L-Carnitine Protects Renal Tubular Cells Against Calcium Oxalate Monohydrate Crystals Adhesion Through Preventing Cells From Dedifferentiation

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Urolithiasis • Dedifferentiation • L-carnitine • Calcium oxalate monohydrate • Adhesion

Abstract
Background/Aims: The interactions between calcium oxalate monohydrate (COM) crystals and renal tubular epithelial cells are important for renal stone formation but still unclear. This study aimed to investigate changes of epithelial cell phenotype after COM attachment and whether L-carnitine could protect cells against subsequent COM crystals adhesion. Methods: Cultured MDCK cells were employed and E-cadherin and Vimentin were used as markers to estimate the differentiate state. AlexaFluor-488-tagged COM crystals were used in crystals adhesion experiment to distinguish from the previous COM attachment, and adhesive crystals were counted under fluorescence microscope, which were also dissolved and the calcium concentration was assessed by flame atomic absorption spectrophotometry. Results: Dedifferentiated MDCK cells induced by transforming growth factor β1 (TGF-β1) shown higher affinity to COM crystals. After exposure to COM for 48 hours, cell dedifferentiation were observed and more subsequent COM crystals could bind onto, mediated by Akt/GSK-3β/Snail signaling. L-carnitine attenuated this signaling, resulted in inhibition of cell dedifferentiation and reduction of subsequent COM crystals adhesion. Conclusions: COM attachment promotes subsequent COM crystals adhesion, by inducing cell dedifferentiation via Akt/GSK-3β/Snail signaling. L-carnitine partially abolishes cell dedifferentiation and resists COM crystals adhesion. L-carnitine, may be used as a potential therapeutic strategy against recurrence of urolithiasis.
Introduction

Although the use of low-invasive surgery such as shock wave lithotripsy and endoscopy has brought about remarkable strides in the treatment of urolithiasis, the high rate of recurrence is still a troublesome problem. Calcium oxalate (CaOx) stone is one of the most frequent types of renal calculi, and exploration of mechanisms of CaOx stone formation, is meaningful for curing and preventing the recurrence of urolithiasis.

Traditionally, urinary supersaturation is essential for crystallization. Calculus patients tend to excrete urine that is more supersaturated than that of non-calculus people, but supersaturation values overlap widely [1], and temporary supersaturated urine may present usually in the kidney of people who are never susceptible to kidney stone [2], which means post-crystallization factors play an even more central role. A widely accepted hypothesis of post-crystallization mechanism is the crosstalk between renal tubular epithelial cells and urinary crystals [3]. Exposure to crystals attachment can injure tubular epithelial cells, evoking a cascade of reactions, such as oxidative stress, inflammatory reaction, apoptosis, phagocytosis, which in turn are able to promote crystals aggregating and adhering [4]. After injury, renal tubular epithelial cells will trigger repair procedure which is characterized by dedifferentiation-regeneration-redifferentiation process, resemble partial epithelial to mesenchymal transition (p-EMT) and partial mesenchymal to epithelial transition (p-MET) [5]. Accumulating evidence obtained from in vivo study suggested that almost all adhesive intratubular crystals were anchored to immature cells [6, 7], moreover, others reported that as a result of re-existing cell injury, tubular epithelial cells injury occurs more easily in crystal-cell interactions [8].

Therefore, we hypothesis that the dedifferentiating period of repair process, carried out by survival epithelial cells after injury, might intensify subsequent CaOx crystals adhesion. We believe that when the balance between cell-transdifferentation during repairing and crystal-adhesion was maintained exactly, crystals would be cleaned up finally. Otherwise, excessive dedifferentiating cells, which failed to transform into redifferentiation, might develop into the anchor points of subsequent crystals adhesion, making kidney be susceptible to stone formation.

L-carnitine is a naturally occurring amino acid derivative synthesized in the liver and kidney, it acts as a carrier for fatty acids acrossing the inner mitochondrial membrane, which is necessary for subsequent β-oxidation and ATP production [9]. The compound has been considered as a specific inhibitor of mitochondrial reactive oxygen species (ROS) [10], and the beneficial effects of which to protect kidney cells against oxidative stress, lipid peroxidation, proinflammatory cytokines, and apoptosis during acute and chronic kidney injury have been documented [11-13]. These pathogenetic factors have also been reported to play pivotal part in renal stone formation [14]. Thus whether L-carnitine is also of value in preventing more CaOx crystals adhesion is examined in our study.

Materials and Methods

Chemicals and Reagents

DMEM (Dulbecco’s Modified Eagle Medium), fetal bovine serum (FBS) and AlexaFluor-488 Ig G were purchased from Thermo Fisher Scientific (CA, USA), Sodium oxalate (Na2C2O4), Anhydrous calcium chloride (CaCl2), calcium oxalate monohydrate (COM) and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO, USA), transforming growth factor β1 (TGF-β1) was purchased from R&D Systems (Minneapolis, USA), E-cadherin and Vimentin antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), Occludin, phospho-Akt, Akt, phosphor-GSK-3β, GSK-3β, Snai1, GAPDH antibodies were purchased from Cell signaling Technology, USA.
MDCK Cells

Madin Darby canine kidney (MDCK) cell line was purchased from ATCC (Manassas, USA). The cell line was maintained in complete DMEM medium with 10% FBS. The cells were maintained at 37°C in humidified atmosphere of 95% air and 5% CO₂.

AlexaFluor-488-tagged COM crystal preparation

5 mmol/L CaCl₂ was mixed with 0.5 mmol/L Na₂C₂O₄ in a buffer containing 90 mmol/L Tris-HCl, 10 mmol/L NaCl and 0.11 μg/ml AlexaFluor-488 IgG (pH 7.4). Then COM crystals were harvested by centrifugation at 2000× g for 5 min. The supernatant was discarded and COM crystals were resuspended in methanol. After another centrifugation at 2000× g for 5 min, methanol was discarded and the crystals were air-dried overnight at room temperature [15].

COM crystal adhesion

After exposure to corresponding treatment for 48 hours, 200 μg/ml AlexaFluor-488-tagged COM crystals were added into medium and followed by maintaining statically for 5 minutes. The non-adherent crystals were removed by rapidly washing the cells three times with PBS, and the rudimental crystals were counted using fluorescence microscope. Then rudimental crystals were dissolved by 2 ml of HCl (5 mol/L), and the calcium concentration of the solution was assessed by flame atomic absorption spectrophotometry (AA-6300, Shimadzu, Japan), to quantitative analyze adherent COM crystals [16].

Western blot analysis

After incubating in the medium containing corresponding treatment for 48 hours, cells were washed three times with cold phosphate-buffered saline (PBS) and then scraped into the extraction buffer (150 mmol/L NaCl; 20 mmol/L tris-(hydroxymethyl)aminomethane; PH 7.4, 0.1% sodium dodecyl sulfate [SDS]; and 1% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride). After centrifugation at 12000 rpm for 15 min at 4°C, the supernatant was obtained and the protein content was measured using the UV spectrophotometer. Then samples were denatured in road buffer (2% SDS; 10% glycerol; 62.5 mmol/L Tris-HCl; PH6.8) in a boiling water bath and separated by SDS-polyacrylamide gel electrophoresis (PAGE; 10% acrylamide) under non-reducing conditions, loading a fixed quantity (80 μg) of the total protein in each lane. The separated proteins were then electrophoretically transferred to nitrocellulose membrane (Millipore Corporation, Billerica). The membrane was incubated in 5% powdered milk to block non-specific binding, then was incubated with antibodies of target proteins, followed by incubation with horseradish peroxidase-conjugated IgG (Amersham Biosciences) as the secondary antibody. Then they were detected using the enhanced chemiluminescence (ECL) detection reagents (Thermo, USA) and exposed to X-ray films (Kodak).

Statistical Analysis

All data are expressed as mean±SD. Statistical analyses were performed using SPSS software version 13.0 (Chicago, IL, USA), and statistical significance was assessed through T-tests and one-way ANOVA with LSD post hoc comparisons. P < 0.05 was considered statistically significant in all analyses.

Results

Adhesion of COM crystals is facilitated by MDCK cell dedifferentiation

To determine whether dedifferentiation facilitated COM crystal adhesion to cells, MDCK cells were treated with transforming growth factor β1 (TGF-β1) (5 ng/ml), a potent stimulus of dedifferentiation, for 48 hours. We monitored cell dedifferentiate states. The phase-contrast microscope was used to observe cell morphology, and the expression of epithelial marker E-cadherin and mesenchymal marker Vimentin were examined by immunoblot analysis. Unstimulated MDCK cells exhibited a pebble-like morphology, but in response to TGF-β1, cells undergo transition into motile, myofibroblast-like morphology (Figure 1A). Results of immunoblot indicated that the expression level of E-cadherin decreased at 48h
after TGF-β1 exposure, in contrast, Vimentin expression was elevated significantly (Figure 1B and C). Thus MDCK cells could be dedifferentiated successfully by TGF-β1 stimulation.

Then we detected adhesion of COM crystals onto cells of different differentiate state. We removed TGF-β1, and 200 μg/ml AlexaFluor-488-tagged COM crystals were added into culture medium, followed by 5 min static time for adhesion. After washing, amounts of rudimental COM crystals were observed by fluorescence microscope to counting. There were more COM crystals adherent to dedifferentiated cells contrasted with normal cells (figure 2A and B). We also dissolved rudimental COM crystals by HCl, and volume of Ca^{2+} was assessed by flame atomic absorption spectrophotometry. Consistent with the results of counting, there was much larger Ca^{2+} harvested from dedifferentiated group (Figure 2C). These results suggested that cell dedifferentiation facilitated COM crystals adhesion.

**Effect of COM attachment on cell dedifferentiation**

We measured expressions of Occludin, E-cadherin and Vimentin after COM (50 μg/cm²) attachment using immunoblot analysis, to detect differentiate states of MDCK cells. Occludin is one of tight junctional proteins, loss of which is considered to be important to cell transdifferentiation. Occludin and E-cadherin expression decreased significantly at 48h (Figure 3A and B), and remain decreased at 72h after COM exposure (Figure 3A and C). Vimentin expression was elevated at 48h, and it remained elevated during experimental period compared to control cells (Figure 3). To sum up, phenotypic markers of dedifferentiation, accompanied with the loss of tight junctional protein, occurred at 48h after COM stimulation.

**Effect of Akt/GSK-3β and Snail signaling on COM attachment-induced dedifferentiation in MDCK cells**

Akt/GSK-3β signaling play an important role in cell transdifferentiation. To determine the mechanism of the COM attachment-induced dedifferentiation, we inspected whether Akt/GSK-3β signaling was involved in this process. As shown in figure 4A and B, COM attachment induced activation of Akt and inactivation (phosphorylation) of GSK-3β. They
were phosphorylated at 15 min and the increased phosphorylations maintained over 30 min past COM treatment. Then we detected Snail expression, which mediates the regulation of GSK-3\(\beta\) on dedifferentiation frequently [17]. COM increased Snail expression significantly (Figure 4 E and F). Pretreatment of LY294002 (5 \(\text{μmol/L}\)), a PI3K/Akt inhibitor, partially abolished the phosphorylation of Akt and GSK-3\(\beta\) (Figure 4C and D), reversed decrease in E-cadherin, increase in Vimentin and Snail expression as well (Figure 4E and F). In summary, Akt/GSK-3\(\beta\) and Snail signaling partially mediate COM attachment-induced dedifferentiation.

\textbf{L-carnitine protects MDCK cells from COM-induced dedifferentiation and subsequent COM crystals adhesion by reducing Akt/GSK-3\(\beta\) signaling activities and Snail expression}

Many disease-related renal fibroblasts originate from transdifferentiated tubular...
epithelia cells at the sites of injury [18], and L-carnitine has been studied for its protective effects against renal oxidative injury and fibrotic process [13, 19], so we set out to determine whether L-carnitine could alleviate COM-induced cell dedifferentiation.

About 2 hours prior to COM exposure, cells were pretreated with L-carnitine (2 mmol/L) and we examined the level of E-cadherin and Vimentin. The E-cadherin level was significantly decreased and the Vimentin level was increased in COM treated cells compared to the control cells, however, we found that these changes were significantly reversed led by L-carnitine pretreatment. E-cadherin and Vimentin levels of L-carnitine alone pretreated cells were similar with control cells (figure 5A and B), turning down the possibility of the effect of L-carnitine on differentiate states of normal cells. These results demonstrated that L-carnitine could ameliorate the degree of cell differentiation in the presence of COM attachment.

Since Akt/GSK-3β and Snail signaling mediated COM attachment-induced dedifferentiation, we investigated the effect of L-carnitine on Akt/GSK-3β and Snail signaling. As shown in figure 5C and D, the levels of p-Akt, and p-GSK3β were increased at 15 min past COM crystals administration, while they were decreased with combined COM and L-carnitine. L-carnitine partially reversed COM-induced upregulation of Snail expression as
well (figure 5A and B). These results indicated that L-carnitine cripples COM attachment-induced dedifferentiation by inactivating Akt/GSK-3β and Snail signaling.

The inhibitory effect of L-carnitine on subsequent COM crystals adhesion was detected also. After COM attached for 48 hours, MDCK cells had a stronger ability to bind subsequent AlexaFluor488-tagged COM crystals as compared to control cells, but L-carnitine pre-treatment attenuated this enhanced ability (Figure 6A). Results of crystals counting and detecting of volume of Ca^{2+} also indicated the inhibitory effect of L-carnitine on COM crystals adhesion (Figure 6B and C).

**Discussion**

CaOx crystals nucleation, aggregation, interaction with renal tubular epithelial cells are basic steps of calcium oxalate stone formation [20], and crystal-cell interaction is defined as an essential role [4]. When the concentrations of calcium and oxalate increased over the level...
of supersaturation, CaOx crystals were precipitated and attached to tubular epithelium or would be swept out by flowing urine. Animal and tissue culture studies have demonstrated that renal tubular epithelial cells injury, caused by risk factors, could promote CaOx crystal retention and developing into nidi, providing possibility of stone formation [21]. Normal renal tubular epithelial cells exist in a highly differentiated state, but after injury, surviving cells will undergo a transient process of dedifferentiation, representing a mesenchymal-like phenotype of immature epithelial cells, with ability of proliferation and migration, to regenerate tubular epithelium. Then they will redifferentiate back to a mature epithelial phenotype, with full polarity and tubular function [5]. In animal study, Asselman M et al. confirmed that the intratubular crystals were observed adherent to immature, regenerating and proliferating cell nuclear antigen (PCNA) positive tubular epithelial cells in ethylene glycol-administered rats [6], and in kidney transplant patients study, Anja Verhulst et al. shown that distal tubular cells proliferating/regenerating preceded crystal retention [7]. However, whether epithelial cell dedifferentiation was a reason for crystal retention, or was evoked by crystals retention, could not be concluded. These prompted us to investigate
the relationship among crystals attachment, epithelia cells differentiate state and further crystals adhesion. We presumed that if cell failed to switch on redifferentiation during epithelium repairing, the sustained dedifferentiate state might plays a crucial role in renal calculus formation.

In this study, we confirmed that COM attachment could induce E-cadherin suppression and Vimentin increasing, which were the molecular characteristics of epithelial cells undergoing dedifferentiation [22], and dedifferentiated cells induced by TGF-β1 bore higher adhesive power to COM crystals compared with the normal cells. These results indicated that COM attachment could induce cell dedifferentiation, and COM crystals could adhere to dedifferentiated cells more easily.

Since tubular epithelial cells dedifferentiation can intensify COM crystals adhesion, we deemed that cell dedifferentiation plays a potential role in CaOx stone development, and components which can modulate dedifferentiation or promote redifferentiation might be efficacious to prevent recurrence. We investigated the effect of L-carnitine on COM attachment-induced dedifferentiation and subsequent COM crystals adhesion. Although there were not any literatures paying attention to L-carnitine in renal tubular epithelial cells dedifferentiation, its protective effects against ROS and NADPH, which are usually activated after COM /oxalate stimulation [23, 24], have been reported abundantly. In current study, we discovered that with pre-treatment of L-carnitine, the loss of E-cadherin induced by COM attachment was inversed, and the increase of Vimentin was attenuated. There were fewer subsequent COM crystals adherent to group with combined COM and L-carnitine pre-treatment than group with COM alone. These results indicated that L-carnitine inhibits the process of COM attachment-induced dedifferentiation, and then prevented subsequent COM crystals adhesion efficaciously.

The mechanisms underlying inhibitory effect of L-carnitine on cell dedifferentiation were investigated as well. Akt/GSK3β/Snail signaling is one of common signaling mediate cell dedifferentiation [25]. We demonstrated that COM attachment activated Akt/GSK-3β and Snail signaling, PI3K/Akt pharmacological inhibitor could abolish these alters and inhibit dedifferentiation, indicating that Akt/GSK3β/Snail signaling plays an overarching role in COM-induced dedifferentiation. Relationships of L-carnitine and Akt/GSK3β have been reported to be contradictory in several pathogenesises [26-28], but whether L-carnitine is able to protect MDCK cells from COM-induced dedifferentiation through Akt/GSK3β/Snail signaling is uncertainty. In our study, L-carnitine attenuated the COM-induced activating of Akt/GSK3β/Snail signaling, considering Akt/GSK3β/Snail signaling as a contributor to the inhibitory effect of L-carnitine on cell dedifferentiation.

In a nutshell, there are two aspects involved: COM attachment-induced cell dedifferentiation and L-carnitine–induced anti-dedifferentiation response, which can be a part of the important hypothesis that tries to explain the mechanisms of COM crystals adhesion and the role of anti-dedifferentiated defense interventions in countering the positive feedback-like COM crystals adhesion, concluding from our investigation. L-carnitine is considered safe since it has been FDA-approved, and products containing L-carnitine are available as dietary supplements in the US [29]. Therefore, L-carnitine may has potential in prevention of renal calculi recurrence.

Conclusions

COM attachment facilitates subsequent COM crystals adhesion because of inducing MDCK cell dedifferentiation via Akt/GSK-3β and Snail signaling, and L-carnitine partially abolishes cell dedifferentiation and resists COM crystals adhesion. L-carnitine, may be used as a potential therapeutic strategy against recurrence of urolithiasis.
Disclosure Statement

The authors declare that they have no competing interests and nothing to disclose.

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