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VEGF alleviates ALS-CSF induced cytoplasmic accumulations of TDP-43 and FUS/TLS in NSC-34 cells

Shubham Shantanu[a], K. Vijayalakshmi[b], S. Shruthi[b], B.K. Chandrasekhar Sagar[c], T.N. Sathyaprabha[b], A. Nalini[a], Trichur R. Raju[b], Phalguni Anand Alladi[b,*]

[a] Department of Neurology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore 560 029, India
[b] Department of Neurophysiology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore 560 029, India
[c] Department of Neuropathology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore 560 029, India

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ABSTRACT

Cytoplasmic mislocalisation and aggregation of TDP-43 and FUS/TLS proteins in spinal motor neurons contribute to the pathogenesis of the highly fatal disorder amyotrophic lateral sclerosis (ALS). We investigated the neuroprotective effect of VEGF on expression of these proteins in the motor neuronal cell line NSC-34 modelled to reminisce sporadic form of ALS. We studied the expression of TDP-43 and FUS/TLS proteins after exposure to ALS-CSF and following VEGF supplementation by quantitative confocal microscopy and electron microscopy. ALS-CSF caused cytoplasmic overexpression of both the proteins and stress-granule formation in the cells. These alterations were alleviated by VEGF supplementation. The related ultrastructural changes like nuclear membrane dysmorphism and p-bodies associated changes were also reversed. However the protein expression did not completely translocate to the nucleus, as some cells continued to show to cytoplasmic mislocalisation. Thus, the present findings indicate that VEGF alleviates TDP43 and FUS pathology by complimenting its role in controlling apoptosis and reversing choline acetyl transferase expression. Hence, VEGF appears to target multiple pathogenic processes in the neurodegenerative cascade of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder which manifests following degeneration of the motor neurons of spinal cord and motor cortex. Vascular endothelial growth factor (VEGF) is the most promising neuroprotectant, verified in experimental models of ALS (Keifer et al., 2014). We have earlier reported that VEGF reversed ALS-CSF induced depletion of choline acetyl transferase expression and attenuated apoptosis in a receptor-dependent manner in NSC-34 motor neuron like cells (Vijayalakshmi et al., 2015). Its influence on TDP-43 and FUS/TLS induced pathology is not known.

Mutation and aggregation of RNA-binding proteins like TAR DNA-binding protein of 43 kDa i.e. TDP-43 and fused in sarcoma/translocated in liposarcoma (FUS/TLS), in ALS suggest disrupted RNA homeostasis as a pathogenic trigger (Neumann et al., 2006; Kwiatkowski et al., 2009). Presence of ubiquitinated TDP-43 and FUS-positive inclusions, in sporadic ALS (SALS), familial ALS (FALS) and ALS with fronto-temporal dementia (Deng et al., 2011) symbolize a mechanistic link between SALS and FALS (Arai et al., 2006; Neumann et al., 2006). Physiological shuttling of TDP-43 between the nucleus and cytoplasm governs homeostatic cellular functions of gene expression, transcriptional regulation, mRNA transport etc (Zinszner et al., 1997; Ayala et al. 2008), while cellular stress affects shuttling and produces cytoplasmic stress granules (Mackenzie et al., 2010). Absence of nuclear localisation, induces neurodegeneration via “loss of function” while cytoplasmic aggregation acts via “toxic gain of function” (Da Cruz and Cleveland, 2011). VEGF is the most promising neuroprotectant, verified in experimental models of ALS (Oosthuyse et al., 2001; Li et al., 2003). In NSC-34 motor neuron like cells, it reversed ALS-CSF induced depletion of Choline acetyl transferase (ChAT) expression and attenuated apoptosis in a receptor mediated manner (Kulshreshtha et al., 2011; Vijayalakshmi et al., 2015). Its influence on TDP-43 and FUS/TLS expression is not known.

* Corresponding author.
E-mail address: alladiphalguni@gmail.com (P.A. Alladi).

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2. Materials and methods

2.1. CSF exposure

The study was approved by the institutional human ethics committee. We collected CSF of ALS patients (definite/probable; n = 6), following diagnosis as per the modified Airlie House El EsCorial criteria and of patients of non-neurodegenerative pathologies (idiopathic intracranial hypertension/normal pressure hydrocephalus; n = 6) by lumbar puncture and snap-froze them (Table 1). We obtained informed consent for collection of patient CSF. The NSC-34 cell line were grown with DMEM with 10% FBS and antibiotics. On the 6th day, the growth medium was supplemented with patient CSF, and was allowed to interact for 48 h (10% v/v). In our earlier studies we found that ALS-CSF exposure caused loss of differentiation in approximately 60% of the NSC-34 cells. The differentiated ones represent the mature motor neuronal phenotype (Vijayalakshmi et al., 2009). Amongst these, approximately 20–30% of the cells showed a multinucleate phenotype with cytoplasmic vacuolation and beading of neurites which is a classical feature of a degenerating cell.

On the 8th DIV, we added recombinant VEGF (Sigma-Aldrich USA; 150 ng/mL) to the culture medium and allowed the interaction for 24 h. The study groups were devised based on CSF exposure and VEGF supplementation namely NC (no CSF); NALS (non-ALS patients); ALS (ALS patients); NC + V; NALS + V and ALS + V (Vijayalakshmi et al., 2015).

2.2. Sequential immunofluorescence and quantification

Sequential immunofluorescence and quantification was performed as per standard protocol (Vijayalakshmi et al., 2015). Briefly, the methanol fixed cells (on coverslips, in duplicates) were unmasked with 2XSSC-Formamide (55 °C, 30 min). Non-specific staining was blocked using 1.5% BSA. The binding of the first protein (rabbit anti-TDP-43; 1:600 dilution; 24 h at 4 °C; Proteintech USA) was identified using anti-rabbit FITC (1:200; 4 h incubation; Sigma-Aldrich, USA). After re-blocking, the cells were labeled for FUS (mouse anti-FUS/TLS; 1:500; 36 h at 4 °C; Santacruz Biotechnology, USA), which was detected by CY3-conjugated anti-mouse IgG (1:200, Sigma-Aldrich USA). TOPRO-3-iodide was the nuclear marker (1:1800; 30 min; Invitrogen, USA). In absence of "exclusively-nuclear/cytoplasmic" localisation, the pattern was classified as: predominantly-nuclear (N), predominantly-perinuclear (P) and predominantly-cytoplasmic (C) and intensity measurements and ratio (N/C) were derived. As appropriate, student's *t*-test and one-way ANOVA with Tukey's post-hoc test (more than 2 groups) were applied for significance. We specifically employed optical sectioning using confocal microscopy so as to be sure about the specific localization of the proteins. Confocal microscopy also provided us with means to examine the differentiated cells specifically, which represent the mature motor neuron phenotype and are largely affected by ALS-CSF (Vijayalakshmi et al., 2015).

Table 1
Details of patients whose cerebrospinal fluid was used in the study.

	ALS-CSF (n = 6)	NALS-CSF (N = 6)
Gender	Male – 4, Female – 2	Male – 4, Female – 2
Age(Mean± SD)	49.56 ± 5.12 (39–64)	44.63 ± 6.76 (33–56)
Age at onset	46.36 ± 4.87 (36–60)	
Duration(Mean± SD)	16.4 ± 11.6 (3–48mth)	
Onset Pattern: Limb	4	
Bulbar	2	
Dysphagia	3	
Dysarthria	4	
Spasticity	6	
Minipolymyoclonus	5	

2.3. Electron microscopy

For electron microscopy, the NSC-34 cells were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 24 h. The pellets were post-fixed in 1% osmium tetroxide (1hr), dehydrated in ethyl alcohol grades and followed by clearing in propylene oxide. For embedding we used araldite CY212 resin (TAAB, UK) and polymerization was done at 60 °C for 48 h. Ultrathin sections were stained using uranyl acetate and lead citrate. The sections were scanned using Tecnai G2 Spirit Bio-twin (FEI Netherlands) at 80 KVA and images were captured using MegaView-III digital CCD camera (Vijayalakshmi et al., 2015).

3. Results

In our study, both TDP-43 and FUS were predominantly localised to the nucleus in the NC (Fig. 1, A–D & Supplementary figure showing low magnification images) and NALS groups, with discrete nuclear pockets of staining. ALS-CSF exposure led to cytoplasmic mislocalisation and stress-granules formation (Fig. 1, E–H, arrows), which were reduced by VEGF supplementation (Fig. 1, I–L). ALS-CSF exposure caused significant rise in cytoplasmic TDP-43 expression (Fig. 1, M–O; $p < 0.001$ NC vs ALS####; $p < 0.001$ NALS vs ALS ****); marked reduction in nuclear staining and hence the N/C ratio ($p < 0.001$ vs NC**** and NALS ####) suggesting mislocalisation. VEGF supplementation partly reversed the cytoplasmic expression in terms of intensity (Fig. 1M; ALS vs ALS+V, $p < 0.05$) and percentage of cells showing nuclear (Fig. 1O, Nuclear $p < 0.01$ **) perinuclear (Fig. 1O, PN $p < 0.01$ ##) and cytoplasmic localisation (Fig. 1O, PN $p < 0.05$$) but not the N/C ratio.

Similarly, ALS-CSF caused cytoplasmic mislocalisation of FUS/TLS protein, where it was also overexpressed (Fig. 1 P&Q; $p < 0.001$ NC vs ALS; $p < 0.001$ NALS vs ALS). We also observed a reduction in nuclear staining intensity (N/C intensity ratio; $p < 0.001$ NC vs ALS****; $p < 0.001$ NALS vs ALS####) and VEGF supplementation caused part reduction in cytoplasmic levels (ALS vs. ALS + VEGF, $p < 0.05$) but not the N/C ratios.

The electron-micrographs showed highly undulated and dysmorphic nuclear membrane in the cells with pathological exposure to ALS-CSF (Fig. 2A, arrows). In addition, several large 'P-body' like structures were seen around the nuclear membrane in the exposed cells (Fig. 2A, arrowheads). Higher magnification images revealed that the 'P-body' like structures were fairly larger and were distinctly nested within the dysmorphic nuclear membrane (Fig. 2B, arrowheads). VEGF supplementation caused a sizeable reduction in the number and size of 'P-body' like structures (Fig. 2C; encircled structures) and looked comparable to the controls (Fig. 2 compare 'C' with 'D').

4. Discussion

We have earlier reported stress on ER and fragmentation of the Golgi apparatus in the motor neurons induced by ALS-CSF, suggesting an impact on protein trafficking machinery (Ramamohan et al., 2007; Vijayalakshmi et al., 2011). Furthermore it has been reported that RNA-binding proteins like FUS and TDP-43 mediate FALS pathogenesis, but their role in SALS and VEGF mediated neuroprotection is not known. Here, we demonstrate that ALS-CSF induces mislocalisation of nuclear proteins TDP-43 and FUS/TLS, to cytoplasm in NSC-34 cells. While a faint cytoplasmic staining of these proteins under normal conditions may be physiological in nature, their over-expression in the