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Neuroserpin polymers cause oxidative stress in a neuronal model of the dementia FENIB

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ABSTRACT

The serpinopathies are human pathologies caused by mutations that promote polymerisation and intracellular deposition of proteins of the serpin superfamily, leading to a poorly understood cell toxicity. The dementia FENIB is caused by polymerisation of the neuronal serpin neuroserpin (NS) within the endoplasmic reticulum (ER) of neurons. With the aim of understanding the toxicity due to intracellular accumulation of neuroserpin polymers, we have generated transgenic neural progenitor cell (NPC) cultures from mouse foetal cerebral cortex, stably expressing the control protein GFP (green fluorescent protein), or human wild type, G392E or delta NS. We have characterised these cell lines in the proliferative state and after differentiation to neurons. Our results show that G392E NS formed polymers that were mostly retained within the ER, while wild type NS was correctly secreted as a monomeric protein into the culture medium. Delta NS was absent at steady state due to its rapid degradation, but it was easily detected upon proteasomal block. Looking at their intracellular distribution, wild type NS was found in partial co-localisation with ER and Golgi markers, while G392E NS was localised within the ER only. Furthermore, polymers of NS were detected by ELISA and immunofluorescence in neurons expressing the mutant but not the wild type protein. We used control GFP and G392E NPCs differentiated to neurons to investigate which cellular pathways were modulated by intracellular polymers by performing RNA sequencing. We identified 747 genes with a significant upregulation (623) or downregulation (124) in G392E NS-expressing cells, and we focused our attention on several genes involved in the defence against oxidative stress that were up-regulated in cells expressing G392E NS (*Aldh1b1*, *Apoe*, *Gpx1*, *Gstm1*, *Prdx6*, *Scara3*, *Sod2*). Inhibition of intracellular anti-oxidants by specific pharmacological reagents uncovered the damaging effects of NS polymers. Our results support a role for oxidative stress in the cellular toxicity underlying the neurodegenerative dementia FENIB.

1. Introduction

The serpinopathies are protein conformational diseases characterised by the polymerisation and intracellular deposition of mutant variants of the serpins (serine protease inhibitors), within the endoplasmic reticulum (ER) of the cells that synthesise the protein. This common pathological mechanism results in liver disease upon polymerogenic mutations in alpha-1 antitrypsin (A1AT), and a rare but fatal neurodegenerative dementia caused by mutations in neuroserpin (NS) [reviewed in Roussel et al., 2011]. The latter is an autosomal dominant condition known as FENIB (familial encephalopathy with neuroserpin inclusion bodies) (Davis et al., 1999), described in patients carrying one of six different mutations in the NS gene: S49P, S52R

Abbreviations: A1AT, alpha1 antitrypsin; Aldh, aldehyde dehydrogenase; ApoE, apolipoprotein E; ER, endoplasmic reticulum; FENIB, familial encephalopathy with neuroserpin inclusion bodies; GPx, glutathione peroxidase; GST, glutathione transferase; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NS, neuroserpin; NFκB, nuclear factor κB; *Prdx6*, peroxiredoxin 6; Scara, scavenger receptor class A; SOD, superoxide dismutase; unfolded protein response, UPR; wild type, WT.

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(Davis et al., 1999); H338R, G392E (Davis et al., 2002); G392R (Coutelier et al., 2008) and L47P (Hagen et al., 2011). In all cases, mutant NS was found to accumulate within affected neurons forming periodic acid-Schiff (PAS)-positive and diastase-resistant inclusion bodies (Collins bodies), most abundant in the cerebral cortex but also present in other regions of the central nervous system (Davis et al., 1999)(Hagen et al., 2011). For the first four mutations, a strong correlation was found between the predicted tendency to polymer formation by each mutant variant of NS and the number of Collins bodies and severity of FENIB (Davis et al., 2002), suggesting a toxic gain-of-function for NS polymers. Polymer formation by mutant NS and its correlation with the disease phenotype was later confirmed in COS-7 and PC12 cell models of FENIB, where overexpression of each mutant variant lead to intracellular accumulation of polymeric NS within the ER to a degree that was proportional to the severity of FENIB in patients (Miranda et al., 2004)(Miranda et al., 2008)(Moriconi et al., 2015).

The toxicity of NS polymers is supported by the phenotypes observed in two animal models of FENIB. Transgenic mice overexpressing S49P and S52R NS showed the formation of abundant intraneuronal Collins bodies, neuronal loss in the cerebral cortex and hippocampus, and pathological phenotypes reminiscent of FENIB during late adulthood (Galliciotti et al., 2007). Overexpression of human S49P, S52R, H338R and G392E NS in *Drosophila melanogaster* led to a decrease in locomotor activity, with decreasing mobility correlating to increased polymer content in the brain (Miranda et al., 2008). Despite these results, the mechanism of toxicity of NS polymers in cell models of disease has been elusive so far. Accumulation of NS polymers within the ER fails to induce a classical unfolded protein response (UPR), contrarily to a truncated version of NS (delta NS) lacking the last third of the aminoacidic sequence, which does not fold properly, does not polymerise and activates the UPR (Davies et al., 2009). Instead, NS polymers activate the ER overload response through a poorly understood signalling pathway that depends on Ca^{2+} and leads to the activation of nuclear factor κB (NFκB) (Davies et al., 2009). Nevertheless, three different cell model systems, transiently transfected COS-7 cells, stable inducible PC12 cells and stable inducible HeLa cells, failed to show clear signs of cell malfunction and death upon NS polymer accumulation (Miranda et al., 2004)(Miranda et al., 2008)(Roussel et al., 2013), precluding a detailed investigation of the cellular mechanisms underlying NS polymer toxicity. This lack of a toxic phenotype could be related to the proliferative nature of these cell lines, but a neuronal model with stable overexpression of NS has not been developed to date. Mouse neural progenitor cells can be isolated from several regions of the mouse foetal brain, propagated *in vitro* as primary cells and differentiated to mature, non-dividing neurons through a well-defined protocol (Conti et al., 2005)(Soldati et al., 2012). They can also be stably transfected for expression of heterologous proteins, making them a suitable system for cellular studies on neuronal function.

Oxidative stress, the imbalance between generation and disposal of reactive oxygen species (ROS), is an important factor in several neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis [reviewed in Cobb and Cole, 2015]. Under physiological conditions, ROS have important roles in signalling and immune defence, and their levels are kept under check by several antioxidant defence systems, including enzymatic (mainly superoxide dismutase, glutathione peroxidase, catalase and thioredoxin reductase) and non-enzymatic (specially glutathione, GSH) mechanisms, which can either scavenge ROS or decrease their formation [reviewed in Li et al., 2013]. Neurons are particularly vulnerable to oxidative stress due to their high energy requirements, to a decrease in antioxidant defences with age and to their terminally differentiated nature, and so oxidative stress is a key player in neurodegenerative diseases, although it is not clear whether oxidative stress is a cause, a consequence or both in these pathologies [reviewed in Gandhi and Abramov, 2012]. The ER, where NS polymer formation takes place, provides an oxidizing environment for correct formation of disulfide bonds during protein folding. Accumulating evidence suggests that ROS can be generated as a by-product of protein oxidation during normal ER function and also upon ER stress due to accumulation of misfolded proteins. Both ER stress and oxidative stress, through ROS generation, may increase the leak of Ca^{2+} from the ER lumen, as well as induce protein and lipid oxidation. High levels of ROS generation within the mitochondria further increase Ca^{2+} release from the ER, generating a vicious cycle of ROS production and cellular oxidative stress [reviewed in Malhotra and Kaufman, 2007].

We report here the generation of a novel cell model system for FENIB, consisting of mouse neural progenitor cells (NPCs) derived from mouse foetal cerebral cortex, stably expressing either a control green fluorescence protein (GFP) or one of three NS variants: wild type (WT), the highly polymerogenic G392E or delta NS, a truncated version of NS lacking the last 134 aminoacids and used as a misfolding protein control (Davies et al., 2009). By RNA sequencing comparison of control GFP and polymer-containing G392E NS cells differentiated to neurons, we have identified several genes related to oxidative stress as relevant for the toxicity of NS polymers in FENIB. The relevance of this pathway was confirmed by an increase in apoptosis of cells expressing G392E NS upon pharmacological inhibition of their anti-oxidant defences.

2. Material and methods

2.1. Reagents and antibodies

Unless stated otherwise, reagents, buffers, culture media and serum for cell cultures were purchased from Sigma-Aldrich (Milan, Italy). Custom-made rabbit polyclonal anti-NS antibody and rabbit polyclonal anti-GAPDH antibody were from Abcam (Cambridge, UK). The mouse monoclonal anti-NS antibodies were made in-house as reported before (Miranda et al., 2008). Anti-KDEL was from Enzo Life Sciences (through 3VChimica S.r.l., Italy), anti-GM130 from BD Transduction Laboratories and anti-catalase from Merk Millipore (both through SIAL S.r.l., Italy). Goat polyclonal anti-rabbit-HRP (horseradish peroxidase) and rabbit anti-mouse-HRP are from Sigma-Aldrich (Milan, Italy). Goat anti-mouse IgG-Alexa Fluor® 488 and -Alexa Fluor® 594, and goat anti-rabbit IgG-Alexa Fluor® 594 were from Life Technologies (Milan, Italy).

2.2. Generation and culture of neural progenitor cell cultures with stable neuroserpin expression

The open reading frame of human NS was amplified by PCR and subcloned into the pTP6 expression vector. This vector contains the CAGG promoter followed by the NS gene, an internal ribosome entry site (IRES) and the gene for puromycin resistance, allowing selection for simultaneous expression of both genes. NPCs derived from the mouse cerebral cortex at embryonic day 13.5 and protocols to culture them *in vitro* were previously described (Soldati et al., 2012). Briefly, NPCs were maintained in expansion medium consisting of basal medium [DMEM/F12 (LifeTechnologies, Milan, Italy), 1% penicillin/streptomycin, 0.1 M L-glutamine (LifeTechnologies, Milan, Italy), 1 mM Hepes, 7.5% $NaHCO_3$, 0.6% glucose] supplemented with 20 ng/ml of human recombinant epidermal growth factor (EGF; R&D System, through Aurogene, Rome, Italy), 10 ng/ml of human recombinant basic fibroblast growth factor (bFGF; R&D System, through Aurogene, Rome, Italy), and 1% N2 supplement (LifeTechnologies, Milan, Italy). NPCs were routinely cultured at 37 °C in a 5% CO_2 atmosphere within flasks previously coated with 10 μg/ml poly-ornithine (for 4 h) and 5 μg/ml laminin (for 12 h), seeding them at a cell density of 0.5–1 × 10^4 cells/cm^2 and passaging them every 3–5 days using Accutase. NPCs expanded for up to 20 passages *in vitro* since their original derivation were used for this work. Early passage NPCs were transfected with pTP6-NS in three different versions: wild type, G392E and delta NS. The transfection was performed with Amaxa Nucleofector