Melamine Induces Human Renal Proximal Tubular Cell Injury via Transforming Growth Factor-β and Oxidative Stress

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Although several reports have failed to observe adverse subchronic renal effects following relatively high melamine exposure, the safety of low and continuous melamine exposure is still debatable. Recent studies suggest that long-term, low-dose melamine exposure is associated with an increased risk of urolithiasis, which has been linked to chronic kidney disease (CKD). CKD is a consequence of nephron loss and is associated with the interaction of inflammation, oxidative stress, and transforming growth factor-β (TGF-β), which increases extracellular matrix genes and cell apoptosis with progression to fibrosis and end-stage renal disease. Thus far, information is still lacking regarding the influence of melamine at the gene and protein levels, which are activated at a much earlier phase than the occurrence of the renal morphological change. In this study, we stimulated human renal proximal tubular HK-2 cells with melamine (0, 125, 250, 500, or 1000 µg/ml) for different time intervals and observed its effects on several well-documented molecular mechanisms of CKD. Here, we demonstrate that melamine can activate mitogen-activated protein kinases, NFκB, and reactive oxygen species, which results in the upregulation of interleukin-6, monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, and TGF-β1 in HK-2 cells. The melamine-stimulated overexpression of TGF-β1 not only promotes fibronectin production but also leads to decreased antiapoptotic (bcl-2, bcl-xL)/proapoptotic (bad, bax) protein ratio, increased caspase-3 and caspase-9 activities, and eventually HK-2 cell apoptosis. Our study suggests that melamine exposure may be a risk factor for the chronic loss of tubular cells and may ultimately lead to tubulointerstitial damage.

Key Words: inflammation; MAPK; NF-κB; fibronectin; apoptosis; caspase.

Melamine (2,4,6-triamino-s-triazine) is a resin widely used in the manufacturing of a variety of tableware for daily use because of its cheap cost and relatively light and unbreakable characteristics (Tyan et al., 2009). Melamine became a public health issue because pet food that was determined to be contaminated with melamine caused many cases of animal renal failure and death in the United States and elsewhere (Brown et al., 2007; Kuehn, 2009). Subsequently, the outbreak of formula milk that had been contaminated with melamine in 2008 raised concerns about its toxicity on the adverse renal outcomes in children worldwide (Guan et al., 2009). Due to the uncertainty of a safe dose of melamine for human intake, both the World Health Organization (WHO) and the U.S. Food and Drug Administration (U.S. FDA) recommended lowering the tolerable daily intake (TDI) of melamine from 0.5 to 0.2 mg/kg body weight/day and from 0.63 to 0.063 mg/kg body weight/day, respectively (U.S. FDA, 2008; WHO, 2009). One study, however, found that several children with melamine-related urolithiasis had an intake even below the updated WHO- or FDA-recommended TDI (Chen et al., 2009). Li et al. (2010) also reported that children exposed to melamine levels below 0.2 mg/kg per day (WHO-recommended TDI) still have a 1.7 times higher risk of developing urolithiasis than those without melamine exposure in a study including 683 child patients who presented with renal stones and 6498 child controls. In adults, we determined that melamine exposure (as measured by urinary melamine levels) was also associated with both uric acid and calcium urolithiasis (Wu et al., 2010). Taken together, these studies suggest that exposure to low levels of melamine may be a risk factor for urolithiasis formation. Urolithiasis is a chronic disease with a 60% chance of recurrence within 10 years of the first episode; it has also been linked to a number of other chronic diseases, such as metabolic syndrome and chronic kidney disease (CKD) (Khan, 2012).

CKD is a condition that is characterized by the progressive and irreversible decline of renal excretory function as a consequence of renal tissue injury and nephron loss, which is likely attributable...
to the death of tubular epithelial cells (García-Sánchez et al., 2010). It is estimated that 10–20% of the adult population have some degree of CKD that may have been caused by a variety of factors, and renal function usually declines over a period of years or decades (De Vecchi et al., 1999; García-Sánchez et al., 2010). The reduction of kidney function is associated with an inflammatory response in CKD, and cytokines contribute to a proinflammatory milieu when renal function declines (Carrero and Stenvinkel, 2010). Oxidative stress is another common pathway leading to CKD (Djamali, 2007; Hsieh et al., 2002; Massy et al., 2009). Excessive oxidative stress modulates the activation of mitogen-activated protein kinases (MAPKs) and various cytokines and transcription factors, which eventually lead to increased expression of extracellular matrix (ECM) genes with progression to fibrosis and end-stage renal disease (Hsieh et al., 2002; Kashihara et al., 2010). Transforming growth factor-β (TGF-β) is one of the factors upregulated by oxidative stress and has been recognized as an important mediator of a variety of glomerular, tubular, and inflammatory processes involved in CKD (García-Sánchez et al., 2010; Hsieh et al., 2006).

Depending on the purification process, melamine may contain a number of structurally related byproducts such as cyanuric acid (Dorne et al., forthcoming). Even though many studies indicate that the action of melamine on renal injury is due to the formation of stones with uric acid or cyanuric acid (Dorne et al., forthcoming), it is still unclear whether melamine itself could be a possible cause of CKD. In addition to consuming formula milk that contained melamine (Guan et al., 2009), the daily use of melamine-made tableware may be another source of melamine ingestion. Recently, we reported that melamine migration can be detected from daily-use melamine-made tableware, even at low temperatures (30°C–40°C). The amount of melamine migration sharply increases after 60°C–70°C (Chien et al., 2011). Although several reports have not observed adverse subchronic renal effects following relatively high melamine exposure (Choi et al., 2010; Kobayashi et al., 2010; Park et al., 2011; Stine et al., 2011), most of the findings on melamine toxicity were based on morphological changes of the kidneys instead of the impact of melamine at the molecular level. In addition, the safety of low and continuous melamine exposure is still debatable because recent epidemiological studies suggested that long-term, low-dose melamine exposure was associated with the risk of urolithiasis (Liu et al., 2011; Wu et al., 2010). Because information is lacking in regards to the influence of melamine at the molecular level, which should display abnormalities much earlier than the occurrence of morphological change, we aimed to investigate whether melamine exposure could affect the inflammatory and fibrotic genes that are related to chronic renal proximal tubular cell injury.

MATERIALS AND METHODS

Reagents and antibodies. Dulbecco’s Modified Eagle’s Medium (DMEM; catalog no. 12320), penicillin/streptomycin, fetal bovine serum (FBS), CM-H₂DCFDA, and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA). Melamine, PD98059 (a p44/42 MAPK inhibitor), SB203580 (p38 MAPK inhibitor), Tiron (an antioxidant), and anti-TGF-β1 antibody were purchased from Sigma-Aldrich, Inc. (St Louis, MO). MnTBAP (a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger) was purchased from Merck KGaA (Darmstadt, Germany). SNS50 (a cell-permeable inhibitory peptide of NF-κB) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). Mouse anti-actin monoclonal antibodies were purchased from Millipore Corporation (Temecula, CA). Phospho-p44/p42 MAPK, p44/p42 MAPK, phospho-p38 MAPK, p38 MAPK, phospho-IκBα, IκBα, phospho-NF-κB p65, NF-κB p65, Bcl-2, Bcl-xl, Bad, Bax, caspase-3, cleaved caspase-3, and cleaved caspase-9 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Oligonucleotides were synthesized by BioBasic, Inc. (Markham, Canada). Human interleukin-6 (IL-6) ELISA kits were purchased from RayBiotech, Inc. (Norcross, GA). Human TGF-β1 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN).

Cell culture. Human renal proximal tubular cells (HK-2 cells; BCRC no. 60097; Bioresource Collection and Research Center, Taiwan, R.O.C.) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. When 80% confluence was reached, the cells were synchronized overnight in 0.5% FBS DMEM before being stimulated. The stimulation was also performed in 0.5% FBS DMEM. Thirty minutes before the addition of melamine, TGF-β1 antibody (300 ng/ml), PD98059 (10⁻⁴ M), SB203580 (10⁻⁴ M), Tiron (10⁻³ M), MnTBAP (10⁻³ M), or SNS50 (30 µg/ml) was added to the cells.

RNA extraction and real-time PCR. To observe the mRNA expression, HK-2 cells were stimulated with different concentrations of melamine (0, 125, 250, 500, or 1000 µg/ml) and harvested after 1, 3, 6, or 24 h. Total RNA was extracted using TRIzol reagent according to the supplier’s protocol (Invitrogen) and quantified using the Quant-iT RNA assay kit (Invitrogen). For each sample, cDNA was synthesized from 2 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit according to the protocol of the supplier (Invitrogen). The cDNA was then diluted with water at a ratio of 1:4, and aliquots were amplified using the Maxima SYBR Green qPCR Master Mix according to the instructions of the supplier (Thermo Fisher Scientific, Inc., Waltham, MA). cDNA and primers were added to the PCR mixture to reach a final volume of 20 µl. PCR reactions were performed using the MiniOpticon real-time PCR system (Bio-Rad Laboratories, Hercules, CA). Primers were designed by Beacon Designer 5 software (Premier Biosoft Int., Palo Alto, CA), and the primer sequences are shown in Table 1. β-actin mRNA expression was measured as an internal control.

SDS-PAGE and Western blot analysis. To analyze the proteins, HK-2 cells were stimulated with different concentrations of melamine (0, 125, 250, 500, or 1000 µg/ml) and harvested after 15 min, 30 min, 6 h, or 24 h. Then, the cells were lysed in the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.) supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany). Protein concentration was measured using the Quant-IT Protein assay kit (Invitrogen). For analysis of each specific protein, the cell lysate was loaded and separated on 7.5, 10, or 15% SDS-polyacrylamide gels. After transference to the PVDF membranes, the proteins of interest were detected using the corresponding antibodies. Images of protein expressions on the blots were quantified by ImageJ software (Rasband, 1997).

Detection of reactive oxygen species. Reactive oxygen species (ROS) produced from HK-2 cells were detected by 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) reagent. HK-2 cells were cultured in a 12-well plate until 80% confluence and then stimulated with different concentrations of melamine (0, 125, 250, 500, or 1000 µg/ml) and harvested after 15 min, 30 min, 6 h, or 24 h. Then, the cells were lysed in the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.) supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany). Protein concentration was measured using the Quant-IT Protein assay kit (Invitrogen). For analysis of each specific protein, the cell lysate was loaded and separated on 7.5, 10, or 15% SDS-polyacrylamide gels. After transference to the PVDF membranes, the proteins of interest were detected using the corresponding antibodies. Images of protein expressions on the blots were quantified by ImageJ software (Rasband, 1997).

Cytotoxicity assays. The cytotoxicity of melamine in HK-2 cells was determined using the WST-1 reagent (Roche Applied Science). WST-1 is a colorimetric assay that provides a convenient and rapid method for the detection of metabolic activities in cultured cells. The principle of the assay is based on the cleavage of a yellow tetrazolium salt (WST-1) into a water-soluble formazan dye upon cleavage by a mitochondrial enzyme, mitochondrial dehydrogenase. The formation of formazan is directly proportional to the number of viable cells. The HK-2 cells were seeded into 96-well plates at a density of 5000 cells per well and allowed to adhere overnight. The next day, the cells were treated with different concentrations of melamine (0, 125, 250, 500, 1000 µg/ml) or 0.01% DMSO (negative control) for 72 h. After incubation, the cells were washed with PBS and then incubated with WST-1 solution (5 µl per well) for 4 h at 37°C. The absorbance at 490 nm was measured using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).
tetrazolium salt that is cleaved to formazan by mitochondrial dehydrogenases in viable cells. To measure the cell viability, HK-2 cells were seeded into 96-well plates. When 80% confluence was reached, the cells were stimulated with different concentrations of melamine (0, 125, 250, 500, or 1000 µg/ml) for 24 or 48 h. At the end of the incubation, WST-1 was added to the cells and the amount of formazan was measured at 450 nm by an ELISA reader.

In addition, cell injury was detected using a Cytotoxicity Detection Kit Plus (Roche Applied Science) that is a colorimetric assay for the quantification of cell death and cell lysis based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells.

Nuclear morphology and apoptosis assays. Hoechst 33342, a cell membrane–permeable dye, was used to monitor the nuclear morphology of cell apoptosis. HK-2 cells were seeded into 12-well plates. When 80% confluence was reached, the cells were stimulated with different concentrations of melamine (0, 125, 250, 500, or 1000 µg/ml). After 24 h, Hoechst 33342 solution (final concentration as 10 µg/ml) was added to the wells, and the cells were incubated for 15 min in the dark at room temperature. After washing with PBS, the nuclear morphology of apoptotic cells was observed under an EVOS fl fluorescence microscope.

Apoptotic cells were also analyzed by a flow-cytometric method. After 24 h of stimulation with melamine, the adherent and detached cells were combined and stained with 50 µg/ml propidium iodide prior to being analyzed by a flow cytometer (Beckman Coulter, Inc., Brea, CA). Apoptotic cells were evaluated by calculating the peak areas of hypodiploid nuclei (sub-G1).

ELISA. To measure the proteins secreted into the medium, HK-2 cells were stimulated with melamine with or without inhibitors, antioxidants, or TGF-β1 antibody for 24 h. Then, the conditioned media from each sample were collected. The concentrations of IL-6 and TGF-β1 in each sample were determined using a Human IL-6 ELISA Kit and a Human TGF-β1 ELISA Kit, respectively.

Statistical analysis. A minimum of three independent experiments were performed per procedure. The data were analyzed by one-way ANOVA followed by the Bonferroni test using GraphPad Prism software. All data are presented as the mean ± SEM, and a probability level of p < 0.05 was regarded as statistically significant.

RESULTS

Melamine Stimulates the Expression of IL-6, MCP-1, and VCAM-1 mRNA

To examine whether melamine could initiate renal tubulointerstitial inflammation, we stimulated HK-2 cells with melamine and detected the mRNA expression of the three candidate inflammatory genes IL-6, monocyte chemoattractant protein-1 (MCP-1), and vascular cell adhesion molecule-1 (VCAM-1). IL-6 is a central cytokine in the inflammatory process; MCP-1 has a crucial role in the attraction, migration, and activation of monocytes; VCAM-1 is closely correlated with monocyte/macrophage infiltration. These three molecules help to maintain the inflammatory milieu in CKD (Triñanes et al., 2012). Figure 1A shows that 250 µg/ml melamine significantly increased the mRNA expression of IL-6 to 200% after 6 h of stimulation and that increase persisted to 24 h; 500 and 1000 µg/ml melamine did not further enhance the expression of IL-6 mRNA (Fig. 1B). The expression of MCP-1 mRNA was increased by 250 µg/ml melamine to more than 300% at 6 h, but the level of expression declined to 200% at 24 h (Fig. 1C). Melamine increased MCP-1 mRNA expression to 150% at 125 µg/ml, and the increase in expression peaked (more than 300%) at 250 µg/ml (Fig. 1D). VCAM-1 mRNA was upregulated by 250 µg/ml melamine to 130% at 3 h, 150% at 6 h, and declined to 120% at 24 h (Fig. 1E). Figure 1F illustrates how the expression of VCAM-1 mRNA was affected by the dosage of melamine. These results suggest that melamine activates several inflammatory genes in HK-2 cells.

Effects of Melamine on the Activation of MAPKs and NF-κB

Some evidence has indicated that MAPKs (e.g., p44/42 MAPK and p38 MAPK) and nuclear factor-kappa B (NF-κB) mediate the inflammation in CKD (Evans et al., 2002; Murali et al., 2007; Yeh et al., 2001). NF-κB is bound to inhibitory IκB proteins in the cytoplasm. After stimulation by a variety of stimuli, NF-κB is released from phosphorylated-IκB and translocates to the nucleus where it binds to its coactivators. As shown in Figure 2A, the levels of phosphorylated p44/42 MAPK and p38 MAPK were increased after 15 min and 30 min of melamine stimulation in a dose-dependent manner. The phosphorylated IκB and NF-κB proteins were significantly increased by the 30-min stimulation of 250 µg/ml melamine (Fig. 2B). Higher concentrations of melamine did not further increase the phosphorylation of IκB and NF-κB; in fact, 1000 µg/ml melamine did not change the phosphorylation levels of the two proteins (Fig. 2B). These results demonstrated that melamine activates MAPKs and NFκB, which may regulate the expression of inflammatory genes.

Effects of Melamine on ROS Generation

Because oxidative stress is a common pathway in the pathogenesis of CKD, we also tested the effect of melamine on ROS generation in HK-2 cells. As shown in Figure 3, the administration of melamine increases the positive staining of CM-H$_2$DCFDA (green fluorescence), indicating the increase in ROS production. Our results show that 250, 500, and 1000 µg/ml melamine significantly increased the ROS generation to
approximately 150, 180, and 280%, respectively (Fig. 3). This finding confirms that melamine induces oxidative stress in HK-2 cells.

The Mechanisms That Mediate Melamine-Stimulated IL-6, MCP-1, and VCAM-1 Expression

To verify whether the melamine-induced increases in IL-6, MCP-1, and VCAM-1 mRNA expression were regulated via the activation of MAPKs and NF-κB and/or increased oxidative stress, we administered PD98059, SB203580, SN50, Tiron, and MnTBAP to HK-2 cells while stimulating them with melamine. As shown in Figures 4A, 4C, and 4D, the expressions of IL-6, MCP-1, and VCAM-1 mRNAs were significantly upregulated by melamine stimulation. The administration of PD98059, SB203580, SN50, Tiron, and MnTBAP blocked the effect of melamine on IL-6, MCP-1, and VCAM-1 mRNA upregulation.
As shown in Supplementary fig. 1, the inhibitory effects were not due to the toxicities of these inhibitors and antioxidants. The trend of IL-6 protein production was consistent with the mRNA expression (Fig. 4B). These findings suggest that melamine may cause inflammation in HK-2 cells via the activation of p44/42 MAPK, p38 MAPK, and NF-κB and also through the generation of ROS.

**Melamine Induces HK-2 Cell Apoptosis**

Guo et al. (2012) have reported that 8mM (~1000 µg/ml) melamine could cause apoptosis in rat kidney epithelial cells. Therefore, we examined whether melamine could induce apoptosis in human proximal tubular cells. As shown in Figure 5A, 500 and 1000 µg/ml melamine significantly decreased HK-2 cell viability after 24 or 48 h.

Cyanuric acid can interact with melamine to form crystals that caused nephrotoxicity and resulted in illness and deaths of human infants and companion animals (Dorne et al., forthcoming). To compare the toxicity of melamine to that of melamine-cyanuric acid crystals, we treated HK-2 cells with 250 µg/ml melamine combined with 250 µg/ml cyanuric acid. Figure 5A shows that the cell viability dramatically decreased to approximately 50 and 12.5% after 24 and 48h, respectively. Melamine
decreased HK-2 cell viability, which was not a result of cellular necrosis because the LDH activity in the culture media was not increased (Fig. 5B). In contrast, treatment with melamine combined with cyanuric acid increased the LDH activity in the medium, indicating that the dramatic decline of cell viability was caused by necrosis of the HK-2 cells (Fig. 5B). Figures 5C and D demonstrate the HK-2 cell morphology under 500 µg/ml melamine stimulation; the results indicate that melamine caused apoptotic morphologies, including DNA condensation and nuclei fragmentation. In Figures 5E and 5F, the combination of melamine and cyanuric acid formed crystals of various sizes in the culture medium, and the number of HK-2 cells was significantly decreased, indicating the formation of the crystals that caused severe cell death. As shown in Supplementary fig. 2, the average pH values of the media are approximately 7.5 and there is no significant difference among these groups. The result indicates that the addition of cyanuric acid did not change the pH value of the medium and the cytotoxicity was not due to the effect of low pH. Figure 5G shows the degree of HK-2 cell apoptosis increased with the dose of melamine.

We also used flow cytometry to confirm and quantify the melamine-induced apoptosis. The results show that an increase in melamine dose caused an increase in the percentage of cells in the sub-G1 phase, suggesting that the cells were apoptotic (Fig. 6A). The quantification is demonstrated in Figure 6B. These findings suggest that even lower doses of melamine could induce renal proximal tubular cell apoptosis.

Melamine Changes the Expression of Bcl-2 Family Proteins and the Activity of Caspase-3/-9

Bcl-2 family members and caspases are key players in the pathway of apoptosis (Kurokawa and Kornbluth, 2009; Sorenson, 2004). To understand the mechanism of melamine-induced apoptosis in HK-2 cells, we detected the protein levels of bcl-2, bcl-xl, bad, bax, cleaved caspase-3, and cleaved caspase-9. As shown in Figure 7A, antiapoptotic protein bcl-2 was downregulated by 500 and 1000 µg/ml melamine and bcl-xl was downregulated by 1000 µg/ml melamine. In contrast, proapoptotic proteins bad and bax were increased by 250, 500, and 1000 µg/ml melamine (Fig. 7A). Cleaved caspase-3 and caspase-9 were increased by 250, 500, and 1000 µg/ml melamine (Fig. 7B). These findings indicate that melamine induces apoptosis via the change of the antiapoptotic/proapoptotic protein ratio and the activation of caspase-3 and caspase-9.

Melamine Stimulates TGF-β1 Expression via ROS and NF-κB

TGF-β1 is well documented as one of the most important profibrotic cytokines in renal damage and is known to promote apoptosis following renal injury in both in vivo and in vitro models (Docherty et al., 2006). Figure 8A shows that the stimulation of HK-2 cells with 250 µg/ml melamine increased TGF-β1 mRNA expression to approximately 200% at 3 h, 6 h, and 24 h. Figures 8B and C show that 250 µg/ml melamine had the strongest potency to augment the expression of TGF-β1 mRNA and protein. Furthermore, melamine-stimulated elevations of TGF-β1 mRNA and protein were mediated via ROS and NF-κB because Tiron, MnTBA, and SN50 inhibited the effect of melamine on TGF-β1 production (Figs. 9A and B). Treatment with TGF-β1 antibody did not influence mRNA
expression, but it neutralized TGF-β1 protein in the culture medium (Figs. 9A and B).

Melamine Induces Apoptosis via TGF-β1 and ROS

As shown in Figure 10A, the percentage of sub-G1 cells was increased by 500 µg/ml melamine, but this increase was suppressed by the addition of TGF-β1 antibody, Tiron, and MnTBAP. In addition, the removal of TGF-β1 and ROS inhibits the effect of melamine on the upregulation of the proapoptotic proteins bad and bax and the activation of caspase-3 and caspase-9 (Fig. 10B). These findings imply that melamine causes HK-2 cell apoptosis via the generation of TGF-β1 and ROS.

Melamine Enhances Fibronectin Production via TGF-β1 and ROS

Tubulointerstitial fibrosis is regarded as a central event in the progression of CKD and is a result of increased deposition of ECM, such as collagen and fibronectin (García-Sánchez et al., 2010). As shown in Figures 11A and B, 250 µg/ml melamine significantly promoted the expression of fibronectin mRNA and protein to more than 200%, but the higher concentrations of melamine did not further enhance the production of fibronectin. Figures 11C and D show that the overproduction of fibronectin was suppressed by the addition of TGF-β1 antibody, Tiron, and MnTBAP, implicating the involvement of TGF-β1 and ROS in the mediation of melamine-enhanced fibronectin expression.

DISCUSSION

This study demonstrates that melamine activates MAPKs and NF-κB and induces the production of ROS, which can result in the upregulation of IL-6, MCP-1, VCAM-1, and TGF-β1 in
FIG. 5. The effect of melamine on cell viability. (A) HK-2 cells were stimulated by melamine (0–1000 µg/ml) with or without cyanuric acid (250 µg/ml) for 24 or 48 h. Cell viability was determined by the WST-1 test and converted to a percentage. (B) HK-2 cells were stimulated by melamine (0–1000 µg/ml) with or without cyanuric acid (250 µg/ml) for 24 h. Necrosis of the cells was determined by LDH activity in the culture medium. Data represent the mean ± SEM of six independent experiments. a: p < 0.001; b: p < 0.01; c: p < 0.05 compared with the group without treatment. (C, D) HK-2 cells were stimulated with melamine (500 µg/ml) for 24 h. (E, F) HK-2 cells were stimulated with melamine (250 µg/ml) and cyanuric acid (250 µg/ml) for 24 h. (C, E) The cells were stained with Hoechst 33342. (D, F) The corresponding cells observed under the light microscope. (G) HK-2 cells were treated with melamine (0, 250, 500, or 1000 µg/ml) for 24 h and then stained with Hoechst 33342. →: apoptotic cells; ←: melamine-cyanuric acid crystals.
human renal proximal tubular cells. The melamine-stimulated overexpression of TGF-β1 not only promotes fibronectin production but also leads to changes in the antiapoptotic (bcl-2, bcl-xl)/proapoptotic (bad, bax) protein ratio and increases in caspase-3 and caspase-9 activities, eventually resulting in proximal tubular cell apoptosis.

The addition of melamine to pet food in the 2007 incident in the United States and infant formula in the 2008 incident in China has had consequences on both animal and human health, resulting in the deaths of thousands of cats and dogs and the hospitalization of over 294,000 infants diagnosed with urinary tract stones and sand-like calculi, which included a number of associated deaths (Dorne et al., forthcoming; Puschner and Reimschuessel, 2011). The incidents raised global concerns on food and feed safety related to the melamine contamination (Gossner et al., 2009; Levinson and Gilbride, 2011). Currently, melamine has not only been detected from many food sources including eggs, chicken, pork, fish, and vegetable protein products but also could...
be released from daily-use melamine-made tableware in a wide range of conditions (Chien et al., 2011; Dorne et al., forthcoming; Gossner et al., 2009; Levinson and Gilbride, 2011). Therefore, it is hard to estimate the duration of exposure and the quantity of daily melamine ingestion for individuals. Melamine was an unknown substance to nephrologists until very recently, and its toxicity has not been fully investigated in cKD.

Tubulointerstitial inflammation, oxidative stress, and TGF-β1 activation play central roles in renal function deterioration and even in the development of CKD. Their combined effects disrupt glomerulus-tubule continuity, cause the development of pathogenic hypoxia, generate myofibroblasts and fibrosis, and impair the protective autoregulation of glomerular blood flow, which leads to glomerulosclerosis. TGF-β1 has been recognized as one of the critical genes in the pathogenesis of CKD. TGF-β1 promotes renal fibrosis through inducing ECM deposition and causes renal cell apoptosis via the activation of caspases. Oxidative stress and TGF-β1 mediate both apoptosis and fibrosis in kidneys (Djamali, 2007; Docherty et al., 2006; Evans et al., 2002; García-Sánchez et al., 2010; Murali et al., 2007; Rodríguez-Iturbe and García García, 2010; Triñanes et al., 2012). Recently, Guo et al. (2012) reported that 8mM (~1000 µg/ml) melamine causes excessive intracellular ROS and results in apoptosis via p38 MAPK activation in a rat kidney epithelial NRK-52e cell line; however, they did not investigate whether melamine could change the expression of genes related to CKD in stages that occur before apoptosis. Here, we found that 250 µg/ml melamine increases the production of ROS and the expression of IL-6, MCP-1, and VCAM-1 within 6h in a human proximal tubular cell line. Through the elevation of oxidative stress and these inflammatory genes, melamine may consequently induce an immune response and cause the accumulation of monocytes and macrophages in the tubulointerstitial region. In addition, 250 µg/ml melamine also increased TGF-β1 and fibronectin, suggesting that melamine may stimulate renal tubular fibrosis. Tiron and MnTBAP blocked the
Melamine-induced overexpression of IL-6, MCP-1, VCAM-1, and TGF-β1. Furthermore, melamine increases fibronectin via ROS and TGF-β1 because Tiron, MnTBAP, and TGF-β1 antibody inhibit the effect of melamine. Here, we offer evidence that melamine activates the critical genes involved in CKD via ROS generation.

We also noted that melamine caused renal proximal tubular cell death in a dose-dependent manner. Melamine induced a programming cell death via decreases in bcl-2 and bcl-xl, increases in bad and bax, and activation of caspase-3 and caspase-9. Blockage of ROS and TGF-β1 attenuated melamine-induced HK-2 cell apoptosis via preventing the upregulation of bad and bax and the cleavage of procaspase-3 and procaspase-9. Our results showed that 1000 µg/ml melamine caused approximately 10% of HK-2 cells to undergo apoptosis. Even 125 µg/ml melamine increased the amount of apoptotic cells approximately 2% compared with that of the control group. Thus far most of the reports indicate that the toxicity of melamine is mainly due to the involvement in the formation of crystals with endogenous uric acid or coexposure to cyanuric acid in renal tubules, resulting in potential acute kidney failure (Dorne et al., forthcoming). In our study, melamine-cyanuric acid crystals dramatically decreased cell viability, caused release of cellular LDH, and resulted in HK-2 cell necrosis.

**FIG. 8.** The effect of melamine on TGF-β1 expression. (A) HK-2 cells were stimulated with 0 or 250 µg/ml melamine for 1, 3, 6, or 24 h. (B) HK-2 cells were stimulated with 0, 250, 500, or 1000 µg/ml melamine for 6 h. (C) HK-2 cells were stimulated with 0, 250, 500, or 1000 µg/ml melamine for 24 h and then the culture media were collected for TGF-β1 protein measurement. Data represent the mean ± SEM of three independent experiments. a: p < 0.001; b: p < 0.01. Each sample was analyzed in duplicate.

**FIG. 9.** The effects of the TGF-β1 antibody, antioxidants, and NF-κB inhibitor on the expression of TGF-β1. HK-2 cells were stimulated by melamine (250 µg/ml) with or without 300 ng/ml TGF-β1 antibody, 10⁻⁴M Tiron, 10⁻⁶M MnTBAP, or 30 µg/ml SN50 for 24 h. (A) The expression of TGF-β1 mRNA. (B) TGF-β1 protein secreted into the media. Data represent the mean ± SEM of three independent experiments, and the measurements were performed in duplicate for each sample. a: p < 0.001 compared with the group without treatment; b: p < 0.001 compared with the group only treated with melamine.
degree and the form of injury caused by melamine and melamine-cyanuric acid crystals seem distinct. Our results suggest that melamine is a substance that may initiate a complex molecular mechanism leading to chronic tubular cell loss.

Activation of NF-κB and MAPKs can upregulate a variety of cytokines, chemokines, adhesion molecules, and growth factors that mediate interstitial infiltration of inflammatory cells, dysregulation of ECM, and apoptosis of kidney cells (Docherty et al., 2006; Evans et al., 2002; García-Sánchez et al., 2010; Murali et al., 2007; Rodríguez-Iturbe and García García, 2010; Triñanes et al., 2012; Yeh et al., 2001). Our study shows that 250 µg/ml melamine stimulated the phosphorylation

![Graph A](image1.png)

**FIG. 10.** The effects of the TGF-β1 antibody and antioxidants on melamine-induced HK-2 cell apoptosis and caspase-3/-9 protein activation. The cells were stimulated by melamine (500 µg/ml) with or without 300 ng/ml TGF-β1 antibody, 10−5 M Tiron, or 10−6 M MnTBAP. (A) Apoptotic cells were detected by flow cytometry after 24 h of treatment and evaluated by calculating peak areas of hypodiploid nuclei (sub-G1). (B) Bad, Bax, Caspase-3/-9 proteins were detected after 6 h of treatment. (C) The quantified results for (B). Data represent the mean ± SEM of three independent experiments. a: p < 0.001.
of p38 MAPK, p44/42 MAPK, IκB, and NF-κB within 30 min. SB203580, PD98059, or SN50 prevented the melamine-stimulated overexpression of IL-6, MCP-1, VCAM-1, and TGF-β1. The results suggest that melamine could rapidly activate these signaling kinases and transcription factors, which would subsequently enhance inflammatory and fibrotic gene expression. The concentration of melamine needed for peak activation of MAPKs and NFκB was 250 μg/ml rather than 1000 μg/ml. The trend of this dose effect in the activation of MAPKs and NFκB is similar to that in the expression of IL-6, MCP-1, and TGF-β1. In contrast, the concentration of melamine needed for peak alteration of the antiapoptotic/proapoptotic protein ratio and caspase activity was 1000 μg/ml. This phenomenon suggests that lower concentrations of melamine may not rapidly damage renal cells but may activate critical mechanisms gradually leading to CKD.

Dorne et al. (forthcoming) comprehensively reviewed the recent advances in the risk assessment of melamine and cyanuric acid in animal models. The review noted that melamine is absorbed from the gastrointestinal tract with nearly no
metabolism and is rapidly excreted in urine with a half-life of 4–6 h in rats and monkeys (Dorne et al., forthcoming). In adult male Fischer 344 rats, over 90% of a single oral dose of [14C]-melamine (0.025 mCi; ~1.3 mg/kg body weight) was excreted in urine, exhaled air, and feces within the first 24 h with 99% total recovery after 96 h. No residual radioactivity was observed in blood and plasma after 24 h although the residual radioactivities in the liver and kidneys were 1.8 and 1.3 μg equivalents/kg tissue, respectively (Dorne et al., forthcoming; Mast et al., 1983). Most of the animal studies had shown that melamine, without combined cyanuric acid or uric acid, would not cause significant renal damage (Dorne et al., forthcoming); however, these animal studies either were conducted using a single-dose treatment or used short observation periods (range from 3 to 15 days) that were not sufficient to observe the formation of CKD. During the 1980s, the National Toxicology Program at NIH conducted studies in which rats were administered melamine orally for 13-week and 2-year periods. The studies demonstrated that after 2 years, melamine exposure increased the incidence (relative to age-matched controls) of chronic renal inflammation in both the low (4500 ppm) and high (9000 ppm) dose groups in female rats, but not in male rats (National Toxicology Program, 1983). In 2009, Hard et al. re-evaluated the kidney histopathology for the original glass histology slides from the National Toxicology Program. They found a constellation of tubule changes extending from the papilla to the cortex, which consistently included tubule dilation and tubule basophilia as salient features at the 13-week time point (Hard et al., 2009). After 2 years of melamine exposure, these lesions had usually resolved into fibrotic scars, in which tubule loss and ECM deposition were prominent, running from the superficial cortex into the medulla (Hard et al., 2009). Using HK-2 cell model, we present the possible mechanisms by which melamine may lead to chronic tubular cell loss and fibrosis. However, long-period animal studies should be conducted in the future to confirm the mechanisms observed in vitro. The study of using the transgenic animal model or kidney-specific gene-deletion strategy is also necessary to elucidate melamine toxicity on some critical genes in human.

Several limitations are present in this study. First, only one HK-2 cell line was used in this research. HK-2 cell line has been widely used to investigate different causes of CKD, such as diabetic nephropathy, so it is an ideal cell line for studying nephrotoxic agents. However, the pathological and toxicological mechanism of melamine on kidney function in different human renal cell lines should be investigated in the near future. Second, the concentrations used in the in vitro study were relatively high, compared with melamine exposure levels in human. However, we cannot ignore that melamine may accumulate in renal tissue if an individual was exposed to melamine daily for a long period of time. Third, we do not know whether melamine can be internalized into HK-2 cells and whether melamine uptake is a critical step to induce renal cell damage. Although we did measure the concentrations of melamine in the medium and the cell lysate after the stimulation and the results showed that nearly 99% of the melamine retains in the medium after 0.5 and 6 h of stimulation (Supplementary table 1), this experiment cannot really confirm that < 1% of melamine has entered into the cell or only attached to the cell membrane. Another probable approach is to develop an innovative method, such as labeling with isotope, fluorescence or nanoparticle, to pursue the fate of melamine.

Figure 12 summarizes that melamine alone, without adding cyanuric acid to form crystals, could be a risk factor for chronic loss of tubular cells and may ultimately lead to tubulointerstitial damage. The present study suggests that food safety related to melamine should be cautiously monitored due to its potential risk in CKD.

**SUPPLEMENTARY DATA**

Supplementary data are available online at [http://toxsci.oxfordjournals.org/](http://toxsci.oxfordjournals.org/).

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