Reduced survival motor neuron (Smn) gene dose in mice leads to motor neuron degeneration: an animal model for spinal muscular atrophy type III

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Spinal muscular atrophy (SMA) is caused by deletion or specific mutations of the telomeric survival motor neuron (SMN) gene on human chromosome 5. The human SMN gene, in contrast to the Smn gene in mouse, is duplicated and the centromeric copy on chromosome 5 codes for transcripts which preferentially lead to C-terminally truncated SMN protein. Here we show that a 46% reduction of Smn protein levels in the spinal cord of Smn heterozygous mice leads to a marked loss of the cytoplasmic Smn pool and motor neuron degeneration resembling spinal muscular atrophy type 3. Smn heterozygous mice described here thus represent a model for the human disease. These mice could allow screening for SMA therapies and help in gaining further understanding of the pathophysiological events leading to motor neuron degeneration in SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is the most common form of motor neuron disease in children and young adults. Clinical symptoms include symmetric muscle weakness and atrophy. At the time of death, severe loss of motor neurons can be observed at all levels of the spinal cord and in the brain stem motor nuclei of the V–XII cranial nerves (1).

At least two candidate genes have been identified in SMA patients. These genes have been named neural apoptosis inhibitory protein (NAIP) (2) and survival motor neuron (SMN) (3). The genomic region containing these two genes is duplicated on the long arm of human chromosome 5. SMN is expressed from both genes (3,4), but gene products from the centromeric SMN copy preferentially lead to alternatively spliced variants lacking C-terminal amino acid sequences (5,6). More than 95% of SMA patients show deletions in the telomeric SMN copy (1,7,8). Expression from the centromeric copy seems to be intact in most affected individuals. Thus, cells derived from SMA patients still express significant levels of SMN protein (9,10), and the question arises whether the disease is caused by reduced SMN dose in motor neurons or by a loss of specific functions associated with structural domains coded by exons 5 and 7, which are lacking in most transcripts coded by the centromeric SMN gene copy.

The gene duplication of SMN is not found in mouse (11–13), and thus represents a relatively recent event during evolution. We have generated mice with disrupted Smn genes by homologous recombination in embryonic stem cells (11). Homozygous inactivation of the Smn gene in mice leads to massive cell death in the early blastocyst stage, corresponding to the initiation of embryonic RNA transcription. This finding supports evidence on the function of Smn in at least two essential cellular processes: in the biogenesis of spliceosomal U snRNPs (14,15) in the cytoplasm and in nuclear pre-mRNA splicing (16). The reason for the relative specificity of the disease for motor neurons is still unknown. In one model it is assumed that motor neurons need more SMN protein than other types of cell for their functional maintenance. Alternatively, and not mutually exclusively with the first hypothesis, SMN may serve additional, so far unknown cell type-specific functions in motor neurons which cannot be compensated by the product of the centromeric SMN gene. In order to investigate the consequences of reduced Smn availability in motor neurons in more detail, we have analyzed motor neurons in mice with a heterozygous inactivation of the Smn gene. These mice were obtained from intercross of mice in which one allele of the Smn gene was disrupted (2). These mice develop a specific form of motor neuron disease characterized by loss of motor neurons between birth and 6 months of age. Smn heterozygous mice described here thus represent a model that resembles the human disease.

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RESULTS

Analysis of Smn protein content in the spinal cord of wild-type and Smn*+/- mice

The Smn protein content in the spinal cord of wild-type mice was investigated by western blot analysis. The highest levels were observed in the embryonic spinal cord. A clear drop in Smn immunoreactivity in spinal cord extracts became apparent between postnatal days 5 and 15. Levels of Smn immunoreactivity were lowest in the spinal cord of 12-month-old mice (Fig. 1a).

Comparison of Smn protein content in the spinal cord of 6-month-old Smn*+/- and Smn*+/- mice showed a reduction of 46.0 ± 9.8% in Smn protein levels (Fig. 1b and c). Similarly, confocal analysis of Smn-immunostained sections of spinal cord from Smn heterozygous mice showed reduced Smn immunoreactivity in the large motor neurons within the ventrolateral part of the lumbar spinal cord (Fig. 1f and g). In these cells, Smn was detectable in the cytoplasm and in specific nuclear structures previously described as gemini of coiled bodies (gems) (17). Interestingly, inactivation of one allele of Smn in mice does not decrease the number of gems in the nucleus. On average, 1.8 ± 0.2 gems were found in motor neurons from wild-type mice whereas Smn*+/- motor neurons contained 2.0 ± 0.3 gems.

Splicing of Ich-1, a candidate mRNA, is not disturbed in the spinal cord of Smn*+/- mice

The amounts of mRNA which could be extracted from spinal cord of Smn*+/- and Smn*+/- mice did not differ significantly (data not shown). In order to investigate whether reduction of Smn protein content in the spinal cord of adult Smn*+/- mice affects splicing of pre-mRNA, we have investigated the specific splicing of a short intron in the Ich-1 pre-mRNA (Fig. 2b). Ich-1 is highly expressed in motor neurons (18, unpublished data). The Ich-1 gene in mice contains an intron of 81 bp which is followed by a short exon of 61 bp (Fig. 2a). This short exon is alternatively spliced and inclusion of this exon leads to an mRNA coding for the short form of Ich-1 (19,20) (Fig. 2a). The ratio of the reverse transcription–polymerase chain reaction (RT–PCR) products corresponding to the pro-apoptotic Ich-1L and anti-apoptotic Ich-1s isoforms is not changed in Smn*+/- mice (Fig. 2b). Moreover, the short intron is properly spliced in both Smn*+/- and Smn*+/- spinal cord, suggesting that splicing of this specific mRNA is not disturbed in 12-month-old Smn*+/- mice.

Quantification of motor neuron loss in Smn*+/- mice

We next analyzed the effect of inactivation of one Smn allele on motor neurons. In the lumbar spinal cord of newborn Smn heterozygous mice, motor neurons appeared normal, both with respect to morphology and cell number. Strikingly, in 6-month-old animals, 40% of the motor neurons were lost in comparison with wild-type mice (Table 1, Fig. 3a and b). Spinal motor neuron loss in 1-year-old mice was >50% (Table 1). However, the loss of spinal motor neurons between 6 and 12 months in Smn*+/- mice was not statistically significant (P > 0.05).

Abnormalities were also detectable in peripheral nerves. In sciatic and phrenic nerves of 6-month-old Smn*+/- mice, a marked increase in the number of degenerating axons could be observed (Fig. 4), as previously identified in patients with SMA (1).
Smn does the typical SMA phenotype become apparent. Similarly, in capability to stand and walk. Only in later stages of development normally during the first months and years after birth and gain the becomes apparent after birth, SMA type III patients develop patients lead to a motor neuron disease which in most cases at least twice as high as the Smn levels in adult mouse spinal cord. A reduction of Smn protein content becomes apparent between postnatal days 5 and 15, and relatively low levels are detectable in the spinal cord of 12-month-old mice (Fig. 1a).

Interestingly, the reduction of Smn protein content in the adult mouse spinal cord leads to severe changes of Smn distribution in the cytoplasm but not in the nucleus (Fig. 1f and g). Recent data have shown that SMN in the nucleus might be involved in the reactivation of spliceosomal complexes, and that overexpression of a truncated SMN (SMNΔN27) leads to severe impairment of splicing of the δ-crystallin pre-mRNA (16). Thus, it was suspected that splicing of pre-mRNAs might be impaired in motor neurons of SMA patients. However, in the spinal cord of adult Smn mice, splicing of Ich-1 is not affected. In the light of this finding and our observation that motor neurons of Smn mice show drastically reduced Smn immunoreactivity in the cytoplasm, it is tempting to speculate that motor neuron degeneration in these animals could result from an impaired function of Smn in U snRNP biogenesis. Alternatively, and not mutually exclusively, reduced levels of Smn may influence other processes such as processing or subcellular transport of specific mRNAs which could be functionally important for the maintenance of these neurons.

Degeneration of motor neurons in Smn mice starts after birth and is progressive until postnatal month 6. However, motor neuron loss in the spinal cord between 6 and 12 months of age is lower than before 6 months of age, and this loss of motor neurons during the late period does not reach statistical significance. Interestingly, this corresponds to observations in SMA type III patients who do not develop motor deficits before an age of 3–6 years. After a critical phase of the disease is over when loss of muscle strength occurs, these patients enter a phase of relatively stable muscle power over an extended time (1).

The start of the disease in lumbar spinal motor neurons occurs earlier than in facial motor neurons. This corresponds to observations that degeneration of facial motor neurons in SMA patients is less pronounced than in spinal motor neurons (1). Thus, Smn heterozygous mice show a phenotype that is reminiscent of the pathophysiological events leading to SMA in patients and therefore could be useful for studying the specific cellular processes leading to motor neuron degeneration in SMA. Furthermore, they could be a useful model for screening of drugs which should prevent motor neuron degeneration in SMA patients.

**MATERIALS AND METHODS**

**Quantification of Smn in spinal cord by western blot analysis**

The spinal cord was freshly dissected from Smn+/- and Smn-/- mice (derived from the same litters), homogenized and dissolved in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). After centrifugation, the protein concentration of the supernatants was observed during the period of physiological cell death between embryonic day 13 and birth (21) or during a subsequent period of several weeks when these neurons are more vulnerable to axonal lesion than in adult animals. This appears inversely correlated to the relatively high expression of the Smn gene during embryonic development. Smn levels in the embryonic mouse spinal cord are at least twice as high as the Smn levels in adult mouse spinal cord.

**DISCUSSION**

Our data show that reduced levels of Smn protein in Smn heterozygous mice leads to postnatal loss of spinal and facial motor neurons. Facial motor neurons are also affected, but motor neuron loss occurs later and is lower in comparison with the lumbar spinal motor neurons. The motor neuron degeneration observed during postnatal development of Smn heterozygous mice reflects specific characteristics of the mild (type III) form of human SMA (1). Thus, although reduced SMN levels in SMA patients lead to a motor neuron disease which in most cases becomes apparent after birth, SMA type III patients develop normally during the first months and years after birth and gain the capability to stand and walk. Only in later stages of development does the typical SMA phenotype become apparent. Similarly, in Smn-/- mice no enhanced cell death of motor neurons was

**Table 1. Number of spinal motor neurons (L1–L6) in Smn+/- and Smn-/- mice**

<table>
<thead>
<tr>
<th>Age</th>
<th>Smn+/-</th>
<th>Smn-/-</th>
<th>Reduction</th>
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<tr>
<td>Postnatal day 1</td>
<td>3180 ± 166 (n = 4)</td>
<td>3119 ± 339 (n = 4)</td>
<td>Not significant</td>
</tr>
<tr>
<td>6 months</td>
<td>3164 ± 431 (n = 4)</td>
<td>1913 ± 239 (n = 4)</td>
<td>-40% (P &lt; 0.05)</td>
</tr>
<tr>
<td>12 months</td>
<td>3229 ± 164 (n = 3)</td>
<td>1458 ± 70 (n = 3)</td>
<td>-54% (P &lt; 0.05)</td>
</tr>
</tbody>
</table>

Similar results were obtained when facial motor neurons were examined. The facial nucleus is a prominent structure in the brain stem of mice, and the number of motor neurons contained in both facial nuclei exceeds the number of motor neurons in the lumbar spinal cord (L1–L6). In 1-year-old Smn+/- mice a significant loss of 23% of the motor neurons became apparent in the facial nucleus (Table 2, Fig. 3c and d).

**Figure 2.** Splicing of Ich-1 mRNA in the spinal cord of 12-month-old Smn+/- and Smn-/- mice. (a) Map of a genomic fragment of the Ich-1 gene containing the upstream exon, a short intron (81 bp) 5' of the 61 bp exon which is alternatively spliced in Ich-1L and Ich-1s, and the downstream exon. The exons are shown as bars. The introns are shown as lines. Arrowheads indicate the position of specific primers used for the RT-PCR assay for detection of alternatively spliced isoforms of the Ich-1 pre-mRNA. (b) Analysis of alternative spliceforms of the Ich-1 pre-mRNA in spinal cord of 12-month-old Smn+/- and Smn-/- mice. PCR analysis reveals two amplification products corresponding to Ich-1s (233 bp) and Ich-1L (172 bp). No PCR product could be amplified at an expected size of 314 bp which would correspond to an amplification product including the short intron 5' of the 61 bp exon which is included in Ich-1s.
determined using the Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA) according to the manufacturer’s instructions. Each supernatant was mixed with the same volume of sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue). The samples were boiled for 2 min and stored at −20°C, and then electrophoresed on a 10% polyacrylamide gel, transferred to Protean membrane (Schleicher & Schuell, Dassel, Germany) with the Biometra Fastblot system B24 at 7 V/cm constant voltage for 1 h in 25 mM Tris pH 8.3, 150 mM glycine, 10% methanol. The Protean membrane was blocked for 30 min with 5% instant milk in Tris-buffered saline (TBS) containing 0.2% Tween (TBS-T). Primary antibody (monoclonal anti-mouse Smn IgG1, 250 µg/ml; Dianova, Hamburg, Germany) was diluted 1:1000, and anti-actin (1 mg/ml; Roche, Mannheim, Germany) diluted 1:1000 in 5% instant milk in TBS-T. The anti-Smn antibody recognizes mouse Smn protein but not truncated Smn lacking the N-terminus with the SIP-1 interaction domain (M. Kralewski and M. Sendtner, unpublished data). The antibodies were incubated with the membrane for 2 h at room temperature. The membrane was washed three times for 15 min at room temperature with TBS-T. Goat anti-mouse horseradish peroxidase-conjugated antibodies (Roche) were used as a secondary antibody at a dilution of 1:10 000 with 5% instant milk in TBS-T. Incubation was for 1 h at room temperature followed by three washing steps. Subsequently, the Smn immunoreactive bands were visualized using enhanced chemiluminescence reagent (ECL; Amersham, Braunschweig, Germany) according to the manufacturer’s instructions. The blots were exposed to the Fuji medical X-ray film (Super RX) for the detection of the chemiluminescent emissions. Each experiment was repeated at least twice, the Smn immunoreactive bands scanned and the intensity quantitated, using the Aida Software 2.0× (Raytest, Straubenhardt, Germany). The intensity of the Smn immunoreactive bands in 6-month-old Smn+/+ mice was 54.0 ± 9.8% (mean ± SD) compared with the bands from Smn+/+ mice on the same membranes. Stripping and reprobing of the blots was done according to the manufacturer’s protocols.

**Immunodetection of Smn in spinal motor neurons**

Six-month-old Smn+/+ and Smn−/− mice from the same litters were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde in phosphate buffer. The lumbar spinal cord was dissected, the L4 segment identified and frozen in Tissuetec (Sakura, Zoeterwonde, The Netherlands). Frozen sections (10 µm) were prepared, blocked with TBS containing 10% bovine serum albumin for 20 min at room temperature, incubated with a mouse monoclonal antibody against Smn (Dianova) at 1 µg/ml, washed three times with TBS, incubated with secondary antibodies (anti-mouse Cy3; Rockland, Gilbertsville, PA), washed again three times with TBS, embedded with 1,4-diazabicyclo[2.2.2]octane (DABCO; Merck, Darmstadt, Germany), and covered with glass slides. Smn immunoreactivity was visualized with a Leica confocal microscope, the settings for pinhole and voltage were identical for the analysis of all sections.
The number of gems was determined in at least 20 motor neurons from Smn+– and Smn+– spinal cord sections in one experiment. This experiment was repeated four times; data shown are from one representative experiment.

Quantification of motor neurons in Smn+– and Smn+– mice

Smn+– and Smn+– mice were derived from the same litters from an Smn+– intercross described previously (11). These mice had been backcrossed five times and subsequently at every third generation with C57/Bl6 mice (Charles River, Sulzfeld, Germany). Mice were housed in cages with protective hoods (filter tops). Sentinels of our mouse colony were tested every 3 months for infection with mouse hepatitis virus, Reo3, Theiler, PVM, Sendai and MVM viruses, and found seronegative. The animals had free access to water and standard diet (Altromin 1314; Altromin, Lage, Germany).

Mice were deeply anaesthetized and transcardially perfused with fixative, as described previously (22) for preparation of paraffin serial sections of the brain stem and spinal cord. Motor neurons were counted in every fifth section of the brain stem and every tenth section of the lumbar spinal cord (L1–L6), and the raw counts were corrected for double counting of split nucleoli as described (22). Semi-thin sections (1 µm) of sciatic and phrenic nerves were prepared from mice which had been perfused with 1% glutaraldehyde and 4% paraformaldehyde according to published methods (22).

Differences between groups were evaluated with Student’s t-test (unpaired, significance level $P < 0.05$), applying the Graphics Prism Program (Graph Pad Software, San Diego, CA).

Detection of alternatively spliced isoforms of Ich-1 by RT–PCR

Spinal cords from 12-month-old Smn+– and Smn+– mice were homogenized in 1 ml of TRIZOL reagent (Life Technologies, Karlsruhe, Germany), the homogenate was then incubated with 0.2 ml of chloroform and centrifuged at 12 000 g for 15 min. The

Table 2. Number of facial motor neurons in Smn+– and Smn+– mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Smn+–</th>
<th>Smn+–</th>
<th>Reduction</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>2308 ± 64 (n = 5)</td>
<td>2486 ± 253 (n = 4)</td>
<td>Not significant</td>
</tr>
<tr>
<td>12</td>
<td>2320 ± 31 (n = 3)</td>
<td>1774 ± 128 (n = 4)</td>
<td>−23% ($P &lt; 0.05$)</td>
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</table>

Figure 4. Axonal pathology in Smn+– sciatic and phrenic nerves. (a and c) Semi-thin sections of sciatic nerves of Smn+– mice; (b and d) the sciatic nerves of Smn+– mice from the same litters. (e and f) Transverse sections of the distal phrenic nerves of Smn+– (e) and Smn+– (f) mice. The number of large fibers appears reduced in both types of nerve. Degenerating axons [arrows in (b), (d) and (f)] and dividing glial cells indicative of Wallerian degeneration [arrowhead in (d)] can be observed. Bar, 25 µm.
aqueous phase was separated and the RNA precipitated with 100% ethanol. The pellet was washed once with 70% ethanol, dried at room temperature and dissolved in 50 μl × TE.

Total RNA (1 μg) was used as a template for each RT–PCR reaction. The first strand cDNA was generated with oligo(dT) primers. The primers used to amplify Ich-1 were: 5′ primer, 5′-ATGCTAATGGTCACAGCTTA-3′, and 3′ primer, 5′-GTCTCATCTTTCAATCC-3′. The following conditions were applied for the PCR reactions: 1× reaction buffer (Genecraft, Münster, Germany), 200 μM dNTPs, 2 μM each primer, 2 U of Taq DNA polymerase (Genecraft); the total volume was 50 μl. The cDNA was denatured for 3 min at 94°C before it was subjected to 25, 28, 30 and 32 PCR cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s, respectively.

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