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Current and New Perspectives on the Molecular and Cellular Mechanisms of Amyloid Formation and Toxicity in Light Chain Amyloidosis

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1. Introduction

Light chain (AL) amyloidosis is a protein misfolding disease characterized by the abnormal proliferation of monoclonal plasma cells that secrete free immunoglobulin light chains (LC) into circulation. These LCs misfold and aggregate as amyloid fibrils in vital organs. The process of amyloid formation causes organ failure, although the exact mechanism is unknown. The most frequently affected organs are the kidneys, heart, liver and peripheral nerves. AL amyloidosis is a devastating disease with a median survival of 12-40 months (Kumar et al., 2011; Wechalekar et al., 2008). The incidence of AL is 9 per million per year in the US, comparable to the incidence of Hodgkin's Lymphoma. Current treatments are harsh and not curative (chemotherapy and autologous stem cell transplantation), targeting the plasma cells producing the protein. There is currently no treatment that targets the misfolding process or the amyloid fibrils.

This chapter will discuss the latest developments in our understanding of the molecular mechanisms of AL amyloidosis including the role of mutations, cellular microenvironment, dimerization structures, different species populated in AL amyloid fibril formation, and light chain-associated cell and tissue toxicity. We will describe the challenges facing AL amyloidosis researchers to develop effective animal models of the disease and to find the best therapeutic strategies to treat this complex, devastating disease.

2. Light chain (AL) amyloidosis – Role of the protein in disease

A LC is composed of an N-terminal variable domain (V_L) and a C-terminal constant domain (C_L). The V_Ls are not uniformly variable throughout their lengths. Three small regions, the hypervariable regions or complementarity determining regions (CDR), show much more variability than the rest of the domain. These regions vary both in size and in sequence among different V_L germline isotypes; they determine the specificity of the antigen-antibody interactions. The remaining parts of the V_L, four framework regions (FRs), have quite similar amino acid sequences. The overall structure of the V_L is an immunoglobulin fold with 9 β-strands (A, B, C, C', C'', D, E, F, and G) packed tightly against each other in two antiparallel β sheets joined together by a disulfide bridge in a form of a Greek key β-barrel. The N- and C- termini strands (A and G, respectively) are parallel (Branden & Tooze, 1999). The CDRs

form three loops between amino acids 24-34, 50-56 and 89-95 that contain the sequence that will recognize the antigen.
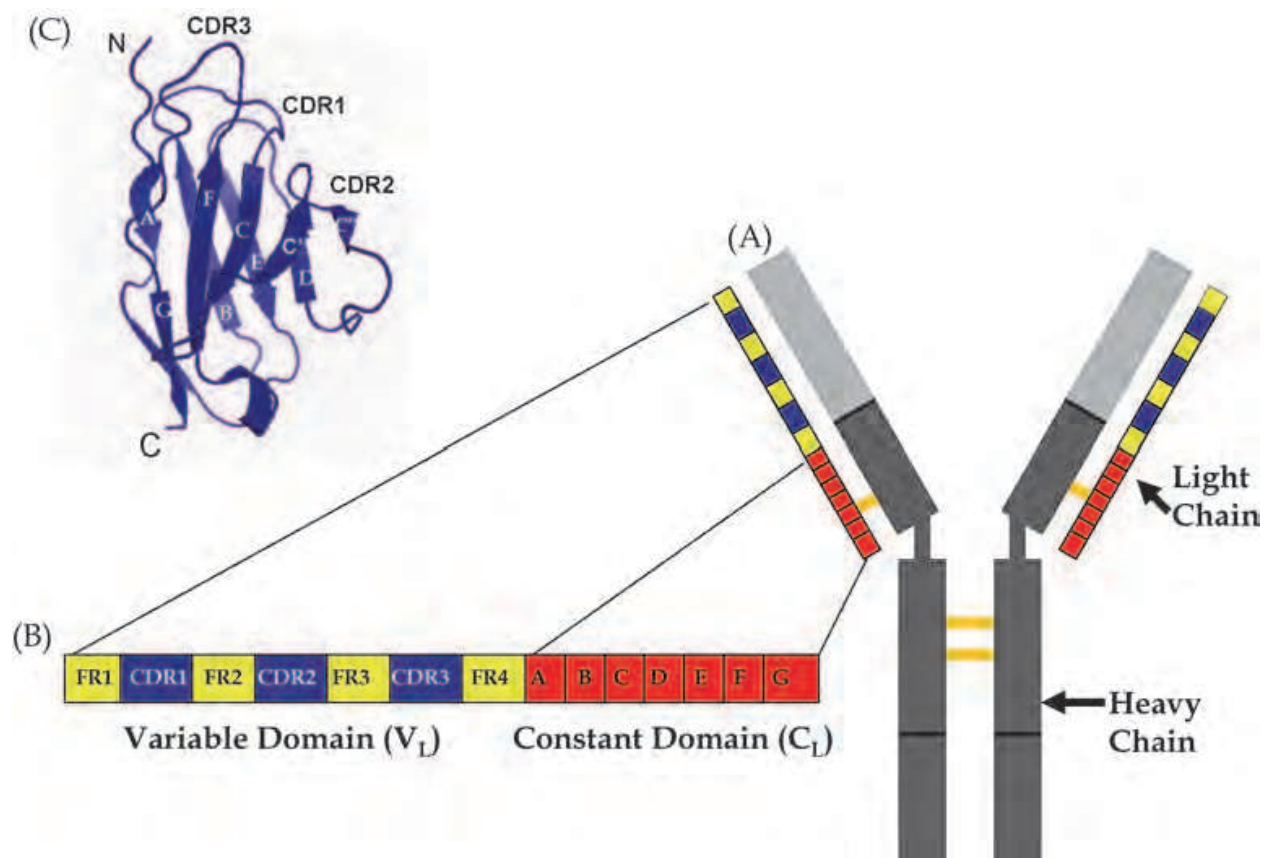

Fig. 1. (A) Immunoglobulin (IgG) structure showing heterotetramer of two light chains and two heavy chains linked by disulfide bonds (B) Schematic representation of a LC showing complementarity determining regions (CDR) and framework regions (FR) (C) V_L structure showing CDR regions, β strands C, C', F, and G involved in heavy/light chain interface, and N and C termini (β strands A and G, respectively).

Immunoglobulin quaternary structure consists of a heterotetramer formed by the LC and the heavy chain (HC) linked together via disulfide bonds. The LC V_L domain interacts with the HC variable domain through β-strands C, C', F and G. The source of sequence variability in LCs comes from combinatorial pairing of the V genes (discussed below) and the J genes (corresponding to strand G or FR4), making it possible to generate about 3000 different LC sequences. In addition, somatic mutations improve the antibody affinity for the antigen, leading to further sequence variation. LCs are secreted and are found in circulation and are sometimes referred to as Bence-Jones proteins. Heavy chains are unable to be secreted alone, so they are always present as part of an intact immunoglobulin molecule as shown in Figure 1.

There are 40 kappa and 33 lambda germline genes available to form a LC variable domain. In AL amyloidosis, there is an overrepresentation of specific germline genes: κI, λ1, λII, λIII, and λVI (Poshusta et al., 2009). The process of somatic hypermutation adds to the complexity of AL amyloidosis because it means that each patient possesses a unique amyloidogenic protein: a combination of different germline genes and different somatic

mutations. These protein sequence differences could result in different propensities to form amyloid fibrils, and may be involved in the different organ involvement found in different AL patients as well as the different degrees of severity of the disease.

Most of the biochemical and biophysical studies reported in the literature have been conducted using the variable domain of AL proteins (Baden et al., 2009). This stems from the fact that variable domain fragments were found in AL amyloid deposits and for 20 years it was thought that the protein underwent limited proteolysis before or after becoming part of the amyloid fibril. However, a recent report describing the laser micro-dissection of amyloid deposits from biopsied tissue followed by mass spectrometry analysis determined that the full length immunoglobulin light chain is present in the amyloid deposits of these patients (Vrana et al., 2009), contradicting the previous assumption that only light chain variable domains are found in amyloid deposits.

2.1 What contributes to the amyloidogenicity of immunoglobulin light chains?

It is generally accepted that LCs from AL patients are prone to aggregation due to a number of factors: certain germline sequences are overrepresented and may be intrinsically more prone to aggregation (see section 2.1.1.); somatic mutations destabilize the protein and may promote conformations that are more favorable for amyloidogenesis (see section 2.1.2) or decrease thermodynamic stability (see section 2.1.3). Finally, the presence of co-factors and the cellular environment play an important role (section 2.1.4).

2.1.1 Sequence determinants of amyloidogenicity

In AL amyloidosis, λ is overrepresented (λ/κ=3:1) as compared to healthy individuals or multiple myeloma (MM, non amyloidogenic control) patients (λ/κ=1:2), especially the λ VI subtype (Kyle & Gertz, 1995). In addition, V_L germline donor gene usage in AL is biased (Abraham et al., 2003; Comenzo et al., 2001; Prokaeva et al., 2007). The Comenzo, Abraham and Prokaeva studies agree that AL V_L germline donor gene usage comprises Vλ I, Vλ II, Vλ III, Vλ VI, Vκ I, while there are slight differences in the sample size, sample selection and the frequency of use of each germline donor gene in each study. Comenzo and co-workers demonstrated that 30% of AL V_L genes used Vλ VI 6a germline donor (Comenzo et al., 2001). Abraham and co-workers found that most κ patients selected for their study used the Vκ I subgroup (77%) (Abraham et al., 2003); a similar observation was made by Prokaeva and co-workers (Prokaeva et al., 2007).

To determine if the germline sequences are prone to generating inherently more amyloidogenic AL proteins, two studies tested κ and λ germline proteins. Baden et al. compared AL-09, an amyloidogenic protein that has 7 somatic mutations, to its germline protein κI O18/O8 (Baden et al., 2008a). The germline protein was more thermodynamically stable than its amyloidogenic counterpart, and although it was able to form fibrils, its fibril formation kinetics were significantly slower than AL-09. Additionally, fibril formation of AL protein BIF and MM protein GAL (also of the κI O18/O8 germline) was compared at 37°C, but only BIF formed fibrils (Kim et al., 2000).

Because the λ6a germline is expressed almost exclusively in AL patients (it is one of the last germline genes screened in the process of selection) and is not expressed in the normal LC repertoire (Abraham et al., 2003; Comenzo et al., 2001; Prokaeva et al., 2007), del Pozo Yauner et al. hypothesized that this germline would be as unstable as AL proteins. However, experiments revealed that the λ6a germline protein was more stable than Wil, an

amyloidogenic protein from that germline with 11 somatic mutations (Del Pozo Yauner et al., 2008). The λ6a germline also had significantly slower fibril formation kinetics than Wil. When compared to AL-09 and κI O18/O8, Wil and λ6a demonstrated a comparable increase in stability, but faster fibril formation kinetics (14 hours for λ6a compared to 216 hours for κI O18/O8 at 37°C). Additionally, λ6a was able to form fibrils in the absence of seeds, while κI O18/O8 required seeds for fibril formation. This may indicate an increase in fibrillogenic propensity for λ6a germline proteins. More studies are necessary to verify that AL-prone germline sequences are more amyloidogenic than normal Ig repertoire germline sequences. The mutational diversity among AL proteins has been well documented. Several studies have compared amino acid sequences of AL proteins, searching for common mutations or mutational regions. In an analysis of 121 κI light chains (37 of which were amyloidogenic), Stevens found four structural features that render a LC protein more likely to be amyloidogenic (Stevens, 2000). All of these involved loss or gain of certain residues, including a mutation that introduces a glycosylation site, mutations of Arg61 or Ile27b and mutations of Pro residues in β-turns.

A more recent analysis of 141 κ and λ AL light chain sequences catalogued the non-conservative mutations in these proteins and modeled their positions onto known LC structures to correlate structural regions (β-strands or loops) with potentially destabilizing mutations (Poshusta et al., 2009). This study confirmed that the total number of non-conservative mutations may be less important than their location as an amyloidogenic determinant for LC proteins. Additionally, the patients' free light chain levels, an indicator of disease progression (Dispenzieri et al., 2008), were also assessed in a subset of the analyzed protein sequences. A correlation between non-conservative mutations in certain regions and free light chain (FLC) levels was revealed, suggesting that patients with initial low FLC levels acquired mutations in their LCs that rendered these proteins to be more amyloidogenic than LCs from patients with higher FLC levels. Analyzing the location of these mutations could further advance understanding of the mechanisms of amyloid formation and lead to a prognostic factor for AL disease progression.

2.1.2 Structural determinants of amyloidogenicity in light chain proteins

Structural studies have shown that most variable domains from AL amyloidosis patients crystallize as monomers or dimers with the expected antiparallel β-sheet immunoglobulin fold. The dimer observed is homologous to the conformation occurring between light and heavy chains in immunoglobulin molecules. The germline κI O18/O8 crystallizes as a canonical dimer while the amyloidogenic protein AL-09 adopts an altered dimer with a 90° rotation with respect to the canonical dimer structure (Baden et al., 2008a). Restorative mutational analysis showed that a single mutation in AL-09 (AL-09 H87Y) stabilized the protein, delayed amyloid formation, and changed its conformation from the altered dimer to the canonical dimer interface (Baden et al., 2008b). We have recently reported that the reciprocal mutant κI Y87H, in which we mutated the germline residue towards the residue found in AL-09, crystallized as a canonical dimer. However, using solution Nuclear Magnetic Resonance (NMR) spectroscopy, we showed that this protein adopts a different dimer interface rotated 180° from the canonical dimer interface and 90° from the AL-09 altered dimer interface (Peterson et al., 2010). The different dimer structures could be compared to the hands on a clock moving in intervals of 90° (Figure 2).
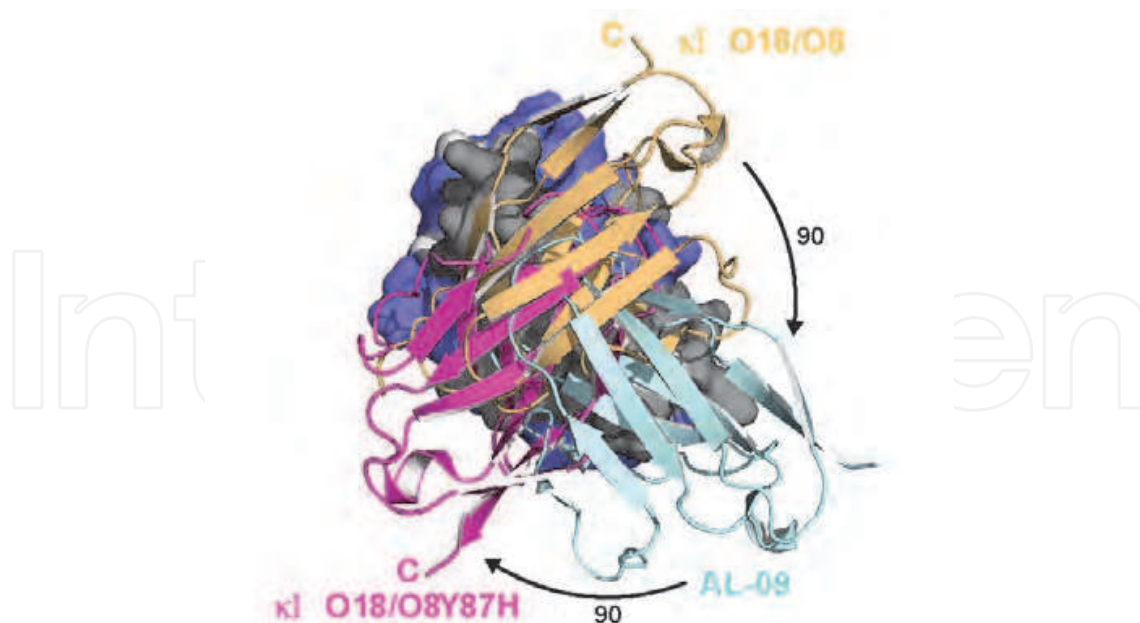
Fig. 2. Overlay of three different dimer orientations found in crystal structures of κI O18/O8, AL-09, and NMR structure of κI Y87H. (Figure adapted from (Peterson et al., 2010, Fig. 4), with permission of Elsevier. Copyright © 2010.)

Sequence alignments of the variable domains of 50 κ and 91 λ AL light chains revealed that non-conservative mutations on the dimer interface, especially Histidine mutations, are very common in AL proteins (Poshusta et al., 2009). Taken together with our structural analysis of AL-09, AL-09 H87Y and κI Y87H, our results suggest that dynamic dimerization could occur frequently in AL proteins. Our structural studies show that light chains are able to dimerize in different conformations; the residues in the dimer interface determine whether or not a dimer conformation will be favored or if the numerous interfaces will be populated at the same time.

2.1.3 Thermodynamic stability as a factor determining amyloidogenicity

Studies using variable domain proteins from AL patients have shown that mutations in the variable domain that reduce the thermodynamic stability are more prone to form amyloid fibrils (Hurle et al., 1994; Stevens et al., 1995; Wetzel, 1997). In an analysis linking mutations and stability, Hurle et al. analyzed 36 sequences (18 κ and 18 λ) in search of rare amino acid replacements that occurred in structurally significant regions of the proteins (Hurle et al., 1994). They then constructed single-point mutants incorporating the rare residues into a non-amyloidogenic Bence Jones LC protein to determine whether the amino acids destabilized the protein significantly enough to induce unfolding. Four of the six mutations were destabilizing, leading to the conclusion that some mutations are involved in amyloidogenicity.

To determine if a single mutation is enough to render a protein amyloidogenic, Davis et al. studied AL protein SMA and MM protein LEN. Only eight residues differ between these two proteins, and each SMA mutation was introduced into LEN to assess the individual effects on fibrillogenesis. Of the mutations tested only P40L, located in a loop region, was able to form Thioflavine T (ThT) positive fibrils in unseeded reactions (Davis 2000). Although stability data were not reported for these mutants, it is likely that the P40L mutant

was less stable than wild-type LEN because Pro40 (very favorable for loops and turns) is conserved among 98% of all κ and λ germline sequences.

In vitro fibril formation studies have revealed that AL proteins form fibrils under a variety of solution conditions with varying kinetics and morphology of fibrils. AL-09 is unique because it forms amyloid fibrils with very similar kinetics across a wide variety of solution conditions (Martin & Ramirez-Alvarado, 2010). Additionally, AL-09's fibril formation kinetics are significantly faster than other AL proteins. We propose that the altered dimer interface populated by AL-09 facilitates the initial misfolding events that trigger amyloid formation, while the other proteins require stochastic conformational fluctuations to populate the appropriate misfolded intermediate that leads the amyloid formation reaction. Incubation of light chains from both κIV amyloidosis and multiple myeloma patients have shown that amyloid formation is enhanced at low pH while amorphous aggregation occurred around neutral pH; all of these reactions populated different partially folded intermediates (Ionescu-Zanetti et al., 1999; Khurana et al., 2001; Souillac et al., 2003; Souillac et al., 2002a,b; Souillac et al., 2002b).

Another link between thermodynamic stability and fibril formation is found in the recently analyzed κI O18/O8 and λ6a germline proteins. These proteins were significantly more stable than all AL amyloidogenic proteins that have been studied to date (Baden et al., 2008a; Del Pozo Yauner et al., 2008). The T_m values (melting temperatures, at which 50% of the proteins are unfolded) for the germline proteins were increased by 15°C and 11.6°C, respectively, over the corresponding AL proteins analyzed in each study. Both κI O18/O8 and λ6a germline proteins had slower fibril formation kinetics than their amyloidogenic counterparts.

Del Pozo Yauner and colleagues incorporated an R25G mutation into the λ6a germline protein (6aJL2-R25G), as this mutation is found in 25% of amyloidogenic λ6 LCs and presumably represents an allotypic variant (Ch'ang et al., 1994; del Pozo Yauner et al., 2006; Del Pozo Yauner et al., 2008). This mutation resulted in a 6°C decrease in T_m value for the mutated protein, and 6aJL2-R25G had a much shorter lag time and faster growth rate than the λ6a germline protein. The authors explain that the R25G mutation may affect the structure of complementarity determining region 1 (CDR1), resulting in an altered conformation and increased amyloidogenicity (del Pozo Yauner et al., 2006).

Further research on the κI O18/O8 germline protein and amyloidogenic AL-09 also connected thermodynamic stability to fibril formation. Baden et al. undertook a systematic restorative mutational analysis of the non-conservative mutations of AL-09, which are all located in the dimer interface (Baden et al., 2008b). Of the three non-conservative restorative mutations (I34N, Q42K and H87Y), restoring the His87 mutation to the Tyr87 residue found in the germline sequence increased the thermodynamic stability and decreased the fibril formation kinetics to the same levels as κI O18/O8. Significant structural alterations were also observed with this restorative mutant (discussed above, shown as a summary in Figure 2). Restoring the Asn34 residue had intermediate effects on stability and fibril formation propensity, while reintroducing Lys42 did not appear to alter the thermodynamics to any extent.

In complementary experiments introducing the His87 residue from the amyloidogenic protein into κI O18/O8, this protein was only destabilized half as much as AL-09. κI Y87H also had intermediate fibril formation kinetics between those measured for κI O18/O8 and AL-09, indicating that this mutation alone may not have been sufficient for the

amyloidogenicity observed in AL-09. However, introducing a second mutation into κI O18/O8 (Ile34, in addition to His87) completely destabilized the protein and exhibited the same fast fibril kinetics as amyloidogenic AL-09. Thus, rather than a single mutation that causes amyloidogenesis, it is probable that a combination of destabilizing and compensatory mutations leads to fibrillogenicity among AL proteins.
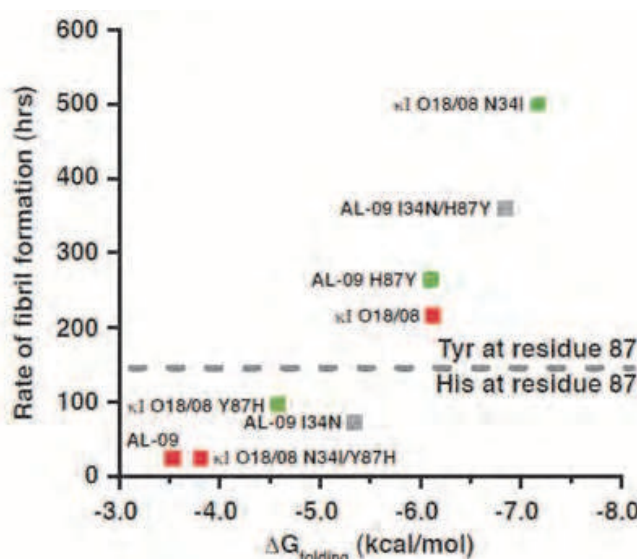

Fig. 3. In restorative AL-09 and reciprocal κI mutants, rates of fibril formation are inversely correlated with $\Delta G_{folding}$. Mutation of Tyrosine 87 to Histidine stabilizes an altered dimer interface and leads to faster fibril formation. HSQC Analysis was used to identify each protein as single-state (green) or promiscuous (red) dimers; others were not determined (grey). (Figure and legend adapted from (Peterson et al., 2010, Fig. 5), with permission of Elsevier. Copyright © 2010.)

Other groups have studied fibril formation using different AL and MM proteins. Jto, an MM protein, and Wil, an AL protein, are both light chain proteins from the λ6a germline that differ by 19 amino acids. Fibrils were formed with both Jto and Wil at 37°C, pH 7.5 (Wall et al., 1999). Jto fibrils appeared more rigid, were shorter and displayed slower kinetics than fibrils formed by Wil.

Certain ionic interactions may affect fibrillogenesis and be crucial to maintain the structure and stability of LC proteins. Wall et al. noted an ionic interaction between Asp29 and Arg68 in MM protein Jto, whereas AL protein Wil has neutral amino acids in these positions (Wall et al., 2004). To test the importance of this ionic interaction, mutations were made to Jto to introduce the neutral residues (from Wil) at these sites (JtoD29A, JtoR68S). The thermodynamic stabilities of these mutants were the same, and the rate of fibril formation for JtoD29A was the same as that for Jto. However, fibril formation kinetics were much faster for JtoR68S, and an X-ray crystal structure of this mutant revealed several side-chain differences compared to Jto and JtoD29A. These differences changed the electrostatic potential surface and increased the amount of solvent-exposed hydrophobic surface for the protein. These results highlight critical structural features such as ionic interactions that participate in the stability and fibrillogenicity of AL proteins.

Studies describing the properties of full length light chains from AL amyloidosis patients have been performed using both urine-derived proteins and recombinant full length

constructs. The constant domain within the λ6a protein AL-01-095 (C_L belongs to LC3* 04) full length protein appears to confer great thermodynamic stability (Klimtchuk et al., 2011), while the kappa unique C_L does not play any role in the stability to the κI O18/O8 protein AL-09 (Olatoye, Levinson, and Ramirez-Alvarado, unpublished observations). Full length proteins isolated from urine samples from MM, Light Chain Deposition Disease (LCDD) and AL patients were studied to determine the type of aggregate formed by each type of protein. Fibril formation reactions were followed at the T_m for each protein for 72 hours. The results indicated that MM proteins formed spherical species, LCDD formed amorphous aggregates and AL proteins formed fibrils (Sikkink & Ramirez-Alvarado, 2008a). Amyloid formation reactions with full length AL-09 and κI O18/O8 show slow rates of amyloid formation with respect to variable domain AL-09 and κI O18/O8. The deposits found using electron microscopy show more disorder within the amyloid fibrils. These results suggest that the presence of the constant domain affects the misfolding pathway for these proteins.

Collectively, these results have shown how the differences between LCs from AL amyloidosis patients (from different germline sequences and with different mutations) can determine the LC thermodynamic stability and fibril formation propensity.

2.1.4 Effect of co-factors and protein modifications in amyloid formation reactions

Amyloid fibril formation is initiated by the accumulation of oligomers to form a critical nucleus during the lag phase. After nucleation, fibril growth occurs during the elongation phase. In addition to studying the characteristics that make a soluble LC protein more amyloidogenic, a tremendous amount of research has and is currently being done with respect to the factors that affect fibril formation *in vitro*. These factors include temperature, pH, ionic strength, agitation, protein concentration, and pressure, which all destabilize the protein in order to populate partially folded states that are prone to aggregation (Chiti et al., 1999). Each AL protein may be affected slightly differently by these co-factors (Figure 4).

2.1.4.1 pH

Experiments at various pH values showed differences between the AL and MM proteins. The rate of fibril formation for AL protein SMA was highly accelerated at pH 2 (Khurana et al., 2001). Both SMA and LEN formed fibrils at pH 2 with agitation, but SMA displayed faster kinetics (Khurana et al., 2003). Amorphous aggregation of SMA was observed in samples from pH 4 to 7, while fibrils were observed in samples at pH ≤3 implying that SMA formed different partially folded intermediates depending on the pH of the solution. At pH 4.5, 30 mM NaCl , SMA formed annular aggregates whereas at high ionic strength, fibrils and amorphous deposits were the predominant species (Zhu et al., 2004). At pH 7, the fibril formation kinetics of LEN was faster with lower protein concentrations and increased concentrations of urea (0 to 3 M) (Souillac et al., 2002a).

Dye binding studies such as Thioflavine T fluorescence are commonly used to monitor fibril formation. However, differentiating between different species formed during fibril formation is not possible with this method. Thus, atomic force microscopy imaging was used to observe the evolution of different fibrillar species during a fibril formation reaction of SMA at pH 2; different filament sizes were found at different time points during the fibrillation. A model was proposed where two filaments combine to form a protofibril and two protofibrils intertwine to form a type I fibril (Ionescu-Zanetti et al., 1999).

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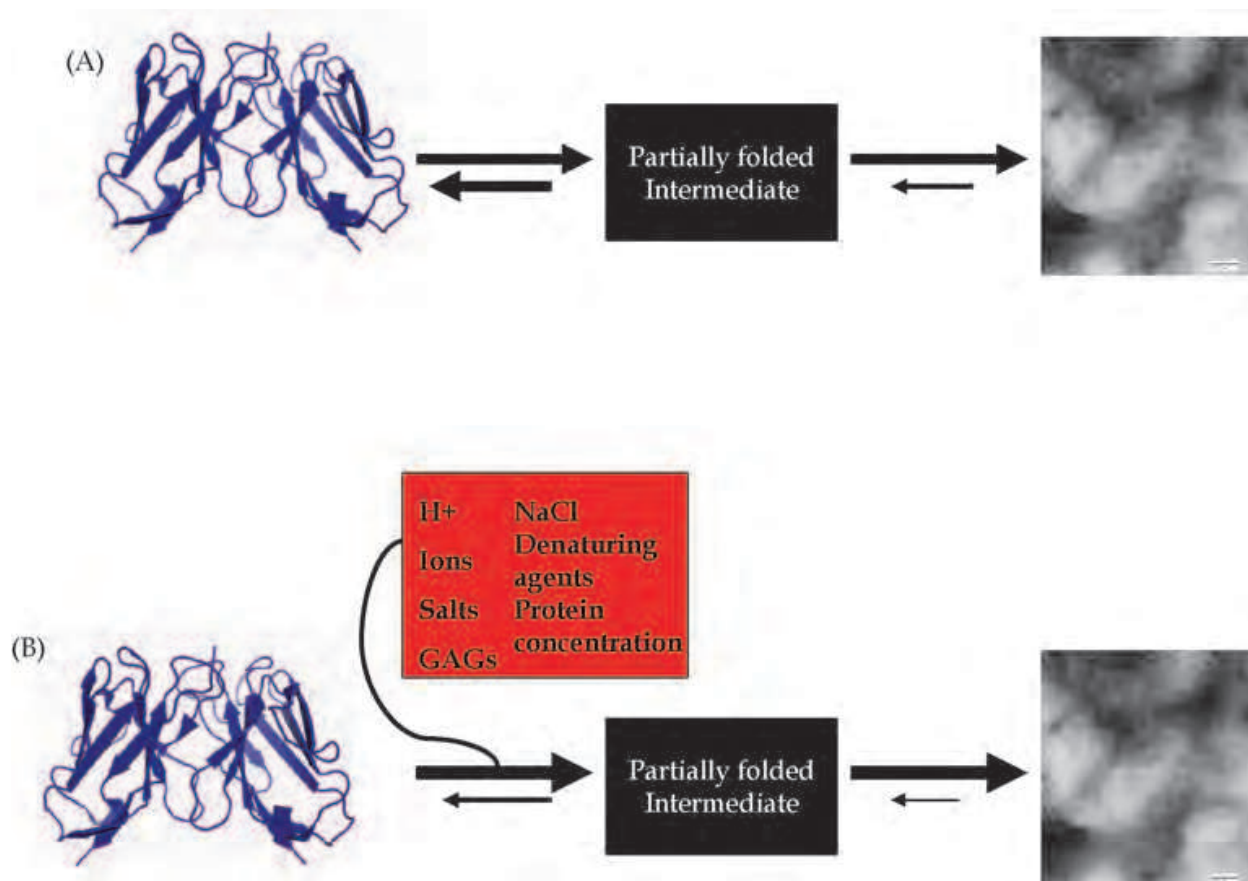Cellular Mechanisms of Amyloid Formation and Toxicity in Light Chain Amyloidosis

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Fig. 4. The role of co-factors in amyloid formation: (A) amyloid formation reaction showing transition from native dimer structure to partially folded intermediate to amyloid fibrils (B) amyloid formation reaction accelerated by the presence of different co-factors. A common co-factor that accelerates fibril formation is low pH (high concentration of H+).

2.1.4.2 Effect of the microenvironment: renal solutes, denaturants, protein concentration, and surfaces

The use of renal solutes shed light onto the destabilizing and compensatory effects that different reagents can have on amyloid formation. Urea, a known protein denaturant, decreased the thermodynamic stability and the fibril formation kinetics of both SMA and LEN while betaine and sorbitol (organic osmolytes) had the opposite effect. A concentration of 1.5 M urea was enough to increase fibril formation of both SMA and LEN (Kim et al., 2001). Conversely, the presence of 0.5 M betaine or sorbitol partially inhibited SMA fibril formation showing the interplay between stabilizing and denaturing forces that may occur in physiological environments.

Other denaturant studies indicate that SMA fibril formation kinetics were dependent on the concentration of guanidine hydrochloride (GdHCl) (Qin et al., 2007). The reaction at 2 M GdHCl had the fastest amyloid formation kinetics and the presence of fibrils was confirmed by electron microscopy, whereas amorphous aggregates were formed at lower concentrations of GdHCl. Additionally, GdHCl affected the intermediate structures in fibril formation, determined by circular dichroism spectroscopy. At 1 M GdHCl, amorphous aggregates were formed by native-like intermediate structures, while at 2 M, amyloid fibrils were generated through an unfolded intermediate.

Protein concentration was another factor that influenced the fibril formation kinetics; low concentrations of LEN had faster kinetics of amyloid formation than higher concentrations (Souillac et al., 2002a,b). At high protein concentrations, LEN produced off-pathway oligomeric species before fibrils were formed (Souillac et al., 2003). At low protein concentrations, the off-pathway species were absent (Souillac et al., 2002b). Adding a "seed" of preformed fibrils to soluble protein solutions to trigger fibril growth also accelerated the kinetics of LEN fibril formation (Harper & Lansbury, 1997). The addition of 5% seeds in a SMA fibril formation reaction decreased the lag time by half when compared to an unseeded reaction (Davis et al., 2000).

Zhu et al. studied the effect of surfaces on SMA amyloid fibril formation. (Zhu et al., 2002). They found that on mica surfaces, the rate of fibrillation was faster and the amount of protein required for the reaction decreased. They also discovered different fibril growth mechanisms; on a mica surface, protofibrils were observed, while in solution, fibrils were present (Zhu et al., 2002). These surface experiments may be relevant *in vivo* since AL amyloid deposits are associated with the extracellular matrix in the basement membrane of tissues.

In an effort to understand the role of components of the basement membrane where fibrils deposit, the role of lipids in amyloid formation for AL was recently reported. The results indicated that a higher protein to lipid vesicles ratio slowed SMA amyloid formation kinetics (Meng et al., 2008). SMA fibrillation was affected by adding cholesterol to the lipid vesicles; specifically, cholesterol concentrations above 10% had an inhibitory effect. Additionally, in the presence of cholesterol and lipid vesicles, higher Ca^{2+} concentrations were shown to decrease SMA fibril formation kinetics The same effect was seen with Mg^{2+} and Zn^{2+} (Meng et al., 2008). This study suggests that amyloid deposition is influenced by the combined effects of cations and membrane surfaces.

2.1.4.3 Hofmeister series

One factor affecting fibril formation is the addition of salts or ions. The Hofmeister series is a tool to understand salt ionic effects that ranks ions according to their ability to stabilize or destabilize a protein (Cacace et al., 1997; Zhang et al., 2005). A proof of principle study was done with the amyloidogenic V_L protein AL-12 to determine the role of physiologically relevant anions and cations from the Hofmeister series on protein stability and amyloid fibril formation. The presence of various salts with AL-12 did not affect the secondary structure of the protein (Sikkink & Ramirez-Alvarado, 2008b), and all salts enhanced amyloid formation. Reactions with SO_4^{2-} and Mg^{2+} showed the largest enhancement of amyloid formation. In addition, we recently performed a systematic analysis of the effect of different concentrations of NaCl on amyloid formation using two similar amyloidogenic light chains. AL-09 readily formed fibrils across a wide range of salt concentrations; however, the amyloidogenic light chain AL-103 (90% sequence identity to AL-09) showed a roughly inverse dependence of the fibril formation rate on salt concentration (Martin & Ramirez-Alvarado, 2010). These studies with various AL proteins and salts will help determine how sulfate ions enhance amyloid formation and will shed light onto the role of glycosaminoglycan sulfation on fibril formation *in vivo*.

2.1.4.4 Glycosaminoglycans

Glycosaminoglycans (GAGs) are a component of the extracellular matrix (Bosman & Stamenkovic, 2003) and have been found extensively in amyloid deposits. They are long,

unbranched, negatively charged heterogeneous polysaccharides formed by disaccharides of N-acetylglucosamine or N-acetylgalactosamine and uronic acid. Ohishi et al. found that GAGs are an integral part of AL amyloid fibrils and that the level of GAGs increased 10-fold in tissues from amyloidosis patients, suggesting that GAGs not only play a role interacting with amyloid fibrils but the presence of the fibrils affect GAG levels (Ohishi et al., 1990). *In vitro* studies using HPLC chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed an interaction between light chain proteins and various GAGs (Jiang et al., 1997). In another *in vitro* study, our laboratory showed that dermatan sulfate accelerated AL-09 amyloid fibril formation, whereas chondroitin sulfate A inhibited fibril formation and yielded a spherical intermediate (McLaughlin et al., 2006). More recently, we have found that GAGs enhance amyloid formation by a transient electrostatic interaction with an early intermediate of the amyloid formation reaction (Martin & Ramirez-Alvarado, 2011).

Further studies of GAG influence on AL fibrillogenesis via the multiple sulfate moieties or its possible crowding effect on the amyloid fibril reaction may reveal important clues about the mechanism of amyloidogenesis and the role that GAGs play in the *in vivo* extracellular matrix deposition.

2.1.4.5 Posttranslational modifications and oxidative stress

Posttranslational modifications (PTMs) are also implicated in amyloidogenicity. Of the amyloidogenic structural risk factors that Stevens identified in κI light chains, N-glycosylation was found in 22 of 121 samples, and 18 of those 22 samples were amyloidogenic (Stevens, 2000). None of the light chain germline genes encode a glycosylation site (N-x-S/T); thus, any putative glycosylation sites are introduced through somatic hypermutation. Of the 18 amyloidogenic glycosylated LCs in Stevens study, most of them (13/18) also had other PTMs (including S-cysteinylation, fragmentation, dimerization and S-sulfonation), so a definitive role for glycosylation is difficult to delineate.

Other studies also implicated glycosylation as an important characteristic among amyloidogenic proteins (Dwulet et al., 1986; Engvig et al., 1998; Foss et al., 1998; Omtvedt et al., 2000), and AL proteins were found to be glycosylated more frequently than circulating non-amyloidogenic free LCs (Holm et al., 1986; Omtvedt et al., 1997). Despite this evidence, the precise role of this PTM has yet to be determined.

A more recent study of nine κI light chains revealed several different PTMs in the full length LC proteins. Each of the proteins studied had at least one type of PTM, and the range of PTMs included N-glycosylation, disulfide-linked dimerization, S-cysteinylation, fragmentation, S-sulfonation, 3-chlorotyrosine formation, and conversion of aspartic acid to pyruvate (Connors et al., 2007). The exact relevance of these modifications to AL pathogenesis is unknown, but cysteinylation of other proteins was suggested to induce conformational changes (Chen et al., 1999; Watarai et al., 2000), which could play a role in misfolding. Additionally, chlorotyrosine residues were linked to oxidative damage (Mohiuddin et al., 2006).

Some PTMs found in AL proteins may actually have a protective role against amyloidogenesis. The two most heavily modified proteins in the aforementioned study (Connors et al., 2007) also included a methionine residue that had been oxidized to methionine sulfoxide. Methionine and cysteine are the most easily oxidized amino acids, and oxidation of a methionine residue could protect other critical residues from damage by reactive oxygen species (ROS) (Levine et al., 2000). A study of MM protein LEN showed that

the methionine-oxidized form of the protein led to the formation of amorphous aggregates instead of fibrils (Hu et al., 2008). Thus, methionine oxidation may be part of a protective mechanism against amyloidogenic fibril formation for AL proteins. However, because methionine oxidation is a fluctuating process, its antioxidant effect could be overcome by a preponderance of other amyloidogenic factors.

Oxidation effects are particularly relevant to the study of AL proteins because oxidative stress has been linked both to amyloid fibril deposits and to the mechanism of cell death (Merlini & Westermark, 2004; Schubert et al., 1995). In a study by Ando and coworkers, AL amyloid deposits stained positively for 4-hydroxy-2-nonenal (HNE), a lipid peroxidation product indicative of oxidative injury (Ando et al., 1997). This result could not differentiate whether oxidative stress was involved in amyloid formation or if the fibrils triggered an oxidative stress reaction after deposition. However, a more recent study indicated that oxidative stress caused by soluble amyloidogenic AL proteins plays a role in cell death. Brenner et al. examined the effects of cardiac AL proteins on cardiomyocytes and found that the presence of the amyloidogenic proteins caused an increase in intracellular reactive oxygen species and upregulation of a redox-sensitive protein (heme oxygenase-1) (Brenner et al., 2004). In addition, the contractility and relaxation of the cardiomyocytes was impaired, directly linking these soluble light chain proteins to cardiomyopathy in AL patients.

Research is still being conducted to understand the mechanism of AL fibril formation and the role of co-factors and the cellular environment on amyloidogenicity. It is important to expand on the currently reported work with additional AL proteins to find commonalities and differences of fibril formation properties for the different AL proteins.

3. Tissue damage in AL amyloidosis-toxic effect of light chains

The most important aspect of AL amyloidosis pathophysiology is the tissue damage associated with the process of amyloid formation. AL amyloidosis is a systemic protein misfolding disease; the site of deposition is distant from the site of protein synthesis and secretion (in this case, bone marrow plasma cells). While there have been some advances in cellular and tissue studies on the effect of light chains in cellular and tissue viability, what happens to the protein while in circulation is unknown. This aspect of the pathophysiology could only be studied with appropriate animal models.

3.1 Cellular toxicity studies

One of the most important questions in amyloidosis research is to determine the most toxic species of the amyloid formation reaction. For years, researchers assumed that the amyloid fibril deposits were highly toxic to the cells near them by blocking the exchange of nutrients, creating a mechanical barrier around the cells, and by attracting macrophages that ultimately caused tissue damage. Later on, experiments conducted with soluble fractions from preparations of amyloid affected tissue showed that soluble species were as toxic as or more toxic than insoluble amyloid fibrils. Recent work done by our laboratory and others has shown that the presence of soluble AL proteins in cell culture induces apoptosis (Shi et al., 2010; Sikkink & Ramirez-Alvarado, 2010). In particular, we were able to demonstrate that the light chain species present in cell culture at the time of maximum apoptotic activity are primarily light chain monomer and dimers.

Internalization studies using immunoglobulin light chain proteins have shown that full length AL-09 internalizes into cardiomyocytes within 24 h, migrating into lysosomal compartments and in certain instances, the nucleus (Figure 5). Full length κI O18/O8 is delayed in this process. Single, restorative full length mutant AL-09 H87Y mimics the full length germline phenotype (Levinson, Olatoye, and Ramirez-Alvarado, unpublished observations). These studies are allowing us to fully characterize the biophysical, biochemical and cellular properties of amyloidogenic light chains to fully determine the role of somatic mutations in the disease process. These cellular internalization studies will reveal more details about the exact mechanism of toxicity by amyloidogenic light chains.
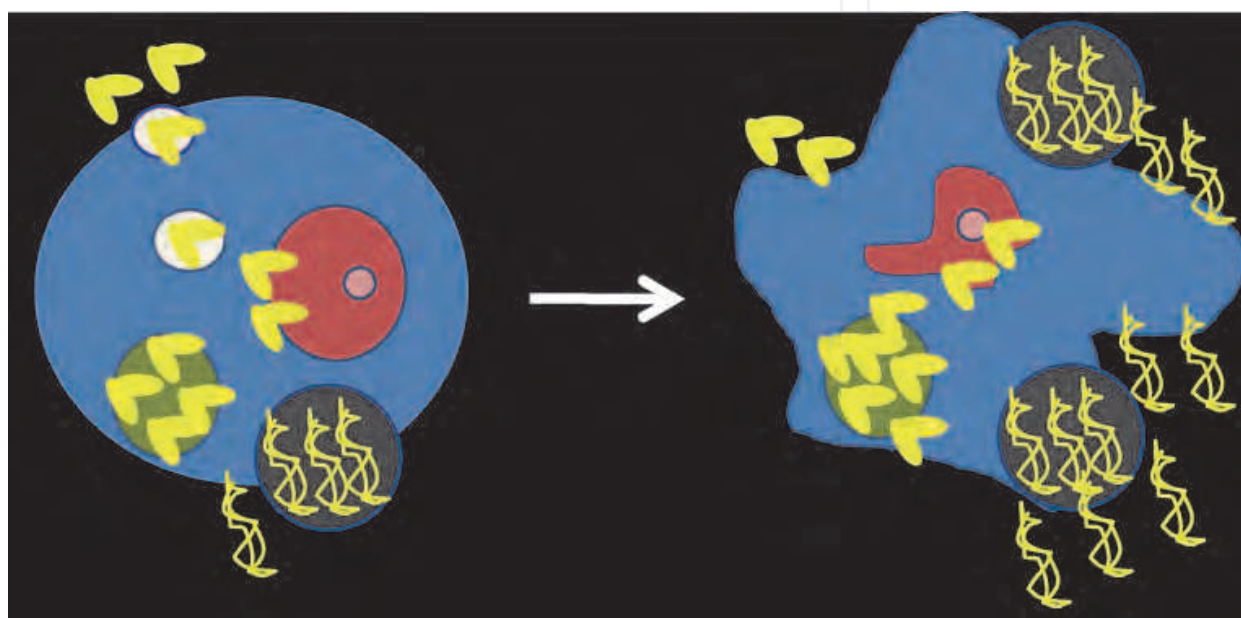

Fig. 5. Proposed model of LC internalization, aggregation, and apoptosis showing internalization through endosomes (light grey) to lysosome (green) and nucleus (red) and removal of amyloid fibrils by exocytosis (dark grey). Amyloid fibril formation may happen intracellularly and could be later excreted into extracellular compartments. Alternatively, the process of cell death may allow fibrils to move to the extracellular matrix.

3.2 Human tissue toxicity studies

The first tissues affected by AL protein deposition are the blood vessels. It was previously shown that AL amyloidosis patients present with early endothelial microcirculatory dysfunction (Berghoff et al., 2003), and that light chain amyloid infiltration in epicardial coronary arteries occurs in almost all of the AL amyloidosis patients analyzed (Wittich et al., 2007).

Another report showed that the presence of light chain is associated with histological evidence of myocardial ischemia (decrease in the blood supply) in the majority of AL patients studied (Neben-Wittich et al., 2005). These findings suggest that microvascular dysfunction is central to AL pathophysiology, yet its underlying mechanism is unknown. Migrino et al. recently reported an increase in protein oxidation in AL amyloidosis patients. When arterioles were exposed to amyloidogenic light chains, they observed higher levels of superoxide and impaired dilation to sodium nitroprusside (Migrino et al., 2010). Human

arterioles are physiologically relevant to early AL pathophysiology and offer an important tissue system to study tissue dysfunction caused by AL light chains.

3.3 Model systems

Arendt and co-workers have established the first amyloidogenic human cell line system, ALMC-1 and ALMC-2 (Arendt et al., 2008). They used plasma cells from an AL patient isolated both pre- (ALMC-1) and post- (ALMC-2) stem cell transplant. These cell lines secrete a full length λ6a LC protein called ALMC. While there is some genetic variation between ALMC-1 and ALMC-2, the protein sequences from both cell lines are 100% identical. The protein secreted from these cell lines was fully folded with a β-sheet structure; it was as stable as other full length proteins (Sikkink & Ramirez-Alvarado, 2008a) and had the ability to form amyloid fibrils *in vitro*. These cell lines are a valuable tool because this is the only human-derived system that secretes a significant amount of protein for biophysical studies. We expect that future studies using these cell lines will advance our understanding of the cellular microenvironment and its possible role in the misfolding of light chain proteins.

Currently, there is no reported animal model for AL amyloidosis that displays the full pathophysiology of the disease. An animal model attempt involved cloning and expression of amyloidogenic light chains using the cytomegalovirus (CMV) promoter. SP2/O Ig null plasmacytoma cell lines were stably transfected with the amyloidogenic light chain vectors and were transplanted into Balb/c and RAG mice, where they grew as plasmacytomas that secrete the amyloidogenic light chains. 4-6 weeks post transplant of these cells, human amyloidogenic light chains were found in the urine of the transfected animals. Some protein casts and granular deposits were found in the tubules of the kidneys of some of the transfected animals. No Congo red staining (indicative of the presence of amyloid fibrils in tissues) was observed with these deposits. (Ward and coworkers abstract included in (Skinner et al., 2007))

Another animal model attempt involved creating a transgenic animal using the CMV promoter and bovine growth hormone polyadenylation signal. The expression of the transgenic protein was not ubiquitous, and the protein levels expressed were 1/10 of the levels found in the transplant model. Immunohistochemical analysis of different tissues showed the presence of the transgenic protein in the stomach gastric pit cells, the squamous epithelial cells of the bladder, the tubule cells in the kidney, in the cardiac cells and the pancreas. Congo red positive aggregation was observed in the lumen of the gastric glands of the stomach. The authors suggest that the low pH found in the stomach promoted amyloid formation of these amyloidogenic light chain after 4-6 months (Ward and coworkers abstract included in (Skinner et al., 2007)).

More recently, Shi and co-workers reported an animal model in which wild type and dominant negative p38α transgenic mice were initially injected with amyloidogenic light chains through the tail vein followed by systemic intravenous infusion via the use of an osmotic minipump for 7 days. Wild type animals with fully active p38α presented an increase in the Bax/Bcl2 ratio and a very modest increase in cellular apoptosis as determined by TUNEL staining (Shi et al., 2010).

Currently, none of the murine transgenic models of any of the systemic amyloidoses exhibit ideal characteristics to study the disease process. Buxbaum proposed that any future successful transgenic animal model of the extracellular amyloidoses should allow more

precise understanding of the pathogenesis and the role of other proteins in facilitating or inhibiting amyloid generation and deposition. The use of worms (*Caenorhabditis elegans*) and flies (*Drosophila melanogaster*) to study amyloidoses has allowed the study of some disease processes, but Buxbaum argued that the relationship between the cellular and molecular phenotype and human disease may be problematic (Buxbaum, 2009).

4. How to ameliorate and eventually eliminate AL associated toxicity?

Current treatments for AL amyloidosis target the malignant plasma cell population in bone marrow. These treatments are somewhat successful, whilst they are poorly tolerated by some AL amyloidosis patients. New therapeutic strategies targeting the amyloidogenic light chains and the AL amyloid fibrils are currently in development and their efficacies are being studied.

4.1 Small molecules

Small molecules have been tested in search of fibril formation inhibitors. Congo red is a histological dye that binds to amyloid fibrils and presents a green birefringence under polarized light (Sipe & Cohen, 2000). AL-09 fibril formation was inhibited by Congo red at a 1:1 molar ratio (McLaughlin et al., 2006). In contrast, Congo red did not inhibit fibril formation of SMA suggesting some specificity in the role of Congo red as an inhibitor (Kim et al., 2003). More research is needed to find effective fibril inhibitors for a variety of AL proteins both *in vitro* and using cell culture systems.

4.2 Antibodies

A murine monoclonal antibody (mAB 11-1F4) that binds to light chain fibrils but not soluble proteins was generated and characterized by Solomon and co-workers (O'Nuallain et al., 2007; Solomon et al., 2003). Immunohistochemical analysis revealed that mAB 11-1F4 recognized light chain fibrils regardless of their V_L subgroup. The specificity of this antibody for AL fibrils (κI, κII, κIV, λ1, λ3, λ6, λ8) was shown by Europium-Linked Immunosorbant Assay (EuLISA) where an EC_{50} value (concentration of antibody at half maximum binding) for binding was ~130 ± 39 nM (O'Nuallain et al., 2007). The interaction of mAB 11-1F4 with native and fibrillar light chain LEN components was also checked by EuLISA and the antibody had similar avidity with both components. However, the fibrils had a ~2 fold reduction in signal (O'Nuallain et al., 2007). Peptide mapping was used to determine the cryptic epitope; it is located in the first 18 amino acids of the variable light chain domain and a prolyl residue at position 8 is necessary. A competition EuLISA was set up with mAB 11-1F4, and recombinant Wil fibrils were inhibited by a 50-fold molar excess of soluble LEN (1-22) peptide (O'Nuallain et al., 2007).

4.3 siRNA

A recent report has shown that small interference RNA (siRNA) can be used to reduce the amount of messenger RNA for amyloidogenic light chains. Phipps and co-workers transfected SP2/O mouse myeloma cells with a construct encoding the λ6 AL light chain Wil under control of the cytomegalovirus promoter, using the l2-producing myeloma cell line RPMI 8226 as a control. The siRNA were designed specifically to the V, J, or C portions of the molecules. Forty eight hrs after exposure to the siRNAs, the authors observed 40%

reduction in messenger RNA and LC production with a greater effect observed in the 8226 cells (Phipps et al., 2010).

5. Conclusion

Our knowledge of the molecular mechanisms of AL has greatly increased as a result of recent research about role of mutations, dimerization structures, different species populated in AL amyloid fibril formation, the cellular microenvironment, and light chain-associated cell and tissue toxicity. However, this complex disease is far from being understood, and each new discovery implicates other pathogenic factors, prompts additional questions, and reinforces the need for innovative research. In particular, the development of effective transgenic animal models and misfolding related therapeutic strategies, especially targeting light chain monomers and dimers, is necessary. A multidisciplinary research effort is required to analyze all aspects of the disease and provide a deeper understanding of its pathogenesis, ultimately leading to a successful therapeutic intervention.

6. Acknowledgments

7. References

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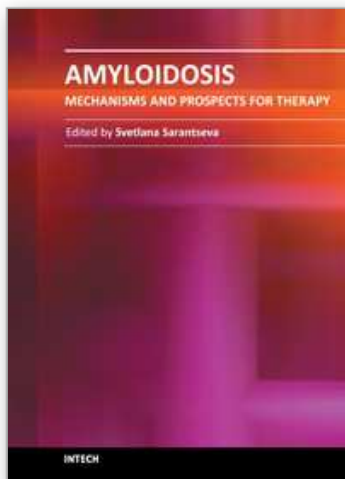
Amyloidosis - Mechanisms and Prospects for Therapy

Edited by Dr. Svetlana Sarantseva

Amyloidoses are a heterogeneous group of diverse etiology diseases. They are characterized by an endogenous production of abnormal proteins called amyloid proteins, which are not hydrosoluble, form depots in various organs and tissue of animals and humans and cause dysfunctions. Despite many decades of research, the origin of the pathogenesis and the molecular determinants involved in amyloid diseases has remained elusive. At present, there is not an effective treatment to prevent protein misfolding in these amyloid diseases. The aim of this book is to present an overview of different aspects of amyloidoses from basic mechanisms and diagnosis to latest advancements in treatment.

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