Human amyloidogenic light chain proteins result in cardiac dysfunction, cell death, and early mortality in zebrafish

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Mishra S, Guan J, Plovie E, Seldin DC, Connors LH, Merlini G, Falk RH, MacRae CA, Liao R. Human amyloidogenic light chain proteins result in cardiac dysfunction, cell death, and early mortality in zebrafish. Am J Physiol Heart Circ Physiol 305: H95–H103, 2013. First published April 26, 2013; doi:10.1152/ajpheart.00186.2013.—Systemic amyloid light-chain (AL) amyloidosis is associated with rapidly progressive and fatal cardiomyopathy resulting from the direct cardiotoxic effects of circulating AL light chain (AL-LC) proteins and the indirect effects of AL fibril tissue infiltration. Cardiac amyloidosis is resistant to standard heart failure therapies, and, to date, there are limited treatment options for these patients. The mechanisms underlying the development of cardiac amyloidosis and AL-LC cardiotoxicity are largely unknown, and their study has been limited by the lack of a suitable in vivo model system. Here, we establish an in vivo zebrafish model of human AL-LC-induced cardiotoxicity. AL-LC isolated from AL cardiomyopathy patients or control nonamyloidogenic LC protein isolated from multiple myeloma patients (Con-LC) was directly injected into the circulation of zebrafish at 48 h postfertilization. AL-LC injection resulted in impaired cardiac function, pericardial edema, and increased cell death relative to Con-LC, culminating in compromised survival with 100% mortality within 2 wk, independent of AL fibril deposition. Prior work has implicated noncanonical p38 MAPK activation in the pathogenesis of AL-LC-induced cardiotoxicity, and p38 MAPK inhibition via SB-203580 rescued AL-LC-induced cardiac dysfunction and cell death and attenuated mortality in zebrafish. This in vivo zebrafish model of AL-LC cardiotoxicity demonstrates that antagonism of p38 MAPK within the AL-LC cardiotoxic signaling response may serve to improve cardiac function and mortality in AL cardiomyopathy. Furthermore, this in vivo model system will allow for further study of the molecular underpinnings of AL cardiotoxicity and identification of novel therapeutic strategies.

amyloidosis; apoptosis; in vivo model cardiovascular disease; p38 mitogen-activated protein kinase

AMYLOID light-chain (AL) amyloidosis is the most common systemic amyloidosis in the developed world and results from a plasma cell dyscrasia with clonal production of amyloidogenic immunoglobulin light chain (AL-LC) proteins (20). Circulating AL-LC proteins cause a wide spectrum of systemic tissue pathology, and over half of AL amyloidosis patients develop AL cardiomyopathy (14). Cardiac amyloidosis is associated with a high mortality rate due to the general refractoriness of AL cardiomyopathy to standard heart failure regimens (11) and to antiplasma cell therapies (20). These issues are further compounded by the limited understanding of the pathophysiology of cardiac amyloidosis and the absence of a suitable in vivo model for the investigation of disease mechanisms and novel treatments.

AL amyloidosis disease pathology was originally thought to explicitly result from extracellular fibril deposition resulting in passive stiffness of the myocardium and presenting as restrictive cardiomyopathy. However, clinical observations have challenged this notion with the finding that varying amyloidogenic proteins result in greatly differing outcomes despite comparable degrees of fibril deposition (9). This was further extended when a direct relationship between circulating serum free LC levels and patient prognosis was observed (7, 23). Collectively, these clinical data suggest that in AL amyloidosis, while tissue infiltration of AL fibrils contributes to gross organ dysfunction, the precursor protein itself may greatly influence disease pathology.

Previous work from our laboratory and others (5, 19, 27) has demonstrated that AL-LC precursor proteins, independent of cardiac fibril formation and passive restriction of cardiac function, trigger a direct cardiotoxic response through noncanonical activation of p38 MAPK signaling, causing impaired cellular function. It is unclear, however, whether this direct cardiotoxic effect contributes to the early mortality seen in patients with AL cardiomyopathy and, consequently, whether antagonizing the cardiotoxic effects of AL-LC may alter the natural history of this disease.

Zebrafish (Danio rerio) have proven to be a valuable model system for the investigation of cardiovascular physiology and disease (3, 24). Here, we describe a novel in vivo model of the acute cardiotoxic effects of human AL-LC protein in zebrafish. We found that this model closely recapitulates prior in vitro observations of AL-LC-induced cardiotoxicity with cardiac dysfunction and cardiomyocyte death, independent of cardiac AL fibril infiltration. Moreover, we found that inhibition of the AL-LC-associated cardiotoxicity through a small-molecule p38 MAPK inhibitor protected against AL-LC-induced cardiac dysfunction and cell death and decreased mortality.

MATERIALS AND METHODS

Experiments performed in this study were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Harvard Subcommittee for Animal Research (Protocol no. 04650).

LC collection and purification. Human AL-LC was isolated from 24-h urine samples obtained from patients with primary amyloidosis referred to the Amyloid Treatment and Research Program of Boston
Zebrafish and cardiac injections. Embryos used were obtained from natural spawning of wild-type Danio fish obtained from Ekkwill Waterlife Resources (Ruskin, FL). The stages (hours postfertilization) described in this report are based on the developmental stages of normal zebrafish embryos at 28.5°C (15). Embryos were grown in E3 embryo water for the duration of the experiments. At 48 h postfertilization, zebrafish were manually dechorionated and anesthetized with a 1:100 dilution of 4 mg/ml tricaine solution for 1 min (MS-222, Argent Chemical Labs). Using a depression mold made from 1% agarose, zebrafish were positioned laterally for injection, and the heart was visualized under a dissection microscope (Fig. 1A). Using a Femtojet microinjector (Eppendorf), a glass micropipette and micromanipulator were used to inject a 1-nl volume of solution containing AL-LC, Con-LC, or vehicle (UltraPure water) directly into the inflow tract of the zebrafish.

The final circulating concentration of Con-LC or AL-LC was estimated to be ~100 mg/l. Phenol red (0.5%) was added to the injection solution for visual confirmation of systemic injection. Immediately after the injection, zebrafish were placed in fresh E3 water for deanesthetization. E3 embryo water was replaced daily during experiments.

Measurement of cardiac function. For cardiac function measurements, embryos were positioned on a glass depression slide in a lateral position to ensure visibility of ventricle and acclimated to heat of illumination for 30 s. Video microscopy was performed with an Axioplan (Zeiss) upright microscope using a ×10 objective lens. Image acquisition was performed with a high-speed digital camera at a rate of 250 frames/s, and sequential image frames were analyzed to calculate heart rates and measure diameters in end-systolic and diastolic phases (28). Ventricular volumes were calculated using the following equation: \((4/3)\pi l^2s^2\), where \(l\) is the radius of the long axis and \(s\) is the radius of the short axis, as shown in Fig. 1B. Stroke volume was defined as diastolic volume minus systolic volume. Cardiac output was defined as heart rate multiplied by stroke volume.

For echocardiographic assessment of cardiac function, 3 days postinjection (5 days postfertilization), zebrafish were embedded in 100 ml of 4% low-melting agarose in E3 water (Invitrogen) in the center of a p35 petri dish. After the embedding procedure, E3 water was added to a height of 2 mm above the agarose. Color Doppler echocardiography was performed using the MS700 probe (Vevo2100, VisualSonics) and used to determine the peak aortic flow velocity at 50 MHz. The color Doppler gate was placed on the dorsal edge of ventricle. The acquisition process was performed within 3 min of embedding to ensure viability of the fish. Maximal velocities of each fish were acquired in a blinded fashion. Six to eight fish were examined in each group.

Immunoblot analysis. Zebrafish embryos were lysed and homogenized with cell lysis buffer (Cell Signaling Technology). Lysates of equal volumes were used for SDS-PAGE followed by transfer to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were blocked with Odyssey Blocking buffer (Li-Cor) for 1 h.
and incubated with primary antibodies at 4°C overnight. Antibodies for active caspase 3 were purchased from Abcam (catalog no. ab13847), GAPDH from R&D (catalog no. 2275-PC-100), and phospho-p38 from Promega (catalog no. V1211). Membranes were washed, incubated with IRDye 700CW donkey anti-rabbit IgG or IRDye 800CW donkey anti-mouse IgG for 1 h, and scanned using the Odyssey Infrared System (Li-Cor).

TUNEL staining. Three days postinjection, hearts were isolated from zebrafish and fixed in 4% paraformaldehyde-PBS for 20 min. Isolated hearts were permeabilized in PBS-0.1% Tween overnight at 4°C. Before being stained, isolated hearts were washed twice in PBS and then labeled with fluorescein-terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) using a kit from Roche (Lewes, UK) according to the manufacturer’s instructions. Hearts were mounted with Vectashield mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei. Labeled nuclei were counted using fluorescence confocal microscopy.

Cardiac-specific annexin reporter system. GFP-CMLC-Ga4 transgenic zebrafish were mated, and embryos were collected immediately. At the single cell stage, embryos were injected with a dsRED-Annexin-UAS reporter construct (31). Forty-eight hours postfertilization, embryos were injected with vehicle, Con-LC, or AL-LC. Hearts were isolated at 5 days postfertilization, fixed in 4% paraformaldehyde for 20 min, and permeabilized with PBS-0.1% Tween overnight at 4°C. Hearts were mounted with Vectashield mounting media containing DAPI, and annexin-positive nuclei were counted using fluorescence confocal microscopy.

Survival. Initial screening of zebrafish for inclusion into experiments was performed 1 h postinjection. Only zebrafish that had regained full mobility were used for further experiments and survival screening, at which point “time 0” was established. Survival was assessed daily, and the survival criterion was the presence of cardiac contraction. Deceased animals were removed, and E3 water was replaced daily. Starting at 8 days postfertilization (6 days postinjection), zebrafish were fed twice a day.

Electron microscopy. At 3 days postinjection (5 days postfertilization), whole zebrafish embryos were preserved in a mixture of 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). For the preparation of samples for examination with transmission electron microscopy, whole zebrafish were embedded in plastic resin and cut into ultrathin (∼60–80 nm) sections. Imaging was performed using the microscope model Tecnai G² Spirit BioTWIN at the electron microscopy facility of Harvard Medical School.

Statistical analysis. All data are reported as means ± SE. Statistical difference between experimental groups was determined using one-way ANOVA. Comparison between individual groups was performed using a post hoc Bartlett’s test. P values of <0.05 were considered significant.

RESULTS

Human AL-LC proteins result in direct cardiotoxicity in vivo in zebrafish. To determine whether the introduction of human AL-LC proteins into zebrafish results in cardiotoxicity in vivo, human AL-LC protein isolated from AL cardiomyopathy patients was directly injected into the zebrafish circulation (19). Vehicle or Con-LC isolated from multiple myeloma patients was similarly introduced as control groups (19). For all groups, LC was delivered directly into the circulation via micropipette injection into the sinus venosus of anesthetized zebrafish at 48 h postfertilization. Phenol red was added to the solution for visual confirmation of successful delivery.

Prior work from our laboratory has suggested that AL-LC directly impairs cardiomyocyte contractile function and induces apoptosis in isolated cellular preparations (27). Cardiac hemodynamics were therefore measured using high-speed video microscopy at 24 h postinjection. Stroke volume was reduced in AL-LC-injected zebrafish compared with control groups (Fig. 1C). Heart rate was comparable among all three groups (Fig. 1D), and, from these parameters, cardiac output was calculated and found to be reduced in AL-LC-injected zebrafish compared with control groups (Fig. 1E).

In conjunction with impaired cardiac function, we observed pericardial edema in AL-LC-injected zebrafish but not with vehicle or Con-LC injection (Fig. 2). Further characterization of embryos at 24 h postinjection. Left: bright-field microscopy of embryos at ×2.5 magnification injected with Veh (A), Con-LC (B), and AL-LC (C). Right: enlargement showing ×10 magnification of the cardiac region.
revealed increased cell death in AL-LC-injected zebrafish compared with controls, as assessed by both expression of the cleaved active form of caspase 3 via Western blot analysis (Fig. 3A) and counting of apoptotic nuclei by TUNEL staining at 72 h postinjection (Fig. 3B). Cardiomyocyte-specific cell death was further quantified using a fluorescent annexin reporter system targeted specifically to cardiomyocytes and was found to be increased after AL-LC injection (Fig. 3C).

To determine if the observed cardiac phenotype was due to the direct cardiotoxic effects of AL-LC protein, independent of AL fibril deposition, we assessed AL deposition using high-resolution transmission electron microscopy 3 days after LC or vehicle injection. While changes in morphology indicated cardiac pathology at the organelle level, our electron microscopy data revealed no evidence of AL fibril deposition in AL-LC-injected samples (Fig. 4). Collectively, these data suggest that the introduction of human AL-LC into zebrafish results in direct cardiotoxicity, marked by impaired cardiac function and cardiomyocyte-specific cell death, independent of AL fibril deposition.

**Direct AL-LC cardiotoxicity results in profound mortality.** To determine the definitive impact of AL-LC-induced cardiotoxicity in vivo in zebrafish, survival was monitored posttreatment. AL-LC injection was associated with a marked increase in mortality, with a median survival of 5 days postinjection, and 100% mortality by 13 days postinjection (Fig. 5A). In contrast, Con-LC- or vehicle-injected zebrafish maintained survival over the same time course. These data suggest that cardiac toxicity induced by circulating AL-LC is sufficient to cause significant mortality. To further verify that the observed phenotype in our model was in fact due to the amyloidogenic properties of LC, rather than specific properties associated with the LC isolated from a single patient, additional LCs derived from multiple patients were examined and found to similarly impair survival after AL-LC injection (Fig. 5B).

The pathological range for serum free LC levels is quite variable among the patient population, and a correlation between patient mortality and serum free LC levels has been observed. To determine whether our model system could recapitulate this clinical observation (18), we examined the
Importantly, we found a clear dose-dependent mortality in zebrafish using 10, 100, and 1,000 µg/ml AL-LC, with the highest concentration of AL-LC causing 100% death at 7 days postinjection. This dose dependence further supports the concept of a direct cardiotoxic effect of AL-LC, which contributes to overall mortality (Fig. 6).

**Antagonism of p38 MAPK protects against cardiotoxicity and early mortality in response to human AL-LC in vivo.**

Prior work has shown that noncanonical p38 MAPK activation is essential to the development of human AL-LC cardiotoxicity (27). To determine whether inhibition of p38 MAPK may prevent cardiac dysfunction and mortality, zebrafish were treated with 10 µmol/l SB-203580, a selective p38MAPK inhibitor (27), at the time of either vehicle, Con-LC, or AL-LC injection. Antagonism of p38 MAPK prevented the decline in cardiac function in response to AL-LC, as assessed by echocardiography 3 days postinjection (Fig. 7, A–C). Furthermore, inhibition of p38 MAPK by SB-203580 was associated with improved survival, prolonging the median survival rate to 12 days postinjection (Fig. 8A). To evaluate whether delayed administration of a p38 inhibitor could also rescue mortality, SB-203580 was administered 5 days postinjection, when a cardiac phenotype was already evident. Delayed inhibition of p38 MAPK also resulted in significant improvement in overall survival (Fig. 8B). In addition to preventing the decline in cardiac function and improving survival after AL-LC injection, inhibition of p38 also decreased cell death, as measured by TUNEL staining (Fig. 8, C and D), and active caspase-3 expression, as observed by Western blot analysis of whole embryo lysates harvested from 15 zebrafish embryos and duplicated in 2 independent experiments (Fig. 8E). These data suggest that therapies targeting the pathways underlying direct AL-LC cardiotoxicity may represent an effective strategy for improving overall survival, even when delivered after the onset of cardiac dysfunction.

**DISCUSSION**

AL cardiomyopathy is a severe form of heart disease associated with a particularly poor prognosis (8, 13, 17). While localized fibril deposition was initially hypothesized to be the pathological basis of AL cardiomyopathy, work from our
A ZEBRAFISH MODEL OF AL CARDIAC TOXICITY

Fig. 6. AL-LC injection leads to loss of survival in a dose-dependent manner. A–C: Kaplan-Meier analysis of survival after the injection of Veh, Con-LC, or AL-LC at varying dose concentrations of 10 µg/ml (A), 100 µg/ml (B), or 1,000 µg/ml (C). Survival was monitored daily. n ≥ 75 zebrafish/group. *P < 0.01.

laboratory and others (21, 22, 27, 30) has established that AL-LC proteins possess an intrinsic cardiotoxicity that results in contractile dysfunction and apoptotic cell death in vitro. While we have previously provided evidence of AL-LC-induced cell death in vivo in the mouse after delivery of human AL-LC (27), such mouse models are not amenable to survival analysis and chemical screening, in part because of the limited availability of human AL-LC proteins. The lack of an appropriate in vivo model of AL-LC cardiotoxicity has precluded investigation of the relationship between AL-LC cardiotoxicity and mortality as well as the evaluation of pharmacological interventions. Here, we report the establishment of an in vivo zebrafish model of direct AL-LC toxicity. AL-LC-injected zebrafish recapitulate AL-LC-induced cardiac pathology, including cell death and contractile dysfunction, similar to that observed in vitro (27), as well as demonstrate early mortality. Importantly, human AL-LC proteins are introduced into zebrafish circulation 48 h postfertilization to specifically circumvent any confounding effects associated with early cardiac and whole organism development. The observed cardiac dysfunction in our model also reiterates findings in a recent study (29) showing cardiomyopathy after transient overexpression of amyloidogenic LC in embryonic zebrafish. Importantly, while both models have suggested a cardiotoxic effect associated with amyloidogenic LC proteins, the direct injection of human AL-LC used here results in profound early mortality, similar to that observed in AL cardiomyopathy patients, which was prevented by inhibition of p38.

Over the past decade, zebrafish have become widely used as a platform for the study of cardiac physiology and cardiovascular disease (1, 3, 4, 6, 10, 26, 32, 33). Relative to mammalian systems, there are several critical advantages inherent to the zebrafish model. The low circulating volume in zebrafish at this stage (10 nl at 48 h postfertilization) allows for the achievement of pathological circulating LC concentrations (2) with a minimal absolute amount of human LC protein. This is essential given the critical limitation in availability of human LC protein. Second, the zebrafish is highly amenable to genetic manipulation using morpholino knockdown of target genes, which will allow for systematic screening of various mechanistic targets. Finally, the zebrafish model allows for screening of a number of small-molecule compounds, including the p38 inhibitor used in this study, which can be done in a high-throughput manner using a large number of animals for survival studies.

In our model, upon AL-LC administration, zebrafish develop cardiac dysfunction and cardiac cell death, which contribute to early mortality starting 2 days postinjection and peaking at 5–7 days postinjection (7–9 days postfertilization), at the time when cardiac function is required for fish survival.

While cardiac amyloidosis has typically been characterized as a disease of impaired diastolic function, this concept is oversimplistic and is based on the assessment of ejection fraction, a load-dependent parameter. AL amyloidosis is characterized by low systolic blood pressure, and the consequent reduction in ventricular afterload maintains a near-normal left ventricular ejection fraction despite contractile dysfunction. Even with preserved ejection fraction, longitudinal ventricular function, as evaluated by Doppler and speckle tracking imaging, has consistently been found to be impaired in patients with cardiac amyloidosis, indicating that ventricular contraction is impaired early in the disease process (16, 25). Consistent with these clinical observations, AL-LC was found to induce contractile dysfunction in our in vivo zebrafish model in the absence of confounding AL fiber deposition. The concentration of AL-LC proteins used for these experiments was selected based on clinical measurements of serum free LC concentrations in patients (7). Three independent doses representing concentrations that were in the low, median, and high ranges of patient concentrations were tested (Fig. 6). The concentration...
chosen (100 µg/ml) for this study is within the midrange of pathological concentrations observed in patients with AL cardiomyopathy. This concentration elicited a marked effect on mortality in zebrafish along with closely recapitulating the in vitro pathology of AL-LC-treated cardiomyocytes, suggesting that this novel in vivo system faithfully models AL-LC-induced cardiotoxicity. Additionally, AL-LC from multiple patient sources was used to demonstrate that the toxicity was not specific to LCs derived from one particular patient (Fig. 5B).

Finally, the use of control human nonamyloidogenic LC proteins isolated from patients with multiple myeloma allowed for the effective control of any nonspecific effects related to immunoglobulin LC protein itself or excessive human protein loading into zebrafish circulation in vivo.

Cardiac function, as measured by high-speed video imaging, demonstrated depressed cardiac output apparent at 2 days after AL-LC injection, when we began to observe early death.

While AL amyloidosis is a systemic disease, and given the systemic introduction of AL-LC, we cannot definitively rule out the possible involvement of other organs systems and cell types in the early mortality. The evidence of cardiac abnormalities (cardiac dysfunction and cardiomyocyte death) and the increased survival associated with the attenuation of AL-LC-induced cardiotoxicity supports the notion that cardiac pathology likely contributed significantly to overall mortality.

Using isolated cardiomyocytes in culture, we have previously demonstrated that AL-LC induces activation of p38 via a noncanonical signaling pathway that is independent of the upstream MAPKK MKK3/6. Instead, AL-LC activates p38 via transforming growth factor-β-activated protein kinase-1 binding protein-1-mediated p38α MAPK autophosphorylation (27). The involvement of noncanonical p38 MAPK activation was also validated in our present zebrafish model of AL-LC-induced toxicity, showing the ability of SB-203580 to inhibit p38 MAPK phosphorylation (Fig. 8F). Importantly, inhibition of p38 MAPK via SB-203580 protected against cardiac dysfunction, decreased cardiac cell death, and augmented survival, highlighting a substantive role for AL-LC cardiotoxicity in the overall disease pathogenesis. The improvement of survival further supports the notion that targeting the cardiotoxic effects
of AL-LC protein might significantly affect prognosis, a concept that is supported by clinical observations in which reduction or elimination of circulating AL-LC proteins through chemotherapy followed by autologous stem cell transplantation resulted in a marked reduction of circulating cardiac biomarkers and significantly improved patient cardiac function and survival, independent of cardiac fibril deposition (12, 18).

While it remains to be determined whether AL-LC proteins affect other organ systems in our zebrafish model, our findings suggest that any additional underlying systemic pathology is likely also dependent on p38 MAPK signaling.

The experiments presented here establish a novel in vivo model of AL-LC cardiotoxicity, implicating the cardiotoxic properties of AL-LC as a causal determinant of survival outcome in AL cardiomyopathy. Additionally, our findings support the potential use of p38 MAPK inhibition as a treatment strategy for AL cardiomyopathy patients. Further use of this zebrafish model will allow for systematic interrogation of the pathophysiological mechanisms of AL cardiomyopathy while providing a system for the high-throughput screening of candidate small molecules as pathway probes or potential therapeutic leads.
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