

Neuronal death is enhanced and begins during foetal development in type I spinal muscular atrophy spinal cord

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Summary

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by mutations in the survival motor neurone gene (*SMN*). The degeneration and loss of the anterior horn cells is the major neuropathological finding in SMA, but the mechanism and timing of this abnormal motor neurone death remain unknown. A quantitative study was carried out comparing neuronal death in controls and SMA foetuses and neonates. Between 12 and 15 weeks of gestational age, a significant increase in nuclear DNA vulnerability, as revealed with the method of *in situ* end-labelling of nuclear DNA fragmentation, was detected in SMA foetuses and was reflected by a decrease in the number of neurones of the anterior horn. Neurones with nuclear DNA vulnerability are no longer detected at the end of the foetal

period and the post-natal period. On the other hand, abnormal morphology of motor neurones, mainly early chromatolytic changes, was observed only after birth. Our findings indicate that in type I SMA, the absence or dysfunction of *SMN* is reflected by an enhanced neuronal death that is already detectable at 12 weeks, the earliest SMA foetal stage analysed. This is associated with a progressive loss of motor neurones towards the neonatal period. Given that a proportion of the remaining SMA motor neurones in the neonatal period appear with pathological findings not detected at earlier stages, it can be hypothesized that type I SMA results in differential age-dependent responses leading to cell death and motor neurone degeneration during development.

Keywords: apoptosis; motor neurone death; motor neurone degeneration; *SMN1*; spinal muscular atrophy

Abbreviations: ELAV = embryonic lethal abnormal visual system; NAIP = neuronal apoptosis inhibitory protein; SMA = spinal muscular atrophy; SMN = survival motor neurone; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end-labelling

Introduction

Childhood spinal muscular atrophy (SMA) is an autosomal recessive disease characterised by degeneration and loss of the anterior horn cells of the spinal cord, resulting in progressive muscular paralysis with atrophy. The childhood SMAs have been divided into three forms 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 neurone (*SMN1*) gene are responsible for SMA. The *SMN1* gene is duplicated alongside a highly homologous copy, *SMN2*, which may act as a phenotypic modifier of the disease (Lefebvre *et al.*, 1995).

Expression of the *SMN* gene has been detected in the motor neurones of the spinal cord as well as in different neuronal populations (Tizzano *et al.*, 1998). The *SMN* protein is part of a complex with various proteins involved in the splicing reaction. This apparently essential function in all cells is critical for motor neurones, which warrants further research to elucidate the mechanism of disease.

During development, a large number of cells degenerate and die by a non-pathological process known as programmed cell death. In many tissues, dying cells display similar changes in morphology and nuclear DNA degradation through an apoptotic process, although it has been suggested

that more than one cell death mechanism may act during development (Schwartz *et al.*, 1993). In SMA, there is degeneration and loss of the anterior horn motor neurones as found in autopsy specimens, but the mechanism and timing of this cell death remain unknown. No description of foetal spinal cord pathology in SMA has been reported so far. Early stage reports refer to neonatal or infant cases (reviewed in Crawford and Pardo, 1996).

The identification of *SMN*, the gene responsible for SMA, allowed us to identify foetuses predicted to have SMA type I in at-risk families and to collect foetal SMA spinal cords for study. We performed a quantitative analysis of neurones and a neuronal death study of the anterior horn of the spinal cord, comparing control and SMA foetuses with infants. The objectives were to determine whether the neurodegenerative process and loss of motor neurones in SMA spinal cord are due to an enhanced cell death through an apoptotic mechanism. We found evidence of a prenatal onset of enhanced motor neurone death and loss in SMA type I spinal cord. After birth, morphological changes were seen in motor neurones suggesting that, in addition to motor neurone loss, motor neurone dysfunction may be also responsible for muscle denervation in SMA.

Methods

Foetal and post-natal specimens

The Ethics Committee of the Hospital Sant Pau and the Research Institute approved the project, and for each case family consent was obtained. Control foetal material from first and second trimesters of gestation was obtained from pregnancies terminated by dilatation and evacuation for social reasons. Gestational age was determined by ultrasound measurements. In total, 58 control samples were collected and analysed. Sixteen SMA foetuses were obtained from abortions after confirmation of homozygous deletion of exon 7 and 8 of the *SMN* gene by chorionic villi DNA analysis (van der Steege *et al.*, 1995). After a careful analysis of all samples, only foetuses with optimal tissue preservation were considered in the present series. Eighteen controls (10–22 weeks) and nine type I SMA specimens (12 weeks, $n = 4$; 15 weeks, $n = 4$; and 22 weeks, $n = 1$) were selected for neuronal counting and analysis of death cells (Table 1). Six of these SMA cases (12 weeks, $n = 2$; 15 weeks, $n = 3$; 22 weeks, $n = 1$) had a homozygous deletion of exon 5 of the neuronal apoptosis inhibitory protein (NAIP). This gene is located in the SMA locus and may be a candidate modifier *SMN* gene (Roy *et al.*, 1995). Also analysed were autopsy post-natal spinal cords from one newborn and three infants (3, 5 and 8 months of age) without SMA that were used as controls, and one newborn and four infants (one at 4 months and three at 9 months of age) affected with SMA type I (homozygously deleted for *SMN1*). Thoracic and lumbar regions of each spinal cord were assigned according to their localization in the macroscopic anatomic identification (i.e. the presence of

Table 1 Number and age of foetuses, neonates and infants studied

	Age group	Weeks/months	No. of samples
Control	1	10	9
	2	12	5
	3	15	3
	4	22	1
	5	1.5 (neonate)	1
	6	3, 5 and 8 months	3
SMA	2	12	4
	3	15	4
	4	22	1
	5	1.5 (neonate)	1
	6	4 (1) and 9 (3) months	4
Total			36

ribs), and immediately fixed by immersion with 4% paraformaldehyde and embedded in paraffin. Sections 5 μ m thick were mounted onto L-lysine 50%-treated slides and stained with haematoxylin–eosin, or processed for immunohistochemistry or TUNEL analysis (*in situ* end-labelling of nuclear DNA fragmentation). Bilateral counts were made of neuronal nuclei at a sampling ratio of every fifteenth or twentieth section in foetal samples. An average of eight sections per thoracic or lumbar region from each age-matched control specimen were analysed and compared with SMA specimens.

Quantitative analysis of neurones of the anterior horn

For quantitative analysis in haematoxylin-stained sections, the area of each one of the ventral horns was defined in square microns, and the number of neurones determined and counted using Visilog 5 (Noesis Vision Inc., St Lauren, Quebec, Canada). The NeuN (Chemicon, Hofheim, Germany) and Hu antibodies were used for the identification of neurones. NeuN immunostaining of neurones at 10 and 12 weeks was patchy and incomplete with respect to Hu (Fig. 1). Data from the later stages showed similar results for both antibodies [see Fig. 2 for Hu, and data not shown for NeuN (available upon request)]. Hu was therefore used to count neurones given that it identifies the neurones of the anterior horn of the spinal cord unambiguously in all stages analysed. The Hu antibody recognizes a nuclear protein with a high degree of homology to ELAV (embryonic lethal abnormal visual system), an important RNA-binding protein for the development of the nervous system in *Drosophila* (Szabo *et al.*, 1991). Previous studies have shown that the Hu antigen is expressed very early on in neuroblast development and, like ELAV, probably plays an important role in the process of neurogenesis (Graus and Ferrer 1990; Marusich *et al.*, 1994).

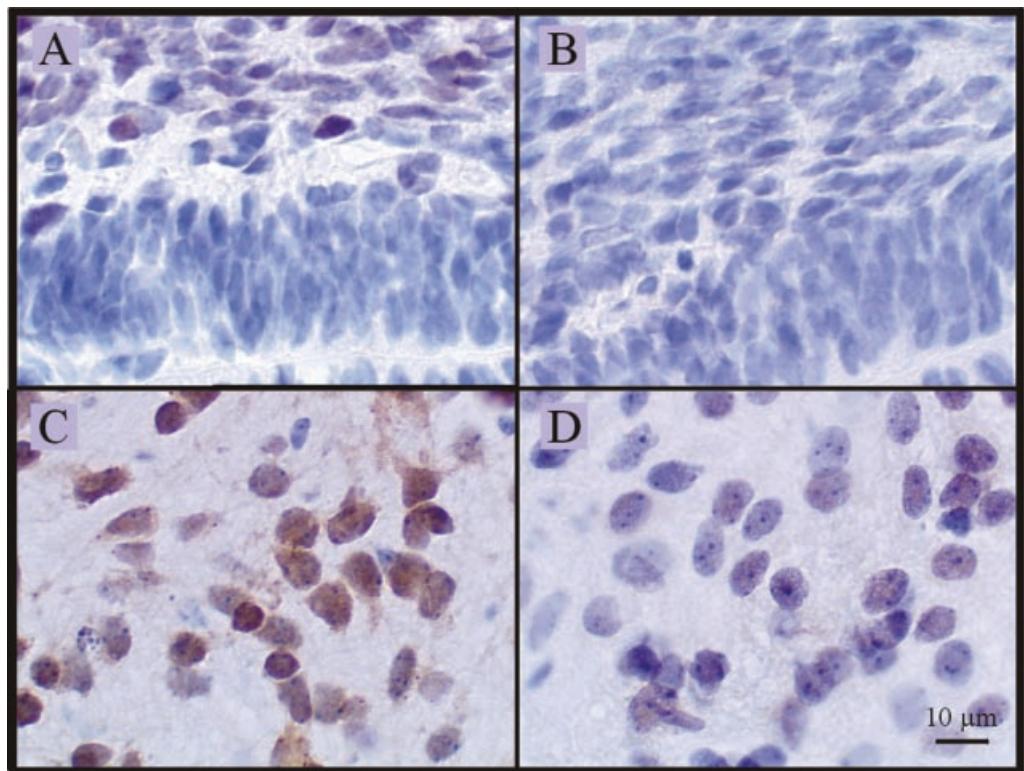


Fig. 1 Comparison of Hu (A and C) and NeuN (B and D) immunostaining in ventral horn spinal cord serial sections of control foetuses at 10 weeks (A and B, same specimen) and 12 weeks (C and D, same specimen). Expression of NeuN at 10 weeks is undetectable in neuroepithelium and neurones of the ventral horn, whereas at 12 weeks expression appears patchy and incomplete with respect to Hu immunostaining. Scale bar: 10 μ m.

The Hu antigen was demonstrated in the nervous system by immunohistochemistry using a purified human biotinylated Hu antibody provided by Dr F. Graus (Service of Neurology, Clinic Hospital, Barcelona, Spain). The serum was obtained from a patient with paraneoplastic encephalomyelitis and recognized bands of 35–40 kDa by western blotting. The purified antibody stained the nuclei of neurones of the central and peripheral nervous system of rats perfused through the heart with 4% paraformaldehyde and processed in paraffin sections. The specificity of the antibody was tested by incubating a few sections without the primary serum (negative staining), demonstrating the abolition of the immunoreactivity by pre-incubation with a different non-biotinylated anti-Hu positive serum. Although the anti-Hu antibody recognizes a nuclear antigen in optimal tissue sections, cytoplasmic staining could be seen in post-mortem material due to cytoplasmic leakage of the antigen (Figs 1 and 2). This situation could also be found when analysing better preserved material. Motor neurones of the foetal anterior horn were identified as those with Hu-positive nuclei and, to a lesser degree, cytoplasmic immunostaining, with a large nucleus and prominent nucleoli surrounded by a thin rim of cytoplasm (Figs 1 and 2). The nuclei of 15 motor neurones by section were traced from each individual to estimate the mean nuclear diameter at different ages.

DNA fragmentation studies in neurones of the anterior horn

In haematoxylin–eosin-stained sections, apoptotic cells from the anterior horn were identified by their morphological appearance, such as extreme chromatin condensation and formation of apoptotic bodies (Fig. 3A and B). *In situ* end-labelling of nuclear DNA fragmentation was carried out using the ApopTag peroxidase kit (*in situ* apoptosis detection kit; Intergen, Purchase, NY, USA) according to the manufacturer's instructions. The peroxidase reaction was visualized with 0.05% diaminobenzidine (Sigma, Alcobendas, Madrid, Spain) and 0.01% hydrogen peroxide. Tissue sections were counterstained with haematoxylin (Fig. 3C–F). As positive controls, brain sections of Sprague–Dawley rats irradiated with a single dose of 2 Gy γ -rays at post-natal day 3 were utilized.

Immunohistochemical procedures

Dewaxed sections were processed for immunohistochemistry following the avidin–biotin peroxidase method (ABC kit, Vectastain; Vector, Burlingame, CA, USA). After blocking endogenous peroxidase with hydrogen peroxide and methanol, the sections were treated with sodium citrate buffer 10 mM pH 6 for 25 min at 100°C and left overnight. Next, the

sections were incubated at 4°C overnight with an affinity-purified, biotinylated, Hu antiserum (1 : 3500 dilution) and normal serum (1 : 100 dilution). Then, the sections were incubated with ABC at 1 : 100 dilution for 1 h at room temperature (ABC Elite kit, Vectastain; Vector, Burlingame, CA, USA). The peroxidase reaction was visualized with 0.05% diaminobenzidine (Sigma, Alcobendas, Madrid, Spain) and 0.01% hydrogen peroxide. The sections were slightly counterstained with haematoxylin.

Double labelling: TUNEL and immunohistochemistry for Hu

To determine the neuronal or glial origin of TUNEL-positive cells, a double procedure of *in situ* end-labelling of nuclear DNA fragmentation and immunohistochemistry for Hu was carried out. First, the *in situ* end-labelling of nuclear DNA fragmentation was performed as described above. Then, immunohistochemistry for Hu was also performed as described above, but the peroxidase reaction was visualized with 0.03% nickelated diaminobenzidine (Sigma, Alcobendas, Madrid, Spain) and 0.01% hydrogen peroxide. Sections were not counterstained.

Processing of the data and statistical analysis

All sections were blindly examined, counted and reviewed by three of the investigators at different magnifications using a Nikon ES-400 microscope, and the images and data were processed using Visilog version 5 (Noesis Vision Inc., St Lauren, Quebec, Canada). For the neuronal count, the interclass correlation coefficient among the observations of the three investigators was 0.818, for the TUNEL-positive cells it was 0.982 and for the mean nuclear diameter it was 0.988. Given that the values obtained by the investigators were very similar, the final result of each item was the mean. In control samples, the total number of neurones was calculated according to the average number of anterior horn sections per number of specimens for each age group. The mean motor neurone nuclear diameter in each age group was also measured. Finally, the apoptotic cells morphologically detected by *in situ* end-labelling of nuclear DNA fragmentation were calculated according to the average of number of anterior horn sections per number of specimens for each age group. The three parameters were compared using analysis of variance and the Scheffé *post hoc* test for multiple comparisons between the groups. We also compared the total number of neurones, the nuclear diameters and the number of apoptotic cells detected by morphology and by *in situ* end-labelling of nuclear DNA fragmentation in control and SMA samples (12, 15 and 22 weeks of gestation) and neonates using *t*-test. *P* values <0.05 were considered significant between the groups (see Table 1 for identification of groups and gestational age, and Figs 4–6 for descriptive statistics).

Results

Studies in control foetuses

In control foetuses, at 10 weeks the total number of neurones in the anterior horn was 842 per $10^5 \mu\text{m}^2$ (data not shown) and declined gradually from 12 to 22 weeks and the neonatal period (Fig. 4) (*P* < 0.0005). There was no statistical difference between the thoracic and lumbar anterior horns (*P* = 0.384). The mean nuclear diameters of motor neurones increased from 8.05 μm at 10 weeks (data not shown) to 8.5 μm at 12 weeks, then to 11.27 μm in the neonatal period (Fig. 5) (*P* = 0.017). There was no statistical difference between thoracic and lumbar nuclei (*P* = 0.566).

Counting of TUNEL-positive cells in the anterior horn of the spinal cord demonstrated an average of 4.65 cells at 10/11 weeks (data not shown) followed by a gradual decrease (*P* = 0.050). All the cells counted had apoptotic morphology (Figs 3 and 6). There was no significant difference in the number of TUNEL-positive cells between thoracic and lumbar anterior horns of the spinal cord (*P* = 0.382; data not shown).

Comparison between control and SMA foetuses

The total number of neurones (per number of square microns) on sections of the ventral horn was decreased in SMA foetuses at 12, 15 and 22 weeks and in the neonatal period when compared with controls, although this failed to achieve statistical significance (*P* = 0.174). This resulted in the lower curve seen in Fig. 4, although similar temporal evolution of both groups occurred (*P* = 0.944). The mean nuclear diameters of SMA neurones were similar to those of controls (*P* = 0.396) (Fig. 5). TUNEL-positive cell counting comparing control and SMA foetuses from 12 and 15 weeks of gestation demonstrated significant differences in the number of cells (*P* < 0.0005) (Fig. 6). The statistical difference was maintained even when comparing gestational age (12 weeks, *P* < 0.0005; 15 weeks, *P* = 0.003), and thoracic (*P* = 0.037) and lumbar regions (*P* = 0.005) separately. Although the number of neurones in the thoracic and lumbar SMA sections was similar (*P* = 0.895), a mean of 2.99 TUNEL-positive cells per anterior horn was detected in the thoracic SMA sections, whereas the mean was 4.67 in the lumbar sections. This tendency of the lumbar sections to present more TUNEL-positive cells was significant (*P* = 0.023). No significant difference was detected between NAIP+ or NAIP- SMA foetuses. In 22-week-old foetal samples we did not detect apoptotic cells by morphological appearance or TUNEL-positive staining in the anterior horns.

Findings in the neonatal and infant period

The number of neurones in sections of the anterior horn of a SMA neonate was approximately 50–60% lower than in a non-SMA neonate. Even though some motor neurones have a normal appearance, a subpopulation of motor neurones showed peripheral displacement of the

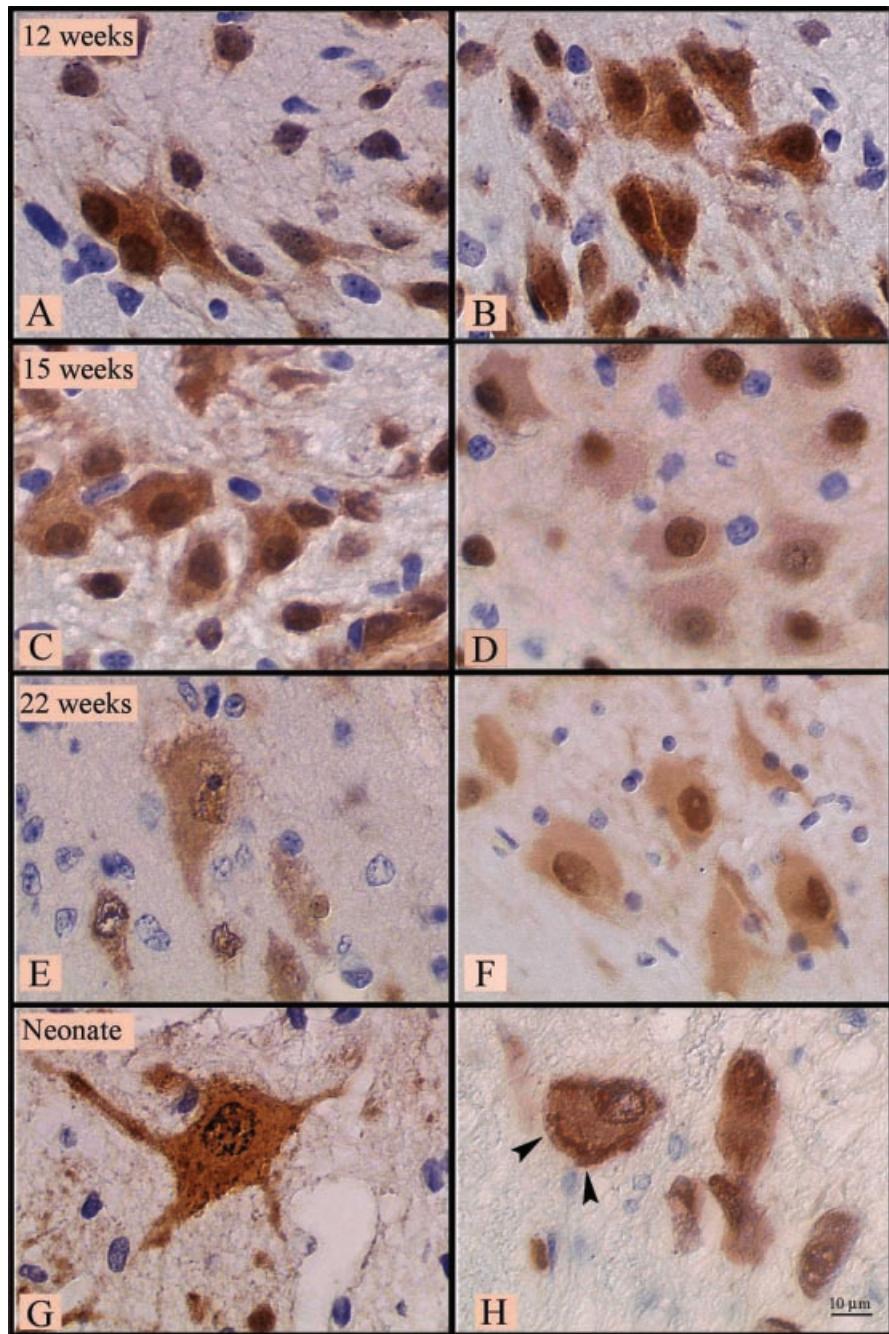


Fig. 2 Ventral horn spinal cord sections of foetuses at different gestational ages and one neonate showing positive staining of neurones with Hu antibody. Motor neurones can be identified as neurones with large nuclei and prominent nucleoli. (**A, C, E and G**) Controls; (**B, D, F and H**) SMA. Note the similar appearance of control and SMA motor neurones in the foetal stages analysed. Arrowheads indicate early chromatolysis in a motor neurone of a SMA neonate with peripheral displacement of the nuclei. Scale bar: 10 μ m.

nuclei and central reduction in the number of Nissl corpuscles, which was consistent with early central chromatolysis (Fig. 2). This was also found in the motor neurones from SMA infants (Fig. 7). At this level of detection, none of these observations were clearly made in SMA foetuses during the prenatal stages

analysed. Analysis of post-natal spinal cords did not show apoptotic cells by morphological appearance or TUNEL-positive staining in the anterior horns. The sparse TUNEL-positive cells identified tested negative for Hu immunoreactivity, therefore we may conclude that these were of glial origin (Fig. 7).

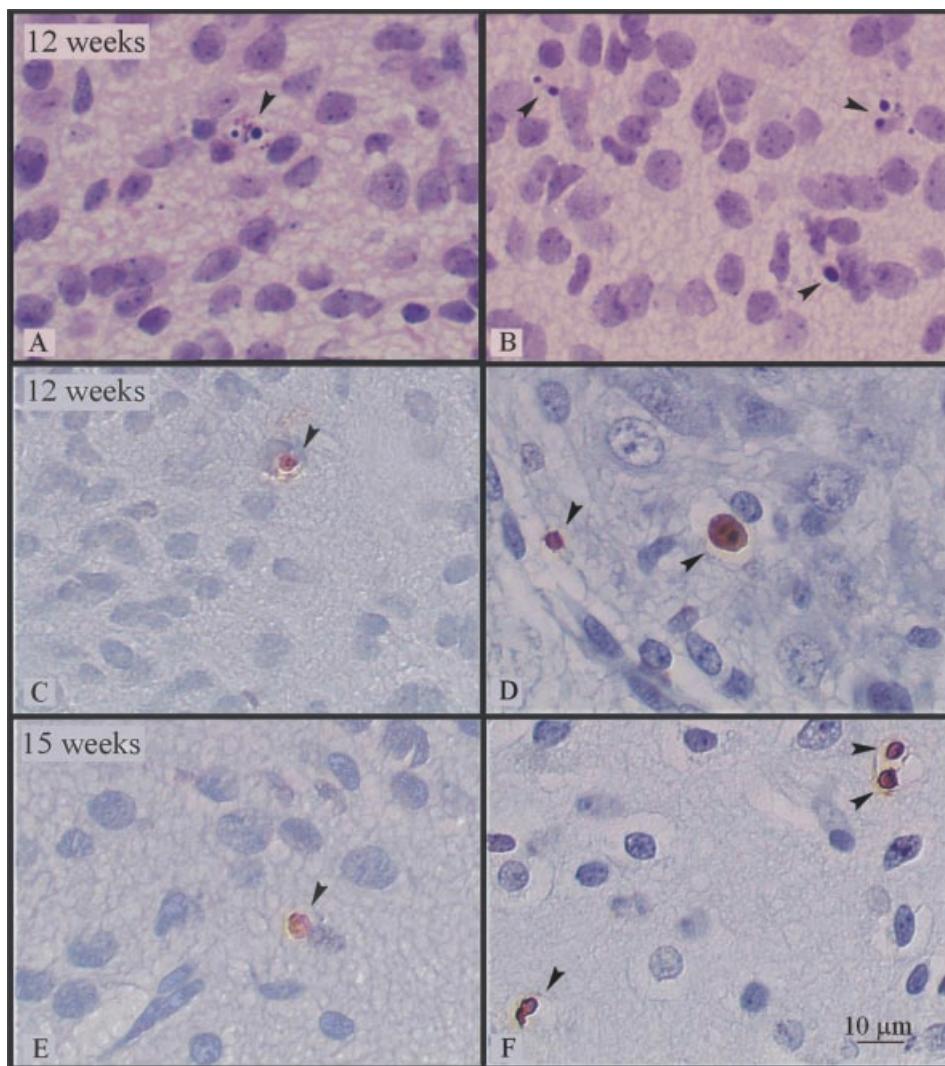


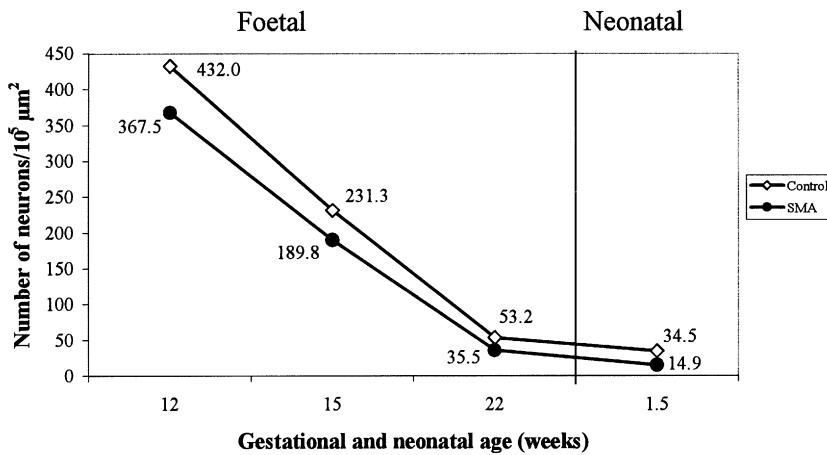
Fig. 3 Apoptotic cells of the foetal anterior horn. (A–B) Haematoxylin–eosin staining of 12-week foetal anterior horn sections. (A) Control foetus. Arrowheads indicate apoptotic cells. (B) SMA foetus. Arrowheads indicate apoptotic cells. (C–D) *In situ* end-labelling of the nuclear DNA fragmentation technique in 12-week anterior horn sections showing the positive apoptotic cells (arrowheads). (C) Control foetus; (D) SMA foetus. (E–F) *In situ* end-labelling of the nuclear DNA fragmentation technique in 15-week foetal anterior horn sections showing positive apoptotic cells (arrowheads). (E) Control foetus; (F) SMA foetus. Scale bar: 10 μ m.

Discussion

To our knowledge, this is the first report to focus on the quantitative analysis of motor neurone loss and neuronal death during development in SMA human spinal cords. In control foetuses, a decrease in the number of neurones in sections of the anterior horn from weeks 10 to 22 was detected in parallel with the presence of TUNEL-positive cells during this period. In agreement with our results, Forger and Breedlove (1987) found a peak of degeneration during human development between weeks 12 and 16, and described a complete absence of pyknotic cells after 25 weeks. They also found there was a dramatic decrease in the number of motor neurones between weeks 11 and 25–26 of gestation. They concluded that motor neurone death during normal

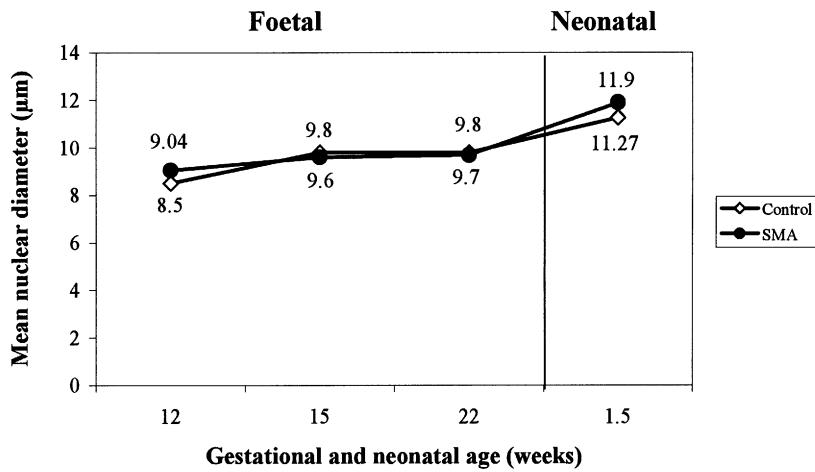
development probably begins at around 8–11 weeks, coincident with the functional neuromuscular contact as reported in other species.

By quantitative analysis of sections of the anterior horn, we found that SMA foetuses had between 15 and 35% fewer neurones when compared with controls. Although the tendency of SMA foetuses to possess fewer neurones failed to reach statistical significance, this was a consistent finding in all samples analysed. It is possible that the variability of the measure (hundreds of cells) together with the limited number of samples decreased the statistical power of the analysis. On the other hand, the number of TUNEL-positive cells represented a small percentage of the total neurones in the anterior horn, and their values were significantly increased at 12 and



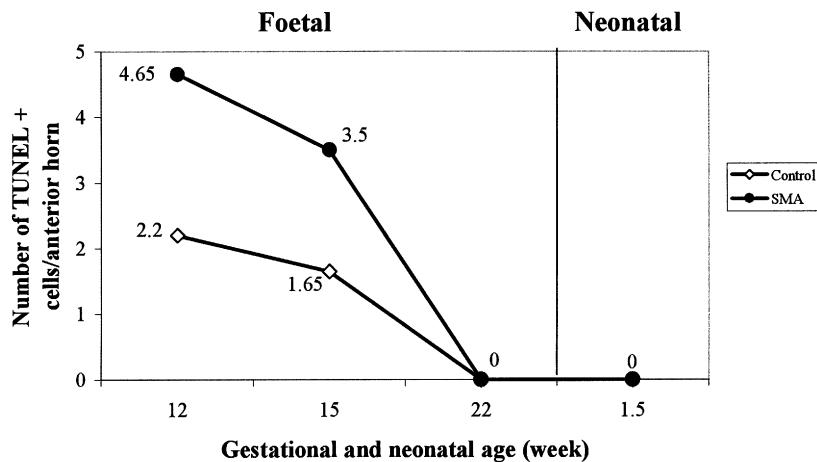
Number of neurons in the anterior horns of foetal spinal cords								
Weeks	12	SD	15	SD	22	SD	1.5	SD
Control	432.0	160.8	231.3	69.6	53.2	8.1	34.5	1.5
SMA	367.5	64.4	189.8	34.1	35.5	2.9	14.9	0.6

Fig. 4 Total Hu-positive neurone counting of sections of the anterior horns from 12–22 weeks and during the neonatal period. There is a gradual decrease from 12–22 weeks in the control group. In SMA the number of neurones is lower but the shape of the curve is similar. Type I SMA neonates showed almost 50% fewer neurones than controls. Given that the mean nuclear diameter of motor neurones was similar in controls and SMA (see Fig. 5), no correction formula was used in the counting.



Mean nuclear diameter (μm)								
Weeks	12	SD	15	SD	22	SD	1.5	SD
Control	8.5	1.2	9.8	1.2	9.8	1.8	11.27	1.6
SMA	9.04	1.3	9.6	1.8	9.7	0.9	11.9	2.2

Fig. 5 Mean nuclear diameter of motor neurones of the anterior horns. There are no significantly different values among them.



Number of TUNEL + cells by anterior horn section of foetal and neonatal spinal cords				
Weeks	12	SD	15	SD
Control	2.2	1.1	1.65	0.5
SMA	4.65	1.2	3.5	1.5

Fig. 6 TUNEL-positive cell counting of the anterior horn from 12–22 weeks and during the neonatal period. There is a gradual decrease in the number of TUNEL-positive cells from 12–22 weeks. In SMA the number is significantly increased at 12 and 15 weeks.

15 weeks in the SMA samples. Taken together, these observations suggest that neuronal death in SMA is already present at 12 weeks (the earliest stage analysed). Interestingly, there were more TUNEL-positive cells detected in the lumbar SMA spinal cord. Given that DNA breaks can occur very early in apoptosis, the TUNEL method detects apoptotic cells prior to recognition based on morphological changes. The increase in the number of cells with their nuclear DNA fragmented in ventral horns of SMA foetuses means that the natural apoptotic process that occurs in the anterior horns during foetal development is enhanced in SMA. Given that TUNEL staining can also detect necrotic cells with their DNA fragmented, other mechanisms of cell death, such as necrosis, autophagic degeneration or non-lysosomal vesiculate degeneration, could also be involved (Clarke, 1990). However, apoptosis is morphologically defined by the presence of early chromatin condensation and the formation of apoptotic bodies, whereas necrosis can be identified by cytoplasmic and nuclear changes. Apoptosis in the present series was considered on the basis of characteristic morphology and positive TUNEL staining.

It has been suggested that the neuronal cell death programme is impaired in SMA, thus resulting in a pathological persistence or reactivation of normally occurring apoptosis (Sarnat, 1983). This hypothesis is strongly supported by the observations reported here, showing an increased number of apoptotic cells in SMA anterior horn during the foetal period. However, it is not clear whether apoptosis plays a role in the post-natal progression of Werdnig–Hoffmann disease. In this work, virtually all post-

natal TUNEL-positive cells identified were negative for Hu immunostaining indicating that these were cells of glial origin. Hu is considered as an early neuronal marker and antigenicity of Hu-immunoreactive cells was demonstrated even in advanced apoptotic stages (Ferrer *et al.*, 1993). Hayashi *et al.* (1998) were unable to show TUNEL-positive cells in post-natal spinal cords in four cases with Werdnig–Hoffmann disease, although some TUNEL-positive cells were observed in the lateral nuclei of the thalamus and, in one case, in the cerebral cortex as well. Homozygous deletion of the *SMN* gene was not confirmed in these cases (Hayashi *et al.*, 1998). However, Simic *et al.* (2000) reported the presence of apoptotic cells in the spinal cords of five children with Werdnig–Hoffmann disease, confirmed by genetic analysis of the *SMN* gene. Interestingly, positive *in situ* nuclear DNA fragmentation was not seen in chromatolytic neurones, but in apparently normal neurones and glial cells. Finally, we were unable to find apoptosis in motor neurones of the anterior horns in infants with Werdnig–Hoffmann disease (Ferrer, 1996) and in Holstein-Friesian calves with SMA (Pumarola *et al.*, 1997), although the same methods were useful in demonstrating apoptosis during the foetal period. Taken together, these data suggest that motor neurone death largely takes place prenatally (in the early events of the disease) but might progress during the post-natal period in a subset of cases with end-stage Werdnig–Hoffmann disease.

The expression of the *SMN* gene and its protein has been shown early on in development (Burlet *et al.*, 1998; Tizzano *et al.*, 1998) and the absence of *SMN1* in this period may be reflected by an enhanced foetal motor neurone death. The

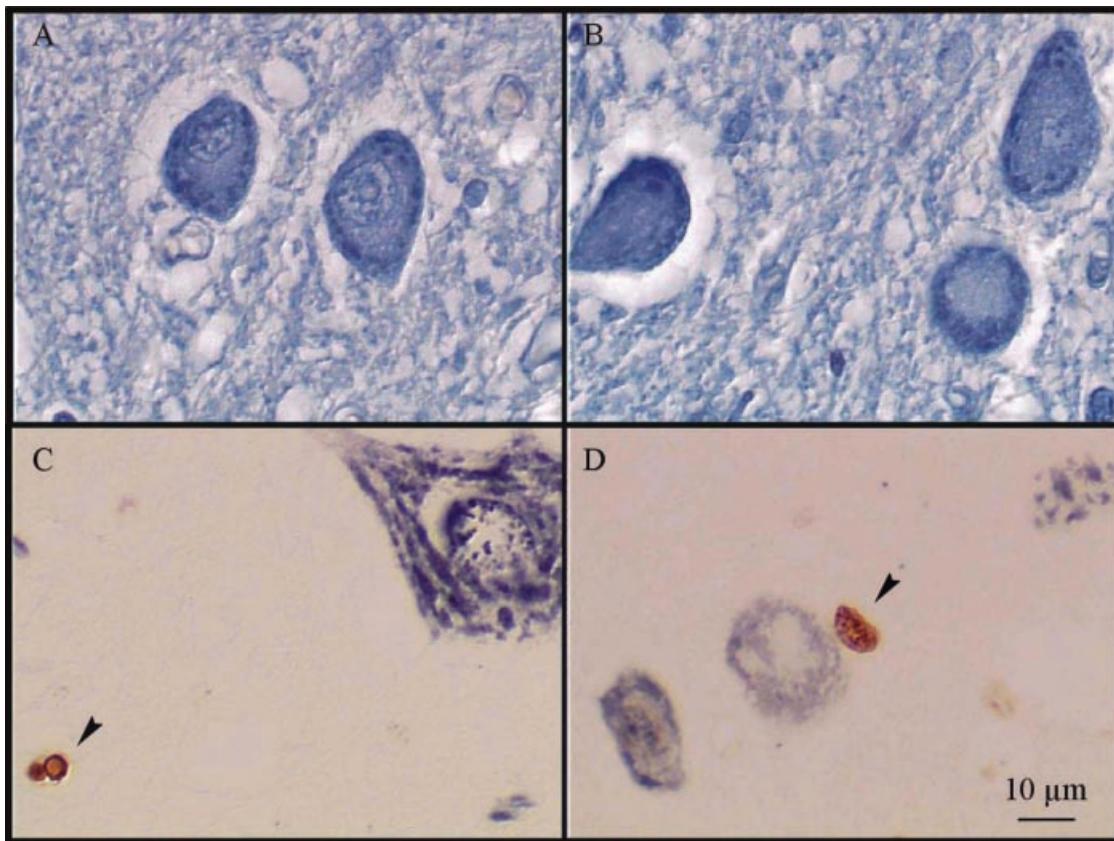


Fig. 7 Motor neurones of post-natal SMA type I cases. (A–B) TUNEL staining of two 9-month-old infants' SMA. These sections show an absence of apoptotic morphology or TUNEL-positive cells, but they also show morphological changes, mainly peripheral displacement of the nuclei and central reduction in the number of Nissl corpuscles, consistent with early central chromatolysis. (C–D) Double-labelling TUNEL (developed with standard diaminobenzidine, brown staining) and Hu (developed with nickelated diaminobenzidine, dark blue staining). The arrowheads indicate TUNEL-positive cells with negative staining for Hu, demonstrating that these cells are not neurones but glial cells. (C) Non-SMA 5-month-old infant showing an Hu-positive motor neurone. (D) SMA 4-month-old infant with chromatolytic Hu-positive motor neurones and a positive TUNEL glial cell. Scale bar: 10 μ m.

beginning and maintenance of this phenomenon could be modulated by other factors such as the functional effect of the number of copies of *SMN2*, the *NAIP* gene (Liston *et al.*, 1996), or other unknown genetic or cellular factors. Experimental results showing that full-length SMN could have a protective anti-apoptotic effect on rat neurones were recently reported. Moreover, it appears that mutant SMN derivatives from patients with SMA (such as SMN Δ 7 or Y272C) accelerate neuronal death (Kerr *et al.*, 2000).

The present findings suggest that motor neurones may degenerate or disappear during the perinatal period through a mechanism different from apoptosis. Since all SMA specimens of our series were related to type I SMA, the most severe form of the disease, it is possible to speculate that type II or III cases may develop this kind of neuronal death subsequently during development or even post-natally. We have not observed, at this level of detection, significant differences in the morphology of foetal SMA and control motor neurones. However, in the neonatal period, neurones with eccentric nucleus and early chromatolytic changes were observed. We detected that the number of motor neurones in

the neonatal period was reduced by 57% compared with controls, a finding in agreement with those reported by Simic *et al.* (2000). Although the loss of motor neurones has been considered the major neuropathological finding in SMA, abnormalities in remaining motor neurones may account for additional signs of muscle denervation.

The *SMN* gene is not duplicated in the mouse and homozygous inactivation of the *Smn* gene in mice leads to massive cell death in the early blastocyst stage (Schrank *et al.*, 1997). Recently, four reports appeared concerning the development of SMN-deficient mice models that mimic human SMA. Hsieh-Li *et al.* (2000) and Monani *et al.* (2000) created mice with a different number of transgenic copies of *SMN2*, developing a phenotype resembling severe or milder forms of the disease. Monani *et al.* noticed that neuronal death appeared after birth in the final stages of the disease. In mice dying at 6 days, there are neither degenerative changes of motor neurones nor signs of skeletal muscle denervation, suggesting that an acute degenerative process might occur. However, since some mice died very shortly after birth, degenerative changes in the motor neurones may begin in the

foetal stages. Jablonka *et al.* (2000) reported a *SMN* heterozygous mouse without signs of denervation or motor defect, showing post-natal loss of spinal and facial motor neurones until month 6, which then decreased. Finally, Frugier *et al.* (2000) developed a conditional deletion of the murine exon 7, generating a mouse with reduced life expectancy consistent with chronic SMA (type II and III). The authors reported severe skeletal muscle denervation with morphological changes in motor neurones, such as indentation in the motor neurone nuclear membranes and nuclear fragmentation despite the absence of motor neurone loss. This last finding suggests the presence of an apoptotic process and that motor neurone loss is a late manifestation of the disease in these animals. These authors noted that these changes had not been reported in human SMA and, probably, that they represented early events of neuronal degeneration that subsequently led later to motor neurone loss (Frugier *et al.*, 2000). The increased neuronal death detected in our SMA foetuses agrees with this hypothesis although it is necessary to obtain evidence for the timing of neuronal death in the milder human forms of the disease.

In conclusion, our observations show that neuronal death in the anterior horn of the spinal cord of SMA type I individuals begins during foetal development. The increased number of TUNEL-positive cells found in our series suggests that this process involves an apoptotic mechanism consistent with enhanced naturally occurring programmed cell death. The absence of apoptotic motor neurones after birth, together with the pathological changes seen in post-natal motor neurones, allows us to hypothesize that another unknown mechanism may affect motor neurones in this period. Therefore, SMA may result in differential age-dependent responses leading to cell death and motor neurone degeneration. The manifestations of type I SMA seem to be the consequence of progressive motor neurone loss during development and dysfunction of the remaining motor neurones in the perinatal–neonatal period, leading to muscle denervation. These observations open new avenues for therapy in SMA. Unravelling of the role of *SMN* and other modifier genes or factors in all of these phenomena will help us in circumventing the molecular defect and in finding an alternative approach to an early intervention to treat or cure this disease.

Acknowledgements

We wish to thank Judith Melki for helpful comments and criticism, Teresa Folip, Jose Luis Alvarez, Agustín Clua, Rosemary Arguelles, Victoria Cusí, Carmen Rodríguez, Juan Parra, María Amenedo and David Chitayat for assistance in obtaining and preparing foetal and post-natal human material, Francesc Graus and Iñigo Rojas for the purified anti-Hu antibody, and Rosa Blanco, Rosa Rivera, Margarita Carmona, Berta Puig, Marta Barrachina and Isabel Domínguez for technical help. This work was funded by Telemarató TV3 Malalties Hereditaries 98-810, and by FIS (Fondo de

Investigación Sanitaria) grant numbers 98-556, 99-1118 and 00-0481.

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Received August 1, 2001. Revised November 27, 2001.

Accepted February 4, 2002