Cardiovascular, Pulmonary and Renal Pathology

Role of Endocytic Inhibitory Drugs on Internalization of Amyloidogenic Light Chains by Cardiac Fibroblasts

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Amyloidosis is a disease of protein misfolding that ultimately impairs organ function. Previously, we demonstrated that amyloidogenic light chains (κ 1, λ 6, and λ 3 subtypes), internalized by cardiac fibroblasts, enhanced sulfation of secreted glycosaminoglycans. In this study, we investigated the internalization and cellular trafficking of urinary immunoglobulin light chains into cardiac fibroblasts. We demonstrate that these light chains have the ability to form annular rings in solution. Internalization was assessed by incubating cells in the presence of light chain conjugated to Oregon Green 488 followed by monitoring with live cell confocal imaging. The rate of light chain internalization was reduced by treatment with methyl- β -cyclodextrin but not filipin. Amyloid light chain did co-localize with dextran-Texas Red. Once internalized, the light chains were detected in lysosomes and then secreted into the extracellular medium. The light chain detected in the cell lysate and medium possessed a lower hydrophobic species. Nocodazole, a microtubule inhibitor, did not disperse aggregates. In addition, internalization and retention of the light chain proteins was altered in the presence of the proteasomal inhibitor MG132. These results indicate that the cell internalizes light chain by a fluid phase endocytosis, which is then modified and ultimately compromises the cell. (*Am J Pathol* 2006, 169:1939–1952; DOI: 10.2353/ajpath.2006.060183)

The dynamics of cellular processing of light chains (LCs) has been of great interest to investigators, but the cellular response is not well understood. Primary (AL) amyloidosis is a disease of protein misfolding. Plasma cell dyscrasia produces amyloidogenic immunoglobulin LCs that circulate through the vascular system and deposit as insoluble fibrils in tissues. The nonbranching fibrils are composed of filaments that form β -pleated sheets, stain with Congo Red, and display an apple-green birefringence with polarizing microscopy.

The LCs are known to disrupt the normal physiology of organs such as heart, kidney, lungs, peripheral nerves, and intestines, with the most common involvement in kidney and heart. Although renal amyloidosis often results in nephrotic syndrome, cardiac involvement occurs in up to 50% of patients with AL amyloidosis and is a leading cause of morbidity.^{1,2} Congestive heart failure is associated with amyloidotic cardiomyopathy.²

Investigators have demonstrated that LCs induce oxidative stress responses in isolated cardiac cells and are associated with a decrease in contractility.³ These data indicate that LC toxicity may contribute to cardiac dysfunction as well as play a role in amyloid fibril deposition. Furthermore, internalization of LCs (κ 1, λ 6, and λ 3 subtypes) by cardiac fibroblasts resulted in enhanced sulfation of secreted glycosaminoglycans (GAGs) with minimal heparan-sulfated proteoglycans localized within the cytoplasm.⁴ These results supported earlier studies demonstrating an increase in GAGs with amyloid deposition.⁵ However, the mechanism of internalization and cellular trafficking of LCs is not well understood.

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Internalization and cellular trafficking has been studied using a number of amyloid systems. Models of AA and A β amyloid suggest that endocytosis and/or pinocytosis may be critical to amyloid protein internalization.^{6,7} In addition, studies using mesangial cells demonstrate that internalization of LCs by cells utilizes a specific receptor complex pathway.^{8,9} In neuronal cells, investigators showed that receptors of advanced glycation end products function as signal-transducing cell surface acceptors for A β .¹⁰ In fact, receptors of advanced glycation end products are proposed by investigators to act as a general mechanism for cellular uptake of misfolded proteins.¹¹ These results may reflect differences in cell types.

Structural analyses of a number of amyloidogenic proteins suggest that internalization of these biomolecules may occur when they adopt pore-like conformations that can penetrate and disrupt the cellular membrane.^{12–18} The oligomers possess a number of morphologies, including annular rings that form on mica at concentrations less than 50 $\mu\text{g/ml}$.¹² Pore-like structures such as these have been identified in other diseases with protein misfolding, ie, Parkinson's,¹⁹ Huntington's, and Alzheimer's^{14,20} diseases and on mitochondrial²¹ and lysosomal²² membranes.

Our goal was to evaluate the internalization and processing of soluble LCs by cardiac fibroblasts. Cardiac fibroblasts were chosen because of their role in the enhanced production of glycosaminoglycans in response to LCs, and the known association of GAGs and amyloid deposits.^{4,23,24} Rat primary cardiac fibroblasts were treated with fluorescently labeled LCs in the presence of endocytic inhibitors, proteasomal inhibitors, and cellular probes. The response of the cells and the localization of the LCs were evaluated. We established that the rate of internalization was concentration and mass-dependent and was reduced by treatment with methyl- β -cyclodextrin (M β CD) but not by filipin. Co-localization is detected with dextran-Texas Red, a marker of pinocytosis. In addition, proteasomal inhibitors altered processing of the LC, resulting in increased retention.^{25,26}

Materials and Methods

LC Purification

LCs were purified from urine collected from patients with AL amyloidosis with the approval of the Institutional Review Board of Boston University Medical Center. A total of four κ 1 and one λ 6 LCs were evaluated. The κ LCs included two monomers, a truncated form, and a dimer. The λ 6 LC was a monomer. All LCs examined here had been evaluated previously and had demonstrated internalization.⁴ Urine samples were dialyzed, lyophilized, and treated with Affi-Gel Blue (Bio-Rad Laboratories, Hercules, CA) to remove albumin. LC proteins were purified by chromatographic separation on a Sephacryl S-200 column (Amersham Pharmacia Bio-Tech, Buckingham, UK). The immunoglobulin LC proteins were subtyped using a number of cellular and molecular analyses and

the sequence examined using both molecular and mass spectrometry.²⁷

Cell Culture

Primary rat cardiac fibroblasts were isolated as previously described.⁴ In brief, ventricles were subjected to digestion in a buffer containing collagenase, hyaluronidase, and trypsin. Cells were cultured in Dulbecco's low-glucose modified Eagle's medium supplemented with 7% calf serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (Life Technologies, Grand Island, NY).⁴ Cells were used in either the first or second passage. For live cell imaging experiments cells were plated at a concentration of 5×10^3 cells/ml on eight-well coverslip chamber slides (Nalge Nunc, Rochester, NY) and cultured for 48 hours. For other assays, cells were plated on P-100s at an equivalent cell density.

Confocal Laser-Scanning Microscopy

Imaging was performed on a Zeiss LSM 510 confocal microscope (Thornwood, NY) as described previously.^{4,28,29} Images were taken using a $\times 63$ objective with an optical slice of 3 μm . Live cell imaging was performed with a $\times 40$ objective with an optical slice ranging from 1 to 3 μm . Z-stacks were taken with an optical slice of 1 μm at an interval of 0.5 μm . Images were collected with $\times 4$ averaging. Detector gain and amplitude offset were determined for each experiment to maximize the linear range without saturation and were kept consistent for comparable experiments. Average fluorescence intensities of internalized LCs were measured in individual cells using Zeiss LSM software for region of interest and graphed throughout time.

Localization of LC

Live cell imaging was performed to monitor the localization and internalization rate of LC proteins. Amyloidogenic LCs were conjugated to Oregon Green 488 (Molecular Probes, Eugene, OR) and purified over a sizing column using the suggested methodology from Molecular Probes and Trinkaus-Randall and colleagues.⁴ The ratio of Oregon Green molecules/LC was determined for each conjugation and used to calculate comparative internalization rates between different LCs. $\text{OG488:LC} = A_{496} \cdot 100/70,000 \cdot \text{Cp}$, where Cp is LC molar concentration. The fluorescently conjugated LCs (1.29 $\mu\text{mol/L}$) were added to cells plated on eight-well glass coverslips and imaged at an excitation of 488 nm for interval time points throughout a period of 24 hours. A single Z-series was taken at each experiment to determine the localization of the LC within the cell compared with a simultaneously acquired differential interference contrast image. The addition of unconjugated Oregon Green 488 to cells was used as a control.

Indirect Immunohistochemistry and Co-Localization

Indirect immunohistochemistry was performed to localize specific proteins after fixation.^{30–32} In brief, cells were fixed with freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.2 for 15 minutes at room temperature, washed with PBS, permeabilized with 0.1% Triton X-100, and blocked with PBS containing 5% bovine serum albumin. The cells were incubated in PBS/1% bovine serum albumin containing appropriate monoclonal antibodies (mAbs) for 18 hours at 4°C. After incubation with the primary antibody, cells were rinsed with PBS, washed in 3% bovine serum albumin/PBS, and incubated with a secondary antibody conjugated to Alexa Fluor 546 or 633 (1:250) (Molecular Probes) for 2 hours at room temperature or co-stained with rhodamine phalloidin (1:50) for 45 minutes at room temperature. Confocal settings were optimized to control for signal crossover. Cells were imaged using the LSM 510 version 3.2 as described.^{32,33}

Inhibitors

The internalization of LCs by cardiac fibroblasts was evaluated in the presence of inhibitors of endocytosis. The following optimal conditions were established to maintain morphological integrity and demonstrate efficacy of the drug: 10 mmol/L methyl- β -cyclodextrin (M β CD), 1 hour pretreatment; 10 μ mol/L cytochalasin D, 30 minutes pretreatment; 1 μ mol/L nocodazole, 1 hour pretreatment; 7.6 μ mol/L filipin, 1 hour pretreatment; and 5 μ mol/L MG132 for 1 hour pretreatment (Sigma, St. Louis, MO). Light Chain-OG was added to cells in the presence or absence of inhibitors and followed throughout time.

Co-Localization Studies

To monitor intracellular trafficking of LCs, fluorescent markers of membrane structures and mechanisms were used. The following fluorescent live cell markers were used in conjunction with LC-OG (488): cholera toxin B (555) 0.877 μ mol/L for 2 hours, wheat germ agglutinin (WGA) (633) 0.105 μ mol/L at several time points at 37°C, LysoTracker DND 99 (577) 50 nmol/L for 30 minutes, Mitotracker 25 nmol/L for 30 minutes, ER Tracker (587) 1 μ mol/L for 20 minutes, transferrin 0.312 μ mol/L with an 8-minute pulse,³⁴ FLAER (633) 10^{-8} mol/L, and FM4-64 8.23 μ mol/L for 8 minutes at 4°C at 37°C (Molecular Probes). Dextran-Texas Red (10 and 40 kd) (Molecular Probes) were added to cells at equimolar concentrations as AL-LC for parallel time course experiments. Co-localization was defined using the Zeiss LSM 510 co-localization co-efficient program, images were scanned at the optimal pixel number (2048 $\times$ 2048) and the crosshair function and scatter region of interest mode were used. Only pixels above background fluorescence were evaluated using LSM 510 co-localization and crosshair functions.³⁵

Figure 1. Formation of annular rings. **Top:** LC was incubated for 8 days (pH 6, 37°C), dried on disks, negatively stained, and viewed using transmission electron microscopy. **Bottom:** LC was incubated for 1 day (pH 2, room temperature), placed on mica, and imaged using atomic force microscopy. Samples represent three independent runs.

Transmission Electron and Atomic Force Microscopy

To assess the formation of annular LC complexes, transmission electron microscopy and atomic force microscopy were used. Five μ l of 1.1 μ mol/L LC solution were applied to 300 mesh carbon-coated copper grids that had been glow discharged rendering the surface hydrophilic. The LC was adsorbed onto the grid for 25 minutes at room temperature, and the excess solution was removed by wicking filter paper. Samples deposited on grids were stained negatively with 1% sodium phosphotungstic acid (NaPT) for 10 seconds. NaPT was removed and the sample air-dried. Grids were visualized with a Philips CM12 transmission electron microscope.

Atomic force microscopy imaging was performed with a commercial MultiMode atomic force microscope controlled by Nanoscope IIIa electronics (Digital Instruments, Santa Barbara, CA) equipped with a 12- μ m scanner (E-scanner). Images were taken in air using a tapping mode on the mica surface. RTESP tapping mode silicon

Figure 2. Internalization of LC using live cell confocal imaging. Cardiac fibroblasts were cultured in eight-well coverslips, and κ 1-LC conjugated to Oregon Green 488 (1.29 μ mol/L) was added to the medium. **A:** Series of images taken from a 1-hour time course of a representative experiment using live cell confocal microscopy (LSM 510). Series of images are taken from Supplemental Movie 1 (see <http://ajp.amj-pathol.org>). Scale bar = 20 μ m. **B:** Cardiac fibroblasts were cultured, and before the addition of LC, cells were incubated in the presence or absence of nocodazole and images taken. Note aggregates (arrow) with nocodazole. $n = 30$.

tips were mounted on triangular 100- μ m-long cantilevers ($k = 20$ to 80 N/m) (Digital Instruments). The tips were exposed to UV light before use. For high-resolution imaging, the microscope head was placed on a vibration-isolated air table. Samples were imaged by adding the same concentration onto freshly cleaved mica. Samples were incubated for 3 minutes at room temperature, washed with dH₂O, and dried under a nitrogen stream.

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

The hydrophobic nature of AL-LC-OG in cell culture media and lysate samples was assessed by RP-HPLC. Cell lysates and medium samples were separated on a gel filtration column (GF-250) to assess for change in mass. Analyses were performed on the samples on a Poroshell 300SB-C8 (5 μ m, 2.1 $\times$ 75 mm) reversed-phase column (Agilent, Palo Alto, CA) using a linear gradient of 0 to 100% solvent B throughout 5 minutes and operating at a flow rate of 1.0 ml/minute (solvent A: 5% CH₃CN, 10

mmol/L Tris, pH 7.4; solvent B: 85% CH₃CN, 10 mmol/L Tris, pH 7.4). Elution profiles were generated with fluorescence monitoring (excitation wavelength, 488 nm; emission wavelength, 530 nm). Peak fractions were collected and dried in a SPD111V centrifugal concentrator (ThermoSavant, Holbrook, NY).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

The presence of LC-OG proteins was evaluated by SDS-PAGE and Western blot analysis. Dried fractions collected from RP-HPLC separations were reconstituted in SDS-PAGE reducing buffer (10 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, 2.5% SDS, 5% β -mercaptoethanol, and 0.01% bromophenol blue), heat-denatured with boiling for 3 minutes, electrophoresed on 10 to 15% polyacrylamide gradient gels, and stained with Coomassie blue or immunoblotted. Nitrocellulose mem-

Figure 3. Internalization and secretion of $\kappa 1$ -LC. **A:** Internalization of $\kappa 1$ -LC-OG488 was determined by measuring fluorescence intensity of a minimum of 20 cells in a field. $n = 3$. Fluorescence intensity was calculated based on conjugation of OG to LC. **A:** Internalization rate is correlated with concentration of LC. **B:** After internalization of LC-OG, movement of LC-OG out of cells was monitored throughout 72 hours in the absence of exogenous LC-OG. $n = 3$.

branes were probed with rabbit anti-human antibodies directed against $\kappa 1$ -LC (Dako Cytomation, Carpinteria, CA) and then incubated with a goat anti-rabbit whole IgG antibody conjugated to alkaline phosphatase (Sigma). Blots were visualized using BCIP/NBT color development substrate (Promega, Madison, WI). SDS-PAGE and transfer of HPLC samples were performed on the PhastSystem (GE Health Care, Giles, UK). Other lysates were evaluated using SDS-PAGE, and the LC-OG was detected using an antibody directed against Oregon Green (Molecular Probes), or the LC-OG was imaged directly on the KODAK Image Station 440 (Eastman-Kodak, Rochester, NY).

Results

Amyloidogenic LCs Form Annular Rings

Formation of pore-like structures have been demonstrated with a number of proteins associated with protein

Figure 4. Internalization rate depends on the mass of the LC. Calculations of internalization were determined by measuring fluorescence intensity of a minimum of 20 cells in a field in five independent experiments. Internalization is demonstrated throughout a period of 4 hours. **A:** Three $\kappa 1$ chains were evaluated (truncated, 12 kd; monomer, 23 kd; dimer, 47 kd). **B:** Rate of internalization of two $\kappa 1$ and one $\lambda 6$ LC monomers.

misfolding. Our goal was to determine whether annular rings formed from $\kappa 1$ -LCs in solution in our system. The change from oligomers to ring-like structures was detected using atomic force microscopy and transmission electron microscopy (Figure 1). The atomic force microscopy image represents an annular ring formed in solution (0.25 mg $\kappa 1$ -LC, pH 2, 25°C). It has an average outer/inner diameter of 68.01 nm/30.07 nm, respectively (Figure 1). At this level of resolution (500 nm $\times$ 500 nm), three subunits were detected. Rings were detected throughout a range of pH (2 to 7) and were a minor species (1%). To image larger surface areas, negative staining transmission electron microscopy was used. The experiments were performed under conditions determined to be optimal for fibril formation (without the addition of other components such as GAGs) (0.25 mg/ml $\kappa 1$ -LC, pH 6, 37°C, rotation of 25 rpm). Two types of ring-like structures were observed; one with a larger ring (average outer/inner diameter, 110.5/42.5 nm) and a second smaller ring with a dimension similar to the atomic force microscopy (average outer/inner diameter, 76.5/36 nm). These data indicate that soluble LCs purified from clinical sources (see Materials and Methods) are capable of forming annular ring structures *in vitro*.

Figure 5. LC does not co-localize with the plasma membrane. Co-localization was determined using the crosshair function and Scatter R01 mode of the Zeiss LSM 510 co-localization co-efficient program. **A–C:** LC-OG488 was added to cells, co-stained with FLAER (**A**), FM4-64 (**B**), and WGA (**C**), and imaged live on the Zeiss LSM 510. **D–F:** LC-OG488 was added to cells and stained with an antibody directed against caveolin (**D**) or incubated in the presence of cholera toxin subunit B (**E**) and transferrin (**F**). Scale bar = 20 μm . $n = 3$.

Dynamics of Amyloid LC Internalization

To determine internalization rate, fluorescence intensity of internalization of $\kappa 1$ -LC-OG was measured as described in the Materials and Methods, reflecting the number of Oregon Green molecules bound per LC. Images taken every minute throughout a period of 1 hour demonstrated a gradual increase in internalized LC-OG signal, shown in representative images (Figure 2A). The $\kappa 1$ -LC displayed a cytoplasmic, punctate pattern with an increase in perinuclear concentration throughout time (Figure 2A). When the images were run continuously as movies, the internalized LCs displayed continual motion (average rate, 0.25 $\mu\text{m}/\text{second}$) (Supplemental Movie 1, see <http://ajp.amjpathol.org>). By 4 hours, the punctae had a mean volume of 0.5 μm^3 . The motion was arrested when cells were treated with nocodazole, a microtubule inhibitor. In addition, the punctae appeared to form small aggregates in the presence of nocodazole, which had a mean volume of 0.75 μm^3 . There was no dispersion of the aggregates detected throughout time in the presence of the microtubule inhibitor.

The internalization depended on concentration of the LC, mass, and temperature. It correlated positively with increasing concentration of $\kappa 1$ -LC. When cells were exposed to increasing concentrations of protein (0 to 5.15

$\mu\text{mol/L}$), the internalization rate (change in intracellular fluorescence intensity) did not plateau throughout a period of 4 hours (Figure 3A). Internalization was verified in cell lysates using HPLC, SDS-PAGE, and Western blot analysis using an antibody directed against the Oregon Green of the conjugated LC-OG (Figures 10 and 11). After 24 hours, there was a decrease in the rate of internalization, which we hypothesized to be attributable to secretion of the LC. To evaluate secretion, cells were incubated in the presence of fluorescently labeled LC for 24 hours, and the medium was replaced with LC-deficient medium. Both the intracellular fluorescence and the fluorescence in the medium were monitored. As predicted, a decrease in intracellular LC fluorescence was detected after the removal of LC-rich medium (Figure 3B). After 3 days, LC-OG was detected in the medium at a concentration of 19.6 nmol/L.

The rate of internalization depended inversely with increasing mass of the LC. Three $\kappa 1$ LCs of varying size (truncated 12 kd, monomer 23 kd, dimer 47 kd) were added to cells at an equivalent molar concentration (1.29 $\mu\text{mol/L}$). Images were taken throughout time, and internalization rate was calculated (Figure 4A). The truncated form was internalized 4.5-fold faster than the monomer, whereas the internalization rate of the dimer was 10-fold

slower than that of the monomer. A comparison of three LC monomers (two $\kappa 1$ and one $\lambda 6$) showed minor variations in rate of internalization (Figure 4B). All of the LCs evaluated to date showed internalization by cardiac fibroblasts.

There was a decrease in rate of internalization when cells were incubated in AL-LC at 4 and 20°C compared to 37°C. Negligible internalization was detected at 4°C throughout a 24-hour period, whereas at 20°C it was less than 50% than that at 37°C. When cells were incubated at 4°C and then switched to 37°C, there was a rapid increase in internalization. No aggregation or accumulation of LC was seen at the membrane at the lower temperatures. For clarity the remaining analyses are limited to a single $\kappa 1$ -LC that is a monomer and was evaluated in Figures 2, 3, and 4.

Association of Amyloid LC with the Plasma Membrane and Co-Localization with Dextran

To evaluate whether LC-OG interacted with proteins found on the plasma membrane, co-localization studies were performed. There was minimal co-localization with the membrane dyes FLAER ($0.6 \pm 0.1\%$), FM4-64 ($5 \pm 2.4\%$), and WGA (used as a membrane marker at 10 minutes) ($4.7 \pm 1.8\%$) throughout a period of 4 hours (Figure 5, A–C). To assess co-localization images were scanned at the optimal pixel number (2048×2048), and only pixels above background fluorescence were evaluated using the Zeiss LSM 510 co-localization co-efficient program. The crosshair function and scatter region of interest mode were used.

Filamentous actin played a role in the internalization and localization of LC within the cell. When cells were evaluated after treatment with cytochalasin D, internalization of LCs was inhibited (Figure 6A). In addition, its localization was altered, and it was found concentrated at the apices of cells. The concentration and incubation period used in the assays were optimized to alter internalization but not lamellipodial extensions. Preliminary experiments demonstrated that higher concentrations resulted in greater inhibition, but it was associated with a marked change in cell architecture. Internalization was not mediated by nocodazole (data not shown). The functional studies correlated with immunohistochemical stains of mAbs directed against microtubules and rhodamine phalloidin to detect F-actin (Figure 12). Only phalloidin showed a 5% co-localization with LC-OG.

Although there was negligible co-localization with FLAER, depletion of membrane cholesterol with the endocytocytic inhibitor, M β CD, inhibited internalization by 44% in comparison to the untreated controls at the same time point (Figure 6B). However, filipin, which disrupts lipid rafts, did not inhibit internalization compared to untreated control (Figure 6C). Co-localization of LC-OG and markers of endocytosis including cholera toxin subunit B ($2.3 \pm 0.3\%$) and transferrin ($1.9 \pm 0.8\%$) was minimal (Figure 5, D–F). Caveolin, which has been shown to play a minor role in pinocytosis, had a co-localization of $8.4 \pm 0.5\%$. Together, these results indicate that LC internaliza-

Figure 6. Internalization rate of LC is reduced by inhibitors. **A:** Preincubation with cytochalasin D (cyto D) (preliminary experiments demonstrated that 10 $\mu\text{mol/L}$ did not alter lamellipodial extensions) reduced internalization. **B:** Preincubation with M β CD (10 mmol/L) abrogated internalization. **C:** Preincubation of cardiac fibroblasts with filipin (7.6 $\mu\text{mol/L}$) did not reduce internalization rate. $n = 3$.

tion is not mediated by lipid rafts, which are important mediators of receptor endocytosis, but is more likely associated with a general pinocytosis.³⁶

Dextran-Texas Red, a classic marker of pinocytosis, was used to test the hypothesis that the AL-LC was internalized by pinocytosis. Co-localization of dextran with internalized LC-OG was detected with a co-localization co-efficient of 45 ± 1.9 SEM in both Z-stacks and time lapse imaging (Figure 7). Together these results support the hypothesis that LC is internalized by bulk flow endocytosis.

Figure 7. Internalization of LC co-localizes with pinocytic marker. Cells incubated in media with both LC-OG and bulk phase marker dextran-Texas Red at equimolar concentrations show co-localization of internalized molecules by 4 hours with confocal imaging. Optical slice = 3 μ m. $n = 5$.

To monitor internalization of LC throughout time, studies were performed with markers for the following organelles: endoplasmic reticulum, Golgi, lysosome, mitochondria, and nucleus (Figure 8). Lysosomes showed substantial co-localization with LC. Co-localization with mitochondria, nucleus, endoplasmic reticulum, and Golgi were less than 10%. Neither did we co-localize LC with an antibody directed against the *trans*-Golgi (data not shown). Although LC-OG did not co-localize with membranes, when parallel live cell differential interference contrast and confocal microscopy were performed, analyses of the merged images demonstrated that the internalized LC-OG was present within vesicles (Figure 9A). To monitor movement of the internalized LC to lysosomal compartments, experiments were conducted at 25°C. The LC-OG appeared in early vesicles distinct from labeled lysosomes (red), and after 5 hours LC was detected in lysosomes (16.6% co-localization). By 48 hours, more than 32% of the internalized LC co-localized with the lysosomes (Figure 9B).

Cells Alter Hydrophobicity of Amyloid LC

To determine whether there was a change in hydrophobicity or mass in the LC after it was internalized or secreted, cells were incubated in LC for 3 days, and media and cell lysates were collected. At day 3, the media was removed and replaced with exogenous media lacking LC, and medium was collected after day 6. When mass of the media and cell lysate fractions were assessed using gel filtration HPLC (GF-250), we did not detect changes in the profile (data not shown). In addition, the cell lysates and medium were eluted from a Poroshell 300SB-C8 reversed-phase column with increasing acetonitrile and analyzed using both UV and fluorescent monitors. Comparison was made to LC-OG that was not internalized. Elution profiles were compared with unbound Oregon Green, unconjugated LC, and LC-OG that was not added to cells. There was a shift to a less hydrophobic species in both the cell lysate and medium compared with the LC-OG purified from the day 0 cell lysate (Figure 10).

Although the LC-OG eluted with its major peak at 4 minutes (data not shown), the major peak of the cell lysate was eluted at 3.8 minutes at day 0 and at 3.4 minutes after 3 days. The shift to a less hydrophobic species was detected in the medium after 6 days. Immunogenicity was verified using Western blot analysis.

Cellular Response

Because amyloidogenic proteins are associated with cellular stress, we hypothesized that exposure to the AL-LC would induce responses that are characteristic of toxicity and cell death. Fibroblasts respond to the stress of the LC within 2 days using the thiazolyl blue (MTT) assay to determine mitochondrial function. The response was calculated as percent change of mitochondrial function compared to cells lacking LC. Cells were plated at the same seeding density and evaluated throughout 8 days. Previous experiments had shown that LC did not alter proliferation. There was an initial 25% increase greater than control for the first 2 days, which was followed by a negative 45% change by day 4.

As we demonstrated that the LC was internalized and secreted, we examined whether the proteasomal inhibitor (MG132) altered internalization or processing of the LC. In our cell culture system, when cells were preincubated with inhibitor there was a fourfold decrease in internalization compared with control (Figure 11A). To determine whether the inhibitor altered the retention of the LC, cells were incubated with LC until it was internalized and then incubated in the presence or absence of inhibitor. When cell lysates were immunoprecipitated with antibody to anti-OG and immunoblotted with anti-OG antibody, there was an increase in the retention of LC in the presence of inhibitor (Figure 11B). The increase detected with Western blot analysis was corroborated by confocal images of cells in the presence and absence of inhibitor (Figure 11C).

However, when the lysates were immunoblotted with anti-ubiquitin antibody, ubiquitin was not detected. Ubiquitin was detected only when total cell lysates were im-

munoblotted with the anti-ubiquitin antibody. Although ubiquitination of the LC was not detected, there was a collapse in actin stress filaments in the presence of inhibitor that was not detected in cells stained for microtubules or vimentin microfilaments (Figure 12). These data indicate that MG132 alters the processing of the LCs within the cell in a complex manner. Further studies will be performed to elucidate the role of the inhibitors on the LC.

Discussion

In this study, we demonstrate that cardiac fibroblasts internalize LC through bulk phase endocytosis. Although the pathology of amyloid disease has been evaluated extensively, the mechanism of internalization and subsequent cellular processing was not well understood. Our previous work demonstrated that exposure of cells to κ and λ LCs caused an increase in both the sulfation of secreted GAGs and the presence of unsulfated cytoplasmic heparan-sulfated proteoglycans. Furthermore, we showed that heparan sulfate associated with a κ 1-LC and caused an alteration in the thermal stability of the LC.⁴

Internalization of κ 1-LCs revealed that a reduction of temperature from 37 to 4°C or inhibition with M β CD reduced the rate and overall uptake. These results were not surprising because lowered temperature and M β CD are both known to reduce endocytosis and decrease membrane fluidity.^{37,38} However, disruption of lipid rafts with filipin did not alter internalization. The lack of disruption by filipin is consistent because it intercalates into cholesterol-rich lipid rafts without altering cholesterol composition and is further supported by the lack of co-localization of LC-OG with markers of clathrin-mediated endocytosis.^{39,40} This is supported by the absence of co-localization with additional membrane probes (FM4-64) and membrane-associated probes (WGA or FLAER), which have been used previously to demonstrate receptor-mediated endocytosis. In our system, there was co-localization of dextran and LC. These results indicate that different cell types such as mesangial cells may have distinct modes of internalization.⁹ Furthermore, when differential interference contrast images were superimposed on confocal images, the LC-OG488 was present within membrane vesicles but was not observed at the plasma membrane. Results from staining with FM4-64 were consistent with these conclusions. In addition, we detected neither aggregation nor pinching off of LC vesicles at the plasma membrane in contrast to groups that have detected receptor-mediated endocytosis with epidermal growth factor.⁴¹ Additional studies with fluoresceinated nonamyloidogenic proteins of similar mass demonstrated a similar internalization (data not shown). Together, these results suggest that internalization occurs via a nonspecific en-

Figure 8. Co-localization of κ 1-LC-OG488 with organelles. Cells were stained with organelle markers, incubated with LC-OG 488, and then imaged and co-localization determined. **A:** LC-OG and Lysotracker DND 99 (577). **B:** LC-OG and WGA 633 (to image Golgi). **C:** LC-OG and Mitotracker. **D:** LC-OG and ER tracker (587). **E:** LC-OG and ToPro. Scale bars = 20 μ m. $n = 3$.

docytotic mechanism in which the LCs remain in a fluid phase when internalized.

Our experimental results demonstrate that internalization is mediated by a number of factors including mass and concentration of the LCs. Although there was a significant decrease in uptake exhibited by an increase in

mass, there were only minor differences in the internalization of the three monomers, which may reflect structural differences in the LCs. In addition, there was no detectable saturation of internalization rate during the first 4 hours of the incubation. At later time points (24 hours), the rate of uptake plateaus, and we hypothesize that this

Figure 10. Change in hydrophobicity of LC-OG after internalization. Cardiac fibroblasts were grown to confluency in 100-mm² plates and exposed to LC-OG for 3 days. Samples were separated on a GF-250 and analyses performed on a Poroshell 300SB-C8 RP column using a linear gradient of 0 to 100% acetonitrile. Elution profiles were generated with fluorescence monitoring (excitation, 488; and emission, 530 nm). Early peaks represent void and unconjugated OG. LC-OG cell lysate at day 0 containing internalized LC (—), pulse of LC-OG in cell lysate after day 3 (---), and pulse chase of LC-OG after exogenous LC removed at day 3 and collected in medium from days 3 to 6 (- · -). *n* = 2.

may be attributable to secretion of the LC. These data agree with pinocytotic and secretory models,^{40,42–44} which hypothesize that internalization rate can be mediated by protein concentration in the fluid phase and adsorption to the membrane surface of each endocytosed vesicle.^{42,44} The models accurately reflect our data in that internalization correlates directly with the concentration of the protein in the surrounding medium and inversely to the mass of the LC (Figures 3 and 4). If the LC was induced to form aggregates and then added to the cells, internalization was not detected. Together, the data indicate that the internalization rate of the LC is not dependent on a finite number of membrane receptors but better fits a fluid phase endocytic model.⁴²

The internalization of LCs may provide an environment that is conducive to the formation of toxic oligomers or pores.⁴⁵ Formation is thought to be favored by high protein concentration, pH, and posttranslational modifications that favor protein destabilization.^{13,15} It has been hypothesized that when LCs are concentrated, they have the ability to form pores that can penetrate or disrupt membrane-bound vesicles.^{46,47} The passage of Alzheimer's A β amyloid protein into membranes was hypothesized to occur via channels.⁴⁸ Our *in vitro* work demonstrates the ability of a κ 1-LC protein to form such conformations in solution. Additional work is necessary to understand if pores form on cell membranes and under what conditions and to compare it to other morphologies that are detected in solution.⁴⁷ Shtilerman and colleagues¹⁵ demonstrated that annular α -synuclein protofibrils are produced in solution or bound to membrane surfaces. We hypothesize that the LCs that are internalized and subsequently released into the extracellular matrix may be modified to facilitate binding to GAGs and formation of Congo Red-positive amyloid fibrils.

Internalization was partially inhibited by disruption to the cytoarchitecture of the cells. The response of LC in the presence of cytochalasin D was monitored because the actin cytoskeleton plays a critical role in recycling associated with pinocytosis.⁴⁹ When cells were cultured

Figure 11. Proteasomal inhibitor alters internalization and retention of AL-LC in cardiac fibroblasts. **A:** MG132 retards rate of LC-OG internalization compared with control using live cell confocal imaging. Internalization was determined by calculating intracellular fluorescence intensity. **B:** MG132 caused retention of LC-OG in cell. Top: Cells cultured in the presence or absence of LC-OG. Cell lysates were immunoprecipitated with anti-OG and immunoblotted with anti-OG antibody. Cell lysate lacking LC-OG served as negative control. Cell lysates in the presence of MG132 showed increased LC-OG after immunoprecipitation and immunoblotting with anti-OG antibody. LC-OG alone was immunoblotted with anti-OG as positive control. Bottom: Confocal images of cells showing internalized LC-OG in the presence and absence of MG132. Ave SI, signal intensity. Scale bar = 20 μ m. *n* = 3.

with cytochalasin D and LC, there was a decrease in internalization as predicted by a pinocytotic model and supported by the co-localization with dextran. In contrast, microtubules do not seem to play a role in internalization. Instead, preliminary results indicate that microtubules may play a role in cellular trafficking. In our model, nocodazole inhibited the intracellular motility, as described by Goldman and colleagues,⁵⁰ and increased the retention of the LC, which resulted in large irregular clusters or aggregates. It has been hypothesized that LCs form aggregates in cells and that the aggregates are dispersed with nocodazole.^{48,51} In our system, the addition of nocodazole did not disperse the large aggregates of LC. Our data support a lysosomal pathway, rather than a

Figure 12. Proteasomal inhibitor alters actin staining. Cells were incubated in the presence of LC-OG and in the presence or absence of MG132. Cells were fixed in 4% paraformaldehyde and stained with rhodamine-phalloidin or probed for expression of tubulin and vimentin using indirect immunohistochemistry. Cells in presence of inhibitor display altered actin staining. Scale bar = 20 μ m. *n* = 3.

cytoplasmic proteasomal pathway implicated in aggregate formation. This is supported by experiments that demonstrate a concentration of LC in lysosomes throughout long-term experiments. The proteasomal pathway may be ultimately used if the LC departs from the cell through pores as suggested by investigators.^{23,52} Together these data indicate that internalization occurs via an active mechanism such as constitutive pinocytosis into an endosomal/lysosomal pathway mediated by microtubules.^{42,44}

The processing of the LC that occurs after internalization is of interest. When macrophages internalize serum amyloid A, the protein is intact initially; however, after 24 hours greater than 90% was degraded.⁶ Although we do not detect degradation in our model system, the amount of protein was low. In fact, there were minor changes in hydrophobicity, and more in-depth analyses will be examined using mass spectrometry.

To evaluate downstream processing pathways, studies were performed throughout time, and co-localization was detected at specific time points in lysosomes but not Golgi or ER. These data are distinct from other studies implicating the role of Golgi in intracellular trafficking of sorLA and the amyloid precursor protein.⁵³ Initially, we

proposed that there was co-localization to the Golgi; however, additional live cell stains demonstrated no localization to the ER and no localization with an antibody directed against the trans Golgi. A similar interpretation was made by Dul and colleagues.⁴⁸ In addition, co-localization with lysosomes increased throughout time which support observations of Teng and colleagues.⁹ In addition, Yang and colleagues^{23,52} have reported that lysosomal damage is an early event in amyloid A β pathogenesis. It is possible that a similar processing mechanism occurs in AL-amyloid. We did not detect any co-localization of LC with mitochondria using Mitotracker, even though it has been reported that amyloidogenic proteins increase the permeability of mitochondrial proteins.⁵⁴ However, there was a detectable change in mitochondrial function in response to LC.

Internalization and cellular trafficking of the LC was altered by the proteasomal inhibitor MG132, a classical competitive inhibitor of chymotrypsin-like activity. MG132 decreased the internalization rate of LC compared to controls. Interestingly, when the inhibitor was added to cells after exposure to LC, there was a decrease in secretion compared to LC alone. The decrease in secretion of LC was accompanied by a collapse in the F-actin filaments. Bortezomib, a modified dipeptidyl boronic acid with a similar structure to MG132, was used in parallel experiments and yielded similar results.^{26,27} We hypothesize that this may be attributable to a decrease in trafficking and processing of LC. Additional studies must be performed to understand the underlying mechanism because we did not detect association of LC with ubiquitin using either biochemical or immunohistochemical methodologies. Together, these data indicate that the LC mediates the cell's response to stress and that it can be altered by a number of pharmacological agents. The downstream responses may ultimately alter cellular homeostasis leading to cellular toxicity, alteration of matrix production, and LC fibrillogenesis.

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