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Cytotoxicity of amyloidogenic immunoglobulin light chains in cell culture

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Light-chain amyloidosis (AL) is a devastating protein-misfolding disease characterized by abnormal proliferation of plasma cells in the bone marrow that secrete monoclonal immunoglobulin light chains that misfold and form amyloid fibrils, thus causing organ failure and death. Numerous reports on different protein-misfolding diseases show that soluble oligomeric species populated by amyloidogenic proteins can be quite toxic to cells. However, it is not well established whether the soluble immunoglobulin light-chain species found in circulation in patients with AL are toxic to cells in target organs. We determined the cellular toxicity of two well-characterized light-chain variable domain proteins from cardiac AL patients and their corresponding germline protein, devoid of somatic mutations. Our results show that the soluble form of the AL proteins we characterized are toxic to cardiomyocytes, and that the species found in cell culture correspond, for the most part, to the species present in circulation in these patients.

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There are >20 human amyloid diseases characterized by misfolding of otherwise native protein precursors that give rise to the formation of amyloid fibrils. These diseases can be divided by localized (Alzheimer's disease (AD)) or systemic (light-chain amyloidosis (AL)) deposition. Historically, the observation of end-stage amyloid deposits in biopsies and postmortem specimens led to the assumption that amyloid accumulation alone can explain the progression of amyloid diseases. However, a large number of reports now suggests that monomeric and soluble intermediate species (usually referred to as protofibrils[1]) are responsible for the major cytotoxicity in amyloid diseases. An early AD study by Hartley *et al.*[2] demonstrated that low-molecular-weight Aβ (LMW Aβ) and protofibrils induced toxicity in mixed brain cultures without an evident increase in fibril formation, suggesting that the neurotoxicity of LMW Aβ and protofibrils cannot be explained by conversion to fibrils.

More recently, Lesne *et al.*[3] found that extracellular accumulation of 56-kDa soluble Aβ oligomers caused memory deficits in middle-aged AD transgenic animals. In the case of systemic, familial amyloid polyneuropathy/cardiomyopathy caused by mutant Transthyretin (TTR), Reixach *et al.*[4] conducted a cell culture-based analysis of the effect of purified TTR species on the viability of cells in culture. Their findings indicate that neither TTR fibrils nor soluble aggregates >100 kDa were toxic to the human neuroblastoma cell line IMR-32.

Pokrzywa *et al.*[5] developed a *Drosophila* model for TTR-associated amyloidosis. The authors showed that flies expressing high TTR levels had a larger proportion of high-molecular-weight aggregates that are assumed to be less toxic. The authors hypothesize that there is an optimal concentration, specific to each mutated variant of TTR that determines the rate of toxic aggregate formation and consequently its effect on the phenotype, a notion consistent with earlier studies in cultured cells.

AL is a devastating amyloid disease characterized by the abnormal proliferation of plasma cells in the bone marrow that secrete free immunoglobulin light chains that circulate as monomers and dimers.[6] These light chains misfold as amyloid fibrils, generating toxic species that affect vital organs such as the kidney, heart, and liver, thus causing organ failure and death.[7] An early observation made by Kyle *et al.*[8] indicated that AL patients can achieve resolution of their nephrotic syndrome after treatment while presenting more extensive amyloid deposition than what was initially observed in the biopsied tissue. Recent studies have reported that soluble light-chain species internalize into renal and cardiac cells and cause alterations in cell homeostasis.[9–14] Brenner *et al.* incubated urine-derived light chains from patients with cardiac AL in the presence of cardiomyocytes. The light chains altered the cellular redox state of cardiomyocytes, with an increase in intracellular reactive oxygen species and upregulation of the cellular stress marker heme oxygenase-1. Impairment of cardiomyocyte contractility and relaxation was also observed.[9] It has been recently shown that urine-derived AL light-chain proteins are toxic to cardiomyocytes by inducing apoptosis through the p38 MAPK pathway. The light chain-induced p38 activation depended on TAB1-mediated p38α MAPK autophosphorylation. Treatment with a selective p38 MAPK inhibitor significantly attenuated AL light chain-induced oxidative stress, cellular dysfunction, and apoptosis.[14]

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Abbreviations: AL, light-chain amyloidosis; AD, Alzheimer's disease; TTR, Transthyretin; LDH, lactate dehydrogenase; FBS, fetal bovine serum; ThT, thioflavine T; MTT, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
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A recent report by Migrino *et al.*[15] using human adipose arterioles shows that the presence of urine-derived AL light chains causes elevated levels of superoxide, impaired dilation in response to sodium nitroprusside, and microvascular dysfunction. All of the aforementioned cell and tissue culture studies with AL proteins have been conducted using urine-derived light chains from different AL patients, for whom the primary sequence, the exact oligomeric state of the light chains, and the folded state of these proteins were not reported.

We hypothesize that soluble AL proteins are more cytotoxic than their germline counterparts because of the presence of somatic mutations in AL proteins that promote sampling of toxic, partially folded states.

In this study, we characterized the cellular toxicity caused by soluble recombinant, amyloidogenic light-chain variable domain proteins, AL-09 and AL-12, and their corresponding germline protein κI O18/O8, previously characterized in our laboratory.[16–18] Both AL-09 and AL-12 are derived from cardiac AL patients with very different survival after diagnosis. Patient AL-09 was a 63-year-old man with a 14-year history of monoclonal gammopathy of undetermined significance.[19] AL-09 developed fatigue, anorexia, and claudication symptoms. The bone marrow presented with 60% of plasma cells; hence, AL-09 was initially diagnosed with straight multiple myeloma. Two months into treatment, he was diagnosed with cardiac AL after showing signs of cardiomyopathy and atrial flutter. Amyloid was confirmed in the cardiac tissue by biopsy. AL-09 survived less than a year. At the time of AL-09 patient diagnosis, the free light-chain assay was not available. We recently determined the free light-chain level from frozen plasma samples stored in our specimen bank.[20] AL-09 κ-light-chain levels in serum were 201 mg/dl.

Patient AL-12 was a 64-year-old woman diagnosed with multiple myeloma/cardiac AL after presenting with symptoms of hoarseness, trouble swallowing, 15 lb weight loss, fatigue, mild compensated congestive heart failure, and epigastric pain. The presence of amyloid in the cardiac tissue was confirmed by biopsy. AL-12 survived 10 years. AL-12 patient did not have the free light-chain assay performed on plasma at the time of her illness. We also recently determined the free light-chain level from frozen plasma samples stored in our specimen bank.[20] AL-12 free κ-light-chain levels in serum were 10.1 mg/dl.

AL-09 protein has seven somatic mutations from germline κI O18/O8 and three nonconservative mutations, whereas AL-12 protein has eight somatic mutations from κI O18/O8 and seven nonconservative mutations. For AL-09 and AL-12, we cloned the variable domain region from the patients' dominant clone light-chain cDNA into the bacterial plasmid pET12a and expressed the variable domains in *Escherichia coli.*[16,18] The variable domains (herein known as AL proteins for simplicity) were purified and characterized extensively in terms of their structure and fibril-formation properties in our previous studies.[17,18,21] We artificially generated a plasmid corresponding to the germline protein κI O18/O8 with a junction region JK2 by site-directed mutagenesis.[17] The germline protein κI O18/O8 has been used in previous studies for comparative analysis in terms of protein structure, protein stability, amyloid-formation properties, and, in this study, cellular toxicity. The results of this study show that AL proteins in solution induced significant toxicity over time. We find that monomers and dimers are the predominant light-chain species under cytotoxic conditions.

Results

Influence of amyloidogenic light chains on cell viability. To detect changes in mitochondrial viability for cardiomyocytes incubated with the three light chains tested, we measure the percentage of living cells as a function of soluble protein concentration (10–20 μM) using the 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 1). There is a weak protein concentration dependence linked to cellular viability for AL-12 and κI O18/O8. Statistically significant differences were found for AL-12 after 72-h incubation between 10 and 14 μM ($P<0.002$), 10 and 16 μM ($P<0.006$), 10 and 20 μM ($P<0.01$), and 12 and 14 μM ($P<0.01$). For κI O18/O8, statistically significant differences were found after 72 h incubation between 10 and 16 μM ($P<0.03$), and 10 and 20 μM ($P<0.004$). No clear concentration dependence was observed for AL-09. The only instances in which there is a

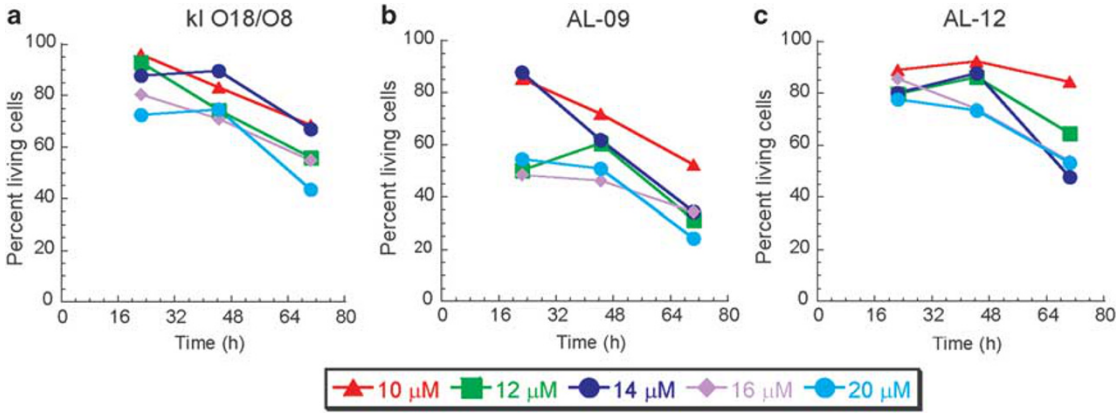

Figure 1 Cell viability in the presence of light chains followed by MTT assay. Protein concentrations from 10 to 20 μM were incubated with HL-1 cardiomyocytes for 24–72 h to determine cell viability. (**a**) κI O18/O8, (**b**) AL-09, and (**c**) AL-12. Samples were set up in triplicate, with average values shown from one assay

statistically significant difference for AL-09 are after 24-h incubation between 10 and 16 μM ($P<0.03$), after 48-h incubation between 10 and 16 μM ($P<0.05$), and after 72-h incubation between 10 and 12 μM ($P<0.02$), and between 10 and 20 μM ($P<0.02$). Using a concentration of 12 μM, we observe differences in cell viability in the presence of the three proteins. The difference in the number of remaining living cells after 24 h of protein incubation between AL-09 and κI O18/O8 is significant ($P<0.03$), although there is a large error associated with the 24 h incubation data for all proteins. No statistically significant difference was seen between AL-09 and AL-12 after 24-h incubation ($P<0.10$).There is an increase in cell viability after 48 h of incubation for all proteins. The increase in cell viability between 24 and 48 h in the presence of AL-09 is the only condition that shows statistical difference ($P<0.03$). After 72 h of incubation (the conditions in which we see the most significant changes between proteins), only 38% of cells remain alive in the presence of AL-09, followed by AL-12 (57% living cells), and κI O18/O8 (52% living cells) (Figure 2). Statistically significant differences were found between the number of living cells in the presence of AL-09 and AL-12, and AL-09 and κI O18/08 ($P<0.004$ and $P<0.02$, respectively) after 72-h incubation. In summary, our MTT assay results suggest that AL-09 causes the largest reduction in the viability of HL-1 cardiomyocytes.

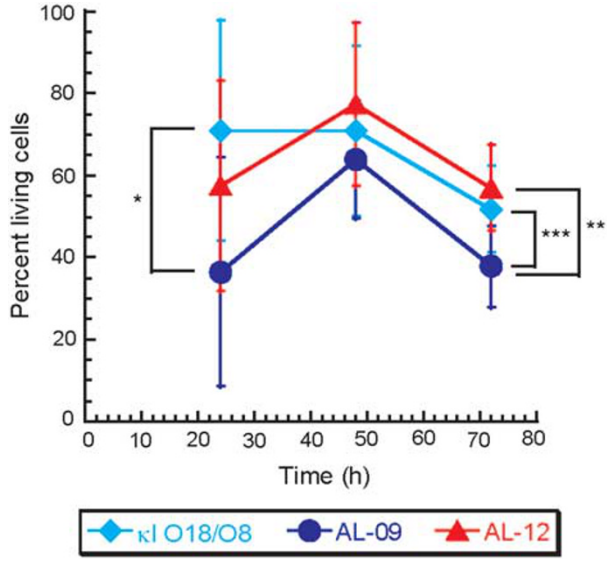

Figure 2 Percentage of living HL-1 cardiomyocytes when incubated with 12 μM soluble light-chain protein followed by MTT assay. Proteins were incubated with cells for 24–72 h. Samples were set up in triplicate in two independent assays with the average values, error bars, and *P*-values shown. *$P<0.03$, **$P<0.004$, ***$P<0.02$

Increased caspase activity in the presence of amyloidogenic light chains. A more direct method to determine induced cell death is to measure caspase activity in the presence of light chains. The assay we use is specific for caspases 2, 3, and 7. We observe no protein concentration dependence on caspase activity in the presence of light chains (Figure 3). No major differences in caspase activity are observed between proteins and protein concentrations after 24 and 48 h of incubation. Seventy-two hours seem to be the best incubation time to observe differences in caspase activity. Some protein concentrations for AL-12 and κI O18/O8 have baseline caspase activity observed with cells alone.

We observe the largest difference in caspase activity with 12 μM protein. After 72 h of incubation with 12 μM protein, AL-09 presents the highest caspase activity, followed closely by AL-12. We observe baseline caspase activity levels with κI O18/O8 (comparable with cells alone, Figure 4). The difference between caspase activity for AL-09 and κI O18/O8 and that between AL-12 and κI O18/O8 is statistically significant ($P<0.008$ and $P<0.05$, respectively). AL-09 and AL-12 present similar caspase activities ($P<0.4$).

Biochemical characterization of cytotoxic species populated by light chains. To determine the exact oligomeric state of the species populated in cell culture, we used size exclusion chromatography. In Figure 5a–c, we observe three common peaks for media from cells incubated with the three proteins. These peaks show apparent molecular mass ~12, 130, and 400 kDa. The 12-kDa peak is only present in samples with amyloidogenic light chains

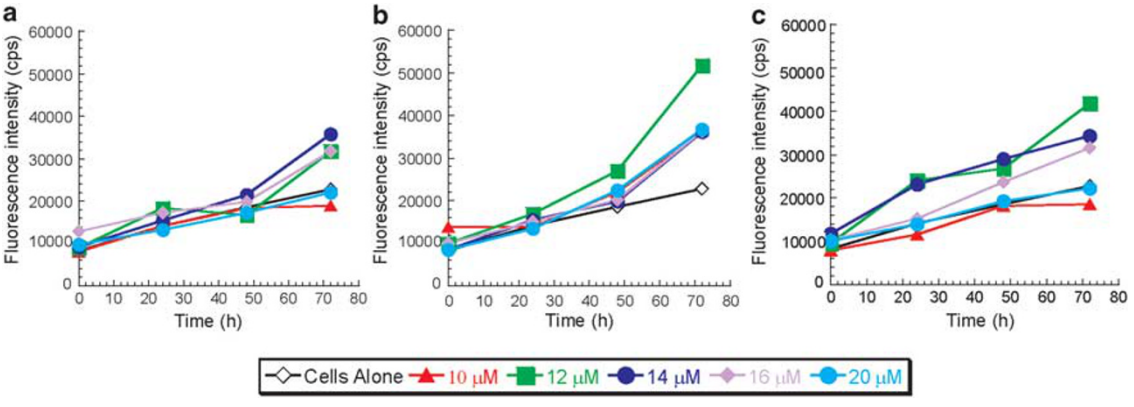

Figure 3 HL-1 cardiomyocytes caspase activity in the presence of light chains. Protein concentrations of 10–20 μM were incubated with cells for 24–72 h. Reagents were added 1 h before reading each plate. Samples were set up in triplicate with the average fluorescence shown. (**a**) κI O18/O8, (**b**) AL-09, and (**c**) AL-12

(denoted as 'monomer peak' hereafter) and elutes at slightly different volumes ~12 kDa for all proteins, possibly due to the different monomer–dimer equilibrium that occurs at this concentration for all proteins based on their dimer dissociation constants.[17] The monomeric and 400-kDa peaks do not show large changes in absorbance over the course of the 72-h incubation. AL-09 and AL-12 show a slight increase in the absorbance of the 130-kDa peak after 72 h, whereas κI O18/O8 shows a similar slight increase in the 130-kDa peak after 48 h. As the media alone contain proteins (as shown in Figure 5a–c), we performed western blot analysis on the fractions corresponding to the different peaks found by size exclusion chromatography to identify the light-chain species observed in cell culture. We observe immunoreactive protein around the 12-kDa (monomer) peak for all proteins. AL-09 is the only protein that clearly showed the presence of 400-kDa immunoreactive protein (corresponding to a 33-mer) after 24 h of incubation (AL-12 shows a faint band corresponding to the 33-mer immunoreactive species after 48 h) (Figure 5d–l). After 72 h of incubation, all immunoreactive light-chain species disappear from the media for AL-09 and AL-12, with some faint bands from the monomer peak for κI O18/O8 still visible. This is possibly because they are binding to the cell surface and/or being internalized into cardiomyocytes, as has been observed for recombinant full-length AL-09 and κI O18/O8 (Olatoye and Ramirez-Alvarado, unpublished observations).

Our western blot results suggest that monomers and dimers are the predominant species in cell culture for the proteins analyzed in this study. The protein peak corresponding

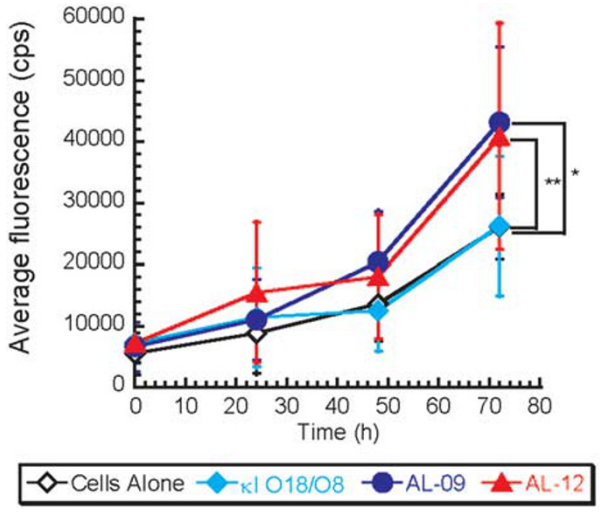

Figure 4 Caspase activity detected in HL-1 cardiomyocytes with a protein concentration of 12 μM. Cells were incubated with protein for 24–72 h. Reagents were added 1 h before reading plate. Average fluorescence, error bars, and *P*-values were calculated from triplicate samples from two independent assays. $^{*}P<0.008$, $^{**}P<0.05$

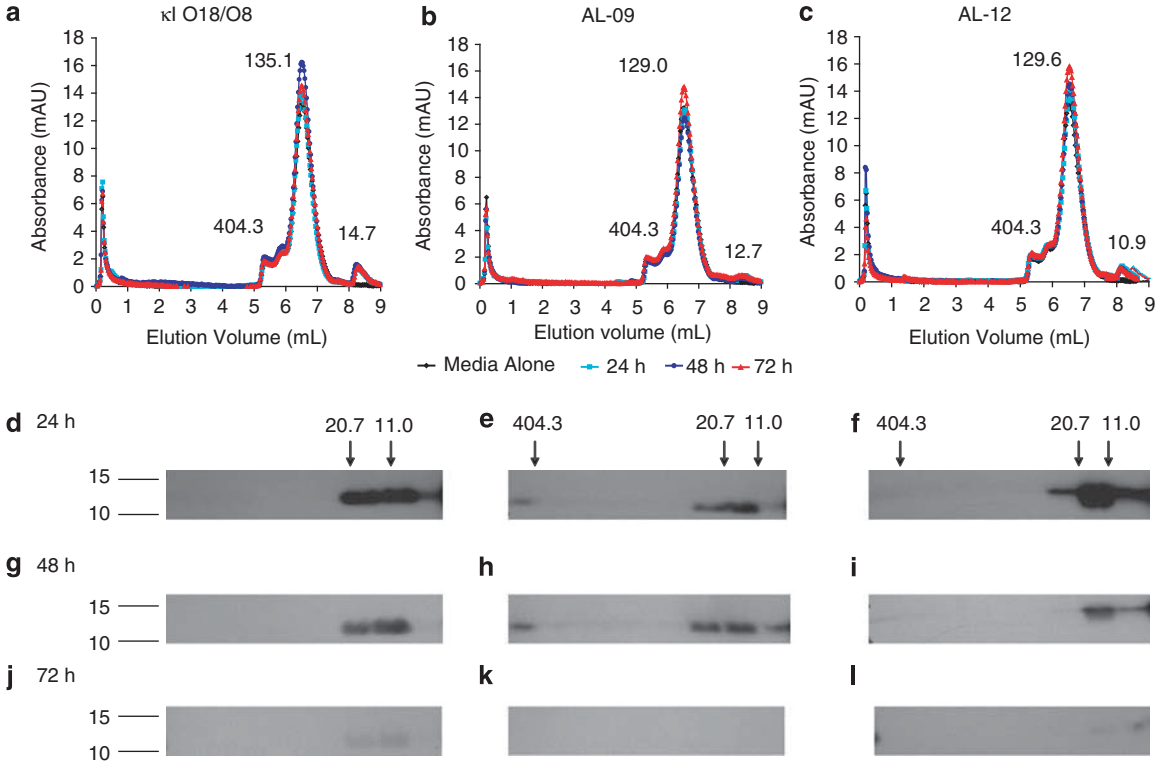

Figure 5 Soluble light-chain species detected in cell culture analyzed by analytical size exclusion chromatography and western blot. HL-1 cardiomyocytes were incubated with 12 μM protein for 24–72 h. Samples were set up in triplicate and combined before injecting onto the analytical size exclusion column. The molecular mass corresponding to the peaks was calculated using a standard curve described in the 'Materials and Methods' section. (**a**) κI O18/O8, (**b**) AL-09, and (**c**) AL-12. The molecular weight corresponding to peaks of interest are shown. Western blot analysis shows immunoreactive κI O18/O8 (**d**, **g**, **j**), AL-09 (**e**, **h**, **k**), and AL-12 (**f**, **i**, **l**) species in cell culture. Molecular weights denoted in the figure are expressed in kDa

to 130 kDa is part of the media and does not contain any light-chain species. Electron microscopy images of cell culture samples in which cytotoxicity was observed do not show any distinguishing features compared with the media alone, confirming our findings that the species found in cell culture are soluble and are of LMW (data not shown). Fibril-formation reactions followed by thioflavine T (ThT) fluorescence in an independent experiment showed no signs of amyloid formation under conditions used in the cellular assays (37°C, 72 h) (Supplementary Figure 1).

Discussion

AL is a devastating amyloid disease characterized by misfolding of light chains as amyloid fibrils, thus causing organ damage and death. The mechanisms underlying the pathophysiology of AL remain largely unknown. In this study, we showed that soluble, recombinant, immunoglobulin light-chain variable domain proteins from AL patients have large effects on cardiomyocyte viability, in agreement with a recent report using urine-derived proteins.[14] We demonstrated that the species populated are of LMW comprising mostly monomers in equilibrium with dimers and soluble 33-mer species. Using the MTT assay reported by others to assess the cytotoxicity of soluble amyloidogenic proteins,[4] we observe large variability between our replicates for all proteins after 24 h incubation and an increase in cell viability after 48 h (significantly different only for AL-09), possibly due to the reported errors associated with the process of solubilization of the dark blue formazan produced in this assay and artifacts associated with the proteins contained in the media.[22,23] Another way to measure cell viability is to determine the lactate dehydrogenase (LDH) enzyme released into the media. However, after an extensive optimization protocol, we were unable to correlate LDH activity with cytotoxicity on HL-1 cardiomyocytes because of the high LDH activity calculated for cardiomyocytes alone in cell culture (see the 'Materials and Methods' section; Supplementary Figure 2), in agreement with observations made in a recent report studying the possible anti-apoptotic effects of L-carnitine on cardiomyocytes.[24] The AL proteins used in this study have 90% sequence identity. After 72 h of incubation with 12 μM protein, AL-09 is the most cytotoxic protein by the two methods used in this paper (MTT and caspase activity). AL-12 does not cause a large effect in cell viability using MTT, but it induces high caspase activity in cell culture close to the activity induced by AL-09. The germline protein κI O18/O8, devoid of somatic mutations, decreases cellular viability slightly, but does not induce any additional caspase activity compared with cells alone. The clinical characteristics of patients AL-09 and AL-12 indicate that both had suffered severe cardiac damage when initially diagnosed with cardiac AL. Patient AL-09 had higher levels of free κ-light chain in serum than did patient AL-12, offering a possible explanation for the difference in the severity of their disease. However, our studies were carried out at the same concentration for both proteins, suggesting that the cytotoxic potential of AL-09 does not depend entirely on a high protein concentration.

Biophysical characterization of the proteins used in this study has shown that although AL-09 and AL-12 present similar thermodynamic stability, both AL proteins are less stable than κI O18/O8. Crystal structures of AL-12 and κI O18/O8 have shown that these proteins form the canonical dimer interface observed in immunoglobulin light chains.[17,18,21] However, AL-09 populates an altered dimer interface[17] and forms fibrils quite readily compared with AL-12 and κI O18/O8 (Supplementary Figure 1). These previous reports suggest that somatic mutations present in AL-09 cause the protein to dimerize in an altered conformation, promoting misfolding and facilitating the formation of amyloid fibrils. The current study adds important information to the characterization of these amyloidogenic light chains. It shows that AL-09 is highly toxic to cardiomyocytes, suggesting that the altered dimer interface allows intramolecular and intermolecular interactions that cause the protein not only to form fibrils quite readily but also most importantly to populate soluble cytotoxic species. We identified light-chain species corresponding to monomer and 33-mer present in cell culture that seem to be responsible for the cytotoxicity observed in this study. The use of size exclusion chromatography coupled with western blotting was particularly useful as the media used for these experiments have a high protein content and there were no distinguishing features in the chromatogram of the cytotoxic reactions other than the presence of the monomeric peak. As we mentioned before, these monomeric species are probably in equilibrium with dimeric species based on the dissociation constants for these proteins.[17] Further studies should be conducted to determine the role of the 33-mer species in cytotoxicity. The monomeric and 33-mer species would need to be purified from the media and re-incubated with cardiomyocytes to establish their exact role in cytotoxicity. It would be important to determine whether these species are made entirely of light chains or whether there are other proteins associated with the 400-kDa assembly. Although we observe small changes in the 130-kDa peak for all proteins, this peak does not contain any light-chain species, and at this point, we cannot conclude whether changes in the media protein components at 130 kDa are causing any change to the light chains and have an influence on cell viability results. The disappearance of light-chain species from the media suggests that the protein is entering into the cells and promoting cell death during the internalization process. In the future, it would be important to study the cytotoxic effects of 72-h media depleted of light-chain protein to prove this hypothesis. In addition, future studies determining the cytotoxicity associated with amyloid fibrils from these proteins will help identify the most cytotoxic species in cell culture for AL proteins.

AL-09 has three nonconservative mutations within or adjacent to the dimer interface (namely N34I, K42Q, and Y87H), whereas AL-12 has seven nonconservative mutations throughout the protein structure (namely Y32H, S65R, D70H, E81A, Q90E, N93Y, and Y96Q), three of them in the dimer interface (Y32H, Q90E, and Y96Q). Previous sequence/structure analysis performed in our laboratory found that the number of nonconservative mutations in amyloidogenic light chains do not determine their amyloidogenicity. Rather, we proposed that the location of mutations confers the proteins with their amyloidogenic potential.[20] The cytotoxicity associated with AL-09 suggests that the specific location of mutations within the dimer interface promotes the formation

of cytotoxic species. Future studies determining the role of specific somatic mutations in cytotoxicity will be necessary to fully understand the origin of cytotoxicity in amyloidogenic light chains.

Materials and Methods

Cells. Mouse HL-1 cardiomyocytes were kindly provided by Dr. William Claycomb.[25] Flasks were coated with fibronectin and gelatin and cells were maintained in Claycomb medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 0.1 mM norepinephrine dissolved in 30 mM ascorbic acid, 2 mM L-glutamine, and 100 Units/ml penicillin–streptomycin. Cells were incubated at 37°C with 5% CO_2 as reported previously.[26]

Protein preparation. κI O18/O8, AL-09, and AL-12 were expressed and purified as reported previously.[16–18] In brief, the proteins were expressed in *E. coli* BL21 (DE3) gold-competent cells at 30°C, 250 r.p.m. The cells were induced with 0.8 M IPTG, harvested, and frozen at −20°C. Thawed cells were treated with osmotic shock. AL-09 was extracted from inclusion bodies using 6 M urea, whereas κI O18/O8 and AL-12 were extracted from periplasmic fractions. The proteins were purified using size exclusion chromatography (HiLoad 16/60 Superdex 75 column) on an AKTA FPLC (GE Healthcare, Piscataway, NJ, USA). Pure fractions were collected, concentrated, and frozen. Proteins were thawed immediately before each assay. After filtration, the protein concentration was determined as reported previously.[18]

Cell viability assays

Mitochondrial viability assay. Cardiomyocytes were plated (10 000 cells per ml) in a 96-well Costar polystyrene plate and allowed to grow for 2 days where they reached 70% confluency before addition of the soluble protein. For these assays, we used supplemented Claycomb media without norepinephrine because the ascorbic acid solution used to prepare norepinephrine interferes with the cell viability reagents described below. Different soluble protein concentrations (10, 12, 14, 16, and 20 μM) were added to the cells. Each condition was set up in triplicate. Plates were assayed 24, 48, and 72 h after addition of the soluble protein. The cell proliferation kit I (MTT) (Roche, Indianapolis, IN, USA) was used for this assay. In brief, 10 μl of MTT reagent was added per well and incubated at 37°C, 5% CO_2 for 4 h. Next, 100 μl of solubilization buffer was added to each well and incubated overnight. Plates were read on a SpectraMax 384 Plate Reader (Molecular Devices, Sunnyvale, CA, USA) at 570 and 690 nm. The 690-nm absorbance reading (reference) was subtracted from the 570-nm absorbance reading for each well, and the percentage of living cells was calculated using the following formula:

$$\%\,\text{living cells} = \frac{(\text{sample absorbance} - \text{blank absorbance})}{\text{control absorbance} - \text{blank absorbance}} \times 100,$$

where blank absorbance is the media alone and control absorbance cells with media alone. The average of the percentage of living cells was then calculated along with S.D. Cells without protein were used as the reference for 100% living cells. Data from two independent experiments were analyzed using the Microsoft Excel 2003 (Microsoft, Redmond, WA, USA) *t*-test for two samples assuming equal variance tool to calculate *P*-values.

LDH assay. The 96-well Costar polystyrene plates were plated with 15 625 cells per ml 1 day before addition of the soluble protein with supplemented Claycomb media (containing 10% FBS). Media were removed and the plate was washed with 1% FBS and norepinephrine-free media. Several soluble protein concentrations (10, 12, 14, 16, and 20 μM) were added to the cells with supplemented Claycomb media containing 1% FBS and no norepinephrine. Samples were set up in triplicate. Cells were incubated with the soluble protein for various time points (24, 48, and 72 h). The assay was performed following LDH cytotoxicity detection kit protocol (Clontech, Mountain View, CA, USA), with a few modifications. In brief, 200 μl of media from each well was transferred to a new 96-well polystyrene plate and spun at 300 × *g* for 10 min. Next, 100 μl of supernatant was transferred to a new plate in which we added 100 μl of reaction mixture to each well, and incubated at room temperature in the dark for 30 min. The plate was read at 492 and 690 nm on a SpectraMax Plus 384 Plate Reader (Molecular Devices). The 690-nm reference absorbance reading was subtracted from the absorbance at 492 nm and the average absorbance and S.D. were calculated for each triplicate. Cell membrane integrity or percentage of LDH released could not be calculated because of high-absorbance readings with cells alone.

Caspase activity assay. Cardiomyocytes were plated at 25 000 cells per ml in a 96-well clear bottom black polystyrene plate with 100 μl of supplemented Claycomb media with 1% FBS and no norepinephrine. Different protein concentrations (10, 12, 14, 16, and 20 μM) were immediately added to the cardiomyocytes. Samples were set up in triplicate. Plates were incubated for 0, 24, 48, or 72 h before following the assay protocol. Each plate was assayed using the homogeneous caspase assay, fluorimetric kit (Roche). In brief, 100 μl of substrate working solution was added to each well and incubated for 1–24 h at 37°C, 5% CO_2. The plate was read on the Analyst AD plate reader (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The average fluorescence and S.D. were calculated for each sample. Data from two independent experiments were analyzed using the Microsoft Excel 2003 Student's *t*-test tool for two samples assuming equal variance to calculate *P*-values.

Analytical size exclusion chromatography. Before proteins were incubated with the media and cells, we performed analytical size exclusion chromatography studies in the buffer to confirm that all proteins were monomeric. The oligomeric state of the protein remaining in the cell culture medium was analyzed on a BioSil HPLC size exclusion column (Bio-Rad, Hercules, CA, USA) on an AKTA FPLC (GE Healthcare). Gel filtration molecular weight standards (Bio-Rad) were used (thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B12). The column was calibrated with 0.1 ml of the standards at a flow rate of 0.1 ml/min using 0.05 M Na_2HPO_4, 0.05 M NaH_2PO_4, 0.15 M NaCl, 0.01 M NaN_3, pH 7.4 buffer. A calibration curve was determined by plotting the log molecular weight of the standards as a function of the elution volume. Media alone and soluble, freshly filtered protein dissolved in media were injected to serve as baseline for our experiments. A line of best fit was calculated to determine the molecular weight of the peaks in the protein samples.

Western blot. After size exclusion chromatography was performed on the protein remaining in the media, the fractions containing protein were frozen at −80°C, thawed at a later date, and run on denaturing 15% SDS-PAGE gels. The gel was then transferred to an Immobilon P (PVDF) membrane as reported previously.[27] The primary antibody used was a sheep anti-human κ-light chain with a dilution of 1 : 500 (The Binding Site Inc., San Diego, CA, USA), and the secondary antibody used was a rabbit anti-sheep polyclonal IgG H and L (HRP) antibody (Abcam Inc., Cambridge, MA, USA). The dilution for the secondary antibody was 1 : 8000 for the 48 and 72 h gels and 1 : 4000 for the 24 h gels.

Fibril formation. Fibril-formation samples were set up in triplicate with 20 μM protein, 5 μM ThT, and 10 mM Tris-HCl buffer, pH 7.4 in a 96-well black polystyrene plate. Plates were incubated at 37°C with constant agitation at 250 r.p.m. The plate was read on the Analyst AD plate reader (Molecular Devices) at an excitation wavelength of 440 nm and emission wavelength of 480 nm. Average values were calculated for each protein. Soluble AL-09 and κI O18/O8 were centrifuged to the sedimentation time of a 0.5S particle[28] before the fibril-formation reaction, whereas AL-12 was filtered before the reaction.

Electron microscopy. A 3 μl aliquot of each sample was placed on a 300-mesh copper/formvar carbon grid and air dried. Grids were negatively stained with 2% uranyl acetate, washed, air dried and examined on a Phillips Tecnai T12 transmission electron microscope (FEI Inc., Hillsboro, OR, USA).

Conflict of interest

The authors declare no conflict of interest.

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