Downregulation of Bcl-2 Proteins in Type I Spinal Muscular Atrophy Motor Neurons During Fetal Development

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Abstract. Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by mutations in the survival motor neuron gene. The degeneration and loss of the anterior horn cells constitute the major neuropathological finding in SMA, although the mechanism and timing of this abnormal motor neuron death remain unknown. It has recently been reported that the fetal SMA spinal cord shows a significant increase in cells with DNA fragmentation, suggesting that the programmed cell death is aberrantly increased in type I SMA during development. We have analyzed 2 antiapoptotic proteins, Bcl-2 and Bcl-X, by Western blot and immunohistochemistry screening for differential expression in control and SMA fetal spinal cords. Expression of these proteins was found in various neuronal populations and structures of the developing spinal cord. At 15 weeks, motor neurons of SMA fetuses showed a marked decrease in the levels of Bcl-2 and a delay in the expression of Bcl-X in comparison with controls. The difference in the pattern and degree of expression is consistent with a role for both proteins in the aberrant programmed cell death observed in type I SMA.

Key Words: Apoptosis; Bcl-2; Bcl-X; Human development; Motor neuron death; SMN1; Spinal muscular atrophy.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by degeneration and loss of motor neurons of the spinal cord, resulting in progressive muscular paralysis with skeletal muscular atrophy. This disease can be divided into 3 groups according to clinical criteria. Type I (Werdnig-Hoffmann disease) is the most severe, with onset occurring before 6 months and death by 2 years. Type II is the intermediate form, with onset before the age of 18 months and with patients never gaining the ability to walk. Type III (Kugelberg-Welander disease) is a milder form, with onset after the age of 18 months and amputation preserved.

The survival motor neuron (SMN) gene, which when mutated causes SMA (1), is expressed in the motor neurons of the spinal cord as well as in various neuronal populations (2). In vitro studies have demonstrated an interaction between the SMN protein and various components of the spliceosomal small nuclear ribonucleoproteins (snRNPs), forming a complex (3, 4). Although SMN plays a crucial role in the biogenesis of mRNA (an essential function for all cells), the reason for the almost exclusive involvement of motor neurons in SMA is still unknown.

In the central nervous system, as in other tissues, more cells are produced than are ultimately required by the organism, and the surplus cells subsequently die by means of programmed cell death at specific times during development (5). In SMA there is a supraphysiological loss of the anterior horn motor neurons, although the mechanism of this pathological cell death is not known. It has been suggested that in this disease the neuronal cell death program is affected, leading to a pathological persistence or reactivation of normally occurring apoptosis (6). Considerable evidence suggests that apoptosis is a mechanism of motor neuron death in SMA. Experimental results showing a protective antiapoptotic effect on rat neurons conferred by full-length SMN and also demonstrating that mutant SMN derivatives (e.g., SMNΔ7 or Y272C) accelerate neuronal death have recently been reported (7). Apoptosis has also been detected in motor neurons of postnatal Werdnig-Hoffmann disease (8), although this may reflect the end stage of the disease. To gain insight into the active mechanism of disease we focused on developmental studies. We found a significant increase in the number of TUNEL-positive cells in the anterior horn of SMA type I fetuses, suggesting an apoptotic mechanism consistent with increased naturally occurring programmed cell death (9). Recently, similar results have been reported elsewhere, reinforcing the evidence of aberrant fetal apoptosis in SMA (10).

The Bcl-2 family of proto-oncogenes encodes proteins that regulate programmed cell death in several physiological and pathological conditions (11, 12). Bcl-2 regulates cell death and survival during the development of the nervous system (13). Bcl-X is a member of this family and may play a role in brain development since “knockout” mice for this gene suffer massive apoptosis of immature cortical and spinal neurons (14).
In the present study we examined the expression of Bcl-2 and Bcl-X in control and SMA spinal cord sections at different fetal developmental stages in order to determine a possible involvement of these proteins in the cell death process in SMA.

MATERIALS AND METHODS

Fetal Specimens

The Ethical Committee of the Hospital Sant Pau and Research Institute approved the project and family consent was obtained for each case. Control fetal material from first and second trimesters of gestation was obtained from elective terminations of pregnancy. Gestational age was determined by ultrasound measurements. Seventeen fetuses with optimal tissue preservation were considered in the present study. Nine controls: 12 weeks (n = 4), 15 weeks (n = 4), and 22 weeks (n = 3) and 8 type I SMA specimens: 12 weeks (n = 4), 15 weeks (n = 3), and 22 weeks (n = 1) were processed for this study. SMA fetuses were obtained from abortions after confirmation of homozygous deletion of exon 5 and 8 of the SMN gene by chorionic villi DNA analysis (15). Six of these SMA cases (12 weeks = 2; 15 weeks = 3; 22 weeks = 1) had homozygous deletion of exon 5 of the neuronal apoptosis inhibitory protein (NAIP), localized also in the SMA locus (16). Thoracic and lumbar regions of each spinal cord were assigned according to their location in the macroscopic anatomic identification (i.e. presence of ribs) and immediately fixed by immersion with 4% formaldehyde, and then embedded in paraffin. Five-μm-thick sections were mounted onto L-Lysine 50%-treated slides and slides were then washed with TRIS-buffered solution pH 7.4 in an ultrasonic bath for 30 min, and finally to streptavidin-peroxidase for 15 min at room temperature. The Ethical Committee of the Hospital Sant Pau and Research Institute approved the project and family consent was obtained for each case.

Immunohistochemical Procedures

Dewaxed sections were processed for immunohistochemistry following the avidin-biotin-peroxidase method (ABC kit, Vector, Burlingame, CA). After blocking endogenous peroxidase with hydrogen peroxide and methanol, the sections were treated with sodium citrate buffer 10 mM pH6 for 25 min at 100°C and left overnight. The sections were then incubated with normal serum for 2 h at room temperature (1:5 dilution). They were next incubated at 4°C overnight with a rabbit polyclonal antibody Bcl-2 (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500. Bcl-2 antibody (N-19) was raised against a peptide mapping at the amino terminus of Bcl-2 of human origin whereas Bcl-XL was directed to the central region of the protein. As negative control reactions, the anti-Bcl-2 antibody (dilution 1:75) was preincubated with an excess of Bcl-2 peptide (dilution 1:75) for 1 h at room temperature and the sections were incubated with the preabsorbed antibody. The peptide of the Bcl-XL antibody from Transduction Laboratories was not available and sections were incubated with the blocking solution for the negative control (data not shown). After the incubation, the sections were exposed to the biotinylated secondary antibody for 15 min, and finally to streptavidin-peroxidase for 15 min at room temperature (HRP Rabbit/Mouse/Goat Universal LSAB®+kit, DAKO). The peroxidase reaction was visualized with 0.05% diaminobenzidine (Sigma) and 0.01% hydrogen peroxide. The sections were slightly counterstained with hematoxylin and eosin for morphologic analysis or processed for immunohistochemistry.

Morphologic Changes in Apoptotic Cells

Apoptosis involves a progressive contraction of cell volume and widespread chromatin condensation but with the initial preservation of the integrity of cytoplasmic organelles. Cells undergoing apoptosis separate into membrane-bound fragments that are rapidly phagocytosed by adjacent cells. In keeping with a study by Clarke (17), we considered apoptotic cells as those with a pyknotic nucleus and a visible clump of nucleolar material and a large soma with substantial cytoplasm. Cells showing condensation of nuclear chromatin, broken spherical profiles apparently engulfed by phagocytes, and rounded masses were also considered.

Immunoblot

Spinal cords from 1 control (15 weeks) and 1 type I SMA fetus (15 weeks) were available and were processed. Samples were homogenized in Nicholson treatment buffer (125 mM TrisHCl, pH 6.8; 4% SDS; 4M urea; 5% mercaptoethanol; 10% glycerol, 0.001% bromphenol blue) (18) and then boiled for 5 min. The extracts were separated by SDS-PAGE using a mini-protein system (Bio-Rad, Madrid, Spain) with low range molecular weight standards (Bio-Rad). Protein analyzed on gel electrophoresis was electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were then washed with TRIS-buffered solution pH 7.4 and 0.1% Tween-20 (TTBS), and blocked with TTBS containing 5% skimmed milk and 1% BSA for 60 min. The membranes were incubated overnight at 4°C with the polyclonal Bcl-2 antibody at a dilution of 1:150. After washing, the immunoblots were incubated with horseradish peroxidase-conjugated antirabbit secondary antibody (1:2,000 dilution; DAKO, Barcelona, Spain) for 1 hour (h) at room temperature, washed again, and developed with the SuperSignal Chemiluminescent Substrate (Pierce, Rockford IL). Stripping of the membrane and subsequent reprobing with a rabbit polyclonal antibody Bcl-XL, the predominant isoform of the Bcl-X gene (Transduction Laboratories, Lexington, KY) at a dilution of 1:5,000 was performed. A final stripping and subsequent reprobing with a monoclonal actin antibody was performed. Preincubation of the Bcl-2 antibody with available peptide was used to test the specificity of the antibody (see below).

Densitometric Analyses

Western blot densitometric analyses were performed by scanning and analyzing the Bcl-2, Bcl-X and β-actin band intensities using the imaging analyser Scion Image (Scion Corporation, Frederick, MD) to calculate their ratios.

Interpretation of the Results

The Bcl-2 and Bcl-X immunoreactivity was compared in each of the gestational stages in control and SMA individuals. Positive immunostaining was considered and quantified visually, comparing the positive zones with the negative control.
Fig. 1. Global immunostaining pattern of Bcl-2 in fetal spinal cord. Twelve-weeks gestational age control (A) and SMA (B) samples immunostained with the Bcl-2 antibody. The expression is seen in the median and lateral anterior horn motor neuron cluster (arrowheads), the postmigratory young neurons of the posterior horn, the neuroepithelial cells of the central canal, and the posterior septum. No differences were noted between controls and SMA samples. Control (C) and SMA (D) 15-weeks gestational age samples. Note the loss of expression in SMA anterior horn compared with controls (arrowheads). Expression is also decreased in the posterior horn. Scale bar = 100 μm.

zones of the same section. The presence of Bcl-2 and Bcl-X differential immunostaining was also studied in apoptotic cells.

RESULTS

Bcl-2 Expression

Bcl-2 expression at 12-weeks gestational age was detected in all the samples analyzed. In the spinal cord, practically all neuronal types showed a positive cytoplasmic immunostaining. Specific cells that showed a more intense reactivity included the median and lateral anterior horn motor neuron cluster, the postmigratory young neurons of the posterior horn, the neuroepithelial cells of the central canal, and the posterior septum. Strong expression was also seen in the axons located in the dorsal root ganglia and the peripheral nerve. No differences were noted between control and SMA samples (Figs. 1, 2). At 15 weeks, whereas positive immunostaining was still detected in the control samples (although to a lesser degree), a marked decrease was seen in anterior horn motor neurons of SMA fetuses (Figs. 1, 2). Expression was detected in the other spinal cord structures and in the dorsal root ganglia and peripheral nerves (Fig. 2). A 22-week SMA sample demonstrated a similar pattern (data not shown). The difference in the amount of Bcl-2 observed by immunohistochemistry in 15-week control and SMA samples was confirmed by Western blot analysis, which showed a 28 kDa Bcl-2 band with an approximately 55% decrease in SMA (Fig. 3).

Bcl-X Expression

At 12-weeks gestational age we found a weak expression of Bcl-X, particularly in motor neurons and the posterior horn neurons, without differences between control
Fig. 2. Detailed immunostaining pattern of Bcl-2 in fetal spinal cord. Panels A–D: Twelve-weeks gestational age. Control (A) and SMA (B) motor neurons of the anterior horn. Control (C) and SMA (D) of peripheral nerve structures. No differences are detected at this stage. The arrowheads in (A) and (B) indicates apoptotic cells. Panels E–H: Fifteen-weeks gestational age. Control (E) and SMA (F) motor neurons of the anterior horn. Note the marked absence of positive immunostaining in SMA motor neurons. Control (G) and SMA (H) of peripheral nerve structures. Scale bar = 10 μm.

and SMA. The expression was stronger in the central canal and in the 2 septa as well as in dorsal root ganglia and peripheral nerves (Fig. 4). At 15 weeks, a strong immunoreactivity was observed in motor neurons of control fetuses together with a positive expression in the other structures. However, this was not the case in the SMA specimens, where a proportion of motor neurons and neurons of the posterior horn did not show expression (Fig. 4). At 22 weeks, we still found several neurons without staining or with a weak staining in a SMA specimen. Thus, the increasing expression of Bcl-X during the fetal development seems to be irregular and delayed in type I SMA. The difference in the amount of Bcl-X observed by immunohistochemistry in 15-week control
and SMA samples was confirmed by Western blot analysis, which showed a 26 kDa band with an approximately 75% decrease in SMA (Fig. 3).

We were unable to detect a differential immunoreactive pattern of the 2 proteins in the apoptotic cells identified by morphology (Figs. 2, 4). The pattern was similar in all of the apoptotic morphological phases from control and SMA samples.

The possibility of antibody cross-reaction between the Bcl-2 and Bcl-X antibodies employed in this study was virtually excluded by the discovery of a consistent differential temporal and spatial pattern of cellular expression between the 2 antibodies.

**DISCUSSION**

In this work we describe the pattern of Bcl-2 and Bcl-X expression by immunohistochemistry in control and SMA fetuses at 12- and 15-weeks gestational age. In control fetuses, Bcl-2 expression is predominant in motor neurons at 12 weeks, but somewhat reduced at 15 weeks. Little or no Bcl-X expression was detected in motor neurons at 12 weeks, with an increase at 15 weeks. Similar results have been reported in fetuses of from 10- to 20-weeks gestational age (19, 20). This period coincides with an increase in physiological motor neuron death and with the establishment of functional neuromuscular synapses (21). Bcl-2 and Bcl-X may both be required for the survival of early postmitotic neurons before appropriate synaptic connections are established. After Bcl-2 is downregulated, Bcl-X expression may be necessary to continued neuronal survival (22), in addition to its role in target-derived neurotrophic factors. We found that the physiological reduction of Bcl-2 observed in control motor neurons at around 15 weeks appeared earlier and to a greater degree in SMA motor neurons. Moreover, Bcl-X, which appears to compensate for the Bcl-2 reduction at this stage, showed a patchy and delayed pattern of expression in most of the SMA motor neurons.

The downregulation of Bcl-2 and the irregular expression of Bcl-X in motor neurons may contribute to the increased neuronal death described during the development of SMA fetuses (9, 10). Thus, these results reflect an insufficient Bcl-2 and Bcl-X protection during this period. Bcl-X is weakly expressed at more advanced gestational ages in SMA, which may explain why the enhanced cell death described at 12 to 15 weeks is no longer detected in SMA at advanced fetal stages (9). In agreement with our observations in fetal specimens, Simic et al described reduced Bcl-2 levels in motor neurons of the spinal cord from postnatal SMA type I cases (8). They suggested that the loss of cytoplasmic Bcl-2 expression in patients with SMA favors apoptotic motor neuron death in type I SMA. Interestingly, the three 15-week SMA samples in our study had homozygous absence of NAIP. Although SMA may be attributed exclusively to the loss of SMN function, it is possible that enhanced apoptosis due to the lack of NAIP may exacerbate the disease process. The NAIP gene was initially considered a possible phenotypic modifier gene (16) but its influence on the SMA phenotype has not yet been demonstrated. Further studies on SMA samples with the presence of at least 1 copy of NAIP could be instructive.

An interaction between SMN and Bcl-2 was reported by Iwahashi et al (23). These authors found that the coexpression of SMN with Bcl-2 produced a synergistic preventive effect on Bax-induced apoptosis, a pro-apoptotic factor that sequesters or binds Bcl-2. SMN and
Bcl-2 proteins appear to bind with each other in regions that are essential to their function (24). However, other investigators have failed to demonstrate this interaction (25). The neuronal degeneration in SMA patients is mainly restricted to anterior horn motor neurons. Our results show that in SMA tissues, other cell types and fibers in the spinal cord, dorsal root ganglia, and peripheral nerves are capable of maintaining the expression of Bcl-2 longer, in agreement with the findings of other authors (13, 20). Moreover, Iwahashi et al postulated that SMN might be crucial only in cells with a decreased Bcl-2 expression, such as motor neurons (23). Motor neurons capable of

Fig. 4. Detailed immunostaining pattern of Bcl-X in fetal spinal cord. Panels A–D: Twelve-weeks gestational age. Control (A) and SMA (B) motor neurons of the anterior horn. Control (C) and SMA (D) of peripheral nerve structures. The expression is not seen in motor neurons, whereas it is present in peripheral nerve structures without significant differences between control and SMA samples. The arrowheads in (B) indicate apoptotic cells. Panels E–H: Fifteen-weeks gestational age. Control (E) and SMA (F) motor neurons of the anterior horn. The expression is detected in control motor neurons whereas it is irregular or absent in most of the SMA motor neurons. Control (G) and SMA (H) of peripheral nerve structures. Scale bar = 10 μm.
expressing Bcl-X may prolong their survival by extending anti-apoptotic activity without SMN. However, it is not possible at this stage to rule out other protective factors that might play a role in this process. It has been suggested that the control of cell death may vary with lineage and stage of development owing to the existence of neurons expressing differing subsets of Bcl-2 family members (26). Moreover, the relative expression level of Bcl-2-related death agonist and antagonist is not the sole parameter for determining susceptibility to apoptosis. Thus, posttranslational modification of Bcl-2-related proteins also has a major impact on apoptosis regulation (27). In this context, the mutated form of the SMN protein may alter the interaction between Bcl-2 and SMN, and thus interfere in its synergistic antiapoptotic activity (23).

These results yield interesting insights into the pathogenesis and mechanism of the disease in SMA and warrant further investigation into the cell death pathway during SMA development. The identification of additional protectors or enhancers of apoptosis could play a key role in the search for an early therapy for this disease.

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