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SMN Protects cells against mutant SOD1 toxicity by increasing chaperone activity

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Abstract

Deletion or mutation of the survival of motor neuron (*SMN1*) gene causes Spinal Muscular Atrophy (SMA), a motor neuron degenerative disease. To study the SMN function, we co-transfected mouse NSC34 cells with *SMN* and mutant superoxide dismutase 1 (*SOD1*) constructs. We demonstrated that SMN protected NSC34 cells against cell death induced by mutant SOD1 under oxidative stress. Further studies indicated that over-expression of wild-type SMN up regulated chaperone activity. In contrast, chaperone activity was decreased in cells expressing SMN mutant Y272C or in cells with SMN suppressed by shRNA. *In vitro* assays using bacteria lysates expressing GST-SMN or purified GST-SMN protein showed that the GST-SMN reduced catalase aggregation, indicating that SMN may possess chaperone activity. We conclude that SMN plays a protective role in motor neurons by its chaperone activity. Our results provide support for the potential development of therapy for SMA and amyotrophic lateral sclerosis (ALS).

Keywords

Spinal muscular atrophy (SMA); the survival of motor neuron (SMN); superoxide dismutase 1 (SOD1); Amyotrophic lateral sclerosis (ALS); M Chaperone

INTRODUCTION

SMA is a devastating neurodegenerative disorder that affects more than 1 in 6000 newborns. The α -motor neurons in the anterior horn cells of the spinal cord are specifically targeted in the SMA patients. There are two highly homologous copies of *SMN* in human beings, *SMN1* and *SMN2* that are located on chromosome 5q 13 as an inverted repeat [1]. In more than 95% of SMA patients, the *SMN1* copy is deleted or mutated [1–4] whereas the *SMN2* predominantly

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produces an exon7-skipped transcript, generating a truncated, unstable protein which fails to compensate for the loss of *SMN1* in SMA patients [1,5].

SMN is a house keeping protein that is essential for cell survival of cells. SMN is distributed in the cytoplasm and in the nucleus of cells and is involved in messenger RNA metabolism. The SMN protein is tightly associated with a group of proteins known as Gemins (Gemin2–8) and forms a large multifunctional complex [6–11]. Moreover, the cytoplasmic SMN is essential for the assembly of Sm protein and Uridine rich small nuclear RNAs (snRNAs) into snRNPs, the essential components of pre-mRNA splicing machinery. In this process, the SMN complex functions like an ATP-dependent molecular chaperone that facilitates the assembly of specific RNA and protein components into ribonucleoprotein particles [12].

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder resulting from selective death of motor neurons in the brain and the spinal cord [13]. Familial ALS (FALS) represents ~10 % of all ALS cases [13]. Approximately 20 % of FALS is linked to a genetic defect on chromosome 21q22 and the superoxide dismutase 1 (SOD1) was identified as a causative gene for the disease. SOD1 is a cellular homodimeric enzyme that is essential for scavenging of superoxide radicals.

It is well established in cell culture models that the mutant SOD1 expression induces cell death [14–16]. To explore the possibility of anti-cell death effects of SMN, we evaluated protective roles of SMN in a cell culture models that over-express mutant SOD1. Our results indicate that SMN protects cells with its chaperone activity.

MATERIALS AND METHODS

Plasmids

The coding sequences of SMN with Myc-tag in the N-terminal and Flag tag in the C-terminal were cloned into pcDNA3. For *SOD1*, the coding sequences were PCR amplified and cloned into pCS2-MT (6X Myc-tagged). All the constructs were verified by sequencing.

Cell culture, transfection, experimentally induced stress and MTT assay

NSC34 cells were grown in DMEM supplemented with 10 % FBS and 2 % P/S at 37° C with 5 % CO₂. Lipofectamine 2000 (Invitrogen) was used for transfection. About 50% transfection efficiency is achieved in all the experiments. The NSC34 cells were treated with 50 µM of freshly prepared H₂O₂ for 20 hours for stress induction and then replaced with fresh medium. The cells were analyzed for cell viability assays by MTT assay. In brief, the MTT (Sigma) was added to cultures at a final concentration of 0.5 mg/ml for 2 hours at 37° C and the resulting purple colored formazan product was solubilized with MTT solubilization solution (Sigma). The dissolved reaction product was spectrophotometrically quantified at 570 nm and corrected against blanks which consisted of media processed in the absence of cells. The reported values are means of three independent experiments with triplicates. The relative cell viability of NSC34 cells was calculated as follows: relative cell viability = (A_e) × 100/(A_c), where ' A_e ' is the experimental absorbance and ' A_c ' is the absorbance of untreated controls. For all the analyses, Student's *t*-test was employed to evaluate the statistical significance between the control and treatments.

GST-Proteins

Cultures of *E. coli* DH5α carrying PGEX4T-1-SMN or its mutants were grown in LB medium with 100 µg/ml Ampicillin until mid-log phase (OD₆₀₀ 0.5–0.6). The cultures were induced aerobically (200 rpm) for 3–4 hrs at room temperature by adding IPTG (0.5 mM). Cultures were then collected with centrifugation and resuspended in Low Salt Association Buffer

(LSAB, 100 mM NaCl, 100 mM Tris, pH 8.0, 1% NP-40) in the presence of 1 mM of PMSF. Cells were maintained on ice and sonicated with 4 pulses of 15 seconds followed by centrifugation at $20,000 \times g$ for 10 minutes at 4 °C (Beckman coulter, Rotor ID: JA-14). The protein concentrations were determined and equal amounts of proteins were used for in vitro chaperone assays. Following purification as described [17], proteins were dialysed in P5-buffer and purified on a Talon affinity column [18]. Following extensive wash, proteins were eluted with P500 and dialysed in storage buffer (50 mM Tris-HCl pH 7.5, 100mM NaCl, 10% glycerol).

Chaperone activity assay

The chaperone activity assay was performed as described by Hook and Harding [19]. Forty micrograms per ml of the solubilized cytosol or bacteria protein that was mixed with 200 µg/ml of catalase (Sigma) was prepared in phosphate buffered saline. The reaction mixture was heated at 55°C, at different time points (0, 5, 10, 15, 20, 30 and 40 minutes) and the aggregation of catalase solution was followed by measuring the light scattering at 360 nm with a spectrophotometer (Beckman Coulter, DU600). The relative initial aggregation velocity was determined using the plots based on Michaelis-Menten constant equation [20] and the reduction in the velocity of catalase aggregation was used as a measure of chaperone activity [19,20]. In order to illustrate chaperone activity more directly, a first order reaction model for protein aggregation was adopted to estimate the relative initial reaction velocity. To plot chaperone activity directly, we normalized the inhibition of catalase aggregation by setting the control (BSA) at 0 % chaperone activity, and the maximal inhibition (by chaperone activity in the homogenate) at 100 % chaperone activity. This plot generated quantitative data with good linearity.

RNA interference

Suppression of SMN was achieved by transfecting HEK293 cells with shRNA plasmids (Open BioSystems, V2Hs_92493: 5'GCTATCATACTGGCTATTATAT3').

Western blot Analysis

The cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1.0 % Nonidet P40, 0.25 %, sodium-deoxycholate, 150 mM NaCl] with cock-tail protease inhibitor (Roche), and 1 mM PMSF. Equal amounts of protein from all the samples were boiled with Laemmli's buffer [21] and separated by 10 and/or 12 % SDS-PAGE, and the resolved gels were blotted on Immobilon P membrane (Millipore). The transblots were probed with antibodies against Sheep anti-SOD1 (Biosdesign), and anti-SMN (Transduction Laboratories) followed by appropriate secondary horse-radish peroxidase (HRP)-conjugated antibodies. In all cases, the blots were stripped with stripping buffer [62.5 mM Tris-HCl (pH 6.7), 2 % SDS and 90 mM 2-mercaptoethanol] and reprobed with anti-tubulin antibody (EMD) for loading controls. The signal was detected by enhanced chemiluminescence (Pierce).

RESULTS

SMN increases cell viability in NSC34 cells expressing mutant SOD1

It is well known that SMN possesses anti-cell death effects [22,23] while expression of mutant SOD1 in cell culture model induces cytotoxicity [14,24,25]. Since both SMA and ALS are motor neuron disorders, we assessed whether SMN was able to inhibit cell death caused by mutant-SOD1-induced cytotoxicity using MTT assay. The cDNA of epitope-tagged human SMN (wild-type and mutant, Y272C), human SOD1 (wild-type and mutants, G93A and A4V), and GFP (green fluorescence protein) were transiently co-transfected into the mouse motor neuron like NSC34 cells. The expression of proteins was assessed by western blot analysis.

Both the level of SMN and SOD1 protein expression was comparable in all the transfected samples (Fig. 1A & 1B).

After 72 hours transfection and treatment with 50 μ M of hydrogen peroxide for six hours, NSC34 cells expressing mutant SOD1, A4V and G93A, two severe familial ALS-linked mutations, showed 30 % reduction in cell survival compared with cells transfected with an empty vector. On the other hand, cells co-expressing wild-type SMN along with the SOD1 mutants increased cell survival and the mean percentage of cell survival in A4V+wild-type SMN transfected sample is 83.3 ± 3.93 % (mean $\pm$ SD). Similarly, ~14 % increase of cell survival was registered in cells co-expressing wild-type SMN with G93A SOD1 transfected cells (Fig. 1C). Cell survival was increased only in cells co-transfected with wild-type SMN but not cells co-expressing the mutant SMN (Y272C). Interestingly, increase of cell viability was also observed in cells expressing either wild-type SMN, or wild-type SOD1 alone after the addition of hydrogen peroxide compared to empty vector transfected cells treated with hydrogen peroxide. It appears that both wild-type SMN and wild-type SOD1 protect cells from oxidative stress (Fig. 1C). On the other hand the cell mortality was increased in mutant SOD1 and mutant SMN expressing cells treated with hydrogen peroxide (Fig. 1C). Taken together, our data suggest that transient expression of wild-type SMN increased cell survival in mutant SOD1 expressed cells.

SMN regulates chaperone activity

A recent report demonstrated that SOD1 mutants in mice reduced chaperone activity [26]. Therefore, we asked whether SMN promotes cell survival against mutant SOD1 cytotoxicity by inducing overall chaperone activity. To test this, wild-type and mutant SMN were over-expressed in HEK293 cells. Conversely, the endogenous SMN in these cells was suppressed by shRNA. The solubilized cytosolic extracts from these cells were tested for chaperone activity using a well established method that determines the ability of chaperones to prevent heat-induced denaturation and aggregation of substrate, catalase [26–28]. The relative chaperone activity was calculated as described in the experimental procedures. The results indicate that wild-type SMN expressing cells significantly increased (115 ± 7.06 %) the chaperone activity compared to the cells transfected with empty vector (which is considered as 100 %). Whereas expression of the disease-associated mutant SMN (Y272C) (59.16 ± 7.76 %) or suppression of the endogenous SMN protein significantly inhibited the chaperone activity (84.67 ± 5.03 %) (Fig. 2A, and Fig. 3C). Three independent experiments with duplicates were conducted and similar results were obtained. To eliminate the possibility that observed differences were due to inconsistency in transfection efficiency or protein expression, the expressed SMN protein was immunoblotted using anti-SMN antibody. The western blot results indicate that the expression level of SMN protein is comparable in both wild-type and mutant SMN transfected samples (Fig. 2B). In cells that were transfected with SMN shRNA, the endogenous SMN was reduced to about 30–40% (Fig. 3A&B). Further more, neither the wild-type nor the mutant SMN showed any light scattering or aggregation by measuring light scattering at 360 nm, indicating that both wild type and mutant SMN maintain similar protein stability at the temperature used in the chaperone assays (data not shown). Taken together, our results suggest that SMN contributes to the protection of cellular proteins by functioning in regulate molecular chaperones.

Bacteria expressed GST-SMN increases chaperone activity

On one hand, it is possible that increase of chaperone activity with over-expression of SMN is due to regulation of other chaperone proteins. On the other hand, the SMN protein may be itself a molecular chaperone. To test this hypothesis, bacteria lysates expressing GST, GST-SMN, and GST mutant SMN proteins were prepared. A total of 40 μ g bacteria protein was mixed with catalase for in vitro chaperone activity assays. We demonstrated that GST-SMN lysate

significantly increased chaperone activity in comparison to GST ($118 \pm 5.85\%$) or GST-SMN $\Delta 7$ (Fig 4B). In contrast, the SMN mutant Y272C didn't prevent aggregation of catalase, instead, promoted denaturation of the protein indicating that the wild type SMN not the mutants possesses chaperone activity. Furthermore, we used purified GST-SMN-His protein. We found that the purified GST-SMN-His protein has a higher chaperone activity ($120.78 \pm 5.03\%$) than BSA (Fig. 4C).

DISCUSSION

Several genetic studies have indicated that there is a genetic link between SMA and ALS diseases [29,30]. Veldink et al showed that reduced SMN protein production from certain SMN genotypes increased susceptibility to severity of sporadic ALS while Corcia et al suggested that SMN1 is a risk factor for ALS [29,30]. To further investigate the potential convergence of SMN and SOD1, we evaluated the cytoprotective effect of SMN against mutant-SOD1-mediated cytotoxicity in NSC34 cells. Transient expression of wild-type SMN protected cell death from mutant SOD1-mediated toxicity (Fig. 1) by up-regulating overall chaperone activity (Fig. 2). By contrast, the disease-associated mutant SMN expression failed to protect from cell death and the chaperone activity is reduced (Fig. 1, Fig 2). More importantly, we showed that the purified GST-SMN but not mutants increased chaperone activity, suggesting the SMN protein may be a chaperone (Fig. 4). Our findings are consistent with recent reports that expression of SMN in stable NSC34 cell lines of mutant SOD1 is reduced [31] and that chaperone activities were reduced in transgenic mice expressing SOD1 mutants [26]. Taken together, we deduce that the accumulated cytoplasmic SMN is perhaps involved in cellular protection through up-regulation of chaperone activity. The inhibition of chaperone activity in motor neurons due to reduced level of SMN protein may contribute to the pathogenesis of cell death of the motor neurons in both SMA and ALS diseases. Therefore, the chaperone activity and anti-oxidant properties of SMN could be exploited for the development of therapy for neurodegenerative disease.

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Fig. 1.

SMN protects cells against cell death induced by SOD1 mutants in NSC34 cells. NSC34 cells were transiently transfected with 6x myc tagged human wild-type and mutant SOD1 and/or double-tagged (Myc tag in the N-terminal and Flag tag in the C-terminal) version of cDNA of human wild type and mutant SMN. After 72 hours the cell lysates were subjected to immunoblot. **A.** Anti-SOD1. The endogenous murine SOD1 is shown in the same blot. The tubulin blot serves as protein loading control. **B.** Anti-hSMN antibody. **C.** Transient expression of human SMN promotes cell survival against mutant SOD1-mediated cytotoxicity in NSC34 cells. Cells were treated with 50 μ M of hydrogen peroxide to induce stress for 20 hours and replaced with fresh medium. The cell viability was measured by MTT assay after 72 hours transfection. Expression of mutant SOD1, A4V significantly inhibited cell survival and co-expression of wild-type SMN significantly protects cell death ($p < 0.01$ compared to untreated cells) and similarly SMN co-transfected cells increased cell survival in G93A-SOD1 expressing cells ($p < 0.05$). However, co-expression of mutant SMN (Y272C) did not protect from cell death caused by both the mutants of SOD1. **D.** Cells expressing mutant SOD1 (A4V or G93A) alone altered the cellular morphology. However, wild-type SMN but not mutant SMN reversed the morphological changes in cells treated with 50 μ M of hydrogen peroxide. The images are representatives of four such images obtained in three independent experiments. Phase-contrast images were taken at a magnification of x100.

Fig. 2.

SMN regulates chaperone activity. A. HEK 293 cells were transfected with empty vector, wild-type SMN, and mutant (Y272C) SMN. After 72 hours of transfection, solubilized cytosolic extracts were prepared and subjected to chaperone assay as described in Materials and Methods. The values are normalized to empty vector transfected cells. The relative chaperone activity was inhibited (~50%) in mutant SMN expressing cells (* $p < 0.01$ compared to empty vector). The wild-type SMN expressing cells increased ~15% in chaperone activity compared to empty vector transfected cells ($p < 0.05$ compared to empty vector control cells). B. The soluble form of cytosolic extract from the transfected samples were analyzed by immunoblot with antibodies against GAPDH, SMN and the α -tubulin.

Fig. 3.

Suppression of SMN down-regulates chaperone activity in HEK 293 cells. HEK293 cells were transfected with shRNA specifically targeting SMN and the control shRNA. A. Expression of SMN protein and tubulin were analyzed by Western blot. B. expression of SMN was quantitated (Panel B). C. Cytoplasmic proteins of HEK293 cells were prepared and in vitro chaperone activity assay were performed as described in Materials and Methods. Suppression of the endogenous SMN reduced chaperone activity ($p=0.057$).

Fig. 4.

GST-SMN increases chaperone activity. GST, GST-WT-SMN, and GST-SMN mutants were induced to express in *E. Coli* DH5 α . The bacteria lysates were prepared for *in vitro* chaperone activity assay. A. Expression of SMN in *E. Coli* DH5 α was analyzed by Western blot with anti-SMN antibody. B. *In vitro* chaperone activity assays suggest that GST-SMN increases chaperone activity in comparison to GST, while GST-SMN (Y272C) reduces chaperone activity. C. GST-SMN protein was purified. *In vitro* assays demonstrated that purified GST-SMN has a higher chaperone activity than control BSA protein.