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Original article

New insights into the pathogenesis of spinal muscular atrophy

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Abstract

To clarify the pathomechanism of spinal muscular atrophy (SMA) with mutations in the gene for survival motor neuron (SMN) protein, postmortem neuropathological analyses were performed on spinal cords obtained at autopsy from 2 fetuses with SMA, 5 infants and a low teenager with SMA type 1, and a higher teenager with SMA type 2; the diagnosis of all of them was confirmed clinically and genetically. Histopathologically, it was noted that lower motor neurons (LMNs) in the SMA cases showed immature profiles characterized by fine Nissl bodies restricted to the periphery of small round somata with a few cell processes in the fetal period, and showed small-sized profiles in the postnatal period. LMNs began to reduce in size and number in the fetal period, ballooned neurons (BNs) appeared postnatally, and the remaining LMNs including BNs diminished with age. BNs were filled with phosphorylated neurofilament protein, and morphologically similar to but smaller than typical chromatolytic neurons as axonal reaction. The population of survived LMNs was relatively preserved in an SMA type 2 case, who lived to 17-year-old, as compared to SMA type 1 cases. Immunohistochemical analysis demonstrated expression of Bcl-2, Bax, activated caspase-3 and SMN in the LMNs prominent in the fetal cases. There was no significant difference in staining for these substances between the control and SMA cases. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay revealed no significant signal in the control and SMA cases. Given that downregulation of SMN leads to a failure in neurite outgrowth and neuromuscular contact of LMNs, the present results suggest the involvement of a fetal developmental maturation error as well as a postnatal retrograde dying-back degeneration of LMNs in SMN-mutated SMA.

Keywords: Spinal muscular atrophy (SMA); Clinicopathological study; Ballooned neuron; Survival motor neuron (SMN); Programmed cell death

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal-recessive neurological disorder characterized by progressive loss of lower motor neurons (LMNs) in the spinal cord and lower brain stem, associated with a resultant neurogenic muscular atrophy [1,2]. SMA is subclassified into three clinical phenotypes: Werdnig-Hoffmann disease (type 1), intermediate form (type 2) and Kugelberg-Welander disease (type 3). SMA type 1 is the most severe form, with clinical onset before 6 months of age; the majority of the affected patients naturally die by 1-year-old (y), although some survive beyond 2 y with artificial ventilation. SMA types 2 and 3 patients are affected before and after 18 months of age, respectively.

The gene for survival motor neuron (SMN) protein, responsible for the majority of SMA cases, was identified in 1995 on human chromosome 5q13 [3]; this gene is duplicated with a telomeric copy (SMN1) and a centromeric analogue copy (SMN2) [3]. The SMN1 gene produces full-length SMN transcript, whereas the SMN2 gene produces mainly SMN transcript lacking exon 7

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and to a lesser extent reduced levels of full-length SMN transcript [3,4]. The reduced levels of full-length SMN transcript result in downregulation of SMN protein, thereby causing SMA. Modifier genes such as the neuronal apoptosis inhibitory protein (NAIP) gene [5] and the p44 gene [6] are located on the same locus to that of the SMN gene. SMN protein is ubiquitously distributed in the fetal period and markedly reduced in the postnatal period, indicating that this substance is required for embryonic-fetal development [7,8]. Studies using immunochemical and immunohistochemical approaches disclosed a significant downregulation of SMN protein in the spinal cords of SMA patients as compared to normal controls [9,10]. The SMN1 gene is homozygously mutated in more than 90% of the SMA patients [3]. Therefore, many of the SMN1-mutated SMA patients have only the SMN2 gene, leading to reduced levels of full-length SMN protein. In particular, deletion of the SMN1 gene, which does not alter the number of SMN2 copies, causes severe SMA phenotype, whereas the conversion of the SMN1 gene to the SMN2 gene, which could increase the number of SMN2 copies, causes the milder SMA 2 or 3 phenotype [11].

Although there have been only a few postmortem studies on SMA spinal cords particularly from types 2 and 3 [12,13], the pathological differences between SMA type 1 and milder phenotypes including SMA fetus have not yet been fully understood. Furthermore, little is known about the precise mechanism underlying various onset ages of the three SMA phenotypes with a common genetic error. While programmed cell death (PCD) has been shown to be involved in the pathogenesis of SMA type 1 [14–16], mechanisms by which LMNs are degenerated in SMA types 2 and 3 remain to be determined. To address this issue, we examined postmortem spinal cords obtained at autopsy from fetal and postnatal SMA cases by morphological approaches to LMN pathology, focusing on histopathological verification and the state of expression of apoptosis-related proteins and SMN as well as DNA fragmentation. We also assessed the significance of our results in comparison with the literature.

2. Materials and methods

2.1. Subjects and tissue preparation

This investigation was carried out on spinal cords removed at autopsy from 9 SMA cases and 13 control cases (Table 1), and performed after obtaining written informed consent from the family members by clinical geneticists of the Japan Society of Human Genetics in our department of genetic counseling, in accordance with the "Guidelines Concerning Genetics Research" of eight gene research-related societies. Of the 9 SMA cases, two were fetuses with unknown type, five were infants with type 1, one was a low teenager with type 1, and the rest was a higher teenager with type 2. The postnatal 7 SMA patients demonstrated the characteristic clinical features of SMA meeting clinical criteria [2]. An SMA type 1 Case S7, who did not underwent genetic examinations, was an elder brother of an SMA type 1 Case S5, who presented deletions in exons 7 and 8 in the gene for SMN.

Multiple 6-μm-thick sections were cut from formalin (10%)-fixed, paraffin-embedded spinal cord materials of each case. Sections were then used for histopathological, immunohistochemical and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) analyses.

2.2. Histopathological and immunohistochemical analyses

The primary antibodies used for immunohistochemistry were mouse monoclonal IgG_1 against phosphorylated neurofilament protein (p-NFP) (Clone No. SMI31; diluted 1:10,000; Sternberger Monoclonals, Baltimore, MD, USA), rabbit polyclonal IgG against ubiquitin (Cat. No. Z458; diluted 1:500; Dako, Glostrup, Denmark), mouse monoclonal IgG_1 against Bcl-2 (Clone No. 124; diluted 1:500; Dako), rabbit polyclonal IgG against Bax (Cat. No. Ab-1; diluted 1:20; Oncogene Research Products, San Diego, CA, USA), rabbit polyclonal IgG against activated caspase-3 (Cat. No. 42835; diluted 1:500; Genzyme, Cambridge, MA, USA), and mouse monoclonal IgG_1 against SMN protein (Clone No. 2B1; diluted 1:100; gifted from Dr. Gideon Dreyfuss) [17].

Sections were deparaffinized, rehydrated, quenched with 3% hydrogen peroxide for 5 min at room temperature to inhibit endogenous peroxidase activity, rinsed in phosphate-buffered saline, pH 7.6 (PBS), pretreated for 30 min at room temperature with 3% nonimmune animal serum to block nonspecific antibody binding, and then incubated overnight at 4 °C with the primary antibodies. Prior to staining for Bcl-2 and SMN protein, antigen retrieval was performed by microwaving sections in 100 mM citrate buffer, pH 6.0 (400 W, 95 °C, 35 min). Immunoreaction products were visualized by the avidin–biotin–immunoperoxidase complex (ABC) method. 3,3′-Diaminobenzidine tetrahydrochloride (DAB) and hematoxylin were used as the chromogen and counterstain, respectively. Sections from which the primary antibodies were omitted served as negative reaction controls. Immunohistochemical localization of the examined substances were observed by light microscopy, and verified by comparison with consecutive sections stained with hematoxylin–eosin (H&E).

2.3. TUNEL analysis

Sections were deparaffinized, rehydrated, digested with 20 μg/mL proteinase K (Sigma Chemical, St.