Calcium oxalate monohydrate crystals stimulate monocyte chemoattractant protein-1 and transforming growth factor β1 expression in human renal epithelial cells

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Abstract. Crystal-cell interactions play a key role in the formation of kidney stones. Few studies have referred to the role of monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor β1 (TGFβ1) in kidney stone formation. Recently, a genome-wide analysis of genes related to kidney stone formation and eliminiation in mice indicated that MCP-1 and TGFβ1 are involved in nephrolithiasis. In this study, in order to verify whether MCP-1 and TGFβ1 are involved in the process of crystal-cell interactions *in vitro*, we observed the effects of calcium oxalate monohydrate (COM) on MCP-1 and TGFβ1 expression in cultured HK-2 cells. HK-2 cells were treated with different concentrations of COM, and a group of untreated cells served as the control. The expression of MCP-1 and TGFβ1 was detected by western blot analysis after treatments with different COM concentrations (300, 500, 700 and 900 μg/ml) for different times (3, 6, 12 and 24 h). We found that the expression of MCP-1 was upregulated by COM treatment in a dose-dependent manner, and was increased initially at the first 6 h of treatment, then slightly decreased over time. Also, COM treatment resulted in a dose-dependent increase in TGFβ1 expression, and the expression levels peaked at 12 h. This study demonstrates that COM stimulates the expression of MCP-1 and TGFβ1 in renal epithelial cells.

Introduction

The formation of kidney stones is a complicated process involving multi-steps, in which the formation and attachment of crystals to the surface of renal tubular epithelial cells plays a key role (1,2). To avoid the formation and attachment of crystals, the kidney has developed several defense mechanisms acting at different levels. Physiologically, a high concentration of calcium itself is able to reduce antidiuretic hormone-stimulated water permeability of the collecting duct through the calcium sensing receptor, inducing an increased urinary volume and a reduced risk of supersaturation (3,4). Biochemically, a number of micro- and macromolecular urinary constituents (such as citrate, magnesium and proteins) increase the urinary supersaturation capacity and delay or prevent crystal formation, growth and aggregation (5-8). At the level of crystal-cell interactions, normal differentiated tubular epithelia have no affinity for crystals, and crystal retention can be prevented by coating crystals with urinary macromolecules (9,10).

However, it would be interesting to note what would happen if these kidney defense mechanisms were not sufficient and the crystals adhered to the tubular epithelium. Recently, Okada *et al* found that mice have a higher tolerance to calcium oxalate (CaOx) stone formation than rats, and the generated calcium stone formation and deposits could be eliminated after several days (11,12). Vervaet *et al* also reported the similar stone-elimination phenomenon in rats and humans and put forward a new concept, stone-elimination ability, as a factor regulating stone formation (13). These studies indicate that the kidney has an additional defense mechanism. Previously, a genome-wide analysis using DNA microarrays indicated the possible association of stone formation with the elimination process by the recruitment of monocytes/macrophages (11). Association analysis demonstrated several genes involved in the stone-elimination process, including chemokine ligand 2 (Ccl2), Cd44, colony-stimulating factor 1 (Csf1), matrix gla protein (Mgp), secreted phosphoprotein 1 (Spp1) and transforming growth factor β1 (Tgfb1) (12). Ccl2 encodes monocyte chemoattractant protein 1 (MCP-1). Umekawa *et al* reported the increased expression of MCP-1 in renal tubular cells exposed to CaOx monohydrate (COM) crystals (14,15). Tgfb1 encodes transforming growth factor β1 (TGFβ1). Few investigations have referred to the role of TGFβ1 in kidney stone formation. In order to verify whether MCP-1 and TGFβ1 are involved in the process of crystal-cell interactions, in this study, we observed the effects of COM on MCP-1 and TGFβ1 expression in cultured HK-2 cells.

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Materials and methods

Antibodies and chemicals. Antibodies for MCP-1, TGFβ1, GAPDH and peroxidase-conjugated secondary antibodies were purchased from Boster (Wuhan, China). COM was from Sigma-Aldrich (San Diego, CA, USA). Enhanced chemiluminescence (ECL) western blotting subtract was obtained from Haoranbio (Shanghai, China).

Cell culture and treatment. HK-2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Bovogen Biologicals (Melbourne, Australia). High glucose DMEM medium was purchased from HyClone (Logan, UT, USA). Serum-free medium was purchased from Invitrogen (Carlsbad, CA, USA). HK-2 cells were cultured in high glucose DMEM medium supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. For cell treatment, HK-2 cells were plated at 6-well plate for 24 h (~70% confluency) and then treated with serum-free medium containing different concentrations of COM (300, 500, 700 and 900 μg/ml) for 6 h. A group of untreated cells served as the control. In the time course treatment, the HK-2 cells were treated with 500 μg/ml COM for different times (3, 6, 12 and 24 h).

Western blot analysis. After treatment, cells were harvested, rinsed with PBS and lysed on ice in radio-immunoprecipitation assay (RIPA) buffer supplied with protease inhibitors. Equal amounts of protein were separated on a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and blotted onto nitrocellulose filter membranes. The membranes were blocked in a Tris-buffered saline solution with 5% non-fat dry milk containing 0.1% Tween-20 and incubated with antibodies overnight at 4˚C. Immunoreactive signals were detected by incubation with horseradish peroxidase-conjugated secondary antibodies followed by chemiluminescent detection using the ECL substrate kit. Band densities on the immunoblots were scanned and the relative band densities were normalized against anti-GAPDH blots. The band density from the control was set at a value of 1.

Statistical analysis. All experiments were repeated three times. Western blot analysis and immunostaining results are from a representative experiment. All measurement data are expressed as the means ± SEM. SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. The unpaired t-test was used for comparison between two means. P-values <0.05 were considered to show statistically significant differences.

Results

COM treatment upregulates MCP-1 expression. As shown in Fig. 1, the expression of MCP-1 was upregulated by COM treatment in a dose-dependent manner. In the time course treatment (Fig. 2), the expression of MCP-1 increased at the first 6 h of treatment. Subsequently, its expression slightly decreased over time; however, the expression of MCP-1 ramained significantly higher compared to the control at end-point treatment (24 h).

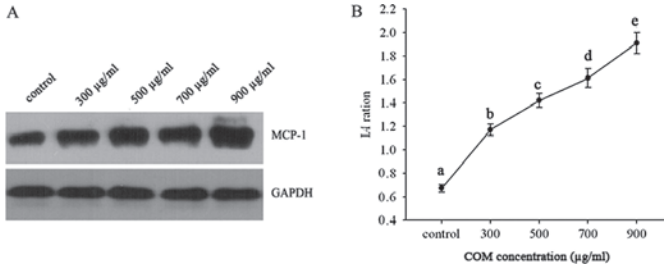

Figure 1. The expression of MCP-1 was upregulated by COM treatment in a dose-dependent manner. (A) Western blot analysis, (B) quantitative analysis. The values not sharing the same letter were significantly different (P<0.05).

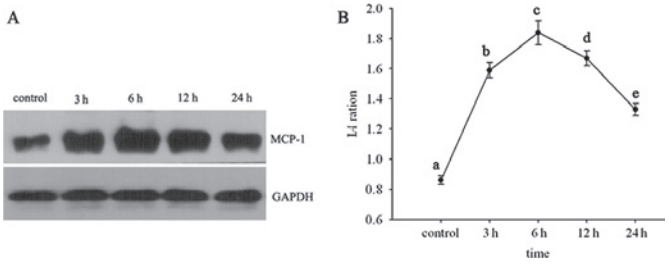

Figure 2. Expression of MCP-1 with different treatment times, which peaked at 6 h. (A) Western blot analysis, (B) quantitative analysis. The values not sharing the same letter were significantly different (P<0.05).

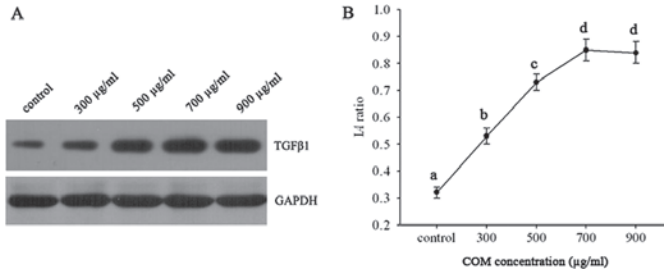

Figure 3. The expression of TGFβ1 was upregulated by COM treatment in a dose-dependent manner. (A) Western blot analysis, (B) quantitative analysis. The values not sharing the same letter were significantly different (P<0.05).

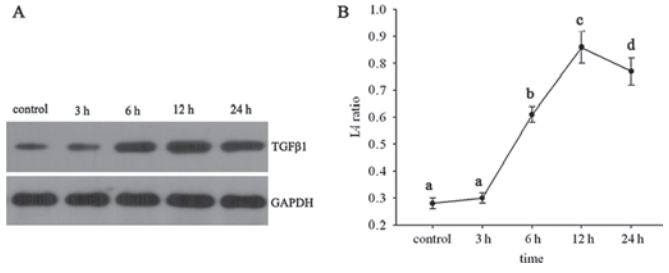

Figure 4. The expression of TGFβ1 with different treatment times, which peaked at 12 h. (A) Western blot analysis, (B) quantitative analysis. The values not sharing the same letter were significantly different (P<0.05).

COM treatment upregulates TGFβ1 expression. Similarly, COM treatment resulted in a dose-dependent increase in TGFβ1 expression (Fig. 3). In the time course treatment, TGFβ1 expression significantly increased at the time-points of 6 and 12 h, and peaked at 12 h. Its expression slightly decreased

at the end-point compared to the time-point of 12 h; however, it remained higher than the control (Fig. 4).

Discussion

Recent studies have revealed a novel defense mechanism of the kidney to avoid stone formation, post-adhesion crystal clearing (11-13). That means the progression of nephrocalcinosis not only depends on the extent, rate and duration of intratubular crystal adhesion, but is additionally countered by a stone-elimination mechanism, which may avoid, slow down or even reverse the progression towards stone formation. *In vivo* studies based on microarray analysis have indicated that monocyte-macrophage interaction participates in this phenomenon, and that the amount of both renal crystals and macrophages are highly associated with several gene expressions, including MCP-1 and TGFβ1 (11,12). CaOx is the most common type of kidney stone, in which COM is the predominant constituent (16). To further confirm the role of MCP-1 and TGFβ1 after crystal formation, we investigated the effects of COM crystals on the expression of MCP-1 and TGFβ1 with HK-2 human renal tubular epithelial cells. We found that HK-2 cells expressed the MCP-1 and TGFβ1 protein, and that the exposure of HK-2 cells to COM crystals resulted in a significant alteration of MCP-1 and TGFβ1 expression. The expression alteration occured in a dose- and time-dependent manner.

Chemokines represent a large family of chemotactic cytokines that are notable for inducing directional cell migration. To date, human chemokines have been classified into four subfamilies known as CXC, CC, XC and CX3C (C represents cysteine, X represents random amino acid), according to the primary structure of the protein. MCP-1 belongs to the CC subfamily, whose function is the recruitment of monocytes and T-cells into the arterial wall of the injured part (17,18). Umekawa *et al* reported the increased expression of MCP-1 in renal tubular cells exposed to COM crystals (14,15). MCP-1 induced the recruitment and migration of immunocytes, especially monocytes/macrophages, to inflammation sites caused by tissue injury. TGFβ1 belongs to the TGFβ superfamily, which is involved in cell growth regulation, extracellular matrix turnover and inflammatory reaction (19). Similarly, TGFβ1, as well as MCP-1, plays an important role in renal glomerular fibrosis (20,21). However, few investigations have referred to the role of TGFβ1 in kidney stone formation. Tsujihata *et al* reported the inhibitory effect of atrovastatin on kidney stone formation and considered that the suppression of TGFβ1-induced tubular damage and interstitial fibrosis could be caused by the inhibition of renal tubular cell injury and oxidative stress (22). Similar findings of TGFβ1-mediated renal damage and interstitial fibrosis were observed in unilateral ureteral obstruction (23).

Our data confirm that MCP-1 and TGFβ1 participates in the crystal-renal tubular cell interactions, which may regulate the kidney stone formation. Also, the expression change pattern between MCP-1 and TGFβ1, which was induced by COM treatment, was slightly different. The expression levels of MCP-1 peaked at 6 h, while the expression levels of TGFβ1 peaked at 12 h. This may be attributed to different activation pathways or sequences. However, the detailed pathways involved require further investigation.

In conclusion, our *in vitro* study provides evidence that MCP-1 and TGFβ1 are involved in the process of crystal-cell interactions, and the findings are in accordance with previous studies *in vivo*. The hypothesis is that MCP-1 and TGFβ1 participate in the post-adhesion crystal clearing mechanism by recruiting monocytes/macrophages. Thus, an *in vitro* study using co-cultured renal tubular cells and macrophages to detect the paracrine systems between cells is warranted.

Acknowledgements

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