Neuroscience 181 (2011) 226–233

SURVIVAL OF MOTOR NEURON PROTEIN OVER-EXPRESSION PREVENTS CALPAIN-MEDIATED CLEAVAGE AND ACTIVATION OF PROCASPASE-3 IN DIFFERENTIATED HUMAN SH-SY5Y CELLS

R. S. ANDERTON,[a*] B. P. MELONI,[a,b] F. L. MASTAGLIA,[a] W. K. GREENE[c] AND S. BOULOS[a]

[a]*Centre for Neuromuscular and Neurological Disorders, University of Western Australia and Australian Neuromuscular Research Institute, QEII Medical Centre, Nedlands, WA, Australia*

[b]*Department of Neurosurgery, Sir Charles Gairdner Hospital, WA, Australia*

[c]*School of Veterinary and Biomedical Sciences, Murdoch University, WA, Australia*

Abstract—Spinal muscular atrophy (SMA), a neurodegenerative disorder primarily affecting motor neurons, is the most common genetic cause of infant death. This incurable disease is caused by the absence of a functional SMN1 gene and a reduction in full length survival of motor neuron (SMN) protein. In this study, a neuroprotective function of SMN was investigated in differentiated human SH-SY5Y cells using an adenoviral vector to over-express SMN protein. The pro-survival capacity of SMN was assessed in an Akt/PI3-kinase inhibition (LY294002) model, as well as an oxidative stress (hydrogen peroxide) and excitotoxic (glutamate) model. SMN over-expression in SH-SY5Y cells protected against Akt/phosphatidylinositol 3-kinase (PI3-kinase) inhibition, but not oxidative stress, nor against excitotoxicity in rat cortical neurons. Western analysis of cell homogenates from SH-SY5Y cultures over-expressing SMN harvested pre- and post-Akt/PI3-kinase inhibition indicated that SMN protein inhibited caspase-3 activation via blockade of calpain-mediated procaspase-3 cleavage. This study has revealed a novel anti-apoptotic function for the SMN protein in differentiated SH-SY5Y cells. Finally, the cell death model described herein will allow the assessment of future therapeutic agents or strategies aimed at increasing SMN protein levels.

Key words: SMN, anti-apoptotic, adenoviral, SMA, caspase-3.

With an incidence of 1 in 6,000 live births, spinal muscular atrophy (SMA), an autosomal recessive disorder characterized by progressive motor neuron loss and muscle weakness, is the leading genetic cause of infant mortality (Lefebvre et al., 1995; Crawford and Pardo, 1996; Feldkotter et al., 2002). Spinal muscular atrophy is caused by mutation(s)/deletion of the *survival of motor neuron 1* (*SMN1*) gene, which leads to a critical reduction in survival of motor neuron (SMN) protein levels. Humans uniquely possess a second centromeric *SMN* gene, termed *survival of motor neuron 2* (*SMN2*), which is derived from a duplication and inversion event on chromosome 5 (Lefebvre et al., 1998; Rochette et al., 2001). The SMN2 gene contains a synonymous base change in exon 7 (C→T) which forces exclusion of exon 7 from approximately 90% of pre-mRNA transcripts, resulting in the formation of a truncated and unstable protein (Lefebvre et al., 1995; Lorson and Androphy, 2000). The clinical phenotype and severity of SMA is categorized into four types, based on the age of onset, motor achievement and SMN2 copy number (Roberts et al., 1970; McAndrew et al., 1997).

Ubiquitously expressed throughout the central nervous system and particularly in the spinal cord, the SMN protein can localize to the nucleus of cells as part of structures known as gems (Liu and Dreyfuss, 1996; Coovert et al., 1997; Lefebvre et al., 1997). In addition, the SMN protein, when localizing to the cytoplasm and nucleus of cells plays an important role in the assembly of small nuclear ribonucleoproteins (snRNPs). However, while the latter function is well understood, SMN's role in motor neuron survival remains unclear (Rossoll et al., 2003). Regardless, evidence suggests that the loss of SMN's anti-apoptotic function is a contributing factor in SMA (Kerr et al., 2000).

Over-expression of SMN protein protects cells against nerve growth factor deprivation in rat PC12 cells, camptothecin-induced apoptosis in human SMA patient fibroblasts and staurosporine-induced death in mouse NSC34 cells (Vyas et al., 2002; Wang et al., 2005; Parker et al., 2008). In this study, adenoviral vectors were used to investigate the survival mechanisms of the SMN protein in both apoptotic and oxidative models in differentiated human SH-SY5Y neuroblastoma cells, and in an excitotoxic model in rat cortical neurons. For the first time, this study also compares the anti-apoptotic mechanisms used by SMN and Bcl-xL, another protein also implicated in SMA (Soler-Botija et al., 2003).

EXPERIMENTAL PROCEDURES

Cloning of human SMN cDNA and construction of adenoviral vectors

Total human RNA was isolated from HEK293 cells, reverse transcribed and amplified by PCR using a gene specific primer pair containing unique restriction sites (bold) and a Kozak sequence

*Correspondence to: R. S. Anderton, Australian Neuromuscular Research Institute, Verdun Street, 4th Floor, A Block, QEII Medical Centre, Nedlands 6009, WA, Australia. Tel: +61-8-9346-7309; fax: +61-8-9346-3487.
E-mail address: randerton@meddent.uwa.edu.au (R. S. Anderton).

Abbreviations: BDNF, brain-derived neurotrophic factor; DIV, on day *in vitro*; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; MOI, multiplicity of infection; PBS, phosphate buffered saline; PI3, phosphatidylinositol-3 kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SMA, spinal muscular atrophy; SMN, survival of motor neuron protein; SMN1, survival of motor neuron 1; SMN2, survival of motor neuron 2.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.02.032

(underlined) as follows: - forward (*KpnI*) 5′**GGTACC**AGATCTGCCACCATGGCGATG-AGCAGCGG3′ and reverse (*HindIII*) 5′**AAGCTT**TTAATTTAAGGAATGTGAGCACC3′. The resulting PCR products were gel purified, ligated into pGEM-Teasy (Promega, Madison, WI, USA) and sequence verified. For subcloning, the SMN cDNA fragment was released by restriction enzyme digestion and ligated into the modified shuttle plasmid pRSV/WPRE/CMV:GFP (Boulos et al., 2006) to generate the vector: pRSV:SMN1/CMV:EGFP.

Recombinant adenoviruses were prepared according to the method of He et al. (1998), with some modifications (Boulos et al., 2006). Briefly, pShuttle plasmid DNA (pRSV:SMN1/CMV:GFP) was linearized by *PmeI* digestion and introduced, by electroporation (Gene Pulser II, Bio-Rad, Hercules, CA, USA) into the *Escherichia coli* strain BJ5183 harboring the pAdeasy plasmid (Zeng et al., 2001). Recombinants were selected on media containing 50 μg/ml kanamycin, and their plasmid DNA analysed by *PacI* digestion. HEK293 cells grown to 90% confluency in 25 cm^2 flasks were transfected with 3 μg of *PacI* linearized recombinant plasmid DNA using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Following the appearance of viral plaques (5–10 days), culture lysates were used for viral amplification in HEK293 cells. The SMN1 expressing adenoviral vector was designated AdRSV:SMN:CMV:GFP (AdRSV:SMN). Adenoviral particles were purified and concentrated from HEK293 cell lysates using the Adeno-X kit (BD Biosciences, San Jose, CA, USA). Viral titres were determined by end-point dilution assay as indicated by enhanced GFP reporter expression. Vectors consisting of an adenovirus expressing only green fluorescent protein (GFP; AdRSV:Empty:CMV:GFP; AdRSV:Empty) and an adenovirus over-expressing Bcl-xL (AdRSV:Bcl-xL:CMV:GFP; AdRSV:Bcl-xL) have been described previously (Boulos et al., 2006).

Rat primary cortical neuronal cell cultures

All animal procedures were approved by the University of Western Australia Animal Ethics Committee. Establishment of cortical cultures was as previously described (Meloni et al., 2001). Briefly, cortical tissue from E18 to E19 Sprague–Dawley rats were dissociated in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 1.3 mM L-cysteine, 0.9 mM $NaHCO_3$, 10 U/ml papain (Sigma, St. Louis, MO, USA) and 50 U/ml DNaseI (Sigma) and washed in cold DMEM/10% horse serum. Culture 96-well plates were coated with poly-D-lysine (50 μg/ml; 70–150K; Sigma) and incubated overnight at room temperature. The poly-D-lysine was removed and replaced with Neurobasal Media (containing 2% B27; 4% FCS; 1% horse serum; 62.5 μM glutamate; 25 μM 2-mercaptoethanol and 30 μg/ml penicillin). Neurons were plated at a density of 40,000 cells per well and cultures were maintained in a CO_2 incubator (5% CO_2; 95% air balance and 98% humidity) at 37 °C. On day *in vitro* (DIV) 4 half the media was removed and replaced with fresh NB/2% B27 (NB2°) containing the mitotic inhibitor cytosine arabinofuranoside. One third of the culture media was removed and replaced with fresh NB2° DIV 9, and the cultures were maintained at 37 °C in 5% CO_2.

Control fibroblasts and SMA patient fibroblasts

Control human fibroblasts (AGO6814; Coriell Institute, Camden, NJ, USA) and SMA type I patient fibroblasts (GMO3813; Coriell Institute) were maintained in DMEM containing penicillin (20 U/ml), streptomycin (20 mg/ml) and foetal calf serum (FCS; 5%; heat-inactivated) and incubated at 37 °C (5% CO_2).

SH-SY5Y cell propagation and differentiation

SH-SY5Y neuroblastoma cells were maintained in DMEM containing penicillin (20 U/ml), streptomycin (20 mg/ml) and FCS (5%; heat-inactivated) and incubated at 37 °C (5% CO_2). To obtain differentiated SH-SY5Y cultures, cells were seeded into a 96 well plate (≈30,000 cells/well) in 100 μl of DMEM (5% FCS) containing all-trans retinoic acid (15 μM; Sigma). After 3 days, half the media was replaced with serum free DMEM and retinoic acid (15 μM). Five days after plating, half the media was replaced with serum free DMEM containing brain-derived neurotrophic factor (BDNF; 2 μM; Sigma), and maintained for a further 3 days before use. Undifferentiated SH-SY5Y cells showed a rounded appearance with short processes, compared to retinoic acid treated SH-SY5Y cells which displayed a more neuronal-like morphology, with cells appearing triangular and showing longer processes (Encinas et al., 2000; Jamsa et al., 2004).

Adenoviral transduction of SH-SY5Y cells in 96 well plates

Adenovirus was diluted in serum free DMEM media containing BDNF (2 μM) and added to differentiating SH-SY5Y cultures 5 days after plating. To ensure uniform transduction of each virus in SH-SY5Y cultures (multiplicity of infection; MOI: 35–50), GFP reporter expression was initially quantitatively measured with a fluorescence plate reader (SPECTROstar; Omega, NC, USA) and subsequently routinely assessed by fluorescent imaging (Olympus IX70; Olympus DP70 digital camera; Olympus, Melville, NY, USA). Cultures were used for experiments 3 days after viral transduction.

Adenoviral transduction of rat primary cortical neurons and fibroblasts in 96 well plates

Adenovirus was diluted in pre-conditioned media and added to cells in culture. To ensure uniform transduction of each virus in both cortical neurons and fibroblasts (MOI: 70–90), GFP reporter expression was routinely assessed by fluorescent imaging (Olympus IX70; Olympus DP70 digital camera; Olympus). Cultures were used for experiments 3 days after viral transduction.

Immunohistochemistry of SMA patient fibroblasts

SMA type I patient fibroblasts (GMO3813; Coriell Institute) grown on cover slips were fixed in ice cold 4% formalin in phosphate buffered saline (PBS) for 1 h. Cover slips were rinsed in PBS-tween (PBS-T), blocked in 10% goat serum and exposed to an anti-SMN (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody diluted in PBS-T (1% goat serum) at 4 °C overnight. Primary antibody was removed by washing in PBS prior to exposure with the secondary antibody (Alexa Fluor 488 goat anti-mouse, 1:400; Invitrogen) diluted in PBS-T at 4 °C for 2 h. Cover slips were rinsed with PBS, mounted onto slides, and viewed using fluorescence microscopy.

Cell injury models

Hydrogen peroxide oxidative stress model. To determine if the SMN protein could protect against oxidative stress, a hydrogen peroxide (H_2O_2) model was used. Bissonnette et al. (2004) has previously shown that in differentiated SH-SY5Y cultures exposed to 270 μM H_2O_2, cell viability was decreased by ≈50%. Differentiated SH-SY5Y cultures were treated with 300 μM H_2O_2 in serum-free DMEM media, and cell death was assessed 16–18 h (37 °C; 5% CO_2) after H_2O_2 addition.

Inhibition of Akt/PI3-kinase cell signalling apoptosis model. To assess the potential anti-apoptotic function of the SMN protein, differentiated SH-SY5Y cultures were treated with a selective phosphatidylinositol-3 kinase (PI3) inhibitor, LY294002 (Sigma), which inhibits BDNF-mediated pro-survival signalling resulting in a predominantly apoptotic cell death (Fujiwara et al., 2006). This model involved replacing media in wells with serum-free DMEM