The Developmental Pattern of Myotubes in Spinal Muscular Atrophy Indicates Prenatal Delay of Muscle Maturation

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Abstract

The loss and degeneration of spinal cord motor neurons result in muscle denervation in spinal muscular atrophy (SMA), but whether there are primary pathogenetic abnormalities of muscle in SMA is not known. We previously detected increased DNA fragmentation and downregulation of Bcl-2 and Bcl-XL expression but no morphological changes in spinal motor neurons of SMA fetuses. Here, we performed histological and morphometric analysis of myotubes and assessed DNA fragmentation and Bcl-2/Bcl-XL expression in skeletal muscle from fetuses with type I SMA (at ~12 and 15 weeks’ gestational ages, n = 4) and controls (at ~10–15 weeks’ gestational ages, n = 7). Myotubes were smaller in the SMA than in control samples at all ages analyzed (p < 0.001) and were often arranged in clusters close to isolated and larger myotubes. Numbers of terminal deoxynucleotidyl transferase dUTP nick end labeling–positive cells in control and SMA fetuses were similar, and no differences in Bcl-2 or Bcl-XL immunostaining between control and SMA muscle were identified. Areas with smaller myotubes and the morphometric analysis suggested a delay in growth and maturation in SMA muscle. These results suggest that spinal motor neurons and skeletal muscle undergo different pathogenetic processes in SMA during development; they imply that muscle as well as motor neurons may be targets for early therapeutic intervention in SMA.

Key Words: Human muscle development, Myotubes, Spinal muscular atrophy.

INTRODUCTION

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease that affects spinal cord motor neurons and results in progressive skeletal muscular atrophy. Childhood SMA has been divided into 3 types based on the age of onset and clinical severity: Type I (severe form, Werdnig-Hoffmann disease); Type II (intermediate form); and Type III (mild/moderate form, Kugelberg-Welander disease). Mutations of the survival motor neuron (SMN1) gene are responsible for SMA, indicating a selective vulnerability of spinal cord and muscle to reduced SMN protein (1). Degeneration and loss of the α-motor neurons of the anterior horn of the spinal cord are the major neuropathologic features of the disease. Signs of muscle denervation usually appear early in most patients. One distinctive feature of SMA is the presence of a few scattered hypertrophic Type I fibers that presumably result from physiological hypertrophy. Fidzianska et al (2–4) reported that muscle biopsy specimens from patients with Type I SMA have a fetal appearance and are morphologically different from those in other denervating diseases such as amyotrophic lateral sclerosis. These observations led to the suggestion that Type I SMA could result from the arrested development of the motor unit and not from a degenerative process.

The simplest explanation for the neuromuscular phenotype in SMA is to assume that insufficient SMN protein causes motor neuron dysfunction and death and that muscle atrophy is a secondary consequence of denervation. Nevertheless, investigations to address the question of whether muscle, nerve and muscle, or just motor neurons are the critical target tissue in SMA are ongoing; the developmental period is believed to be a critical period in SMA pathogenesis. The SMN is normally expressed in the cytoplasm of myotubes and in most of their nuclei during development. By contrast, fetal SMA muscle has shown weaker SMN expression, with very sparse nuclei exhibiting positive immunostaining (5). Increased neuronal DNA fragmentation has been reported in the spinal cord during fetal development in Type I SMA, suggesting an alteration in programmed cell death (6, 7). Moreover, our group reported downregulation of Bcl-2 and Bcl-XL protein expression in SMA fetal spinal cords, thereby linking these proteins with the aberrant programmed cell death observed (8). Furthermore, neuropathologic descriptions of SMA largely come from necropsy postnatal samples, making assessment of earlier events more difficult (9).

To obtain further evidence for primary muscle involvement in SMA, in this study, we compared morphology, DNA fragmentation, and Bcl-2 and Bcl-XL expression in the skeletal muscle from control and type I SMA samples during fetal development.

MATERIALS AND METHODS

Fetal Specimens

The project was approved by the Ethics Committee and the Research Institute at the Hospital of Sant Pau, and family
consent was obtained for each case. Control fetal material from first and second trimesters of gestation was obtained immediately after elective terminations of pregnancy for social reasons. Gestational ages were determined by ultrasound measurements. Prenatal SMN tests performed at approximately 11 to 13 weeks permitted identification of fetuses that would have developed SMA in families with a previous patient affected by Type 1 disease. The SMA fetuses were collected from therapeutic abortions after confirmation of a homozygous deletion of Exons 7 and 8 of the SMN gene by chorionic villi DNA analysis (10). Our group has previously studied spinal cord samples from these specimens (5, 7, 8). After a careful analysis of all samples, 7 controls aged 10 (n = 2), 12 (n = 3), and 15 (n = 2) weeks and 4 Type I SMA specimens aged 12 (n = 2) and 15 (n = 2) weeks were selected for the study. Upper and lower limbs of each of the fetuses were isolated and immediately fixed by immersion in 4% paraformaldehyde and routinely processed for paraffin embedment. Five-micrometer-thick transverse sections were mounted onto lysine 50% for paraffin embedment. Five-micrometer-thick transverse sections were mounted onto 1-lysine 50%-treated slides and stained with hematoxylin and eosin, processed with the method of in situ end-labeling of nuclear DNA fragmentation (i.e. terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL]), or treated for immunohistochemistry to detect Bcl-2 and Bcl-XL. Additional samples were collected and snap frozen for immunoblot analysis.

Morphometric Analysis of Myotubes
All transverse sections were examined under a Nikon ES-400 microscope (60× objective lens). Myotubes were measured after their identification as cells with a large cytoplasm and small central nucleus surrounded by undifferentiated myoblasts. Their nuclei and cytoplasm diameters were measured by 3 investigators using Visilog version 5 (Noesis Vision, Inc, St Laurent, Quebec, Canada). To maximize accuracy of the myotube measurements, we adapted the method of the lesser diameter, defined as “the maximum diameter across the lesser aspect of the muscle fiber” described by Dubowitz and Sewry (11). For the cytoplasm diameters, the interclass correlation coefficient among the observations of the 3 investigators was 0.8. Because the values obtained by 2 of the investigators were very similar (interclass correlation coefficient, 0.827), the final result described was the mean. We attempted to measure myotubes in longitudinal sections, although this is more difficult to do accurately than cross-section measurements. To estimate the number of nuclei in longitudinal sections, the distances between nuclei in controls and SMA fibers were compared. All data were collected and analyzed, testing the mean with a 2-way analysis of variance test. Values of p < 0.01 were considered significant between the groups (control and SMA samples at 10, 12, and 15 weeks of gestation). Calculations were undertaken with SPSS14.0.

In Situ End-Labeling of Nuclear DNA Fragmentation
To detect cell death by TUNEL staining, we used the ApopTag peroxidase kit (in situ apoptosis detection kit; Intergen, Purchase, NY), following the supplier’s instructions. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Tissue sections were counterstained with hematoxylin. Brain sections of Sprague-Dawley rats irradiated with a single dose of 2-Gy γ rays at Postnatal Day 3 were used as positive controls. Sections were examined under a 60× objective lens to define the fields for the detection of TUNEL-positive cells.

Immunoblot
Skeletal muscle from control and SMA fetuses was homogenized in Nicholson treatment buffer (125 mM Tris HCl, pH 6.8; 4% sodium dodecyl sulfate; 4 M urea; 5% mercaptoethanol; 10% glycerol, 0.001% bromophenol blue) (12) and then boiled for 5 minutes. The extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a mini-protein system (Bio-Rad, Madrid, Spain) with low-range molecular weight standards (Bio-Rad). Protein analyzed on gel electrophoresis was electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were then washed with PBS (pH 7.4, Sigma, St. Louis, MO) and blocked with PBS containing 5% skim milk for 60 minutes. The membranes were incubated overnight at 4°C with the monoclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250. After washing, the immunoblots were incubated with rabbit peroxidase–conjugated anti-mouse secondary antibody (1:300 dilution; DAKO, Barcelona, Spain) for 1 hour at room temperature, washed again, and developed with the SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL). Stripping of the membrane and subsequent reprobing with a rabbit polyclonal antibody Bcl-XL (Transduction Laboratories, Lexington, KY) at a dilution of 1:100 was performed. The secondary antibody was a goat peroxidase–conjugated anti-rabbit (1:1000 dilution, DAKO). A final stripping and subsequent reprobing with a monoclonal β-tubulin antibody were performed.

Densitometric Analyses
Western blot densitometric analyses were performed, scanning and analyzing Bcl-2, Bcl-XL, and β-tubulin band intensities using the imaging analyzer Quantity One 4.1.1 version (Bio-Rad) to calculate their ratios.

Immunohistochemistry
Dewaxed sections of skeletal muscle of control and SMA fetuses were processed for immunohistochemistry as previously described (8). Briefly, after blocking endogenous peroxidase with hydrogen peroxide and methanol, the sections were treated with sodium citrate buffer 10 mmol/L, pH 6, for 25 minutes at 100°C and left for 1 hour at room temperature. Next, the sections were incubated with normal serum for 2 hours at room temperature (1:5 dilution). They were then incubated at 4°C overnight with a rabbit polyclonal antibody to Bcl-2 [N-19] (Santa Cruz Biotechnology) at a dilution of 1:75 or with anti–Bcl-XL (Transduction Laboratories) at a dilution of 1:500. For negative-reaction controls, we incubated the sections with the blocking solution.
only. After incubation, the sections were exposed to the biotinylated secondary antibody for 15 minutes and finally to streptavidin-peroxidase for 15 minutes at room temperature (HRP Rabbit/Mouse/Goat Universal LSAB+Kit, DAKO). The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. The sections were slightly counterstained with hematoxylin. All the sections were blindly examined at various magnifications with a Nikon ES-400 microscope.

RESULTS

Histology

In cross sections, the myotubes were larger and more evenly distributed in the control than in the SMA muscle samples (Figs. 1A–J). In some areas of the SMA muscle, small myotubes arranged in clusters with a well-preserved architecture were seen (Figs. 1D, F, H, J). We also detected SMA myotubes that resembled control myotubes in size and were aggregated in clusters (Figs. 1F, H). There were also larger isolated myotubes in SMA muscles close to the smaller myotubes (Fig. 1J). These features were similar at the 2 gestational ages of SMA muscle analyzed (12 and 15 weeks).

In longitudinal sections, the myotubes appeared thinner in SMA than in control muscle in some regions (Fig. 2). The appearances of the SMA and control muscles were similar in upper and lower limbs and in proximal and distal segments.

Morphometric Analysis of Myotubes

The mean nuclear diameter of 423 control myotubes in the 3 periods analyzed (10, 12, and 15 weeks) was 4.03 ± 0.58 μm (SD), whereas the mean nuclear diameter for 253 SMA myotubes at 12 and 15 weeks was 3.77 ± 0.57 μm (p < 0.001). The mean diameter of the control myotubes was 12.11 ± 1.98 μm, whereas for SMA myotubes, it was 9.64 ± 2.15 μm (p < 0.001). The differences were statistically significant when the gestational ages of the control and SMA muscles were separately compared (12 weeks, p < 0.001; 15 weeks, p < 0.001) (Figs. 3A, B). The distributions of the values at 12 weeks were clearly different between the control and SMA groups with some overlap (Fig. 4A). Between 12
and 15 weeks, no differences were observed in the evolution of the control and SMA myotube nuclear sizes (Figs. 3A, B). The diameter of the control myotubes tended to decrease, however, whereas the diameter of SMA myotubes tended to increase during this period (Fig. 4B). This finding indicates the different behaviors over time in the 2 groups. Inspection of the slope in SMA between 12 and 15 weeks suggests a behavior similar to the slope of controls between 10 and 12 weeks (Fig. 4C). A statistical comparison of both slopes demonstrated a tendency to increase with time. Nevertheless, the value for the control slope was 3.31 ± 0.23, whereas the SMA slope was 2.07 ± 0.24 (p < 0.001). These data indicate that the increase in size of the myotubes in SMA is delayed because at 12 weeks, the SMA myotubes showed values lower than those of the controls at 10 weeks.

The distances between nuclei in longitudinal sections were determined in control and SMA 12-week samples. A total of 403 values (199 in controls and 204 in SMA) was obtained. The mean value for controls was 38.7 mm ± 13.24 mm, whereas in SMA samples, it was 40.1 mm ± 12.79 mm (t-test, p = 0.290).

**TUNEL Analysis**

A total of 95 control and SMA muscle sections of upper and lower limbs were stained by TUNEL. For control tissues, 212 fields (defined by a 60× objective lens) were studied, and a total of 164 TUNEL-positive cells were detected. The average number of positive cells per field was 0.773. In the SMA sections, we analyzed 165 fields and detected 98 TUNEL-positive cells. The mean value was similar to those of the controls (0.593 positive cells per field). Thus, isolated TUNEL-positive cells were found in both groups, with no qualitative or semiquantitative differences between them identified. The isolated TUNEL-positive cells were detected within groups of clustered and nonclustered myotubes and in connective tissue (Fig. 5).

**Bcl-2 and Bcl-X<sub>L</sub> Expression**

Immunohistochemical studies showed no significant differences between control and SMA muscle with respect to Bcl-2 and Bcl-X<sub>L</sub> expression in the 2 sample ages analyzed (12 and 15 weeks). Positive immunostaining was observed mainly in the cytoplasm of the myotubes and myoblasts with...
both antibodies. Expression in nuclei was barely detected (Fig. 6A). Immunoblot studies revealed no quantitative differences between control and SMA muscle (Fig. 6B).

DISCUSSION

We systematically analyzed several features of skeletal muscle (one of the most critically affected tissues in SMA) in control and Type I SMA fetuses at 12 and 15 weeks’ gestational age. During skeletal muscle development, myoblasts fuse into myotubes (i.e. centrally multinucleated cells) that subsequently become muscle fibers (13). Primary myotubes are usually present by 8 weeks of development and are regarded as classic myotubes with chains of central nuclei. Secondary myotubes with smaller diameters and more widely spaced nuclei usually appear after 13 to 14 weeks (14). Myotube diameters in humans tend to decrease between 12 and 15 weeks of gestation; similarly, this also occurs in mice between Days E18 and E20 (15). We detected a decrease in the overall values of control myotube diameters toward Week 15 that could be attributed to the progressive appearance of smaller secondary myotubes. The increase in myotube diameters in SMA samples at Week 15 suggests that primary myotubes are still very abundant in SMA at this stage.

We also found that the developmental patterns of SMA myotubes differ from those in controls with respect to myotube size, gestational age, and pattern of change over time. The SMA myotubes had significantly smaller nuclei and cytoplasm at all stages analyzed compared with controls. The greatest difference in size between control and SMA myotubes occurred in the control and SMA muscle at 12 weeks, whereas SMA myotubes showed a gradual increase in diameter over time. The mean myotube sizes in WT samples begin to decrease after 12 weeks, whereas SMA myotubes show a gradual increase in diameter over time (values represent the mean). (C) Overlapping of myotube behavior in SMA between 12 and 15 weeks and in controls between 10 and 12 weeks. The no. 1 in the x axis represents the mean of the values at 10 weeks in control and 12 weeks in SMA. The no. 2 in the x axis represents the mean of the values at 12 weeks in control and 15 weeks in SMA. Both slopes tended to increase with time. Note that the values and the slopes of the SMA samples at 12 to 15 weeks are similar to the control samples at 10 to 12 weeks, but the value for the control slope was higher than the SMA slope (p < 0.001).
Muscle fibers have been described in Kugelberg-Welander patients (16). Moreover, several developmental proteins (e.g., neural cell adhesion molecule, developmental heavy chain myosin, desmin, and vimentin) have been observed in atrophic muscle fibers from SMA patients (17, 18). Based on observations in these studies of postnatal SMA muscle as well as our present observations in prenatal (i.e., presymptomatic) SMA, it is reasonable to postulate that delayed myogenesis is an early manifestation of SMA during the fetal period.

Apoptosis occurs during normal skeletal muscle histogenesis; at the myotubule stage, undesired cells are eliminated by apoptosis, thereby reducing the total numbers of muscle fibers (19). Unlike previous observations of increased numbers of TUNEL-positive neurons in the spinal cord during development of Type I SMA fetuses (6, 7), we were unable to detect differences in DNA fragmentation between the fetal SMA and control muscle samples using the same TUNEL protocol. These observations suggest that apoptosis is not increased in SMA muscle during early development. The window of development that we studied (from 12 to 15 weeks), however, does not exclude the possibility that the number of apoptotic cells subsequently increases during development in SMA.

Fidzianska et al (3) observed that numerous muscle cells underwent removal by apoptosis in an SMA patient aged 8 weeks and hypothesized that increased apoptosis could be a pathological mechanism in the skeletal muscle of SMA patients. The authors proposed that the elimination of muscle fibers could result in secondary motor neuron death. Other investigators have described an increase in TUNEL-positive cells in muscle fibers of SMA patients (18, 20). This increase may not only indicate apoptosis, but also delayed development of myocytes and the presence of defective innervation (20, 21). All of these studies were carried out in postnatal samples and in the terminal period of the disease in most cases, whereas the present study addresses changes that occur during SMA development in a preclinical stage. Interestingly, in a mouse model in which the Smn deletion is targeted to the muscle, no apoptotic cells were observed;

**FIGURE 6.** Bcl-2 and Bcl-XL expression in fetal muscle. Immunohistochemical staining of fetal control and spinal muscular atrophy (SMA) muscle. (A, B) There is an intense Bcl-2 signal in the cytoplasm of the control (A) and SMA (B) muscle samples from 12 weeks’ gestational age. (C, D) Immunohistochemical staining for Bcl-XL in 15-week fetal muscle. The expression of Bcl-XL is patchy and irregular in controls (C) and SMA (D). Scale bar = 10 μm. (B) Immunoblot of Bcl-2 and Bcl-XL in fetal muscle. The ratios are similar between control (dark gray) and SMA (light gray).

was detected at 12 weeks, a period when primary myotubes are predominant. At this age, the values of SMA myotubes, albeit lower, resembled those of the controls at 10 weeks, indicating a delay in growth and maturation in SMA muscle. The superimposition of the 10- to 12-week values in controls with the 12- to 15-week values in SMA shows similar slopes of the increase in myotube size. The values obtained were even lower in SMA, reinforcing the hypothesis of muscle delay in development (Fig. 4). No significant differences were observed in the distance between nuclei in longitudinal sections, suggesting that myotube elongation is similar between controls and SMA.

Immature cells akin to myotubes have been observed in muscle samples of Type I SMA patients (4), and immature muscle fibers have been described in Kugelberg-Welander patients (16). Moreover, several developmental proteins (e.g., neural cell adhesion molecule, developmental heavy chain myosin, desmin, and vimentin) have been observed in atrophic muscle fibers from SMA patients (17, 18). Based on observations in these studies of postnatal SMA muscle as well as our present observations in prenatal (i.e., presymptomatic) SMA, it is reasonable to postulate that delayed myogenesis is an early manifestation of SMA during the fetal period.

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**TABLE.** Comparison of Spinal Cord and Muscle in Fetal SMA at Approximately 12 to 15 Weeks of Development

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Histology LM</th>
<th>TUNEL + Cells</th>
<th>Bcl-2/Bcl-XL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal cord</td>
<td>No detectable differences between SMA and controls</td>
<td>Significantly increased in SMA compared with controls</td>
<td>Downregulation in SMA</td>
</tr>
<tr>
<td>Muscle</td>
<td>SMA myotubes smaller than in controls</td>
<td>No differences detected between SMA and controls</td>
<td>No detectable differences between SMA and controls</td>
</tr>
</tbody>
</table>

LM, light microscopy; MN, motor neuron; SMA, spinal muscular atrophy; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Compiled from Soler-Botija et al (5, 7, 8) and this work.

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the authors did, however, detect necrotic fibers, a low proportion of regenerating myocytes, and a definite muscular dystrophy (22). Furthermore, in a similar mutant mouse, a limited capacity of satellite cells to regenerate skeletal muscle has been shown (23). In these models, the authors did not observe muscle denervation or morphological changes in motor neurons as had been detected in another mouse model, in which the deletion was targeted at motor neurons (24).

We were unable to detect differences in the expression of Bcl-2 and Bcl-XL, proteins involved in antiapoptotic mechanisms (25). Tews and Goebel (20) found weak Bcl-2 expression in postnatal Type I SMA muscle biopsies and suggested that muscle fibers are unable to express sufficient levels of Bcl-2 because of immaturity. Our finding that Bcl-2 expression in SMA fetal muscle appeared similar to that in controls suggests that Bcl-2 makes a limited contribution to the survival of SMA muscle during development. Dominov et al (26) analyzed the expression of the Bcl-2 family of proteins during muscle development and detected expression in myoblasts, myotubes, and muscle stem cells of embryos, fetuses, and newborns. Our study is the first to describe the pattern of expression of the Bcl-2 proteins in developing SMA muscle.

Based on our earlier reports on spinal cord of fetuses predicted to develop Type I SMA (7, 8), comparison with muscle in the same developmental period reveals some striking differences between the 2 tissue types (Table). The SMA motor neurons show no demonstrable alterations under light microscope examination (7), whereas myotubes of affected fetuses tend to be smaller and arranged in clusters. On the other hand, SMA motor neurons showed greater DNA fragmentation than controls, whereas SMA muscle displays a rate of isolated TUNEL-positive cells similar to that of controls. Moreover, downregulation of Bcl-2 and Bcl-XL proteins has been observed in SMA spinal cord (8), but here we found no differences from controls in these proteins at the same gestational age (present work). Thus, spinal cord and muscle differ in their pathogenic responses during development in SMA. A study of terminal peripheral nerves and neuromuscular junctions could identify possible pathogenetic links between the 2 tissues (work in progress). In a recent study using embryonic SMA mice, McGovern et al (27) found no defects in axonal formation or outgrowth, but they did find a significant increase in synapses lacking motor axon input.

Our results support the view of SMA as a developmental disorder and the hypothesis that similar to motor neurons, skeletal muscle could play a major role in SMA pathogenesis. In fact, lower amounts of SMN in developing muscle (5) could affect its growth and maturation regardless of denervation. For example, myotube diameters in the murine aneural soleus muscle during development do not differ from those of the myotubes of innervated muscles (15). In rats, it has been demonstrated that immature muscle fibers can survive denervation for longer periods, but their development into mature muscle fibers is dependent on functional innervation (28). It has also recently been reported that the SMN complex localizes to the sarcomeric Z-disk, and that myofilaments from a severe SMA mouse model display morphological defects consistent with Z-disk deficiency (29). Studies of cocultures of rat embryo spinal cord with muscle from SMA patients have shown that SMA muscle fibers were unable to prevent motor neuron death by apoptosis. Therefore, a defect in the neurotrophic function of SMA muscular cells has been assumed (30). In line with our present results, these authors noted a subtle difference in the size of myonuclei, with those of SMA myotubes appearing smaller (30). Furthermore, cultured muscle cells from SMA patients failed to cluster acetylcholine receptors and presented abnormalities that may affect neuromuscular junction installation (31). Thus, it is reasonable to assume that the dynamic interaction between motor neurons and muscle is disrupted in SMA disease, where lower SMN protein (5) could be involved in dysregulation of developmental changes in motor neurons, neuromuscular junctions, and muscle (32). Based on postnatal studies in mouse SMA models, Kariya et al (33) recently demonstrated that insufficient SMN protein arrests the postnatal development of the neuromuscular junction, impairing maturation of acetylcholine receptor clusters.

In conclusion, our results highlight the need for further research into SMA muscle during development and the need to consider both motor neurons and muscle as targets for early intervention and new therapies (34) in SMA.

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