Uric Acid-induced Phenotypic Transition of Renal Tubular Cells as a Novel Mechanism of Chronic Kidney Disease

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Abstract

Recent experimental and clinical studies suggest a causal role of uric acid in the development of chronic kidney disease. Most studies have focused on uric acid-induced endothelial dysfunction, oxidative stress and inflammation in the kidney. Direct effects of uric acid on tubular cells have not been studied in detail, and whether uric acid can mediate phenotypic transition of renal tubular cells such as epithelial-to-mesenchymal transition (EMT) is not known. We therefore investigated whether uric acid could alter E-cadherin expression and EMT in the kidney of hyperuricemic rats and in cultured renal tubular cells (NRK cells). Experimental hyperuricemia was associated with evidence of EMT before the development of significant tubulointerstitial fibrosis at 4 weeks, as shown by decreased E-cadherin expression and an increased α-smooth muscle actin (α-SMA). Allopurinol significantly inhibited uric acid-induced changes in E-cadherin and α-SMA with an amelioration of renal fibrosis at 6 weeks. In cultured NRK cells, uric acid induced EMT which was blocked by the organic anion transport inhibitor, probenecid. Uric acid increased expression of transcriptional factors associated with decreased synthesis of E-cadherin (snail and slug). Uric acid also increased the degradation of E-cadherin via ubiquitination, which is of importance since downregulation of E-cadherin is considered to be a triggering mechanism for EMT. In conclusion, uric acid induces EMT of renal tubular cells decreasing E-cadherin synthesis via an activation of snail and slug as well as increasing the degradation of E-cadherin.

Uric Acid; Chronic Kidney Disease; Renal Tubular Cells; epithelial-to-mesenchymal transition; E-cadherin
HYPERURICEMIA in chronic kidney disease (CKD) has long been viewed as a consequence of decreased renal function rather than a risk factor for the development or progression of renal disease (8). However, recent epidemiologic studies suggest that uric acid per se might predict the development of kidney disease in healthy individuals and subject with impaired renal function (2, 9, 18, 35, 42, 50). Pilot treatment studies also reported that lowering serum uric acid levels decreased blood pressure and retarded the aggravation of renal function in patients with established kidney disease (13, 25, 46). While further clinical trials are necessary before implementing uric acid-lowering therapies in subjects with CKD. In addition, more basic science evidence is also needed to identify potential mechanism(s) by which uric acid may induce renal disease.

Many factors involved in the causation of renal disease have direct effects on renal tubules. Some profibrogenic factors induce phenotypic transition of renal tubules in vitro, which is termed epithelial-to-mesenchymal transition (EMT). EMT is characterized by a decrease in the expressions of E-cadherin and zonula occludens protein (ZO-1) with an acquisition of de novo α-smooth muscle actin (α-SMA) expression, and is associated with the development of renal fibrosis (29, 30, 55). EMT has also been demonstrated in vivo by Strutz et al. (47) as well as others (14, 22, 52, 56), although this has been challenged by some investigators (14, 55). Various stimuli such as high glucose, hypoxia, profibrotic cytokines and inflammatory mediators are known to trigger the signals to activate transcription factors for down-regulation of the expression of epithelial cell markers in tubular epithelial cells, which in turn induce phenotypic transformation (12, 17). Importantly, EMT is one of the earliest phenomena of renal fibrosis and has been considered a therapeutic target due to its reversible characteristic (12, 14, 30).

To further understand the mechanism of uric acid-associated kidney disease, we investigated the effect of uric acid on phenotypic transition of renal tubules in an animal model of hyperuricemia and also cultured renal tubular cells with an elucidation of the mechanism of E-cadherin down-regulation. We also examined whether uric acid-lowering therapy ameliorated uric acid-induced EMT in the kidney.
MATERIALS AND METHODS

Reagents. All chemicals and tissue culture plates were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and Nunc Labware (Waltham, MA, USA), unless otherwise stated. Hyperuricemia was induced by feeding the rats with the diet containing 2% oxonic acid (OA, hepatic uricase inhibitor; Sigma, St. Louis, MO, USA). Allopurinol (xanthine oxidase inhibitor, Schein Pharmaceutical, Florham Park, NJ, USA) was dissolved in drinking water at the concentration of 13 mg/dL. Uric acid (Ultrapure, 1-6 mg/dL, Sigma) was dissolved in warmed media and filtered. Crystals were not detectable (polarizing microscopy), nor did they develop during cell incubation. Endotoxin was not detectable in all batches of uric acid (Limulus Amebocyte Assay, BioWhittaker Inc, Walkersville, MD), which indicated <0.015 endotoxin U/ml. Mycoplasma contamination was also excluded (Immu-Mark Myco-Test, ICN Biomedicals, Irvine, CA). Uric acid level of control media containing 5% fetal bovine serum (FBS) was below the detection limit (<0.22 mg/dL) of uric acid assay kit (Quantichrome™ Uric Acid Assay Kit, Bioassay Systems, Hayward, CA).

In-vivo experimental protocol. Fifty-four male Sprague-Dawley rats (200-240 g; Central Lab. Animal Inc., Seoul, Korea) were randomized into 3 groups (n=18 per each group) after measuring body weight, blood urea nitrogen, creatine and uric acid (Control group with normal rat chow; hyperuricemia group with 2% OA; hyperuricemia with allopurinol group). 2% OA could be given without apparent toxicity to rats according to our previous studies (26, 31), and there was no mortality during the study period. The dose of allopurinol was determined on the basis of the uric acid-lowering effect in previous studies (26, 31). The mean daily intake of allopurinol was 24.5 ± 7.5 mg/kg/day. At 2, 4 and 6 weeks, 6 rats per each group were sacrificed for an assessment of renal function and histologic evaluation of kidney. All animal procedures were approved by the Animal Care Committees of Ewha Womans University School of Medicine.

Uric acid, proteinuria, and renal function. Serum uric acid concentration was determined by a carbonate phosphotungstate method and uric acid standard (26, 31).
Blood urea nitrogen and creatinine were determined colorimetrically utilizing a commercial kit.

**Renal morphology and immunohistochemistry.** Tissue for light microscopy and immunoperoxidase staining was fixed in Methyl Carnoy’s solution and embedded in paraffin. Four-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. Indirect immunoperoxidase staining of 4-µm sections was performed as described previously (26), with monoclonal antibodies directed to the following antigens: E-cadherin with mouse monoclonal (BD Biosciences, Franklin Lakes, NJ), α-smooth muscle actin (α-SMA) (Sigma, St. Louis, MO), vimentin (Millipore, Billerica, MA) and collagen III (Sigma, St. Louis, MO). Controls included omitting the primary antibody and substitution of the primary antibody with preimmune mouse serum. All analyses were performed blinded. Tubulointerstitial fibrosis score (0 to 5) was evaluated on the basis of PAS staining as described previously (26). Percent positive area of the expression of E-cadherin, α-SMA and vimentin in the tubules of renal cortex was analyzed using computer image analyzer (OPTIMAS 6.5, MediaCybernetics, Bothell, WA). Vascular α-SMA and glomerular vimentin expressions were excluded in analyzing the positive area for α-SMA and vimentin.

**Cell morphology and fluorescent immunocytochemistry.** Rat renal tubular epithelial cells (NRK-52E cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). NRK-52E cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% FBS, 60 μg/ml penicillin. Cells were maintained at 37°C in a humidified 5% CO2/95% air atmosphere incubator. Fresh growth medium was added to the cells every 2 days until cells reached an adequate confluency for each experiment. The uric acid concentration for the in-vitro experiments was determined by the consideration of normal level of uric acid in rats and the results of the cell proliferation and LDH assay. Uric acid at the concentrations of 1-6 mg/dL did not induce the changes in cell proliferation and LDH release (data not shown). After the stimulation of cells with uric acid for 1 to 4 days, morphological changes of the NRK-52E cells were examined under an inverted phase contrast microscope (Axiovert 200;
Carl Zeiss, Oberkochen, Germany), and the images were obtained by digital camera (AxioCam HRC; Carl Zeiss, Germany). For immunofluorescence staining, cells were washed and fixed in 4% phosphate-buffered paraformaldehyde [25 min at room temperature (RT)] and permeabilized with 0.2% triton X-100 in phosphate-buffered saline (PBS) (15 min at RT). After washing with PBS and blocking with 5% BSA for 1 h, cells were incubated with primary antibodies specific for E-cadherin as a marker of epithelial cell or α-SMA as mesenchymal cell marker in 5% BSA overnight at 4°C, followed by an incubation with goat anti-mouse IgG-FITC-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature in the dark. The nucleus was counter-stained with DAPI, and the cells were visualized under the Axiovert 200 fluorescence microscope (Carl Ziss, Oberkochen, Germany).

**Western blot analysis.** Protein samples isolated from cell lysate or homogenates of renal cortex were mixed in reducing buffer, boiled, resolved on 10% SDS-PAGE gels, and transferred to a polyvinylidene difluoride membrane by electroblotting. Membranes were blocked in 5% wt/vol nonfat milk powder in Tris-buffered saline for 30 min at room temperature. Then, blots were incubated overnight in blocking solution with primary antibodies at 4°C. Antibodies to E-cadherin (BD Biosciences, Franklin Lakes, NJ), α-SMA, Snail (Cell Signaling Technology, Beverly, MA), Slug (Santa Cruz Biotechnology, Santa Cruz, CA) and Twist (Santa Cruz Biotechnology, Santa Cruz, CA) were used. After washing the blot with Tris-buffered saline with Tween 20, the blots were incubated with Horseradish peroxidase–conjugated secondary antibodies corresponding to each primary antibody followed by enhanced chemiluminescence detection (Santa Cruz Biotechnology, Santa Cruz, CA). Positive immunoreactive bands were quantified by densitometry and compared with the expression of human β-actin.

**Real time PCR.** The levels of transcripts were determined by real time PCR (RT-PCR) on the ABI PRISM 7000 sequence detection system using SYBR Green I as a double-stranded DNA-specific dye according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). The PCR reaction was carried out in 5 µM of cDNA, 10 µM of SYBR Green PCR master mix, and 5 pM of sense and antisense primers of E-cadherin (Forward primer: AACGAGGGCATTTCTGAAAACA, Reverse
primer: CACTGTC ACGTGCAGAATGTACTG) or α-SMA (Forward primer: GACCCTGAAGTATCCGATAGAACA, Reverse primer: CACGCGAAGCTCGTTA TAGAAG) for a final volume of 20 µM per reaction. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. The relative mRNA expression levels of the target genes in each sample were calculated using the comparative CT method. The CT value is the cycle number at which the fluorescence signal is greater than a defined threshold. At least three independent PCR procedures were performed to allow statistical analysis. The amount of PCR products was normalized with the house-keeping gene, β-actin, to determine the relative expression ratios for each mRNA in relation to the control group.

Detection of E-cadherin ubiquitination and degradation. NRK-52E cells were seeded overnight in 5% FBS/DMEM, and then cells were rinsed twice with phosphate-buffered saline before switching to uric acid-containing DMEM. At 24 and 48 hours of uric acid exposure, whole cell lysates were subjected to immunoprecipitation using an anti-E-cadherin antibody. Ubiquitinated E-cadherin was detected by Western blot with anti-ubiquitin antibody.

Statistical analysis. All data are presented as mean ± SD. Differences in parameters at each time point and concentration of uric acid were compared by paired t test. Differences in the various parameters between groups were evaluated by two-way ANOVA followed by correction for multiple comparisons. Significance was defined as $P < 0.05$. 

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RESULTS

Serum uric acid, BUN, creatinine and renal pathology in OA-treated rats. The administration of the uricase inhibitor, oxonic acid (OA, 2%) to rats resulted in a mild increase in serum uric acid level from 2 weeks of OA treatment with significant hyperuricemia at 4 weeks (2.48 vs. 0.97 mg/dl, OA vs. control, \( p < 0.01 \), Fig. 1A). Blood urea nitrogen and creatinine levels were significantly increased at 6 weeks in the hyperuricemic rats, and were ameliorated with allopurinol treatment (Fig. 1, B and C). The kidneys of OA-treated rats showed no significant pathologic changes on PAS staining at 4 weeks, however focal interstitial fibrosis with tubular dilatation and shrinkage was observed at 6 weeks of OA treatment (Fig. 2). Interstitial fibrosis was evident in immunohistochemistry of type III collagen (Fig. 2, E-G). An increase in tubulointerstitial fibrosis score in hyperuricemic rats was prevented in rats treated with allopurinol (Fig. 1D and Fig. 2).

EMT in the kidneys of OA-treated rats: Expression of E-cadherin, \( \alpha \)-SMA and vimentin. The expression of the epithelial cell marker, E-cadherin, was evident both in cell membrane and cytoplasm in renal cortical tubules of normal rats as shown in Figure 3A. OA-treated rats demonstrated a decrease in E-cadherin expression at 4 weeks when tubulointerstitial changes were not evident yet (Fig. 3B). A decrease in E-cadherin expression was more evident in proximal tubules which was identified by the presence of brush border and a lower number of nuclei per unit area (denotes in P in Fig. 3A), however E-cadherin expression was also decreased in other cortical tubules in hyperuricemic rats (percent positive are of E-cadherin in renal cortex; 48.5±11.9% vs. 12.5±5.5%, control vs. hyperuricemic rats, \( p < 0.01 \)). At the same time, some tubules showed \textit{de novo} staining of \( \alpha \)-SMA and vimentin (Fig. 3, F and J), the markers of mesenchymal cells. These findings suggested uric acid-induced phenotypic transition of renal tubular cells before the development of significant tubulointerstitial fibrosis. At 6 weeks of OA treatment, an altered expression of epithelial and mesenchymal cell markers became more evident (Fig. 3, C, G, and K), which were inhibited by allopurinol treatment (Fig. 3, D, H, and L). Interestingly, the expression of \( \alpha \)-SMA and vimentin...
was found both in renal tubules and in the interstitium, suggesting an accumulation or migration of myofibroblasts into interstitial areas. Consistent with IHC staining, Western blot of the renal cortex showed an altered expression of E-cadherin and α-SMA (Fig. 4), suggesting the presence of EMT before the development of significant tubulointerstitial fibrosis. Allopurinol significantly ameliorated uric acid-induced changes in E-cadherin and α-SMA.

Effect of uric acid on cell morphology of NRK cells. Uric acid (6 mg/dL) induced the changes in cell morphology of NRK cells from 24 hours of stimulation from a typical cobblestone shape to elongated spindle-shaped cells with a loss of cell-to-cell contact (Fig. 5), which became more evident with prolonged exposure to uric acid.

Effect of uric acid on the expression of epithelial and mesenchymal cell markers. In association with morphologic transition, we also examined the expression of E-cadherin as a marker of epithelial cell and α-SMA as an indicator of mesenchymal phenotype. There was abundant cellular expression of E-cadherin primarily at the cell membrane in unstimulated renal tubular cells (Fig. 6A) whereas both membranous and cytoplasmic E-cadherin expressions were significantly decreased in uric acid-stimulated cells (Fig. 6B). In addition, uric acid induced de novo expression of α-SMA (Fig. 6D) in contrast to almost negative staining in untreated cells (Fig. 6C). Western blot analysis also demonstrated uric acid-induced alteration in the expression of E-cadherin and α-SMA. With 3 mg/dl of uric acid, the expressions of E-cadherin were decreased to approximately 40% of those in control cells at 48 hours (p< 0.05 vs. control) (Fig. 7, A and D). In addition, uric acid markedly up-regulated α-SMA expression from 48 hours in a dose- and time-dependent manner (p<0.05 vs. control) (Fig. 7, A, B, D, and E). Importantly, pre-treatment with probenecid (1 mM) significantly blocked the uric acid-induced changes in the expression of E-cadherin and α-SMA in NRK-52E cells (p<0.05 vs. uric acid-treated cells) (Fig. 7, C and F), suggesting that uric acid per se entering into cells was responsible for uric acid-induced EMT. Consistent with the changes in protein expression of E-cadherin in uric acid-stimulated renal tubular cells, mRNA expression of E-cadherin was decreased in NRK cells 6 hours (Fig. 8A). There was also

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a significant increase in α-SMA mRNA expression in NRK cells exposed to uric acid at 6 and 24 hours (Fig. 8, C and D).

*Effect of uric acid on the expression of Snail, Slug, Twist and E-cadherin degradation.* To understand the mechanism of uric acid-induced down-regulation of E-cadherin, we examined the changes in the expression of snail, slug and twist, which are major transcription factors of E-cadherin. Uric acid increased the expression of snail and slug whereas it did not induce the change in twist expression (Fig. 9A). Uric acid also increased the degradation of E-cadherin (Fig. 9B). E-cadherin degradation products, shown as two lower molecular weight protein bands, were found in uric acid-stimulated cells. We next tested whether E-cadherin was ubiquitinated on uric acid stimulation. Significant ubiquitination of immunoprecipitated E-cadherin was observed in NRK cells exposed to uric acid for 24 hours, suggesting uric acid-induced E-cadherin degradation via ubiquitination can be another mechanism of E-cadherin down-regulation in addition to a decreased transcription of E-cadherin.
DISCUSSION

The novel finding of our study is that uric acid induces phenotypic changes in cultured tubular epithelial cells and in the renal tubules of an animal model of hyperuricemia. Previous studies from our group and others demonstrated uric acid-induced endothelial dysfunction and local inflammation in the kidney as major mechanisms of renal disease (6, 11, 26, 31, 54). This study suggests that uric acid also has direct effects on renal tubules as another potential mechanism of uric acid-induced renal disease. Probenecid, which blocks the entry of soluble uric acid into cells, inhibited uric acid-induced EMT, and allopurinol treatment also ameliorated an alteration of E-cadherin and α-SMA expression in oxonic acid-treated animal, which suggested uric acid *per se*, was responsible for phenotypic transition of renal tubular cells.

Experimental and clinical studies support a role for uric acid in the progression of renal disease independent of urate crystal deposition or hypertension (19, 26). Epidemiological studies have shown that serum uric acid is an independent risk factor for the development of chronic kidney disease. In one Japanese study, hyperuricemia conferred a 10.8-fold increased risk in women and a 3.8-fold increased risk in men for the development of CKD compared to those with normal uric acid levels (21). The higher relative risk in the hyperuricemic subject was independent of age, body mass index, blood pressure, total cholesterol, serum albumin, glucose, and proteinuria.

Previous studies have suggested that hyperuricemia may induce renal injury by causing afferent arteriolar thickening and rarely hyalinosis of the preglomerular arterioles of the kidney, often accompanied by glomerular hypertrophy (26, 31, 33). Further studies showed that uric acid induced endothelial dysfunction via an activation of local renin-angiotensin system (RAS) with an induction of oxidative stress and local inflammation (27, 28, 49, 54). Collectively, uric acid-induced renal disease has been explained by endothelial dysfunction which is associated with glomerular hypertension with a reduction in renal plasma flow that could lead to renal injury (43).

To date, few studies have considered whether there could be direct tubular effects of uric acid. One study showed uric acid-induced inhibition of tubular cell proliferation *in vitro* (16) and a second study demonstrated an up-regulation of ERK,
BAX and α-SMA in HK-2 cells exposed to uric acid using SILAC coupled to LC-MS (39). Therefore, our observation of early development of EMT of renal tubules provides an important insight regarding the mechanism of progression of renal disease in hyperuricemia. In this study, phenotypic transition of renal tubules was found before the development of renal dysfunction and significant tubulointerstitial fibrosis in hyperuricemic rats, and ameliorated by uric acid-lowering therapy. After 4 weeks of OA treatment, the expression of α-SMA and vimentin was observed in the cytoplasm of some renal tubules along with a decrease in E-cadherin expression suggesting a phenotypic transition to mesenchymal cells from epithelial cells, and it became evident at interstitial area with the development of fibrosis in 6 weeks. In cultured renal tubular cells, a loss of cell adhesion was the first finding observed as early as 24 hours of uric acid exposure, which became evident with morphologic change into elongated fibroblastoid cells. Phenotypic transition of NRK cells was associated with de-novo acquisition of α-SMA as well as a significant decrease in E-cadherin.

Although our data suggests uric acid is responsible for the EMT of renal tubules, the possibility of oxonic acid-related phenotype transition in animal model of hyperuricemia cannot be ruled out. However, our in-vitro data showed oxonic acid (up to 1 mM of concentration) did not induce EMT in several different renal tubular cell lines (data now shown). In addition, the beneficial effect of xanthine oxidase inhibitor, allopurinol, could be attributed to lowering uric acid or other effects of allopurinol including its anti-oxidant action. Furthermore, we have previously reported that both xanthine oxidase inhibitors and uricosuric agents can ameliorate renal fibrosis in the hyperuricemic remnant kidney model(26). Therefore, our data suggests that it is not oxonic acid but rather uric acid that is responsible for EMT, and lowering uric acid by inhibiting uric acid synthesis or by enhancing uric acid excretion alleviates EMT of renal tubules.

E-cadherin down-regulation was originally regarded as a marker of EMT; however it is now thought to be an important initial step in the transdifferentiation of epithelial cells to a mesenchymal phenotype (3, 37). Expression of E-cadherin is a hallmark of a fully differentiated epithelium where it functions to maintain cell-cell junctions, thereby inhibiting aberrant cell proliferation and migration. Indeed, a loss of E-cadherin function via siRNA-mediated knockdown resulted in loss of cell contacts.
and cell scattering with an acquisition of elongated, fibroblast-like morphology in mammary epithelial cells (15, 34, 37, 51). Importantly, genetic inactivation of E-cadherin per se induced an expression of mesenchymal protein such as N-cadherin and vimentin (37). Genetic or epigenetic silencing of E-cadherin gene of cancer cells was shown to promote the acquisition of invasive phenotype, thereby driving systemic metastasis via EMT (3, 15, 34, 37, 51). These findings suggest the loss of E-cadherin acts as a pleiotropic regulator of cell phenotype, enabling it to function as a master regulator of cell behavior. Therefore, uric acid-induced down-regulation of E-cadherin of renal tubular cells may have a critical role in initiating the EMT process and causing renal fibrosis.

Expression of E-cadherin is regulated by complex network in transcription, translation and post-translational modification. Several transcription factors have been reported to be involved in EMT via repression of E-cadherin, which include Snail, Slug and Twist (4, 5, 36, 44). Previous studies have shown that overexpression of Snail and Slug lead to a reduction of E-cadherin expression (4, 5, 36, 44). Snail and slug are snail family of zinc finger transcription factors which have been shown to play a critical role in EMT to determine cancer invasiveness and chemo-responsiveness (1, 48). Similarly, an over expression of Twist, a transcription factor containing a helix-loop-helix DNA binding domain, also results in decrease of E-cadherin expression (41). In this study, E-cadherin down-regulation in uric acid-stimulated NRK cells was associated with a decreased transcription of E-cadherin mRNA and an increased activation of Snail and Slug, but not with Twist overexpression. TGF-β is reported to down-regulate the expression of E-cadherin by inducing snail, slug and twist in various epithelial cells and cancer cells (7, 53, 58), however differential regulation of these E-cadherin repressors has been demonstrated in different cells exposed to various stimuli (32, 37, 58).

We also demonstrated enhanced degradation of E-cadherin by uric acid. A decrease in E-cadherin expression in uric acid-stimulated NRK cells was associated with an increase in the degradation of E-cadherin. During EMT, an initial decrease in E-cadherin levels likely occurs by lysosomal degradation (23, 38, 45). Lysosomal degradation was known to be associated with ubiquitination of E-cadherin (45), which was also found in uric acid-stimulated NRK cells in this study. Our data demonstrated
uric acid-induced downregulation of E-cadherin mRNA as early as 6 hours, followed by E-cadherin ubiquitination and degradation at 24 and 48 hours, respectively.

Although EMT of renal tubular cells has been regarded as one of the earliest phenomenon of renal fibrosis, there remain controversies whether EMT is actually a source of interstitial fibroblasts (55, 57). It is mainly due to an insufficient in-vivo evidence of EMT (10, 14, 20), and is also related to a misinterpretation of EMT which can be traced to the belief that an epithelial cell undergoing EMT should become a new fibroblast. However, the definition of EMT does not necessarily require the completion of phenotype transition to a specific cell type. EMT is defined by the process of phenotypic and functional changes that are reminiscent of mesenchymal cells. From this point of view, there is sufficient evidence to assert that at least some EMT markers are expressed during renal fibrosis including in human kidney disease (12, 14, 17, 24, 40, 56). Such biomarkers are very useful compared with conventional histology, because they are sensitive and are expressed at an early stage of the disease. Therefore, uric acid-induced EMT in renal tubules may represent one of the earliest findings in patient with hyperuricemia, which could help in identification of patients at high-risk for developing renal fibrosis. Future studies are needed to determine if EMT in the kidneys of hyperuricemic patients could also represent either a therapeutic target or an indicator of therapeutic response.

In conclusion, uric acid, at physiological concentrations, can induce phenotypic transition of renal tubular cells via both a decreased synthesis and an enhanced degradation of E-cadherin, thereby initiating EMT of renal tubules and interstitial fibrosis. In addition to well-recognized mechanisms of uric acid-induced renal disease including endothelial dysfunction, altered glomerular hemodynamics and an enhanced local inflammatory reaction in the kidney, uric acid-induced EMT of renal tubular cells may be another mechanism explaining the association of hyperuricemia and progression of renal disease.
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GRANTS
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DISCLOSURES
Dr. Richard J Johnson discloses that he has a patent for allopurinol in the treatment of hypertension and also has patent applications related to lowering uric acid as a means for treating diabetes, obesity and diabetic nephropathy. He is also author of the book, The Fat Switch (Mercola.com) that discusses the potential role of fructose and uric acid in the epidemics of obesity, diabetes, and kidney disease.

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Figure legends

Fig. 1. Changes in serum uric acid, BUN, creatinine and renal fibrosis in hyperuricemic rats. Serum uric acid level increased from 4 weeks after oxonic acid with an elevation of BUN and creatinine at 6 weeks. Tubulointerstitial fibrosis became evident from 6 weeks. Allopurinol treatment significantly ameliorated the changes in uric acid and renal fibrosis in oxonic acid-fed rats. (N=8). *p<0.05 vs. 0 and 2 weeks, #p<0.05 vs. 0, 2 and 4 weeks.

Fig. 2. Renal pathology in hyperuricemic rats. In oxonic acid (OA)-fed rats, there were no significant morphologic changes except mild glomerular hypertrophy at 4 weeks (B) compared to control rats (A). Linear interstitial fibrosis with tubular dilatation and shrinkage became evident from 6 weeks (C), which was markedly ameliorated in allopurinol (Allo)-treated rats (D). Collagen type III immunohistochemistry revealed mild interstitial fibrosis at 6 weeks (G) compared to control (E), OA 4 weeks (F), OA 6 weeks+allopurinol (H). Magnification X100 (A-D), X200 (E-H).

Fig. 3. Phenotypic transition of renal tubules in hyperuricemic rats. Representative immunohistochemistry of E-cadherin (A-D), α-SMA (E-H) and vimentin (I-L) staining in control rats (A, E, I) and rats in 4 weeks (B, F, J) and 6 weeks (C, G, K) of OA feeding. E-cadherin staining in renal tubules was markedly decreased in proximal tubules (P) and other cortical tubules shown as either faint cytoplasmic or membranous staining in 4 weeks (B) and 6 weeks (C) compared to control rats (A). α-SMA was expressed in vascular smooth muscle of arteriole (arrowheads) in control (E) and hyperuricemic rat kidney (F, G). However, some tubular cells expressed α-SMA in cytoplasm of renal tubules (F, arrows) and interstitial area (G, arrows). Vimentin was also expressed in tubulointerstitial area in hyperuricemic rats (J, K) in contrast to control rats. Allopurinol treatment ameliorated an altered expression of E-cadherin (D), α-SMA (H) and vimentin (L) in hyperuricemic rats (6 weeks).

Fig. 4. Expression of E-cadherin and α-SMA in renal cortex. Representative western blot (A) and its quantitation analysis (B) demonstrated a decreased E-cadherin

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expression in 4 weeks of OA feeding (OA4) with an increased α-SMA, which was more
evident in 6 weeks of OA (OA6). Allopurinol (Allo) ameliorated the changes in E-
cadherin and α-SMA. *p<0.05 vs. control (C) and OA6, #p<0.05 vs. others, †p < 0.05 vs.
C and OA6+Allo.

Fig. 5. Effect of uric acid on cell morphology in NRK-52E cells. NRK-52E cells
displayed typical cobblestone appearance at different stages of confluency from day 1 to
day 6 after seeding (A-D). Uric acid (6 mg/dl) induced a dissociation of cell adhesion at
day 1 (E) and day 2 (F), followed by a phenotypic transition to elongated fibroblast-like
cells from cuboidal clustered epithelial cells, which was more evident at day 4 (H).
Magnifications: × 100.

Fig. 6. Fluorescence immunocytochemistry for E-cadherin and α-SMA in NRK-
52E cells. NRK-52E cells exhibited abundant E-cadherin expression along cell
membrane (A, red) and negligible α-SMA expression (B). Following treatment with uric
acid for 48 hours, E-cadherin immunoreactivity was decreased (C) whereas de-novo
expression of α-SMA was substantially increased in cytoplasm of NRK-52E cells (D,
red). Nuclei were counter-stained with DAPI. Magnifications: × 100.

Fig. 7. Dose- and time-dependent effect of uric acid on the expression of E-
cadherin and α-SMA in NRK-52E cells. Representative western blot demonstrated
uric acid-induced alteration in E-cadherin and α-SMA (A, B). Uric acid induced a dose-
dependent changes in the expressions of E-cadherin and α-SMA from 48 h (N=5).
Probenecid (P) significantly inhibited uric acid-induced changes in E-cadherin and α-
SMA (C). Quantitative analysis was shown in D-F. Con, control; UA, uric acid. *p<
0.05 vs. 0NA expression of E-cadherin and α-SMA in NRK-52E cells. E-cadherin
mRNA expression was significantly decreased at 6 (A) and 24 hours (B) in uric acid-
stimulated cells whereas α-SMA mRNA was up-regulated at 6 (C) and 24 hours (D,
N=5). *p<0.05 vs. 0 and 1 mg/dL of uric acid, #p<0.05 vs. 0, 1, 3 mg/dL of uric acid.

Fig. 9. Effect of uric acid on the expression of snail, slug, twist and E-cadherin
degradation. Uric acid increased snail and slug expression from 3 and 6 mg/dL of uric
acid, respectively at 6 hours (A). Twist expression was comparable in control and uric acid-stimulated cells (A). Uric acid also increased the degradation of E-cadherin (arrows, B) at 48 hours and ubiquitination (C) at 24 hours. Representative Western blottings of E-cadherin, snail, slug and twist are shown. *p<0.05 vs. 0 mg/dL of uric acid.
Figure 1

(A) Serum Uric Acid (mg/dL) vs. Weeks
(B) BUN (mg/dL) vs. Weeks
(C) Creatinine (mg/dL) vs. Weeks
(D) Tubulointerstitial Fibrosis Score vs. Weeks

Figure 1
Figure 2
Figure 3
Figure 4

A

E-cadherin

α-SMA

β-Actin

Control | OA, 4 weeks | OA, 6 weeks | OA+Allo, 6 weeks

B

E-Cadherin (% control)

α-SMA (% control)

C | OA4 | OA6 | OA6+Allo

C | OA4 | OA6 | OA6+Allo

* | # | *

† | † | #
Figure 5
Figure 7
Figure 8
Figure 9