Spinal muscular atrophy: from animal model to clinical trial

Edmar Zanoteli, MD, PhD
Jessica Ruivo Maximino, PhD
Umbertina Conti Reed, MD, PhD
Gerson Chadi, MD, PhD

* Department of Neurology, Medical School of the University of São Paulo (FMUSP), São Paulo, Brazil
** Neuromuscular Section, Associação de Assistência à Criança Deficiente (AACD), São Paulo, Brazil

Corresponding author: E. Zanoteli
Faculdade de Medicina da Universidade de São Paulo (FMUSP)
Av. Dr. Arnaldo, 455, 2nd floor, room 2119
São Paulo, Brazil 01246-903
E-mail: zanoteli@terra.com.br

Summary

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration and loss of lower motor neurons in the spinal cord and brainstem. Clinically, SMA has been classified into four types, according to the maximum function attained. The disease is caused by deletion or mutation of the telomeric copy of the SMN gene (SMN1), and the clinical severity is in part determined by the copy number of the centromeric SMN gene (SMN2). The SMN2 mRNA lacks exon 7, resulting in reduced production of the full-length SMN protein. Treatment of SMA consists of supportive care, although many drugs have been demonstrated to improve muscle strength and motor function of patients. The development of animal models of SMA has led to better interpretation of the physiopathology of the disease and testing of potential drug targets. Several mechanisms have been targeted in SMA drug trials, including neuroprotection, neurogenesis, energy metabolism improvement, anabolic stimulation and increment of SMN2 transcripts. Gene therapy and cell transplantation have also been tested in murine SMA.

KEY WORDS: animal models, clinical trials, SMN gene, spinal muscular atrophy

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease of childhood characterized by degeneration and loss of lower motor neurons in the spinal cord anterior horn cells and somatic motor nuclei in the brainstem, causing progressive proximal symmetrical weakness and atrophy of skeletal muscle. It is the most common inherited neuromuscular disease of childhood with a prevalence of 1 in 6,000 to 10,000 (1,2). Clinically, SMA has been classified into four types on the basis of age at onset and maximum function attained (3). Type I SMA is characterized by onset within the first six months of age, and affected children never achieve the ability to sit without support. Type II SMA is characterized by onset of muscle weakness and hypotonia usually after six months of age; these children are able to sit but not to walk unaided. In type III SMA, the initial manifestations usually occur after 10 months of age, and these patients achieve the ability to walk. Finally, type IV SMA is a mild form with onset of muscle weakness in adulthood. Patients with type IV SMA usually manifest muscle weakness in the second or third decade of life.

In this review of SMA, we focus on the molecular basis of the disease and on the more recent therapeutic strategies.

Clinical aspects and prognosis

Type I SMA (Werdnig-Hoffmann disease) is characterized by severe muscle weakness starting before six months of age. Affected children are not able to sit without support, and the major clinical manifestations include symmetrical proximal muscle weakness, lack of motor development and hypotonia. In the neonatal period and during the first months of life, affected infants have sucking or swallowing problems and severe respiratory distress with abdominal breathing. The diaphragm is not involved until late in the course of the disease, and the muscles of the face are relatively spared (4). Fasciculation of the tongue can be seen in some children, and a postural tremor of the fingers is very occasionally observed (5). Most children with type I SMA die before the age of two years (4), however, patients receiving respiratory support may live longer (5). Children with type II SMA usually present the first symptoms between six and 12 months of age (Fig. 1, over). The maximum motor milestone these children achieve is the ability to sit independently when placed in a sitting position. Although poor muscle tone may be evident at birth or within the first few months of life, individuals with type II SMA may gradually reach motor milestones (5). Finger trembling and hypotonia are common. Type II patients usually lose the ability to sit independently in the second decade of life (6), and approximately 68% of them are alive at 25 years of age (7). These children have normal intelligence. Patients with type III SMA (Kugelberg-Welander disease) manifest the first symptoms after one year of age and, for a period of their life at least, are able to walk independently. Muscles of the proximal portions of the limbs, especially in the legs, are more severely affected. The prognosis of this group of patients generally correlates with the maximum motor function attained (6).
individuals who develop normal walking ability prior to the onset of weakness can maintain this ability until the third or fourth decade of life. In a study on the natural history of the disease, the life expectancy of individuals with SMA III was not found to differ from that of the general population (7). Of the patients studied by Russman et al. (8) over a period of 18 months, none lost strength in the individual muscle groups evaluated, although some lost functional abilities. Whether the loss of motor function observed in individuals with SMA is caused by loss of motor units or other factors, such as scoliosis, progressive contractures and pulmonary insufficiency, is difficult to determine.

Ancillary examinations

Electromyography in SMA patients reveals denervation and diminished motor action potential amplitude. A reduced interference pattern is seen with maximal effort; polyphasic waves, positive sharp waves, and fibrillations are present in all individuals with SMA (9). Motor and sensory nerve conduction velocities are normal. Muscle biopsy reveals grouped muscle fiber atrophy (Fig. 2) and fiber type grouping as opposed to the normal checkerboard pattern. Angulated and large type 1 fibers are also seen (9). The nerve biopsy is usually normal; however, hypomyelination and axonal degeneration have been described in the severe prenatal form (10). The serum creatine-quinase level does not exceed five times the normal upper limits. The abovementioned examinations are useful if molecular genetic testing of the SMN1 gene is unavailable or normal.

Molecular genetics

Approximately 95%-98% of individuals with a clinical diagnosis of SMA lack exon 7 in both copies of the SMN1 gene (Fig. 3), while approximately 2%-5% are compound heterozygotes for deletion of SMN1 exon 7 in one allele and an intragenic mutation of SMN1 in the other allele (11,12). The human SMN gene is located on chromosome 5 (5q), and exists in two copies, SMN1 and SMN2. SMN1 is the telomeric copy and produces a full-length survival motor neuron (SMN) protein necessary for normal lower motor neuron function (11). The centromeric SMN2 copy mostly encodes a protein that is lacking in exon 7 due to alternative splicing, and thus produces a less stable protein. The amount of functional SMN protein produced by SMN2 is not sufficient to prevent progressive motor neuron degeneration when SMN1 is absent. The SMN2 gene copy number is variable, ranging from zero to five. Several studies have demonstrated a strong inverse correlation between the number of SMN2 copies and SMA severity (12-15). Most type I SMA patients carry two SMN2 copies, whereas type II SMA patients carry three, and those with type III

Figure 1 - Muscular atrophy and osteoskeletal deformities in a patient with type II SMA.

Figure 2 - Grouped muscle fiber atrophy (arrow) in the muscle biopsy of the SMA patient shown in figure 1 (Hematoxylin & Eosin staining).

Figure 3 - Single strand conformation polymorphism analysis of the PCR-amplified exon 7 fragment of the SMN gene showing the lack of SMN1 in SMA patients. C = normal control, P = SMA patient (shown in figure 1).
SMA carry three or four SMN2 copies. Individuals with a deletion on the SMN1 gene and with five copies of SMN2 are asymptomatic (14). Due to their disease-modifying properties, SMN2 activation and/or modulation of the SMN2 splicing pattern to boost full-length SMN protein levels have been used as strategies for SMA treatment. Another probable modulator of the clinical severity of SMA is DNA methylation. Hauke et al. (16) have demonstrated that the SMN2 gene is subject to gene silencing by DNA methylation. Their analysis of SMN2 methylation in patients suffering from severe versus mild SMA carrying identical SMN2 copy numbers revealed a correlation between CpG methylation and disease severity (16). These results, related to differences in susceptibility to DNA methylation, provide evidence that SMN2 alleles are not functionally equivalent. Quantitative PCR is currently used for the accurate determination of SMN2 copy number. A PCR-based dosage assay, called “SMA carrier testing” or “SMN gene dosage analysis”, can determine the number of SMN1 gene copies, thus allowing highly accurate carrier detection (13).

**Animal models of SMA**

The most recent advances in SMA research have been achieved thanks to the development of an animal model of SMA, which has not only yielded important insights into the pathogenesis of the disease, but also provided essential in vivo systems for identifying and validating the efficacy of potential treatments (17).

Mice have a single Smn gene that is similar to the human SMN1: it contains a C nucleotide at the +6 position of Smn exon 7 and is not alternatively spliced; it is located on mouse chromosome 13 in the region syntenic to human chromosome 5q13 (18). This information has been used in different strategies to genetically engineer mice to model SMA and study the disease process. The first strategy used homologous recombination technology to generate a knockout allele by creating an in-frame fusion between exon 2a of Smn and the Escherichia coli lacZ gene (19). This strategy abolished Smn expression in the targeted allele and placed lacZ under Smn transcriptional and translational control. Intercrosses that produce homozygous mutant embryos lacking a functional Smn gene (Smn−/−) die before implantation in the uterus (19).

To summarize the genetic situation seen in SMA patients and test the hypothesis that human SMN2 acts as a disease modifier through the small amount of functional SMN protein that each copy produces, two groups of researchers generated transgenic mice that contained the entire human SMN2 locus (20,21). The generated animals had a similar phenotype to that seen in severe type I SMA children, and thus provided a mouse model for this disease. In addition, transgenic mice harboring SMN2 in the Smn−/− knockout background showed pathological changes in the spinal cord and skeletal muscles similar to those observed in SMA patients. These experiments demonstrated that an increase in the copy number of the SMN2 gene results in a milder phenotype in mice, and that motor neuron loss is a late-onset phenomenon that occurs after birth (20,21). The severity of the pathological changes in these mice correlated with the amount of SMN protein that contained the region encoded by exon 7. Interestingly, it has been noted that the level of expression from eight copies of the SMN2 gene on the Smn−/− knockout background completely corrects the SMA phenotype (21).

The SMNΔ7 SMA mouse model, which accurately mimics the human disease, has been used extensively to test potential SMA therapies (22). In the absence of treatment, the reported average lifespan of SMNΔ7 SMA mice ranges from 13 to 18 days (23). The original breeding pairs from the SMNΔ7 SMA mice (SMN2−/−;SMNΔ7−/−;Smn+−) on the FVB background are generated from males and females of the genotype SMN2−/−; SMNΔ7−/−; Smn−/− [FVB.Cg-Tg (SMN2−/Δ7) 4299 Ahmb Tg(SMN2)89 Ahmb Smn(+/−)]. These breeding pairs are phenotypically normal and do not exhibit symptoms of neuropathology. The Tg(SMN2−/Δ7) 4299 Ahmb allele consists of SMA cDNA lacking exon 7 whereas the Tg(SMN2)89 Ahmb allele consists of the entire human SMN2 gene. Offspring resulting from the mating of breeder pairs can have the following genotypes: homozygous for both transgenes and homozygous for the targeted mutation (25%); homozygous for both transgenes and heterozygous for the targeted mutation (50%) and homozygous for both transgenes and wild type at the Smn1 locus (25%). This latter model is a triple mutant mouse that harbors two transgenic alleles and a single targeted mutant. Mice that are homozygous for the targeted mutant Smn allele and homozygous for the two transgenic alleles exhibit symptoms and neuropathology similar to patients. At birth, triple mutants are noticeably smaller than normal littermates. By day 5, signs of muscle weakness are apparent and become progressively more pronounced over the following week as the mice display an abnormal gait, shakiness in the hind limbs and a tendency to fall over. Mean survival is approximately 13 days, and fibers isolated from the gastrocnemius muscle of a 14-day old triple mutant clearly show evidence of atrophy (http://jaxmice.jax.org/strain/005025.html). The SMN2 transgene from the Burghes laboratory, SMN2 (89 Ahmb), contains only the SMN2 genomic locus, whereas the SMN2 transgene from the Li laboratory contains SMN2 (2Hung), the SERF1 gene, and a portion of the NAIP gene. Although there are minor differences between these transgenic models, the essential observations are the same: SMN2 is able to rescue the embryonic lethality of Smn−/− mice and an increase in SMN2 copy number correlates with a milder disease course.

**Pathogenesis**

The pathogenesis of SMA is still under investigation. SMN protein is present in the nucleus and cytoplasm. Nuclear SMN is largely associated with so-called gems, cell structures thought to have a role in mRNA metabolism (24). SMN is also believed to play a role in spliceosome assembly and to function as a specificity factor preventing potentially deleterious non-specific RNA binding (25). Cytosolic SMN is found in axons, and postsynaptically at the neuromuscular junctions, suggesting that the pathogenesis of the disease does not exclusively involve the motor neuron cell body (26). Oprea et al. (27) have demonstrated that unaffected SMN1-deleted
females exhibit significantly higher expression of plastin 3 (PLS3) than their SMA-affected counterparts. The authors showed that PLS3 is important for axonogenesis because it increases the F-actin level. Overexpression of PLS3 rescued the axon length and outgrowth defects associated with SMN down-regulation in motor neurons of SMA mouse embryos and in zebrafish (27). This study suggests that defects in axonogenesis are the major cause of SMA, thereby opening up new therapeutic options for the disease.

Drug treatment

No effective therapies are currently available for SMA, and supportive care remains the principal treatment, which, when provided in the earlier stages of the disease, might prolong survival and reduce morbidity in affected children. In general, the strategies to improve motor neuron function in SMN deficiency include the increase of the SMN protein level, neuroprotection, neurogenesis, energy metabolism improvement, anabolic stimulation, gene therapy and cell replacement.

Increasing SMN protein level

Increasing SMN2 gene expression is one of the major strategies currently under consideration. The modulation of SMN2 expression is in part controlled by acetylation and deacetylation of histones in the promoter region. Thus, histone deacetylase (HDAC) inhibitors, such as phenylbutyrate and valproic acid (VPA), could promote acetylation of the DNA, increasing the gene expression and the level of full-length SMN protein (28). Many studies have demonstrated that phenylbutyrate, a drug used in the treatment of urea acid cycle disorders, and VPA, increase SMN2 transcripts and full-length SMN protein levels in skin fibroblasts and white blood cells (29-31).

Tsai et al. (32) have demonstrated that in type III SMA-like mice, VPA raises SMN protein levels in the spinal cord through SMN2 promoter activation and, probably, restoration of correct SMN2 pre-mRNA splicing. In this study, VPA-treated SMA mice showed better motor function, larger motor evoked potentials, less degeneration of spinal motor neurons, less muscle atrophy, and better neuromuscular junction innervation than non-treated SMA mice. The authors also showed that VPA increases the level of anti-apoptotic factors Bcl-2 and Bcl-xL in the spinal neurons, which may reduce motor neuron apoptosis and can induce neurogenesis in the spinal cord of SMA mice by activation of ERK44/42 (32). However, the number of newly formed neurons was small and there were no newly formed motor neurons. The authors believe that the practical therapeutic contribution of VPA, if any, is more likely to be in terms of increased neuroprotection rather than neurogenesis (32). Although there are many studies showing the favorable effect of VPA on SMN protein expression, a recent one has shown that treatment with VPA, as well as increasing SMN expression, also led to reduced growth cone size and reduced excitability in axon terminals of motoneurons from SMA mice, probably due to an inhibitory function of VPA on voltage-gated Ca2+ channels (33). The authors concluded that VPA treatment might, as a result of different mechanisms, aggravate disease-specific symptoms in SMA patients.

New HDAC inhibitors have recently been tested. Avila et al. (34) demonstrated that trichostatin A, a specific hydroxamic acid class of HDAC inhibitors, when administered after disease onset, activates SMN2 gene expression in vivo, promoting increases of SMN level and improvements in motor unit pathology, motor function and survival in a mouse model of SMA. Pathological analysis of mice treated with trichostatin A showed increased myofiber and anterior horn neuronal size (34). More recently, the same group of researchers showed that early trichostatin A administration in SMA mice combined with aggressive nutritional support results in a remarkable and sustained increase in median survival compared with trichostatin A alone (35), indicating that an optimized nutrition regime combined with HDAC inhibitors may be more effective than either approach alone. Furthermore, Garbes et al. (36), in a recent report, showed that administration of thydroxyamic acid LBH589 (an HDAC inhibitor used in cancer clinical trials) induced in human SMA fibroblasts up to 10-fold elevated SMN levels, accompanied by a markedly increased number of gems without significant cytotoxic effects. Full-length SMN2 expression levels were increased two- to three-fold by transcription activation via SMN2 promoter H3K9-hyperacetylation and restoration of correct splicing via elevated levels of hTRA2-beta1, a protein that binds directly to the specific sequences on DNA, facilitating the inclusion of exon 7. The positive effect was noted even in those cells non-responsive to VPA (36). The authors concluded that this drug is a highly promising candidate for SMA therapy.

Yuo et al. (37) have demonstrated that treatment with an Na+/H+ exchanger inhibitor, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), can decrease intracellular pH, and significantly enhances SMN2 exon 7 inclusion and SMN protein production in SMA lymphoid cell lines. The intracellular pH homeostasis of many mammalian cell types is controlled by the plasma membrane Na+/H+ exchanger. The authors showed that EIPA increases the number of nuclear gems in SMA cells, and may promote SMN2 exon 7 inclusion through upregulation of the splicing factor SRp20 in the nucleus (37). This study, showing that the cellular pH microenvironment can modulate pre-mRNA alternative splicing in vivo, provided a new direction for the development of drugs for SMA treatment (37). However, it is still not clear whether the SMN splicing effects of amiloride are due to other activities of the drug, unrelated to the intracellular pH modulation.

Following the recent development of a steric block antisense oligonucleotide (AO) that blocked an intrinsic splice suppressor element and enhanced SMN2 exon 7 inclusion in SMA patient fibroblasts, Williams et al. (38) showed that periodic intracerebroventricular delivery of this AO in transgenic mice resulted in increased SMN expression in brain and spinal cord, concomitantly with improved bodyweight throughout the lifespan of SMA animals. Treatment of SMA mice with AO also provided partial correction of motor deficits. According to the authors, these results confirm that AOs that abrogate aberrant SMN2 splicing can be considered promising compounds for the treatment of SMA (38).
Hydroxyurea (HU), a medication that enhances the expression of human fetal hemoglobin, also modifies gene expression and increases SMN levels in skin fibroblasts from individuals with SMA (39). Full-length mRNA level and gem number increased significantly, and hnRNP A1 protein decreased in lymphoid and fibroblast cell lines from SMA patients treated with HU at different concentrations (40). Lunn et al. (41) showed that indoprofen, a non-steroidal anti-inflammatory drug and cyclooxygenase inhibitor, is capable of increasing endogenous SMN protein by specific activation of the SMN2 promoter, bringing about a significant increase in the number of nuclear gems in fibroblasts from SMA patients. The mechanisms by which these classes of drugs elevate transcriptional SMN2 gene activity should be better demonstrated, and their efficacy for SMA therapy awaits clinical confirmation.

**Gene therapy and cell replacement**

Considering that neurotrophic factors might have a protective role in SMA, Lesbordes et al. (42) demonstrated that intra-muscular injection of adenoviral vector expressing cardiotrophin-1, a neurotrophic factor belonging to the IL-6 cytokine family, improves median survival, delays motor defects in mutant mice and exerts a protective effect against loss of proximal motor axons and aberrant cytoskeletal organization of motor synaptic terminals. A recent study demonstrated positive effects, on the mouse SMA phenotype, of transplantation of a class of stem cells (spinal cord neural stem cells – NSCs): promotion of neurogenesis and induction of neuroprotective mechanisms (43). The authors showed that NSCs intrathecally transplanted into SMA mice produced improvement of neuromuscular function, increased life span and improved motor unit pathology (43). It was seen that the NSCs, after injection, migrated into the parenchyma and generated a small proportion of motor neurons. Another significant finding of this study, based on global gene expression analysis of laser-capture-microdissected motor neurons from treated mice, was that transplantation of NSCs produced a modification of the SMA phenotype towards the wild-type pattern. The same group of authors showed recently that pluripotent stem cells from embryonic stem cells have the same potential therapeutic effects as those derived from spinal cord and offer great promise as an unlimited source of neural stem cells for transplantation (44). The authors concluded that stem cell transplantation, alone or in combination with other molecular and pharmacological approaches, could prove beneficial to SMA patients in the future.

**Clinical trials**

Pre-clinical evaluations have set out to evaluate the efficacy of HDAC inhibitors in SMA (45). In a pilot trial, orally administered phenylbutyrate led to a short-term motor function improvement in 10 patients with type II SMA without producing any significant side effects (46). Although another study has also shown promising results with the use of phenylbutyrate (47), Mercuri et al. (48), observing 107 patients with SMA from 10 different centers, found no significant improvement in motor function or strength after a 13-week drug treatment. Thus, a clear efficacy of phenylbutyrate in SMA remains to be demonstrated. Valproic acid has been tested in SMA with promising results. However, controlled double-blind and randomized studies are still in progress to better clarify its efficacy in this disease. Brichta et al. (29) observed a 13-fold increase in the blood level of full-length SMN protein in seven out of 10 VPA-treated SMA children. An improvement of muscle strength was reported by Weihl et al. (49) in seven adult patients with SMA type III and IV during VPA treatment. Tsai et al. (50) observed a significant increase in global strength during six months of VPA treatment in type II SMA children. In an open-label study, Swoboda et al. (51) assessed 42 SMA individuals under treatment with VPA over 12 months, and observed a significant improvement that was almost entirely restricted to participants under five years old. These authors also concluded that VPA can be used safely in SMA subjects over two years of age, but that carnitine status must be closely monitored (51). In our service, 22 type II and III SMA children undergoing one year of VPA treatment presented a slight improvement of motor abilities, as measured by the Hammersmith scale. This beneficial effect was more pronounced in the children younger than six years of age (unpublished data).

Another HDAC inhibitor that has already been tested in SMA patients is HU. Liang et al. (40) showed slight increases in muscle strength and full-length SMN mRNA after eight weeks’ treatment of 33 SMA patients (types II and III) with different HU dosages, these finding indicating that this drug is another HDAC inhibitor with potential for use in SMA treatment. Although other drug classes have been tested in SMA patients, no significant improvement was demonstrated in any of them. Considering that cell bodies of bulbar and spinal motor neurons are particularly exposed to glutamate, a potential neurotoxic excitatory amino acid neurotransmitter, inhibitors of glutamate release such as riluzole have been tested in SMA patients. Russman et al. (52) tested riluzole in a small number of SMA infants and observed only slight benefits in some children. Miller et al. (53) showed that gabapentin, another inhibitor of glutamate release, is not beneficial in SMA. A multicenter randomized unblinded and uncontrolled trial with gabapentin in 120 individuals with SMA types II and III showed benefit in terms of muscle strength but no improvement in motor or respiratory function (54). Another drug that has been tested in these patients, on account of its potential neurotrophic effect, is thyrotropin-releasing hormone (TRH); however, no clear difference between treated patients and controls was demonstrated (55). Recently, oral administration of the TRH analogue taltireline hydrate in a patient with SMA type III was associated with significant increases in muscle strength (56). A favorable effect of oral β2-adrenoreceptor agonist on SMN levels in SMA fibroblasts was recently demonstrated (57). Furthermore, salbutamol treatment resulted in significant increases in muscle strength, forced vital capacity (58), and functional scores in children with SMA (59).
Concluding remarks

Spinal muscular atrophy is a motor neuron disease characterized by severe and progressive motor functional impairment caused by mutations in the SMN gene. Ever since the discovery of the related gene and its causative effect on SMA, many studies have identified ways in which SMN protein expression might improve the phenotype, however effective clinical therapies are still not available. Activation of the centromeric SMN2 gene, which is capable of producing at least 10% of the full-length protein, by regulation of the gene promoter or the splicing of exon 7 of the gene are some of the targets of drug therapy. Whereas these approaches seem to trigger benefits in experimental animal models, the results have not yet been fully translated into clinical practice. Furthermore, cell/gene therapies and drugs with potential neuroprotective effects have also been proposed. It seems reasonable to conclude that treatment of SMA requires the development of strategies to complement the multidisciplinary approach and rehabilitation offered nowadays. In addition, it is worth bearing in mind that innovative technologies for neurological rehabilitation have offered solutions to improve the health of patients, increase their level of independence, and give them a better quality of life (60,61).

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