Preplaced cell division: a critical mechanism of autoprotection against S-1,2-dichlorovinyl-L-cysteine-induced acute renal failure and death in mice

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Submitted 23 September 2005; accepted in final form 18 February 2006

Preplaced cell division: a critical mechanism of autoprotection against S-1,2-dichlorovinyl-L-cysteine-induced acute renal failure and death in mice. Am J Physiol Renal Physiol 291: F439–F455, 2006. First published February 21, 2006; doi:10.1152/ajprenal.00384.2005.—Previous studies have shown that renal injury initiated by a lethal dose of S-1,2-dichlorovinyl-L-cysteine (DCVC) progresses due to inhibition of cell division and hence renal repair, leading to acute renal failure (ARF) and death in mice. Renal injury initiated by low to moderate doses of DCVC is repaired by timely and adequate stimulation of renal cell division, tubular repair, restoration of renal structure and function leading to survival of mice. Recent studies have established that mice primed with a low dose of DCVC (15 mg/kg ip) 72 h before administration of a normally lethal dose (75 mg/kg ip) are protected from ARF and death (nephro-autoprotection). We showed that renal cell division and tissue repair stimulated by the low dose are sustained even after the lethal dose administration resulting in survival from ARF and death. If renal cell division induced by the low dose is indeed the critical mechanism of this autoprotection, then its ablation by the antimitotic agent colchicine (1.5 mg CLC/kg ip) should abolish autoprotection. The present interventional experiments were designed to test the hypothesis that DCVC autoprotection is due to stimulated cell division and tissue repair by the priming low dose. CLC intervention at 42 and 66 h after the priming dose resulted in marked progressive elevation of plasma blood urea nitrogen and creatinine resulting in ARF and death of mice. Light microscopic examination of hematoxylin and eosin-stained kidney sections revealed progression of renal necrosis concordant with progressively failing renal function. With CLC intervention, S-phase stimulation (as assessed by BrdU pulse labeling), G1-to-S phase clearance, and cell division were diminished essentially abolishing the promitogenic effect of the priming low dose of DCVC. Phospho-retinoblastoma protein (P-pRB), a crucial protein for S-phase stimulation, and other cellular signaling mechanisms regulating P-pRB were investigated. We report that decreased P-pRB via activation of protein phosphatase-1 by CLC is the critical mechanism of this inhibited S-phase stimulation and ablation of autoprotection with CLC intervention. These findings lend additional support to the notion that stimulated cell division and renal tissue repair by the priming dose of DCVC are the critical mechanisms that allow sustained compensatory tissue repair and survival of mice in nephro-autoprotection.

colchicine; phospho-retinoblastoma; tissue repair

ACUTE RENAL FAILURE (ARF) is the generic term for an abrupt and sustained decrease in renal function resulting in retention of nitrogenous (urea and creatinine) and non-nitrogenous waste products (36, 59). Development of drug-induced ARF in hospital settings continues to be associated with poor outcomes (3, 4, 9, 13, 26, 34, 37, 44). An estimated 5–20% of critically ill patients experience an episode of ARF during the course of their illness, in many cases accompanied by multiorgan dysfunction syndrome (7, 10, 26). It is evident that ARF can result from alterations in renal perfusion, changes in glomerular filtration, and tubular dysfunction and correction of these factors can ameliorate the effects of ARF (17, 56, 57). Over the last three decades, several experimental models have identified pathophysiological mechanisms associated with ARF and have enhanced our understanding of the disease (27, 48, 62, 63). Clearly, a greater understanding of the potential mechanisms to prevent acute tubular necrosis, ARF, and death is needed. Although little is known about recovery from ARF (21, 33), early detection and intervention to stimulate renal tubular repair to restore the structure and function may lead to 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.

Previous studies showed that proximal tubular injury initiated by a lethal dose of S-1,2-dichlorovinyl-L-cysteine (DCVC) progresses because of inhibited cell division and renal tissue repair that lead to ARF and death in mice (63). Proximal tubular injury initiated by low to moderate doses of DCVC is repaired by timely and adequate stimulation of cell division and renal tissue repair, restoration of renal structure and function, and survival of mice. Prior administration of a low dose of DCVC primes renal cell division such that tissue repair is no longer inhibited even after exposure to a normally lethal dose of DCVC (64). This autoprotection model may offer a unique opportunity to understand the molecular mechanisms behind stimulation of life saving-sustained renal tissue repair even in the presence of renal injury that normally leads to ARF. Sustained stimulation of phosphorylated ERK1/2 is thought to be a key to persistent tubular repair and recovery (65). The existence of a dynamic interface between the repair pathways and the cell-death pathways is being recognized. Phosphorylation events crucial to cell division are known to reside in the cyclins, cyclin-dependent kinases, phosphatases, cdk inhibitors, and activators that regulate their activities. The critical role of these cellular signaling mechanisms downstream of...
ERK 1/2 in sustaining renal tissue repair in the renal autoprotection model was documented recently (23). We showed that transactivational events of renal cell division cycle were up-regulated by the low priming dose and these signaling events were sustained even after exposure to a normally lethal dose in this renal autoprotection model (23).

The present work was conducted with two distinct objectives: first, to test the “tissue repair” hypothesis of autoprotection by antimitotic intervention with colchicine (CLC); second, to identify any critical changes in cell signaling events associated with this antimitotic intervention. Apart from its well-known antimitotic effects by microtubular perturbations (29), CLC also inhibits S-phase DNA synthesis by inhibition of thymidine kinase and thymidylate synthetase (61). CLC intervention in autoprotection has been previously employed to ascertain the role of low dose-stimulated cell division in autoprotection against CCl4-induced hepatotoxicity (46). If primed renal cell division by a low dose of DCVC is indeed the critical mechanism for the recovery from ARF-bound renal injury of a normally lethal dose of DCVC, then antimitotic intervention should result in abolition of autoprotection due to progression of renal injury, ARF, and animal death.

Our results indicate that CLC intervention abolishes renal autoprotection by inhibiting low dose-stimulated renal cell division confirming that it is the critical mechanism of autoprotection. Phosphorylation of pRB, a crucial event in S-phase progression and cell cycle progression, was prevented by CLC intervention of autoprotection. These findings suggest that pharmacological stimulation of promitogenic and protissue repair events may be helpful in averting the progressive course of ARF-bound renal injury.

METHODS

Animals and treatment. Male Swiss Webster (SW) mice (25–29 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and were maintained in our central animal facility under 21 ± 1°C temperature 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 acclimatized 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 National Institutes of Health and were approved by our Institutional Animal Care and Use Committee (IACUC).

Chemicals. Unless stated otherwise, all chemicals and biochemi-
cals were purchased from Sigma (St. Louis, MO). DCVC was provided by Syngenta Central Toxicology Laboratory (Maclesfield, Cheshire, UK) and was >99.5% pure. Mouse monoclonal anti-cyclin D1 (DCS6) and anti-phospho-protein phosphatase-1 (pTh320) were purchased from Cell Signaling Technology (Beverly, MA), and mouse monoclonal anti-cyclin-dependent kinase 4 (Cdk4), Cdk6, p16, p21, and protein phosphatase-1 (PP-1), and anti-phospho-retinoblastoma protein (P-pRB) (pS785) were purchased from Sigma. Loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-goat, antimouse secondary antibodies conjugated with horseradish peroxidase, and anti-S3’-bromo-2’-deoxyuridine (BrdU) monoclonal antibody were purchased from Sigma. Anti-rabbit secondary antibody conjugated with horseradish peroxidase was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). 3,3’-Diaminobenzidine tetrahydrochloride was purchased from Dako (Carpinteria, CA). A Bio-Rad protein assay kit and nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules, CA).

CLC intervention protocol. If survival advantage against a lethal dose of DCVC-induced ARF and death comes from robust, low dose-stimulated cell division and nephrogenic tubule repair in the autoproected mice, blocking cell division should lead to loss of this advantage. CLC was administered at 42 and 66 h after a low dose of DCVC (15 mg/kg) to investigate the effect of antimitotic intervention in autoprotection. These time points were selected for CLC administration for two reasons: first, well before this time, the process of β-lyase-mediated bioactivation, a critical step in DCVC-induced renal injury, would be complete (63); second, 15 mg DCVC/kg cause S-phase stimulation which peaks at 48 and 72 h, and administration of CLC 6 h before these critical time points is known to be effective in blocking DNA synthesis and cell division (24). The objective was to block renal cell division and tubule repair at 48 and 72 h and see whether DCVC-initiated renal injury progresses to ARF resulting in increased mortality.

CLC intervention experiments have been previously used to examine the role of cell division in the auto- and heteroprotection models of hepatotoxicants (8, 30–32, 46, 47). Recent work showed that advancement of proximal tubular epithelial cells to the S phase in mice with type I diabetes is a critical event in mitigating the progression of DCVC-initiated renal injury by upregulation of tissue repair, leading to survival of the diabetic mice by averting ARF (12). Blocking the entry of cells into S phase by antimitotic intervention using CLC (1.5 and 2 mg/kg) abolished stimulated nephrogenic tissue repair and nephropoietic even after these two doses of CLC, the treated mice were devoid of any adverse effects on renal function and histology (12). In the present study, preliminary experiments suggested that administration of CLC (1.5 mg/kg) at 42 and 66 h is required to effectively inhibit the renal cell division stimulated by a low dose of DCVC. Even at 1.5 mg/kg, CLC is devoid of toxicity and does not cause any adverse effects on renal structure or function other than the intended antimitotic effects.

For lethality experiment, mice were divided into five groups (n = 10 per group). Groups I and II received 15 mg DCVC/kg ip in distilled water (DW; 10 ml/kg ip) on day 0 followed by either 10 ml/kg DW ip (group I) or 1.5 mg CLC/kg (group II) at 42 and 66 h, respectively. Mice in both the groups further received 75 mg DCVC/kg ip in DW (10 ml/kg) at 72 h. Groups III and IV were injected with 15 mg DCVC/kg ip in DW (10 ml/kg) on day 0 followed by DW (10 ml/kg; group III) or 1.5 mg CLC/kg (group IV) at 42 and 66 h, respectively. On day 3 (72 h) mice in both the groups (III and IV) received 10 ml DW/kg ip. Mice in group V (controls) were injected DW (10 ml/kg ip) on day 0 followed by CLC (1.5 mg/kg ip) at 42 and 66 h. These mice received 10 ml DW/kg ip at 72 h. Mice were observed twice daily for 14 days and survival/mortality was recorded. A separate time course study (n = 3) per time point. Group II also consisted of three mice (n = 3) at all time points except for the 24-h time point after high dose where it consisted of five mice (n = 5) to ensure a minimum of three surviving mice because mice started dying between 96 and 120 h (24–48 h after the high dose) in the lethality experiment (Table 1).

For these mice, renal dysfunction was assessed by plasma blood urea nitrogen (BUN) and creatinine (Sigma Kit no. 555-A) and the extent of renal cell division was measured by BrdU labeling. Renal histopathology was conducted using 10% buffered formalin-fixed, paraffin-embedded, 4-μm-thin kidney sections stained with hematoxylin and eosin (H and E). In addition to the qualitative histopathological evaluation, renal injury was quantitated from the H and E-stained kidney sections. Three ×200 microscopic fields were randomly chosen from corticomedullary region (CMR) encompassing the outer stripe of outer medulla (OSOM) from each mouse (n = 3 per group); the CMR encompassing OSOM was the principally targeted region by
DCVC. The proportion of area per field composed of necrotic tubules was estimated and recorded. Tubules were considered necrotic if they were lined with profiles of amorphous cells with coagulated eosinophilic cytoplasm and ghostly silhouettes of karyolytic nuclei or exfoliated cells, detritus, or both.

Renal cell division. BrdU, a thymidine analog, is incorporated into cellular DNA during S-phase DNA synthesis. Immunohistochemical technique using a monoclonal antibody to BrdU was employed to estimate cell division (50). Briefly, a 20-mg/ml solution of BrdU (Sigma) was prepared in PBS with stirring at 37°C followed by filter sterilization. Mice were administered BrdU (100 mg/kg ip) 2 h before termination. Kidneys from controls and treated mice were washed with ice-cold normal saline (0.9% NaCl), cut transversely into 2- to 3-mm slices, and then fixed into 10% phosphate-buffered formaldehyde for 48 h. Specimens were embedded in Formula R (Surgipath, Richmond, IL), sectioned at 4 μm, and mounted on positively charged glass slides. Sections were then digested with 0.05% protease for 20 min at room temperature. Endogenous peroxidases were inhibited with 1% hydrogen peroxide (20 min). Slides were incubated for 1 h at room temperature with an anti-BrdU monoclonal antibody (Sigma). After incubation with the primary antibody, the slides were incubated for 30 min at room temperature with biotinylated anti-mouse IgG diluted 1:200 in PBS. Slides were then incubated with an avidin-biotin peroxidase complex for 30 min at room temperature. The BrdU incorporation was localized by a final incubation with 3,3'-diaminobenzidine tetrahydrochloride. Cells in S phase were identified by their characteristic brown nuclear staining. A total of 27 microscopic fields at x200 magnification were randomly chosen from the CMR in the

**Table 1. Effect of CLC intervention on DCVC-induced autoprotection**

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Treatments and Sequence</th>
<th>Number of Mice</th>
<th>% Mortality</th>
<th>Time of Death, h*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Low dose + DW + DW + high dose</td>
<td>10</td>
<td>0</td>
<td>24–48</td>
</tr>
<tr>
<td>Group II</td>
<td>Low dose + CLC + CLC + high dose</td>
<td>10</td>
<td>10</td>
<td>120</td>
</tr>
<tr>
<td>Group III</td>
<td>Low dose + DW + DW + DW</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>Low dose + CLC + CLC + DW</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Group V</td>
<td>DW + CLC + CLC + DW</td>
<td>10</td>
<td>0</td>
<td></td>
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</tbody>
</table>

Effect of colchicine (CLC) intervention at 42 and 66 h after administration of the low dose (15 mg/kg) of S-1,2-dichlorovinyl-l-cysteine (DCVC) on the DCVC-induced renal autoprotection in male Swiss Webster mice. Groups I and II received 15 mg DCVC/kg ip in distilled water (DW; 10 ml/kg) on day 0 followed by either 10 ml/kg DW ip (group I) or 1.5 mg/kg CLC (group II) at 42 and 66 h, respectively. Mice in both the groups further received 75 mg DCVC/kg ip in DW (10 ml/kg) at 72 h. Groups III and IV were injected with 15 mg DCVC/kg ip in DW (10 ml/kg) followed by either DW (10 ml/kg) or CLC (1.5 mg/kg) at 42 and 66 h. On day 3 (72 h) mice in both these groups further received 10 ml DW/kg ip. Mice in group V (controls) were injected 10 ml DW/kg ip on day 0 followed by CLC (1.5 mg/kg) ip at 42 and 66 h. These mice further received 10 ml DW/kg ip at 72 h. Mice were observed twice daily for 14 days and survival/mortality data were recorded. All deaths occurred between 24 and 48 h after the high dose of DCVC in CLC-intervened autoprotection group (group II). *Time after the fourth treatment.

**Fig. 1.** Renal dysfunction measured by plasma creatinine elevation in mice treated with low dose with or without colchicine (CLC) followed by a high dose. Four groups of male Swiss Webster (SW) mice were used. Mice (n = 3) were injected with 15 mg S-1,2-dichlorovinyl-l-cysteine (DCVC)/kg ip in distilled water (DW; 10 ml/kg) with or without 1.5 mg CLC/kg at 42 and 66 h ip followed by either 75 mg DCVC/kg or DW ip at 72 h. One more group received vehicle DW (10 ml/kg) with 1.5 mg CLC/kg at 42 and 66 h ip followed by DW (10 ml/kg) ip at 72 h. Data are expressed as means ± SE (n = 3). *Statistically significant from respective 0-h controls. †Significantly different from autoprotected group that did not receive CLC (group I) at the corresponding time point (P ≤ 0.05),
Fig. 2. Renal injury following a priming dose of DCVC or vehicle (DW) with or without CLC intervention followed by vehicle (DW). Representative photomicrographs of hematoxylin and eosin (H and E)-stained kidney sections from A: 0-h control mice. B and C: mice treated with 15 mg DCVC/kg showed moderate necrosis in proximal tubules at 36 and minimal damage at 96 h, respectively. D: mice treated with 15 mg DCVC/kg followed by 1.5 mg CLC/kg ip (at 42 and 66 h) showed no adverse effect on kidney structure at 96 h. E, F, and G: mice received 10 ml DW/kg ip with 1.5 mg CLC/kg ip at 42 and 66 h followed by DW (10 ml/kg) ip at 72 h showed no renal damage at 48, 72, and 96 h, respectively. All fields were chosen from the CMR encompassing OSOM. N, area of necrosis; G, glomerulus. Original magnification: ×200.
kidney sections per time point from three mice per group (n = 3). BrdU-positive cells were counted and represented graphically as the mean number of positive BrdU cells per group as an indicator of S-phase DNA synthesis and renal cell division.

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 phenylmethylsulfonylfluoride, 1 mM HEPES, pH 7.6, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) 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 with bovine serum albumin as the standard. Proteins (50 µg) were separated on either 7.5 or 12% SDS-polyacrylamide gels and transferred to immunoblot nitrocellulose membranes. Membranes were blocked in 5% dried milk in PBST (0.1% Tween 20

Fig. 3. Renal injury in the autoprotection regimen using a low dose of DCVC with or without CLC intervention followed by a high dose. Representative photomicrographs of H and E-stained kidney sections from A: mice pretreated with 15 mg DCVC/kg received 10 ml DW/kg at 42 and 66 h and showed completely recovered proximal tubular structure at 72 h just before the administration of a high dose (group I). B and C: mice after pretreatment with 15 mg DCVC/kg and receiving 10 ml DW/kg at 42 and 66 h followed by 75 mg DCVC/kg at 72 h showed significant renal damage characterized by intensely eosinophilic necrotic tubular epithelium with some exfoliation of cellular detritus into tubular lumens (*) at 84 h (B) and 96 h (C), respectively. Additionally, robust tubular repair was also evident, characterized by squamous to low cuboidal reepithelialization of tubules (@) with typical cytoplasmic basophilia and slight karyomegaly (arrows) of the newly regenerated epithelium. D: mice pretreated with 15 mg DCVC/kg received 1.5 mg CLC/kg. Marked tubular necrosis is present at 72 h just before the administration of high dose (group II). E and F: mice pretreated with 15 mg DCVC/kg received 1.5 ml CLC/kg ip at 42 and 66 h followed by 75 mg DCVC/kg at 72 h showed extensive coagulative necrosis of proximal tubules at 84 h (E*) and 96 h (F*), respectively. All fields were chosen from the CMR encompassing OSOM. Original magnification: ×200 for A and D; ×400 for B, C, E, and F to show the pathomorphological characteristics.
in 1:200 PBS) and incubated with 1:200 dilution of primary antibodies against cyclin D1, 1:1,000 dilution of cdk4, cdk6, p16, PP-1, phospho-retinoblastoma protein (P-pRB; pS795), 1:500 dilution of phospho-protein phosphatase-1 (Phospho-PP-1, pTh320), and 1:100 GAPDH 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 an ECL system (Pierce Biotechnology, Rockford, IL). Data in all immunoblot studies for the proteins [cyclin D1, cdk4, cdk6, p16, P-pRB, PP-1, and phospho-PP-1 (Th320)] in the respective experimental groups were collected from three such independent studies (n = 3) and a representative blot from the three replicates illustrated. Expression data (n = 3) for all the proteins were normalized to GAPDH which served as the internal control and quantitated (quantitation shown only for selected proteins). Only the representative GAPDH is shown for the respective groups (see Fig. 7).

**Phospho-pRB immunohistochemistry.** Formalin-fixed, paraffin-embedded tissues were sectioned at 4 μm and placed onto slides. After being heated at 55°C for 1 h, the sections were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (H2O2) in methanol for 10 min. For antigen retrieval, specimens were microwaved in 0.01 mol/l of sodium citrate (pH 6.0) for three changes of 5 min each, followed by cooling in PBS. The sections were incubated in 3% bovine serum albumin made in PBS for 1 h at room temperature. The anti-phospho-retinoblastoma protein (P-pRB) (pS795) antibody was applied at a 1:500 dilution in 1% bovine serum albumin/PBS and incubated in a humidity chamber at 4°C overnight. After the primary antibody incubation, a secondary horseradish peroxidase linked anti-mouse antibody was applied at a 1:300 dilution in 1% bovine serum albumin in PBS for 1 h at room temperature, followed by the application of diaminobenzidine with H2O2 for 10 min, according to the manufacturer’s instructions. The slides were washed in DW and lightly counterstained with hematoxylin.

**Data and statistical analysis.** Data (BUN, creatinine levels, and quantitative assessment of the expression of proteins) were expressed as means ± SE (n = 3) for all the experiments. Statistical significance between CLC-intervened autoprotected group and nonintervened autoprotected group at the same time point was analyzed by Student’s t-test. Comparison between different time points for each group was determined by one-way ANOVA followed by Tukey’s honestly significantly different and Duncan’s multiple range tests using SPSS (SPSS, Chicago, IL). In all cases, P ≤ 0.05 was used as the statistical criterion to determine statistically significant differences.

**RESULTS**

**Lethality studies.** Previous studies showed that 90% of the mice receiving the high dose of DCVC (75 mg/kg) die between 36 to 48 h after dosing (23, 63, 64). In the present study, a priming low dose of DCVC (15 mg/kg) given 72 h before the administration of a normally lethal dose of DCVC (75 mg/kg) afforded 100% protection (Table 1). CLC intervention at 42 and 66 h after administration of the priming low dose of DCVC in the autoprotection protocol (low dose/CLC high dose) led to 100% mortality. Renal failure and mortality seen in CLC-intervened autoprotected mice were comparable to results from our previous study showing renal dysfunction and mortality caused by a high dose of DCVC alone in the absence of a sublethal priming dose (23).
Renal injury. Kidney injury was examined by H and E-stained light microscopic examination of the renal sections from mice in all groups. Renal morphology in mice receiving CLC alone (group V) was indistinguishable from the kidneys of normal mice (0 h of all groups in Fig. 2) and see Fig. 4) at any time point (48, 72, and 96 h) after CLC administration (Fig. 2, E, F, G and see Fig. 4). A priming low dose of DCVC alone (in groups I, II, III, and IV) caused mild proximal tubular necrosis damaging up to 35% of the nephrons in the CMR that peaked at 36 h (Fig. 2B and see Fig. 4). This injury regressed noticeably by 96 h in groups III and IV (Fig. 2, C and D, and see Fig. 4). With CLC intervention after the low priming dose (group IV), proximal tubular necrosis progressed after 36 h reaching a maximum at 72 h (Figs. 3D and 4) causing 40% damage in the CMR but then regressed exhibiting very mild damage at 96 h (as shown in previous Figs. 2D and 4). Ninety percent of these mice recovered and survived (Table 1). As expected, primed mice that received CLC before 75 mg DCVC/kg (group II) manifested extensive proximal tubular necrosis at 84 h characterized by amorphous, intensely eosinophilic tubules with ghostly silhouettes of karyolytic nuclei (Figs. 3E and 4), and this injury progressed through 96 h damaging almost 70% of the CMR (Figs. 3F and 4). This
Fig. 7. Immunoblot analysis of cyclin D1, cdk4, cdk6, and p16 in autoprotected mice with or without CLC intervention. Treatment details: Autoprotected group (group I): male SW mice (n = 3) receiving 15 mg DCVC/kg ip in DW (10 ml/kg) on day 0 followed by 10 ml/kg DW ip at 42 and 66 h, respectively. Mice in this group further received 75 mg DCVC/kg ip in DW (10 ml/kg) at 72 h. A: representative Western blot showing cyclin D1, cdk4, cdk6, and p16 protein expression in autoprotected group. CLC intervention in autoprotection (group II): male SW mice (n = 3) receiving 15 mg DCVC/kg ip in DW (10 ml/kg) on day 0 followed by 1.5 mg CLC/kg at 42 and 66 h, respectively. Mice in this group further received 75 mg DCVC/kg ip in DW (10 ml/kg) at 72 h. B: representative Western blot showing cyclin D1, cdk4, cdk6, and p16 protein expression in CLC-intervened autoprotection group. Controls (group V): male SW mice (n = 3) receiving 10 ml DW/kg ip followed by 1.5 mg CLC/kg ip at 42 and 66 h. These mice further received 10 ml DW/kg ip at 72 h. C: representative Western blot showing cyclin D1, cdk4, cdk6, and p16 protein expression in control group. D: densitometric analysis of p16 protein expression in groups I, II, and V. Data were normalized by using GAPDH as internal control. Only representative blot for GAPDH is shown for the respective groups here. Data are expressed as means ± SE (n = 3). *Statistically significant from respective 0-h controls. !Statistically significant from autoprotected group which was not intervened with CLC (group I) at that particular time point (P ≤ 0.05).

Fig. 6. Renal tissue repair in the autoprotection regimen with or without CLC intervention. Representative photomicrographs of BrdU immunohistochemistry of kidney section from A: 0-h control mouse. B: mice pretreated with 15 mg DCVC/kg received 10 ml DW/kg ip at 42 and 66 h. Intense staining of BrDU in the nuclei (arrow) of regenerating renal proximal tubular epithelial cells that are in S phase at 72 h just before the administration of a high dose (group I). C and D: mice pretreated with 15 mg DCVC/kg received 10 ml DW/kg ip at 42 and 66 h and then received 75 mg DCVC/kg at 72 h. These photomicrographs are representative of kidney sections from mice at 12 and 24 h, respectively, after receiving 75 mg DCVC/kg (84 and 96 h after receiving 15 mg DCVC/kg). E: mice pretreated with 15 mg DCVC/kg receive 1.5 mg CLC/kg ip at 42 and 66 h and later received 75 mg DCVC/kg at 72 h showing sparsely distributed BrdU-positive cells. F and G: mice pretreated with 15 mg DCVC/kg received 1.5 mg CLC/kg ip at 42 and 66 h and later received 75 mg DCVC/kg at 72 h. These photomicrographs are representative of kidney sections from mice at 12 and 24 h, respectively, after receiving 75 mg DCVC/kg (84 and 96 h after receiving 15 mg DCVC/kg); there is marked inhibition of S-phase DNA synthesis. S, DAB-labeled S-phase cells with characteristic brown nuclear staining. Original magnification: ×400.
DCVC-induced injury progressed to affect the inner medullary and outer cortical regions at later time points and finally led to 100% mortality in group II by 120 h after the first dose (48 h after the high dose; Table 1). The proximal tubular necrosis was significantly high with minimal to no appearance of repair 84 h onwards (12 h after 75 mg DCVC/kg) in group II compared with autoprotected group that did not receive CLC (group I; Figs. 3, B and C, and 4). In group I, there was clear evidence of renal tubular repair characterized by marked renal tubule basophilia and squamous to low cuboidal reepithelialization inside residual tubular basal lamina which began 84 h onwards (12 h after the high dose treatment; Fig. 3, B and C). This culminated in regression of renal injury by 96 h (24 h after the high dose; Fig. 4).

S-phase DNA synthesis and renal cell division. S-phase stimulation as an index of renal cell division and tissue repair was assessed by BRDU incorporation into the DNA of tubular epithelial cells in the autoprotected mice with or without the CLC intervention. S-phase DNA synthesis decreased significantly at 72, 84, and 96 h after the low dose in CLC-intervened mice on the autoprotection regimen (group II, low dose + CLC + CLC + high dose) compared with autoprotected group (group I) that did not receive CLC (Fig. 5). BRDU immunohistochemical staining in the kidney sections taken from these respective groups (Fig. 6, B, C, D, E, F, and G) showed the effectiveness of CLC intervention in inhibiting S-phase DNA synthesis and renal cell division.

Role of key proteins regulating G1/S checkpoint clearance cyclins and cdks. Cyclin D1, a member of D-type cyclins, is synthesized in the early G1 phase and binds to and activates either CDK4 or CDK6 as cells leave the quiescent phase (19, 41, 42, 54). The active CDK4/6 phosphorylates retinoblastoma protein (pRb) in the late G1 phase of renal cell cycle, thereby stimulating S phase (35). In this study, we investigated the role of cyclin D1 and its kinases in cell cycle regulation and S-phase stimulation with CLC intervention in autoprotection. Consistent with the previous report (23), in the autoprotected mice without CLC intervention, cyclin D1 levels increased markedly after the high dose of DCVC and remained elevated throughout the time course (75 to 96 h in Fig. 7A). The cyclin D1 expression profile seen after the high dose in CLC-intervened autoprotected mice was not different from autoprotected group without CLC (group II; Fig. 7B). Even after the high dose in this group, cyclin D1 levels progressively increased from 6 to 24 h (78 to 96 h in Fig. 7B).

CLC alone (DW + CLC + CLC + DW) caused a transient increase in cyclin D1 at 6 h after CLC (48 h in Fig. 7C) before declining to basal levels at 72 h. Cd4k protein expression in the autoprotected group increased even after the high dose (Fig. 7A). This indicates that higher cd4k expression induced by the priming dose was further upregulated by the administration of the lethal dose. Cd4k expression was found to be transiently higher after lethal dose in CLC-intervened autoprotected mice (Fig. 7B). However, CLC alone caused a slight increase in cd4k expression at 48 and 72 h before declining to the basal level at 96 h (Fig. 7C). Cd6k protein level (Fig. 7A) decreased at 12 h after the low dose and remained higher thereafter until 96 h in the autoprotected mice that did not receive CLC. With CLC intervention in autoprotection, the cd6k expression was not significantly less (quantitation not shown) compared with expression observed in autoprotection group without CLC from 3 to 24 h after the lethal dose (Fig. 7, A and B, 72 h onwards). CLC alone did not cause any significant change in cd6k levels compared with 0-h controls at any time point (Fig. 7C).

Negative regulators of CDK activity. p16INK4a is a cyclin-dependent kinase inhibitor (CKIs). It associates with CDKs (cdk4/6), thereby inactivating cdk4/6 (35, 54), predominantly cdk4 (55). p16 Expression (Fig. 7, A and D) decreased from 3 to 12 h, increased at 24 h, and then transiently decreased until 60 h (Fig. 7, A and D). Later on, from 24 h onward it remained high until 96 h (24 h after high dose) in the autoprotection regimen without CLC (Fig. 7, A and D). As a result of overexpression of p16 in the nonintervened autoprotected group after the high dose, cd4k may have been active only temporarily until 72 h. Therefore, cd6k compensated for the lack of cdk4 activity and thereby maintained the phosphorylated state of pRB. This was evident by sustained and enhanced expression of cd6k (Fig. 7A). With CLC intervention, the pattern of p16 expression is nearly the same as seen in the autoprotection regimen without CLC except at 48 h, and 72 to 78 h (Fig. 7, B and D). CLC intervention did not change p16 levels significantly at any time point compared with 0-h controls (Fig. 7, C and D), signifying lack of any effect by CLC intervention.

Phosphorylation of p-RB and S-phase stimulation. It has been shown that phosphorylation of p-RB in the proximal tubules and mesangial cells of the kidney is critical for the passage of cells from G1-to-S phase (42, 52). We previously showed that phosphorylation of p-RB is critical for S-phase
stimulation in the DCVC-induced autoprotection model (23). Therefore, in this study our objective was to test the state of phosphorylation of p-RB with CLC intervention. Phosphorylation of p-RB started increasing from 3 h after the administration of the high dose in the autoprotected group (75 h onwards in Fig. 8, L and N). There was a statistically significant increase in P-pRB at all times after the high dose of DCVC (Fig. 8M) in autopsotected mice without CLC intervention. P-pRB protein levels were substantially and progressively lower at 72 h onwards with CLC intervention in the autoprotection regimen (Fig. 8, M and N). The P-pRB levels were markedly lower at 12 and 24 h after the high dose (84 and 96 h in Fig. 8, M and N) in mice in the autoprotection regimen with CLC intervention. These findings are consistent with P-pRB immunohistochemical staining in the CMR in the kidney sections (Fig. 8). Strong nuclear and cytoplasmic localization of phosphorylated-pRB was noticed at 72, 84, and 96 h after low dose in nonintervened autopsotected mice (Fig. 8, E, F, and G) compared with CLC-intervened autopsotected mice at the same time points (Fig. 8, H, I, and J). When the low dose-stimulated cell division was not blocked in the autoprotected mice, P-pRB was expressed more prominently in the regions of the CMR. In the course of renal regeneration after a high dose, groups of P-pRB-expressing cells were recruited in the inner stripe of the outer medulla and these cells migrated to the regions of damage in S3 segments of CMR encompassing the outer stripe of the outer medulla. CLC administered at 42 h (DW + CLC + CLC + DW) to mice receiving DW at 0 h inhibited P-pRB at 96 h, although there was a slight rebound in the phosphorylation of pRB at 48 h but this declined after receiving CLC at 66 h (Fig. 8, B, C, D, and K).

Protein phosphatase-1 as the key regulator of p-RB phosphorylation. Expression of protein phosphatase-1 (PP-1), a specific P-pRB phosphatase, was found to be significantly lower 3 h onward after high dose (75 to 96 h in Fig. 9, B and D) in the autopsotected mice thereby favoring the maintenance of the phosphorylated state of pRB. Compared with normal autoprotected mice, with CLC intervention in autoprotection, PP-1 expression significantly increased at 48 h and then showed a temporary decrease until 72 h (Fig. 9, C and D). From 72 h onwards after the high dose, PP-1 expression increased significantly compared with the nonintervened autoprotected group (Fig. 9, B, C, and D), thereby preventing the maintenance of pRB phosphorylation (Fig. 8, L, M, and N). CLC alone did not cause any significant change in PP-1 expression at 48, 72, and 96 h after its administration compared with 0 h (Fig. 9, A and D), indicating that CLC did not affect phosphorylation of PP-1 and thereby leaving intact PP-1 enzyme active, which led to downregulation of P-pRB, inhibiting S-phase stimulation. These molecular signaling events were consistent with BrdU immunohistochemical staining indicating inhibition of S-phase DNA synthesis.

PP-1 activity. Previous studies showed that cdc2 kinase phosphorylates threonine 320 (Th320) in PP-1 (23). The phosphorylation of Th320 of PP-1 is reduced by the cyclin-dependent protein kinase inhibitor, olomoucine, and increased by the PP-1 and PP-2A inhibitor, calycin A (25). We analyzed phosphorylation of PP-1 using the phospho-Th320-specific antibody in the autoprotected mice with or without CLC intervention. The highest level of phosphorylation of Th320 of PP-1 was detected from 6 to 24 h (78 to 96 h after the priming dose in Fig. 9, F and H) after the lethal dose in autoprotected mice that were not intervened with CLC. Phosphorylation inactivated PP-1 which led to higher P-pRB and S-phase stimulation. With CLC intervention in autoprotected mice, the levels of phosphorylated PP-1 were severely inhibited from 48 h (6 h after the first dose of CLC) after the priming dose and these inhibited phosphorylated PP-1 (Th320) levels were persistent thereafter even after the high dose (Fig. 9, G and H). These findings suggest that CLC activates PP-1 by blocking its phosphorylation at threonine 320. Under these conditions, P-pRB is dephosphorylated and normal cell division is hindered. CLC alone did not cause any change in phosphorylated PP-1 (Th320) levels at 48, 72, and 96 h after its administration compared with 0 h (Fig. 9, E and H), indicating that CLC did not affect phosphorylation of PP-1 at threonine 320 and this appears to be the mechanism of activation of PP-1.

DISCUSSION

Prior administration of a low dose of DCVC protects mice against a subsequently administered normally lethal dose of DCVC at 72 h (23, 64). This protection is neither due to lower bioactivation of DCVC by the renal enzyme cysteine conjugate β-lyase nor due to altered renal function. Protection is due to the priming dose stimulating proximal renal tubule repair which intervenes with the progression of renal injury allowing the mice to recover (64). We showed that cell signaling events such as phosphorylation of p-RB downstream in the ERK 1/2 pathway are critical for enabling sustained stimulation of tissue repair (23). The objectives of the present study were twofold: first, to test the tissue repair hypothesis of autoprotection mechanism by antimitotic intervention with CLC; and second, to investigate the changes in critical cell signaling events (Fig. 10) associated with ablation of cell division by CLC antimitotic intervention.

The mechanism of protection against lethal renal injury by prior injury or preconditioning is an area of increasing investigational interest (5, 18, 23, 64, 65). Some of the candidate mediators of preconditioning are heat shock protein molecular chaperones (6), activation of the nitric oxide synthase (38), stimulation of PPAR receptors (43), induction of endoplasmic reticulum and stress proteins (18), activation of ERK 1/2 (39), and activation of PI3 kinase Akt/PKB pathway (1). Recently, we reported that mice receiving a low dose of DCVC exhibit stimulated compensatory tissue repair via a sustained increase in P-pRB enabling clearance of cells through the G1-to-S checkpoint (23). Maintenance of the phosphorylated state of P-pRB is achieved by cooperative coordination of cyclin D1/cdk4-cdk6 which leads to a persistent increase in P-pRB thereby enhancing S-phase DNA synthesis (23).

CLC intervention in autoprotection model. Antimitotic intervention with CLC has been shown to abolish protection by the priming dose of hepatotoxics against the lethal dose of the same toxicant (32, 46) or another toxicant (8). In the present study, we used CLC to test whether the protective dose-stimulated tubular cell division and tissue repair are the mechanisms of renal autoprotection to support our earlier hypothesis (23). CLC is known to exert its antimitotic effects by blocking S-phase DNA synthesis via inhibition of thymidine kinase and thymidylate synthetase and by microtubular...
perturbations (29, 61). CLC intervention led to a significant suppression of S-phase DNA synthesis, renal cell division, and tissue repair, leading to escalation of injury and deteriorating renal function followed by 100% mortality in the autoprotection regimen. CLC alone did not cause any adverse effect on the kidney function or histology at any time point, precluding the possibility that CLC itself is potentiating DCVC nephrotoxicity in the CLC-intervened autoprotection group. This is evident from the fact that the renal dysfunction after administration of a normally lethal dose of DCVC at 72 h in the autoprotection regimen was significantly different from the CLC-intervened group.

Fig. 9. Immunoblot analysis of protein phosphatase-1 (PP-1) protein expression in autoprotected mice with or without CLC intervention. Treatment details as described above in Fig. 8. A: representative Western blot showing PP-1 protein expression in kidneys of control group. B: representative Western blot showing PP-1 protein expression in kidneys of autoprotected group. C: representative Western blot showing PP-1 protein expression in kidneys of CLC-intervened autoprotected group. D: densitometric analysis of PP-1 protein expression in groups I, II, and V. Data were normalized by using GAPDH as the internal control. Data are expressed as means ± SE (n = 3). *Statistically significant from respective 0-h controls. †Statistically significant from autoprotected group that was not intervened with CLC (group I) at that particular time point (P ≤ 0.05). Immunoblot analysis of phosphorylated protein phosphatase-1 (Th320) protein expression in autoprotected mice with or without CLC intervention. E: representative Western blot showing phospho-PP-1 (Th320) protein expression in kidneys of control group. F: representative Western blot showing phospho-PP-1 (Th320) protein expression in kidneys of autoprotected group. G: representative Western blot showing phospho-PP-1 (Th320) protein expression in kidneys of CLC-intervened autoprotected group. H: densitometric analysis of phospho-PP-1 (Th320) protein expression in groups I, II, and V. Data were normalized by using GAPDH as the internal control. Data are expressed as means ± SE (n = 3). *Statistically significant from respective 0-h controls. †Statistically significant from autoprotected group that was not intervened with CLC (group I) at that particular time point (P ≤ 0.05).
autoprotected groups with or without CLC intervention is not different until 6 h later (until 78 h in Fig. 1). It has been reported that the elimination half-life of DCVC from the plasma in the male SW mouse is 23 min (12) and F344 rats is 2.5 h (15). By 6 h and later, very little DCVC, if any, would be present in the circulation of these mice. Because DCVC has a very short plasma half-life, all the distribution and bioactivation processes are likely to be completed. Therefore, any modification of renal injury would be due to other factors, such as tissue repair, that are independent of DCVC bioactivation. It is evident from our results that S-phase stimulation (assessed by BrdU incorporation into renal nuclear DNA) is severely inhibited with CLC intervention in autoprotection (Figs. 5 and 6, E, F, and G) leading to inhibition of renal tissue repair, progression of injury, ARF, and death.

Renal cell division is well orchestrated by various signaling events (23, 65). 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 (14). With growth factor-induced ERK1/2 stimulation, cyclin D1 binds to and activates CDK4/6, as cells leave the quiescent phase (53, 58). The activated CDK4/6 phosphorylates p-RB (pRb) in the late G1 phase of renal cell division cycle, thereby releasing the E2F-DP complex that acts as a transcription factor for various S-phase-related genes (14, 19, 22, 28, 35, 51, 54, 55). In the present study, P-pRB levels were significantly low with CLC intervention in the autoprotection group causing substantial decrease in S-phase DNA synthesis compared with nonintervened autoprotected group. Decreased P-pRB is an interesting observation because it is not mediated via inhibition of the cyclin D1/cdk4-cdk6 system. With growth factor-induced ERK1/2 stimulation, cyclin D1 binds to and activates CDK4/6, as cells leave the quiescent phase (53, 58). The activated CDK4/6 phosphorylates p-RB (pRb) in the late G1 phase of renal cell division cycle, thereby releasing the E2F-DP complex that acts as a transcription factor for various S-phase-related genes (14, 19, 22, 28, 35, 51, 54, 55). In the present study, P-pRB levels were significantly low with CLC intervention in the autoprotection group causing substantial decrease in S-phase DNA synthesis compared with nonintervened autoprotected group.

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istration might be due to activation of MAPK (ERK 1/2) resulting from microtubular perturbations caused by CLC. Most likely it is an adaptive mechanism of microtubular repair resulting in subsequent activation of cyclins (2).

Despite increased protein expression of cyclin D1/cdk4-cdk6 with CLC intervention in autoprotection, pRB phosphorylation was substantially decreased. This finding suggested the possibility of activation of certain phosphatases by CLC intervention, preventing the maintenance of the phosphorylated state of phospho-pRB. This hypothesis is consistent with existing reports which suggest that antiimitotic agents like colcemid, a methylated derivative of CLC, activate PP-1, a direct P-pRB phosphatase, and lower the P-pRB level (16, 45).

Previously, it has been shown that BRCA1, a nuclear phosphoprotein that is preferentially expressed and phosphorylated during cell cycle progression, becomes hyperphosphorylated near the G1-to-S phase boundary and remains phosphorylated during S phase (49). However, studies have shown that BRCA1 protein is hypophosphorylated in MCF7 cells by CLC and this dephosphorylation is mediated via activation of PP-1 (60, 66). Therefore, it would not be unreasonable to hypothesize that CLC-mediated decrease of pRB phosphorylation, the critical downstream event responsible for the progression of cells from G1-to-S phase in the autoprotection group, may well be through activation of PP-1 (Fig. 10).

PP-1 is known to be a critical component of eukaryotic cell cycle progression. In vitro, previous studies showed that cdc2 kinase phosphorylates threonine-320 (Th320) in PP-1 and that this leads to inhibition of enzyme activity (25). The phosphorylation of Th320 of PP-1 was reduced by the cyclin-dependent protein kinase inhibitor, olomoucine, and increased by the PP-1 and PP-2A inhibitor, calyculin A (25). These observations suggest that phosphorylation by cdc2 kinase and hence inhibition of PP-1 activity are likely to contribute to the increased phosphorylation of proteins that are critical to the initiation of normal cell division.

The present study shows that with CLC intervention in autoprotected mice, phosphorylation of Th320 of PP-1 was severely inhibited compared with nonintervened autoprotected mice. This renders PP-1 to be active throughout the time course in CLC-intervened autoprotected mice. In contrast, when stimulated cell division is not blocked by CLC, cell cycle-dependent phosphorylation of PP-1 is achieved to meet the demand for cell division. The phosphorylation of Th320 of PP-1 with CLC alone is not affected significantly at any time point compared with the 0-h controls (Fig. 9E). It is clear that CLC intervention in the autoprotection model inhibits phosphorylation of Th320 of PP-1, thereby activating PP-1. Therefore, increased PP-1 activity in the CLC-intervened autoprotected group appears to be the mechanism of decreased pRB phosphorylation and hindered S-phase DNA synthesis. When cell division is stimulated, PP-1 activity is also likely to be regulated during the cell cycle by other mechanisms apart from cdc2 kinase. For example, other regulators such as inhibitor-2, sds22 or its homologs, could be involved in controlling PP-1 activity (25). However, the present work indicates that inhibition of replicative DNA synthesis (S phase) by CLC is due to activation of PP-1 that prevents achieving sufficient levels of P-pRB needed to clear cells from G1 to S phase. Also, the role of protein phosphatase 2A (PP-2A) in the inhibition of cell division following CLC treatment would be worthwhile investigating (20, 67). Identification of the protein phosphatases involved in dephosphorylation of pRB might provide additional insight into the role of pRB during renal cell cycle progression. A better understanding of the role of these phosphatases, their endogenous inhibitors (inhibitor-2 and nuclear inhibitor of protein phosphatases), their mechanism(s) of action, activation, and consequences of phosphorylation/dephosphorylation events in response to renal injury and ARF may lead to strategies for pharmacological intervention to stimulate renal recovery and avert ARF.

When cell division is 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. Recent evidence shows that progression of renal injury may be mediated via the action of proteolytic enzymes such as calpain released from cells dying from cytotoxicity. New cells are resistant to the lytic action of these enzymes as they overexpress endogenous inhibitors of these proteolytic enzymes (11, 12, 27a). Hence, appearance of newly dividing/divided proximal tubular cells in the tubules would work to curb any progression of renal injury. While this notion requires further testing and verification, it is an attractive concept that explains why injury no longer progresses in the presence of timely cell division and nephrogenic tissue repair.

Overall, our findings with CLC intervention are consistent with the notion of the critical role of renal cell division and tissue repair in the autoprotection model. These protective signaling mechanisms are particularly intriguing because they suggest the possibility of pharmacological manipulation of signaling events/molecules to stimulate compensatory cell division/nephrogenic repair mechanisms in the kidney to avert drug/toxicant-induced ARF. Furthermore, the antimitotic action of CLC via inhibition of S phase appears to be mediated via activation of PP-1 that decreases the phosphorylation of p-RB.

GRANTS
These studies were made possible through National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-61650. This work was partly supported by the Louisiana Board of Regents Support Fund through the University of Louisiana at Monroe, Kitty DeGree Chair in Toxicology.

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