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***In vitro* co-expression of human amyloidogenic immunoglobulin light and heavy chain proteins: a relevant cell-based model of AL amyloidosis**

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Abstract

Immunoglobulin (Ig) light chain (LC) amyloidosis (AL) is characterized by the overproduction and tissue deposition of monoclonal LC in various organs and tissues. The plasma circulating monoclonal LC is believed to be the precursor of the deposited protein and *in vitro* studies aimed at understanding AL pathobiology have mainly focused on LC and its variable domain. While 33% of patients have free circulating monoclonal LC, ~40% feature LC complexed to heavy chain (HC) forming a monoclonal intact Ig; the significance of free vs. bound LC in the amyloid forming pathway is unknown. To address this issue, we developed a cell-based model using stable mouse plasmacytoma Sp2/0 cells that co-express patient-derived amyloidogenic LC and HC proteins. The system was designed using amyloidogenic kappa and lambda LC, and gamma HC sequences; stable production and secretion of either free LC and/or intact Ig were accomplished by varying the LC to HC ratios. This novel cell-based system provides a relevant tool to systematically investigate LC and HC interactions, and the molecular events leading to the development of AL amyloidosis.

Keywords

AL amyloidosis; immunoglobulin light and heavy chains; plasma cell-based model; protein cloning; expression and secretion

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Introduction

Immunoglobulin (Ig) light chain (LC) amyloidosis (AL) is a plasma cell disorder characterized by the overproduction and tissue deposition of monoclonal Ig LC (IGL) and its fragments in various organs and tissues. The amyloid deposits contain highly organized, non-branching fibrils that are rich in cross- β -sheet structure and are thought to originate from aggregated forms of circulating monoclonal LC. Numerous studies have reported the presence of both monomeric full-length and C-terminally truncated forms of LC in ex vivo amyloid fibrils [1,2]. In addition, several reports have demonstrated that amyloid deposits can also contain either Ig heavy chain (HC) or HC fragments along with LCs [3–6]. The role of LCs as the major components of fibrillar deposits in AL amyloidosis is well established, yet the effect of HCs remains unclear. The ultimate goal of our research is to establish the effect of HC and its association with LC in the development of AL amyloidosis.

Normally, intra-cellular assembly of LC and HC leads to formation of competent Ig antibodies that are secreted into circulation. An intact Ig molecule is a heterotetramer assembled from two identical LCs and two HCs connected through disulphide bonds [7]. The assembly and secretion of intact Ig depend on a variety of factors including amino acid sequence, stoichiometric ratio and proper folding of LCs and HCs [8,9]. *In vitro* studies aimed at elucidating the molecular determinants of AL amyloidosis have been focused exclusively on LC and its fragments. However, clinical data indicate that circulating monoclonal free LC alone is found only in 33% of AL patients, whereas intact monoclonal Ig is present in 42% as evidenced by serum immunofixation electrophoresis (SIFE) [10]. Importantly, the nature of the monoclonal LC, circulating either in free form or in complex with HC, may be linked to survival as patients with no identifiable HC have a lower survival rate compared to those featuring HC [11]. The mechanisms underlying predominant generation of either free LC or monoclonal intact Ig in AL amyloidosis are not well defined. Amyloidogenesis could be related to specific LC and/or HC features including transcription and expression levels of LC and/or HC genes, sequence-specific differences in the intracellular assembly of Ig, variability in secretion of intact Ig vs. free LC, or differences in the structural stability and susceptibility to proteolysis between LC and HC proteins.

Previously reported cell-based studies have used patient-based plasma cell lines or HEK cells that secrete amyloidogenic free LC or LC variable fragments to analyse the role of intra- and extracellular events involved in the generation and aggregation of LC [12–14]. The aim of the current study was to create a relevant, stable cell-based model that could express intact Ig using various patient-derived amyloidogenic LC and HC sequences. Such a model system would provide a platform to systematically investigate intra- and extra-cellular factors affecting folding, stability and misfolding of amyloidogenic LC associated with HC. These important aspects of AL amyloidosis have not been addressed in previous studies.

Methods

Sample selection, clinical features and laboratory characteristics

Bone marrow aspirates, clinical information and laboratory data were obtained from the sample repository and patient database maintained by the Boston University Amyloidosis

Center. Informed consent for sample and data collection was obtained from all patients at presentation with the approval of the Boston University Medical Center Institutional Review Board.

For *in vitro* co-expression, immunoglobulin LC V (IGLV) genes were selected from the list of 304 amyloidogenic bone marrow derived IGL sequences that have been previously cloned in the Gerry Amyloidosis Research Laboratory. The following selection criteria were applied: (i) no treatment prior to initial evaluation; (ii) presence of intact IgG in circulation as indicated by SIFE; (iii) usage of either IGLV6–57 or IGKV1–39, the most common germline genes in the rearrangement of the amyloidogenic IGL [14]; and (iv) complete IGL J-C gene sequence coverage. After amyloidogenic IGL sequences were selected, the cloning and sequencing of paired IGH V-D-J-C genes were performed. Three samples met all selection criteria and were chosen for *in vitro* expression; these are designated as AL-222, AL-204 and AL-221. Of the three specimens, a population of CD138+ cells was available and used for AL-221, while unselected bone marrow plasma cells from AL-222 and AL-204 were utilized in the study. Table 1 lists the demographic, clinical and laboratory features of three AL patients with cloned IGL and IGH genes that were used for *in vitro* co-expression. All cases had low bone marrow plasma cell burden; two patients presented with an LC λ and one with an LC κ restriction. An intact IgG clonal band was identified on SIFE in each patient. Patient AL-221 had abnormal free LC ratio; patients AL-222 and AL-204 had abnormal IgG heavy light chain ratios. All patients featured renal involvement with nephrotic range proteinuria and elevated creatinine; none of the patients was dialysis dependent. Other involved organs included liver, autonomic neuropathy and soft tissue.

Cloning and sequencing of the patient-derived amyloidogenic Ig LC (IGL) and Ig HC (IGH) genes

Cloning and sequencing of the IGL genes was performed as previously described [15].

For IGH gene cloning, the bone marrow aspirate cells were treated with ammonium chloride to lyse red blood cells as detailed elsewhere [15]. Total RNA was extracted using Trizol reagent (Fisher, Waltham, MA), and complementary DNA was synthesized from mRNA using the iScript™ Select cDNA Synthesis kit with OligodT primers (Bio-Rad, Philadelphia, PA). A portion of the IGH gene, between the upstream framework region 1 (FR1) of the IGH variable region (IGHV) and the downstream IGH joining region (IGHJ), was PCR amplified using the IGH Somatic Hypermutation Assay v2.0 (In VivoScribe, San Diego, CA) with AmpliTaq Gold polymerase (Fisher, Waltham, MA). The resulting amplicon, approximately 350 bp in size, was purified from an agarose gel, sub-cloned and sequenced. The clonal sequence was determined by a consensus of at least 50% of 12 independently cloned and sequenced products. Sequences were compared using Jellyfish gene analysis software (Field Scientific, Lewisburg, PA). Of 12 IGH V-D-J clonal sequences obtained for each of the three bone marrow specimens, 85–100% were identical.

Once the IGHV gene was identified, nucleotide sequence errors introduced by FR1 primers were corrected by additional PCR amplification with 5′ primers for the appropriate IGHV leader region and a 3′ universal primer for all four IGG isotypes of the IGH constant region. Primer sequences are shown in Table 2. IGH genes with the corrected FR1 sequence were

evaluated for homology to the corresponding germline donor sequences using the International Immunogenetics Information system (IMGT/V-QUEST, <http://imgt.cines.fr>) [16]. The assignment of the germline gene counterpart was based on the maximal homology of the nucleotide sequences. Homology with germline sequence was determined for each gene used in the rearrangement of IGH and a single monoclonal IGH sequence in every case was confirmed. As expected, somatic mutations were found in all IGH V-D-J sequences; all IGHC genes were found to be of the IGG1 isotype. The V-(D)-J-C genes, used in rearrangement of the monoclonal IGL and IGH in three cloned cases, are shown in Table 3. All IGL and IGH sequences were submitted to the GenBank database with the following accession numbers: KY432415 (AL-222H), KY432416 (AL-222L), KY432414 (AL-204H), EU599347 (AL-204L), KY432408 (AL-221H) and KY432409 (AL-221L).

Cloning of recombinant IGH and IGL

Two plasmids, containing the mammalian vectors pMAZ-IGL to express human κ LC and pMAZ-IGH to express human IgG1 HC (kindly provided by Prof. I. Benhar, Tel-Aviv University, Israel), were used to produce human IgG1 antibodies in mammalian cell culture. Vectors are described in detail elsewhere [17]. Briefly, each vector carried the germline constant region sequences for the respective LC and HC chain genes. To enable double drug selection for stable transfectants, the hygromycin B restriction cassette was incorporated into the pMAZ-IGL plasmid and the neomycin expression cassette for G418 selection was incorporated into the pMAZ-IGH plasmid.

Sample-derived cDNA from each of the three bone marrow specimens was used as a template to amplify previously cloned LC and HC genes for sub-cloning into the pMAZ-IGL and pMAZ-IGH vectors. Flanking restriction sites were introduced at the 5' and 3' ends of the corresponding amplicons by employing PCR with sequence-specific primers listed in Table 4. Specific methods for individual samples are detailed in the sections which follow.

AL-222: The full-length LC-encoding DNA fragment, IGL V-J-C, which contained *Bss*HII and *Xba*I flanking restriction sites, was amplified for sub-cloning into the pMAZ-IGL vector. The PCR-amplified IGH V-D-J genes containing flanking restriction sites for *Bss*HII and *Nhe*I were sub-cloned into the pMAZ-IGH vector. The IGL V-J-C sequence was digested with *Bss*HII and *Xba*I restriction endonucleases and ligated into the purified pMAZ-IGL vector cleaved with *Bss*HII and *Xba*I. The IGH V-D-J sequence was digested with *Bss*HII and *Nhe*I restriction endonucleases and ligated into the purified pMAZ-IGH vector cleaved with *Bss*HII and *Nhe*I. The constructs were verified by DNA sequencing. The resulting plasmids were termed AL-222L/pMAZ-IGL and AL-222H/pMAZ-IGH.

AL-204: A procedure similar to that used for the AL-222 was employed with IGL V-J-C and IGH V-D-J sequences to generate plasmids AL-204L/pMAZ-IGL and AL-204H/pMAZ-IGH.

AL-221: IGK V-J and IGH V-D-J genes were amplified in two separate PCR reactions. *Bss*HII and *Bs*WI restriction sites were incorporated into IGK V-J, and *Bss*HII and *Nhe*I restriction sites were incorporated into IGH V-D-J amplicons. The DNA encoding IGK V-J

was cleaved with *Bss*HII and *Bs*WI restriction endonucleases and was ligated into the purified pMAZ-IGL vector that was enzymatically cleaved with *Bss*HII and *Bs*WI. The DNA encoding IGH V-D-J was digested with *Bss*HII and *Nhe*I restriction endonucleases and was ligated into the purified pMAZ-IGH vector cleaved with *Bss*HII and *Nhe*I. The constructs were verified by DNA sequencing. The resulting plasmids were termed AL-221L/pMAZ-IGL and AL-221H/pMAZ-IGH.

Nucleofection of Sp2/0 cells with AL LC/pMAZ-IGL and AL HC/pMAZ-IGH expression vectors

The mouse plasmacytoma Sp2/0 cell line, which does not express endogenous Ig, was selected for use to generate a stable source of amyloidogenic LC and HC expression. Plasmids AL LC/pMAZ-IGL and AL HC/pMAZ-IGH were linearized with *Xba*I, an enzyme used to prevent disruption of the gene of interest during integration. Nucleofection was performed using the Amaxa Nucleofector I apparatus (Amaxa Inc., Gaithersburg, MD) according to manufacturer's protocol. Briefly, 5×10^6 cells per sample were cultured in Hybridoma-SFM media (Gibco, Carlsbad, CA) supplemented with penicillin/streptomycin and 5% foetal bovine serum (FBS, Aleken Biologicals, Nash, TX). The cells were pelleted, resuspended in 100 μ L Nucleofector Solution (Lonza, Allendale, NJ), and mixed with 5 μ g of linearized DNA. An AL LC/pMAZ-IGL to AL HC/pMAZ-IGH plasmid ratio of 3:2 was selected for the co-nucleofection. The cell suspension was placed into an Amaxa-certified cuvette, nucleofected in the Amaxa apparatus using program A-27, mixed immediately with 500 μ L of pre-incubated media, and transferred into a 12-well plate for incubation at 37 °C and 5% CO₂ for 24 h. Cell cultures were subsequently transferred into a six-well plate, incubated for 24 h, and then placed into small flasks containing antibiotics for cell selection. To select for LC-expressing cells, we used 0.2mg/mL of hygromycin; for HC-expressing cells, 0.8mg/mL of G418 was used.

IgG expression in Sp2/0 cells

To obtain a monoclonal cell culture, colonies from single stably-transformed Sp2/0 cells were selected by serial dilution. Culture media were gradually depleted of FBS over the period of two cell passages to avoid interference from bovine Ig in the detection of expressed human Ig. Selected cell cultures were grown in serum-free medium (Hybridoma-SFM, Gibco, Carlsbad, CA) at 37 °C and 5% CO₂ for 24, 48 or 72 h; supernatants were tested for LC, HC and IgG protein expression and secretion by immunoblot analysis. Briefly, proteins were separated by 12% SDS PAGE under non-reducing conditions and transferred to a nitrocellulose membrane. For LC detection, primary antibodies included polyclonal rabbit anti-human Ig λ LC antibodies or polyclonal rabbit anti-human Ig κ LC antibodies (Bethyl Laboratories, Inc., Montgomery, TX). For HC detection, polyclonal rabbit anti-human IgG antibodies (DAKO, Carpinteria, CA) were used. Subsequently, membranes were probed with HRP-conjugated goat anti-rabbit polyclonal IgG and were developed using an enhanced chemiluminescence system, ECL (Thermo Scientific, Waltham, MA).

Cell viability was assessed at 24, 48 and 72 h by performing an MTT assay. A 96-well plate was used to seed 2.5×10^5 cells in 100 μ L of Hybridoma-SFM into each well, and 20 μ L of a 5 mg/mL MTT stock solution was added to each culture. After 3–4 h of incubation at

37 °C and 5% CO₂, supernatants were removed and substituted with 100 µL of DMSO for cell solubilization. After another hour of incubation at 37 °C and 5% CO₂, cell viability was determined spectroscopically on an ELx405 plate reader (BioTek, Winooski, VT) by measuring the difference in the optical density, $OD = OD_{570nm} - OD_{690nm}$.

Purification of IgG from cell culture supernatant using protein A agarose resin

Sp2/0 cells stably expressing monoclonal IgG1 comprised of LC and HC corresponding to each of three samples were cultured in Hybridoma-SFM media (Gibco, Carlsbad, CA) supplemented with hygromycin (0.2 mg/mL) and G418 (0.8 mg/mL) at 37 °C and 5% CO₂ for 72 h. Secreted intact IgG1 was purified from the cell supernatant on a Protein A Agarose (Abcam, Cambridge, MA) column according to manufacturer's recommendations. IgG1 was eluted with 0.1 M citric acid at pH 3 and the solution was neutralized to pH 7 with 1.5 M Tris. Serum-free media were used to allow visualization of LC and Ig production in cell cultures by immunoblotting. Secreted proteins were separated by 8-16% SDS-PAGE performed under non-reducing conditions and visualized by immunoblotting; rabbit anti-human polyclonal κ or λ LC antibody (Bethyl Laboratories, Inc., Montgomery, TX) or rabbit anti-human IgG (DAKO, Carpinteria, CA) was used as the primary antibody, and HRP-conjugated goat anti-rabbit IgG as a secondary antibody. Membranes were developed using an ECL system.

RNA preparation from cell cultures and cDNA synthesis

Each Sp2/0 cell line was cultured in Hybridoma-SFM media (Gibco, Carlsbad, CA) supplemented with 5% FBS, in three passages. Total RNA was purified from 5×10^6 cells of each culture with RNeasy Plus Mini kit (Qiagen, Hilden, Germany) using a robotic workstation QIAcube (Qiagen, Hilden, Germany). RNA was quantified spectroscopically at 260 nm and 500 ng of RNA was used for cDNA synthesis. The QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) was used to eliminate genomic DNA prior to the reverse transcription reaction, which yielded 500 ng of cDNA (25 ng/µL concentration).

Quantitative analysis of LC and HC gene expression by qPCR

LC and HC copy numbers in mRNA samples derived from bone marrow specimens and the corresponding cultured cell lines were determined by quantitative real-time PCR using the SYBR Green PCR Master Mix system (Applied Biosystems, Foster City, CA). PCR product formation was detected by monitoring the fluorescence of SYBR Green, which occurred upon dye binding to double-stranded DNA. Human or murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control to normalize sample-to-sample variations. Table 5 lists individual primers that were designed to amplify IGL V-J and IGH V-D-J genes for each specimen by using OligoPerfect Designer software (Invitrogen/Thermo Fisher Scientific, Inc., Waltham, MA). The primer specificity was verified by the presence of a single peak in the melting curve of the realtime PCR product, and by the presence of the appropriately sized DNA amplicons on agarose gel electrophoresis. PCR reactions were performed in a CFX96 Real-Time System thermocycler (Bio-Rad, Hercules, CA).

Each 15 μ L of the PCR reaction mixture contained 6.25 ng of DNA, 3.75 pmol of forward and 3.75 pmol of reverse primers, and 2 $\times$ SYBR Green Master Mix. The mixtures were denatured upon initial incubation at 90 $^{\circ}$ C for 10 min, followed by incubation at 90 $^{\circ}$ C for 15 s and at 60 $^{\circ}$ C for 1 min; the last two steps were repeated 50 times. All samples were analysed in triplicates, and all experiments were independently performed twice, for a total of six replicates. Statistical analysis was performed using EXCEL software.

Human GAPDH gene was used to normalize the LC and HC mRNA expression in the bone marrow-derived specimens, and the mouse GAPDH gene was used as a housekeeping gene for the Sp2/0 cell derived samples. The human and mouse GAPDH plasmids were purchased from Sino Biological Inc. (Beijing, China). Quantification of LC, HC and GAPDH gene expression was accomplished using an absolute standard curve method. Standard curves were generated for each transcript from a dilution series of synthesized plasmid cDNA standards as indicated in Table 5.

Results

Expression of IGL and IGH genes from patient-derived bone marrow samples

The appropriate genes used in rearrangements of IGL and IGH were identified, and bone marrow-derived mRNA was used to assess IGL and IGH gene expression. The IGL and IGH transcript expression ratios in the three specimens are shown in Table 6. Levels of IGL expression exceeded those of IGH expression in all cases.

Cell-based model co-expressing amyloidogenic LC and HC proteins *in vitro*

Selected stable cell lines with co-transfected recombinant sample-derived gene sequences showed expression of LC and HC proteins. To optimize protein expression, a time-dependent increase in the secretion of LC (Figure 1(A)) and HC (data not shown) was assessed by immunoblotting; continuous cell proliferation was also observed at the selected time intervals (Figure 1(B)). On the basis of these observations, the incubation conditions at 37 $^{\circ}$ C for 72 h with subsequent culture harvesting were selected for analyses. For each reported cell model system corresponding to a particular AL patient, several clones were generated: eight clones for AL-222, seven clones for AL-204 and eight clones for AL-221. Examples of selected clones for each of the three cell lines are shown in Figure 2. IGL and IGH gene expressions were measured using mRNA derived from cultured cell lines. The LC transcript level was more abundant compared to that of the HC in all cell lines. Individual clones showed various LC to HC gene expression ratios ranging from 2 to 24, which roughly reflected the relative amounts of secreted proteins.

The expression levels of LC and HC could be potentially affected by the incorporation of the transfected plasmids into various parts of the gene. Therefore, to express and purify IgG, we selected the cell line that exhibited an LC to HC gene expression ratio closest to that measured in the corresponding bone marrow-derived cDNA specimen. LC and HC with sequences specific to those identified in AL-222 were expressed, assembled into an IgG1 molecule, and secreted as an intact Ig heterotetramer; the latter was demonstrated by SDS-PAGE and immunoblotting (Figure 3). Similar profiles were obtained for AL-204 and

AL-221 cell lines (data not shown). Along with the intact Ig, free LC was also secreted by the cell system; SDS-PAGE and immunoblot of cell supernatant showed LC present as dimers and monomers (Figure 3), reflecting the presence of these protein species in the patients' serum.

Discussion

The current study describes the first cell-based model that co-expresses patient-specific amyloidogenic LC and HC, and competently secretes intact IgG1 molecule. For *in vitro* expression, we have focused on IGLV6–57 and IGKV1–39, the most common genes used in the rearrangement of IGL in AL amyloidosis [14]. To generate this model, we chose a mouse plasmacytoma Sp2/0 cell line that is particularly relevant to AL amyloidosis, the disorder where native monoclonal Ig protein is produced by clonal plasma cells residing in the bone marrow. Since Sp2/0 cells do not express or secrete endogenous Ig, our cell model mimics expression and secretion of monoclonal proteins by clonal bone marrow plasma cells. This model can be used to represent the wide variety of LC and HC sequences featured in patients with AL amyloidosis.

Another novel aspect of the current study is that we have measured the LC to HC gene expression ratios in the patient-derived bone marrow specimens and showed that in each sample the expression levels of LC exceed those of the HC by a factor of 1.7–3.8 (Table 6). To our knowledge, relative gene expression levels of LC to HC in AL patients or in healthy subjects have not been previously reported. A previous study that used protein urea concentrations as a proxy for secretion ratios in healthy subjects reported that 60% of LC was secreted into the serum as intact Ig, while 40% was secreted as free LC [18]. However, levels of protein secretion could be affected by multiple factors including, but not limited to, protein expression [19]. A range of LC to HC gene expression ratios measured in the current work provides a direct reference for future studies of the effects of the relative gene expression on the secretion of either amyloidogenic LC or intact Ig by clonal plasma cells in AL amyloidosis.

We envision several other applications for our cell system in future studies of AL amyloidosis. This system can be used to determine how specific LC and HC sequences influence the assembly and secretion of amyloidogenic proteins. In fact, previous cell-based studies, which have been focused on the LC with sequences representative of multiple myeloma, have shown that single point mutations in the LC variable region may block the expression of LC and secretion of intact Ig by the cells [8,20]. These findings suggest that multiple factors, such as the amino acid sequence, stoichiometric ratio and proper folding of both LC and HC determine the secretion of intact Ig [21]. Our cell-based model enables one to identify and systematically analyse the roles of these important factors in LC and Ig secretion, and thereby distinguish the role of free vs. complexed LC in the generation and ultimate deposition of LC in AL amyloidosis. Furthermore, our system could be used to study the effects of drugs on Ig homeostasis; to avoid potential effects of nutrient starvation on plasma cell biology and Ig expression [22], such studies should be carried out in serum-enriched media followed by proteomic profiling using mass-spectrometry.

This cell-based model can also be used to express various patient-derived and model LC and HC sequences in different combinations to generate a wide array of recombinant Ig molecules representative of AL amyloidosis. The proteins generated in this manner can be used in structural and aggregation assays, e.g. to determine the sequence-specific effects of LC and HC interactions on the fibrillation of LC protein. In addition, the intact Ig generated by our cell system can be used to determine the role of thermodynamic and kinetic structural stability in protein misfolding. Previous studies by our group and others have established the kinetic stability of LC alone and its likely role in protein misfolding in AL [2,23]. The model system described in this report enables us to extend these studies to intact Ig, and thereby determine the potential role of kinetic barriers in the misfolding of both LC and HC in AL amyloidosis.

In summary, we have developed a cell-based model using stable mouse plasmacytoma Sp2/0 cells that co-express patient-derived amyloidogenic LC and HC proteins. Results from our studies demonstrate that these stably transfected cells produce and secrete intact human amyloidogenic IgG1 comprised of monoclonal LC and HC mimicking those expressed in the bone marrow of patients with AL amyloidosis.

While this paper was under review, a new cell-based study was published wherein cultured patient-derived plasma cells have been used to reveal that the production of amyloidogenic LC is a cell stressor and to identify stress-response pathways as potential therapeutic targets for AL [24].

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Abbreviations

AL immunoglobulin light chain amyloid protein

ECL	enhanced chemiluminescence
FBS	foetal bovine serum
FR1	framework region 1
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
HC	heavy chain
Ig	immunoglobulin
IGH	immunoglobulin heavy chain
IGHV	immunoglobulin heavy chain variable region
IGHJ	immunoglobulin heavy chain joining region
IGL	immunoglobulin light chain
IGLV	immunoglobulin light chain variable region
IGL V-J-C	immunoglobulin light chain variable-joining-constant region
IGH V-D-J-C	immunoglobulin heavy chain variable-diversity-joining-constant region
LC	light chain
MTT	3-(4,5-dimethylthiazol-2yl) –2,5-diphenyltetrazolium bromide
SFM	serum-free media
SIFE	serum immunofixation electrophoresis

Figure 1. Optimization of the cell system for expression of IgG with amyloidogenic sequences specific for patients 1 (AL-222), 2 (AL-204) and 3 (AL-221). (A) SDS-PAGE and immunoblot of spent media shows secretion of monomeric and dimeric LC at indicated time points. (B) Cell viability at indicated time points was assessed in an MTT assay.

Figure 2.

Selected monoclonal Sp2/0 cell lines for expression of IgG with amyloidogenic sequences specific for patients 1 (AL-222), 2 (AL-204) and 3 (AL-221). Three clones for each patients' cell line are shown: immunoblots with (A) anti-human λ LC (for patients 1 and 2) and anti-human κ LC (for patient 3), and (B) anti-human IgG.

Figure 3.

Intact human amyloidogenic IgG1 protein expressed in Sp2/0 cell system. The protein specific to AL-222 patient was purified using protein A agarose column. After incubation at 37 °C for 72 h, the media were applied to the protein A agarose column to isolate IgG1 protein, followed by SDS-PAGE and immunoblotting. Immunoblots with (A) anti-human λ LC and (B) anti-human IgG are shown.

Table 1

Demographic, clinical and laboratory characteristics of three patients with AL amyloidosis at initial evaluation.

Characteristics	AL-222	AL-204	AL-221	Reference range
Age, years	71	70	67	
Age at death, years	78	78	68	
Bone marrow plasma cells, %	5	5	10	
LC restriction	λ	λ	κ	
SIFE	IgG	IgG	IgG	
FLC κ	26.3	14.5	111	3.3–19.4
FLC λ	82	30.9	37.8	5.7–26.3
FLCr	0.32	0.47	2.9	0.26–1.64
IgG HLC κ	1.69	2.70	8.57	3.84–12.07
IgG HLC λ	8.26	12.91	3.55	1.91–6.74
IgG HLCr	0.20	0.21	2.41	1.12–3.21
Affected organs				
Kidney				
Serum creatinine, mg/dL	3.2	2.9	2.3	0.7–1.3
24-h urine protein, mg	8296	2554	6095	
Soft tissue	–	–	+	
Liver	–	+	–	
Alkaline phosphatase, U/L	87	234	80	25–100
Autonomic neuropathy	–	+	–	
Treatment prior to evaluation	–	–	–	

LC: light chain; SIFE: serum immunofixation electrophoresis; FLC: free light chain; FLCr: free light chain ratio; Ig: immunoglobulin; HLC: heavy light chain.

Table 2

Primers used for cloning of the rearranged monoclonal IGH V-D-J-C genes from three AL bone marrow specimens.

Access code	Forward primer sequence	Reverse primer sequence
AL-222H	5'-ATGGAGTTTGGGCTGAGCTGG	5'-CGAGAGCCCGGGGAGCGGGG
AL-204H		
AL-221H	5'-ACCTGGAGGTTCTTGTGGT	5'-CGAGAGCCCGGGGAGCGGGG

Table 3

V-(D)-J-C genes used in the rearrangement of monoclonal IGL and IGH in three AL bone marrow specimens.

Access code	IGL genes	IGH genes
AL-222	IGLV6-57 – IGLJ2 – IGLC2	IGHV3-30 – IGHD3-3 – IGHJ6 – IGHG1
AL-204	IGLV6-57 – IGLJ3 – IGLC3	IGHV3-15 – IGHD4-23 – IGHJ5 – IGHG1
AL-221	IGKV1-39 – IGKJ2 – IGKC	IGHV1-69 – IGHD5-24 – IGHJ4 – IGHG1

Table 4

Primer sequences used for amplification of LC and HC genes for sub-cloning into pMAZ/IgL and pMAZ/IgH vectors.

Access code	DNA domain		Primer sequence ^a
AL-222	IGLλ6	Forward	5'-TCCACAGGCGCGCACTCCAATTTCTGCTGACTCAGCCC
		Reverse	5'-GGGCCCTCTAGATTATTATGAACATTCTGTAGGGGCCA
	IGHV	Forward	5'-TCCACAGGCGCGCACTCCCAGGTGCAGCTGGTGGAGT
		Reverse	5'-ATATATGCTAGCTGAGGAGACGGTGACCGTG
AL-204	IGLλ6	Forward	5'-TCCACAGGCGCGCACTCCAATTTTATGCTGACTCAACCGC
		Reverse	5'-GGGCCCTCTAGATTATTATGAACATTCTGTAGGGGCCA
	IGHV	Forward	5'-TCCACAGGCGCGCACTCCGAGGTGCAGCTGGTGGAGT
		Reverse	5'-ATATATGCTAGCTGAGGAGACGGTGACCAGG
AL-221	IGLVκ1	Forward	5'-TCCACAGGCGCGCACTCCGACATCCTGATGACCCAGTCT
		Reverse	5'-ATATATCGTACGTTTGAAATCCACCTTGGTCC
	IGHV	Forward	5'-TCCACAGGCGCGCACTCCCAGGTCCGGCTGGTGCAAT
		Reverse	5'-ATATATGCTAGCTGAGGACACGGTGACCAGGG

^aPrimer-introduced restriction sites are underlined.

Table 5

Plasmid DNA and corresponding primer sequences used in qPCR analyses.

Target gene	Plasmid DNA	Forward primer	Reverse primer
Mouse GAPDH	pUC19-mGAPDH	5'-GGTGAAGGTCGGTGTGAACG	5'-CTCGCTCCTGGAAGATGGTG
Human GAPDH	pGEM-hGAPDH	5'-CGAGATCCCTCCAAAATCAA	5'-TGTGGTCATGAGTCCTTCCA
AL-222L	AL-222L/pMAZ-IgL	5'-CCCACTCTGTGTCGGAGTCT	5'-AGCCTCGTCCTCAGTCTTCA
AL-222H	AL-222H/pMAZ-IgH	5'-GCTGGATTCAGTTCAGTGAT	5'-CCCCAGACGTCCATAGTGTT
AL-204L	AL-204L/pMAZ-IgL	5'-CTGACTCAACCGCACTCTGT	5'-CAGCCTCGTCCTCTGTCTTC
AL-204H	AL-204H/pMAZ-IgH	5'-TGTGCAGCCTCTGGTTTTC	5'-CCCCAATCACCCATCATAGT
AL-221L	AL-221L/pMAZ-IgL	5'-AGTCTCCACCCTCCCTGTCT	5'-GGGGGATTGTAAGTCTGTTGA
AL-221H	AL-221H/pMAZ-IgH	5'-ATCTGGGGCTGAGGTGAAG	5'-TACATTGCCGTGTCCTGAGA

Table 6

Ratios of LC to HC transcript expression levels in three bone marrow-derived plasma cell mRNA.

Patient	Access code	IGL:IGH ^a
1	AL-222	17 ± 0.2
2	AL-204	3.8 ± 0.5
3	AL-221	2.2 ± 0.3

^aIGL:IGH expression ratios are presented as a mean of six independent measurements ± STD.