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Expression of kidney injury molecule-1 (Kim-1) in relation to necrosis and apoptosis during the early stages of Cd-induced proximal tubule injury

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Abstract

Cadmium (Cd) is a nephrotoxic industrial and environmental pollutant that causes a generalized dysfunction of the proximal tubule. Kim-1 is a transmembrane glycoprotein that is normally not detectable in non-injured kidney, but is up-regulated and shed into the urine during the early stages of Cd-induced proximal tubule injury. The objective of the present study was to examine the relationship between the Cd-induced increase in Kim-1 expression and the onset of necrotic and apoptotic cell death in the proximal tubule. Adult male Sprague-Dawley rats were treated with 0.6 mg (5.36 μ moles) Cd/kg, subcutaneously, 5 days per week for up to 12 weeks. Urine samples were analyzed for levels of Kim-1 and the enzymatic markers of cell death, lactate dehydrogenase (LDH) and alpha-glutathione-S-transferase (α -GST). In addition, necrotic cells were specifically labeled by perfusing the kidneys in situ with ethidium homodimer using a procedure that has been recently developed and validated in the Prozialeck laboratory. Cryosections of the kidneys were also processed for the immunofluorescent visualization of Kim-1 and the identification of apoptotic cells by TUNEL labeling. Results showed that significant levels of Kim-1 began to appear in the urine after 6 weeks of Cd treatment, whereas the levels of total protein, α -GST and LDH were not increased until 8–12 weeks. Results of immunofluorescence labeling studies showed that after 6 weeks and 12 weeks, Kim-1 was expressed in the epithelial cells of the proximal tubule, but that there was no increase in the number of necrotic cells, and only a modest increase in the number of apoptotic cells at 12 weeks. These results indicate that the Cd-induced increase in Kim-1 expression occurs before the onset of necrosis and at a point where there is only a modest level of apoptosis in the proximal tubule.

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Conflict of Interest Statement

Dr. Bonventre is co-inventor on Kim-1 patents. None of the other authors had any conflicts of interest pertaining to the work described in the manuscript.

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Keywords

cadmium; Kim-1; necrosis; apoptosis; proximal tubule

Introduction

Cd is an important industrial and environmental pollutant that can exert pleiomorphic effects on multiple organ systems (for reviews see ATSDR, 2003; Jarup et al., 1998). With the chronic, low-level patterns of exposure that are common in humans, the primary target of toxicity is the kidney where Cd causes a generalized dysfunction of the proximal tubule characterized by polyuria and increases in the urinary excretion of low-molecular-weight proteins, electrolytes, amino acids and glucose (Friberg, 1984; Jarup et al., 1998; Kjellstrom, 1986; Lauwerys et al., 1984; Piscator, 1986). These nephrotoxic effects are thought to occur when circulating Cd that is bound to metallothionein or other low-molecular-weight materials in plasma is delivered to the epithelial cells of the proximal tubule and taken up to such an extent that the renal cortical Cd concentration exceeds a critical threshold concentration of 150–200 µg/g of tissue that causes injury and death of the epithelial cells (Bridges and Zalups, 2005; Dudley et al., 1985; Goyer et al., 1989; Kjellstrom, 1986; Klaassen and Liu, 1997; Sudo et al., 1996; Suzuki, 1980).

Both necrotic and apoptotic mechanisms have been implicated in the pathways leading to death of proximal tubule epithelial cells during exposure to Cd. High levels of Cd are clearly capable of causing proximal tubule necrosis (Brzoska et al., 2003; Dudley et al., 1985; Goyer et al., 1989; Kjellstrom, 1986; Sudo et al., 1996; Peereboom-Stegeman et al., 1979). However, other studies indicate that the early stages of Cd nephrotoxicity primarily involve apoptosis of proximal tubule epithelial cells, with little evidence of necrosis (Aoyagi et al., 2003; Hamada et al., 1991; Tanimoto et al., 1993; Yan et al., 1997). Moreover, results of several recent studies suggest that the early stages of Cd-nephrotoxicity may involve changes in cell adhesion molecule function and cytoskeletal organization that occur before the onset of either necrotic or apoptotic death of proximal tubule epithelial cells (Jacquillet et al., 2006; Prozialeck et al., 2003; Prozialeck et al., 2007; Sabolic, 2006; Thevenod, 2003).

As a result of the widespread use of Cd in industry and its extensive dissemination in the environment, much attention has been focused on the identification of sensitive urinary biomarkers of the early stages of Cd-induced proximal tubule injury (for reviews see Bernard, 2004; Mueller et al., 1998). Some of the biomarkers that have been used for this purpose include the Cd-binding protein metallothionein (Shaikh et al., 1990) and various low molecular weight proteins such as β_2 -microglobulin (Bernard, 2004; Lauwerys et al., 1984) and Clara cell protein-16 (CC-16) (Bernard et al., 1994). These low molecular weight proteins are readily filtered by the glomerulus, but are normally reabsorbed by the proximal tubule. As Cd accumulates in the epithelial cells of the proximal tubule, the reabsorption of these proteins is decreased and the resulting increase in their urinary excretion is characteristic of the early stages of Cd-induced proximal tubule injury.

Kim-1, which is also known as hepatitis A virus cellular receptor 1 (Havcr1) (Feigelsstock et al., 1998), is a type I transmembrane protein that is not detectable in normal kidney tissue but is expressed at high levels in dedifferentiated proximal tubule epithelial cells after ischemic or toxic injury (Han and Bonventre, 2004; Ichimura et al., 2004; Vaidya et al., 2008). Kim-1 functions as a regulator of cell-cell adhesion and endocytosis at a time when the dedifferentiated regenerating cells of the injured proximal tubule relocate to denuded patches of the basement membrane and reform a continuous epithelial layer (Bailly et al., 2002; Ichimura et al., 2008). This process is associated with the proteolytic cleavage of the ectodomain of Kim-1

into the urine (Bailly et al., 2002). The Kim-1 ectodomain is stable in urine and can be detected in the urine of humans with acute kidney injury (Han and Bonventre, 2004), and in a variety of nephrotoxic models in animals (Amin et al., 2004; Ichimura et al., 2004; Vaidya et al., 2006; Vaidya et al., 2008). Kim-1 has been shown to be a sensitive marker of proximal tubule injury caused by a variety of chemical agents including cisplatin, S-(1,1,2,2-tetrafluorethyl)-L-cysteine, folic acid (Ichimura et al., 2004; Vaidya et al., 2006), gentamicin (Zhou et al., 2008), Hg (Zhou et al., 2008) and cyclosporine (Perez-Rojas et al., 2006).

In recent studies utilizing a sub-chronic model of Cd exposure in the rat, we showed that Kim-1 is a very early urinary marker of Cd-induced kidney injury (Prozialeck et al., 2007 and the accompanying publication by Prozialeck et al. in this journal). Urinary levels of Kim-1 were found to be elevated 3–4 weeks before the appearance of metallothionein and Clara cell protein-16, which are traditional markers of Cd toxicity, and 4–5 weeks before the onset of overt polyuria and proteinuria. In our original report (Prozialeck et al., 2007), we presented preliminary evidence suggesting that at the time Kim-1 first appeared in the urine, there was little evidence of proximal tubule necrosis. The objective of the present study was to further examine the relationship between the Cd-induced increase in Kim-1 expression and the onset of necrotic and apoptotic cell death in the epithelial cells of the proximal tubule. This study entailed the use of a well-established sub-chronic model of Cd exposure in rats and the analysis of a panel of urinary, histologic and genetic markers of Kim-1 expression, necrosis and apoptosis.

Methods

Animals and Tissues

The tissues and urine samples that were used in the present studies were obtained from adult male Sprague-Dawley rats that has been treated with Cd as part of our previous study on the urinary excretion of Kim-1 (Prozialeck et al., 2007) as well as studies described in the accompanying paper by Prozialeck et al. in this journal. Animals in the Cd treatment group received daily (Monday-Friday) subcutaneous injections of CdCl₂ at a Cd dose of 0.6 mg (5.36 μmoles)/kg in 0.3–0.4 ml isotonic saline for up to 12 weeks. Control group animals received daily injections of the saline vehicle alone. One day each week, 24h urine samples were collected. The animals were allowed free access to water at all times. Food was also available *ad libitum*, except during the period in which the urine samples were being collected. Each treatment protocol included 6 control and either 6 or 12 Cd-treated animals. The treatment protocol was repeated 3 times and the data from the different protocols were pooled whenever possible. The entire treatment protocol was reviewed and approved by Midwestern University's Animal Care and Use Committee.

Determination of Urinary Kim-1, LDH and α-GST

Immediately after the collection period, urine samples were aliquoted. Fresh, non-frozen samples were immediately sent to a commercial veterinary laboratory (Antech Diagnostics, Oakbrook, IL) for the determination of lactate dehydrogenase (LDH) activity. Other aliquots were frozen and stored at –80°C and later analyzed for Kim-1 using a Microsphere-based Luminex xMAP™ assay developed in the Vaidya/Bonventre laboratories (Prozialeck et al., 2007; Vaidya et al., 2005). Other aliquots of urine were first stabilized in a special buffer (Argutus Medical, Dublin, Ireland) prior to freezing and then later assayed for alpha glutathione-S-transferase (α-GST) activity using commercially available kits (#BIO 64 RAT Argutus Medical, Dublin, Ireland). For all analyses, the urine samples were coded so that individuals performing the analyses were blinded as to the identity of the samples.

Real-time RT-PCR Analysis of Kim-1 Expression

RNA was isolated from samples of renal cortex with TRIzol reagent (Invitrogen, Carlsbad, CA), and purified with RNeasy mini-kit (Qiagen, Valencia, CA). Primers for the *Kim-1* gene (NM_173149, forward primer: TGGCACTGTGACATCCTCAGA; reverse primer: GCAACGGACATGCCAACATA) and the house keeping *GAPDH* gene (NM_017008, forward primer: CCTGGAGAAACCTGC- CAAGTAT; reverse primer: AGCCCAGGATGCCCTTTAGT) were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA). Total RNA was reverse transcribed with ABI High-Capacity cDNA Reverse Transcription kit and then subjected to real-time PCR using ABI SYBR green PCR master mix (Applied Biosystems, Cheshire, UK). The cycle time values of *Kim-1* were normalized with *GAPDH*, and the relative differences between control and Cd groups were calculated and expressed as percentage of controls.

Labeling of Necrotic Cells in the Proximal Tubule

Necrosis of proximal tubule epithelial cells was evaluated by perfusing the left kidney in situ with the cell-membrane impermeable nuclear fluorochrome, ethidium homodimer, using a procedure recently developed and validated by Edwards et al. (2007). This fluorochrome is normally excluded from viable cells, but labels the nuclei of dead or dying cells with an intense red/orange fluorescence. Following the perfusion procedure, the left kidney was removed and cryosectioned at a thickness of 5 μm . Sections were mounted on glass slides, fixed, permeabilized in -20°C methanol, and stained with 0.3 μM 4,6 diamidino-2 phenylindole (DAPI), to label all nuclei. Fields were viewed under both phase contrast and fluorescent illumination using a 40X objective. The number of total nuclei (DAPI-labeled) and necrotic cells (ethidium-labeled nuclei) were viewed in random fields of the outer renal cortex. Each field contained approximately 300 cells in an area of $9.1 \times 10^4 \mu\text{m}^2$. Digital images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) using the Image-Pro Plus software package (Media Cybernetics, Silver Spring, MD) as described by Edwards et al. (2007). Some images were further processed using the Adobe Photoshop CS Program (Version 8.0, Adobe Systems).

In some studies, Kim-1 was visualized in the tissue sections from the ethidium perfused kidneys, a procedure that allows for the visualization of Kim-1 and necrotic cells in the same tissue section. Briefly, cryosections of the ethidium-perfused kidneys were mounted on glass slides and then fixed and permeabilized in -20°C methanol. Sections were incubated for 10 minutes in 10% goat serum to block nonspecific labeling. The samples were then incubated with the primary antibody (a mouse anti-Kim-1 generated in the Bonventre laboratory) at a 1:50 dilution for 45 minutes at 37°C . After a brief rinse in PBS, the samples were incubated in the secondary antibody, a 1:50 dilution of FITC-conjugated goat-anti-mouse IgG for 30 minutes (Pierce, Rockford, IL). Following a rinse in PBS and water, the samples were mounted under coverslips with aqueous mounting medium.

Identification of Apoptotic Cells

Apoptotic cells in either cryosections or formalin-fixed paraffin-embedded tissue sections were visualized by terminal deoxynucleotide transferase mediated dUTP nick end-labeling (TUNEL) using ApoTag kits (#S7100 and S7100 TRITC, Chemicon, Temecula, CA) with either immunofluorescence or immunoperoxidase visualization according to the manufacturer's protocol.

In some studies, a dual labeling procedure was used to visualize Kim-1 and apoptotic cells in the same cryosection. After apoptotic cells were labeled with the ApoTag Kit reagents and fluorescence identified with tetramethyl rhodamine iso-thiocyanate (TRITC) detection, the samples were blocked for 10 minutes with 10% goat serum in PBS and then incubated with

the primary antibody, a 1:50 dilution of the mouse-anti-Kim-1. Following a rinse in PBS, the samples were incubated with the secondary antibody, a FITC-conjugated goat-anti-mouse IgG for 30 minutes. Samples were then rinsed in PBS and water and mounted under coverslips.

Determination of Tissue Levels of Cd

Levels of Cd in representative samples of renal cortex were determined by Chemical Solutions Inc. (Mechanicsburg, PA) using a DRCII inductively-coupled plasma mass spectrometer to analyze nitric acid extracts of ashed tissue samples.

Statistical Analysis

Statistical analyses were performed using the Sigma Stat computer program (Version V2.03, Systat Software Inc., Point Richmond, CA). Data for the various urinary and histological parameters were analyzed by the non-parametric Kruskal-Wallis test and Dunn's posthoc test for multiple comparisons. The nonparametric test was used because analysis of the data by the Kolmogorov-Smirnov test (normality) and Levene's test (homogeneity of variances) showed that much of the data did not meet the criteria that are necessary for the use of standard parametric tests.

Results

The effects of the Cd treatment regimen on body weight and a panel of urinary and blood parameters have been described in detail elsewhere (Prozialeck et al., 2007) and in the accompanying papers by Prozialeck et al. and Edwards et al. in this journal. The key findings from those analyses were that after 9–10 weeks of exposure, the Cd-treated animals developed significant polyuria and proteinuria, with no change in urinary creatinine excretion, effects that are characteristic of a mild level of Cd-induced proximal tubule injury. For the convenience of the reader, the data for urine volume and urinary protein excretion (expressed as percent of control) are shown in Figure 1. The figure also shows the time course for the urinary excretion of Kim-1 and the proximal tubule-derived enzyme α -GST. As may be seen, significantly elevated levels of Kim-1 were present in the urine after 6 weeks of Cd treatment. By contrast, significant elevations in urinary levels of α -GST were not evident until 8 weeks.

Effects of Cd on Proximal Tubule Morphology

Figure 2 shows representative images of H+E stained sections of renal cortex from control and Cd-treated (0.6 mg/kg/5days per week for 6 or 12 weeks) animals. Note that the proximal tubule epithelial cells in the control samples exhibited cuboidal shapes, well-defined nuclei, and a uniform cytoplasm, with no spaces or gaps between the cells. The only significant changes in the samples from the 6 week Cd-treated animals (top center and top right) were that the epithelial cells showed an irregular appearance, with gaps between the cells. The cells remained attached to the basement membrane, however, and showed little or no overt evidence of necrosis or apoptosis. Analyses of the glomeruli and distal segments of the nephron revealed no evidence of pathology (not shown). The kidneys from the animals that had been treated with Cd for 12 weeks showed essentially the same changes that were evident at 6 weeks, except that isolated proximal tubules showed some detachment of cells and congestion (arrows). Analysis of similar tissue sections from 6 animals from each treatment group indicated that these changes, or lack thereof, were remarkably consistent from one animal to another.

Visualization of Necrotic Cells

To specifically identify necrotic cells in the proximal tubule, kidneys from representative animals were perfused *in situ* with the cell death marker ethidium homodimer and the number of ethidium-labeled nuclei in cryosections of the outer cortex was determined. As a positive

control to demonstrate the labeling of necrotic cells, an additional animal was treated acutely with HgCl₂ (1.75 mg Hg/kg, intraperitoneally), a treatment that produces extensive proximal tubule necrosis within 24 hours (Edwards et al., 2007). Representative micrographic images from this study are shown in Figure 3. The photos in the top row show phase-contrast images of the microscopic fields of the outer renal cortex; those in the middle row show the DAPI-labeled nuclei (total cells) in the same fields and those in the bottom row show the ethidium labeling (necrotic cells) in the same fields. Note that few, if any, ethidium-labeled nuclei are present in the samples from the Cd-treated animals, whereas many ethidium-labeled nuclei are visible in the sample from the Hg-treated animal. To quantify these observations, random fields of the renal cortex were counted by trained observers who were blinded as to the identity of the samples. The cell count data are summarized in Table 1. Note that the number of ethidium-labeled nuclei in the samples from the Cd-treated animals was not significantly different from the controls, whereas the number of labeled cells in the sample from the Hg-treated animals was much higher. Interestingly, there was a small, but statistically significant, reduction in the total number of nuclei that were visible in the fields of the sections from animals that had been treated with Cd for 12 weeks, suggesting that some cells may have been shed into the urine.

Visualization of Apoptotic Cells

Apoptotic cells in formalin-fixed paraffin-embedded tissue sections of the outer renal cortex were visualized by TUNEL and the numbers of apoptotic cells in random microscopic fields were determined. As may be seen from the images in Figure 4, only occasional, isolated apoptotic cells (arrows) were visible in the sections from the control animals and the sections from the animals that had been treated with Cd for 6 weeks. However, there did appear to be an increase in the number of apoptotic cells (arrows) in the sections from the animals that had been treated with Cd for 12 weeks. The cell count data summarized in Table 2 confirm these observations. While there appeared to be a slight increase in the number of apoptotic cells after 6 weeks, it did not reach a level of statistical significance. However, after 12 weeks, there was a statistically significant increase in the number of apoptotic cells in the proximal tubules.

Localization of Kim-1 in Relation to Necrotic and Apoptotic Cells

To evaluate the pattern expression of Kim-1 in relation to the localization of necrotic cells in the proximal tubule, cryosections of the outer renal cortex from kidneys that had been perfused with ethidium homodimer were processed for the immunofluorescent visualization of Kim-1. Results of these analyses are summarized in Figure 5. In the images, Kim-1 is identified by green fluorescence, whereas the nuclei of necrotic cells fluoresce red. Note that in the sample from the 6 week Cd-treated animal, Kim-1 was present on the surface of the epithelial cells in about 25% of the tubules. However, few if any necrotic cells were visible in the sample. Likewise, in the sample from the animal that had been treated with Cd for 12 weeks, Kim-1 was present in more than half the tubules even though no necrotic cells were evident. The samples from a Hg-treated animal (positive control) showed a large number of necrotic cells as well as many Kim-1 positive tubules. It is worth noting that in this sample, Kim-1 is present at highest levels in tubules that show only modest levels of ethidium labeling (long arrows). Kim-1 is generally present at lower levels in those tubules that show the most intense ethidium labeling (short arrows). This observation is consistent with the hypothesis that Kim-1 is mainly expressed in non-lethally injured cells as part of the phagocytic and repair mechanisms in response to injury (Bailly et al., 2002; Ichimura et al., 2008).

Another dual labeling procedure was used to visualize both Kim-1 and apoptotic cells in the same cryosections. This procedure involved the labeling of apoptotic cells by TUNEL with fluorescence visualization and the immunofluorescence labeling of Kim-1 in the same section. Results of these studies are summarized in Figure 6. In these images, apoptotic cells are identified by red fluorescence, whereas the presence of Kim-1 is reflected by green. Note that

after 6 weeks of Cd exposure, there were only a few apoptotic cells present in isolated tubules but that Kim-1 was expressed on cells in about 25% of the proximal tubules. After 12 weeks, there was an increase in both the number of apoptotic cells and in the expression of Kim-1. Interestingly, while Kim-1 was present in all of the tubules that exhibited apoptotic labeling (short arrows), it was also present in many tubules that contained no apoptotic labeling (long arrows).

Correlation of Morphologic and Biochemical Analyses

In conjunction with the morphologic analyses described above, we have attempted to determine how the morphologic findings correlate with the various biochemical, biomarker and gene expression analyses that we have conducted over the past 2 years. Results of these analyses are summarized in Figure 7. The two graphs show the increases in the expression and urinary excretion of Kim-1 in relation to changes in markers of necrosis (ethidium labeling and excretion of LDH and α -GST), and the number of apoptotic cells in the proximal tubules after 6 weeks and 12 weeks of exposure to Cd. Note that after 6 weeks, the level of Kim-1 expression, as assessed by real-time RT-PCR analyses, was increased by 5 fold and the urinary excretion of Kim-1 was increased by 10 fold. However, at this point in time, there was no change in the levels of ethidium-labeling, the urinary excretion of LDH and α -GST, or in the number of apoptotic cells in the proximal tubule. At 12 weeks, the levels of Kim-1 expression and excretion were further increased by 23 fold and 140 fold respectively. Interestingly, while there was no increase in the number of ethidium labeled cells at 12 weeks, there was a significant increase in the urinary excretion of LDH (2.5 fold) and even more pronounced increase in the urinary excretion of α -GST (15 fold). Moreover, there was a significant increase (about 9 fold) in the number of apoptotic cells in the proximal tubule.

Tissue Levels of Cd

After 6 and 12 weeks, the levels of Cd in the renal cortex were 175 ± 9 (n=4) and 183 ± 16 $\mu\text{g/g}$ of tissue (n=4) respectively. These tissue levels are comparable to those reported by other investigators employing similar Cd treatment protocols and are in the range that is generally regarded as the threshold for the onset of proximal tubule injury in the rat (Aoyagi et al., 2003; Dudley et al., 1985; Goyer et al., 1989; Suzuki, 1980; Tanimoto et al., 1993).

Discussion

In considering the implications of the present findings, it is important to note that the Cd dose and treatment protocol that were employed in these studies are similar to those that have been used by many other investigators (Aoyagi et al., 2003; Dudley et al., 1985; Goyer et al., 1989; Peereboom-Stegeman et al., 1979; Shaikh et al., 1999; Suzuki, 1980; Tanimoto et al., 1993). This Cd treatment regimen results in consistent changes in a wide variety of parameters including: body weight, urine volume, urinary protein excretion and renal Cd levels. The time course and magnitude of the changes in these parameters that we observed are essentially identical to those reported in many previous studies (Dudley et al., 1985; Goyer et al., 1989; Shaikh et al., 1999; Suzuki, 1980). With this treatment regimen, the classic early signs of Cd-induced proximal tubule injury (i.e. polyuria and proteinuria, with no change in creatinine excretion) do not appear until 9–10 weeks of exposure and even after 12 weeks, the level of proximal tubule injury is relatively mild. The delayed onset of overt dysfunction makes this model very useful for identifying the early stages of Cd-induced proximal tubule injury.

The results of the present studies indicate that the increase in the expression of Kim-1 and the shedding of the ectodomain into the urine, which occur after as little as 6 weeks of Cd exposure, represent very early events in the pathophysiology of Cd-induced proximal tubule injury. These results also indicate that at this 6-week time point, there is little evidence of either necrotic or

apoptotic cell death in the proximal tubule. This conclusion is based both on morphologic observations and on the complete absence of enzymeuria (α -GST, LDH) at 6 weeks. Even after 12 weeks, of Cd exposure, there is little evidence of proximal tubule necrosis. At this point however, there are statistically significant increases in the number of apoptotic cells in the proximal tubule and in the urinary excretion of α -GST.

The absence of overt evidence of proximal tubule necrosis after 6 weeks with this Cd treatment protocol is consistent with published observations by many other laboratories (Aoyagi et al., 2003; Dudley et al., 1985; Goyer et al., 1989; Tanimoto et al., 1993). Moreover, our general observation that Cd can eventually trigger apoptosis of proximal epithelial cells is consistent with previous observations (Aoyagi et al., 2003; Tanimoto et al., 1993). However, it is important to note that the time course that we observed for the onset of apoptosis (between 6 and 12 weeks) is different from time courses reported by other investigators using similar treatment protocols. Tanimoto et al. (1993) reported that after 4 weeks of treatment with Cd, a very low level of apoptosis (<1% of visible cells) was evident in the straight segments of the proximal tubule. In a more recent study, Aoyagi et al. (2003) noted an increase in the number of TUNEL-labeled cells in the renal cortex of Cd treated rats after 4 and 5 weeks of exposure, but that the level of apoptotic labeling was much less pronounced after 6 and 8 weeks of exposure. However, no quantitative data was included in their analysis. In the present study, we found no evidence of apoptosis after 6 weeks of treatment with Cd but did find a significant increase in the number of apoptotic cells at 12 weeks.

The reasons for the differences from the various studies remain unclear but may be related to differences in the methodologies that were employed. For example, Tanomoto et al. (1993) focused their analyses on the S-3 segment of the proximal whereas our present analysis focused on the S-1 and S-2 segments. We did this because our previous analyses indicated that these regions were especially sensitive to the Cd-induced alterations in cell adhesion molecule localization (Prozialeck et al., 2003) and preliminary analysis of the current results indicated that the S-1 and S-2 segments showed the most pronounced increase in Kim-1 expression. In addition, the Cd treatment protocols that were used in the various studies were slightly different. In the studies by Aoyagi et al. and Tanimoto et al., the animals received injections of 0.6 mg Cd/kg in 1 ml saline 6 days per week, whereas in our studies the animals were treated with 0.6 mg Cd/kg in 0.3–0.4 ml saline only 5 days per week. Moreover, the Tanimoto study utilized rats that were significantly larger and older than those used in the present study; Aoyagi et al. did not specify the age or weight of the animals that were used. In considering this issue, it is important to note that both Tanimoto et al. (1993) and Aoyagi et al. (2003) reported increases in the urinary excretion of Cd and other changes in renal function that occurred within the same time frame (4–5 weeks) that apoptotic cells were identified in the proximal tubules. We, too, observed increases in the urinary excretion of Cd and other indicators of renal injury at the same time that apoptotic cells were identified in the proximal tubules, but the changes were not evident until 6–12 weeks. The fact that the onset of apoptosis in all of the studies coincided with the onset of proximal tubule dysfunction suggests that the differences in the time course in the various studies are probably due to methodologic differences. These findings also suggest that apoptotic cell death may be important in the sequence of events leading to Cd-induced proximal tubule dysfunction. However, it is also noteworthy that in all of these studies the number of cells undergoing apoptosis appears to be remarkably small, i.e. less than 1%. It remains to be determined how such a low level of apoptotic cell death contributes to the profound alterations in proximal tubule function that occur during this stage of Cd-induced injury.

One caveat that should be mentioned in considering the present findings is that the TUNEL technique that was employed labels the fragmented DNA that is generated during the latter stages of apoptosis. The earlier stages of apoptosis involve a complex sequence of events that

include alterations in mitochondrial function, gene regulation and the activation of enzymes such as caspases and calpains (Gobe and Endre, 2003; Lee and Thevenod, 2008; Padanilam, 2003). It is possible that some cells in the early stages of apoptosis may have been present at the time Kim-1 was first expressed. Complicating this issue is the fact that results of in vitro studies have shown that Cd has complex, and sometimes opposing, effects on the cascade of events that are involved in apoptotic cell death (Gunawardana et al., 2006; Lee and Thevenod, 2008). In conjunction with the present studies, we examined the levels of expression of caspases 3 and 8 by real time RT-PCR and found no change in the levels of expression of these proteins after 6 weeks of Cd exposure. Moreover, results of immunofluorescent labeling studies (not shown) revealed no changes in the level of active caspase-3 in the proximal tubules after 6 weeks of Cd exposure. Nevertheless, the present studies do not completely rule out the possibility that some cells in the early stages of apoptosis may be present in the proximal tubules at the time Kim-1 expression begins to increase. If apoptosis is occurring at 6 weeks, however, the number of cells involved must be exceedingly small.

One of the more interesting findings from the present study relates to the time course for the Cd-induced increase in α -GST excretion. α -GST is a cytosolic enzyme that is expressed at high levels in the epithelial cells of the proximal tubule, but not in other segments of the nephron (Ferguson et al., 2008; Sundberg et al., 1994; Vaidya et al., 2008). The excretion of α -GST in the urine is classically thought to result from the loss of cell membrane integrity that occurs when proximal tubule epithelial cells undergo necrosis (Ferguson et al., 2008; Sundberg et al., 1994; Vaidya et al., 2008). However, our results show that during the time period in which α -GST first appears in the urine (8–12 weeks), there is little evidence of necrosis in the proximal tubule.

There are several possible explanations for this phenomenon. The first possibility is that the leakage of α -GST may occur at a very low level of necrosis that could be below the level of detection of the methods used in the present study. This possibility is supported by a study by Kharasch et al. (1998) who found that following treatment of rats with the nephrotoxic metabolite of sevoflurane (compound A), increased (i.e. doubled) urinary excretion α -GST was evident at a point in time when only about 1% of proximal tubules exhibited evidence of necrosis, as assessed by standard brightfield microscopy techniques. In addition, other studies have shown that enzymuria precedes tubular proteinuria in other models of acute renal injury (Bernard et al., 1987; Fauconneau et al., 1997; Gibey et al., 1981). On the other hand, the ethidium perfusion technique that was used to label necrotic cells in the present study is extremely sensitive and can label individual necrotic cells within a given tubule (Edwards et al., 2007). Extensive analysis of multiple tissue sections of ethidium perfused kidneys from the Cd treated animals revealed no increase in the number of ethidium labeled cells.

An alternative explanation for the current findings is that the appearance of α -GST in the urine could result from the shedding of viable or apoptotic cells into the urine. Cd has been shown to disrupt cell adhesion molecules in the proximal tubules (Jacquillet et al., 2006; Prozialeck et al., 2003) and the cell count data in Table 1 suggest that cells are being shed into the urine of the Cd-treated animals. It seems likely that any such cells that might be present in urine could die during the 24 collection period when the samples are at room temperature. In a similar manner, the membrane-coated vesicles that would be shed into the urine from apoptotic cells could also leak their contents into the urine. The potential for lysis of the cells and cellular debris would be enhanced by the freezing and thawing of the samples prior to the α -GST analyses. In the present studies, the urine was not examined for the presence of cells or cellular debris. However, this would certainly be something to explore in future studies.

A key issue that we have been trying to address in our ongoing research is to identify primary molecular mechanisms that are involved in the early stages of Cd-induced proximal tubule

injury. Taken together, the results of the present studies strongly indicate that the early stages of Cd-induced kidney injury involve a sublethal injury or functional alterations in the epithelial cells of the proximal tubule. In this context, the observation that these early Cd-induced effects are associated with changes in the localization and function of cell adhesion molecules such as N-cadherin (Prozialeck et al., 2003) and claudin-2 (Jacquillet et al., 2006) could be especially significant. The fact that the initial changes in the localization of N-cadherin are evident at about the same time that Kim-1 appears in the urine (i.e. 6 weeks) raises the possibility that a loss of cell-cell adhesion, may be the event that triggers the up-regulation of Kim-1 expression. This too, would seem to be interesting area for future studies.

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Figure 1. Effects of Cd on urine volume and urinary excretion of protein, Kim-1, α -GST
 Data for the various parameters are from the accompanying paper by Prozialeck et al. in this journal, and have been recalculated here as fold increases over time-matched controls. Values represent the mean $\pm$ SE. An * denotes that $p < 0.05$ as determined by the non-parametric Kruskal-Wallis test and Dunn's posthoc test for multiple comparisons. $n=9-36$ for the various data points.

Figure 2. Effects of Cd on kidney morphology

Rats were treated with Cd (0.6 mg/kg/5 days per week) for 6 to 12 weeks and representative sections of the renal cortex were processed for hematoxylin and eosin staining.

Figure 3. Visualization of necrotic cells with ethidium homodimer

Animals were treated with either Cd (0.6 mg/kg, subcutaneously 5 days a week for 6 or 12 weeks) or HgCl_2 (1.75 mg/kg of Hg, intraperitoneally). The left kidneys were perfused with ethidium homodimer as discussed in the Methods. Cryosections of the kidneys were then fixed, permeabilized and labeled with DAPI to identify total nuclei. a–d are phase contrast images corresponding to DAPI-labeled panels; (e–h) and ethidium homodimer-labeled panels (i–j). Samples from Hg-treated animals showed widespread necrosis (j), as indicated by intense ethidium labeling. No differences in ethidium homodimer labeling were detected in 6 week Cd^{2+} -treated samples (k) or 12 week Cd-treated samples (l) compared with 12 week saline-treated control (i).

Figure 4. Labeling of apoptotic cells

Animals were treated with Cd for either 6 weeks or 12 weeks and then formalin-fixed, paraffin embedded tissue sections were processed for the visualization of apoptotic cells by TUNEL, as described in the Methods section. The brown labeling highlighted by the arrows represents apoptotic cells.

Figure 5. Dual labeling of necrotic cells and Kim-1

Cryosections from the ethidium-perfused kidneys were processed for the immunofluorescent visualization of Kim-1 as described in the Methods. In the images, Kim-1 is identified by green fluorescence, whereas nuclei of necrotic cells fluoresce red. The images in the left column show the ethidium labeling in a given field and the images in the center column show the Kim-labeling in the same field. The photos on the right show merged images of the ethidium and Kim-1 labeling.

Figure 6. Dual labeling of apoptotic cells and Kim-1

Kidney cryosections were processed for the dual visualization of apoptotic cells by TUNEL and the immunofluorescent visualization of Kim-1 as described in the Methods section. In these images, apoptotic cells are identified by red fluorescence whereas Kim-1 fluoresces green. The images in the left column show apoptotic cells and the images in the middle show the Kim-1 labeling in the same field. The photos in the right column show the merged apoptotic and Kim-1 labeling in the same field.

Figure 7. Effects of Cd on Kim-1 expression and excretion in relation to necrosis, apoptosis and urinary enzyme excretion

Levels of Kim-1 expression were determined by real time RT-PCR and the urinary levels of Kim-1, α-GST and LDH were determined as described in the Methods and are expressed as fold increase over control values. In addition, the cell count data, for apoptotic and necrotic cells in Tables 1 and 2 were recalculated as fold increases. An * indicates a significant increase from control values ($p < 0.05$).

Table 1

Ethidium Cell Count Data

Treatment	Total Nuclei/Field	Eth.-labeled Nuclei/Field	Percent Dead Cells
6 Week Control	358±16.3	0.9±0.5	0.3±0.1
6 Week Cadmium	394±10.8	1.7±0.4	0.6±0.1
12 Week Control	309±13	1.5±0.8	0.5±0.3
12 Week Cadmium	*250±20	2.9±1.0	1.4±0.5
Hg	262±34.9	*64±11.0	*24.4±8.9

Necrotic cells in the proximal tubule were visualized by perfusing the kidneys with the membrane impermeable nuclear fluorochrome (Eth) ethidium homodimer. The total number of nuclei in each field was determined by DAPI labeling. To validate this novel method for assessing cell viability/membrane integrity in the kidney, 3 additional animals were treated acutely with HgCl₂ (1.75 mgHg/kg, i.p.), a treatment that produces necrosis of the proximal tubule within 24 hours. Values represent the mean ± SE. n=5–6 animals per treatment group. The values for each animal represent the mean number of labeled nuclei in 3 fields from 4 separate tissue sections from that animal. An * denotes $p < 0.05$.

Table 2

ApopTag Cell Count Data

Treatment Group	Labeled Cells/Field
Control (pooled values for 6 and 12 weeks)	1.4 ± 0.3
Cd 6 week	2.9 ± 0.6
Cd 12 week	*9.2 ± 2.0

Apoptotic cells in formalin-fixed paraffin embedded tissue sections were visualized as described in the Methods and then viewed using a 40x objective on the microscope. Labeled cells in random fields of renal cortex were determined by trained observers who were blinded as to the identity of the samples. Values in the table represent the mean ± SEM of the number of labeled cells in 4 random fields in sections from 4 animals in each treatment group. An * denotes $p < 0.05$.