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Neurofilament protein aggregation in a cell line model system

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Abstract

Protein aggregates are associated with many diseases and even aggregates of proteins that have no role in disease are inherently toxic to both neuronal and non-neuronal cells. We have developed a model system to explore the mechanism of protein aggregation using a mouse muscle cell line expressing chimeric neurofilament (NF) proteins, a constituent of the protein aggregates in ALS, Lewy body dementia, and Charcot-Marie-Tooth disease. Formation of protein aggregates in these cells leads to reduced cell viability and activated caspases. Aggregates contained both chimeric NF proteins and ubiquitin by immunolocalization and were predominately cytosolic when proteins were expressed at low levels or for shorter periods of time but were present in the nucleus when expression levels increased. This system represents a flexible, new tool to decipher the molecular mechanism of protein aggregation and the contributions of aggregation to cell toxicity.

Keywords: Protein aggregation diseases; Neurofilaments; Caspase activation; Ubiquitin

Neurodegenerative diseases (ND) have a common feature: aggregation of proteins linked to pathology. While the involvement of protein aggregation in ND is well established, the mechanisms by which aggregation leads to cell death are poorly understood. Although it has been demonstrated that that aggregates of non-disease related protein can be cytotoxic to non-neuronal cells [1], there is still some controversy as to whether aggregates are a cause or an effect of the disease process and whether they are cytopathic or cytoprotective [2,3]. A better understanding of the mechanism responsible for this cytotoxicity is likely to lead to a better understanding of a wide variety of diseases involving protein aggregation [1,4].

Therefore, we have developed a cell line model system designed to study neurofilament protein aggregation and the possible toxicity of aggregates to cells. This system uses chimeric NF which form aggregates when expressed in mouse tissue culture cells. The original motivation for this study was to investigate the roles of the head, rod, and tail domains in neurofilament assembly; for this purpose we constructed chimeric NF that combine domains from two of the three NF subunits (medium neurofilament (NFM) and light neurofilament protein (NFL)). However, as these chimeric NF proteins formed aggregates, and because of the increasing recognition of the importance of protein aggregation to pathogenesis, we shifted our focus to the investigation of the process of NF aggregation in these cells.

A number of diseases are associated with aggregation of intermediate filament (IF) proteins, including both liver and skin diseases [4–7], myopathies [8], and ND [9–12]. However, the involvement of NF aggregation in amyotrophic lateral sclerosis (ALS), a disease characterized by motor neuron degeneration, is most relevant to this work. Both the familial and sporadic forms of ALS share many pathological features including neurofilament protein aggregation. NF aggregation appears to be an early event in pathogenesis [13–15] and aggregates contain SOD1, ubiquitin, HSP70, and peripherin (another neuronal IF protein) [13,16,17]. Although HSP70 and ubiquitin may be acting physiologically in protein clearance, cellular

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events involving both SOD1 and NF proteins may be involved in ALS pathogenesis [14,16]. Mutations in NFL and NFH [14,15] and decreased levels of mRNA for NFL are seen in some ALS cases [18], and transgenic mice over-expressing any of the three NF subunits show evidence of motor neuron disease [19–21]. Thus, NF aggregation is clearly linked to ALS pathogenesis.

The cell model system we have developed allowed the contribution of several variables to protein aggregation and their relationship to cytotoxicity to be assessed. First, with the Ecdysone promoter system, protein expression is directly proportional to the amount of inducer added so that dose-response and time-course experiments are possible [22]. Second, a variety of chimeric NF proteins, each containing a head, rod and tail from either NFL or NFM, can be expressed to address the contribution of the NF domains to aggregation. Third, the chimeric NF proteins are expressed in two matched cell lines: mouse muscle cell lines derived from control animals and vimentin knock-out mice that provided an IF negative background, which is important as NF proteins co-assemble with other IF proteins to form filaments [23–26] or disassemble a pre-existing IF network [23,27].

Our results show that the chimeric NF proteins form aggregates that contain ubiquitin, that cells containing aggregates have reduced viability, and that caspases are active under conditions which cause aggregates to form. This suggests that the aggregates are cytotoxic and lead to apoptosis. Finally, we have investigated the mechanism of protein aggregation, specifically addressing the role of expression levels and duration of expression in the aggregation process.

Materials and methods

Construction of chimeras. Standard cloning methods were used to combine the head, rod, and tail domains of NFL and NFM of cDNAs (generously provided by Michael Lee and Don Cleveland) modified to contain an NdeI site at the 5′ end of the coding region, a PvuI site at the head–rod junction, a HindIII site at the rod–tail junction, and a BamHI site at the 3′ end of the coding sequence. Three constructs combining the tail domain from NFL with the head or rod domain from NFL or NFM were made (e.g. LML, MML, and MLL indicating the combinations of the three domains from NFL or NFM). The cDNAs for chimeric neurofilaments were expressed using the Ecdysone Inducible Expression System (Invitrogen, Carlsbad CA).

Cell lines and immunoblot analysis. Chimeric constructs were transfected into mouse muscle cell lines derived from wild-type (MFT-6) and vimentin knock-out (MFT-16) mice (courtesy of Robert Evans, University of Colorado) to form a total of six cell lines named according to the domain structure of the chimeric NF protein and the parental cell line (e.g. LML−/−, LML+/+, MML−/−, MML+/+, MML−/−, MML+/+). Cell lines were cultured in DMEM supplemented with 5% FBS, 50 μg/ml gentamycin, 100 μg/ml kanamycin, 25 μg/ml G418, and 5 μg/ml zeocin. Dose-dependent induction was verified by immunoblotting using standard methods. For each experiment, previously uninduced cells were treated with 1.25–20 μM ponasterone A for 24–72 h.

Statistical analysis of cell viability. Cells were plated at approximately 20% confluency and cultured in the presence of 0, 2.5, and 10 μM ponasterone A for 24 h. Cultures were then washed with PBS to remove debris and stained with trypan blue. The number of viable cells was quantitated by counting the number of adherent cells that excluded trypan blue from each of three replicate cultures. Means of the groups were compared using one-way ANOVA. Post hoc comparisons were done using the Bonferroni correction procedure. All analyses were conducted using Microsoft Office Excel 2003 and statistical significance was taken to occur when $p < 0.05$ (except when using the Bonferroni correction).

Assay for caspase activation. Caspase activation was measured using the cell permeable sulforhodamine-labeled fluoromethyl ketone peptide probe (SR-VAD-FMK, Chemicon International) that covalently binds to a reactive cysteine residue on the active heterodimer so that the level of fluorescence is directly proportional to the caspase activity at the time the reagent is added. The signal generated by cells induced with ponasterone A was compared with samples treated with camptothecin (DNA topoisomerase I inhibitor that triggers apoptosis, courtesy of Carleton Jones, Midwestern University) and untreated cells.

Immunofluorescence staining. Cells grown on coverslips were fixed with 2% paraformaldehyde and permeabilized with ice cold 20% methanol in PBS. After blocking with 2% BSA, the coverslips were incubated with monoclonal anti-vimentin, anti-ubiquitin (1:1,000 dilution in 1% BSA in PBS) or polyclonal anti-NF antibodies (1:500 dilution in 1% BSA in PBS) according to the recommendations of the suppliers followed by incubation with fluorescently-labeled second antibody (1:10,000 dilution in 1% BSA in PBS). Coverslips were then mounted with prolong anti-fade (Invitrogen, Carlsbad, CA) on slides and visualized using fluorescence microscopy.

Results

Expression of NF proteins reduces cell viability

NF proteins have two extremely different organizational structures: filaments and aggregates. Unexpectedly, none of the chimeric NF proteins formed filaments when expressed in either MFT-6 or MFT-16 cells but aggregated instead (see immunofluorescence data below). Since protein aggregates are often toxic, we asked if NF aggregation led to reduced cell viability. The number of viable cells (LML+/+) after incubation for 24 h with 2.5 and 10 μM ponasterone A was compared to untreated control cells. The number of viable cells per grid unit decreased from 9.24 ± 0.33 for untreated cells to 8.21 ± 0.36 after treatment with 2.5 μM ponasterone A and 6.70 ± 0.38 for cells treated with 10 μM ponasterone A (p value < 0.025).

Cells expressing NF proteins undergo apoptosis

Apoptosis in ALS is well documented and involves the activation of caspases 1 and 3 [28,29]. To demonstrate that the reduced cell viability is due to apoptosis, the activation of caspases was measured using an *in situ* assay detecting fluorescence from SR-VAD-FMK, a peptide inhibitor of caspases. Briefly, cells were grown in the presence or absence of ponasterone A and camptothecin for 24 h. Phase contrast shows that treatment with either 2.5 or 10 μM ponasterone A (Fig. 1B and C) or 1 μM camptothecin (Fig. 1D) reduced cell density as compared to untreated control (Fig. 1A). Using a uniform two second exposure, untreated cells show no labeling (Fig. 1E). Cells treated with increasing amounts of ponasterone A showed increased labeling (Fig. 1F and G) at levels similar to