Molecular mechanisms of enhanced renal cell division in protection against S-1,2-dichlorovinyl-L-cysteine-induced acute renal failure and death

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First published March 1, 2005; doi:10.1152/ajprenal.00418.2004.—Sustained activation of ERK 1/2 by a low dose (15 mg/kg ip) of S-1,2-dichlorovinyl-L-cysteine (DCVC) 72 h before administration of a lethal dose of DCVC (75 mg/kg ip) enhances renal cell division and protects mice against acute renal failure (ARF) and death (autoprotection). The objective of this study was to determine correlation among extent of S-phase DNA synthesis, activation of transcription factors, expression of G1/S cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors downstream of ERK 1/2 following DCVC-induced ARF in autoprotection. Administration of the lethal dose alone caused a general downregulation or an unsustainable increase, in transcriptional and posttranscriptional events thereby preventing G1→S transition of renal cell cycle. Phosphorylation of IκBα was inhibited resulting in limited nuclear translocation of NF-κB. However, cyclin D1 expression was high probably due to transcriptional cooperation of AP-1. Cyclin D1/cyclin-dependent kinase 4 (cdk4)-cdk6 system-mediated phosphorylation of retinoblastoma protein was downregulated due to overexpression of p16 at 24 h after exposure to the lethal dose alone. Inhibition of S-phase stimulation was confirmed by proliferating cell nuclear antigen assay (PCNA). This inhibitory response was prevented if the lethal dose was administered 72 h after the low priming dose of DCVC due to promitogenic effect of the low dose. NF-κB-DNA binding is not limited if mice were pretreated with the priming dose. Cyclin D1/cdk4-cdk6 expression stimulated by the priming dose of DCVC was unaltered even after the lethal dose in the autoprotected group, explaining higher phosphorylated-pRB and S-phase stimulation found in this group. These results were corroborated with PCNA immunohistochemistry. These findings suggest that the priming dose relieves the block on compensatory tissue repair by upregulation of promitogenic mechanisms, normally blocked by the high dose when administered without the prior priming dose.

cyclin D1; extracellular signal-regulated kinases 1/2; phospho-retinoblastoma; tissue repair

ACUTE RENAL FAILURE (ARF), a side effect of therapeutic interventions, environmental insults, and exposure to toxicants, is generally characterized as a marked change from baseline renal function manifested as elevations in blood urea nitrogen (BUN) levels, serum creatinine, and a decrease in urine output (38, 39, 56, 60, 63). The incidence of ARF has ranged from 0.15 to 25% in recent studies and its incidence in critically ill patients has ranged from 4 to 8% (48, 56). Also, with advancing age, loss of renal function may be exacerbated by drug-induced or other unknown etiologies culminating in life-threatening ARF. Between 1990 and 2001 in the US, cases of kidney failure rose from 697 to 1,424 per 1 million, according to the Centers For Disease Control and Prevention (CDC) (11). There is considerable interest in preventing such structural and functional decline and possibly restoring kidney function, regardless of the cause. Although little is known about recovery from ARF (30), early detection and acceleration of this process could lead to significant reductions in mortality rates. Therefore, understanding the mechanisms of renal tissue repair is critical to the development and evaluation of pharmacological strategies to prevent and/or treat ARF.

Recovery from ischemia, advancing age- and toxicant-induced acute tubular injury involve recruitment of cells, which may be important sources of paracrine growth factors, as well as proliferation and differentiation of surviving cells to form polarized renal epithelial tubule cells (7, 35). MAPK are important signal transducing enzymes stimulated by growth factors promoting renal regeneration in model systems (4, 10, 13, 36, 39, 65). Recently, it has been shown that activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) increases DNA synthesis in response to renal injury (15, 26, 28, 40, 58, 65). However, downstream of ERK1/2 activation, transcriptional and posttranscriptional mechanisms of S-phase DNA synthesis during active renal regeneration are not well understood. Several intrinsic mechanisms are brought to bear by the kidney on exposure to toxic or ischemic insult, which protect it against subsequent exposure to a toxicant (65) or ischemia (8). Although precise reasons for ARF may or may not be known, it might be possible to understand the mechanisms by which renal structure and function could be restored after injury. In this regard, drug-induced or chemical-induced ARF models might be useful tools. One such model is S-1,2-dichlorovinyl-L-cysteine (DCVC)-induced ARF (63, 64). Prior administration of a low dose of DCVC which primes cell division such that tissue repair is not inhibited even after the administration of a normally lethal dose (64). Sustained stimulation of phosphorylated-ERK1/2 is thought to be a key response enabling this persistent tubular repair, leading to recovery (65).

A number of cell cycle-regulated proteins have been identified and categorized as either cyclins, cyclin-dependent kinases (CDKs) or cyclin-dependent kinase inhibitors (CKIs) (27, 53, 54). Upregulation of these pathways in kidney may...
account for the spatial differences of DNA synthetic activity and cell cycle in outer medulla after renal injury (44–46). The activity of CDKs controls cell cycle progression; this activity is regulated positively and negatively by the association of CDKs with cyclins and CKIs, respectively (37). Several CKIs including p16INK4a, p27Kip1, and p21Cip/Waf1 play roles in coordinating temporal activation of cdk4 and cdk2 by D-type and E-type cyclins, respectively (54). ERK1/2 acts upstream of transcription factors like NF-κB, AP-1 (scheme 1)

and these transcription factors mediate progression of cells from G1 to S-phase by regulating cyclin D1 expression (16, 23, 61). With growth factor and ERK1/2 stimulation, D-type cyclins are synthesized in the early G1 phase, and they bind to and activate CDK4, as cells leave the quiescent phase (27, 53). The active CDK4 phosphorylates endogenous retinoblastoma protein (pRb) in the late G1 phase of renal cell cycle, thereby releasing E2F-DP complex that acts as a transcription factor for various S-phase-related genes (37). It has been shown that phosphorylation of pRb in proximal tubules and mesangial cells of kidney is considered critical for the passage of cells from G1 to S-phase (46, 52). However, to date the regulation of renal cell cycle at transcriptional and posttranscriptional level by various signaling molecules has not been investigated in animals surviving or dying from ARF induced by a lethal dose of DCVC. The objective here was to determine the molecular events following the administration of DCVC that may explain the lack of tissue repair and hence mortality. Therefore, time points were selected and the same mice were maintained in our central animal facility under conditions of 21 ± 1°C and 50 ± 10% relative humidity at all times. They were maintained on a 12:12-h light-dark cycle, over wood chips free of any known chemical contaminants. The mice received commercial rodent Chow (Teklad rodent diet no. 7012) and water ad libitum and were acclimated for 1 wk before use. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by our Institutional Animal Care and Use Committee.

Mice (n = 3 per group) were divided into three groups. Group I (low/priming dose) received one dose of 15 mg DCVC/kg ip in distilled water (DW; 10 ml/kg) on day 0 and the same mice received a single administration of 10 ml DW/kg ip on day 3. Mice in group II (high dose) were injected with 10 ml DW/kg ip on day 0 and a single administration of 75 mg DCVC/kg ip on day 3. Mice in group III (autoprotection group) were treated with one dose of 15 mg DCVC/kg ip on day 0 and a single administration of 75 mg DCVC/kg ip on day 3. BUN was measured as a marker of renal dysfunction to confirm that these mice suffer from ARF. Renal failure assessed by BUN levels was corroborated by histopathology using 10% formalin-fixed, paraffin-embedded, 4-μm-thin kidney sections stained with hematoxylin and eosin (H&E).

**Chemicals.** Unless stated otherwise, all chemicals and biochemicals were purchased from Sigma (St. Louis, MO); DCVC was provided by Syngenta (Central Toxicology Laboratory, Macclesfield, UK) and was >99.5% pure. Mouse monoclonal anti-cyclin D1 (DCS6), rabbit polyclonal anti-IκBα, and anti-phospho-IκBα (Ser32) were purchased from Cell Signaling (Beverly, MA); mouse monoclonal anti-Cdk4, Cdk6, p16, and anti-phospho-pRb (P-pRB) (pS795) were purchased from Sigma. Loading control GAPDH was purchased from Santa Cruz Biotechnology. Anti-rabbit and antimouse secondary antibodies conjugated with horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and Sigma, respectively.

**Time course study.** Mice in group I received one dose of 15 mg DCVC/kg (low dose) ip in DW (10 ml/kg) on day 0 and the same mice received a single administration of 10 ml DW/kg ip on day 3. Mice in group II (high dose) were injected with 10 ml DW/kg ip on day 0 and a single administration of 75 mg DCVC/kg ip on day 3. Mice in group III (autoprotection group) were treated with one dose of 15 mg DCVC/kg (priming dose) on day 0 and a single administration of 75 mg DCVC/kg ip on day 3. Mice from group I and group III (n = 3 per time point) were terminated under diethyl ether anesthesia at 3, 6, 12, 24, 72, 75, 78, 84, and 96 h after the low-dose administration. The same time course was followed for mice in group II (n = 3 per time point) after the administration of DW. Previous studies have shown that 90% of the mice receiving only the high dose of DCVC (group II) die between 108 to 120 h after DW administration (63, 64) and therefore the objective here was to determine the molecular events following the administration of DCVC that may explain the lack of tissue repair and hence mortality. Therefore, time points were selected

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1 Scheme 1. Schematic of hypothesized signaling mechanisms driving the increased renal tubule cell division after challenge with a priming dose of S-1,2-dichlorovinyl-L-cysteine (DCVC) in DCVC autoprotection. DCVC treatment increases plasma IL-6, which activates ERK 1/2 (65) leading to stimulation or initiation of cell division via transactivating mechanisms involving NF-κB and AP-1. NF-κB translocates into the nucleus by phosphorylation of its endogenous inhibitor IκBα through ERK 1/2-mediated IκBα kinase (16). ERK 1/2 also stimulates the activity of AP-1 via phosphorylation of the transcription factor p300 (303-304). NF-κB and AP-1 transcription factors mutually cooperate with each other and bind to the promoter region of DNA, which initiate transcription of cyclin D1 gene. On translation in the cytosol, cyclin D1 complexes with cyclins and CKIs, respectively (37). Several CKIs including p16INK4a, p27Kip1, and p21Cip/Waf1 play roles in coordinating temporal activation of cdk4 and cdk2 by D-type and E-type cyclins, respectively (54). ERK1/2 acts upstream of transcription factors like NF-κB, AP-1 (scheme 1)
for this group until 96 h after exposure to DW. In all cases, the plasma was assessed for BUN levels. Four-micrometer-thin kidney sections that were fixed in 10% formalin and embedded in paraffin were stained with H&E. These sections were used to confirm renal damage by histopathology.

Cell lysates and nuclear extracts were made from treated kidneys for immunoblotting and gel shift assays, respectively, for further experiments. Renal cell division was confirmed by proliferating cell nuclear antigen (PCNA) assay (21).

**Plasma enzymes.** Plasma was separated by centrifugation. BUN levels (Sigma cat. no. 63–25; procedure no. 63-UV) were measured as a biomarker for renal dysfunction using a commercially available kit from Sigma.

**Renal histopathology.** Kidneys from control and treated mice were washed with ice-cold normal saline (0.9% NaCl), cut transversely into thin slices, and then fixed into 10% phosphate-buffered formaldehyde for 48 h. The tissues were then transferred into 70% ethyl alcohol, processed, and embedded in paraffin wax. Kidney sections (4-μm thin) were stained with H&E for histological examination under a light microscope. Unstained kidney sections were prepared for PCNA immunohistochemistry.

**Renal cell division.** PCNA assay was employed to estimate cell division (21). Briefly, antigen retrieval was achieved using 1% zine sulfate solution. Blocking was achieved using 0.5% casein and then reacted with monoclonal antibody (1:5,000) to PCNA (PC10. DAKO, Carpinteria, CA) for 1 h. The antibody was then linked with biotinylated goat anti-mouse IgG antibody, which was then labeled with streptavidin-conjugated peroxidase to 3,3'-diaminobenzidine (DAB). The sections were counterstained with hematoxylin. Cells in S-phase were identified by their characteristic brown nuclear staining.

**Preparation of cell lysates and Western blot analysis.** Kidney samples were homogenized in 5 vol of protein lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonfluoride, 1 mM HEPES, pH 7.6, 1 μg/ml leupeptin, and 1 μg/ml aprotonin) using a polytron homogenizer. The homogenate was stored on ice for 10 min and then centrifuged at 13,000 × g for 15 min at 4°C. The supernatant was collected and protein was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. Proteins (50 μg) were separated on either 7.5 or 12% SDS-polyacrylamide gels and transferred to immuno-blot PVDF membranes (Bio-Rad Laboratories). Membranes were blocked in 5% dried milk in PBST (0.1% Tween 20 in 1× PBS) and incubated with 1:200 dilution of primary antibodies against cyclin D1, IxBo, phospho-IxBo at Ser32 (Cell Signaling), 1:1,000 dilution of cdk4, cdk6, p16, phospho-pRB (P-pRB, pS795) (Sigma), and 1:100 GAPDH (Santacruz Biotechnology) overnight at 4°C. After incubation for 2 h at 25°C with secondary antibodies (1:1,000) conjugated with horseradish peroxidase, membranes were detected by ECL system (Pierce, Rockford, IL). Data in all the immunoblot experiments for low-dose, high-dose, and autoprotected groups were normalized to GAPDH, which served as the internal control. Only the representative GADPH is shown for all the three groups in Fig. 4 (others not shown).

**Nuclear extract preparation and EMSA.** Nuclear proteins were prepared from mouse kidney tissue using previously described methods (18). Double-strand DNA probes were prepared using NF-kB and AP 1 binding sequences from the class I major histocompatibility enhancer element (H2K) (Promega, Madison, WI). The probes were prepared by annealing the complementary oligonucleotide in a thermal cycler in 50 mMol/l Tris, pH 8.0, 1 mMol/l EDTA. Annealed oligonucleotides were purified by polyacrylamide gel electrophoresis and then end labeled with [γ-32P]ATP by using T4 polynucleotide kinase (68). Aliquots of nuclear protein (20 μg) were incubated with 0.2 ng of [γ-32P]end labeled double-strand oligonucleotide probe. The mixture was incubated for 30 min at room temperature and then separated by electrophoresis on nondenaturing 5% polyacrylamide tris-boric acid EDTA gels. The specificity of these DNA-binding proteins for the putative binding site is established by cold competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest. If the complex is specific, the addition of unlabeled specific competitor (cold competitor) should decrease the intensity of the band. The gels were dried and exposed to Kodak (Rochester, NY) X-AR film from 2 h to 2 days.

**Statistics.** Data (BUN levels and quantitative assessment of the expression of various proteins) are expressed as means ± SE (n = 3) for all the experiments. Statistical differences were determined by one-way ANOVA followed by Tukey’s honest significant difference and Duncan’s multiple range tests to determine which means were significantly different from each other or from controls using SPSS (Chicago, IL). In all cases, P ≤ 0.05 was used as the statistical criterion to determine statistically significant differences.

**RESULTS**

**Studies with a low priming dose of 15 mg DCVC/kg.** In this series of experiments, mice (n = 3) were given a low nonlethal dose of DCVC (15 mg/kg) by intraarterial administration. This dose decreased renal function as revealed by increased BUN levels, which peaked at 24 h after dosing before returning to the normal range (Fig. 1A). Histological examination of kidney confirmed that this dose caused a mild renal proximal tubular necrosis that peaked at 24 h (Fig. 2B). As the present study examines transactivational mechanisms that involve immediate genes (6), we selected early time points for collecting
kidney tissue samples for analysis. NF-κB-DNA-binding activity (Fig. 3A) was increased after 3 h of dosing and sustained until 72 h postdosing. IκBα is a regulator protein known to complex with NF-κB, thereby decreasing the availability of NF-κB for nuclear binding. Total IκBα protein (Fig. 4A) started decreasing after 3 h of dosing with DCVC, the decrease being most marked 6 to 24 h before increasing by 72 h and remained until 96 h. It should be noted that the increase in protein expression of several proteins studied in this group 72 h onward is the influence of the priming dose but not because of the stress factor or DW at the time of second injection. Analysis of the IκBα protein present in phosphorylated form (P-IκBα) indicated that when compared with 0-h controls, P-IκBα levels (Fig. 4D) peaked at 6 h and remained elevated until 75 h before returning to basal levels. Treatment with a priming dose of DCVC also caused a small increase in the levels of cyclin D1 (Fig. 5A) from 3 to 24 h, increasing markedly by 72 h and thereafter. Activated protein 1 (AP-1) DNA-binding activity measured by gel shift mobility assay was slightly elevated at 3, 6, and 12 h after DCVC compared with controls and remained elevated until 84 h after dosing with DCVC (Fig. 6A). Cyclin D1 is known to complex with its partners cdk4/cdk6, and the complex permits progression of cells from G1 to S-phase via phosphorylation of pRb. p16 Negatively regulates phosphorylation of retinoblastoma by inactivating cdk4 (54). Therefore, cdk4 (Fig. 7A) levels were measured by immunoblotting. Cdk4 levels were high in the controls and did not differ after treatment with DCVC until 72 h postdosing where cdk4 protein levels were significantly higher compared with the controls. p16 Expression (Fig. 7D) decreased from 3 to 12 h, peaked at 72 h, and remained high until 96 h compared with 0-h controls. Cdk6 protein levels (Fig. 7G) decreased from 3 to 12 h, peaked at 72 h, and remained higher thereafter compared with 0-h controls. Following DCVC treatment, phosphorylation of pRb was increased 12 to 96 h after dosing (Fig. 8A). S-phase DNA synthesis in renal cells was confirmed by PCNA immunohistochemical staining, which revealed an increase in the number of S-phase cells at 72 h after dosing with the priming dose of DCVC (Fig. 8E). In all of the immunoblotting experiments in
the low-dose group, data were normalized to GAPDH protein that served as the internal control (only the representative GAPDH is shown in Fig. 4A).

Studies with a high lethal dose of 75 mg DCVC/kg. With 75 mg DCVC/kg, mice experienced marked and progressive renal failure as assessed by significant elevations in BUN levels over a time course of 3 to 24 h (75 to 96 h in Fig. 1B). Histopathological examination of the H&E-stained kidney sections revealed extensive proximal renal tubular necrosis at 24 h (Fig. 2C). The number of tubules with denuded proximal tubular epithelial cells were significantly higher after the treatment with 75 mg DCVC/kg alone. This injury progressed throughout the kidney and 90% of the mice died around 36–48 h after administration of lethal dose, i.e., 108–120 h after DW administration, consistent with previous report (64). Administration of DW did not alter BUN levels (Fig. 1B), normal architecture of the kidney (Fig. 2A), and the expression of any protein compared with the 0-h controls. Following administration of a high dose of DCVC, NF-κB-DNA-binding activity was remarkably decreased over the time course (Fig. 3B). Total IkB protein (Fig. 4B) remained unchanged at all time points. The phosphorylated form of IkB peaked 6 h after dosing (78 h in Fig. 4E) and before returning to baseline (96 h in Fig. 4E). However, cyclin D1 levels increased 3 to 24 h after DCVC administration (75 to 96 h in Fig. 5B), being markedly elevated 6 and 24 h after the high-dose treatment (78 to 96 h in Fig. 5B). This increase in cyclin D1 expression could be explained by higher AP-1 DNA-binding activity from 3 to 12 h of postdos-
Cyclin D1 levels increased markedly above control values (0 h in Fig. 5C) 3 h after the high dose of DCVC (75 h in Fig. 5C) and remained elevated throughout the time course (96 h in Fig. 5C). Surprisingly, AP-1-DNA-binding activity was undetectable in the autoprotected group after the lethal dose challenge (Fig. 6C). Levels of cdk4 in the autoprotected group did not change even after the high dose (Fig. 7C). This indicates that higher cdk4 expression induced by the priming dose was unaffected by the administration of the lethal dose. p16 Expression in the autoprotected group increased after a high dose (Fig. 7F), indicating temporal activation of cdk4 and thereby maintaining the phosphorylated lethal dose challenge (Fig. 2D). Light microscopic observation of H&E-stained kidney sections revealed considerable reepithelialization of the proximal tubules, with marked basophilia (Fig. 2D), and renal structural restoration was evident. NF-κB-DNA-binding (Fig. 3C) activity increased 3 h after the administration of a lethal dose (75 h in Fig. 3C) in the autoprotected group and remained high until 24 h (96 h in Fig. 3C). There was a marked decrease in the total IkBα protein 6 h after administration of the high dose of DCVC (78 h in Fig. 4C) that persisted throughout the remaining time course. Phospho-IkBα levels increased in the autoprotected group until 24 h after the exposure to a lethal dose (96 h in Fig. 4F) and the phosphorylation was persistent until 24 h of dosing with a lethal dose in this autoprotected group. Cyclin D1 levels increased markedly from 3 to 24 h (75 to 96 h in Fig. 7) after administration of a lethal dose (75 h in Fig. 7) and remained elevated throughout the time course (96 h in Fig. 7). This indicates that higher cdk4 expression induced by the priming dose was unaffected by the administration of the lethal dose.
state of hypophosphorylated pRB. This was evident by sustained and enhanced expression of cdk6 (Fig. 7I). Compared with 0-h controls, cdk6 levels increased from 6 to 24 h after the high dose (78 to 96 h in Fig. 7I) peaking at 6 h (78 h in Fig. 7I). The levels were significantly high at 24 h (96 h in Fig. 7I) after the high dose in the autoprotected group compared with 24 h (96 h in Fig. 7H) after a lethal dose alone. Phosphorylation of pRb was increased 3 h after the administration of the high dose in the autoprotected group (75 h in Fig. 8C). There was a statistically significant increase in P-pRB at all times after the high dose of DCVC (Fig. 8C) in autoprotected mice. PCNA immunohistochemistry showed a number of positively stained S-phase nuclei at 24 h (Fig. 8G) after the administration of a lethal dose in the autoprotected group (96 h after the challenge with priming dose). Most of the S-phase cells were observed in the outer strip of outer medulla where the maximal damage occurred (Fig. 2D) but overall these cells were scattered all over the cortex and medulla. In all of the immunoblotting experiments in the autoprotected group, data were normalized to GAPDH protein, which served as the internal control (not shown).

DISCUSSION

Prior administration of a low dose of DCVC protects mice against a normally lethal dose of DCVC. This protection is neither due to lower bioactivation of DCVC by the renal enzyme cysteine conjugate β-lyase nor mitigated impairment
of renal function (64). We suggest the protection is due to stimulation of proximal tubular tissue repair by the priming dose, which permits the mice to recover from the extensive injury. We showed that activation of ERK 1/2 pathway is an initial step enabling tissue repair to be triggered (65). The objective of the present work was to investigate the role of transactivational mechanisms downstream of ERK 1/2 (scheme 1) in this protection against DCVC-induced ARF and death.

Why does a high dose of DCVC prevent renal tubule cell division? Following a high dose of DCVC, tissue repair is blocked owing to downregulated phosphorylation of pRb (P-pRB) 24 h after dosing (96 h in Fig. 8B), causing inhibition of S-phase stimulation. With a lethal dose of DCVC, limited nuclear NF-κB-DNA binding occurs until 12 h primarily because of the dampened and transient phosphorylation of IκBα protein. Consequently, IκBα protein remains unphosphorylated, retaining NF-κB in the cytoplasmic compartment of the cells at the time of critical need. NF-κB is known to mediate G1 to S progression by regulating cyclin D1 expression (16, 23). Nonetheless, cyclin D1 expression is markedly enhanced from 3 to 24 h (Fig. 5B). The demand for higher cell division may also be met by DNA binding of another transcription factor,
AP-1, to achieve increased expression of cyclin D1 (61). Accordingly, the AP-1 DNA-binding activity is significantly higher until 12 h compared with any time point in mice treated with the low dose of DCVC or in the 0-h controls. These results are consistent with previous reports where transient loss of NF-κB-DNA-binding activity in acetaminophen hepatotoxicity is known to be compensated through uncommitted AP-1 DNA-binding (6). Recently, the cyclin D1 promoter has been shown to be regulated by transcription factors other than NF-κB and AP-1, like SP-1 or E2F-1 (1, 22, 67). The fact that the cyclin D1 promoter contains binding sites for all of these transcription factors indicates the possibility of multiple cooperative interactions. However, despite this backup system to circumvent the block at the G1 checkpoint, our findings indicate further problems downstream of cyclin D1.

Narrow individual thresholds are known to be critical for key cell cycle regulators to exert their effects (54). Hence, subtle changes in the expression of key signaling molecules can influence cell cycle progression. The cyclin D1/cdk4/cdk6 system is known to be critical in the progression of cells from G1 to S-phase by phosphorylating pRB (19). p16 Deregulates this phosphorylation of pRB via inactivation of cdk4 (54). P-pRB is substantially downregulated at 24 h (96 h in Fig. 8B), a critical time point after the administration of the lethal dose, resulting in inhibited S-phase DNA synthesis (Fig. 8F). p16 Was found to be overexpressed with a lethal dose challenge. Recent reports suggest that expression of p16 (INK4a) and p27 (Kip1) CDK1 genes is increased in cortical cells of the aging human kidney (14). From our findings, it seems likely that even in toxicant-induced nephrotoxicity, CKIs like p16 (INK4a) are overexpressed. Nevertheless, P-pRB levels increase dramatically through 12 h (through 84 h in Fig. 8B) and decreases at 24 h (96 h in Fig. 8B) despite similar levels of cdk4 at 84 and 96 h (Fig. 7B). It is known that cdk6 plays a role in phosphorylation of pRB to compensate when cdk4 is inactive. Mouse embryonic fibroblasts prepared from cdk4-null embryonic day 12.5 embryos proliferate normally, suggesting that cdk6 compensates for cdk4 deficiency (55). Consistent with this report, we found cdk6 levels were high until 12 h after the lethal dose challenge (84 h in Fig. 7E). However, 24 h after a lethal dose even cdk6 was significantly downregulated leading to inhibited phosphorylation of pRB and S-phase inhibition. Previous reports suggested that the cyclin D1/cdk4 system is highly active in regenerating proximal tubules. In renal ischemia, CDK4 activities increase markedly and significantly in total kidney (7-fold) and outer medulla (33-fold) (46). A lethal dose of DCVC causes renal tubular necrosis that rapidly progresses to extensive injury at 24 h (Fig. 2C) and mice die between 36 and 48 h after the administration of a lethal dose (63, 64). By 24 h (96 h in Fig. 7B), the CDK4 system may not be able to phosphorylate pRB due to marked tubular necrosis mitigating promitogenic signaling. Other possible mechanisms by which the lethal dose of DCVC inhibits renal repair could be activation of protein phosphatases, like protein phosphatase 1 (29, 49), and inhibition of cyclin E/cdk2 complexes, which are responsible for maintaining the phosphorylated state of hypophosphorylated pRB (31). Whether the lethal dose dampens transcriptional cooperation and inhibits other NF-κB-mediated cell proliferating genes remains to be investigated.

Why does a low dose of DCVC stimulate renal cell division and repair? It is known that exposure to low to moderate doses of toxicants leads to prompt stimulation of cell division and tissue repair in a variety of organs and tissues such as lung (5), liver (12), bone marrow (17, 51), and kidney (63, 64). The cellular signaling mechanisms responsible for this efficient tissue repair are a subject of intense investigation (3, 59). In this study, in contrast to the lethal dose, mice administered a low dose of DCVC exhibited prompt renal tissue repair by timely and adequate stimulation of transactivational mechanisms of cell cycle progression. When a low dose of DCVC is given, the total IκBα protein decreases from 6 to 24 h (Fig. 4A). Most of the IκBα is phosphorylated, which is consistent with the unhindered NF-κB-DNA-binding after exposure to a low dose. The increased levels of IκBα 72 h onward can be explained by the fact that NF-κB present in the nucleus autoregulates the IκBα gene and maintains its levels (62). Prompt and increased phosphorylation of IκBα during the entire time course suggests a sustained increase in nuclear translocation of NF-κB that begins as early as 3 h. Consequently, alternative compensatory AP-1 DNA binding was minimal. The stimulated and sustained NF-κB-DNA binding leads to increased transcription of cyclin D1. Our findings are consistent with an earlier report suggesting the role of NF-κB in cell cycle regulation via transcripational stimulation of cyclin D1 expression (23). Cyclin D1 complexes with its partners cdk4/cdk6 and the complex phosphorylates pRB which releases the transcriptional repression of various S-phase genes (52). Our findings suggest that increased cyclin D1 and cdk4 protein expression throughout the time course leads to higher P-pRB when the mice are challenged with a low dose of DCVC. However, p16 expression increased from 24 h onward and peaked at 72 h after the low dose, suggesting inhibition of cdk4 activity (54). Nonetheless, stimulation of phosphorylation of pRB remains until 96 h after low dose treatment. This finding of sustained phosphorylation of RB in low dose-treated mice despite higher p16 expression might be due to the compensatory role of cdk6 (Fig. 7G) or cyclin E-cdk2 ultimately leading to increased S-phase DNA synthesis (Fig. 8E) and complete recovery from the renal injury. It is known that activity of RB protein, the crucial gatekeeper during the G1 phase, is modulated primarily through phosphorylation by G1 CDKs. The sequential and combined phosphorylation of RB by cyclin D1 and cyclin E-dependent CDKs contributes to inactivation of RB (32, 69). It would be relevant to investigate the role of cyclin E-cdk2 in this sustained stimulation of pRB phosphorylation with a low dose of DCVC.

Why is renal cell division and repair not inhibited if a low priming dose of DCVC is given 3 days before the normally lethal dose? The possibility of protection against subsequent lethal injury by prior injury or preconditioning maneuvers has been an area of increasing interest (5, 8, 26, 33, 34, 59, 64). Although autoprotection has not been demonstrated in the kidney using survival as the end point until recently (64), literature suggesting the existence of such a phenomenon for renal toxicants is available (41, 57). Some of the candidate mediators of preconditioning are heat shock protein molecular chaperones (9), activation of the nitric oxide synthase pathways (42), stimulation of PPAR receptors (47), induction of endoplasmic reticulum and stress proteins (24), activation of ERK 1/2 (43), and activation of a phosphatidylinositol 3-kinase Akt/PKB pathway (2). When a toxicant causes acute renal tubular necrosis, the remaining viable quiescent tubular epithelial cells are triggered to enter the cell cycle, which eventually
leads to reepithelialization of denuded basement membranes (20). Several investigators examined the production and localization of growth factors and their ability to stimulate cell growth and proliferation in regenerating proximal tubules after chemical or ischemia/reperfusion injury (4, 7, 25, 35, 40, 50). The precise mechanisms by which growth factors and other mitogens direct this type of renal proximal tubular repair and regeneration remain largely unknown.

Our results indicate that mice receiving a low dose of DCVC exhibit stimulated compensatory tissue repair via sustained increase in P-prB to clear the cells through the G1 to S checkpoint. When a high dose of DCVC is given to mice receiving the priming dose, the total IkBα protein decreases from 3 to 24 h (75 h to 96 h in Fig. 4C). Phosphorylation of IkBα increases, which is consistent with the unhindered NF-kB-DNA binding, after exposure to a high dose in the autoproected mice. The alternative compensatory AP-1 DNA-binding activity was undetectable at any time point after the administration of a high dose to primed mice (Fig. 6C). The need for this alternative to increase cyclin D1 is obviated due to enhanced and sustained NF-kB-DNA-binding activity in autoproected animals. Similar findings have been reported previously (6). This renal cell cycle is controlled by overexpression of p16 after the promitogenic signaling by the low dose. Maintenance of the phosphorylated state of hypophosphorylated prB is achieved by sustained and enhanced expression of cdk6. Therefore, cyclin D1/cdk4/cdk6 coordinate with each other and lead to persistent and increased P-prB thereby increasing S-phase DNA synthesis (Fig. 8G). It should be noted that initial renal injury as assessed by azotemia, glucosuria, elevations in BUN levels, and histopathological examination is the same or nearly the same in mice receiving the normally lethal dose of DCVC, regardless of whether they receive a priming dose (64). Therefore, the differences in protein levels indicative of cell signaling events between these two groups cannot be because of a differential in the number of surviving cells. When stimulated by the priming low dose, renal cells are able to maintain the cell division cycle stimulatory events even after the high dose and animals survive because of enhanced nephrogenic cell division and repair.

Overall, our findings provide a molecular explanation for inhibition of stimulated compensatory cell division by high doses of DCVC. Promitogenic signaling is disrupted after administration of a lethal dose of DCVC to naïve mice. However, in the autoprotection model, this disruption is prevented due to sustained upregulation of these pathways, initiated by the priming dose of DCVC. This finding is particularly intriguing because it suggests that it should be possible to manipulate signaling events/molecules to prevent the disruptive effects of high doses of drug/toxicants to preserve compensatory cell division/nephrogenic repair mechanisms in the kidney. These extensive in vivo studies provide a basis to set up a significant hypothesis that can be tested with proximal tubular cells in vitro.

Research into mechanisms of ARF has begun to reveal molecular targets for possible therapeutic intervention. Much useful knowledge into the causes and prevention of this syndrome has been gained by the study of animal models. Autoprotection is one such model enabling understanding of the role of increased cell cycle activity after renal injury and the role of proteins regulating this nephrogenic cell division and tissue repair.

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