Krüppel-like factor 5 associates with melamine-cyanurate crystal-induced nephritis in rats

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ABSTRACT

Background. Melamine and cyanuric acid (M/CA), when orally administered together to rats, can induce crystal formation within renal tubules and cause acute kidney injury. Methods. To investigate the pathomechanism of crystal-induced nephritis, melamine and/or cyanuric acid were administered to 3-week-old (young) and 8-week-old (adult) rats, respectively. Results. Crystal formation, blood urea nitrogen elevation, tubular cell injury and macrophage infiltration were noted in rats fed with M/CA, but not in rats fed with vehicle, melamine or CA alone. These parameters were significantly higher in young rats than those in adult rats fed with M/CA 200 mg/kg body weight (BW) for 3 days. Krüppel-like factor 5 (KLF5) was expressed on distal tubule cells, especially when crystals deposited within the lumens. Both mRNA and protein levels were higher in young rats than those in adult rats fed with M/CA (200 mg/kg BW). KLF5 expression has been shown to modulate renal tissue cytokine production, and we found that proinflammatory cytokines like monocyte chemoattractant protein-1 and interleukin-6 were increased in kidney tissues of young rats fed with M/CA for 3 days. In contrast, interleukin-10, an anti-inflammatory cytokine, was upregulated in kidneys of adult rats fed with M/CA for 3 days. Conclusions. Crystals are prone to deposition in distal tubules of young rats fed with M/CA. M/CA Crystal-related nephritis might be induced by the KLF5 expression, which modulated macrophage recruitment and proinflammatory cytokine production, subsequently leading to renal tubular injury and interstitial inflammation.

INTRODUCTION

Adulteration of a powdered formula with melamine led to a global panic in 2008 [1], and the kidneys of infants and children were severely injured [2–4] but rarely harmed in adults [5]. It has been studied that consumption of melamine with cyanuric acid (M/CA) in animals led to renal calculi and obstructive nephropathy. Investigators also found that acute nephritis, characterized by intratubular crystal depositions and tubular epithelial cell necrosis occurred following administration of M/CA to fish, pigs, dogs, cats and mice [6–9]. Although the pathomechanism of M/CA-related nephritis...
may be incomparable with that in humans, the potential impact of nephrotoxicity on young victims remains uncertain.

In the hyperoxaluric rat model, calcium oxalate crystals within the renal tubules induced monocyte–macrophage migration into the interstitium of kidney [10, 11]. Macrophage is the major cell that accumulates around tubules in rodent kidneys after renal injury [12], enhances cytokine secretion and regulates tissue remodelling during nephritis [13, 14]. Recently, Fujiu, Manabe and Nagai [15] found that Krüppel-like factor 5 (KLF5), mainly expressed in collecting duct epithelial cells, plays a pivotal role of a regulator in response to the renal injury. KLF5 initiates the accumulation of M1 macrophages and regulates the inflammation of tubulointerstitium in the unilateral ureteral obstruction (UUO) mouse model through the production of the secretory proteins SI100A8 and SI100A9. We, therefore, conducted a series of experiments to investigate the age-related nephrotoxicity of M/CA in Sprague–Dawley rats aged 3 and 8 weeks old. We further assessed the pathogenic inter-relation between KLF5 expression in distal tubular cells and M/CA crystal-induced nephritis.

**Materials and Methods**

**Ethical approval**

All procedures were approved by the ethics committee of Institutional Animal Care and Use Committee of National Yang-Ming University, Taipei, Taiwan (IACUC approval no.: 970408).

**Animals**

Melamine or CA was administered to the 3- and 8-week-old male Sprague–Dawley rats by oral gavage of a daily dose of 10, 50, 100 and 200 mg/kg BW in 1% carboxymethylcellulose (CMC) for 3 days (Days 0, 1 and 2). Rats fed with 1% CMC solution were served as the vehicle group. Rats in all groups were then sacrificed under anaesthesia on the third day (Figure 1A). Animal experiments were performed by W.Y.Y., and kidney, blood and urine specimens were tested by H.L.H., who was blinded to the study design. Whole blood was collected for the measurement of blood urea nitrogen (BUN) using an Olympus AU-2700 autoanalyzer (Olympus, Tokyo, Japan). Urinary kidney injury molecule (KIM)-1 concentrations were measured using the commercially available ELISA kit (R&D system; Minneapolis).

**Histology and tubular injury score**

Kidneys were embedded in paraffin wax as described previously [16]. The sections were then stained with haematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) reagent (Sigma, MO). Tubular injury was defined as renal tubular dilation, brush border loss and tubular epithelial cell necrosis or loss. The injury score was graded in each PAS-stained section from 0 to 4 (0, no change; 1, changes affecting <25%; 2, changes affecting 25 to <50%; 3, changes affecting 50 to <75%; 4, changes affecting 75–100% of the sections) [16]. Twenty randomly selected cortical fields per rat were assessed at a magnification of ×400.

**Immunohistochemistry**

Immunohistochemical staining was performed as described previously [17]. Sections were incubated with primary antibodies for ED-1 (Serotec, Oxford, UK) or KLF5 (Abcam, Cambridge, MA), respectively. Afterwards, sections were incubated with the Envision avidin–biotin-free horseradish peroxidase (HRP)-labelled polymer (Dako Cytomation, Glostrup, Denmark). Macrophages were assessed by counting ED-1–positive cells in 20 randomly chosen cortical fields at a magnification of ×400 for each section.

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted from the rat kidney using TRIzol reagent (Invitrogen, CA), and then used in a complementary DNA reaction as described previously [18]. The levels of mRNA for β-actin, iNOS, MCP-1, IL-6, Arg-1, IL-10 and KLF5 in the rat kidneys were measured by the quantitative real-time PCR using a LightCycler 1.5 Instrument (Roche, Mannheim, Germany). Primers and probe were designed by the Universal Probe Library Assay Design Centre (Roche).

**Western blot**

Kidney tissue was processed by western blot [18], primary rabbit antibodies for iNOS (Santa Cruz, CA), Arg-1 (Santa Cruz), MCP-1 (Abcam) and primary mouse antibodies for IL-10 (Serotec), KLF5 (Abcam) and GADPH (Sigma) were used. Afterwards, species-directed secondary antibodies, goat anti-rabbit or anti-mouse HRP-conjugated IgG, were used. Data were normalized to GADPH expression.

**Statistics**

Data were presented by means ± standard errors of means (SEM). Statistical analysis was performed using unpaired Student’s t-test between two-group comparisons. For comparison of three or more groups, we used one-way analysis of variance (ANOVA), followed by Duncan’s multiple-comparison post-hoc test. Statistics were analysed by the computer software Statistical Package of Social Science (version 16.0; SPSS, IL, Chicago). A P value of <0.05 was considered statistically significant.

**Results**

**Administration of M/CA caused renal dysfunction in rats**

We initially assessed the respective renal injury effects of melamine alone, CA alone, or M/CA at various doses in 3- and 8-week-old rats (Figure 1A). BUN (Figure 1B) and urinary KIM-1 (Figure 1C) at baseline showed no difference between 3- and 8-week-old rats. When compared with the vehicle group fed with 1% CMC, BUN and urinary KIM-1 levels had no significant changes with oral administration of melamine (200 mg/kg BW) or CA (200 mg/kg BW) to 3- and 8-week-old rats for 3 days. However, a significant increase in BUN and urinary KIM-1 in both 3- and 8-week-old rats fed with M/CA at a dose ≥100 mg/kg BW of each compound was observed, and were
significantly higher in 3-week-old rats than that in 8-week-old rats at a dose of 200 mg/kg BW of each compound.

**Melamine-cyanurate crystals associated with renal tubular cell injury**

In the cortex, tubular dilation with brush border loss, tubular epithelial cell necrosis and loss were noted only in rats fed with M/CA for 3 days in a dose-dependent manner when compared with the vehicle group, but not in rats fed with melamine or CA alone (Figure 2A and C). Intriguingly, crystals in the lumens of renal tubules, particularly in the inner medulla, were observed in 3- and 8-week-old rats fed with M/CA, but not in rats fed with 1% CMC, melamine or CA alone (Figure 2B). On the third day, the number of crystal formation was significantly increased in rats fed with M/CA for 3 days in a dose-dependent manner when compared with the vehicle group (Figure 2D). Moreover, the tubular injury score and crystal number were significantly higher in 3-week-old rats than those in 8-week-old rats ($P < 0.05$) at a dose of 200 mg/kg BW of M/CA.

**Macrophage recruitment in kidney of rats fed with melamine/CA**

Macrophage is the major cell accumulated around the tubules in rodent kidneys after renal injury [12]. ED-1 immunostaining was used to detect macrophages in the kidney sections. In renal interstitium, an influx of ED-1-positive cells was increased on the third day in 3- and 8-week-old rats fed with melamine/CA (200 mg/kg BW), but not in rats fed with 1% CMC, melamine or CA alone. Macrophages infiltrated in all areas of injured kidney, especially nearby the injured tubules of the cortex and outer medulla (Figure 3A). Infiltrated macrophages were significantly higher in 3-week-old rats than those in 8-week-old rats fed with M/CA (Figure 3B).

**KLF5 expression on epithelial cells of distal tubules with crystal deposition**

KLF5, mainly expressed in collecting duct epithelial cells, has been known as a pivotal regulator in response to renal injury [15]. We assessed the association between melamine-
cyanurate crystal formation in the renal medulla and KLF5 expression. Crystal depositions were observed in the lumens of distal tubules located in the renal medulla of 3- and 8-week-old rats fed with M/CA (200 mg/kg BW) for 3 days, but not in the vehicle group (Figure 3C; upper panel). Meanwhile, KLF5 was highly expressed within the dilated tubular epithelial cells of renal medulla (Figure 3C; lower panel), especially in the crystal formation regions. When compared with the vehicle group, KLF5 mRNA were increased in 3- and 8-week-old rats fed with M/CA (200 mg/kg BW) for 3 days but not in rats fed with melamine or CA alone (Figure 3D). The increased KLF5 mRNA expression in 3-week-old rats

![Figure 2: M/CA caused tubular cell injury and crystal formation. PAS staining of renal cortex sections (A) and H&E staining of renal medulla sections (B) in the 3- and 8-week-old rats fed with vehicle (1% CMC), melamine (200 mg/kg BW), cyanic acid (CA, 200 mg/kg BW) or M/CA (50 to 200 mg/kg BW) for 3 days (magnification of ×400). Tubular injury score (C) and crystal number (D) in the 3- and 8-week-old rats were assessed, respectively. Data are expressed as means ± SEM (n = 6–10 in each group). *P < 0.05 versus vehicle group of 3-week-old rats; **P < 0.05 versus vehicle group of 8-week-old rats. P < 0.05 reported on the top of the bars denote differences between young and old rats.](image-url)
were significantly higher than that in 8-week-old rats (Figure 3D).

**Nephritis-related cytokine gene expressions in rats fed with M/CA**

In kidney tissues, mRNA levels of proinflammatory cytokines including iNOS (Figure 4A), IL-6 (Figure 4B) and MCP-1 (Figure 4C) were increased in 3-week-old rats fed with melamine/CA (200 mg/kg BW) for 3 days when compared with the vehicle group, but not in 8-week-old rats fed with M/CA. In contrast, mRNA levels of anti-inflammatory cytokines like Arg-1 (Figure 4D) and IL-10 (Figure 4E) were increased in 8-week-old rats fed with M/CA (200 mg/kg BW) for 3 days when compared with the vehicle group, but not in 3-week-old rats fed with M/CA. Protein levels of iNOS, MCP-1, Arg-1 and IL-10 were consistent with the data of the corresponding mRNA levels in each groups (Figure 4F).

**DISCUSSION**

Melamine or CA appeared to have low nephrotoxicity, but induced extensive renal crystal formation when administered together [9]. Kobayashi et al. [19] found that daily administration of >24 mg/kg M/CA for 14 days caused the crystal formation and nephritis in rats. The cumulative dose of M/CA in the study by Kobayashi et al. was 336 mg/kg BW [19], but it ranged from 30 to 600 mg/kg BW in our study. We found that the cumulative dose of M/CA ≥150 mg/kg caused crystal formation, but acute kidney dysfunction occurred when the cumulative dose increased to ≥300 mg/kg.

In general, 3-week-old rats are nearly equal to 2-year-old children, and 8-week-old rats already reach sexual maturity. For assessing the age-related susceptibility of kidneys to M/CA in humans, we, therefore, used 3-week-old and 8-week-old rats to reflect the young and adult animal models. The dose of M/CA at 200 mg/kg BW of each compound caused markedly severe nephritis in young rats when compared with adult rats, in terms of higher BUN level, more crystal formation and severe renal tubular injury. Therefore, the M/CA-induced renal toxicity in young rats is not proportional to dose accumulation but is related to other mechanisms. The rodent kidney has only 50% of nephrons and no inner medulla at birth [20]. The medulla expands in the second and third postnatal weeks by cell proliferation and apoptotic remodelling in the loop of Henle and collecting ducts with a concomitant surge in urine.
Therefore, the kidney is not well developed in rats younger than 3 weeks. Immaturity might be one of the reasons for more crystal accumulation in kidneys of 3-week-old rats, consequently causing more kidney damage.

We further found an association between macrophage recruitment and increased KLF5 expression in response to melamine-cyanurate crystal deposition within the distal renal tubules (Figure 3). Members of the KLF family are important transcription factors of development, cellular differentiation and growth in cancers and cardiovascular disease [22]. Recently, the expression of KLF5 on collecting duct epithelial cells has been shown to be essential for the development of tubulointerstitial nephritis [15]. Through binding to S100a8 and S100a9 promoters, KLF5 modulates the early accumulation of monocytes, and further regulates the differentiation towards M1 macrophage in UUO-induced nephritis of mice. The early infiltrated M1 macrophages mainly produced IL-1, IL-6, MCP-1 and TNF-α and then led to kidney injury [23]. In our study, we observed the overexpression of KLF5 on distal tubule cells with the simultaneous increased production of proinflammatory cytokines in kidneys of young rats fed with M/CA (Figure 4). These changes were consistent with more severe tubular cell injury and interstitial inflammation (Figure 2). In response to the kidney injury, a later switch to M2 macrophages, amplifying their anti-inflammatory mediators like Arg-1, IL-10 and TGF-β, enhances tubular reepithelialization [24]. Accordingly, in our study, the less expression of KLF5 in adult rats fed with M/CA reduced the IL-6 and MCP-1 production and increased the IL-10 production. Another reason for less severe nephritis in adult rats is presumably due to the less susceptibility to the toxins and the less expression of KLF5 expression when compared with young rats.

Some limitations should be acknowledged. The kinds of cytokines and chemokines (Figure 4) are evidence of the severity of tissue inflammation in kidneys. The results cannot really prove the existence of macrophages with such polarities. However, our study showed that M/CA crystal-induced acute nephritis is correlated with KLF5 expression. Moreover, KLF5 expression has been shown to modulate the differentiation towards M1 macrophage during renal injury [15]. In the future, a KLF5-deficient mouse study or KLF5-RNAi study is indispensable to prove KLF5-regulated macrophage polarization in M/CA crystal-induced nephritis.

In summary, the present study demonstrates that melamine-cyanurate crystals caused acute nephritis through the production of proinflammatory cytokines like IL-6, TNF-α and MCP-1, which might be associated with the KLF5 expression on epithelial cells of the collecting duct in response to crystal formation within its lumen. We proposed a new concept that KLF5 expression and macrophage recruitment may be the essential regulators of the inflammatory process in melamine-cyanurate crystal-induced nephritis. KLF5 might be a target for therapeutic strategy in the management of crystal-induced acute kidney injury.
REFERENCES


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