LACK OF THE C-TERMINAL DOMAIN OF NEBULIN IN A PATIENT WITH NEMALINE MYOPATHY

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Nemaline myopathy is a structural congenital myopathy associated with the presence of rod-like structures (nemaline bodies) within the muscle fibers and with an estimated incidence of 2 in every 100,000 live births.12 Clinically, it is characterized by the presence of hypotonia as well as proximal and facial weakness and skeletal deformities. Depending on the degree of muscle weakness and age at onset, and based on correlations from the international database on nemaline myopathy, five forms were clinically defined by the Nemaline Consortium of the European Neuromuscular Center.18

Nemaline myopathy may show both autosomal dominant and autosomal recessive inheritance patterns, and mutation in at least five genes has been identified in affected patients: the gene for slow α-tropomyosin 3 (TPM3) at 1q22–23,9,16 the nebulin gene (NEB) at 2q21.1-q22,14 the actin gene (ACTA1) at 1q42,13 the β-tropomyosin (TPM2) gene at 9p13,2 and the slow skeletal troponin gene (TNNT1).7 The typical autosomal recessive form appears to be the most common and is caused by mutations in the nebulin gene.14

Nebulin is a large protein (700–900 kDa) found in skeletal muscles, making up 3–4% of the total myofibrillar proteins.19 The C-terminal region of nebulin is located at the Z lines, whereas its N-terminal end is at the pointed end of the thin filament.4,8,11,19 It has been suggested that nebulin acts as a molecular ruler for the regulation of thin filament lengths and is important for the assembly and integration of Z-discs with the sarcomere.8,14 The correlation between mutations in the nebulin gene and the expression of the protein in muscle is still under investigation.

We have characterized the nebulin protein from eight patients with nemaline myopathy both by immunofluorescence microscopy and Western blots. Using antibodies against four different domains of nebulin, we identified one patient who showed a specific loss of the C-terminal SH3 domain of nebulin, whereas three other more N-terminally located epitopes from the N2, M176, and serine-rich domains of nebulin were present. This patient showed the phenotype of the typical form, and a pattern of small and diffusely distributed rods.

PATIENTS AND METHODS

We studied eight patients (from unrelated families) with a diagnosis of nemaline myopathy. The diagnosis was based on clinical examination, course of the...
disease (using the protocol of the International Nemaline Myopathy Consortium,\textsuperscript{18} family history, serum creatine kinase levels, electromyography, and muscle biopsy.

Muscle samples were obtained from biceps or deltoid biopsies, frozen in liquid nitrogen immediately after removal, and stored at $-70^\circ$C until use. Routine histological and histochemical procedures were performed.\textsuperscript{3} The analysis of the proportion of type I/II fibers, number of fibers containing rods, and the type of rods was carried out as described previously.\textsuperscript{5}

Immunohistochemical staining of frozen sections was done through single and/or double-labeling reactions,\textsuperscript{17} using a rabbit polyclonal antibody for $\alpha$-actinin 2 (kindly provided by Dr. A. Beggs) diluted 1:100, as a marker for rod structure. Four different antibodies for nebulin were used (Table 1): a mouse monoclonal antibody directed to an I-band epitope near the N2 line region, diluted 1:200 (Sigma, St. Louis, MO), and three rabbit polyclonal antibodies raised to the expressed SH3 domain, the serine-rich domain, and to the M176–M181 domains.\textsuperscript{11–14} The polyclonal antibodies were diluted 1:10. As second antibodies, anti-rabbit and anti-mouse IgG antibodies, both FITC and CY3 conjugated, were used. The sections were analyzed under a Zeiss Axiophot or confocal microscope with epifluorescence using filters for fluorescein and rodamine.

Western blots were done as described previously.\textsuperscript{6} The blots were incubated first with antibodies specific for nebulin and subsequently with an antibody against dystrophin (Dy4/6D3, kindly provided by Dr. L.V.B. Anderson). The incubations with primary antibodies were done overnight, and bound primary antibodies were detected using alkaline phosphatase-conjugated secondary antibody.

The index patient (Case 2) is a 14-year-old girl with a typical clinical course. Clinical manifestations started during the neonatal period with severe hypotonia and proximal weakness. During the first years of life, she had repeated respiratory infections. Her motor milestones were delayed. Thus, she sat at 3 years of age, stood up at 3.5 years, and walked at 4 years of age. Physical examination revealed facial and proximal muscle weakness, reduced tendon reflexes, severe hypotonia, and moderate muscle atrophy. She had a high-arched palate and pectum excavatum, but there was no tendinous retraction. Serum creatine level was mildly elevated. The electromyographic study revealed a myopathic pattern characterized by short-duration, small-amplitude polyphasic motor unit potentials; motor and sensitive conduction velocity were normal. In the last 2 months, she has experienced severe respiratory insufficiency requiring mechanical ventilation. The muscle biopsy was performed at 6 years of age and showed a typical myopathic pattern, fiber size variation, and a slight degree of connective tissue infiltration. ATPase reaction showed total type I fiber predominance, and Gomori staining revealed the presence of diffuse rods in 83% of the fibers (Fig. 1).

**RESULTS**

Immunohistochemical analysis in six of the eight patients was reported previously.\textsuperscript{5} Briefly, muscle from all patients showed the presence of nebulin in myofibrils. Some differences relating to the rod structure were observed. The majority of the largest subsarcolemmal rods were not labeled by the N2 nebulin antibody (I band epitope), and an indistinct pattern of labeling with the two antibodies directed to the Z-band portion of nebulin (epitopes M176–M181 and serine-rich domain) was observed. Diffuse rods were not revealed using the three antibodies.\textsuperscript{5}

Western blot analysis showed a band of the expected molecular weight in eight patients with the

| Table 1. Data on nebulin using four different antibodies and obtained through immunofluorescence and Western blot methodologies. |
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| Patient | Sex | Age (at biopsy) | Immunofluorescence | Western blot |
| | | | N2 | 101–102 | 176–181 | N2 | SH3 | Dystrophin |
| 1 | F | 3 | + | + | + | + | + |
| 2 | F | 6 | + | + | + | + | − |
| 3 | M | 14 | + | + | + | + | + |
| 4 | F | 9 | + | + | +/m | + | + |
| 5 | F | 3 | + | + | + | + | + |
| 6 | F | 18 | + | + | + | + | + |
| 7 | M | 5 | + | + | + | + | + |
| 8 | M | 4 | + | +/r | +/r | + | + |

*Rare negatively labeled fibers; m, mosaic of positive/negative fibers; −, negative labeling; +, positive labeling.*
FIGURE 1. Patient 2. Characteristic face (A) and phenotype (B), and pattern of rods in the modified Gomori reaction (C) and using antibody against α-actinin 2 (D). Original magnification in (C) and (D), ×400.

FIGURE 3. Schematic representation of the nebulin protein, and the positive pattern of immunofluorescence in Patient 2, using antibodies against the N2, the M176–181, and the serine-rich domains of nebulin. The position of the epitopes in the protein is shown at the top.
N2 antibody (Fig. 2). Interestingly, in one patient, an immunoreactive band was detected with the N2 antibody which did not react with the C-terminal SH3 antibody (Patient 2, Fig. 2). The same blot was incubated with the dystrophin antibody and a strong band was observed in this and other tested patients. The blot with the reaction for the SH3 antibody was re-reacted with antibody for the N2 domain of nebulin, and the patient with the deficient SH3 pattern showed the presence of the N2 band, thus confirming the first result. The intensity of the N2 band in this patient was slightly reduced.

On immunofluorescence, the sarcomeric pattern of labeling was observed with the antibodies raised to the N2, M176, and serine-rich domain. Unfortunately, the SH3 C-terminal antibody failed to work on sections, so that we were not able to confirm the lack of this epitope with immunofluorescence methodology (Fig. 3).

**DISCUSSION**

Nemaline myopathy is a congenital muscle disease, which presents with high clinical heterogeneity. In 1999, a new clinical classification was proposed. Based on age of onset and severity of weakness, five clinical forms were defined: severe congenital, intermediate, typical, juvenile, and adult. The typical form is the most common and is frequently caused by mutations in the nebulin gene. In this report, we have described a patient with clinical characteristics of the typical form, and therefore a strong candidate for mutations in the nebulin gene.

Nebulin is a skeletal muscle-specific giant sarcomeric protein, which accounts for 3–4% of the total myofibrillar proteins. The C-terminal region is part of the Z-disc structure, whereas its N-terminal end is an integral component of the thin filament. About 97% of the nebulin sequence consists of 185 copies of a 35-repeats module (called M1 to 185). The N-terminal region of nebulin consists of a 10 kDa acidic domain which interacts with tropomodulin and therefore is likely to be involved in thin filament length regulation. Similarly, nebulin’s C-terminal SH3 domain may have assembly regulatory functions, as suggested by sequence homology to other SH3 domains. Antibodies against these N- and C-terminal domains have been developed, allowing the immunohistochemical study of nebulin-specific regions.

In 1999, the first mutations in the nebulin gene were described. With the exception of one patient, patients with mutations in the M165–181 segments of the nebulin gene presented the nebulin protein in the fibers, as determined by studies with two different antibodies, M176–181 and SH3 nebulin domains.

Among the eight patients here studied, one (Case 2) revealed a lack of the epitope in the SH3 domain of nebulin, through the Western blot study. A band with the expected size was present, using antibody for the N2 domain, suggesting no total protein deficiency. Immunohistochemical analysis confirmed the presence of the epitopes at N2, repeats M176–181, the serine-rich domain, suggesting that a
probable mutation in the nebulin gene would be present after this region. Unfortunately, the antibody for the SH3 domain failed to work appropriately on immunofluorescence, so we were unable to confirm a lack of this region by this means.

To exclude a possible degradation artifact, we confirmed the preservation of high molecular weight proteins in the muscle extract through the concomitant use of antibodies for dystrophin, indicating that our failure to detect the nebulin SH3 epitope in Patient 2 was not the result of nonspecific muscle protein degradation during extraction or in the myofibril. Based on our results, this patient is a strong candidate for mutations in the nebulin gene, specifically in the SH3 domain. DNA analysis is currently being performed to investigate this possibility.

In a previous study, a patient with a lack of the SH3 domain was detected. However, this patient showed a homozygous mutation in the ninth codon of exon 185. The mutation changes a glutamic acid (GAG) to a stop codon (TAG) and should result in loss of the last 134 amino acids from the beginning of the serine-rich domain in the C-terminal region of nebulin. It remains to be seen whether future studies with specific antibodies for this region will show a protein with a smaller size.

Our patient presented a homogeneous immunohistochemical reaction with the three other nebulin antibodies, showing that the possible mutation was not related to any abnormalities in these domains. A mutation 3′ of the serine-rich domain can cause a lack of expression only in the nebulin C-terminal region. Consistent with a mutation very close to the C-terminal end of nebulin, the Western blot analysis showed no abnormalities in the size of the protein. The nebulin band detected with the N2 antibody was slightly fainter, however, which suggests increased degradation of the proteolysis-sensitive nebulin. In both cases, i.e., primary loss of the SH3 domain by a mutation or secondary loss by proteolysis, an improved understanding of the physiological roles of the nebulin SH3 domain is likely to be relevant to an appreciation of the molecular basis of nemaline myopathy.

The normal pattern of immunofluorescence reaction using the M176–181 antibody shows slow fibers more intensely labeled than fast fibers. Yet, our patient showed homogeneous M176–181 reaction in all fibers. This feature is explained by the predominance of type I fibers in her muscle.

In nemaline myopathy, the organization of the rods within fibers can show different patterns. In the majority of our patients, both large subsarcomally localized rods and small diffuse rods were detected in the fiber in a high proportion of fibers. A total predominance of diffuse rods was observed in 83% of the fibers of our Case 2, in a pattern detected only in this patient. This suggests a possible correlation of rod distribution with a specific type or region of mutations in the nebulin gene.

Our results show the importance of performing nebulin immunohistochemical studies associated with Western blot analysis, because they may be complementary. Molecular studies of the nebulin gene are very complex. The gene is quite large, with mutations distributed along the whole coding region, which makes the DNA study difficult, time-consuming, and expensive. The initial characterization of nebulin at the protein level may be helpful to direct further screenings for mutations on the genomic level. Additional studies on this patient may provide further insights into the role(s) of nebulin’s C-terminal SH3 domain, which appears to be involved in linking together alpha-actinin and nebulin filaments.

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