Myeloma light chains induce epithelial-mesenchymal transition in human renal proximal tubule epithelial cells

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Abstract

Background. To determine the role of epithelial–mesenchymal transition (EMT) as a potential mechanism contributing to the characteristic tubulointerstitial renal fibrosis in multiple myeloma, we examined whether myeloma light chains (LCs) directly induce EMT in human renal proximal tubule epithelial cells (PTECs).

Methods. As positive controls we used TGF-β1 and cyclosporine A (CsA), two agents known to induce EMT in PTECs. Human LCs were isolated and purified from the urine of myeloma patients with modest renal insufficiency without evidence of glomerular involvement. HK-2 cells were exposed to kappa LC (25 μM) for periods up to 72 h.

Results. LCs induced marked cellular morphological alterations in PTECs, accompanied with increased expression levels of profibrotic TGF-β1, FSP-1 and extracellular matrix components. Using semiquantitative immunoblotting and RT-PCR, we observed that the expression of E-cadherin decreased after 24 h, while the expression of α-SMA increased in PTEC after continuous exposure to κ-LCs. Human serum albumin (HSA; 160 μM) had less potent effect on the expression of EMT-related molecules. Neutralizing TGF-β1 antibody blocked CsA-induced EMT but had no effect on LC-exposed cells. LC-induced EMT and the secretions of IL-6 and MCP-1 were, however, markedly attenuated by p38 MAPK interference. The use of bone morphogenetic protein-7 or pituitary adenylate cyclase-activating polypeptide (PACAP) induced the formation of cell aggregates, and the reacquisition of E-cadherin expression and renal proximal tubule epithelial morphology within the confluent cell monolayer during and after LC exposure.

Conclusions. These findings demonstrate that LC is a direct stimulus for EMT in PTECs. LC-induced EMT involved multiple cytokines, is modulated by p38 MAPK, but appeared independent of the action of TGF-β1. LC-induced EMT may be an important mechanism of kidney injury associated with myeloma and may be reversible upon the administration of exogenous PACAP.

Keywords: cytokines; MAPK; myeloma kidney; PACAP; renal fibrosis; TGF-β1

Introduction

Kidney damage from multiple myeloma is an important cause of renal failure and carries a poor prognosis [1]. More than 50% of patients with myeloma will experience impairment of renal function at sometime during their illness [2,3]. Despite the use of conventional and high-dose chemotherapies for treating the underlying malignancy, the results of specific therapies to prevent or reverse myeloma nephropathy have been disappointing and the results of corticosteroid therapy remain modest at best [4].

The characteristic renal histologic lesion in patients with multiple myeloma, myeloma kidney, is a chronic tubulointerstitial nephropathy with marked tubular atrophy, laminaed intratubular casts and extensive interstitial fibrosis suggesting that like many other kidney diseases, renal fibrogenesis is a major mechanism of kidney impairment in multiple myeloma [5–8]. We previously reported that proximal tubular endocytosis of immunoglobulin light chains (LCs) causes cellular protein overloading and activates a sequence of inflammatory processes through nuclear transcription factors, nuclear factor kappa B (NFκB) and AP-1 (c-fos and c-jun) complexes. These transcription factors induce the synthesis of inflammatory cytokines and activate signalling pathways, such as mitogen-activated protein kinases (MAPK), extracellular signal regulated kinase 1/2 (ERK1/2), Jun kinase (JNK) and p38 MAPK, thus identifying potential mechanisms for interstitial inflammation and fibrosis in myeloma kidney [9–12].

Interstitial fibroblast activation is believed to play a central role in the pathogenesis of renal interstitial fibrosis [13–15]. Although the exact origins of these myofibroblasts remain uncertain, recent evidence suggests a significant fraction may be derived from tubular epithelial cells by an epithelial-to-mesenchymal transition (EMT) process under pathological conditions [16–20]. EMT in renal fibrosis is a form of reverse embryogenesis, when highly specialized...
tubular epithelial cells will respond to injury with losing their epithelial characteristics and functions and regaining characteristics of the cells from which they originated. Renal EMT has recently emerged as a major mechanism of renal fibrogenesis in various models of kidney diseases, but its role in myeloma kidney has not been examined. Here we performed studies to test whether the nephrotoxic effects of LCs could be in part due to the induction of EMT, possibly through LC-induced up-regulation of proinflammatory cytokines. Our findings show in vitro experimental evidence that LCs can directly induce EMT in human renal proximal tubule epithelial cells (PTECs) and may potentially be responsible for renal fibrosis in myeloma.

Subjects and materials

Cell cultures

HK-2 human renal PTECs were purchased from the American Type Culture Collection (Rockville, MD) and were maintained in Gibco Keratinocyte-Serum-Free Medium supplemented with 5 ng/ml recombinant EGF and 0.05 mg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA). These cells are not passaged forward beyond about 25–30 passages and were routinely cultured at 37 °C in a humidified atmosphere of 95% air–5% CO₂ and nourished at intervals of 3–4 days. At desired level of confluence (~80%) as the experimental protocol requires, the culture medium is aspirated, the cultures are rinsed with phosphate-buffered saline (PBS), the cells are removed by trypsin/ethylene-diamine tetraacetic acid digestion and re-seeded into 6-well or 12-well plates for experiments.

Isolation and purification of LCs

The LCs used in these experiments were isolated and purified from the urines of multiple myeloma patients with myeloma kidney as described previously [21,22]. Only urines with minimal or no albuminuria were selected for isolation, thus excluding patients with significant glomerulopathy. Nine species of LCs, six κ and three λ, were from eight male and one female patients aged 55–79 and serum creatinine values ranging between 1.5 and 3.9 mg/dl. Thus, as they are obtained from patients without albuminuria all the LCs used here must be considered ‘tubulopathic’. The LCs used in the present study are previously shown to undergo endocytosis by PTECs [21], bind to cubilin [22], and megalin [23], and induce cytokines through phosphorylation of MAPKs and activation of NFkB [11,12]. The purity of LCs is confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the immunologic identity reported from the clinical laboratory is confirmed by western blotting using goat anti-human κ and λ antibodies (Sigma, Saint Louis, MO). LC preparations were tested for endotoxin using the chromogenic Limulus amebocyte lysate test (Charles River Laboratories, Charleston, SC), and found that the LCs used in these experiments are essentially endotoxin free (<3.7 EU/mg). Furthermore, the LC preparations did not contain detectable quantities of interleukin (IL)-1, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 or tumour necrosis factor (TNF)-α.

Cell treatment, viability and preparation of cell lysates

LCs were prepared as a stock solution (1 mM) by dissolving 250 mg of the lyophilized powder in 10 ml serum-free base media and sterilized by 0.2 µm filter. LC concentration (25 µM) in this study was chosen to approximate the concentration range reached in the glomerular ultrafiltrate in patients with modest proteinuria and was previously demonstrated to elicit cytokine responses in human PTEC in vitro [11]. Cell viability was measured by CellTiter 96 Aqueous One solution Cell Proliferation Assay (Promega, Madison, WI). For neutralizing antibody studies, the cells were pre-treated for 1 h prior to the addition of LCs and subsequently co-treated with a monoclonal anti-transforming growth factor β1 (TGF-β1) antibody (R&D Systems, Minneapolis, MN) at a concentration of 30 µg/ml for 8–72 h [24]. To evaluate the transition of PTECs to fibroblasts, we used HK-2 cells cultured in supplement-free fresh media, in the presence or absence of various treatments. Treatment with recombinant human bone morphogenetic protein-7 (BMP-7) (R&D Systems) or synthetic pituitary adenylate cyclase-activating polypeptide (PACAP38) (American Peptide Co., Sunnyvale, CA) was started with κ-LC for 48 h or for 60 h after 48 h LC pre-treatment. After exposure to the TGF-β1, cyclosporine A (CsA), κ-LC, human serum albumin (HSA) or control medium for 8–72 h, culture supernatants were harvested and stored at −70°C for cytokine assays and kinase analysis. The media were removed, the cellular proteins were extracted by lysing the cells with Mammalian Cell Lysis Reagents (Sigma). Protein concentration in the cellular lysates was determined by the Bradford method using DC protein assay reagents (Bio-Rad, Hercules, CA).

Western blot analysis

Protein extracts (20 µg) were separated by 12.5% SDS-PAGE, and the separated proteins were electrophoretically transferred onto a nitrocellulose membrane. Non-specific background was blocked by incubating the membrane in Tris buffered saline-0.1% Tween-20 (TBST) buffer containing 5% skim milk overnight at 4°C. The membrane was then incubated for 1 h at room temperature with an appropriate dilution of the primary antibody (1:500 monoclonal anti-E-cadherin [G-10, Santa Cruz Biotechnology, Santa Cruz, CA], 4.2 µg/ml monoclonal anti-actin, α smooth muscle (α-SMA) [clone 1A4, Sigma] or 0.9 µg/ml monoclonal anti-actin [clone AC-40, Sigma]). After four washes in TBST buffer, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare, Piscataway, NJ), and the antibody complexes were visualized by the Amersham ECL detection system (GE Healthcare) as directed by the manufacturer.

Cytokine immunoassays

A sensitive immunometric assay was adopted for the quantification of human TGF-β1, IL-6, MCP-1 and soluble...
E-cadherin using 96-well plate colorimetric formats. The assays were performed to measure cytokines from cell culture media. The levels of human-activated TGF-β1, IL-6, MCP-1 and sE-cadherin in the media of human renal HK-2 cell cultures, in the presence or absence of various treatments, were measured using a commercial human sandwich enzyme immunoassay ELISA kit (Quantikin, R&D Systems). The absorbance of each well was read on a Dynex Opsys MR microtitrator plate reader (Dynex Technologies, Chantilly, VA) and unknown samples determined from the plotted standard curve. All experiments were performed in quadruplicate.

RNA extraction and reverse transcription polymerase chain reaction

Quantification of gene-specific mRNA for E-cadherin, α-SMA, TGF-β1 and fibroblast-specific protein-1 (FSP-1) as well as extracellular matrix (ECM) components collagen III and fibronectin expression in HK-2 cells cultured in the presence of various treatments was performed by RT-PCR analysis. Total RNA was purified from the cells by extraction with the RNeasy Mini kit (Qiagen, Valencia, CA). One-half micrograms of total RNA from each sample was used for RT-PCR, which was performed by SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen). The pairs of primers used for amplification of human E-cadherin, α-SMA, FSP-1, fibronectin and collagen III were designed with the MacVector program (MacVector Inc., Cary, NC) based on the reported sequences. The validity of the PCR products was verified by sequencing at SeqWright DNA Technology Services (Houston, TX) and the real-time quantitative RT-PCR analysis was performed by using Stratagene Brilliant II SYBR Green QRT-PCR reagents on Stratagene Mx3000P QPCR System (Agilent Technologies, La Jolla, CA).

RNA interference of MAPKs

Ambion siPORT amine transfection agent (Ambion, Austin, TX) was used in siRNA interference experiments for suppression of ERK1/2, p38 and JNK MAPK genes in HK-2 cells. siRNA SMARTpool MAPK1(ERK2), p38 MAPK, JNK and siRNA-negative control were purchased from Upstate (Millipore, Billerica, MA). Cultured HK-2 cells were also transfected by a heterogeneous Short-Cut siRNA Mix specific for the p38 MAPK (21–23 bp) using TransPass R2 Transfection Reagent (New England BioLabs, Beverly, MA). The transfection of siRNA technique has been optimized to obtain maximum silencing of a MAPK gene in HK-2 cells. Cells at 40–60% confluence were prepared into a six-well tissue culture plate with base medium on the day of transfection (~24 h prior to LC treatment, following the manufacturer’s instructions. After transfection, assays for target genes’ activity by western blot, RT-PCR and ELISA in control or LC-exposed cells were performed.

Image and statistical analyses

The levels of protein or RNA expressions were semiquantified using UN-SCAN-IT gel 6.1 analysis program (Silk Scientific, Orem, UT). The density of each band for the protein or transcript was digitized and defined in terms of ‘pixels’ (PSL), which is the total pixel minus the background. For each sample, the signal strength of the protein or transcript was normalized for the corresponding actin or GAPDH signal, respectively. The results were expressed as the mean ± SEM. Multiple comparisons were made with an ANOVA and Tukey-Kramer post hoc tests (InStat, GraphPad, San Diego, CA). Statistical analyses, curve fitting, and calculations were done using GraphPad Prism 4.0c. Statistical significance was defined as P < 0.05 for all analyses.

Results

Effect of LCs on PTEC cellular viability and morphology

Exposure of HK-2 cells to 25 μM κ-LC produced no significant effects on viability at 8, 24, 48 or 72 h (Figure 1a and f show how photoimages of cells at 48 h). A significant decrease in cell viability (>50%) was seen at the higher doses of ≥50 μM LC after 48 h (Figure 1b and f), similar to our previous report [25]. The control cells showed a typical epithelial cuboidal shape, with a cobblestone morphology (Figure 1e); however, upon exposure to LCs, distinct morphological changes are seen. At 25 μM κ-LCs there was evidence of cellular elongation (Figure 1a). Cells treated with 50 μM κ-LCs showed gross elongation with filopodia formation (Figure 1b). These findings were more obvious as the concentration of LC and the duration of treatment increased (data not shown).

LC-induced EMT in human renal PTECs

The ability of LCs to induce EMT in HK-2 cells was investigated in a time-course manner (8–72 h). For this purpose, we tracked the expression of E-cadherin, a marker for the epithelial phenotype, and α-SMA, a marker for the myofibroblast phenotype [26,27]. We observed that in HK-2 cells exposed to 25 μM of κ-LC, the expression of E-cadherin decreased after 24 h, while the expression of α-SMA increased by western blotting analysis (Figure 2), showing that these cells lost their epithelial characteristics and acquired mesenchymal cell properties. We used TGF-β1 and CsA, agents known to induce EMT in epithelial cells [24,28,29], as positive controls and similarly observed a distinct α-SMA induction and E-cadherin suppression in the cells during the 8–72 h incubation period. Treatment of cells with either 40 μM CsA or 25 μM κ-LC for 48 h resulted in marked decreases in E-cadherin mRNA expression, and similarly pronounced increases in α-SMA mRNA expression (Figure 3a and b). HSA, used as a protein control, also produced similar effects, but at much higher concentrations. At 160 μM, HSA had a significant effect on the expression of E-cadherin at 72 h and induced α-SMA expression as early as 8 h (Figures 2a, b and 3a, b). Pretreatment of the cells with TGF-β1 neutralizing antibody blocked the appearance of markers of EMT in CsA-exposed cells (Figure 4b and c). But the same treatment for 1 h prior to LC treatment and subsequent co-treatment did not restore the expression levels of E-cadherin. Treatment of the cells with TGF-β1 neutralizing antibody did not reduce...
Light-chain-induced EMT in human renal PTECs

Fig. 1. Effect of LC treatment on the morphology of human renal PTECs. HK-2 cells were grown to 80% confluency (e) and treated with 25 µM (a) and 50 µM (b) of κ-LCs for 48 h. Cells were visualized using a charge-coupled Nikon Coolpix 995 digital CCD camera attached to a Nikon Diaphot inverted phase-contrast microscope (Nikon, Tokyo, Japan) with 10×/0.30 numeric aperture objective showing the presence of distinct elongation/filopodia formation (a and b). HK-2 cells treated with 500 ng/ml BMP-7 (c) or 10⁻⁸ M PACAP38 (d) resulted in the formation of epithelial cell aggregates/condensation within the 70–80% confluent gross elongated and filopodia-formatted cell monolayer induced by 50 µM κ-LC exposure. (f) The number of viable cells in culture was determined by Promega MTS Cell Proliferation/Cytotoxicity Assay. Mean and SE of absorbance at 490 nm in six replicates was expressed in each group. **P < 0.01 compared with the 50 µM κ-LC-exposed cells.

LC induces alterations in FSP-1 and TGF-β1 expressions

The cytokine inducement of FSP-1 apparently is an important early event in the EMT pathway [30–32]. HK-2 cells treated with κ-LC or CsA displayed increased expression of FSP-1 transcript (Figure 3a and c). Pre-treatment of these cells with TGF-β1 neutralizing antibody for 1 h prior to LC exposure and subsequent co-treatment slightly reduced the expression levels of FSP-1 mRNA (data not shown). FSP-1 may be a downstream consequence of human LC.
endocytosis. The effect of 25 µM κ-LC on the expression of TGF-β1 transcripts was increased over the 72 h exposure period by real-time quantitative RT-PCR analysis (Figure 3c). The secretion of TGF-β1 following treatment with κ-LCs in HK-2 cells was also determined by ELISA. The cells were treated with 40 µM CsA, 25 µM κ-LC or 160 µM HSA for 8–72 h and TGF-β1 levels were determined quantitatively in culture media. Time-dependent
Light-chain-induced EMT in human renal PTECs

Fig. 3. Effect of LC treatment on the expressions of E-cadherin and α-SMA transcripts in human renal PTECs. (a) RT-PCR analysis of the expression of E-cadherin and α-SMA mRNAs, and FSP-1 and TGF-β1 mRNAs, as well as extracellular matrix components (fibronectin and collagen III mRNAs) in response to LC exposure in HK-2 cells. The cells were treated with 25 µM of κ-LCs or were treated with 40 µM CsA (positive control) or 160 µM HSA (protein control), for 8–72 h. The images are representative of at least four independent experiments performed. (b) Relative quantification of E-cadherin and α-SMA mRNA expressions in the κ-LC-treated HK-2 cells. The band intensity levels of mRNA were quantified in arbitrary units and are shown as the intensity of the signal in ‘pixels’ (PSL) normalized to the corresponding GAPDH signal. Each value represents the mean ± SE of four replicates/group. **P < 0.01 and *P < 0.05 compared with the cell cultured alone without treatment (None). (c) Relative quantifying FSP-1 and TGF-β1 mRNA expression by 25 µM κ-LC exposure for up to 72 h in HK-2 cells were performed by real-time quantification RT-PCR analysis. These data are expressed in baseline subtracted fluorescent reading normalized to the reference dye (dRn). The figures represent the mean ± SE of three experiments producing similar results.

increases in TGF-β1 secretion were detected after CsA or κ-LC exposure (Figure 4a). CsA-induced decrease in the expression of E-cadherin mRNA and increase in the α-SMA mRNA were reversed in the presence of anti-TGF-β1 antibody (Figure 4b and c). However, pre-treatment of the cells with TGF-β1 neutralizing antibody had no effect on the LC-induced expression of EMT-related molecules in all treatment groups (Figure 4b and c).

LC induces expression of ECM components

The effect of LC treatment on the expression of ECM components (fibronectin and collagen III) was examined in the HK-2 cells. Addition of κ-LCs, 25 µM, in the medium increased the mRNA expression of collagen III and fibronectin at 24, 48 and 72 h (Figure 3a). Slight increases in the mRNA levels of ECM components were also observed in cells exposed to 160 µM of HSA. The increase in mRNA levels of collagen III was greater than that of fibronectin.

Pre-treatment of these cells with TGF-β1 neutralizing antibody for 1 h prior to LC treatment had no effect on the expression levels of fibronectin and collagen III in treatment groups (data not shown).

The role of MAPKs in LC-induced EMT

We first examined the effects of selective gene silencing of ERK, JNK, and p38 MAPKs on LC-induced cytokine responses in human renal PTECs using siRNA products specific for the MAPKs. We found that transfection by their respective siRNAs leads to suppression of all three MAPK expressions (Figure 5a). Effective silencing of MAPK is achieved at low siRNA concentration 48 h post-transfection. We then measured the production of IL-6 and MCP-1 by ELISA and compared the effect of the same κ-LC in control cells, in cells transfected with either non-specific siRNA or p38 MAPK siRNA. These experiments showed that p38 MAPK silencing almost completely...
Fig. 4. CsA and TGF-β1 in human renal PTECs. (a) Effect of CsA and κ-LC treatments on TGF-β1 secretion in HK-2 cells. The production of TGF-β1 levels in cell culture medium increased significantly for 24–72 h in both 25 μM LC and 40 μM CsA exposed cells. Each value represents the mean ± SE of three experiments. **P < 0.01 and *P < 0.05, compared to the respective control without added protein (None). (b) Representative RT-PCR and immunoblotting analyses showing TGF-β1 neutralizing anti-body at 30 μg/ml, prevented CsA-induced loss of E-cadherin and induction of α-SMA mRNA and protein expression in HK-2 cells (upper strips), but interestingly had no effect on κ-LC-induced changes in E-cadherin and α-SMA (lower strips). The images are representative of at least three independent experiments. (c) Graphical representation of western blot illustrates the effect of TGF-β1 antibody on EMT marker expressions triggered by CsA and κ-LC in PTECs. Each value represents the mean and standard error of three independent experiments.

Reversal of LC-induced EMT by BMP-7 and PACAP38

E-cadherin is an epithelial cell-specific intercellular adhesion molecule. Using a quantitative E-cadherin immunoassay designed to measure human-soluble E-cadherin released into cell culture supernates, we observed that HK-2 cells exposed to 50 μM κ-LC for 48 h showed significant increasing level of soluble E-cadherin in media (Figure 6), which is an important determinant for losing maintenance of the epithelial phenotype. Figure 6a shows that HK-2 cells co-treated with κ-LC and BMP-7 (5–500 ng/ml) or PACAP38 (10⁻⁸–10⁻⁶ M) for 48 h and figure 6b shows that the cells firstly exposed to the LC for 48 h, the cell were then co-treated with 500 ng/ml BMP-7 or 10⁻⁸ M PACAP38 for an additional 60 h, both have significantly reacquired E-cadherin expressions within the confluent cell monolayer. The human renal PTECs treated with BMP-7 or PACAP38 at the optimum dose of 10⁻⁸ M or 500 ng/ml, respectively, resulted in the formation of epithelial cell aggregates/condensation within the 70–80% confluent gross elongated and filopodia-formatted cell monolayer induced by 50 μM κ-LC exposure (Figure 1b–f).

Discussion

Myeloma kidney, a chronic tubulointerstitial disease characterized by tubule atrophy and fibrosis is the most common type of renal injury associated with multiple myeloma. The pathophysiology of this lesion is linked to overproduction of LC immunoglobulins and their toxic effects on PTECs, and the propensity of LCs to bind to Tamm–Horsfall proteins and form casts [5,8,12]. The presence of marked tubular atrophy and the existence of extensive fibrosis in histologic examinations of human renal biopsies from patients with myeloma [5] suggest that like many other chronic kidney diseases with proteinuria, renal fibrogenesis is a major mechanism of kidney involvement in myeloma [33–35]. In the present studies we demonstrate that LC excess may induce directly EMT in vitro suggesting that this may be an important mechanism of renal fibrogenesis in myeloma kidney.

There are four key events that are crucial in the process of EMT [16,17,19]. These include: (i) the loss of epithelial adhesion properties, (ii) de novo expression of α-SMA and actin re-organization, (iii) disruption of the tubular basement membrane and (iv) enhanced cell migration and invasion. Our studies demonstrate that LCs are capable of inducing tubular epithelial cells to undergo three of the four of these steps. Although we did not perform cell...
motility and migration assays, LC-induced morphologic alterations, which included elongation and filopodia formation, are consistent with improved migratory capability of the transformed cells.

We observed that the expression of the epithelial cell adhesion molecule E-cadherin decreased after 24 h, while the expression of α-SMA, a marker for myofibroblasts, increased after continuous exposure to 25 μM κ-LCs in HK-2 cells, indicating that these cells lose their epithelial characteristics and acquire mesenchymal cell properties. LC-induced EMT appears to be associated with multiple cytokine, chemokine and growth factor responses including IL-1, IL-6, IL-8, MCP-1/4, FSP-1, TIMP-1/2, EGF, VEGF and TNF-α/β protein syntheses by cytokine antibody array in the PTECs (data not shown). Among several pathways that may lead to EMT in the kidney, the most prominent is TGF-β1 [19,36]. Moreover, many other proscleerotic factors have effects on EMT indirectly, via induction of TGF-β1. We therefore examined the possible role for TGF-β1 in LC-induced EMT in PTEC cultures [19,37]. We analysed the level of TGF-β1 transcript and protein expression in these cells and found it to be significantly elevated at 24 h persisting through 72 h. Subsequently, we examined the effect of blocking TGF-β1 expression in the cells treated with κ-LCs

![Fig. 5. Effect of MAPK interference on cytokine production and EMT molecules in LC-treated human renal PTECs. (a) Western blotting (left panel) and RT-PCR (right panel) analyses of ERK1/2, p38 MAPK and Jun 1/2 expressions after 24 h siRNA transfection of ERK, p38 MAPK or JNK1/2 in HK-2 cells. (a) Control; (b) non-specific siRNA; (c) specific siRNA. siRNA transfection effectively silenced or successfully suppressed all three MAPKs' protein and mRNA expression. Actin RNA is used as a control for RNA loading and tubulin as a control for protein loading. (b) Effect of κ-LC on IL-6 (upper panel) and MCP-1 (lower panel) production in p38 MAPK siRNA-transfected HK-2 cells. Transfection by p38 MAPK siRNA completely blocked both IL-6 and MCP-1 secretion compared to control cells (**P < 0.01). Bars represent the mean ± SE of three replicate determinations. (c) RT-PCR analysis and relative quantification of E-cadherin and α-SMA mRNA expressions in the LC-exposed HK-2. Cells were treated with 25 μM κ-LCs with or without p38 MAPK siRNA for 1 day prior to the addition of LC. The band intensity levels of mRNA were quantified in arbitrary units and are shown as the intensity of the signal in 'pixels' (PSL) normalized to the corresponding GAPDH signal. Each value represents the mean ± SE of four replicates/group. **P < 0.01 and *P < 0.05, compared to their corresponding control LC-exposed cells.
by using TGF-β1 neutralizing antibodies. Surprisingly, this manoeuvre failed to restore the LC-induced reduction in E-cadherin and α-SMA expression, or the amount of ECM components, collagen III and fibronectin [28], although it did reverse CsA-induced loss of E-cadherin and induction of α-SMA expression in HK-2 cells. CsA, used as positive control in these studies, is an agent known to induce EMT in renal tubule epithelial cells through TGF-β1, and this is believed to be a major mechanism of calcineurin inhibitor-associated renal fibrosis in transplant patients [38]. Thus, the LC-induced EMT pathway appeared to be different from that of CsA-induced EMT and independent of the associated increase in TGF-β1 protein and mRNA expression.

In our previous studies we have found that many of the cytotoxic effects of LCs on PTECs are mediated through MAPKs [11,25]. Interestingly, both p38 MAPK and JNK are also involved in EMT pathways [16,39]. We, therefore, explored the role of MAPKs in LC-mediated EMT, and found that the silencing of the p38 MAPK gene almost appeared to have effect on the expression of either E-cadherin or α-SMA, and its effect in most experiments was similar to those observed in LC-exposed PTECs. Although less marked and consistent than LC, on the expression of E-cadherin and collagen III, but we found HSA at equimolar or greater concentrations much less potent than LCs in inducing EMT responses in human renal PTECs.

There is considerable variability among the LCs associated with the progression of chronic renal disease. Small amounts of LCs may be associated with severe nephropathy in some patients while LC proteinuria up to 8–9 gm/day or greater may be associated with minimal or no renal dysfunction in others. We use supernormal concentrations of LC in these experiments, because despite variability among LCs, excessive quantity is a necessary condition for LC nephrotoxicity. LC concentration (25 µM) in this study was chosen to approximate a concentration in the lower range in a typical myeloma patient with modest LC proteinuria. Moreover, this is a concentration that could conceivably be achieved in the tubule fluid of a patient without myeloma, i.e. without increased production of LC but with glomerular proteinuria with increased glomerular sieving coefficient to LC [40]. Thus, our studies on the cytotoxic effects of LCs in vitro may be relevant not only to patients with myeloma but also to patients with glomerular proteinuria.

The LC-induced cytokine responses are in many respects similar to the effects of albumin on renal proximal tubule cells reported extensively during the recent years and have been attributed to protein overloading of cell trafficking, known as ‘the protein-overload’ hypothesis [41–43]. According to this hypothesis, in proteinuric states excessive protein reabsorption by the proximal tubules evokes these inflammatory and proinflammatory cytokine responses that can be responsible for a chronic tubulointerstitial inflammation and explain the progressive nature of chronic renal disease associated with proteinuria. As shown in this study, the treatment with excess (160 µM) human albumin appeared to have effect on the expression of either E-cadherin or α-SMA, and its effect in most experiments was similar to those observed in LC-exposed PTECs. Although less marked and consistent than LC, on the expression of EMT markers, TGF-β1 and collagen III, but we found HSA at equimolar or greater concentrations much less potent than LCs in inducing EMT responses in human renal PTECs.

EMT has recently emerged as a potentially important mechanism in renal fibrosis [33,35]. The importance of EMT has been demonstrated in experimental animal models, where blockade of EMT attenuates renal fibrosis [17,44]. Direct demonstration of EMT is impossible in human kidneys without serial biopsies using immunocytochemical tools. It is plausible to postulate that some of the fibrosis typically seen in kidney biopsies from myeloma patients must derive from EMT. Our observations in vitro provide experimental evidence in support of this idea that EMT might be occurring in the progression of human kidney disease.
It is relevant to note that in our previous studies we found PACAP38, a modulator of p38 MAPK, effective in countering nephrotoxic effects of myeloma LCs in PTECs and in rats in vivo [8,25,45–47]. In these preliminary studies treatment of the cells exposed to k-LC for 48 h with PACAP38 seemed to reverse LC-induced EMT. PACAP may play an important counterregulatory role both to prevent EMT and stimulate uncommitted cells to regain their tubular phenotype (mesenchymal–epithelial transition). Furthermore, as EMT is a complex series of events, combination therapies, which can target several key steps in the development of EMT, may prove to be more beneficial than mono-target therapies. Such combination therapies could include the use of BMP-7 or hepatocyte growth factor (HGF), which has been suggested to antagonize TGF-β1 action [48–50], as well as PACAP38 to target the downstream effects of LC-induced cytokine signalling including p38 MAPK [25,45]. Such inhibitors represent a potential therapeutic approach, offering a mechanism to slow or even redress established renal fibrosis.

The totality of our studies provides evidence that LC-provoked cytokine responses and LC-induced EMT may contribute to the characteristic tubulointerstitial kidney disease seen in multiple myeloma. The examination of signalling pathways suggests that LC-induced EMT is different from, for example, CsA-induced EMT, and it is independent of TGF-β1 but mediated through p38 MAPK. These observations identify potential therapeutic targets that could prove useful in the treatment of not only myeloma kidney, but other proteinuric kidney diseases as well.

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