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Mesenchymal stromal cells protect human cardiomyocytes from amyloid fibril damage

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Abstract

Background aims. 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 (MSCs) can promote wound healing and tissue remodeling. The objective of this study was to evaluate MSCs to protect cardiomyocytes affected by AL amyloid fibrils. *Methods.* We used live cell imaging and proteomics to analyze the effect of MSCs 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. MSCs 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. *Conclusions.* This important proof of concept study will inform future rational development of MSC therapy in cardiac LC amyloid.

Key Words: *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 fibril deposition, which 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 κI O18/O8 (IGKV 1–33, hereafter called κI 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, whereas 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

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with Wil fibrils inducing complete growth arrest of AC16 cells.

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 (MSCs) are multipotent cells defined by their ability to differentiate into osteocytes, chondrocytes, and adipocytes [12]. MSCs 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, MSCs have a beneficial effect on tissue regeneration that results in decreased scarring and increased global improvement of cardiac function such as left ventricular ejection fraction [13,15]. In Alzheimer disease, caused by abnormal deposit of a different type of amyloid protein limited to the central nervous system, MSCs 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 MSCs can protect cardiomyocytes from AL protein-induced apoptosis through similar mechanisms. In this study, we sought to examine the therapeutic potential of MSCs in cardiac AL amyloidosis.

Methods

Protein preparation

The V_L sequences for κI 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. κI O18/O8 VL was extracted from the periplasmic space by breaking the cells through one freeze-thaw cycle using phosphate buffered saline (PBS; pH 7.4). AL-09 VL was extracted from solubilized inclusion bodies using 5 mol/L urea and refolded by dialysis against 10 mmol/L Tris-HCl, pH 7.4. FL proteins were expressed in *E. coli* Rosetta Gami competent cells. AL-09 and κI FL were extracted from solubilized inclusion bodies using 5 mol/L urea and refolded by dilution (1:10) in ice-cold refolding buffer (10 mmol/L Tris/HCl, 1 mol/L L-arginine, 7 mmol/L reduced glutathione (GSH), 0.7 mmol/L oxidized glutathione (GSSG), 2.5 mmol/L ethylenediaminetetraacetic acid and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) protease inhibitor, pH 8.5) for 48 h at 4°C. κI 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 mmol/L 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 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and their protein concentration was determined by ultraviolet 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 κI VL and FL proteins, respectively; 13 610 and 24 660 cm^{-1} M^{-1} for AL-09 VL and FL proteins, respectively). Far ultraviolet CD scan and thermal unfolding were obtained as a protein quality control. Proteins were aliquoted at concentration below 100 μmol/L, 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 μmol/L protein, 150 mmol/L NaCl, 10 μmol/L Thioflavin T (ThT; Sigma-Aldrich), 0.02% NaN_3 in 10 mmol/L 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 before the fibril formation assay following the protocol described by tanzo 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) 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 by centrifugation at 14 000 rpm, 10 min at room temperature. Supernatant was removed and quantified to determine the concentration of soluble protein left after fibril formation. Final fibril concentration (50 μmol/L) was adjusted to that number with PBS.

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 Dulbecco's Modified Eagle's Medium/F12 medium (Life Technologies) supplemented with 12.5% fetal bovine serum (Mediatech) 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. 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 polymerase chain reaction. We have also tested the cells every 6 months for *Mycoplasma* contamination.

MSCs were derived from lipo-aspirates obtained from consenting healthy donors (donors 1–3, MSC D1, MSC D2 and MSC D3, respectively) with approval from the Mayo Clinic Institutional Review Board following the protocol by Dudakovic et al [27]. Samples 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 μm followed by 40 μm; BD Biosciences). The resulting cell fraction was incubated at 37°C in 5% CO_2 at a cell density of 1.0–2.5 × 103 cells/cm^2 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 mmol/L 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 4 days after plating). The authentication and potential contamination of the MSCs follows the protocol by Dudakovic, and it is performed regularly in the laboratory.

Cell growth assay

Cell viability experiments were carried out as described previously [5]. Briefly, RFP-AC16 cardiomyocytes were plated at a concentration of 2000 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% CO_2 at 37°C; Essen Bioscience). The next day, cell culture medium was replaced with fresh medium, with or without LC fibrils (final concentration 1 μmol/L). 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

Experimental setup was followed as described for the cell growth experiments, except that during fresh medium change the next day after RFP-AC16 seeding, MSCs (from three healthy donors) were added at the same time as fibrils at a final concentration of 200, 400, 800 and 1600 cells/well and 2000 cells/well (MSC:RFP-AC16 ratio was 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

Cultures were set up using the ClearView membrane transwell with 96 laser-etched pores with a diameter of 8 μm within the Incucyte ZOOM incubator (Essen Bioscience). Unlike co-culture experiments, MSCs and RFP-AC16 cells were cultured in different chambers in a non-migrating transwell plate that do not allow cell-to-cell contact.. Cell culture medium alone was added on the lower reservoir plate (or bottom compartment) as control. RFP-AC16 cells, in AC16 cell culture medium, were added to the upper chamber at a concentration of 1000 cells/well and allowed to grow overnight for cell attachment (<20 h) in the IncuCyte ZOOM incubator (5% CO_2 at 37°C). Cell culture medium of the lower compartment was replaced with fresh medium containing MSCs at a final concentration of 400, 800, 1800 and 2000 cells/well (MSC:RFP-AC16 ratio was 0.2:1, 0.4:1, 0.8:1 and 1:1, respectively). At the upper compartments, medium was replaced with fresh medium containing LC fibrils (final concentration 1 μmol/L) 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 with baseline count at the time of medium change was calculated.