Loss of Clusterin Expression Worsens Renal Ischemia Reperfusion Injury

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Running title: Clusterin protects the kidney from injury

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ABSTRACT

Prevention of ischemia-reperfusion injury (IRI) is a challenge in clinical care of the patients with kidney transplants or acute kidney injury, and understanding of the intrinsic mechanisms of resistance to injury in the kidney will lead to a novel therapy. Clusterin, a secreted glycoprotein, is an anti-apoptotic protein in cancer cells. Our study is to investigate the role of clusterin in renal IRI. Renal IRI in mice was induced by clamping renal vein and artery for 45 or 50 minutes at 32°C. Apoptosis of renal tubular epithelial cells (TECs) was determined by FACS analysis. Clusterin expression was examined by Western blot or immunohistochemistry. Here, we showed that clusterin protein was induced in TECs following IRI, and more tubules expressed clusterin in the kidneys following ischemia at higher temperatures. In human proximal TEC HKC-8 cultures, clusterin was up-regulated by removal of serum/growth factors in medium, and was down-regulated by TNF-α/IFN-γ mixture. The levels of clusterin were positively correlated with cell survival in these conditions. Knockdown or knockout of clusterin expression enhanced the sensitivity of TECs to apoptosis. In experimental models of renal IRI, deficiency in clusterin expression worsened the injury, as indicated by a significant increase in renal tissue damage with higher levels of serum creatinine and BUN, and a poorer recovery from the injury in clusterin-deficient mice as compared to those in wild-type mice. Our data indicate that the reduction of inducible expression of clusterin results in an increase in TEC apoptosis in the cultures, and renders mice susceptibility to IRI, implying a protective role of clusterin in kidney injury.
45 **Keywords:** clusterin; kidney ischemia; acute kidney injury; transplantation
INTRODUCTION

Renal ischemia reperfusion injury (IRI) is an inevitable event in kidney transplantation, and contributes to early kidney transplant dysfunction (4, 39, 40). In native kidneys, it is a common cause for acute kidney injury (AKI) in patients (29), who are undergoing cardiac surgery (2) or critical ill (27). The pathogenesis of renal IRI is not completely understood, but has been demonstrated to associate with interstitial inflammatory leukocyte infiltration (15, 62) and renal cell death (37, 45). To date, there is no effective therapy available for renal IRI.

Clusterin (CLU, Apolipoprotein J; SP-40,40; TRPM-2; SGP-2; pADHC-9; CLJ; T64; GP III; XIP8) is a secreted glycoprotein from either epithelial boundary cells in many organs or tissues (e.g. gallbladder, urinary bladder, kidney distal convoluted tubules, testis) or non-epithelial secretory cells (e.g. synovial lining cells, and ovarian granulosa cells) (1, 24). Secreted clusterin (sCLU), originated from cytoplasmic clusterin (cCLU) after posttranslational modification, is a major protein in physiological fluids: plasma, milk, urine, cerebrospinal fluid and semen (24), in which it associates with various molecules and displays various biological functions, such as sperm maturation, membrane recycling, lipid transportation, tissue remodeling, complement inhibition, cell-cell or cell-substratum interactions and programmed cell death (24, 55). Other studies show that it acts as a form of secreted heat-shock protein or molecular chaperone (31, 59). However, it remains unclear whether CLU is a multifunctional protein, or it has a
primary activity for all these physiological effects. Interestingly, the data from in vivo studies using genetic overexpression or knockout mice show the two faces of CLU: pro-apoptotic, as indicated by that the absence of CLU reduces cell death in hypoxia-ischemia-induced brain damage (20), and anti-apoptotic, as shown by an increase in autoimmune myocardial damage in CLU knockout mice (30), or inhibition of post-ischemic brain injury is seen in the mice with overexpression of CLU (58). Therefore, it is possible that CLU can either promote or inhibit cell death, depending on where and how much it is produced.

In renal tissues, including renal tubular epithelial cells (TECs), CLU expression is upregulated following a variety of renal injury, including unilateral ureteral obstruction (3, 48), IRI (43, 60) and rejected renal allografts in patients (10). However, the role of CLU in renal cell death has not been investigated to date. In a model of antibody-mediated glomerular injury, kidneys perfused with CLU depleted plasma develop significantly greater proteinuria at all time points when compared to control kidneys (46), and in vitro CLU at concentrations of 20-50 mg/ml partially protects LLC-PK1 cells (porcine TECs) from H$_2$O$_2$-induced cell death (50). The aim of our present study is to investigate the impact of renal expression of CLU in renal IRI.

**MATERIALS AND METHODS**

**Animals and cell cultures**

Both wild type C57BL/6 (B6) and CLU KO mice in B6 background (B6-Clu$^{-/-}$) were
received from the breeding colonies in the animal facility at the Jack Bell Research Centre (Vancouver, BC). CLU KO mice were generated by Dr. Bruce Aronow’s group (University of Cincinnati, Cincinnati, Ohio, USA) in the Swiss Black outbred genetic background (30), which were subsequently backcrossed into C57BL/6 mice (B6-Clu⁺⁻) for ten generations in our facility for this study. Genotype of mice was determined by PCR as described previously (30). All the animals (males, 8-10 weeks old) for the experiments were cared in accordance with the Canadian Council on Animal Care guideline under the protocols approved by the Animal Use Subcommittee at the University of British Columbia.

Human proximal TEC line HKC-8 was kindly provided by Dr. Daniel L. Sparks (Ottawa, ON, Canada) under the permission from Dr. Lorraine Racusen (41). T-HMC, a human mesangial cell line, was a gift from Dr. Tara McMorrow (35). Murine TECs were isolated from the kidney cortex as described previously (23), and immortalized with origin deficient SV40 DNA. In brief, renal cortex was collected and minced in HBSS containing penicillin-streptomycin. The tissue fragments were washed twice with HBSS and then digested with 1 mg/ml of collagenase V (Sigma-Aldrich Canada, Oakville, ON) in HBSS at 37°C for 15 min with intermittent agitation. The digested tissue was sieved through a 40μm Cell Strainer (BD Falcon™, BD Biosciences, Mississauga, ON). After washed with HBSS and complete K1⁺/+ medium as described previously (7), the sieved cells were seeded and grown in complete K1⁺/+ medium in a collagen-coated flask. Finally, the confluent monolayer was immortalized by transfection with origin deficient SV40 DNA, and TEC clones were identified by their expression of E-cadherin and CD13.
(Alanine aminopeptidase) in FACS analysis. All TEC lines (HKC-8, cloned MKC-1 from a B6 mouse and MKC-Clu from a B6-Clu<sup>−/−</sup> mouse) were grown in complete K1<sup>+/+</sup> medium.

**Western blot**

CLU protein in cell extracts of TECs was examined by Western blot as described previously (7). Briefly, protein samples (100-150 µg/sample) were fractionated by 10% SDS-PAGE, then transferred onto nitrocellulose membrane. The expression of CLU or active caspase-3 was identified with goat polyclonal anti-clusterin-α (C-18) (Santa Cruz Biotech, Santa Cruz, CA) or with rabbit polyclonal anti-activated caspase-3 (Asp175) antibodies (Cell Signaling Tech, Danvers, MA). The protein-antibody bands on the blot were visualized by an enhanced chemiluminescence assay (ECL, Amersham Pharmacia Biotech, Buckinghamshire, England). Blots were re-probed using anti--actin IgG (Sigma-Aldrich Canada) for confirmation of loaded protein in each sample.

To quantitate the CLU expression in Western blot analysis, the density of CLU (sCLU) and re-probed β-actin bands was measured by a densitometry. In each blot, the relative level of sCLU in each sample was calculated by normalization with its density of β-actin. The final change of CLU level (CLU-sample) in treated TECs was determined based on its relative to the basal level of CLU in untreated TECs (sCLU-basal), which was supposed to be the same in all separate experiments. CLU-sample was calculated by the
following equation: $\text{CLU-sample} = \frac{(s\text{CLU-sample}/\text{actin-sample})}{(s\text{CLU-basal}/\text{actin-basal})}$. Thus, the basal CLU level in TECs in K1$^{+/+}$ medium was equal to 1 unit.

**Apoptosis analyses**

Apoptosis in TEC cultures was measured by fluorescence-activated cell sorter (FACS) analysis with Annexin-V conjugated with phycoerythrin (Annexin-V-PE) for early apoptosis and 7-amino-actinomycin D (7-AAD) for late apoptosis staining following the manufacturer’s protocol (BD Biosciences). Thus, in graph, non-apoptotic (viable) cells were in low left quadrant, late apoptotic cells in upper left quadrant (7-AAD positive only), apoptotic cells in upper right quadrant (both Annexin-V and 7-AAD positive) and early apoptotic cells in lower right quadrant (Annexin-V positive only). Briefly, monolayers of TEC were released by a brief incubation with Trypsin-EDTA solution (Sigma-Aldrich), and then incubated with Annexin-V-PE in 1 X binding buffer for 15 min. The intensity of fluorescence of apoptotic cells was measured by a flow cytometry and analyzed as compared to background controls using CELLQUEST software (BD Biosciences). The apoptosis in cell cultures, measured by FACS analysis, was also confirmed by the levels of active form of caspase-3, which was determined by anti-active caspase-3 antibody in Western blot analysis as described above.

**Immunohistochemical analysis**

The kidneys harvested from mice were perfused with PBS prior to formalin fixation,
paraffin embedding and section. The levels of CLU protein in kidney sections were assessed by a standard immunohistochemical method. Briefly, after deparaffin and rehydration buffered-formalin-fixed sections were treated with 3% H₂O₂ in Tris buffer saline (TBS) (pH 7.4) for 30 min at room temperature (RT) to quench endogenous peroxidase, followed by permeabilization with 0.2% Triton X-100 for 10 min at RT. After being washed with TBS containing 0.1% Tween 20 (TBS-T) and blocked with 2% normal rabbit serum, the sections were incubated with goat polyclonal anti-clusterin-α (C-18) (Santa Cruz Biotech) (1:200 dilution) overnight at 4°C. The immune complexes of CLU and anti-CLU antibody on the tissue section were detected using anti-goat Ig antibody conjugated with biotin, and visualized with using a 3,3’-diaminobenzidine (DAB) peroxidase substrate kit (Vector Labs, Burlington, ON). The control negative staining included the sections incubated with normal goat IgG instead of anti-clusterin antibody or the sections of the kidneys from CLU KO mice.

To quantitate the CLU expression in the kidney in the immunohistochemical analysis, the number of CLU expressing tubules was counted in each view, which was randomly selected in the region of renal cortex under 400x magnification (high power field, hpf). The levels of CLU expression in each kidney were quantitated by averaging at least 20 nonoverlapping fields in two serial sections.

**Stable expression of anti-CLU shRNA**

ShRNA expression vector (pHEX-siRNA) was developed in our laboratory from a
modified herpes simplex virus (HSV) expression vector pHEX6300 as described previously (8). Synthetic oligonucleotide sequences, for generation of shRNA targeting CLU mRNA (5’-GCA GCA GAG TCT TCA TCA T-3’) and non-specific control (scrambled) (5’-AAT CGC ATA GCG TAT GCC GTT-3’) (8, 26), were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) and ligated into pHEX-siRNA to create pHEX-Clu or pHEX-control. The vectors were transfected into HKC-8 cells using Lipofectamine2000 (Invitrogen-Gibco, Carlsbad, CA, USA) following the manufacturer’s protocol. Transfected cells were grown in the presence of Zeocin (up to 500 µg/mL) (Invitrogen-Gibco) and selected by cell sorting using FACS for green fluorescence protein (GFP). More than 95% of control or siRNA transfected cells showed strong green fluorescence by either flow cytometry or microscopy.

Renal IRI Model

Both B6 and B6-Clu−/− mice were anesthetized with sodium pentobarbital (40 mg/kg) and isoflurane as needed. The kidneys were exposed through a flank incision. Ischemia was induced in left kidney by clamping renal pedicles at different temperatures (under the ice, room temperature, 32 or 37°C) for 45 or 50 minutes. After the clamps were released, reperfusion of the kidneys was confirmed visually. In the examination of CLU expression in the kidney, the non-ischemic right kidney was kept for life support. In the study of renal injury and survival, it was removed, and the uni-nephrectomized mice were included as sham-operated controls. Renal tissues and sera were harvested at 24 or 48 hrs after ischemia.
Determination of Renal function and semi-quantitative assessment of renal injury

Function of the kidneys was determined by the levels of serum creatinine or blood urea nitrogen (BUN), which were measured by QuantiChrom™ creatinine assay kit (BioAssay Systems, Hayward, CA) or QuantiChrom™ urea assay kit (BioAssay Systems). Histological assessment of tubular injury in kidney sections was performed in a blinded fashion. Formalin-fixed and paraffin-embedded sections (5µm thickness, longitudinal) were stained by both hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) methods. The percentage of damaged tubule (combined both necrosis and vacuolization) in total of tubules was counted in each view, randomly selected in the region of renal cortex under 400x magnification, and was averaged at least of 20 nonoverlapping fields for each kidney. In similarity, the number of tubules containing proteinaceous cast formation in the cortex was counted in each randomly selected view under 100x magnification and was averaged at least 10 nonoverlapping field for each kidney.

Statistical Analysis

One-way analysis of variance (ANOVA) or t-tests (one-tailed distribution) in Microsoft Excel software were used as appropriate for comparisons between groups. Animal survival was compared using Log-rank (Mantel-Cox) test in Prism survival analysis software. Data were collected from individual experiment or mouse in each study for statistical analysis. A p value of ≤ 0.05 was considered significant.
RESULTS

Up-regulation of CLU protein in kidney cells in the response to injury and in vitro culture

Up-regulation of CLU transcript has been noted in the kidneys with IRI (43), and associates with apoptosis (49), but the expression of CLU protein in the kidneys following ischemia at different temperatures and its impact on renal injury are not seriously investigated. The expression of renal CLU protein was examined by immunohistochemistry in tissue samples of the kidneys with IRI in a life-supporting model, in which the non-ischemic kidney was not removed. As shown in Fig. 1A, both naïve kidneys and the kidneys following ischemia under the ice had the similar staining of CLU in glomeruli and some tubular cells. When the ischemia was performed at room temperature (RT), 32°C or 37°C, a higher level of CLU expression, as indicated by remarkable staining, was noted in renal tubules of both cortex and medulla (proximal and distal tubules, Henle’s loop). Some of these tubules were obviously damaged as evidenced by the loss of brush border and dilatation, while other CLU-positive tubules were normal in morphology. As demonstrated in Fig. 1B, following the increase in temperature during ischemia, more tubules were stained CLU-positive, as indicated by an increase in the number of CLU expressing tubules per view from 3.27±1.97 in ice cold-ischemic kidneys to 20±0.85 in RT-ischemic kidneys (p<0.01, n=3), which was further increased to 27.46±3.93 in the kidneys following 32°C ischemia (vs. RT-ischemic
kidneys, p=0.05, n=3). There was a trend of increase in the CLU expressing tubules in 37°C-ischemic kidneys as compared to those in 32°C-ischemic kidneys, but this difference was not statistically significant in this limited number of mice. These data suggest that the high temperatures during the ischemia correlate with higher severity of renal IRI and more tubules with CLU expression.

As demonstrated by Western blot in Fig. 2, both HKC-8 and T-HMC cells constitutively produced CLU in cultures, as seen both cCLU (pre-secreted CLU) and sCLU in cell extracts, and sCLU in culture supernatants, while these two protein bands were absent in TECs (MKC-Clu) isolated from CLU knockout (KO) mice. Other four bands in the low molecular weight range were seen in all three samples including CLU deficient (Fig, 2A), suggesting that the bands were non-specifically stained. Since the phenotype of CLU-expressing cells in the glomeruli was not characterized as shown above, the expression of CLU in human mesangial cell line T-HMC in cultures suggests that CLU in mesangial cells may be spontaneous or upregulated within the glomerulus in these kidneys following IRI.

Regulation of CLU expression and its correlation with cell apoptosis in renal tubular epithelial cells (TECs) in cultures

In HKC-8 cells, CLU expression was up-regulated by the removal of growth factors and serum in K1 culture medium (K1⁻ medium), and was down-regulated by the stimulation with mixture of TNF-α (5 ng/ml) and IFN-γ (10 ng/ml) (Fig. 3A). In Western
blot analysis, when 1 unit represented the basal level of CLU in HKC-8 cells, which were grown in complete K1+/+ medium, its level was declined to 0.525±0.195 by cytokine treatment in K1+/+ medium (p=0.0047, n=4) and was up-regulated to 1.603±0.506 by the change of K1+/+ to K1−/− culture medium (p=0.0307, n=4) (Fig. 3B). Similar down-regulation of CLU was seen by the cytokine treatment in K1−/− medium. Interestingly, the levels of cell apoptosis were conversely correlated with CLU expression in these cultures (Fig. 3C-D), as indicated by that TECs in K1−/− medium had less cell apoptosis (4.7±1.29%) as compared to those (7.09±0.8%) in K1+/+ complete medium (p=0.0471, n=4), and at the same time, the treatment with cytokines resulted in an increase in cell apoptosis in both K1−/− (4.7±1.29% in untreated versus 11.42±1.86% in treated cells, p=0.0072, n=4) and K1+/+ culture media (7.09±0.8% in untreated versus 14.34±2.8% in treated cells, p=0.0106, n=4). The apoptosis was further confirmed by the measurement of activation of caspase-3, an effector caspase for cell apoptosis, in cell extracts; the higher percentage of Annexin-V-stained cells in FACS analysis was positively correlated with the high levels of pro-caspase-3 cleavage (active form of caspase-3) in Western blot analysis (Fig. 3E). These data suggest that the decrease in endogenous CLU expression is associated with cell death in inflammatory cytokine-stimulated TECs.

To further confirm whether specifically reduced CLU expression resulted in an increase in cell apoptosis in TEC cultures, CLU expression in HKC-8 cells was genetically silenced by stable expression of anti-CLU shRNA, and cell apoptosis was examined by FACS analysis. As shown in Fig. 4A, the levels of CLU protein (both cCLU and sCLU) in HKC-8-Clu cells, which were stably expressing anti-CLU shRNA, were
significantly reduced as compared to those in parental HKC-8 cells or cells with stable expression of control shRNA (HKC-8-control). Under the same culture conditions, the reduced CLU expression by shRNA resulted in an increase in spontaneous apoptosis, as indicated by 15.88±1.09% of Annexin-V-positive cells in HKC-8-Clu in K1+/+ culture medium, significantly more than 9.99±1.37% in HKC-8-Control cells (p=0.0006, n=4). In addition, less CLU increased apoptosis in TECs in the response to the stimulation of TNF-α and IFN-γ mixture, as reflected by 21.41±1.83% of Annexin-V positive cells in the cytokines-treated HKC-8-Clu cells, compared to 15.13±2.88% in HKC-8-Control cells under the same treatment (p=0.0309, n=4) (Fig. 4B-C). Taken all together, the data suggest that constitutive expression of CLU in TECs enhances cell survival in culture conditions, as indicated by the significant correlation of lower levels of CLU, seen in cultures in the presence of inflammatory cytokines and growth factors, with higher levels of cell apoptosis.

Deficiency in CLU expression increased tubular damage and apoptosis in ischemia-reperfusion injury (IRI)

As demonstrated above, expression of CLU protein was induced in renal tubules in the kidneys following IRI. To examine the impact of up-regulated CLU protein on kidney tissue injury or damage, the severity of renal IRI in CLU KO mice was compared to that in wild type (WT) controls. The presence of tubular damage was examined by renal histology in Haematoxylin and Eosin (H&E) and Periodic acid-Schiff (PAS)-stained kidney sections. The most prominent renal damage was seen in the outer medulla or
boundary zone in both WT and CLU KO kidneys, as indicated by tubular cell necrosis and detachment. However, the area of this damage in CLU KO kidneys was often seen to extend further to cortex along pars radiata. By a semi-quantitative method to measure the severity of renal tubular injury in renal cortex, the percentage of combined tubular necrosis and vascularization in CLU KO kidneys was significantly higher than those in WT controls (33.86±15.18% in KO versus 6.95±1.68% in WT, p=0.0085, n=5) (Fig. 5A), and the similar results were also observed in the count of intratubular cast formation in both groups (6.2±2.36 in KO versus 1.8±2.37 in WT, p=0.0489, n=5) (Fig. 5B). All these data indicate that the deficiency in renal expression of CLU increases kidney susceptibility to IRI.

To confirm the contribution of cell apoptosis to the high severity of tissue damage in CLU deficient kidneys, the apoptosis of CLU KO TECs (MKC-Clu) was examined as compared to that in WT cells (MKC-1) following stimulation with pro-inflammatory cytokines. As shown in Fig. 6A, no CLU protein was detected in MKC-Clu cells by Western blot analysis. Under the same culture conditions (Fig. 6B), the spontaneous apoptosis was significantly higher in MKC-Clu cultures than those in WT MKC-1 cultures (18.54±3.87% in MKC-Clu versus 10.68±2.8% MKC-1, p=0.0008, n=6-8). Following the stimulation with mixture of TNF-α and IFN-γ, the levels of apoptosis was significantly increased in MKC-1 cultures as compared to those in medium controls (16.85±5.76% in cytokines-treated versus 10.68±2.8% in medium control cells, p=0.0177, n=8), but surprisingly, no significant difference was seen in MKC-Clu cultures (19.35±6.45% in cytokines-treated versus 18.54±3.87% in medium control cells,
Furthermore, the apoptosis in cytokines-treated MKC-1 was no significantly lower than that in cytokines-treated MKC-Clu (p=0.2024, n=8-10). These data indicate that the deficiency in renal expression of CLU results in an increase in spontaneous apoptosis, which may be induced by sublethal oxidative stress in untreated cultures, but has no effect on apoptosis in cytokines-treated cultures. In CLU deficient TECs, the cytokine stimulation does not induce additional apoptosis. All these suggest that cytokines-dependent apoptosis may be mediated by their down-regulation of CLU in TECs as shown in Fig. 3.

The severity of injury was positively correlated with the dysfunction of CLU deficient kidneys

To confirm the correlation of the severity of renal tissue damage in CLU deficient kidneys with their renal function in mice, the levels of serum creatinine and blood urea nitrogen (BUN) were measured in KO mice versus WT controls following IRI and sham-operation. As shown in Fig. 7A, the levels of serum creatinine in mice with renal IRI were significantly higher in KO group (1.12±0.47 mg/dL) than those in WT controls (0.22±0.09 mg/dL) (p<0.0001, n=12). Similar results were seen in the measurement of BUN, as indicated by the higher levels of BUN in KO mice (226.01±50.18 mg/dL) as compared to those in WT controls (107.61±82.11 mg/dL) (p=0.0038, n=12) (Fig. 7B). Although the levels of both creatinine and BUN in KO mice trended to be higher than those in WT mice following sham-operation, statistical significance was not reached (n=0.8, n=4). These data indicate that the high levels of IRI contribute to more severe
dysfunction of the kidneys in CLU-deficient mice.

**The expression of CLU improved ischemia-reperfusion injury recovery**

Renal IRI is acute injury and its recovery results from tubular repair, characterized by remodeling of the basement membrane, cellular proliferation, hypertrophy, and differentiation of new functional proximal tubule cells (21, 44). To further examine the effect of CLU expression on renal self-healing or repair after IRI, renal IRI was induced by 50 minutes of clamping at 32°C. Mice recovered or survived from the promotion of renal repair in this lethal model of renal IRI. As shown in Fig. 8, four out of 20 mice survived in WT group, while all 20 mice in KO group died within 3 days. The recovery or survival of animals from renal IRI was statistical difference between these two groups (p=0.0055, KO versus WT), suggesting that the expression of CLU have beneficial effect on not only protection of the kidney from the injury (Fig. 5) but also promotion of renal tubular repair.

**DISCUSSION**

Renal IRI is a primary cause for AKI in native kidneys, and largely contributes to kidney transplant dysfunction. So far no effective treatment is available for the AKI. It has been recognized for a while that the nature of renal IRI consists of not only cell death (necrosis or apoptosis) but also sublethal injury causing cell dysfunction (12). Therefore, understanding of renal protection from sublethal injury toward complete cell death could lead to prevent IRI in clinic. Our current study demonstrates that kidney cells including
tubular and mesangial cells express CLU in cultures or in vivo in the response to rejection or IRI. In TEC cultures, down-regulation of CLU expression by the treatment with pro-inflammatory cytokines is positively correlated to cell apoptosis, and knockdown of CLU expression results in an increase in pro-inflammatory cytokine-induced cell apoptosis. In a mouse model of renal IRI, as compared to CLU KO mice CLU expression associates with the reduction of renal IRI and dysfunction of the kidneys and also contributes to the promotion of renal repair. These results suggest that local expression of CLU may protect renal cells from cell death in AKI as well as kidney transplant rejection.

Several previous studies have demonstrated that up-regulation of CLU expression is seen in both tubular and mesangial cells in the kidneys with either predominant glomerular or tubular injury (6, 22, 51, 61), and is not necessary to associate with primary site of tissue injury, as indicated by that CLU in TECs is induced in the kidneys with nephritis (6) or at a site distant from the primary injury in cisplatin nephrotoxicity (51). Similarly, our study shows that CLU expression is up-regulated in both glomerular cells and TECs in the kidney following IRI, in which the majority of tubular injury occurs in the outer medulla. The number of CLU-expressing tubules, characterized by either normal morphology or dilatation, is increased following higher severity of renal IRI induced by 32-37°C-ischmia and are spreading in both cortex and medulla but not only limited to the primary region of injury (Fig. 1). All these studies suggest that kidney cells expressing CLU are the response to the injury stress but may not be necessary to the
programmed to irreversible cell death.

To date, the molecular basis of CLU expression in kidney cells are not investigated yet. Our data show that constitutive expression of CLU protein is seen in both TECs and mesangial cells in cultures (Fig. 2) as well as in the kidneys experiencing IRI but not very much in naive kidneys (Fig. 1), suggesting that sublethal stress may induce CLU protein biosynthesis in kidney cells. Cells in culture could behave differently from cells in vivo in many ways, one of which is that cells in cultures impose a state of sublethal oxidative stress (19), implying that oxidative stress may be an inducer for CLU expression in kidney cells. Indeed, up-regulation of CLU has been reported in many types of cells in vitro following exposure to sub-lethal oxidative stress (9, 53, 57), and in vivo tubular expression of CLU is induced in the kidneys in both acute glycerol-induced renal failure and chronic vitamin E/selenium deficiency, two kidney models of in vivo oxidant injury (34). The hypothesis of oxidation-induced CLU expression is further supported by the observations; CLU gene transcription is regulated by heat shock protein (HSF) transcription factor 1 (31), and in ureteral obstruction, the kinetics of CLU expression in the kidneys is very similar to that of HSP70, both reaching maximum levels 48 h after the beginning of obstruction (47). All these indicate that the transcription and post-transcriptional processing of CLU gene may be activated by heat shock or oxidative stress.

Induction of CLU in oxidative-stressed kidneys may be either involved in the process of cell death or prevent cells death as a feedback response within a hostile environment,
where cellular destruction potentially occurs. Indeed, in the models of neural injury, the absence of CLU reduces cell death in hypoxia-ischemia-induced brain damage (20), and in cultures of striatal cells CLU mediates H$_2$O$_2$- and amyloid beta-peptide-induced cell death (13), suggesting the pro-apoptotic activity of CLU in brain tissue. By contrast, deficiency in CLU expression increases autoimmune myocardial damage (30) and renal IRI in this study (Fig. 5), and CLU protects LLC-PK1 kidney cells from H$_2$O$_2$-induced cell death in cultures (50). Furthermore, the increases in cell apoptosis are seen in TEC cultures with a lower level of CLU (Figs. 3 and 4) or in MKC-Clu (Fig. 6). All these data indicate that cardiac/renal CLU expression has a protective role in cell death. This observation is strongly supported by other studies in a variety of cancer cells; high CLU expression is associated with progression of many types of cancers in patients (25, 28, 38, 52), and antisense oligodeoxynucleotides targeting CLU expression in cancer cells increases cell death or decreases cell survival in the response to the treatment with chemotherapeutic drugs, growth factor withdrawal and oxidative H$_2$O$_2$, respectively (16, 17, 32, 56). To date, the mechanisms by which CLU acts differently in neural cells versus cardiac/renal cells are not known.

Based on the data presented by this study, it may be postulated that following oxidative stress induced by ischemia-reperfusion (18, 36), CLU expression in kidney cells, in particular TECs, precedes the development of cellular destruction and protects sublethal-stressed cells from cell death (apoptosis and necrosis), supporting the hypothesis that an increase in CLU expression may be a physiological defense from sublethal-stressed cells to reduce further cell damage and to maintain cell viability during periods of increased
oxidative stress. Our results also show that pro-inflammatory cytokines (IFN-γ and TNF-α) attenuate CLU expression in TEC cultures (Fig. 3), and it seems that the apoptosis induced by pro-inflammatory cytokine TNF-α and IFN-γ is dependent on their effect on the reduction of CLU expression in TECs, as indicated by no significant difference in the apoptosis between cytokines-treated and untreated CLU deficient cells (Fig. 6). These data suggest that renal pro-inflammatory cytokines, accumulated from activated infiltrating leukocytes and perhaps renal resident cells, induce cell death of stressed TECs and mesangial cells by disruption of their CLU expression. However, the molecular mechanisms by which the cytokines down-regulate CLU expression in kidney cells remain further investigation.

In addition to anti-apoptotic function, CLU may act as an inhibitor of complement-mediated cytotoxicity, an important pathological factor for IRI (54, 63). Early studies have shown that CLU binds to membrane attack complex (MAC), resulting in inhibition of complement-mediated attack. The deposition of CLU associated with C3 and C5b-9 complex of complement has been found in renal biopsies from all forms of renal disease (11, 14, 33), and its expression is independent of the presence of an intact complement system (5). An interesting study demonstrates that aging mice deficient in CLU develop a progressive glomerulopathy, characterized by the deposition of immune complexes in the mesangium, and in young KO animals, the development of immune complex lesions is accelerated by unilateral nephrectomy-induced hyperfiltration, and the injected immune complexes are localized to the mesangium of KO but not WT mice (42), suggesting a protective action of CLU against kidney injury at least in part through its prevention of
immune complex disposal in the kidneys.

In conclusion, renal IRI is one of problems related to a majority of patients admitted to the intensive-care unit and the dysfunction of kidney transplants. Unfortunately, treatment for this complex syndrome is as yet lacking, and understanding of it is limited. Our present study clearly demonstrates that renal expression of CLU is induced following IRI or by the exposure to oxidative environments in cultures. Reduced CLU expression results in an increase in cell death or renal tissue injury. The mechanisms by which CLU prevents renal injury are not clarified yet, but anti-apoptosis and inactivation of complements by up-regulated renal CLU may contribute at least in part to its renal protective action. Our data imply the potential of CLU in the prevention of both tubular and glomerular injury seen in patients with AKI and kidney transplant rejection.

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GRANTS

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DISCLOSURE

The authors have nothing to disclose
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FIGURE LEGENDS

**Fig. 1:** Induction of clusterin protein in renal tubules following renal IRI.

Renal IRI was performed in life-supporting male B6 mice, in which the non-ischemic kidney was not removed. Kidney tissues were harvested after 24 hours of reperfusion following 45 minutes of ischemia under different temperatures (ice cold, room temperature, 32°C or 37°C). The tissues were fixed in 10% buffered formaldehyde, embedded in paraffin, and then sectioned for immunohistochemical staining with goat polyclonal anti-clusterin-α (C-18) antibody. Brown color in immunohistochemical staining indicated the presence of clusterin protein. (A) The data are presented as a typical image of CLU staining in renal cortex from each group: (a) Naïve kidneys without renal IRI, (b) ice slush was applied to the kidneys when ischemia was performed, (c) ischemia was performed at room temperature (RT) without heat pad, (d) Ischemia was performed at 32°C, and (e) Ischemia was performed at 37°C. The control negative staining included the sections stained with normal IgG as primary antibody or without primary antibody, and clusterin KO kidney tissue sections stained with anti-clusterin antibody. (B) CLU expressing tubules in renal cortex were counted at 400x magnification (high power field, hpf) in a blinded manner. Data are presented as mean ± SD (standard derivation) in each group (n=3). P<0.01 (Ice cold vs. RT); P=0.05 (RT vs. 32°C).

**Fig. 2:** Spontaneous expression of clusterin protein in human kidney cells in cultures.

Total protein extract was prepared from T-HMC (human mesangial cell line), HKC-8
(proximal tubular epithelial cell line) and TECs (MKC-Clu) isolated from clusterin KO mice as control. (A) The presence of clusterin proteins in cellular extracts was detected by western blot with anti-clusterin-α antibody, and β-actin was reprobed in the same blot with anti-β-actin IgG antibody. (B) Secreted clusterin in culture supernatants was detected by western blot. cCLU: cytoplasmic clusterin (or pre-secreted clusterin); sCLU: secreted clusterin. The data are representative of three independent experiments.

Fig. 3: Negative correlations of clusterin levels with cell death in TECs in cultures.
HKC-8 cells were incubated in K1 complete (K1+/+) or K1 serum/growth factors-free (K1−/) medium in the absence or presence of IFN-γ (10 ng/m) and TNF-α (5 ng/ml) mixture for 48 hours. (A) The levels of clusterin proteins in cellular extracts was detected by western blot with anti-clusterin-α antibody, and β-actin as protein loading controls was reprobed in the same blot with anti-β-actin IgG antibody. The data are presented as a typical image from four separate examinations. cCLU: cytoplasmic clusterin; sCLU: secreted clusterin. (B) The relative change of sCLU to its basal level in HKC-8 cells in K1+/+ medium in Western blot was quantitated, and statistically compared in total four separate experiments (n=4). Data are presented as mean ± SD in each group. P=0.0307 (K1+/+ vs. K1−/− in the absence of cytokines); P=0.0047 (untreated vs. cytokines-treated in K1+/+ medium); P=0.0144 (untreated vs. cytokines-treated in K1−/− medium). (C) Cell apoptosis was determined by FACS analysis with 7-AAD (late apoptosis) and Annexin-V conjugated with phycoerythrin (early apoptosis) staining. Cells with double-negative staining were viable. The data are representative of four separate examinations. (D) Statistical comparison of cell apoptosis, as indicated by total Annexin-V positive
staining, in all four examinations. Data are presented as mean ± SD (standard derivation) in each group. P=0.0471 (K1^{+/+} vs. K1^{-/-}); P=0.0072 (untreated vs. cytokines-treated in K1^{-/-} medium); P=0.0106 (untreated vs. cytokines-treated in K1^{+/+} medium). (E) The levels of activated caspase-3 in cellular extracts was detected by western blot with anti-activated caspase-3 antibody, and β-actin as protein loading controls was reprobed in the same blot with anti-β-actin IgG antibody. The data are presented as a typical image from three separate examinations.

Fig. 4: Increase of apoptosis in TECs expressing anti-clusterin shRNA.

HKC-8 cells were stably transfected with pHEX-control (HKC-8-control) or pHEX-Clu (HKC-8-Clu) in the presence of zeocin. (A) Reduction of clusterin protein in TECs expressing anti-clusterin shRNA, the clusterin proteins in cellular extracts was detected by western blot with anti-clusterin-α antibody, and β-actin as protein loading controls was reprobed in the same blot with anti-β-actin IgG antibody. cCLU: cytoplasmic clusterin; sCLU: secreted clusterin. (B) Both HKC-8-Clu and HKC-8-control cells in K1^{+/+} medium were treated with IFN-γ (10 ng/ml) and TNF-α (5 ng/ml) mixture for 48 hours, cell apoptosis was determined by FACS analysis with 7-AAD (late apoptosis) and Annexin-V conjugated with phycoerythrin (early apoptosis) staining. Data are representative of four separate examinations. (C) Statistical comparison of cell apoptosis, as indicated by total Annexin-V positive staining, in all four examinations. Data are presented as mean ± SD in each group. P=0.0006 (HKC-8-Clu vs. HKC-8-control in culture medium only); P=0.0309 (HKC-8-Clu vs. HKC-8-control in cytokines-treated cultures).
Fig. 5: More severe renal damage in clusterin KO mice following renal IRI.

Clusterin KO and WT C57BL/6 male mice were subjected to renal ischemia–reperfusion at 32°C of body temperature for 45 min. (A) Tubular necrosis and vacuolization of renal cortex were scored by a semi-quantitative histological analysis with periodic acid-Schiff staining in a blinded manner. Percentage of damaged tubules (necrosis and vacuolization) in each section of renal cortex was counted at 400x magnification and averaged for each mouse with no less than 20 viable fields per slide. Data are presented as mean ± SD in each group (n=5). P=0.0085 (KO vs. WT). (B) The number of tubules containing proteinaceous cast formation in the cortical substance was counted in each randomly selected view under 100x magnification and was averaged at least 10 nonoverlapping field for each kidney. Data are presented as mean ± SD in each group (n=5). P=0.0489 (KO vs. WT).

Fig. 6: Increase in spontaneous but not cytokines-mediated apoptosis of clusterin deficient TECs in cultures.

TECs were isolated from the kidneys from WT (MKC-1) or clusterin KO mice (MKC-Clu) and incubated in K1+/+ medium. (A) Clusterin protein was absent in TECs isolated from clusterin KO mice (MKC-Clu), as determined by Western blot as described in Fig. 2. (B-C) Both WT and KO TECs were treated by mixture of IFN-γ and TNF-α (10 ng/ml each) in K1−/− medium for 48 hours. Cell apoptosis was determined by FACS analysis with 7-AAD (late apoptosis) and Annexin-V conjugated with phycoerythrin (early apoptosis) staining. The data are representative of eight separate examinations (B);
statistical comparison of cell apoptosis, as indicated by total Annexin-V positive staining, in all eight examinations, and data are presented as mean ± SD in each group (C). P=0.0008 (KO vs. WT in culture medium only); P=0.2024 (KO vs. WT in cytokines-treated cultures).

Fig. 7: Increase in dysfunction of the kidneys in clusterin KO mice following renal IRI.

Both WT and clusterin KO mice were subjected to renal IRI as described above. Sera were collected after 48 h of reperfusion. Kidney function was determined by measurement of serum creatinine and urea (BUN). (A) Serum creatinine levels. Data are presented as mean ± SD of 12 mice in each group. P<0.0001 (KO vs. WT). (B) BUN levels. BUN was measured in the same sera. Data are presented as mean ± SD. P=0.0038 (KO vs. WT).

Fig. 8: Beneficial effect of clusterin expression on recovery of mice from renal IRI.

Clusterin KO and WT C57BL/6 male mice were subjected to renal ischemia–reperfusion at 32°C of body temperature for 50 min. The mortality of mice was closely monitored (twice a day) in this lethal model. The survival of mice in KO group versus WT group was analyzed by Log-rank (Mantel-Cox) test (p=0.0055, n=20 each group).
Figure 2

A

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kDa
- 80
- 58
- 39
- 17

B

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kDa
- 72
- 55
- 36
- 28
Figure 3A

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Relative sClu level
Figure 3C-D

C

K1+/+

Cytokines in K1+/+

K1−/

Cytokines in K1−/

D

P=0.0471

P=0.0106

P=0.0072

Annexin-V positive cells (%)

K1+/+

Cytokines in K1+/+

K1−/

Cytokines in K1−/−
Figure 3E

Active Caspase-3

β-Actin

E

K1+/+  K1−/−

Cytokines

K1+/+  K1−/−

kDa 39 17
Figure 4A

A

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kDa

- 80
- 58
- 39
- 17
Figure 4B-C

B

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C

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Annexin-V positive cells (%)
Figure 5

Intratubular cast formation /field

Tubular necrosis/vacuolization (%)

B

KO

WT

A

KO

WT

P=0.0085 (KO vs. WT)
Figure 6A

A

MKC-1  MKC-Clu  MKC-Clu

kDa

80

58

39

17

cCLU  sCLU

β-Actin
Figure 6B-C

B

MKC-1

Medium  Cytokines

MKC-Clu

Medium  Cytokines

7-AAD

Annexin-V

C

Annexin-V positive cells (%)

P=0.0008

P=0.2024

Cytokines  

MKC-1  MKC-Clu
Figure 7

A

Serum creatinine (mg/dL)

KO | WT | KO | WT
---|---|---|---
IRI | | | P<0.0001
Sham | | | P=0.8

B

BUN (mg/dL)

KO | WT | KO | WT
---|---|---|---
IRI | | | P=0.0038
Sham | | | P=0.8
Figure 8

![Survival Graph](image)

Survival (%) vs. Days

- **WT**
- **KO**

P=0.0055 (Log-rank)