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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

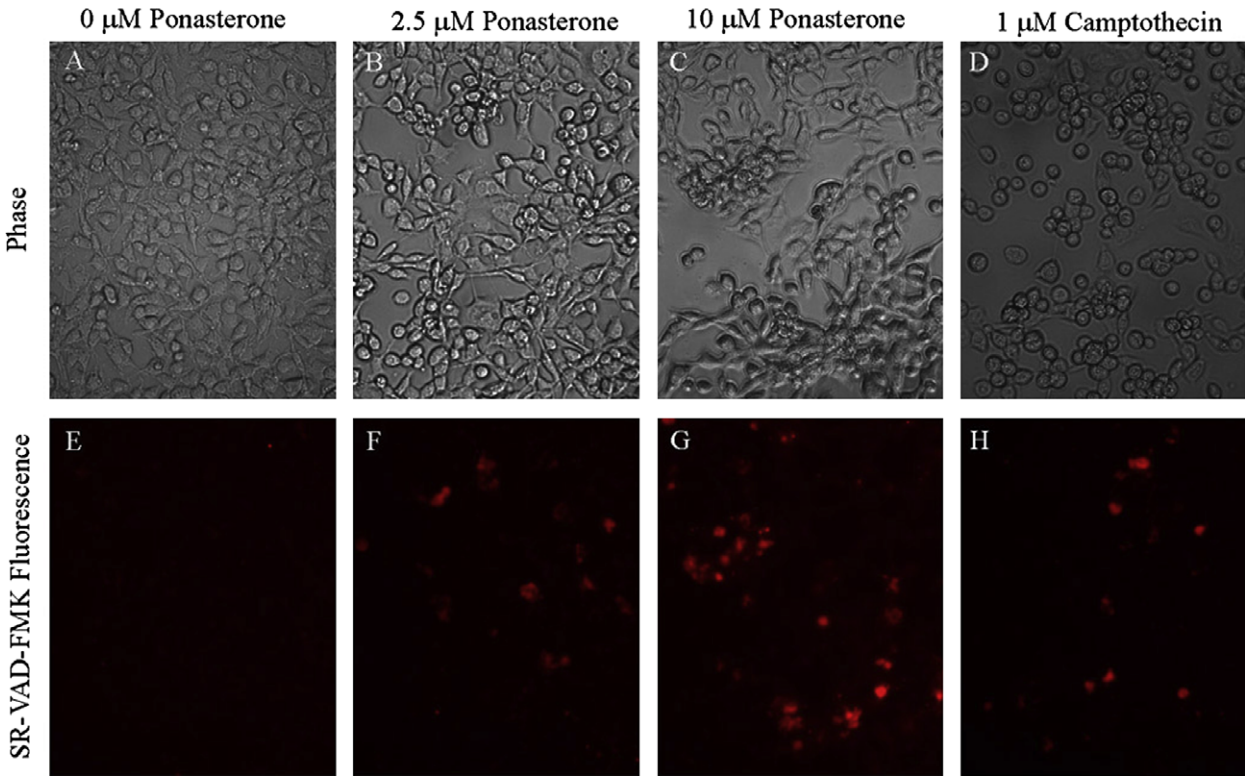

Fig. 1. Caspase activation depends on ponasterone A dose. Cells treated with 2.5 (panels B and F) or 10 μM ponasterone A (C and G) or 1 μM camptothecin (D and H). Phase (A–D) indicates reduced cell densities after treatment. Caspase activation demonstrated by *in situ* fluorescent labeling with cell permeable SR-VAD-FMK (E–G).

camptothecin (Fig. 1H). Similar results were found with all cell lines in this study.

Similar labeling of cells and lysates by anti-ubiquitin and anti-NF antibodies

If expression of chimeric NF proteins triggered apoptosis, we postulated that the chimeric NF protein would be present in ubiquitinated aggregates. Immunofluorescence localization of both NF and ubiquitin changed with increasing levels of NF expression induced by increasing levels of ponasterone A for all constructs expressed in either MFT-6 or MFT-16 cells. As shown in Fig. 2 for LML−/− cells, the majority of the chimeric NF protein was not filamentous; there may be a few areas where chimeric NF protein may have formed filaments (Fig. 2A, arrow). Ubiquitin did not co-localize in these regions (Fig. 2D). As the dose of ponasterone A was increased, NF and ubiquitin were then localized in a punctuate pattern in the perinuclear region (Fig. 2B and E). As the dose of ponasterone A was increased further, the immunolocalized NF and ubiquitin containing proteins appeared in the nucleus (Fig. 2C and F). In general, intensely staining NF aggregates were also stained with ubiquitin and areas more diffusely stained with the anti-NF antibody did not co-localize with signal from the staining from anti-ubiquitin antibody.

The co-localization of ubiquitin and chimeric NF in aggregates by immunolocalization suggested that the chimeric NF may be ubiquitinated. Immunoblots of cell lysates from LML+/+ cells grown in the presence of 5 μM ponasterone A for 24 h probed with anti-NFL polyclonal antibody revealed a diffusely staining band of approximately 68–70 kDa (Fig. 2, lane 1) while a duplicate lane, probed with anti-ubiquitin monoclonal antibody, revealed a sharp band of approximately 70 kDa (Fig. 2, lane 2). These data indicate that a band of the appropriate molecular weight reacts with both anti-ubiquitin and anti-NF antibodies.

Thus, it is likely that the ubiquitin immunoreactive band corresponds to LML protein that has one or more ubiquitin modifications (or other post-translational modification).

Change in cellular localization of vimentin and NF

Immunolocalization experiments on uninduced cells showed little labeling with anti-NF antibodies (Fig. 3A and I). Addition of 2.5 μM ponasterone A led to NF expression, but the expressed protein failed to form filaments (Fig. 3B). This is particularly clear when NF localization in Fig. 3B is compared to the classic filamentous morphology seen with anti-vimentin staining in uninduced cells (Fig. 3E). Addition of ponasterone A led to

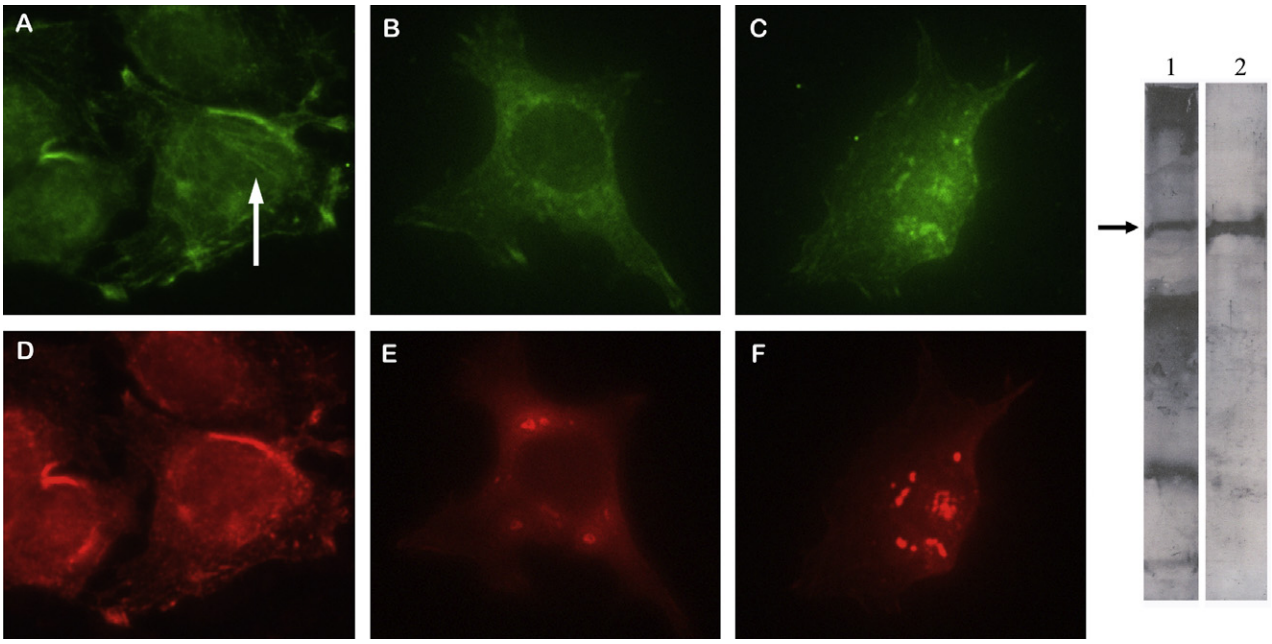

Fig. 2. Similar labeling with anti-ubiquitin and anti-NF antibodies. Immunofluorescence of LML−/− cells induced for 24 h with 2.5 μM (A, D), 5 μM (B, E), and 20 μM (C, F) ponasterone A and double-labeled with anti-NF antibody (A–C) and anti-ubiquitin antibody (D–F). Filaments may be visible with NF but not with ubiquitin staining (arrow). Immunoblot of lysates made from LML+/+ cells induced for 24 h with 5 μM ponasterone A with anti-ubiquitin (1) or anti-NFL (2) antibody. Arrow indicates bands at approximately 70 kDa.

disassembly of the endogenous vimentin network (compare Fig. 3E and F) and eventual co-localization with NF protein in aggregates (Fig. 3D and H).

Advantages of this cell line model system include inducible chimeric NF protein expression with little background expression and protein expression levels that are proportional to the amount of inducer added [22]. We exploited these aspects of the model system to ask whether the localization of NF proteins varied in dose-response and time-course experiments. Fig. 3, panels A–H, shows the localization of NF and ubiquitin in MML+/+ cells grown in the presence of 0–20 μM ponasterone A for 24 h and double-labeled with anti-NF and anti-vimentin antibodies. Without induction, there was little signal with anti-NF antibodies (Fig. 3A), while vimentin formed filaments (Fig. 3E). At 2.5 μM ponasterone A, the immunoreactive proteins were largely cytoplasmic, with the appearance of filaments (Fig. 3B and F). The localization of the proteins shifted to the perinuclear region at 5 μM ponasterone A (Fig. 3C and G) and then to largely intranuclear staining at 20 μM ponasterone A (Fig. 3D and H).

A similar redistribution of chimeric NF proteins was seen in a dose-response experiment. When cells were grown in the presence of 5 μM ponasterone A for 1 day, both filaments and perinuclear punctate staining were seen (Fig. 3J). As the duration of induction was increased, the immunolocalization of the chimeric NF protein became perinuclear at 2 days (Fig. 3K) and then nuclear after 5 days (Fig. 3L).

Discussion

The cell lines described here represent a useful model system for the study of protein aggregation. Protein aggregation is intimately associated with neurodegeneration and these cell lines recapitulate key aspects of this process, including aggregation of neurofilament protein, co-localization with ubiquitin, and reduced cell viability by apoptosis. In developing an experimentally flexible model system, we have used a protein that is associated with ND expressed in non-neuronal cells.

The importance of protein aggregation in disease pathology is increasingly clear. Published data support the toxicity of aggregates formed by non-disease related proteins [1]. Furthermore, there is an increasing appreciation of the role protein aggregation in non-neuronal tissues plays in disease. Transgenic mice no longer exhibited an ALS-type phenotype when mutant SOD1 expression was limited to neurons [30] and mutated huntingtin caused an increase in muscle cell apoptosis [31].

Not only do the proteins that comprise aggregates vary in different diseases processes, but there is heterogeneity in the aggregates within the same disease. For example, both nuclear and cytoplasmic aggregates are seen in Huntington's disease, with aggregates localized in the nucleus being more cytotoxic [32] and occurring later in the disease process [33]. For ALS, there are differences between the aggregates seen in patients with sporadic versus familial ALS [34], and NF aggregates in the axon have greater toxicity than those in the perikarya in a model system [35].

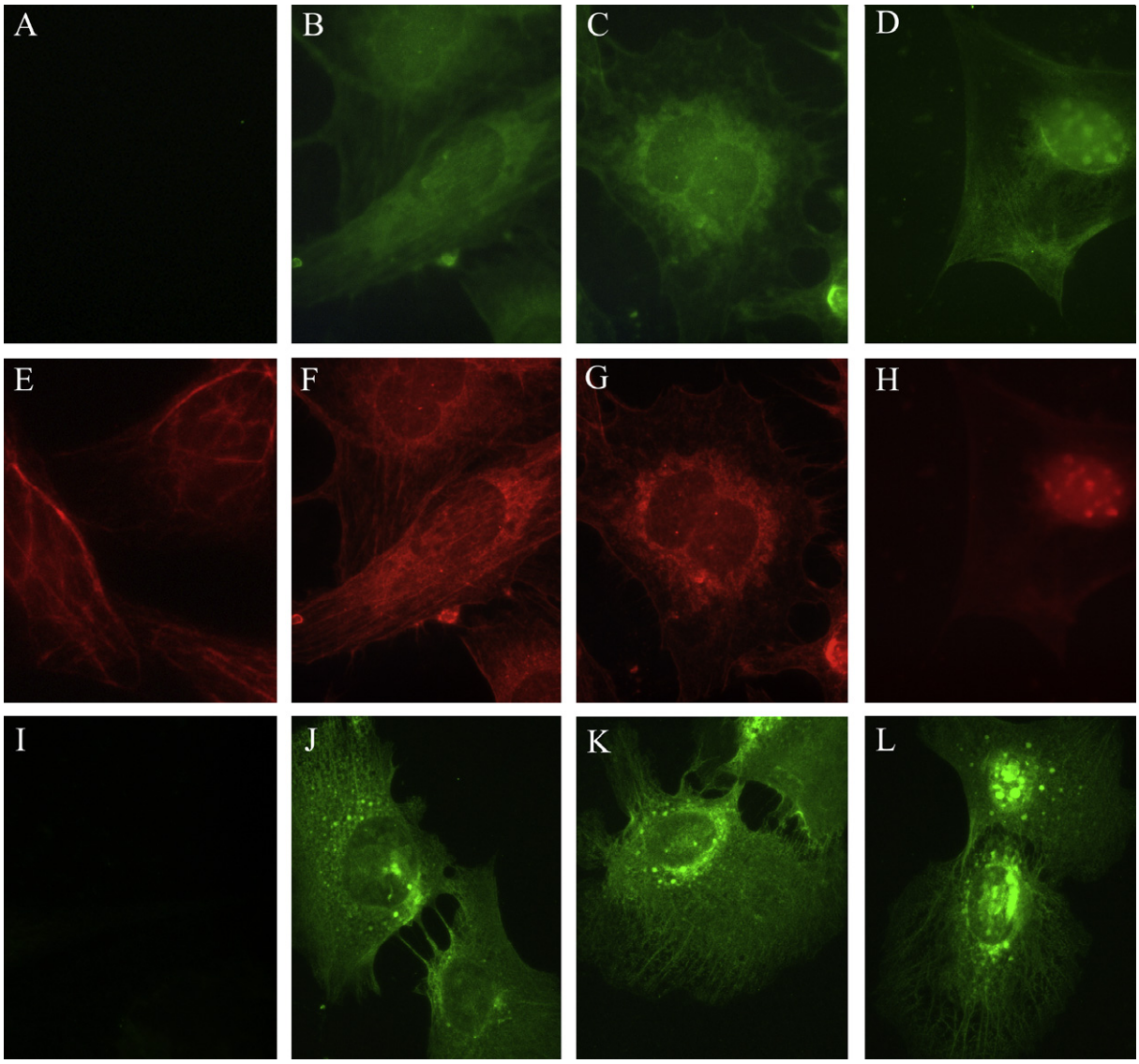

Fig. 3. Immunofluorescence localization of NF aggregates depends dose (A–H) of inducer and time-course (I–L) of induction. MML+/+ cells induced for 24 h with zero (A, E), 2.5 (B, F), 5.0 (C, G), or 20 μM (D, H) ponasterone A and double-labeled with anti-NF (panels A–D) or anti-vimentin antibody (panels E–H). (I–L) Cells induced with 5.0 μM ponasterone A for zero (I), one (J), two (K), or five (L) days and labeled with anti-NF antibody.

The data presented here assess the effect of protein expression levels on the formation of aggregates. Although the overall behavior of each of the chimeric NF proteins was similar in each parent cell line, there are subtle differences between parent cell lines expressing the same chimeric NF. More NF protein appeared localized to thin filaments at low doses of ponasterone A in MFT-6 cells as compared to MFT-16 cells; the nuclear aggregates appeared to be larger in MFT-6 cells at high doses of ponasterone A, suggesting that vimentin, after network disassembly, adds to the size of the aggregates. These data are consistent with published work: expressed NF proteins may co-polymerize with endogenous IF proteins or disassemble a pre-existing IF network [23,27] into aggregates [19–21,35]. Both time-course and dose-response experiments suggest that aggregates form when NF proteins accumulate beyond a threshold level, which could be due to an overload of the proteasomal degradation system.

The current system is designed to be an experimentally flexible model system to study the sequence of events from the onset of protein aggregation to eventual cell death. This system recapitulates several key aspects of ALS and ND in general: aggregates form, they are toxic to cells, promote apoptosis, and contain ubiquitin. As IF protein aggregates that contain ubiquitin and HSP 70 are seen in ND and in mallory bodies associated with a variety of liver diseases [4], use of this model system to explore the mechanism of protein aggregation may, in the future, explain why aggregates are toxic and how they contribute to pathogenesis of a wide variety of diseases.

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