Magnesium improves cisplatin-mediated tumor killing while protecting against cisplatin-induced nephrotoxicity

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Magnesium improves cisplatin-mediated tumor killing while protecting against cisplatin-induced nephrotoxicity. Am J Physiol Renal Physiol 313: F339–F350, 2017. First published April 19, 2017; doi:10.1152/ajprenal.00688.2016.—Approximately 30% of all cancer patients treated with cisplatin, a widely used broad-spectrum chemotherapeutic agent, experience acute kidney injury (AKI). Almost all patients receiving cisplatin have magnesium (Mg) losses, which are proposed to aggravate AKI. Currently, there are no methods to successfully treat or prevent cisplatin-AKI. Whereas Mg supplementation has been shown to reduce AKI in experimental models and several small clinical trials, the effects of Mg status on tumor outcomes in immunocompetent tumor-bearing mice and humans have not been investigated. The purpose of this study was to further examine the effects of Mg deficiency (∆Mg supplementation) on cisplatin-mediated AKI and tumor killing in immunocompetent mice bearing CT26 colon tumors. Using a model where cisplatin alone (20 mg/kg cumulative dose) produced minimal kidney injury, Mg deficiency significantly worsened cisplatin-mediated AKI, as determined by biochemical markers (blood urea nitrogen and plasma creatinine) and histological renal changes, as well as markers of renal oxidative stress, inflammation, and apoptosis. By contrast, Mg supplementation blocked cisplatin-induced kidney injury. Using LLC-PK1 renal epithelial cells, we observed that Mg deficiency or inhibition of Mg uptake significantly enhanced cisplatin-induced cytotoxicity, whereas Mg supplementation protected against cytotoxicity. However, neither Mg deficiency nor inhibition of Mg uptake impaired cisplatin-mediated killing of CT26 tumor cells in vitro. Mg deficiency was associated with significantly larger CT26 tumors in BALB/c mice when compared with normal-fed control mice, and Mg deficiency significantly reduced cisplatin-mediated tumor killing in vivo. Finally, Mg supplementation did not compromise cisplatin’s anti-tumor efficacy in vivo.

cisplatin; colon cancer; hypomagnesemia; acute kidney injury

Cisplatin leads to tubular epithelial cell injury and apoptosis through multiple mechanisms and pathways, including the formation of toxic DNA adducts, and renal inflammation and oxidative stress, as well as mitochondrial dysfunction and depletion of renal ATP; all of which contribute to irreversible renal failure if cisplatin is not terminated (35, 43). The kidneys serve as the primary site of magnesium (Mg) reabsorption and homeostasis (47, 58, 69), and therefore, it is not surprising that hypomagnesemia occurs in most patients treated with cisplatin (65). In addition, hypomagnesemia is proposed to exacerbate cisplatin-induced kidney injury (34).

Mg is an essential nutrient required for optimal metabolic functioning (23, 47). Previous studies by our laboratory and others have shown that Mg deficiency significantly promotes renal injury in animal models of cisplatin-induced acute kidney injury (AKI) (34, 64, 77). Recently, our laboratory reported that Mg supplementation after Mg deficiency significantly blocked cisplatin-AKI using a human ovarian tumor (A2780) xenograft model in immunocompromised mice without reducing the anti-tumor efficacy of cisplatin (65). Although the results of this work were consistent with those obtained from several small human clinical trials showing the efficacy of Mg supplementation on reducing cisplatin-AKI (30, 50, 86), the conclusions were limited because this model lacks T lymphocytes, which have been implicated previously in mediating cisplatin-induced AKI (39).

Numerous approaches for protecting against cisplatin-mediated nephrotoxicity have been investigated. However, they have failed to reproducibly and successfully protect the kidneys without injurious side effects, and many therapies examined were reported to compromise cisplatin’s anti-neoplastic effects (71, 72, 74). In this study, we extended our previous findings using immunocompromised human tumor-bearing mice and investigated the effect of Mg deficiency (∆Mg supplementation) on cisplatin-induced AKI and cisplatin-mediated tumor killing using the CT26 colon cancer model in immunocompetent BALB/c mice.

METHODS

Animals and cell lines. The Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research approved the animal studies (IACUC no. 2015-002). Female BALB/c mice (10–12 wk old; Taconic Farms, Germantown, NY) were acclimated before receiving cisplatin experience significant kidney injury (61). Cisplatin leads to tubular epithelial cell injury and apoptosis through multiple mechanisms and pathways, including the formation of toxic DNA adducts, and renal inflammation and oxidative stress, as well as mitochondrial dysfunction and depletion of renal ATP; all of which contribute to irreversible renal failure if cisplatin is not terminated (35, 43). The kidneys serve as the primary site of magnesium (Mg) reabsorption and homeostasis (47, 58, 69), and therefore, it is not surprising that hypomagnesemia occurs in most patients treated with cisplatin (65). In addition, hypomagnesemia is proposed to exacerbate cisplatin-induced kidney injury (34).

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matized under normal environmental conditions and allowed free access to standard chow and tap water for 1 wk before experimentation. The CT26 WT (CT26) N-nitroso-N-methylurethane-induced, undifferentiated mouse (BALB/c) colon carcinoma (female) and LLC-PK1 porcine (male) renal epithelial cell lines were purchased from ATCC (Manassas, VA).

Mouse model of colon cancer combined with cisplatin-induced AKI. Female BALB/c mice (n = 8–12/group) were randomized to receive either a 1) control diet (Ctrl; normal chow containing 100% of the recommended Mg) or 2) Mg-deficient (MgD) diet (containing 10% of the recommended amount of Mg, prepared by Teklad/Harlan, Madison, WI) beginning on day 0, as described by Solanki et al. (65). This diet was chosen because it produces moderate Mg deficiency in mice over the study time frame. CT26 colon cancer cells grown in RPMI 1640 media containing 10% FBS, penicillin (P), streptomycin (S), and glutamine (Q) were washed, resuspended (2 × 106 cells in 100 μl of saline), and injected subcutaneously (sc) on the right hind flank on day 10. Saline or cisplatin (3.33 mg/kg ip) was injected on days 14–19 (before euthanasia on day 22) (cumulative cisplatin dose: 20 mg/kg). In addition, one group of mice (n = 10, Mg-supplemented group) received the Mg-deficient diet starting on day 0 through day 12, followed by the control (100% Mg) diet, along with 0.3% MgThr (wt/vol) in the drinking water until day 22. This group also received MgSO4 (100 mg·kg−1·day, s.c.) twice daily from day 17 (after the 4th dose of cisplatin) until euthanasia due to CO2 asphyxiation/exsanguination on day 22. Heparinized blood was collected by cardiac puncture; isolated plasma was frozen at −80°C until analysis. One kidney (outer medulla and cortex only) was flash-frozen in liquid N2, and the second kidney was fixed in 10% formalin. Tumors were removed, gently stripped of nontumor tissue, and weighed.

Table 1. Mouse quantitative PCR primers

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tr>
<td>Apaf1v1</td>
<td>5′-AGA GAT CCA CAC AGG AGG CCA TC-3′</td>
<td>5′-CAA TCA CAG CCA AAT GGT CA-3′</td>
</tr>
<tr>
<td>Apaf1v2</td>
<td>5′-GAC AAA ATA ATA GTC GCA CCA AAA-3′</td>
<td>5′-CAA TCA CAG CCA AAT GGT CA-3′</td>
</tr>
<tr>
<td>Bax</td>
<td>5′-GTC GGC TGG TGG TGG-3′</td>
<td>5′-GTC GGC TGG TGG TGG-3′</td>
</tr>
<tr>
<td>Bak1</td>
<td>5′-GGG ATG CCT GGT AAG TCT TCA-3′</td>
<td>5′-CCA GGT AGT GAT CCT CAA-3′</td>
</tr>
<tr>
<td>H6</td>
<td>5′-GCT ACC AAA CTC GAT ATC ATG CCA A-3′</td>
<td>5′-CCA GGT AGT GAT CCT CAA-3′</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5′-GAG CAC AAG GGC TGA CCA TCA-3′</td>
<td>5′-CAT ATT TCT GGT TCA CAC C-3′</td>
</tr>
<tr>
<td>Ngal</td>
<td>5′-CCA TCT ATG AGC TAC AAG AGA ACA AT-3′</td>
<td>5′-TCT GAT CCA GTC GCA ACA GC-3′</td>
</tr>
<tr>
<td>Nflf</td>
<td>5′-GGA CAC CCT CAT GCG TCA-3′</td>
<td>5′-CTG CCA CTT AAC CAG GAA CAT-3′</td>
</tr>
<tr>
<td>Tnfα</td>
<td>5′-CTG TAG GGC AGG TGA TAG G-3′</td>
<td>5′-TTG AGA TCC ATG CGG TGTG-3′</td>
</tr>
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Apaf1v1 and Apaf1v2, apoptotic peptidase activating factor 1, variants 1 and 2, respectively; Bax, BCL2-associated X; Bak1, BCL2 antagonist/killer 1; Ngal, neutrophil gelatinase-associated lipocalin; Nflf, neutrophil cytosolic factor 1.

Real-time quantitative PCR. High-quality RNA was isolated from frozen kidneys using RNeasy Universal Plus Mini kit (Qiagen, Valencia, CA). The purity and concentrations of total RNA were assayed using the Nanodrop spectrophotometer (Wilmington, DE). Quantitative qPCR reactions using specific primers and Roche Universal Probe Library (see Table 1) were performed in triplicate using the Eurogentec One Step RT qPCR Mastermix, 50–100 ng of RNA (260/280 > 1.8), and the Roche 480 Light Cycler under the following conditions: 48°C for 30 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Relative changes in gene expression were calculated as fold changes using the comparative Ct (ΔΔCT) method; mouse Gapdh was used as the housekeeping gene for normalizing transcript levels, as described previously (65). Normalization of samples across several plates was performed according to previously described methods (76). Data are shown as relative fold changes in gene expression (means ± SE).

Western blot analysis. Renal cortex tissues were homogenized in lysis buffer (Tris-buffered saline, pH 7.3, containing 0.25% Triton X-100 and protease and phosphatase inhibitor cocktail); protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Proteins (50 μg/lane) were separated by NuPAGE electrophoresis (Invitrogen, Carlsbad, CA) and then transferred to PVDF membranes (Millipore, Billerica, MA). After blocking for 1 h, the membranes were then incubated with each primary antibody (ERK1/2, p-ERK1/2, STAT3, p-STAT3 Tyr 05, p53, p53 Ser 15 and GAPDH) overnight at 4°C. After washing, the blots were then incubated with the appropriate near-infrared fluorescently labeled secondary antibody (1:15,000; LI-COR Biosciences, Lincoln, NE) for 1 h and washed before the bands were revealed using the Odyssey infrared imaging system (LI-COR Biosciences). A representative image is shown for each set of proteins (ERK, STAT3, and p53). Band loading was determined using GAPDH protein; quantification was determined using ImageJ Software (National Institutes of Health). Data from 4 blots are presented as mean ratios of each phosphoprotein to total protein (± SD).

Assessment of renal cell apoptosis and histological assessment of renal injury. Formalin-fixed kidneys were embedded in paraffin and sectioned (5 μm). Renal apoptosis (in the cortex and outer medulla regions) was assessed by TUNEL staining using the ApoTag kit (Millipore, Temecula, CA). The TUNEL-stained slides were scored by a reviewer blinded to the experimental conditions by counting the number of densely stained apoptotic cells per high power field (using >5 random fields/section, ≥5 mice/group). Similarly, sections were stained with hematoxylin and eosin and scored using a semiquantitative scale designed to assess AKI-associated tubular injury (tubular epithelial cell loss, necrosis, tubular epithelial simplification, intratubular debris, and casts) by a pathologist (M. Solanki) unaware of the experimental groups (using >5 random fields/section, 4–5 mice/group), as described previously (64, 65). Tubular injury scores (ranging between 0 and 4) were based on the percentage of tubules affected (0: <10%; 1: 10–25%; 2: 26–50%; 3: 51–75%; 4: >75%). Data are downloaded from www.physiology.org/journal/ajprenal (052.011.211.149) on October 24, 2019.
shown as means ± SD. Additional markers of renal apoptosis [e.g., apoptotic peptidase activating factor 1 (Apaf1), BCL2 antagonist/killer 1 (Bak1), and BCL2-associated X (Bax) mRNA] were analyzed by qPCR (as described above).

In vitro cisplatin-induced LLC-PK1 cytotoxicity assay. LLC-PK1 cells were grown in M199 media containing 10% FBS and PSQ; media were replaced with either 10% Mg or 100% Mg-MEM media supplemented with nonessential amino acids, 0.2 g/l CaCl2, PSQ, and 5% FBS (100% Mg is the amount found in M199 growth media or 97 mg/l, as MgSO4 and 10%Mg-MEM media contained 9.7mg/l MgSO4). The cells were incubated for 3 days. A portion of the cells maintained in 10% Mg media were supplemented with 90% Mg (final = 100% Mg, using MgSO4) 1 day before cisplatin treatment. MEM or cisplatin (prepared from a stock solution of 1 mg/ml in prewarmed saline and diluted in MEM at the doses indicated) was added, and the cells were assayed for cytotoxicity (n = 4/condition), using the MTT assay (OD570/690) 24 h after cisplatin addition. The half-maximal inhibitory concentrations (IC50 values or doses of cisplatin required to kill 50% of LLC-PK1 cells under various conditions) were calculated using nonlinear regression to fit the data to the log (inhibitor [cisplatin dose]) vs. response (variable slope [percent viable]) curve using GraphPad Prism (GraphPad Prism Software, San Diego, CA).

In vitro cisplatin-mediated CT26 tumor cell killing. CT26 cells were grown in RPMI-1640 media containing 10% FBS and PSQ in 96-well plates. The medium was replaced with MEM medium containing either 100 (49 mg/dl) or 10% Mg (4.9 mg/dl), supplied as MgSO4, 0.2 g/l CaCl2, nonessential amino acids, and PSQ, and the cells were incubated for 3 days. A portion of the cells maintained in 10% Mg media were supplemented with 90% Mg (final = 100% Mg, as MgSO4) ~24 h before cisplatin treatment. MEM or cisplatin (diluted in MEM, at the doses indicated) was added, and cells were assayed for cytotoxicity (n = 4/condition) using the MTT assay, as described above.

Blockade of cellular Mg uptake using spermine. LLC-PK1 and CT26 cells were grown as described above and then pretreated with spermine (0–50 μM) for 3 h, followed by the addition of either MEM (vehicle) or cisplatin (40 μM) overnight. Cytotoxicity was analyzed by MTT assay (n = 6/condition). Cellular Mg uptake was determined using Mag-Fluo 4-AM. Briefly, LLC-PK1 and CT26 cells were grown in 10% Mg medium; 16 h later, medium was replaced with 100% Mg-MEM medium, and various concentrations of spermine were added to each T25 flask. Next, Mag-Fluo 4-AM prepared according to the manufacturer’s instructions was added to each flask after being washed once with HBSS. The cells were incubated at 37°C for 30 min. Cells were dissociated withAccutase and washed once with Ca2+ and Mg2+-free HBSS, and an equal number of cells were plated for each condition in a 96-well plate (n = 6/condition). Intracellular Mg2+ concentration was measured as relative fluorescence units (RFU) using the Genios Pro Fluorescence plate reader (Tecan, Männedorf, Switzerland) at 485 nm Ex/535 nm Em. Data are shown as means ± SD. Additional analyses of intracellular Mg concentrations were performed using CT26 cells. Briefly, CT26 cells were maintained in 10, 25, and 100% Mg-containing medium as described above and treated with vehicle or cisplatin (125 μM). After 8 h, cells were collected and assayed for intracellular Mg levels by inductively coupled plasma mass spectrometry, as described previously (57). Intracellular Mg concentrations were reported as micrograms per 100 milligrams of protein (means ± SD).

Statistical analyses. Experiments were performed at least twice, and data are expressed as means ± SD (or ± SE), as indicated. One-way ANOVAs were used for multiple comparisons, followed by Bonferroni post hoc testing using GraphPad Prism (GraphPad Software, San Diego, CA). P values of <0.05 were considered significant.

RESULTS

Mg status modulates cisplatin-induced AKI in a syngeneic mouse model of colon cancer. To confirm that Mg deficiency enhances cisplatin-AKI and that Mg supplementation following Mg deficiency protects, we examined the effect of Mg status using a cisplatin-AKI model with BALB/c mice bearing CT26 tumors. Control-fed and Mg-deficient mice with CT26 tumors showed no evidence of renal damage when examined by BUN (Fig. 1A) and Cr (Fig. 1B) in the absence of cisplatin treatment. Treatment of control-fed mice with cisplatin alone showed no significant cisplatin-mediated injury, as determined by BUN (Fig. 1A) and Cr levels (Fig. 1B). By contrast, cisplatin treatment of Mg-deficient mice significantly enhanced BUN (Fig. 1A) and Cr concentrations when compared with cisplatin-treated control-fed mice and untreated Mg-deficient mice (Fig. 1B). The enhanced kidney injury observed in the Mg-deficient tumor-bearing mice following cisplatin treatment was significantly blocked by Mg supplementation, as determined by BUN (Fig. 1A) and Cr (Fig. 1B) levels.

When renal injury was analyzed by histological scoring of kidney sections, neither untreated control nor Mg-deficient mice showed significant renal injury, and cisplatin-treated control-fed tumor-bearing mice showed minimal renal injury when compared with untreated control mice (Fig. 1, C and D). Similar to the findings observed with biochemical markers (BUN and Cr), cisplatin-treated Mg-deficient mice showed moderate to severe renal injury when compared with control-fed mice treated with cisplatin and untreated Mg-deficient mice (Fig. 1, C and D), and this effect was significantly blocked by Mg supplementation (Fig. 1, C and D).

Mg deficiency enhances markers of renal oxidative stress and inflammation following cisplatin; Mg supplementation reverses this effect. Next, we examined the effect of Mg status on markers of renal oxidative stress. Cisplatin alone and Mg deficiency alone slightly increased renal Ncf1 mRNA expression when compared with untreated control kidneys of CT26 tumor-bearing mice (Fig. 2A). Cisplatin treatment significantly induced neutrophil cytosolic factor 1 (Ncf1) mRNA expression in the kidneys of Mg-deficient mice when compared with control-fed cisplatin-treated mice and untreated Mg-deficient mice (Fig. 2A). Mg supplementation significantly ameliorated renal Ncf1 mRNA expression observed in cisplatin-treated Mg-deficient mice (Fig. 2A). Although cisplatin alone did not significantly increase renal MPO levels in the kidneys (Fig. 2B), cisplatin combined with Mg deficiency significantly increased renal MPO levels (Fig. 2B), and this was blocked by Mg supplementation (Fig. 2B).

In the absence of cisplatin, Mg-deficient mice showed no Il6 mRNA expression in the kidneys when compared with control-fed mice (Fig. 2C). Il6 mRNA expression in the kidneys was somewhat elevated when control-fed mice with CT26 tumors were treated with cisplatin (Fig. 2C). This was dramatically increased following cisplatin treatment of Mg-deficient mice bearing CT26 tumors (Fig. 2C). Mg supplementation significantly blocked cisplatin-induced Il6 mRNA expression in the kidneys of Mg-deficient mice (Fig. 2C). Renal Tnfa mRNA expression was increased following cisplatin alone (Fig. 2D), and although not significant, Mg deficiency increased renal Tnfa mRNA expression following cisplatin (Fig. 2D). When compared with Mg deficiency alone, cisplatin treatment com-
Mg deficiency enhances cisplatin (Cis)-induced kidney injury, and Mg supplementation protects against this effect in a syngeneic mouse colon cancer model. Control (Ctrl; 100% Mg), Mg-deficient (MgD), and Mg-supplemented mice (MgS) bearing CT26 tumors (as described in METHODS) were treated with saline or cisplatin (3.33 mg/kg ip) on days 14–19 after the diets were started (n = 8–12 mice/group). CT26 colon cancer cells were injected subcutaneously (sc) in the right flank on day 10. Mice were euthanized on day 22. A and B: blood urea nitrogen (BUN; A) and plasma creatinine levels (B) were determined. Data are shown as means (indicated by horizontal lines) ± SD mg/dL. Fixed kidney tissues were stained with hematoxylin and eosin and evaluated for histology. C: representative images for each group are shown (×400 magnification). D: histological damage scores (ranging between 0 and 4) were based on the percentage of tubules affected (0: <10%; 1: 10–25%; 2: 26–50%; 3: 51–75%; 4: >75%) (n = 4–5 mice/group). Data are shown as individual data points with means (indicated by horizontal lines) ±SD. Scale bar, 20 μm (C). ¥¥¥ P < 0.001 vs. Ctrl; ***P < 0.001 vs. MgD Cis; †††P < 0.001 vs. MgD Cis; ‡‡‡P < 0.001 vs. MgD Cis.

Assessment of signaling pathways associated with oxidative stress and inflammation within the kidneys showed similar results. Control-fed and Mg-deficient tumor-bearing mice showed no significant phosphorylation of STAT3 and ERK1/2 in the kidneys in absence of cisplatin (Fig. 2, F and G, respectively). Cisplatin treated control-fed mice exhibited slightly (but not significantly) elevated ERK1/2 activation (Fig. 2F) but no STAT3 activation (Fig. 2F) in their kidneys. When cisplatin was administered to Mg-deficient CT26-bearing mice, both STAT3 and ERK1/2 activation in their kidneys were dramatically increased when compared with untreated Mg deficient mice and cisplatin control-fed mice (Fig. 2, F and G). Cisplatin-mediated activation of both STAT3 and ERK1/2 pathways in the setting of Mg deficiency was significantly abrogated by Mg supplementation (Fig. 2, F and G).

Mg supplementation significantly reduces cisplatin-mediated apoptosis in the kidneys of Mg-deficient mice. The p53 signaling pathway is critically involved in cisplatin-mediated renal cell injury and apoptosis. In this tumor-bearing mouse model, activation of p53 in the kidneys was not observed with cisplatin alone or Mg deficiency alone (Fig. 3A). Activation of p53 in the kidneys of cisplatin-treated tumor-bearing mice was significantly enhanced by Mg deficiency, and this effect was reduced by Mg supplementation (Fig. 3A). Cisplatin-treated control-fed mice bearing syngeneic CT26 tumors showed significantly greater renal Apaf1 mRNA expression (Fig. 3B), a target of p53 (56). Similarly, cisplatin alone significantly induced Bax and Bak1 mRNA expression (Fig. 3C) in the kidneys and renal apoptosis when compared with untreated control-fed mice, as determined by TUNEL staining (Fig. 3, D and E). These markers of renal apoptosis (p53 activation, Apaf1, Bax, and Bak1 mRNA and TUNEL staining) were significantly enhanced when cisplatin was combined with Mg-
Mg deficiency upregulates cisplatin (Cis)-induced markers of oxidative stress and inflammation in the kidneys, and Mg supplementation abolishes this effect. Control (Ctrl; 100% Mg), Mg-deficient (MgD), and Mg-supplemented mice (MgS) bearing CT26 tumors (treated ± Cis; as described in METHODS and Fig. 1) were analyzed for renal cortical mRNA expression of Ncf1 (oxidative stress marker; A) and myeloperoxidase (MPO) activity (B). Kidney cortex tissues were analyzed for inflammatory markers Il6 (C), Tnfa (D), and neutrophil gelatinase-associated lipocalin (Ngal; E) proteins are shown, with quantitation of band densities of p-STAT3/total STAT3 and p-ERK1/2/total ERK1/2 (means ± SE) relative fold change (vs. naïve control-fed LLC-PK1 cells). Representative Western blots for total STAT3, phospho-STAT3 Tyr305 (p-STAT3), and GAPDH (housekeeping gene) are shown, with quantitation of band densities of p-STAT3/total STAT3 and p-ERK1/2/total ERK1/2 (means ± SD; n = 4–6/group) shown below the Western blots (F and G, respectively). **P < 0.01 vs. Ctrl; ***P < 0.001 vs. MgD Cis; †††P < 0.001 vs. Ctrl Cis; ††P < 0.01 vs. Cis; †P < 0.05 vs. MgD Cis.

deficiency (Fig. 3, A–E), and Mg supplementation reversed these effects (Fig. 3, A–E).

Cisplatin-treated CT26 tumor-bearing mice exhibit decreased plasma Mg concentrations, which is worsened by Mg deficiency. Next, we measured the circulating levels of Mg in the tumor-bearing BALB/c mice. Cisplatin-treated control-fed mice showed significantly lower plasma Mg concentrations when compared with naïve control-fed mice (Fig. 4). Mg concentrations found in the cisplatin control-fed mice were similar to those observed in untreated Mg-deficient mice (Fig. 4), which were significantly lower than plasma Mg levels in untreated control-fed mice (Fig. 4). When Mg-deficient mice were treated with cisplatin, their plasma Mg levels dropped significantly below that observed in cisplatin-treated control-fed mice (Fig. 4); this effect was significantly attenuated by Mg supplementation (Fig. 4).

Mg deficiency promotes cisplatin-induced renal epithelial cell killing in vitro; Mg supplementation protects. Next, we determined the IC50 for cisplatin killing of LLC-PK1 cells (a renal epithelial cell line) grown in control media (containing 100% Mg, as found in normal growth media) vs. LLC-PK1 cells grown in Mg-deficient media (±Mg supplementation). Mg deficiency significantly reduced the IC50 for cisplatin [24.7 μM (Mg-deficient) vs. 61.6 μM (control-100% Mg)], whereas Mg supplementation was protective when compared with Mg-deficient cells (47.1 μM) (Fig. 5A).

Similar to the dramatic increase in ERK activation observed in the kidneys when cisplatin was combined with Mg deficiency in vivo, LLC-PK1 renal epithelial cells grown in Mg-deficient media showed higher ERK1/2 phosphorylation following cisplatin treatment when compared with control cells treated with cisplatin and Mg deficiency alone, both of which showed some ERK1/2 activation (Fig. 5B). The effect of Mg deficiency combined with cisplatin on ERK activation was significantly abrogated by Mg supplementation (Fig. 5B). Consistent with these results, both cisplatin alone and Mg deficiency alone increased TNF production by LLC-PK1 cells (Fig. 5C), and in the presence of Mg-deficiency, cisplatin further
upregulated TNF production when compared with cisplatin alone or Mg deficiency alone (Fig. 5C). Mg supplementation of renal epithelial cells blocked TNF production induced by cisplatin in the setting of Mg deficiency (Fig. 5C).

Reduction in Mg uptake by renal epithelial cells promotes cisplatin-mediated cytotoxicity. To determine whether blocking Mg uptake by renal epithelial cells promotes cisplatin-induced cytotoxicity, we used spermine, an inhibitor of transient receptor potential cation channel, subfamily M, member 7 (TRPM7) (68). TRPM7 is one of the major regulators of Mg transport into cells, and in renal epithelial cells TRPM7 may be functionally linked to TRPM6, another major Mg transporter, to regulate Mg uptake (10). As shown in Fig. 6A, pretreatment of LLC-PK1 cells with spermine increased cisplatin-mediated cytotoxicity in a dose-dependent manner (Fig. 6A). Except for the highest dose (50 µM), spermine did not promote LLC-PK1 cytotoxicity in the absence of cisplatin (Fig. 6A). In parallel experiments, spermine at concentrations of ≥25 µM, which promoted cisplatin-mediated cytotoxicity (Fig. 6A), significantly blocked Mg uptake by LLC-PK1 cells (Fig. 6B).

Neither Mg deficiency nor Mg supplementation affect cisplatin-mediated CT26 tumor cell killing in vitro. Whereas Mg deficiency enhanced cisplatin-mediated LLC-PK1 renal epithelial cell cytotoxicity (Fig. 5A) and Mg supplementation protected LLC-PK1 cells against cisplatin-mediated cytotoxicity (Fig. 5A), neither Mg deficiency nor Mg supplementation altered cisplatin-mediated CT26 tumor cell cytotoxicity in vitro (Fig. 7A). Likewise, blockade of Mg uptake using spermine, an inhibitor of TRPM7, had no effect on cisplatin-mediated CT26 tumor cell killing in vitro (Fig. 7B), nor did it affect Mg uptake.
by CT26 tumor cells (Fig. 7C). To confirm this effect, we further assessed Mg concentrations in CT26 cells (maintained for 3 days in 10, 25, and 100% Mg media) 8 h post-cisplatin (125 μM) treatment and found similar intracellular Mg concentrations regardless of extracellular Mg concentrations (10% Mg = 44.56 ± 8.39; 25% = 51.81 ± 15.01; 100% Mg = 57.99 ± 7.20 μg/100 mg protein). Thus, the CT26 tumor cells were able to maintain their intracellular Mg levels despite Mg concentrations being restricted in the culture media.

**Mg deficiency enhances CT26 tumor weight in vivo and reduces cisplatin-mediated tumor killing.** As expected, treatment of control-fed CT26 tumor-bearing mice with cisplatin significantly reduced final tumor weights when compared with control-fed untreated mice (Fig. 7D). We observed that both tumor weights and the anti-tumor efficacy of cisplatin were significantly modulated by the animals’ Mg status. Mg deficiency alone enhanced CT26 tumor weight by ~50% in vivo when compared with control-fed untreated CT26-bearing mice (Fig. 7D). In addition, tumors were significantly larger in the Mg-deficient CT26-bearing mice treated with cisplatin than those in cisplatin-treated control-fed mice (Fig. 7D). Cisplatin killing of CT26 tumors in Mg-deficient mice was not reduced by Mg supplementation (Fig. 7D) but rather slightly (but not statistically) improved (Fig. 7D).

**DISCUSSION**

Cisplatin is one of the most widely used chemotherapeutics because of its relative success in treating many malignancies (e.g., breast, ovarian, testicular, bladder, and lung cancer, as well as advanced colon cancer), its ease of administration, and its relatively low cost (13, 18). Colorectal cancer is the fourth most common form of cancer in the US (48). Colon cancer mortality fell by almost 3% per year between 2004 and 2013 (48), which was in part due to improved treatments and combination therapies that often include cisplatin for patients with advanced disease. Unfortunately, approximately one-third of all cancer patients treated with cisplatin experience kidney injury (15, 30). Because of its widespread use, thousands of cancer patients around the world are at risk for developing cisplatin-mediated AKI. Several potential renoprotective agents (e.g., amifostine and antioxidants) have been examined for preventing and/or treating cisplatin-AKI (52, 84). However, their success has been limited by their ineffectiveness, adverse side effects, costs, and potential to limit cisplatin’s anti-tumor efficacy (71, 72, 74, 84). For example, amifostine, which protects the kidneys by detoxifying platinum-DNA adducts and binding to and neutralizing free radicals, is the only FDA-approved agent for reducing cisplatin-associated nephrotoxicity. Yet its use is constrained by its side effects and cost (70) as well as its potential to limit cisplatin’s anti-tumor efficacy by reducing platinum-DNA adducts (71, 72, 74). Thus, the risk of irreversible renal injury persists among patients receiving cisplatin, supporting the need to explore additional strategies for protecting against cisplatin-mediated nephrotoxicity without compromising its ability to kill tumors.

Our earlier studies using non-tumor-bearing immunocompetent mice demonstrated that Mg deficiency promoted cisplatin-AKI at doses that had minimal renal effects under normal conditions, whereas Mg supplementation protected against cisplatin-induced AKI (64). However, these studies did not address the effect of Mg status on tumor development or
cisplatin-mediated tumor killing. Our more recent studies in immunodeficient mice bearing A2780 human ovarian tumors confirmed that Mg deficiency dramatically increased renal injury following cisplatin and that Mg supplementation could suppress these effects (65). In the previous model, we observed a slight but significant improvement in cisplatin’s efficacy when early Mg supplementation was provided during cisplatin therapy (65). Although the use of the A2780 immunocompromised mouse model permitted us to use a human ovarian tumor cell line, our conclusions regarding the role of Mg status on cisplatin-induced kidney injury and tumor killing were somewhat limited by the absence of T lymphocytes, which have been shown to play a role in mediating cisplatin-induced kidney injury (32, 39). The present study was designed to (1) extend our previous findings using tumor-bearing immunodeficient mice, (2) confirm the effects of Mg deficiency (±Mg supplementation) on cisplatin-mediated AKI in immunocompetent mice, and (3) further explore the effects of Mg status on tumor growth and cisplatin-mediated tumor killing in immunocompetent mice.

We chose the murine CT26 colon cancer model for testing the effects of Mg status on cisplatin-induced AKI and cisplatin-mediated tumor killing for several reasons: (1) the model employs immunocompetent BALB/c mice; (2) CT26 tumors are sensitive to cisplatin-mediated killing (26); (3) immune, genome, and transcriptome analyses support their origin from colonic epithelia, sharing some features with aggressive, undifferentiated human colorectal carcinomas (7, 36); (4) the CT26 model is one of the most widely used syngeneic mouse tumor models; and (5) an inverse correlation between Mg intake and colon cancer in humans has been reported (9, 41, 78). We chose female mice because 1) female sex is a risk factor for cisplatin-induced AKI (43); 2) our previous studies examining Mg status on cisplatin-AKI were performed using female mice (64, 65); (3) CT26 cells are reported to be of female origin (7); and (4) colon cancer affects men and women similarly (74a).

Using CT26 colon tumor-bearing mice, we demonstrate that Mg deficiency significantly enhances cisplatin-mediated kidney injury (e.g., BUN, Cr, and tubular injury scores) when compared with control-fed cisplatin-treated CT26 tumor-bearing mice, which show minimal evidence of cisplatin-induced kidney injury at the cumulative dose used (Fig. 1). By contrast, Mg supplementation following Mg deficiency significantly blocked the toxic effects of cisplatin on the kidneys (Fig. 1). These data are consistent with our previous studies using immunodeficient nude mice bearing A2780 ovarian tumors (65) and strongly support early and aggressive Mg supplementation to protect against cisplatin-mediated AKI.

Immune cells, including neutrophils, dendritic cells (DCs), and T lymphocytes, traffic to the kidneys following cisplatin (43). Some of these cells are injurious, whereas others are protective. DCs are protective because they reduce neutrophil infiltration and increase anti-inflammatory IL-10 levels (66). Likewise, CD4⁺/CD25⁺ Tregs adoptively transferred to nude mice reduce cisplatin-AKI, supporting their defensive role (37). Mg deficiency in our model may compromise the protective roles of Tregs and DCs, as Mg is an important intracellular second messenger in immune cells (38, 67). By contrast, the absence of T cells in nude mice significantly reduces cisplatin-mediated tubular injury, renal cytokine levels, and neutrophil infiltration, and the adoptive transfer of CD3⁺ T cells promotes kidney injury (39). Previous studies have shown that T lymphocytes isolated from Mg-deficient animals produce exaggerated levels of proinflammatory cytokines following stimulation (75). Furthermore, Mg deficiency exacerbates inflammatory responses by increasing IL-6 production by immune cells (42, 54) and by enhancing reactive oxygen species production by neutrophils (5). Cisplatin-induced markers of oxidative stress in the kidneys were enhanced by Mg deficiency (Fig. 2, A and B), and this effect was blocked by Mg supplementation (Fig. 2, A and B). Consistent with these studies, we observed increased Ngal mRNA expression in the kidneys following cisplatin treatment, which was enhanced in the setting of Mg deficiency and reduced by Mg supplementation (Fig. 2E). Neutrophil gelatinase-associated lipocalin (NGAL) was originally described as a component of neutrophil granules (33, 79). Renal neutrophil infiltration is observed following cisplatin (16, 64) and may contribute to the observed renal Ngal mRNA expression along with damaged nephrons that also produce NGAL (53). Urinary NGAL was once considered a specific biomarker of cisplatin-induced AKI in rodents and humans (21, 44). However, more recent studies suggest that NGAL is associated with numerous inflammatory conditions, including sepsis and inflammatory bowel disease (49, 51), and thus it is considered...
an “inflammatory marker.” In summary, based on our findings that Mg deficiency enhances markers of oxidative stress and inflammation following cisplatin, it is possible that Mg deficiency contributes to cisplatin-induced AKI by promoting immune cell-mediated inflammation in the kidneys.

Mg deficiency significantly increased numerous signaling pathways associated with inflammation and apoptosis (e.g., p53, STAT3, and ERK1/2) and markers of apoptosis (e.g., Apaf1, Bax, and Bak1) mRNA expression and TUNEL staining) in the kidneys of cisplatin-treated CT26 tumor-bearing mice when compared with tumor-bearing controls treated with cisplatin. In addition, we observed enhanced mRNA expression of 

Apaf1, a downstream target of p53, along with increased Bax and Bak mRNA expression and TUNEL staining in the kidneys following cisplatin alone (Fig. 3, B–E), in the absence of elevated p53 activation (Fig. 3A). This discrepancy might be due to differences in the techniques used (Western blotting vs. qPCR and TUNEL staining), the timing of assessment, and/or the sensitivity of the methods. Furthermore, renal Apaf1 mRNA expression during cisplatin-AKI can be induced through p53-independent pathways (e.g., E2F1) (85). Similarly, both Bax and Bak can promote cisplatin-mediated renal epithelial cell apoptosis independent of p53 through the mitochondrial pathway (28). It is important to note that cisplatin’s effects on all of these markers and pathways leading to AKI were greatly enhanced in the setting of Mg deficiency and that the effects of Mg deficiency were almost completely blocked by Mg supplementation.

Cyclic cisplatin therapy causes hypomagnesemia in almost all patients because of reduced Mg reabsorption and increased Mg losses, which in turn exacerbate cisplatin-mediated AKI and hypomagnesemia (34, 64, 65) (Fig. 8). As expected, cisplatin treatment alone significantly lowered plasma Mg levels, and this was further exacerbated in the setting of Mg deficiency (Fig. 4). Plasma Mg levels in Mg-deficient animals treated with cisplatin were significantly improved following Mg supplementation, but they were not completely restored to basal (untreated) levels (Fig. 4). However, kidney function (i.e., BUN and Cr levels) in the Mg supplemented group was similar to that observed in untreated mice (Fig. 4). This discrepancy may be explained by the fact that circulating Mg levels do not completely reflect tissue Mg stores, which were not measured in this study and thus limit our conclusions. Our observations strongly support the link between hypomagnesemia and cisplatin-AKI. Mg intakes have decreased by ~50% in the American diet over the past century (17). It is estimated that only ~40% of the US population consumes the recommended daily intake of Mg (19), and the elderly, those

Fig. 7. Cisplatin (Cis)-mediated CT26 colon cancer cell cytotoxicity in vitro is unaffected by Mg deficiency or Mg supplementation. Mg deficiency enhances tumor growth and reduces cisplatin’s tumor-killing efficacy in vivo, and Mg supplementation does not compromise Cis’s efficacy in vivo. A: in vitro, CT26 cells were grown in either 100% Mg media, 10% Mg media, or 10% Mg media, followed by Mg supplementation (± Cis), and assayed for cytotoxicity (n = 4/condition) using the MTT assay, as described in METHODS. IC50 values for Cis killing are shown. B: spermide, to block Mg uptake, did not alter Cis-mediated cytotoxicity of CT26 cells in vitro. Data are shown as mean %control (± SD). C: CT26 cells grown in 100% Mg media were treated with spermine (0 and 50 μM), and intracellular Mg concentrations were analyzed as described in METHODS (n = 6/condition). Spermine did not affect intracellular Mg concentration. Data are shown as mean relative fluorescence units (RFU) ± SD. D: in vivo, CT26 tumors were removed from control (Ctrl; 100% Mg), Mg-deficient (MgD), and Mg-supplemented mice (MgS; treated ± Cis, as described in METHODS and Fig. 1; n = 8–12/group). Tukey-Kramer post hoc test was used to determine statistical significance. Data are shown for each individual mouse with means (indicated by horizontal lines) ± SD.

Fig. 8. Hypomagnesemia exacerbates cisplatin-mediated kidney injury, which further compounds hypomagnesemia, producing a vicious circle. Mg status also affects tumor outcomes.
most likely to be diagnosed with cancer [90% of colon cancer is diagnosed in those over age 50 (55)], are at increased risk for Mg deficiency (1). In addition, numerous factors compromise Mg status, including age, medications that prevent Mg absorption or promote Mg losses, kidney health, and malabsorption disorders (4, 20, 25, 29, 45, 63).

Using LLC-PK1 cells, a porcine renal tubular epithelial cell line used most commonly to study nephrotoxic drugs and mechanisms to block drug-induced nephrotoxicity (24), we showed that exposure to Mg deficiency in vitro promoted cisplatin-mediated cytotoxicity and inflammation, and Mg supplementation blocked this effect (Fig. 5). To further explore the role of cellular Mg status in cisplatin killing of renal epithelial cells, spermine, shown previously to inhibit TRPM7 (68), was used in the LLC-PK1 cell-based cisplatin-cytotoxicity assay. Spermine (≥25 μM) significantly enhanced the killing of LLC-PK1 cells by cisplatin (Fig. 6A) and reduced cellular uptake of Mg (Fig. 6B). These doses are similar to the previously reported IC_{50} of spermine (22 μM) for Mg uptake by ventricular myocytes using similar conditions (68).

In contrast to the effects of Mg-deficient media on LLC-PK1 renal epithelial cells in vitro, exposure of CT26 colon cancer cells to Mg-deficient culture conditions did not affect their sensitivity to cisplatin killing (Fig. 7A). Likewise, Mg supplementation had no effect on cisplatin-mediated CT26 cell killing in vitro (Fig. 7A). Consistent with these observations, pretreatment of CT26 cells with spermine to block Mg uptake had no effect on cisplatin-mediated cytotoxicity (Fig. 7B) or cellular Mg uptake (Fig. 7C). Although the precise transporters that regulate Mg uptake by CT26 tumor cells are not known, these data support previous reports of tumor cells acting as Mg sinks, which are capable of maintaining intracellular Mg levels despite restricting Mg concentrations (6). However, because the exact transporters responsible for Mg uptake by CT26 tumor cells are not known, it is possible that the absence of an effect by spermine in CT26 cells is due to the regulation of Mg homeostasis by other transporters. Conversely, Mg deficiency alone and Mg deficiency combined with cisplatin promoted CT26 tumor growth in vivo when compared with control-fed mice and cisplatin-treated control-fed mice, respectively (Fig. 7D). These findings are inconsistent with our in vitro studies showing no effect of Mg status on cisplatin-mediated CT26 cell killing in vitro and might be due to the additional cell types that accompany CT26 tumors in vivo and because Mg status can regulate TRPM7 activity in some of these cell types (2). Decreased intracellular Mg concentrations increase TRPM7 activity, and there are reports linking increased TRPM7 activity to M2 polarized macrophages (62), which are well known for their tumor-promoting activity (46). The link between Mg status and colon cancer is strongly supported by a recent meta-analysis showing the inverse correlation between Mg intakes and the risk of colon cancer (9). In addition, the risks of colorectal adenomas (12), breast cancer (59), and prostate cancer (11) are enhanced by higher serum Ca/Mg ratios. Furthermore, higher Mg levels in drinking water are associated with a reduced risk of gastric cancer (81), prostate cancer (82), esophageal cancer (83), and decreased morbidity associated with liver cancer (73). Finally, we found that Mg deficiency impaired cisplatin-mediated killing of CT26 tumors in BALB/c mice (Fig. 7D). Overall, these findings confirm the link between cisplatin, Mg deficiency, and nephrotoxicity but also reveal the role of Mg status on tumor growth and susceptibility to cisplatin-mediated killing (Fig. 8).

Together, these observations, along with our previous studies in nude mice bearing human ovarian tumors (65), strongly support assessing Mg status before cisplatin treatment and early and more aggressive Mg supplementation during cisplatin treatment to protect the kidneys from cisplatin-induced AKI without compromising its anti-tumor efficacy. Numerous small clinical trials support the beneficial effects of providing supplemental Mg during cisplatin therapy in testicular (77), ovarian (3), and head/neck cancer (27). More recently, Yamamoto et al. (80) reported that hydration with Mg (vs. hydration without Mg) significantly reduced cisplatin-induced nephrotoxicity in cancer patients when provided before cisplatin chemotherapy, and Saito et al. (60) revealed that pretreatment with intravenous Mg sulfate protected against cisplatin-mediated nephrotoxicity. These studies had small sample sizes (<100 subjects) and lack long-term followup to assess the beneficial role of Mg on tumor-related outcomes, including success of chemotherapy, metastases, tumor progression, and/or survival. The results of our studies support further investigations of the effects of Mg status during cisplatin therapy for renoprotection and studies of the role of Mg status on tumor growth, metastasis, and sensitivity to cisplatin killing in experimental models using numerous tumor types. Likewise, our results support larger, randomized clinical trials of early and aggressive Mg supplementation for cancer patients with a range of tumor types to examine the short-term and long-term effects of Mg supplementation on cisplatin-induced AKI, tumor growth and metastases, and patient survival.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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Mg REDUCES CISPLATIN-AKI WITHOUT COMPROMISING TUMOR KILLING


