Abnormalities in Early Markers of Muscle Involvement Support a Delay in Myogenesis in Spinal Muscular Atrophy

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Abstract

Spinal muscular atrophy (SMA) is characterized by loss of motor neurons in the spinal cord that results in muscle denervation and profound weakness in affected patients. We sought evidence for primary muscle involvement in the disease during human development by analyzing the expression of several muscle cytoskeletal components (i.e., slow, fast, and developmental myosin, desmin, and vimentin) in fetal or postnatal skeletal muscle samples from 5 SMA cases and 6 controls. At 14 weeks’ gestation, SMA samples had higher percentages of myotubes expressing fast myosin and lower percentages of myotubes expressing slow myosin versus control samples. Desmin and vimentin were highly expressed at prenatatal stages without notable differences between control and SMA samples, although both proteins showed persistent immunostaining in atrophic fibers in postnatal SMA samples. We also studied the expression of Pax7-positive nuclei as a marker of satellite cells and found no differences between control and SMA prenatal samples. There was, however, a significant increase in satellite cells in postnatal atrophic SMA fibers, suggesting an abnormal myogenic process. Together, these results support the hypothesis of a delay in muscle maturation as one of the primary pathologic components of SMA. Furthermore, myosins and Pax7 may be useful research markers of muscle involvement in this disease.

Key Words: Human development, Myosin, Pax7, Satellite cells, Skeletal muscle, Spinal muscular atrophy, Survival motor neuron 1 gene.

INTRODUCTION

Spinal muscular atrophy (SMA) is the leading genetic cause of death in infants. It is characterized by degeneration and loss of motor neurons in the spinal cord (1), which result in muscle denervation and weakness in affected patients. Childhood SMA is divided into 3 main types based on the onset and severity of the disease: Type I, severe form; Type II, intermediate form; Type III, milder form (2). Outliers of these main types include Type 0 (congenital form) and Type IV (adult form). All types are caused by mutation of the survival motor neuron 1 (SMN1) gene localized on chromosome 5q (3). Another homologous gene localized in the same chromosomal region, SMN2, encodes around 10% to 50% of the same SMN protein as SMN1 (4-5), an amount that is insufficient to prevent the disease.

Although SMA usually manifests at postnatal stages, several reports have shown that the pathologic alterations of the most severe forms (Type 0 and Type I) start during prenatal development. These studies have reported an increase in motor neuron death (5-7), a decrease in myotube size (8), and aberrant synaptic contacts at neuromuscular junctions, followed by an increase in presynaptic vesicles at terminal nerves (9).

Studies in postnatal SMA patients have shown constitutive abnormalities in muscle cultures and altered expression of several proteins, including myosins, desmin, and vimentin (10-17). Myosins are a group of motor proteins known for their role in muscle contraction and their involvement in a wide range of eukaryotic motility processes. Myosin II is generated in skeletal muscle and consists of 4 light chains and 2 heavy chains. Myosin heavy chains (MHCs) are found in muscle fibers; they are important for muscle cell architecture and structure during development (18, 19). On the other side, desmin and vimentin are intermediate filament proteins found in muscle fibers; they are important for muscle cell architecture and structure during development (19). Finally, satellite cells (SCs), which are considered to be muscle stem cells located between the plasma membrane and the basal lamina of muscle fibers, compose the major regenerative population in skeletal muscle. Satellite cells are usually identified by the expression of the paired box protein Pax7 in nuclei and proliferate during early development (20-22).

The role of muscle in SMA pathogenesis remains controversial. Thus, to find evidence of early muscle involvement in the disease, we aimed to determine the expression of myosins, desmin, and vimentin and to quantify Pax7-positive cells as a marker of SCs in muscle from human SMA developmental samples and controls. The systematic prenatal and postnatal analyses of these proteins in different SMA muscles are essential to establish the time and degree of impairment of muscle function in the disease and to define the necessity to consider both motor neurons and muscle as targets for new therapies.
MATERIALS AND METHODS

Human Samples

The project was approved by the Ethics Committee and the Research Institute at Hospital de Sant Pau and was conducted in accordance with the Declaration of Helsinki. Family consent was obtained accordingly. Fetal samples from the second trimester of gestation were obtained immediately after elective termination of pregnancy. Gestational age was determined from the last menstrual period and from ultrasound measurements. Prenatal SMN tests performed at around 11 to 13 weeks of postmenstrual age allowed us to identify fetuses predicted to develop SMA in families with a prior affected patient. Spinal muscular atrophy fetuses were collected from abortions after DNA analysis of chorionic villi had confirmed a homozygous deletion of exons 7 and 8 of the SMN gene. The samples collected included 3 Type I SMA specimens, 1 Type II SMA specimen, and 3 control fetuses (Table 1). Muscle from upper and lower limbs was isolated, collected on gelatin-coated slides and dried at room temperature before immunostaining. Postmortem diaphragm, intercostal, and quadriceps muscles were obtained from a Type I SMA postnatal patient (aged ~11 to 12 months) and from 3 controls (1 intercostal muscle, aged 11 months; diaphragm and quadriceps muscles, aged 2 months; intercostal and quadriceps muscles, aged 1 month) to compare muscle involvement (Table 1). Immunohistochemical procedures were performed following the same protocol for the fetal samples.

Immunofluorescence

Serial sections of skeletal muscle were processed for immunofluorescence as previously described (9). For double immunofluorescence, sections were incubated overnight at 4°C with mouse anti-myosin antibodies to fast MHC (fMHC), slow MHC (sMHC), and developmental MHC (dMHC) isoforms (the latter recognizes the embryonic and neonatal isoforms) (Novocastra, Newcastle, United Kingdom) or, alternatively, with mouse anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa City, IA) and rabbit anti-laminin (Sigma, St Louis, MO) antibodies. Because neutral cell adhesion molecule is expressed in developmental muscle and muscle denervation, we used mouse anti-human neutral cell adhesion molecule antibody (BD Biosciences, San Jose, CA) to complement our study. After incubation, sections were washed and incubated for 1 hour in Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch, Newmarket, United Kingdom) and Alexa488-labeled goat anti-rabbit IgG (Invitrogen, Eugene, OR). For single labeling, samples were incubated with mouse anti-desmin or mouse anti-vimentin antibodies (Dako, Barcelona, Spain) and Cy3 goat anti-mouse antibodies as secondary antibody. Sections were counterstained with 4',6-diamidino-2-phenylindole (Invitrogen) for nuclear localization. Slides were then washed and mounted in Fluoprep (Biomerieux, Madrid, Spain). All sections were blindly examined at various magnifications using a Nikon ES-400 microscope and an SP5 Leica confocal microscope.

Expression Analysis of Myotubes

In prenatal samples, transverse serial sections were examined at various magnifications under a Nikon ES-400 microscope and an SP5 Leica confocal microscope. Given the similar results obtained in upper and lower limbs, data from these sections were pooled for each sample. For fMHC and sMHC expression analysis, a total of 33,181 control myotubes (15,675 Type II SMA myotubes and 26,846 Type I SMA myotubes) were counted. For Pax7, we evaluated a total of 6,756 control nuclei (2,200 Type II SMA nuclei and 6,934 Type I SMA nuclei).

In each of the postnatal samples, a total of 50 fields (at 40× magnification) were counted to measure the frequency distribution of Pax7 nuclei per field. All data were collected and analyzed by determining the mean ± SE using 1-way analysis of variance and Gabriel post

| TABLE 1. Gestational Age, Phenotype, and Molecular Data of the Fetal and Postnatal Patient Samples Analyzed and Clinical Data Regarding Previous Children in Each Family Who Had SMA |
|-----------------|----------------|----------------|----------------|----------------|
| **Fetal subjects** | **Age** | **Phenotype** | **SMN1 Exons 7–8** | **SMN2 Copies** |
| Sample 1 | 13 weeks | Control | +/- | 2 |
| Sample 2 | 14 weeks | Control | +/- | 2 |
| Sample 3 | 14 weeks | Control | +/- | 2 |
| Sample 4 | 14 weeks | Type I SMA | +/- | 2 |
| Sample 5 | 14 weeks | Type I SMA | +/- | 2 |
| Sample 6 | 13 weeks | Type I SMA | +/- | 2 |
| Sample 7 | 14 weeks | Type II SMA | +/- | 3 |
| **Postnatal subjects** | **Age** | **Phenotype** | **SMN1 Exons 7–8** | **SMN2 Copies** |
| Sample 1 | 13 months | Control | +/- | 2 |
| Sample 2 | 2 months | Control | +/- | 2 |
| Sample 3 | 1 month | Control | +/- | 2 |
| Sample 4 | 11 months | Type I SMA | +/- | 2 |

*SMN2 copies were identical in the corresponding affected child. NA, not applicable.
hoch test. Values of p < 0.001 were considered significant between SMA and control samples. Calculations were made using the statistical program SPSS21.

RESULTS

Expression of Fast and Slow Myosin in SMA Prenatal and Postnatal Samples

Fast myosin (fMHC) was detected in secondary and tertiary myotubes (the latter being the smallest in diameter) (Fig. 1A), whereas slow myosin (sMHC) was found in primary myotubes (Fig. 1B). In SMA and control fetuses, developmental myosin (dMHC) was expressed throughout muscle tissue (data not shown). In SMA, we found a slight but significantly higher proportion of myotubes expressing fMHC (79% ± 0.57% in controls, 76% ± 1.08% in Type II SMA, and 84% ± 0.99% in Type I SMA; p < 0.001 between controls and Type I). Slow myosin was detected in fewer myotubes in SMA than in controls (47% ± 1.21% in controls, 41% ± 3.07% in Type II SMA, and 30% ± 1.25% in Type I SMA; p < 0.001 between controls and Type I SMA) (Fig. 1C). Considering both isoforms together, the percentage of cells ranges from 114% to 117% in SMA to 126% in controls, indicating the presence of hybrid fibers representing the differences between the SMA group and controls (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A591).

In postnatal Type I SMA muscle, quadriiceps showed a prominent pattern of hypertrophic fibers expressing slow myosin, whereas all atrophic fibers showed mosaicism of slow and fast fibers, with the latter being more abundant (Fig. 2). The diaphragm showed mostly similarly sized fibers expressing sMHC predominantly (Fig. 2); very few atrophic dMHC fibers were interspersed between them (not shown). Intercostal muscle exhibited a high amount of atrophic fibers positive for sMHC (Fig. 2). As expected, in all the 3 types of control muscles, normal fibers showed restricted patterns of MHC staining but with slight differences in the expression of slow and fast myosin according to the muscle analyzed (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A592).

Expression of Desmin and Vimentin Is Downregulated After Birth in Controls but Is Maintained in SMA Patients

In prenatal SMA and control muscles, no differential expression of desmin or vimentin was observed. Desmin was localized in the sarcoplasmic cytoskeleton of myotubes, and vimentin was localized in cells of mesodermal origin: fibroblasts and endothelial and smooth muscle cells of blood vessels (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A593). In postnatal control samples, expression of both proteins was downregulated, whereas postnatal SMA muscle maintained positive immunoreactivity to desmin and vimentin in atrophic fibers (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A593).

Pax7 Expression in Prenatal and Postnatal SMA Muscles

In SMA and control prenatal samples, we observed a Pax7-positive signal in nuclei inside the basal lamina of myotubes and in the surrounding endomysium of muscle fibers outside the basal lamina (Fig. 3A). In the prenatal period, SCs accumulate on the surface of myotubes even before a basal lamina forms (20, 21); thus, the cells inside the basal lamina were considered to be precursors of SCs. We counted Pax7-positive nuclei per total nuclei and the numbers of Pax7-positive nuclei inside and outside the muscle fibers. When considering the total number of SCs per total nuclei, SMA samples showed significant differences from control samples in the proportions of SCs. However, the distributions of these cells inside and outside the muscle fibers were similar in both groups. In all samples, approximately 20% of all Pax7-positive nuclei were observed in the connective tissue (Fig. 3B). Immunohistochemistry for neutral cell adhesion molecule showed a similar pattern of expression in the cell membranes of prenatal SMA and control muscles (Figure, Supplemental Digital Content 4, http://links.lww.com/NEN/A594).

In SMA and control postnatal samples, Pax7-positive nuclei were mostly observed under the basal lamina of muscle fibers. Quantification of fields in each cross section of the different SMA muscles studied showed greater numbers of SCs in quadriceps and intercostal muscles (the most affected muscles in SMA) than in the diaphragm (p < 0.001). Pax7-positive nuclei were more abundant in the atrophic areas of these muscles (Fig. 4).

DISCUSSION

In this systematic analysis, we observed findings compatible with primary muscle involvement in SMA. In the prenatal stages of SMA muscle, the numbers of myotubes expressing fast and slow isoforms of MHC were different from those in controls. In atrophic fibers of postnatal SMA muscle, we found a persistent expression of desmin and vimentin and increased numbers of SCs.

Several myofibrillar proteins have been studied as markers of disease severity in children with SMA. One of the most studied proteins has been the family of MHCs. These proteins have been described by immunohistochemistry and by their expression at the molecular level (12, 16, 17, 23). As far as we know, most of the SMA studies were conducted in postnatal quadriiceps femoris muscles. According to these reports, hypertrophic fibers usually expressed sMHC, whereas atrophic fibers expressed both sMHC and fMHC. Developmental MHC has been found in atrophic fibers and a few hypertrophic fibers, a finding that has previously been interpreted as a feature of immature fetal phenotype (16, 23). Here, we present for the first time a systematic comparison of the patterns of different MHC isoforms in SMA muscles between prenatal and postnatal stages (Table 2).

During development, MHC isoforms are expressed in a sequential manner. Early primary myotubes express an embryonic form that is later replaced by fast or slow forms that will define the profile of the muscle fibers. Beyond the neonatal period, most fibers derived from primary myotubes express only slow myosin. On the other hand, secondary and tertiary myotubes are mixed fibers that express a variable combination of developmental, fast, and slow myosins (18, 19).
We found that the proportions of myotubes expressing fMHC and sMHC differed between Type I SMA and control samples (Fig. 1). Slow MHC was notably decreased in Type I SMA fetuses in comparison with Type II SMA fetuses and controls because growth of primary myotubes could be delayed during Type I SMA development (8). We also detected

**FIGURE 1.** Expression and quantification of fMHC and sMHC in control, Type II SMA, and Type I SMA muscles at 14 weeks of development. (A) Expression of fMHC (red). The most positive signal is located in secondary (low expression) and tertiary smaller (strong expression) myotubes. Laminin (green) marks the basal lamina. (B) Expression of sMHC. Positive fibers in these samples correspond to primary myotubes. (C) Histogram showing percentages of positive fMHC and sMHC myotubes in control and SMA muscles. Note the tendency in SMA to have decreased percentages of sMHC-positive cells. Data are expressed as mean ± SE. *** p < 0.0001. Scale bars = 50 μm (lower magnification); 20 μm (higher magnification). WT, control.

**FIGURE 2.** Expression of sMHC, fMHC, and dMHC in postnatal SMA muscles. Double immunofluorescence of laminin (green) and slow, fast, or developmental myosin (red) in quadriceps, diaphragm, and intercostal postnatal SMA muscles. Note the different patterns of expression in the different muscles analyzed. In diaphragm and intercostal SMA muscles, slow fibers predominate, whereas in quadriceps muscles, hypertrophic fibers show a slow phenotype and atrophic fibers show a mosaic between slow and fast patterns. Scale bar = 50 μm.
a slight but significant increase in the number of Type I SMA myotubes expressing fMHC. This might be correlated with a possible delay in the emergence of secondary and tertiary myotubes in SMA, in agreement with our previous finding of a smaller diameter in SMA myotubes (8). These data add further evidence to the idea of a delay in muscle maturation during SMA development (24).

Formation of the first generation of primary myotubes and synthesis of slow myosin are thought to be nerve-independent processes. However, the formation of secondary myotubes expressing fMHC or sMHC is believed to be nerve-dependent (18). In our previous studies, we observed that prepatterned acetylcholine receptor clustering is not affected by the disease because early muscle innervation takes place in

FIGURE 3. Expression and quantification of the paired box protein Pax7 in nuclei in control and SMA prenatal muscle samples. (A) Triple immunofluorescence of 4',6-diamidino-2-phenylindole for nuclear staining (blue), Pax7 (red), and laminin (green) in developing control, Type II SMA, and Type I SMA muscles. Two populations of Pax7-positive (Pax7⁺) nuclei were identified: one located inside the basal lamina and the other located outside the muscle fibers in interstitial tissue (arrow). (B) Histogram showing percentages of total Pax7-positive nuclei (left) and distribution inside or outside the basal lamina (right). Type I SMA muscles tend to show a higher proportion of Pax7-positive nuclei, although the distributions of these nuclei inside or outside the basal lamina are roughly the same in all samples. Scale bars = 50 μm (lower magnification); 20 μm (higher magnification); *** p < 0.0001.
Type I SMA in humans. However, as early as 14 weeks, the nerve-dependent mechanisms needed for the maintenance of neuromuscular junctions seem to fail in SMA (9). Thus, it is reasonable to assume that the alterations that we observed in primary myotubes are independent of innervation defects. Subsequently, secondary and tertiary SMA myotubes can be smaller because of the failure to maintain the initial innervation (8, 9).

Studies in SMA mouse models have also shown a lack of myofiber maturation, and that loss of innervation can be attributed to defects in synapse maintenance. These findings agree with the hypothesis of altered neuromuscular development and primary muscle involvement in human SMA (25, 26).

In postnatal human muscle, most fibers express either sMHC or fMHC isoforms corresponding to histochemical Type 1 and Type 2 fibers, respectively (19). In SMA samples, we observed that the pattern of MHC expression differed depending on the muscle analyzed. Quadriceps and intercostal muscles showed increased atrophic areas expressing fMHC and sMHC fibers and hypertrophic areas filled with sMHC fibers. On the other hand, the diaphragm showed mostly fibers

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**FIGURE 4.** Expression and quantification of Pax7-positive nuclei in control and SMA postnatal muscle samples. Top: Triple immunofluorescence of laminin (green), Pax7 (red), and 4',6-diamidino-2-phenylindole (blue) in diaphragm, intercostal, and quadriceps muscles. The areas with atrophic fibers in quadriceps and intercostal SMA muscles show an increase in Pax7-positive nuclei per field versus control intercostal and diaphragm SMA muscles. Scale bar = 50 μm. Bottom: Box plot of the number and distribution of Pax7-positive nuclei per field in each muscle type. Control intercostal and SMA diaphragm muscles have similar distributions, whereas the vulnerable SMA intercostal and quadriceps muscles tend to show more nuclei per field (p < 0.001).
of the same size expressing sMHC predominantly and rare atrophic fibers. This is in agreement with the fact that the diaphragm is less involved in SMA than are quadriceps and intercostal muscles (27) and that the 3 muscles are differentially innervated and respond differently to paralysis or denervation in adulthood (28). Indeed, in quadriceps and intercostal muscles, hypertrophic fibers are characterized by a sustained expression of sMHC, suggesting that these fibers may originate from primary myotubes. These primary myotubes would retain advantages in size and innervation over secondary and tertiary myotubes, which are smaller and become innervated later (18, 29, 30). Mouse models have also shown vulnerability and resistance to denervation in a subset of muscles, suggesting that some motor units are able to bypass the pathology of the disease (26). The intrinsic mechanisms causing the involvement of some particular muscles in SMA warrant further study.

Desmin and vimentin are abundant in immature and developing fibers but are downregulated as muscle matures; vimentin is detected on vascular tissue, and desmin is observed at the sarcolemma and reorganized at the Z line (19). In some neuromuscular diseases (and after muscle trauma), these proteins are altered and used as markers of muscle alterations. A previous study by Sarnat (14) in normal developing human muscle showed that desmin and vimentin persisted in myotubes until the last months of fetal development, when their expression decreased. We did not observe clear differences between the expression of these 2 proteins in prenatal SMA and control muscle samples; in postnatal control samples, we confirmed a downregulation in their expression. In contrast, postnatal SMA muscle continued to show positive desmin and much higher vimentin immunoreactivity in atrophic fibers. Taken together, these observations indicate that, when maturation in muscle is arrested, desmin and, particularly, vimentin maintain their expression, as observed in fetal myotubes (14).

Satellite cells are responsible for forming new myofibers during embryonic development and for regenerating muscle after muscle trauma. Reports on muscle cell cultures of SMA patients have shown deficiencies in myoblast fusion and signs of degeneration during myogenesis related to a reduction of SMN expression (10, 11). Studies in SMA mouse models have also shown deficiencies in the ability of SCs to form differentiated myotubes (31, 32), thereby highlighting their importance in muscle regeneration and survival (33). This is the first report on the expression of Pax7 to identify SCs in SMA cases. We did not observe significant differences in the number of SCs in SMA muscle in comparison with control samples at the prenatal stages analyzed (approximately 14 weeks of development). In contrast, we found a postnatal increase in the number of SCs in SMA patients compared with controls. This was particularly noticeable in quadriceps and intercostal muscles versus the diaphragm (Fig. 4), thus reinforcing the characteristic vulnerability or resistance of muscles to the disease. Previous ultrastructural studies in SMA postnatal samples have also shown an increase in SCs compared with control infants (34, 35). These reports were based on morphologic findings, such as heterochromatic nuclei and scant cytoplasm. In the present study, we quantified SCs by positive expression of Pax7 and found a higher number of these cells in the atrophic areas of muscle sections. The large number of SCs may have several explanations, including muscle immaturity (34), regeneration processes, or innervation defects of muscle fibers (36). A relative increase in SCs is also possible as a consequence of the larger number of atrophic fibers observed per field. The increase in Pax7-positive cells in atrophic areas resembles the scenario observed in our SMA and control prenatal muscles, which showed an abundance of SCs (Fig. 3A). Indeed, SCs are most abundant during development and decrease in number thereafter as muscle matures (20). The increase in SCs observed in SMA supports the idea of arrested maturation of muscle fibers, as proposed by van Haelst (34) and later by Saito (35). A more thorough assessment should be performed to confirm this hypothesis. It would be of interest to study the expression of Pax7 and other key factors involved in the process of myogenesis at later stages (37) to evaluate the progression of muscle development during the disease course of SMA and to understand the role of SCs in a nerve-dependent or nerve-independent manner.

We conclude that one of the first findings in SMA myogenesis is a significant decrease in the number of sMHC-positive fibers in the prenatal period, which may be associated with a reduction of primary myotubes. This re-
duction may be a consequence of SCs failing to induce fusion and growth of muscle fibers, leading to an increase in SC numbers at later postnatal stages. Altogether, these results support the hypothesis that a failure or delay in the maturation of muscle fibers is pivotal in SMA pathogenesis. Our study has identified markers of muscle involvement that are useful for a deeper characterization of SMA muscle and myogenesis research. In view of current investigations on early intervention and genetic therapies in SMA (38), our findings contribute to the understanding of SMA pathogenesis in humans and helps to define not only motor neurons but also muscle as potential targets of therapy for this disease.

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