clinical Research Group of Muscular Dystrophy, Japan, 1984, pp 179–185 (in Japanese, author's translation)
21. Tsukagoshi H, Matsumoto H, Sano M, Ishiura S, Sugita H: [Clinical and morphological studies and lysosomal enzyme activities in distal myopathy], Annual Report of Clinical Research Group of Muscular Dystrophy, Japan, 1984, pp 190–194 (in Japanese, author's translation)
22. Osame M, Iwamasa T, Nakagawa M, Fukunaga H, Hokato Y, Yamano T, Igata A: Debrancher deficiency myopathy associated with rimmed vacuoles. Neurol Med 1984, 20:637–644 (in Japanase, with English title)
23. Yunis EJ, Samaha FJ: Inclusion body myositis. Lab Invest 1971, 25:240–248
24. Sato T, Anno M: Inclusion body myositis and rimmed vacuole. Neurol Med 1984, 20:631–636 (in Japanese, with English title)
25. Whisnant JP, Espinosa RE, Kierland RR, Lambert EH: Chloroquine myopathy. Staff Meet Mayo Clin 1963, 38:501–513
26. MacDonald RD, Engel AG: Experimental chloroquine myopathy. J Neuropathol Exp Neurol 1970, 29:479–499
27. Sugita H, Sano M, Ishiura S, Hagiwara K: [Experimental chloroquine myopathy—its relevance to distal myopathy with rimmed vacuole], Annual Report of Clinical Research Group of Muscular Dystrophy, Japan, 1984, pp 195–197 (in Japanese, author's translation)
28. Katunuma N, Kominami E: Structures and functions of lysosomal thiol proteinases and their endogenous inhibitor. Curr Top Cell Reg 1983, 22:71–101
29. Takio K, Towatari T, Katunuma N, Teller DC, Titani K: Homology of amino acid sequences of rat liver cathepsins B and H with that of papain. Proc Natl Acad Sci USA 1983, 80:3666–3670
30. Noda T, Isogai K, Hayashi H, Katunuma N: Susceptibilities of various myofibrillar proteins to cathepsin B and morphological alteration of isolated myofibrils by this enzyme. J Biochem (Tokyo) 1981, 90:371–379
31. Katunuma N, Kominami E, Noda T, Isogai K: Lysosomal thiol proteinases and muscular dystrophy, Muscular Dystrophy: Biochemical Aspects. Edited by S Ebashi, E Ozawa. Tokyo, Japan Scientific Society Press; Berlin, Springer-Verlag, 1983, pp 247–256
32. Takio K, Kominami E, Wakamatsu N, Katunuma N, Titani K: Amino acid sequence of rat liver thiol proteinase inhibitor. Biochem Biophys Res Commun 1983, 115: 902–908
33. Takio K, Kominami E, Bando Y, Katunuma N, Titani K: Amino acid sequence of rat epidermal thiol proteinase inhibitor. Biochem Biophys Res Commun 1984, 121: 149–154