Published in final edited form as:

Cytotherapy. 2017 December ; 19(12): 1426–1437. doi:10.1016/j.jcyt.2017.08.021.

Mesenchymal stromal cells protect human cardiomyocytes from amyloid fibril damage

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Abstract

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Author contribution

YL designed, conducted experiments, analyzed data, wrote the paper

MMA designed, conducted experiments, analyzed data, wrote the paper

CJD conducted experiments, analyzed data, wrote the paper

KRR conducted experiments

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GWB and MCD provided technical expertise for experiments with MSC

BJM conducted experiments

AW conducted experiments

JSW analyzed data, wrote the paper

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Competing interests

YL, MMA, CJD, KRR, LBM, PB, GWB, MCD, BJM, AW, JSW, MRA- no competing interests

BACKGROUND: Light chain (AL) amyloidosis is a protein misfolding disease characterized by extracellular deposition of immunoglobulin light chains (LC) as amyloid fibrils. Patients with LC amyloid involvement of the heart have the worst morbidity and mortality. Current treatments target the plasma cells to reduce further production of amyloid proteins. There is dire need to understand the mechanisms of cardiac tissue damage from amyloid to develop novel therapies. We recently reported that LC soluble and fibrillar species cause apoptosis and inhibit cell growth in human cardiomyocytes. Mesenchymal stromal cells (MSC) can promote wound healing and tissue remodeling. The objective of this study was to evaluate MSC to protect cardiomyocytes affected by AL amyloid fibrils.

METHODS: We used live cell imaging and proteomics to analyze the effect of MSC in the growth arrest caused by AL amyloid fibrils.

RESULTS: we evaluated the growth of human cardiomyocytes (RFP-AC16 cells), in the presence of cytotoxic LC amyloid fibrils. MSC reversed the cell growth arrest caused by LC fibrils. We also demonstrated that this effect requires cell contact and may be mediated through paracrine factors modulating cell adhesion and extracellular matrix remodeling. To our knowledge, this is the first report of MSC protection of human cardiomyocytes in amyloid disease.

DISCUSSION: This important proof of concept study will inform future rational development of MSC therapy in cardiac LC amyloid.

Keywords

amyloid; cellular toxicity; immunoglobulin light chain; light chain amyloidosis; mesenchymal stromal cells; stem cells

Introduction

Light chain (AL) amyloidosis is a protein misfolding disease characterized by the abnormal proliferation of monoclonal plasma cells that secrete immunoglobulin light chains (LC) that are prone to misfolding, causing amyloid fibrils deposition that can lead to organ failure and death. LC full length (FL) proteins are comprised of the variable (V_L) and constant (C_L) domains. The mutations in the V_L play a role enhancing aggregation in proteins from patients with AL amyloidosis [1, 2]. Patients with cardiac AL amyloidosis have the worst prognosis, with a median survival of less than a year [3, 4].

We recently reported the mechanism of internalization of an amyloidogenic AL-09 protein and the control germline protein κ I O18/O8 (IGKV 1-33, hereafter called κ I for simplicity) into human cardiomyocytes (AC16 cell line). Fibrillar aggregates and, soluble LC proteins induce cytotoxicity in cultured AC16 cells; however, the toxic effect was mediated via different mechanisms: soluble protein induces apoptosis but allows the cells to grow while the fibrils arrest cell growth and cause morphological changes in the cells [5]. For the toxicity experiments, we used two additional lambda proteins and their corresponding fibrils (AL-T05 [6] and Wil [7–9]). We have observed the most toxic effect with Wil fibrils inducing complete growth arrest of AC16.

Current conventionally available treatments for AL amyloidosis target the light chain producing plasma cells with myeloma-based chemotherapy or stem cell transplant. However, patients with severe cardiac involvement may be too fragile to tolerate even plasma cell-direct chemotherapy or transplant, contributing to these patients' increased morbidity and mortality. No approved therapy targets the cellular damage caused by the amyloid fibrils in affected organs [10]. Two monoclonal antibodies targeting amyloid already deposited in organs are currently tested in clinical trial [10];[11]. More treatment options are desperately needed to improve the direct damage of the amyloid to the involved organs.

Mesenchymal stromal cells (MSC) are multipotent cells defined by their ability to differentiate into osteocytes, chondrocytes, and adipocytes [12]. MSC have been shown to protect heart and kidney from hypoxia-induced injuries and promote wound healing and tissue remodeling [13–18]. In ischemic heart disease and congestive heart failure, MSC have a beneficial effect on tissue regeneration which results in decreased scarring and increased global improvement of cardiac function such as left ventricular ejection fraction [13, 15]. In Alzheimer's disease, caused by abnormal deposit of a different type of amyloid protein limited to the Central Nervous System (CNS), MSC were shown to protect neurons from apoptosis via paracrine secretion of extracellular matrix proteins and growth factors [19] and recruit macrophages to degrade the abnormal proteins, leading to symptomatic improvement in an animal model [19]. We hypothesize that MSC can protect cardiomyocytes from AL protein-induced apoptosis through similar mechanisms. In this study, we seek to examine the therapeutic potential of MSC in cardiac AL amyloidosis.

Materials and methods.

Protein preparation.

The V_L sequences for κ I O18/O8 and AL-09 have been deposited previously under the GenBank accession numbers EF640313 and AF490909, respectively [20]. There is only one κ C_L sequence (protein accession number P01834). V_L domains and FL proteins were expressed and purified as previously described [8, 20–22]. Briefly, V_L domains were expressed in Escherichia coli BL21 (DE3) Gold competent cells. κ I O18/O8 VL was extracted from the periplasmic space by breaking the cells through one freeze-thaw cycle using PBS buffer, pH 7.4. AL-09 VL was extracted from solubilized inclusion bodies using 5 M urea and refolded by dialysis against 10 mM Tris-HCl, pH 7.4. FL proteins were expressed in E. coli Rosetta Garni competent cells. AL-09 and κ I FL were extracted from solubilized inclusion bodies using 5M urea and refolded by dilution (1:10) in ice-cold refolding buffer (10mM Tris/HCl, 1 M L-arginine, 7mM GSH, 0.7mM GSSG, 2.5mM EDTA, and 1mM PMSF protease inhibitor, pH 8.5) for 48 h at 4 °C. κ I FL sequence was mutated at position C214S and the C214 position was kept for AL-09 FL because it displayed a better protein expression and higher extraction yield without changing any other biochemical and biophysical properties. All proteins were purified using size exclusion chromatography in 10 mM Tris buffer, pH 7.0, at 4 °C (HiLoad 16/60 Superdex 75 column) on an AKTA FPLC (GE Healthcare). Eluted fractions were checked by SDS-PAGE, and their protein concentration was determined by UV absorption at 280 nm using an extinction coefficient (ϵ) calculated from the amino acid sequence (14,890 and 25,940 cm⁻¹ M⁻¹ for κ I

VL and FL proteins, respectively; 13,610 and 24,660 $\text{cm}^{-1} \text{M}^{-1}$ for AL-09 VL and FL proteins, respectively). Far UV CD scan and thermal unfolding were obtained as a protein quality control. Proteins were aliquoted at concentration below 100 μM , flash frozen, and stored at -80°C . Proteins were thawed at 4°C , filtered, and/or ultracentrifuged before they were used for each study.

Amyloid Fibril Formation.

Fibril formation assays were performed in triplicate using black 96-well polystyrene plates and shaken continuously at 300 rpm at 37°C in a New Brunswick Scientific Innova40 incubator shaker. Each well contained 260 μl of 20 μM protein, 150 mM NaCl, 10 μM Thioflavin T (ThT) (Sigma-Aldrich, St. Louis, MO, USA), 0.02% NaN_3 in 10 mM sodium acetate, boric acid, and sodium citrate buffer at pH 2.0 or pH 3.0 for V_L domains and FL proteins, respectively. Because the presence of preformed aggregates may accelerate the fibril formation kinetics, protein samples were ultracentrifuged prior to the fibril formation assay following the protocol described by DiCostanzo *et al.* [23]. ThT-fluorescence was used to follow the fibril formation kinetics on a triplicate well [24, 25], and was monitored daily on a plate reader (Analyst AD, Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 440 nm and an emission wavelength of 480 nm, until the reaction reached the plateau (~600-800 h). Triplicate wells containing buffer and ThT were included in our reactions as control.

At the end of fibril formation reaction, fibrils were collected, pelleted and washed three times with PBS buffer by centrifugation at 14,000 rpm, 10 min at RT. Supernatant was removed and quantified in order to determine the concentration of soluble protein left after fibril formation. Final fibril concentration (50 μM) was adjusted to that number with PBS buffer.

Cell Culture.

AC16 human primary ventricular cardiomyocytes were purchased from Dr. Mercy Davidson at Columbia University. This cell line has been immortalized by fusion with SV40 transformed fibroblast cell line devoid of mitochondrial DNA [26]. Cells were maintained with DMEM/F12 media (Life Technologies, Carlsbad, CA, USA) supplemented with 12.5% FBS (Mediatech, Manassas, VA, USA) and 1% Penicillin/Streptomycin (Invitrogen). AC16 cells co-transfected with plasmid expressing red fluorescent protein (RFP) in the nucleus were used (RFP-AC16 cells). Cell culture experiments were carried out under sterile conditions. AC16 cells are not listed in the database of commonly misidentified cell lines maintained by The International Cell Line Authentication Committee (ICLAC). As a control of viability and differentiation, cell morphology was always checked before each experiment and the number of cell passages after thawing was limited to 20. RFP-AC16 is authenticated every 6 months in our laboratory with the appropriate markers by western blot and PCR. We have also tested the cells every 6 months for Mycoplasma contamination.

MSC cells were derived from lipo-aspirates obtained from consenting healthy donors (donor 1 (MSC D1), donor 2 (MSC D2), donor 3 (MSC D3) with approval from the Mayo Clinic Institutional Review Board (IRB) following the protocol by Dudakovic *et al.* [27]. Samples

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were obtained from consenting normal patients that underwent elective removal of subcutaneous adipose tissue. Fat tissue was enzymatically digested using collagenase (Type I at 0.075 %; Worthington Biochemicals) for 1.5 h at 37°C. Adipocytes were separated from the stromal vascular fraction by low speed centrifugation (400 *g* for 5 min). After the adipose supernatant was removed, the cell pellet was rinsed with PBS and passed through cell strainers (70 μ m followed by 40 μ m) (BD Biosciences). The resulting cell fraction was incubated at 37°C in 5% CO₂ at a cell density of $1.0\text{--}2.5 \times 10^3$ cells/cm² in standard culture medium (Advanced MEM) with 5% PLTMax (a clinical grade commercial platelet lysate product [EMD Millipore]), 2 U/mL heparin (hospital pharmacy), 2 mM L-glutamine (Invitrogen) and antibiotics (100 U/mL penicillin, 100 g/mL streptomycin). Cells were harvested while still actively proliferating or when they reached confluence (typically four days after plating). The authentication and potential contamination of the MSC follows the protocol by Dudakovic and it is performed regularly in the laboratory.

Cell growth assay.

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Cell viability experiments were carried out as described previously [5]. Briefly, RFP-AC16 cardiomyocytes were plated at a concentration of 2,000 cells/well in a 96-well Corning polystyrene plate and allowed to grow overnight for cell attachment (<20 h) in the IncuCyte ZOOM incubator (5% CO₂ at 37°C) (Essen Bioscience, Ann Arbor, MI, USA). Next day, cell culture media was replaced with fresh media, with or without LC fibrils (final concentration 1 μ M). The changes in cell growth were analyzed by red counts per well every 4 h until cells become over confluent (> 60 h).

Cell-to-cell co-culture contact assays.

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Experimental setup was followed as described for the cell growth experiments, except that during fresh media change the next day after RFP-AC16 seeding, MSC cells (from three different healthy donors) were added at the same time as fibrils at a final concentration of 200, 400, 800, 1600 cells/well and 2000 cells/well (ratio MSC:RFP-AC16 is 0.2:1, 0.4:1, and 1:1 respectively). The changes in cell growth were analyzed by red counts per well every 4 h for more than 150 h. Each condition was set up in triplicate.

Contact-independent transwell assays.

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Cultures were set up using the ClearView membrane transwell with 96 laser etched pores with a diameter of 8 μ m within the IncuCyte ZOOM incubator (Essen Bioscience, Ann Arbor, MI, USA). Unlike co-culture experiments, MSC and RFP-AC16 were cultured in different chambers in a non-migrating transwell plate that do not allow cell-to-cell contact. Cell culture media alone was added on the lower reservoir plate (or bottom compartment) as control. RFP-AC16 cells, in AC16 cell culture media, were added to the upper chamber at a concentration of 1,000 cells/well and allowed to grow overnight for cell attachment (<20 h) in the IncuCyte ZOOM incubator (5% CO₂ at 37°C). Cell culture media of the lower compartment was replaced with fresh media containing MSC cells at a final concentration of 400, 800, 1800 and 2000 cells/well (ratio MSC:RFP-AC16 is 0.2:1, 0.4:1, 0.8:1 and 1:1 respectively). At the upper compartments, media was exchanged with fresh media containing LC fibrils (final concentration 1 μ M) to the attached RFP-AC16 cells. Each condition was set up in triplicate. Red fluorescent counts per well were measured every 4 h

for more than 150 h. Fold growth compared to baseline count at the time of media change was calculated.

Proteomics Analysis.

Proteomic analysis was performed using the supernatants at the end of the incubation for both co-culture with the three MSC donors and the transwell with MSC D1, with or without the presence of Wil fibrils. At the end of each cell growth assay, cell culture supernatants were removed from the 96-well plate and stored at -20°C for a future analysis. Samples were thawed at 4°C , and centrifuged at 13,000 rpm for 10 min to pellet the cell debris and any amyloid fibrils remaining on the sample. The SDS-PAGE gel bands are prepared for mass spectrometry analysis using the following procedures. Bio-Rad Bio-Safe Commassie stained gel bands are destained in 50% acetonitrile/50 mM Tris pH 8.1 until clear, and the proteins reduced with 50mM TCEP/50 mM Tris pH 8.1 at 55°C for 30 min, followed with alkylation using 25 mM iodoacetamide/50 mM Tris pH 8.1 at room temperature for 30 mins in the dark. Proteins are digested in-situ with 0.15 μg trypsin (Promega Corporation, Madison WI) in 25 mM Tris pH 8.1 / 0.0002 % Zwittergent 3-16, at 37°C overnight, followed by peptide extraction with 2 % trifluoroacetic acid and acetonitrile. The pooled extracts are concentrated and the proteins are identified by nano-flow liquid chromatography electrospray tandem mass spectrometry (nanoLC-ESI-MS/MS) using a Thermo Scientific Q-Exactive Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Thermo Ultimate 3000 RSLCnano HPLC system. The digest peptide mixture is loaded onto a 250nl OPTI-PAK trap (Optimize Technologies, Oregon City, OR) custom packed with Michrom Magic C18, 5 μm solid phase (Michrom Bioresources, Auburn, CA). Chromatography is performed using 0.2 % formic acid in both the A solvent (98% water / 2% acetonitrile) and B solvent (80% acetonitrile / 10% isopropanol/10% water), and a 5% B to 45% B gradient over 78 min at 400 mL/min through a PicoFrit (New Objective, Woburn, MA) 100 $\mu\text{m} \times 35\text{ cm}$ column handpacked with Agilent Poroshell 120 EC C18 packing. The Q-Exactive mass spectrometer set up was a FT full scan from 340-1500 m/z at resolution 70,000 (at 200m/z), followed by HCD MS/MS scans on the top 15 ions having a charge state of +2, +3, or +4, at resolution 17,500. The selected ions on an exclusion list for 30 sec. The MS1 AGC target is set to $1\text{e}6$ and the MS2 target is set to $1\text{e}5$ with max ion inject times of 50 ms for both.

Database searching.

Tandem mass spectra were extracted by msconvert version 3.0.9134. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0) and X! Tandem (The GPM, thegpm.org; version X! Tandem Sledgehammer (2013.09.01.1)). Mascot and X! Tandem were set up to search the UniProt November2015 human and bovine database (191756 entries) assuming the digestion enzyme *strictlytrypsin* and with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 PPM. Glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, oxidation of methionine and carbamidomethyl of cysteine were specified in X! Tandem as variable modifications. Oxidation of methionine and carbamidomethyl of cysteine were specified in Mascot as variable modifications.

Criteria for protein identification.

Scaffold (version Scaffold_4.6.2, Proteome Software Inc., Portland, OR) is used to validate MS/MS based peptide and protein identifications.

Peptide identifications are accepted if they can be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [28]. Protein identifications are accepted if they can be established at greater than 95% probability and contain at least 2 identified peptides. Protein probabilities are assigned by the Protein Prophet algorithm [29]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. For the protein biological pathway comparison and analysis, the FunRich software was used (<http://www.funrich.org/>).

Results.

Light chain fibrils arrest the growth of human cardiomyocytes but not mesenchymal stromal cells.

We tested the toxicity of amyloid fibrils on RFP-AC16 human cardiomyocytes proliferation *in vitro* using real-time live-cell imaging [5]. Fibrils from five recombinant light chain proteins were examined: the germline control κ I O8/O18 (κ I) V_L and FL; patient proteins κ I AL-09 V_L and FL proteins; and rV λ 6Wil protein (hereafter called Wil for simplicity), belonging to the V λ 6 6a (IGLV 6-57) family. AL-09 is the most well characterized κ I protein in our laboratory [2]. Wil protein has been extensively studied by Wall and co-workers [7–9].

Consistent with our recent publication [5], amyloid fibrils caused growth arrest of RFP-AC16 human cardiomyocytes (Figure 1). This effect was more pronounced with the V_L fibrils, particularly with Wil fibrils inducing a complete growth arrest. κ I FL and AL-09 FL fibrils have no effect on the cell growth (Figure 1). RFP-AC16 cells were observed to cluster around Wil fibrils by the first assessment around 4 hours after addition of fibrils (Figure S1). Cell morphology changes are almost instantaneous after exposure to fibrils. RFP-AC16 clustered around Wil fibrils not only had growth arrest (Figure 1), but also distorted membranes, collapsed cell bodies and abnormal nuclei, all morphologic changes associated with extensive cellular damage (Figure S1). Based on these observations, we decided to examine more in depth the capacity of MSC to protect RFP-AC16 from Wil fibrils toxicity.

MSCs derived from adipose tissue of three healthy donors [30, 31] were used in this study to address the potential functional variability of these primary cells. Triplicate from each donor was used for each experiment. Interestingly, MSC exposed to Wil fibril alone do not show morphologic changes as the ones observed for RFP-AC16. In addition, their growth capacity is not affected in the presence of Wil fibrils (Figure 2). This suggests capacity of MSC to resist the mechanisms of damage by LC fibrils.

MSC protects human cardiomyocytes from Wil fibril growth arrest

RFP-AC16 cells were exposed to Wil fibrils with or without co-culture with MSC. Wil fibrils were added at the previously published concentration of 1 μ M that had demonstrated cell growth arrest. The seeding ratio of MSC to RFP-AC16 was 1:5 (or 0.2:1) to avoid overgrowth of MSC in culture. Representative fluorescent microscopy images of co-culture when AC16 was labeled with green fluorescent protein (GFP) and MSC was labeled with red fluorescent dye Dil demonstrated close cell contact but no fusion of the cells between MSC and GFP-AC16 (Figure 3A).

MSCs from all three donors protected RFP-AC16 cells from the complete cell growth arrest induced by Wil fibrils (Figure 3B). While there were variations in the magnitude of protection among the different donors, with MSC D3 inducing the best protection, the variations among the three donors were not statistically significant. The protective effect was not immediate and became first apparent after an average of 65 hr. This may be due to, in part, the time needed for MSC to adhere in culture before taking on other functions. The protective effect from MSC was dose dependent as increasing ratio of MSC seeded relative to RFP-AC16 resulted in increasing improvement in RFP-AC16 growth (Figure 4). This cell growth recovery was not complete, suggesting that there may be additional mechanisms of fibril damage that cannot be reversed by a single dose-exposure of MSC.

After an extended culture period, RFP-AC16 co-cultured with MSC and Wil fibrils had less cell clustering around the fibrils and improved morphology compared to culture alone with Wil fibrils (Figure 5, videos 1 and 2). Both videos show the changes in the distribution of Wil fibrils as the incubation time increases. The difference observed in video 2 is that the MSCs (unlabeled elongated cells) move across the well capturing amyloid fibrils and cellular debris, creating a larger section of the culture that is fibril free, allowing RFP-AC16 to grow and proliferate. Representative image of RFP-AC16 alone with fibrils showed pyknotic-like appearance suggesting dying process, while RFP-AC16 co-cultured with MSCs have re-adopted the more polygonal shape of normal RFP-AC16 without the Wil-induced morphologic changes.

MSC protection of RFP-AC16 from amyloid fibril toxicity is contact-dependent

To determine whether the growth arrest protection observed in the presence of MSC is cell-contact dependent, we examined the cytotoxicity of Wil fibril on RFP-AC16 when the cells are cultured in a different chamber (non-migrating transwell) from the MSC. The fibrils were evenly dispersed throughout the transwell chamber after initial addition to the chamber, but migrate and congregate in different regions of the chamber over time (Figure 6). Similar to co-culture experiment, RFP-AC16 in the transwell chamber clustered around Wil fibrils and underwent morphological changes. However, different from the co-culture experiments, the presence of MSC in transwell chamber did not induce any recovery of RFP-AC16 growth from Wil fibrils (Figure 5, 6). The lack of protective effect was seen for the same ratio of MSC:RFP-AC16 seeding as co-culture experiment. Since the growth and viability of MSC is density dependent and MSCs seeded in transwell chamber would be more sparse than those in co-culture, we also examined transwell culture for higher MSC seeding density (ratio 1:1, 0.4:1, 0.2:1 MSC:RFP-AC16 ratio in the transwell chambers) (Figure 4 and video

3). Again, no protective effect was seen. This indicates that MSC contact with AC16 is needed to preclude the growth arrest.

MSC protective effect is associated with secreted factors

MSC have been shown to secrete growth factors to support nerve regeneration in Alzheimer's disease, a localized amyloid disease [32, 33]. Neuregulin-1 is a glycoprotein related to epidermal growth factor family and has been shown to suppress oxidative stressed-induced cardiomyocyte apoptosis via activation of PI3K/Akt pathway [34]. MSC has been found to express neuregulin-1 under certain conditions such as serum deprivation and hypoxia [35, 36]. Based on these reports, we hypothesized that neuregulin-1 may be upregulated in our studies. We examined culture supernatant for Neuregulin-1 levels by ELISA and did not find any difference in the supernatants between Wil fibrils exposed RFP-AC16 with and without MSC (data not shown).

We then examined other protein changes with a global analysis of the proteomes of the culture supernatants (Figure 7, figures S2–S3, tables S1–S4). Bovine proteins used in the media for all culture conditions were excluded from analysis. Compared to cells incubated in media (control), supernatants of RFP-AC16 exposed to Wil fibrils had changes in extracellular matrix proteins (heparan sulfate proteoglycan and chondroitin sulfate proteoglycan, proteins known to be involved in cell signaling and become upregulated and hyperphosphorylated in the presence of amyloid fibrils in cell culture [37]) and cell adhesion (atractin, vitronectin, and desmoplakin). These changes in extracellular matrix and cell adhesion proteins were consistent between RFP-AC16 cultured alone in either the co-culture experiments or transwell chambers.

Supernatants from RFP-AC16 co-cultured with MSC in the presence of Wil fibrils showed changes in the levels of fibronectin and collagen, considered biomarkers for cardiac fibrosis [38]. These changes were reproduced using western blot for fibronectin (data not shown). Co-culture with MSC2 resulted in the largest reduction of these biomarkers of fibrosis (figure S3, table S2). Proteome changes in co-culture with MSC3 had the most difference to that from the other two donors (figure S3, table S3). A detailed analysis of the proteins that are unique in the supernatants of MSC3 co-cultures in the presence and absence of Wil fibrils show that these unique proteins are involved in cell adhesion, plasma membrane protein modification, and apoptosis (Figure 7, figure S2). Interestingly, supernatants from transwell did not show changes in the same proteins for the corresponding culture conditions (Figure 7, figure S3, table S4). The transwell cultures did not rescue the cell growth arrest of RFP-AC16 (Figure 6), suggesting a role for these proteins in contact-dependent paracrine effect of proteins mediating cell adhesion and extracellular matrix modification.

Discussion

Within the amyloid field, particularly within localized amyloid diseases such as Alzheimer's disease, it has been widely accepted that oligomeric species are potentially more toxic than mature fibrils [39–41]. However, toxicity associated with amyloid fibrils has been observed for a number of systemic amyloid diseases (type II diabetes with IAPP fibrils, familial renal amyloidosis with lysozyme fibrils) and is pathologically relevant [42, 43]. In the context of

light chain amyloidosis, LC amyloid deposits are proposed to be the most common cause of amyloid cardiomyopathy [3]. We recently reported the effects of both soluble and fibrillar species from FL and V_L proteins were compared by using real-time live-cell image analysis. Using endocytic inhibitors, we elucidated the mechanism of internalization of soluble LCs and fibrils as actin-mediated macropinocytosis. Cell mediated seeding of FL and V_L proteins were shown by incubating preformed aggregates with soluble proteins. We demonstrated that κ I, λ 1, and λ 6 LC amyloid fibrils interact with RFP-AC16 causing cell clustering and cell growth arrest. High concentrations of soluble LCs and fibrils cause metabolic dysfunction and apoptosis [5, 7]. Complete growth arrest is seen with amyloid fibrils, limiting our ability to assess for apoptosis with the same enzymatic assay previously used. Cardiomyocyte morphology changes seen with amyloid fibrils are consistent with severe damage, suggestive of morphologic changes described in process of apoptosis or cell death. However improvement in cardiomyocyte morphology and recovery of cell growth when co-cultured with MSC suggest that the damage from the fibrils is reversible. Potential mechanisms of fibril damage through apoptosis or senescence are under investigation.

We have demonstrated that co-culture with MSCs protects RFP-AC16 from growth arrest induced by Wil (λ 6) fibrils. This effect requires cell contact and may be mediated by paracrine secreted factors. To the best of our knowledge, this is the first study that demonstrates the capacity of MSC to reverse the toxic effect of LC amyloid on human cardiomyocytes. We report here a useful *in vitro* model that can be used to facilitate more in-depth study on the MSC mechanisms of action. In this study, we have begun to examine the secretome from the MSC cardiomyocyte interaction. Mechanisms of MSC cardiomyocyte cell contact and downstream signaling changes inside cardiomyocytes are other ongoing areas of investigation. It is possible that MSC from different organ sites such as the bone marrow versus the adipose tissue have different functional capacities. This will be examined in future investigations. From a clinical translation feasibility standpoint, adipose-derived MSC is a more readily accessible source of MSC for clinical trial testing.

Two monoclonal antibodies are currently in clinical trials targeting the amyloid fibrils with promising results already reported [10]. A proposed mode of action for these monoclonal antibodies is through macrophage-mediated opsonization of fibrils [44]. Besides the direct effect of MSC on cardiomyocytes, we can also examine the role of MSC in recruiting and modulating macrophage opsonization functions. The combination of MSC and antibodies may have synergy to deepen the organ specific benefit of therapy targeting amyloid fibrils deposited in the heart.

Stem cells have enormous potential in wound healing and organ regeneration/remodeling applications. The adipose derived MSC studied here are readily accessible and expands abundantly for clinical application [30, 31]. Treatment with stem cells has shown beneficial effects such as improvement of cardiac regeneration and remodeling and increased myocardial performance [45]. Phase I/II clinical trials for these indications have demonstrated safety: no ectopic tissue formation, no immune reaction to allogeneic MSC transplant, and for intramyocardial delivery, no arrhythmia [13, 46–48]. Additional investigations for therapeutic strategies using MSC in cardiac amyloid are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the Ramirez-Alvarado and the IMPACT (immune progenitor and cell therapy) laboratories as well as the Amyloid Interest Group at Mayo Clinic for helpful discussions and feedback.

Funding sources

M.R.-A. was funded by R01 GM 071514, the Seidler Professorship, the Mayo Foundation, and the generosity of amyloidosis patients and their families. Y.L. was funded by Center for Regenerative Medicine Development Project. J.S.W. was funded by R01 DK079984.

Abbreviations

AL	amyloidosis-light chain amyloidosis
C_L	immunoglobulin light chain constant domain
LC	light chain
MSC	mesenchymal stromal cells
V_L	immunoglobulin light chain variable domain
RFP	red fluorescent protein
FL	full length LC protein
CNS	central nervous system
Wil	lambda 6 immunoglobulin light chain variable domain from a light chain amyloidosis patient

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Figure 1. LC fibrils caused human cardiomyocyte growth arrest.

RFP-AC16 cells were incubated with amyloid fibrils (1 μ M monomer concentration). The number of adherent RFP-AC16 cells per well over time were quantified by image analysis for red fluorescent nucleus counts. Wil and AL-T05 fibrils induced near complete arrest of RFP-AC16 growth. AL-09 V_L and κ I V_L fibrils also showed a significant inhibitory effect, whereas both AL-09 FL and κ I FL fibrils did not inhibit cell growth (as reported in Marin-Argany, et al., 2016). (Samples were set up in triplicate for each experiment. Data is shown from four independent experiments conducted in triplicate (n = 12) with the average values and error bars as a mean $\pm$ s.e.m.)

Figure 2. MSC exposed to Wil fibrils have preserved normal morphology and growth capacity. Representative images are shown from MSC D1 cultured alone or with Wil fibrils (1 μ M monomer concentration) at t=0 h and after 48 h of culture. Although amyloid fibrils seeding can be seen in these images, MSC have preserved their morphology in the presence of Wil fibrils in contrast to cardiomyocytes. Their growth capacity was also preserved with similar rate of confluence over time. Scale bar=300 μ m

Figure 3. MSC reversed human cardiomyocyte growth arrest caused by Wil fibrils in cell-to-cell contact experiments.

Representative phase contrast (A) and immunofluorescent images of fluorescently stained MSC (red cytoplasmic fluorescence) and GFP-AC16 (green nuclear fluorescence). The two types of cells remain in close contact with each other in co-culture, but no cell fusion is seen (lack of yellow co-localization). (B) RFP-AC16 cells were incubated with Wil fibrils (1 μ M monomer concentration) either alone or co-cultured with MSC cells (ratio MSC to RFP-AC16=1:5) from three different donors. Ratio of cell count over time compared to baseline (RFP-AC16 with Wil fibrils shown in black) is shown (n=9 for each MSC donor, three biological replicate samples with three technical replicates, mean $\pm$ s.e.m.). Despite the complete growth arrest seen with Wil fibrils on RFP-AC16 alone, co-culture with MSC partially restored RFP-AC16 growth. There is a delay in culture before RFP-AC16 growth is detected, however the protective effect is seen uniformly across MSC from all three donors.

Figure 4. Cell contact is required for MSC protective effect.

RFP-AC16 and MSC D1 co-culture (cell-to-cell contact) effect was compared with transwell experiments that do not allow cell migration. RFP-AC16 and MSC D1 cells were cultured in the top and bottom chambers of transwell, respectively. For each type of experiment, 0.2:1, 0.4:1, and 1:1 MSC to RFP-AC16 ratios were examined. At the end of the culture period (> 150 h), no growth protection of RFP-AC16 was seen in transwell conditions contrary to what was observed in co-culture (cell-to-cell contact) conditions. The fold growth rescue increased at the higher MSC to RFP-AC16 ratios. (Three biological replicates with three technical replicates per experiment, $n=9$, mean $\pm$ s.e.m; two-tailed t test; * $p<0.05$ between co-culture and transwell for each ratio tested; ** $p<0.05$ between co-culture at 1:1 ratio compared to co-culture at 0.2:1 ratio).

Figure 5. Co-culture with MSC restored the human cardiomyocyte morphological changes caused by Wil fibrils.

Representative images of RFP-AC16 cells incubated with Wil fibrils (1 μ M monomer concentration) alone and in the presence of MSCs from three different donors (ratio MSC:RFP-AC16=1:5) are shown. Images are taken at the end of cell growth experiments (t=150 h). MSC (white arrow) were seen in close proximity to RFP-AC16. External aggregates were seen on all four conditions (black puncta). Wil fibrils can be seen on RFP-AC16 cells (yellow arrows). All RFP-AC16 cells cultured alone with Wil fibrils showed an abnormal morphology (yellow arrow). In contrast, RFP-AC16 cells co-cultured with MSCs showed a fraction colony with more normal fusiform and polygonal morphology (blue arrows). Scale bar 150 μ m

Figure 6. Cell contact is required for MSC to restore cell growth and human cardiomyocyte morphology.

Representative images of RFP-AC16 cells incubated with Wil fibrils (1 μ M monomer concentration), cultured at the upper chamber, with MSC D1, cultured at the bottom chamber of transwell are shown. Baseline (t=0 h, A-C) conditions and t=96 h (D-F) are compared. Consistent with co-culture experiments, RFP-AC16 incubated alone with Wil fibrils showed altered morphology and the cells cluster around the fibrillar aggregates. The presence of MSC D1 (ratio MSC:RFP-AC16=1:5) in the bottom chamber of transwell, allow sharing of paracrine factors but not cell migration. These non-contact conditions did not restore RFP-AC16 morphology. Scale bar 150 μ m

Figure 7. Proteome changes in the microenvironment is different between co-culture and transwell with MSC.

Culture supernatants from co-culture and transwell experiments shown previously were analyzed for proteome profile by mass spectrometry. **(A)** Venn diagram for shared versus unique proteins for each culture conditions are shown from a representative co-culture (top) and transwell (bottom) experiment. **(B)** Comparison of biological pathways involved in the supernatant proteome showed very different processes involved in Wil fibril exposure to RFP-AC16 alone (yellow) compared to RFP-AC16 co-cultured with MSC (blue). **(C)** Less dramatically, differential distribution of biological pathways are involved in the supernatant proteome of RFP-AC16 and MSC when exposed to Wil fibrils in co-culture (blue) versus in transwell (yellow).