Magnesium protects against cisplatin-induced acute kidney injury without compromising cisplatin-mediated killing of an ovarian tumor xenograft in mice

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1Elmezzi Graduate School of Molecular Medicine, Manhasset, New York; 2Center for Immunology and Inflammation, Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, New York; 3Hofstra North Shore-LIJ School of Medicine, Manhasset, New York; 4Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts; 5Division of Nephrology, Medical College of Wisconsin, Milwaukee, Wisconsin; and 6Biostatistics Unit, Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, New York

Submitted 5 March 2015; accepted in final form 2 May 2015

Solanki MH, Chatterjee PK, Xue X, Gupta M, Rosales I, Yeboah MM, Kohn N, Metz CN. Magnesium protects against cisplatin-induced acute kidney injury without compromising cisplatin-mediated killing of an ovarian tumor xenograft in mice. Am J Physiol Renal Physiol 309: F35–F47, 2015. First published May 6, 2015; doi:10.1152/ajprenal.00096.2015.—Cisplatin, a commonly used chemotherapeutic for ovarian and other cancers, leads to hypomagnesemia in most patients and causes acute kidney injury (AKI) in 25–30% of patients. Previously, we showed that magnesium deficiency worsens cisplatin-induced AKI and magnesium replacement during cisplatin treatment protects against cisplatin-mediated AKI in non-tumor-bearing mice (Solanki MH, Chatterjee PK, Gupta M, Xue X, Plagov A, Metz MH, Mintz R, Singhal PC, Metz CN, Am J Physiol Renal Physiol 307: F369–F384, 2014). This study investigates the role of magnesium in cisplatin-induced AKI using a human ovarian tumor (A2780) xenograft model in mice and the effect of magnesium status on tumor growth and the chemotherapeutic efficacy of cisplatin in vivo. Tumor progression was unaffected by magnesium status in saline-treated mice. Cisplatin treatment reduced tumor growth in all mice, irrespective of magnesium status. In fact, cisplatin-treated magnesium-supplemented mice had reduced tumor growth after 3 wk compared with cisplatin-treated controls. While magnesium status did not interfere with tumor killing by cisplatin, it significantly affected renal function following cisplatin. Cisplatin-induced AKI was enhanced by magnesium deficiency, as evidenced by increased blood urea nitrogen, creatinine, and other markers of renal damage. This was accompanied by reduced renal mRNA expression of the cisplatin efflux transporter Abcb6. These effects were significantly reversed by magnesium replacement. On the contrary, magnesium status did not affect the mRNA expression of cisplatin uptake or efflux transporters by the tumors in vivo. Finally, magnesium deficiency enhanced platinum accumulation in the kidneys and renal epithelial cells, but not in the A2780 tumor cells. These findings demonstrate the renoprotective role of magnesium during cisplatin AKI, without compromising the chemotherapeutic efficacy of cisplatin in an ovarian tumor-bearing mouse model.

hypomagnesemia; nephrotoxicity; renal protection

THE FOOD AND DRUG ADMINISTRATION (FDA) approved cisplatin or cis-diamineplatinum (II) dichloride for the treatment of both testicular and ovarian cancer in 1978. Today, cisplatin is commonly used to treat numerous cancers, including bladder, lung, and head and neck cancers, as well as testicular and ovarian cancers (6, 21, 28, 34). Ovarian cancer is the fifth leading cause of cancer death among women in United States (4) and the most lethal gynecological malignancy (3). Each year, ~20,000 new cases of ovarian cancer are identified, and in 2011 there were more than 14,000 deaths from ovarian cancer (4). Today, ovarian cancer is commonly treated with a combination of surgery, radiation, and chemotherapy with cisplatin or carboplatin and paclitaxel (induction therapy), depending on the stage and grade of the tumor (25, 26).

Despite its success as an antineoplastic agent, cisplatin causes dose-limiting nephrotoxicity in approximately one third of patients (21, 28, 34). Women are more likely to experience cisplatin-induced acute kidney injury (AKI) than men (11, 21, 34). Cisplatin invariably causes hypomagnesemia in ~90% of treated patients, which may persist even after treatment discontinuation (17). Hypomagnesemia has been linked to the nephrotoxic effects of cisplatin (17).

As an essential nutrient, magnesium (Mg) is required in our daily diet. However, <50% of the US population consumes the recommended amount (300–400 mg/day) of Mg (13, 32). Mg is required for various critical cellular processes and enzymatic reactions; it is the cofactor required for ATP activity and is critical for cell growth and the synthesis of DNA, RNA, and protein, as well as mitochondrial respiration (1, 23, 31). Hence, Mg has been proposed to play a role in tumor growth and metastasis (38). Recently, we reported that Mg deficiency enhances renal damage mediated by cisplatin in a non-tumor-bearing mouse model of cisplatin-induced AKI and that Mg replacement after deficiency offers significant renoprotection (35). In the same study, we showed that Mg supplementation protected against cisplatin-induced AKI by regulating renal platinum accumulation with changes in cisplatin efflux transporter expression by the kidneys. These observations are consistent with recent retrospective and prospective clinical studies showing the renoprotective effects of Mg supplementation in cancer patients taking cisplatin (2, 16, 22, 37, 43). Together, these studies clearly support maintaining Mg homeostasis during cisplatin treatment. However, none of the studies published to date have examined whether Mg status modulates tumor growth and/or tumor killing by cisplatin in vivo. In this study, we extended our initial studies to investigate the role of host Mg status on cisplatin’s chemotherapeutic efficacy using...
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Mg REGULATES CISPLATIN-INDUCED AKI IN TUMOR-BEARING MICE

Table 1. Mouse qPCR primers

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qPCR, quantitative PCR.

a human ovarian tumor xenograft model and cisplatin-induced AKI in mice.

METHODS

Animals and cell lines. All animal studies were submitted to and approved by The Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research (IACUC no. 2012-009). Foxn1+/Foxn1− female athymic nude mice (Harlan, Indianapolis, IN) were acclimated under normal environmental conditions and allowed free access to standard chow and water for 1 wk before experimentation. The LLC-PK1 porcine renal epithelial cell line was purchased from ATCC (Manassas, VA). The A2780 human ovarian carcinoma line was purchased from ATCC (Manassas, VA). The A2780 human ovarian carcinoma cell line was obtained from T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA).

Model of ovarian tumor xenograft combined with cisplatin-induced AKI. Female athymic nude mice (n = 7–8/group, 6–8 wk old) were randomized to receive either 1) control diet (normal chow containing 100% of the recommended Mg) or 2) a Mg-deficient diet (containing 10% of the recommended amount of Mg; prepared by Teklad/Harlan, Madison, WI) on day 1, to produce mild-moderate Mg deficiency as previously described (35). A2780 human ovarian carcinoma cells, grown in RPMI 1640 media containing 10% FBS, penicillin (P), streptomycin (S), and glutamine (Q), were washed, resuspended (5 × 10^6 cells in 100 μl saline), and injected subcutaneously (sc) in the right flank on day 2, as previously described (27). Saline or cisplatin [10 mg/kg intraperitoneally (ip)] were injected on days 12, 19, and 21 (before euthanasia on day 22). In addition, one group of mice (n = 8, Mg replacement group) received the Mg-deficient diet starting on day 1 through day 15 followed by the control (100% Mg) diet along with 0.3% MgCl₂ (wt/vol) in their drinking water until day 22. This group also received MgSO₄ (100 mg/kg⁻¹·day⁻¹ sc) twice daily from day 19 (after 2nd dose of cisplatin) until euthanasia by CO₂ asphyxiation/exsanguination on day 22. All mice were weighed during the entire study, and tumor sizes/volumes were recorded on days 13, 15, 19, 21, and 22. This model is similar to Pabla et al. (27), except 1) the duration between cisplatin treatments was shorter in our model, and mice were euthanized 24 h after the last dose of cisplatin (compared with weekly cisplatin treatment for 4 wk, with euthanasia 7 days post-cisplatin treatment) (27); and 2) Mg status was assessed in our model. As predicted from our previous study (35), consumption of a Mg-deficient diet by tumor-bearing athymic nude mice for 22 days reduced plasma Mg levels by 30% in the presence of cisplatin (cisplatin: 1.58 ± 0.14 mg/dl vs. Mg-deficient+cisplatin: 1.1 ± 0.05 mg/dl, means ± SD, P < 0.05); this decline was significantly reversed by Mg supplementation (Mg-deficient, Mg-supplemented+cisplatin: 2.26 ± 0.18 mg/dl, P < 0.0001). Heparinized blood was collected by cardiac puncture; isolated plasma was frozen at −80°C until analysis. Kidneys were collected and either flash frozen in liquid N₂ (outer medulla and cortex only) or fixed in 10% formalin. Tumors were collected, flash frozen, and stored at −80°C until analysis.

Antibodies and reagents. MRP4 (ABCC4, rabbit anti-human) and GAPDH (rabbit anti-human) antibodies were purchased from Cell Signaling Technology (Danvers, MA), CTR1 (rabbit anti-human), MRP2 (rabbit anti-human), and MRP6 (rabbit anti-human) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Cisplatin [cis-diamineplatinum (II) dichloride] was purchased from Acros Organics (Pittsburgh, PA), MgCl₂·6H₂O and MgSO₄ (anhydrous) were purchased from Thermo Scientific (Waltham, MA).

Determination of plasma Mg, blood urea nitrogen, and creatinine levels. QuantiChrom Magnesium, Urea and Creatinine Assay kits were used to determine plasma Mg²⁺ (mg/dl), blood urea nitrogen (BUN; mg/dl), and creatinine (mg/dl) levels, respectively, according to the manufacturer’s instructions (BioAssay Systems, Hayward, CA).

Real-time quantitative PCR. Quantitative PCR (qPCR) was performed using high-quality RNA isolated from frozen kidneys and A2780 tumors (in vivo) using an RNeasy Universal Plus Mini kit (Qiagen, Valencia, CA), as previously described (35). RNA preps with optical density (OD) 260/280 and OD 260/230 ratios ≥1.9 were used. The qPCR were performed in duplicate/triplicate using specific primers (Tables 1 and 2), as previously described (35). Relative changes in gene expression were calculated as fold-changes using the comparative Ct (ΔΔCt) method (8). Mouse Gapdh and human

Table 2. Human qPCR primers

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GAPDH were used as housekeeping genes for normalizing transcript levels in mouse kidneys and human tumors, respectively.

Histological assessment of renal cell apoptosis and renal injury. Formalin-fixed kidneys were embedded in paraffin and sectioned (5 μm). Renal apoptosis (in the cortex and outer medulla regions) was measured by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) using an ApopTag kit (Millipore, Temecula, CA). The slides were scored by a reviewer blinded to experimental conditions by counting the number of densely stained apoptotic cells per high-power field (using >5 random fields/section, 4–5 mice/group). AKI-associated tubular injury (tubular epithelial cell loss, necrosis, tubular epithelial simplification, intratubular debris, and casts) was scored by a pathologist (I. Rosales) blinded to the experimental groups. Tubule injury scores (ranging between 0 and 4) were based on the percentage of tubules affected as follows: 0 = <10%, 1 = 10–25%, 2 = 26–50%, 3 = 51–75%, and 4 = >75%, as previously described (35).

Assessment of cisplatin transporter expression by A2780 cells. A2780 (human ovarian tumor) cells were grown in RPMI 1640 media containing 10% FBS and PSQ as described above until 60% confluent; media was replaced with “complete” MEM, Ca (2.6 g/dl), nonessential amino acids, and PSQ containing either 100% Mg (4.9 mg/dl) or 10% Mg (0.49 mg/dl), supplied as MgSO4. After 3 days, one set of A2780 cells maintained in 10% Mg media were supplemented with 90% Mg (final = 100% Mg) ~36 h before cisplatin treatment. MEM or cisplatin (diluted in MEM, 25 μM final) was added for 4 h, and the cells were collected, washed, and lysed in RIPA buffer. Lysates were Western blotted using primary antibodies (MRP4 and GAPDH) and 1:300 for Santa Cruz antibodies (CTR1, MRP2, MRP6) and appropriate near-infrared fluorescently labeled secondary antibodies (1:15,000, LI-COR, Lincoln, NE), as previously described (35). Band densities, determined using Image J Software (National Institutes of Health), were normalized using GAPDH as a loading control. Representative blots from n = 4–7 experiments are shown in the figures.

Quantification of platinum accumulation in the kidneys. Renal tissue platinum (Pt) levels were measured by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500cx. All assays were performed at the University of North Carolina at Chapel Hill, as previously described (35). Renal Pt levels were normalized to kidney weight in milligrams.

Determination of cellular Pt accumulation in LLC-PK1 and A2780 cells. LLC-PK1 (renal epithelial) cells were grown in M199 media containing 5% FBS, PSQ in T75-flasks until 60% confluent. Thereafter, the media was replaced with complete MEM containing 5% FBS, Ca (2.7 g/dl), nonessential amino acids, PSQ, and either 100% Mg (9.63 mg/dl for M199) or 10% Mg (0.963 mg/dl), supplied as MgSO4. The cells were incubated for 4 days. One set of the cells maintained in 10% Mg media were supplemented with 90% Mg (final = 100% Mg) ~36 h before cisplatin treatment. For assessment of Pt uptake, MEM or cisplatin (diluted in MEM, 50 μM final) was added to the cultures. The dose and time point were based on prior experiments. After 6.5 h, the cells were collected, washed, and cellular Pt levels were measured by ICP-MS using the Agilent 7500cx, as described above. Cellular Pt levels were normalized to protein concentration using the micro BCA Protein Assay (Thermo Scientific).

A similar experimental protocol was followed for the A2780 human ovarian cancer cells, except they were grown in RPMI 1640 medium containing 10% FBS and PSQ and after they were 60% confluent, media was replaced with complete MEM media containing either 100% Mg (4.9 mg/dl MgSO4 for RPMI media) or 10% Mg (0.49 mg/dl MgSO4) (±90% MgSO4, as Mg supplement). The cisplatin dose used for the A2780 cells was 25 μM, which was based on preliminary experiments testing the cytotoxicity of cisplatin.

Statistical analyses. Experiments were performed at least twice, and data are expressed as means ± SE (or ± SD), as indicated. ANOVA followed by Bonferroni post-hoc testing using GraphPad Prism (GraphPad Software, San Diego, CA) was used to examine those factors that were measured at a single time point. For analyses of tumor growth over time, data were log transformed and analyzed using repeated measures ANOVA (RMANOVA). A “Bonferroni-like” adjustment, such that P < 0.01 was considered significant, was used for prespecified pairwise comparisons. These analyses were carried out using SAS Version 9.3 (SAS Institute, Cary, NC). For all omnibus tests, P < 0.05 was considered significant.

RESULTS

Mg status does not affect tumor growth in vivo, and Mg supplementation does not impair cisplatin-mediated tumor killing in vivo. In the absence of cisplatin administration, A2780 tumor cells implanted in control (100% Mg) and Mg-deficient mice grew slowly over the 3-wk period (Fig. 1, A and B). No significant differences were found in A2780 tumor growth over the course of the study in untreated mice, irrespective of their Mg status (Fig. 1, A and B). As expected, we observed reduced ovarian xenograft tumor growth in all cisplatin-treated mice (±Mg deficiency, ±Mg supplementation) compared with saline-treated tumor-bearing mice (±Mg deficiency, ±Mg supplementation), with significantly reduced tumor growth on day 22 in cisplatin alone mice compared with saline-treated controls (Fig. 1, A and B, P < 0.01). However, there was no significant effect of Mg deficiency on ovarian xenograft tumor growth in cisplatin-treated mice compared with 100% Mg cisplatin-treated mice (Fig. 1A). Tumor growth in Mg-supplemented mice treated with cisplatin did not significantly differ from the cisplatin control mice until after day 21, when the tumors in the cisplatin-treated Mg-supplemented mice showed considerably less growth when measured on day 22 (P < 0.01) compared with tumors in cisplatin-treated 100% Mg-fed controls on day 22 (Fig. 1A). Similarly, tumor growth in cisplatin-treated Mg-supplemented mice was significantly lower than that observed in the cisplatin-treated Mg-deficient mice when measured on day 22 (Fig. 1A, P < 0.01).

Mg status modulates cisplatin-induced AKI in a human ovarian tumor xenograft model. Cisplatin alone (10 mg/kg; 3 doses given on days 12, 19, and 21 after start of the Mg-deficient or control diet) did not enhance plasma BUN or creatinine levels in tumor-bearing female mice compared with saline-treated controls (Fig. 2, A and B). However, when combined with Mg deficiency, the same regimen of cisplatin significantly enhanced BUN (Fig. 2A, P < 0.001) and creatinine levels (Fig. 2B, P < 0.05) compared with cisplatin alone. As shown in Fig. 2, A and B, Mg replacement during cisplatin treatment significantly reduced plasma BUN and creatinine levels compared with Mg-deficient mice treated with cisplatin (Fig. 2, A and B, respectively, P < 0.01).

Assessment of histological kidney damage revealed that cisplatin-treated Mg-deficient mice showed significantly increased renal tubular injury compared with cisplatin alone (Fig. 2, C—E), while Mg replacement was associated with significantly reduced renal tubular injury compared with kidneys obtained from Mg-deficient mice treated with cisplatin (Fig. 2, C—E). Renal inflammation has been implicated in cisplatin-mediated kidney damage (21, 30, 34). Cisplatin alone and Mg deficiency alone did not affect the renal expression of inflammatory genes, including Ccl2 (Fig. 2F) and Cxcl10 (Fig. 2G), compared with untreated control mice bearing A2780 ovarian tumors. However, cisplatin-treated Mg-deficient tumor-bearing...
mice had significantly enhanced Cxcl2 (Fig. 2F, \( P < 0.0001\), ~2.5 fold over control) and Cxcl10 mRNA expression in their kidneys (Fig. 2G, \( P < 0.0001\), ~3-fold over control) compared with cisplatin-treated controls. Mg replacement during cisplatin treatment significantly reduced renal Cxcl2 and Cxcl10 mRNA expression compared with cisplatin-treated Mg-deficient mice (Fig. 2, F and G, respectively, \( P < 0.01\)).

Cisplatin-mediated renal cell apoptosis in tumor-bearing mice is regulated by Mg status. Renal Bak mRNA expression in tumor-bearing mice was not affected by either cisplatin alone or Mg deficiency alone compared with untreated controls (Fig. 3A). However, when Mg deficiency was combined with cisplatin, renal Bak mRNA expression was increased by about threefold compared with kidneys obtained from tumor-bearing mice treated with cisplatin alone or Mg deficiency alone (Fig. 3A, \( P < 0.0001\)). This effect was significantly reversed by Mg replacement during cisplatin treatment compared with Mg-deficient mice treated with cisplatin (Fig. 3A, \( P < 0.01\)).

Based on TUNEL staining, significant renal cell apoptosis was not observed in control athymic tumor-bearing mice in the absence of cisplatin or in untreated tumor-bearing Mg-deficient mice (Fig. 3, B and C). Cisplatin treatment of normal-fed mice slightly enhanced renal cell apoptosis (although not significantly), and this was further and significantly increased when cisplatin was accompanied by Mg deficiency (Fig. 3, B and C, \( P < 0.0001\)). The kidneys of Mg-supplemented tumor-bearing mice treated with cisplatin showed significantly less apoptosis compared with cisplatin-treated Mg-deficient tumor-bearing mice (Fig. 3, B and C, \( P < 0.001\)).

Mg status regulates gene expression of renal cisplatin uptake and efflux transporters. Next, we investigated the role of Mg status on the expression of several well-established cisplatin uptake transporters by the kidneys. In ovarian tumor-bearing female mice, Mg deficiency alone did not significantly affect renal Oct1 (Fig. 4A), Oct2 (Fig. 4B), orCtr1 (Fig. 4C) mRNA expression compared with untreated controls. However, cisplatin alone significantly reduced renal Oct2 (Fig. 4B, \( P < 0.01\)) and Ctr1 mRNA expression (Fig. 4C, \( P < 0.05\) ) compared with untreated controls. When cisplatin was combined with Mg deficiency in tumor-bearing mice, renal Oct1 (Fig. 4A, \( P < 0.05\) ) and Oct2 (Fig. 4B, \( P < 0.0001\) ) mRNA expression was significantly reduced compared with cisplatin treatment alone. Surprisingly, Mg replacement during cisplatin treatment significantly enhanced renal Oct2 mRNA expression (Fig. 4B, \( P < 0.05\) ) and slightly increased Oct1 mRNA expression (Fig. 4A, not significant) compared with cisplatin-treated Mg-deficient tumor-bearing mice. By contrast, renal
Fig. 2. Severity of CIS-induced acute kidney injury (AKI) in a human ovarian tumor xenograft model is modulated by Mg status. Groups are defined as in Fig. 1. CTRL, MgD, and MgD MgS mice bearing A2780 tumors were treated with cisplatin (CIS) (as described in METHODS and Fig. 1). Mice were euthanized 24 h after the last dose of CIS (or saline) on day 21. Blood urea nitrogen (BUN; A) and plasma creatinine levels (B) were determined. Values are means ± SE (mg/dl). Fixed kidney tissues were stained with hematoxylin and eosin and evaluated for histology. C and D: representative images for each group (200 magnification; C) and (400 magnification; D). Scale bar = 50 μm (C) and 20 μm (D). E: histological damage scores (ranging between 0 and 4; means ± SE) were based on percentage of tubules affected using 200 magnification (0: <10%; 1: 10–25%; 2: 26–50%; 3: 51–75%; and 4: >75%). F and G: renal cortical mRNA expression of inflammatory markers Cxcl2 (F) and Cxcl10 (G) was measured by quantitative (q) PCR. Values are means ± SE fold-change (vs. Gapdh, housekeeping gene). †P < 0.05 vs. CIS. ††P < 0.001 vs. CIS. †††P < 0.0001 vs. CIS. ‡‡P < 0.01 vs. MgD CIS.
Ctr1 mRNA expression in cisplatin-treated mice was not significantly altered by Mg status (Fig. 4C).

When we examined the kidneys for mRNA expression of cisplatin efflux transporters, including Abcc2 (which encodes MRP2), Abcc4v1 and Abcc4v3 (variants 1 and 3 which encode MRP4), as well as Abcc6 (which encodes MRP6) in tumor-bearing mice following cisplatin (±Mg deficiency and supplementation), we found that only renal Abcc2 mRNA expression was significantly enhanced by cisplatin alone compared with controls (Fig. 4D, P < 0.001), and this was not affected by Mg status (Fig. 4D). Renal Abcc4v1 mRNA expression was similar in all the groups of tumor-bearing mice, irrespective of Mg status or cisplatin treatment (Fig. 4E). Cisplatin alone significantly reduced renal Abcc4v3 mRNA expression (Fig. 4F, P < 0.05) compared with controls. This decline was further (slightly) reduced by Mg deficiency; however, this decrease was not significant, nor was it reversed by Mg replacement (Fig. 4F). Renal mRNA expression of efflux transporter Abcc6 was not altered by either Mg deficiency alone or cisplatin alone compared with saline-treated controls, but it was significantly

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**Fig. 3.** CIS-mediated renal cell apoptosis in tumor-bearing mice is regulated by Mg status. CTRL, MgD, and MgD MgS mice bearing A2780 tumors (as described in METHODS and Fig. 1) were euthanized 24 h after the last dose of CIS (or saline) given on day 21. Renal cortical mRNA expression of Bak (A) was measured by qPCR. Values are means ± SE fold-change (vs. Gapdh, housekeeping gene). Renal cell apoptosis was measured by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining, and representative photomicrographs are shown (×400 magnification) in B. Scale bar = 20 μm. Apoptosis was determined by counting the number of TUNEL-positive cells per high power field (HPF) using ≥15 random sections, and the mean apoptosis scores (±SE) are shown in C. Arrows indicate TUNEL-positive cells. ††††P < 0.0001 vs. CIS. ‡‡P < 0.01 vs. MgD CIS. ‡‡‡P < 0.001 vs. MgD CIS.
reduced when they were combined (Fig. 4G, P < 0.0001). Mg replacement after Mg deficiency and during cisplatin treatment significantly enhanced renal Abcc6 mRNA expression in tumor-bearing mice (Fig. 4G, P < 0.05) compared with cisplatin-treated Mg-deficient tumor-bearing mice.

Mg status does not affect cisplatin uptake or efflux transporter gene expression by A2780 tumors grown in vivo. Next, we investigated the effect of Mg status on the mRNA expression of cisplatin uptake and efflux transporters expressed by human A2780 tumors grown in athymic nude mice. Neither OCT1 nor OCT2 mRNA expression by the tumors was observed (data not shown). By contrast, A2780 tumor tissue expressed CTR1 mRNA, and its expression was similar in all the groups of tumor-bearing mice, irrespective of Mg status or cisplatin treatment (Fig. 5A). A2780 tumors obtained from the mice expressed numerous cisplatin efflux transporters, including ABCC2 (which encodes for MRP2, Fig. 5B), ABCC4 (variants 1 and 2 which encode MRP4, Fig. 5, C and D), and ABCC6v2 (which encodes for MRP6, Fig. 5E). With the exception of ABCC6v2 mRNA, which was upregulated by cisplatin (Fig. 5E), tumor efflux transporter mRNA expression was not significantly altered by Mg deficiency, Mg supplementation, and/or cisplatin treatment (Fig. 5, B—D).

Mg status does not affect cisplatin uptake or efflux transporter protein expression by A2780 cells grown in vitro. Because adequate tumor specimens were not available, particularly following cisplatin treatment, for the analyses of uptake and efflux transporters at the protein level, we examined the effect of Mg status on their expression using untreated and cisplatin-treated A2780 cells grown in 100% Mg and 10% Mg media (±Mg supplementation) in vitro. Mg deficiency alone and cisplatin treatment alone significantly reduced the protein expression of uptake transporter CTR1 by A2780 cells (grown in 10% Mg) compared with A2780 cells grown in 100% Mg media (Fig. 6, A and B, P < 0.001). However, Mg status did not modulate the effect of cisplatin on CTR1 expression by A2780 cells in vitro (Fig. 6, A and B). For MRP2 (Fig. 6, A and C) and MRP6 (Fig. 6, A and D), no significant differences were observed in protein expression by A2780 cells following exposure to various Mg conditions or cisplatin treatment in vitro. Similar to that observed with CTR1, MRP4 efflux protein expression by A2780 cells was significantly reduced by Mg...
deficiency alone and cisplatin alone compared with control cells (100% Mg media) (Fig. 6, A and E, P < 0.0001), whereas Mg status (Mg deficiency ± Mg supplementation) did not alter the effect of cisplatin on MPR4 expression (Fig. 6, A and E).

Renal Pt accumulation in A2780 tumor-bearing mice and cellular Pt accumulation in LLC-PK1 renal epithelial cells are increased by Mg deficiency, but Pt accumulation in A2780 tumor cells is not. Our recent study in non-tumor-bearing older female mice showed enhanced renal Pt accumulation following Mg deficiency (35). Herein, we confirmed that renal Pt accumulation was significantly enhanced in Mg-deficient nude mice treated with cisplatin (Fig. 7A, P < 0.001) compared with control cisplatin-treated nude mice. Mg replacement of Mg-deficient mice during cisplatin treatment significantly reduced renal Pt accumulation compared with cisplatin-treated Mg-deficient mice (Fig. 7A, P < 0.05).

Similarly, we confirmed the effect of Mg status on renal epithelial cell Pt accumulation in vitro using untreated and cisplatin-treated LLC-PK1 renal epithelial cells grown in either 100% Mg or 10% Mg media (±Mg supplementation). As expected, cisplatin treatment increased cellular accumulation of Pt compared with untreated LLC-PK1 cells (Fig. 7B, P < 0.0001). Similar to our in vivo results, we observed that LLC-PK1 renal epithelial cells grown in 10% Mg media had significantly higher cellular Pt concentration compared with LLC-PK1 cells grown in 100% Mg media (Fig. 7B, P < 0.001).

Mg supplementation of LLC-PK1 cells modestly but significantly reduced the Pt accumulation compared with Mg-deficient LLC-PK1 cells treated with cisplatin (Fig. 7B, P < 0.01).

Adequate tumor specimens were not available for analyzing Pt accumulation in the A2780 ovarian xenograft tumors in mice because cisplatin treatment dramatically reduced tumor growth in vivo. Therefore, we examined the effect of Mg status on Pt accumulation in vivo using untreated and cisplatin-treated A2780 human ovarian cancer cells, as described for the LLC-PK1 cell line. As expected, cisplatin treatment of A2780 cells led to cellular Pt accumulation compared with untreated A2780 cells (Fig. 7C, P < 0.0001). However, we did not observe any significant difference in the cellular Pt accumulation by cisplatin-exposed A2780 human ovarian cancer cells grown in 100% Mg vs. 10% Mg (±Mg supplementation) (Fig. 7C).

**DISCUSSION**

The current study aimed to explore the effects of Mg status (Mg deficiency ± Mg supplementation) on tumor growth in vivo and the chemotherapeutic efficacy of cisplatin in a physiologically relevant tumor-bearing mouse model. Using a previously established human ovarian tumor xenograft model in mice combined with cisplatin-induced AKI (with a reduced time between the last dose of cisplatin and euthanasia, as
described in METHODS) (27), we show for the first time that Mg status does not affect tumor growth in vivo and that Mg supplementation does not compromise the chemotherapeutic efficacy of cisplatin using A2780-tumor-bearing mice (Fig. 1). These results complement our previous observations revealing the beneficial role of Mg supplementation in cisplatin-induced AKI in non-tumor-bearing physiologically relevant older C57BL/6 female mice in vivo (35), as well as our in vitro data showing that Mg supplementation did not impair cisplatin killing of the A2780 human ovarian, MCF-7 human breast, or H460 human lung tumor cell lines (35).

Although little is known regarding Mg deficiency (± Mg supplementation) and cancer, previous studies have connected a high serum Ca/Mg ratio with an increased risk of breast cancer (33) and prostate cancer (9). Furthermore, a recent and extensive meta-analysis reported that higher intakes of Mg were associated with a decreased risk of colon cancer (5). Similarly, higher Mg levels in drinking water correlate with lower risks of gastric cancer (40), prostate cancer (41), and esophageal cancer (42), decreased morbidity associated with liver cancer (36), and a decreased risk of death from ovarian cancer (7). Together, these data suggest that Mg deficiency is associated with an increased cancer risk and sufficient Mg intakes correlate with tumor-protective effects.

Several studies also report the inhibition of primary tumor growth in Mg-deficient animals (24, 44). Mg is required for the

Fig. 6. Mg status does not affect CIS uptake or efflux transporter protein expression by A2780 tumor cells grown in vitro. A2780 human ovarian tumor cells were maintained in media containing either 100% Mg or 10% Mg (Mg-deficient), as described in METHODS. One group of Mg-deficient cells was switched to 100% Mg media (10%/100% Mg) before the addition of CIS (25 μM) for 4 h. Cell lysates were analyzed by Western blotting for the expression of CIS uptake and efflux transporters. A: representative Western blots for tumor cell CTR1, ABCC2 (MRP2), ABCC6 (MRP6), and ABCC4 (MRP4), as well as housekeeping protein (GAPDH) expression. Quantitation of band ratios CTR1/GAPDH (B), ABCC2 (MRP2)/GAPDH (C), ABCC6 (MRP6)/GAPDH (D), and ABCC4 (MRP4)/GAPDH (E) are also shown (mean band density ± SE). ***P < 0.001 vs. 100% Mg. ****P < 0.0001 vs. 100% Mg.
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Activity of NM23-H1, a metastasis suppressor gene (19), and while decreasing primary tumor growth, Mg deficiency was also associated with increased metastasis and higher mortality (24). However, the A2780 human ovarian tumor xenograft model is not a metastatic tumor model. Using the A2780 human ovarian tumor xenograft model, we observed that Mg deficiency had no effect on tumor growth in the presence or absence of cisplatin compared with appropriate control mice. There are several differences between our model and previous models investigating the effect of Mg deficiency on tumor growth in vivo (24, 44), including, the origin, type, and site of the tumor, the species (mouse vs. rat models), the mouse strain (athymic nude mice vs. other strains), and the severity of Mg deficiency (our model has mild-moderate Mg deficiency which was considerably less severe). Furthermore, unlike previous studies which only investigated the effect of Mg deficiency on tumor growth (24, 39, 44), we also examined the effect of Mg deficiency (±Mg supplementation) on the chemotherapeutic efficacy of cisplatin and cisplatin-induced AKI. In our model, tumor growth was not significantly affected by Mg status in saline-treated mice (Fig. 1, A and B). Similarly, cisplatin-induced A2780 ovarian tumor growth inhibition in vivo was not affected by Mg deficiency (Fig. 1, A and B). In fact, in Mg-supplemented mice, A2780 ovarian xenograft tumors grew significantly more slowly after day 21 compared with the control cisplatin-treated and the cisplatin-treated Mg-deficient mice (Fig. 1A). Consistent with these observations, we have previously shown that the efficacy of cisplatin-mediated killing of A2780 ovarian tumor cells in vitro was not impaired by either Mg deficiency or Mg supplementation (35).

Athymic nude mice are commonly used in studies investigating human tumor growth in vivo because they have a paucity of functional T cells and therefore do not exhibit acute graft vs. host rejection and thus allow the growth of human tumors. However, the absence of T cells in these mice complicates a complete understanding of the mechanisms and effects which involve T cell functionality, including cisplatin-mediated AKI. Mice lacking T cells exhibit significantly less cisplatin-mediated AKI than other immunocompetent mouse strains (18), emphasizing the importance of T cells in cisplatin-induced nephrotoxicity. This may explain why cisplatin treatment (administered in multiple doses) alone did not significantly affect kidney function in tumor-bearing female athymic nude mice in this study and one by Pabla et al. (27), while cisplatin at a similar single dose did induce AKI in female C57BL/6 mice (with functional T cells) (35). However, other strain-related differences may account for this effect.

In addition, this is the first report to reveal the augmentation of cisplatin-mediated kidney injury by Mg deficiency in tumor-bearing mice, and the improvement of cisplatin-induced AKI provided by Mg supplementation (Fig. 2, A—E). Following a similar cisplatin-dosing strategy, as previously described for the A2780 ovarian tumor xenograft model in athymic nude mice (27), we found that cisplatin administered in three doses (10 mg/kg ip on days 12, 19, and 21 after start of the Mg-deficient and control diets) to tumor-bearing athymic nude female mice did not significantly affect kidney function, as determined by BUN (Fig. 2A) and creatinine levels (Fig. 2B), as well as renal tubular damage (Fig. 2, C—E), compared with saline-treated tumor-bearing controls (when measured 1 day after the third dose of cisplatin). These results are consistent with a previous study where cisplatin-induced AKI in athymic nude mice was not observed until 7 days after the third dose of cisplatin (27). However, when combined with Mg deficiency, cisplatin treatment caused significant kidney damage in tumor-bearing athymic nude mice, as evidenced by enhanced plasma BUN and creatinine levels (Fig. 2, A and B, respectively). Mg deficiency has been linked to increased inflammation, as well as increased inflammatory cytokines in rodent models, including cisplatin-induced kidney inflammation (35). Similar to our
previous study (35), Mg deficiency in ovarian tumor-bearing female mice was accompanied by higher expression of both proinflammatory (Cxcl2 and Cxcl10 mRNA) (Fig. 2, F and G) and proapoptotic markers (Bak mRNA) and apoptosis (Fig. 3, A–C) in the kidneys following cisplatin administration compared with cisplatin-treated control (100% Mg) ovarian tumor-bearing female mice; these effects were reversed by Mg replacement.

The beneficial effects of Mg supplementation on kidney damage induced by cisplatin in patients with testicular, ovarian, lung, thoracic, and head/neck cancer have been shown by three small clinical trials (2, 16, 37) and retrospective studies (22, 43). In all of these studies, cancer patients received cisplatin as a part of their chemotherapeutic regimen with or without Mg supplementation. Significantly better kidney function was observed in the Mg-supplemented patients compared with controls (2, 16, 22, 37, 43). Overt consequences of Mg supplementation on overall long-term survival, tumor progression, and metastases were not assessed in these studies. We have confirmed the nephroprotective role of Mg in cisplatin-induced AKI, as previously shown in non-tumor-bearing mice (35), in an ovarian tumor xenograft model in mice (Fig. 8, A and B). However, the potential effect of Mg on cell membrane lipid content and fluidity (20) and, further, on additional renal cisplatin uptake mechanisms, including diffusion, cannot be eliminated. This may explain why increased Pt accumulation is observed despite reduced expression of uptake transporters.

Removal of cellular cisplatin via various efflux transporters also contributes to cisplatin levels in the kidneys. Renal mRNA expression of Abcc6 (which encodes MRP6) was significantly reduced in cisplatin-treated Mg-deficient mice, and this was reversed by Mg supplementation (Fig. 4G). These results are consistent with the increased renal cisplatin accumulation observed in Mg-deficient mice and decreased renal cisplatin accumulation observed in Mg-supplemented mice. The expression of other renal efflux transporters was not significantly altered in Mg-deficient mice following cisplatin treatment (Fig. 4). Again, the effect of Mg status on additional cisplatin efflux transporters in the kidneys cannot be ruled out.

Cisplatin uptake and efflux transporters are differentially expressed in tumors, and they are modulated to reduce cisplatin uptake, enhance cisplatin efflux, and in extreme cases promote cisplatin resistance (reviewed in Ref. 10). Consistent with reports describing CTR1 as the main cisplatin influx transporter expressed by human tumors, we observed constitutive CTR1 mRNA expression by A2780 tumors grown in vivo (Fig. 8). Proposed model. Mg supplementation using an ovarian tumor xenograft model ameliorates CIS-induced AKI without compromising the antitumor efficacy of CIS. Saline treatment of athymic nude mice bearing A2780 human ovarian tumor cells has no effect on renal or tumor outcomes, irrespective of Mg status. CIS treatment of MgD tumor-bearing mice enhances renal Pt accumulation, reduces Pt efflux transporter expression by the kidneys, and results in renal damage and AKI. These renal effects are attenuated by Mg supplementation after deficiency (MgD-MgS). By contrast, Mg status does not affect Pt accumulation in A2780 tumor cells in vitro or Pt transporter expression in tumors in vivo and in vitro. Tumor growth (in vivo) is unaffected by MgD, while MgD-MgS improves the antitumor efficacy of CIS.
5A) in the absence of OCT1 and OCT2 mRNA expression (data not shown). CTR1 mRNA expression by the A2780 tumors was not significantly altered by either cisplatin treatment or Mg status (Fig. 5A). Similarly, no significant differences in efflux transporter expression were observed at the mRNA level in the A2780 tumors obtained from the various groups of mice, except for ABCC6v2 mRNA (which encodes MRP6) expression, and this was enhanced by cisplatin alone (Fig. 5, B—E). ABCC6v1 mRNA expression was not consistent across tumors obtained from the same group of mice (and therefore, not included in the analysis). These results in combination with our tumor growth data suggest that Mg deficiency and/or supplementation does not affect gene expression of uptake or efflux transporters in the A2780 tumors, nor does Mg status affect A2780 tumor progression. Unfortunately, due to the success of tumor killing by cisplatin, one of the limitations of this study is the lack of adequate tumor tissue available with which to examine the protein expression of cisplatin uptake and efflux transporters, as well as accumulation of tumor Pt, particularly in cisplatin-treated mice, where final tumor sizes were quite small. In addition, Pt accumulation within the tumors on day 22 would be difficult to assess following cisplatin-induced killing due to loss of membrane integrity and intracellular release. Therefore, we investigated the effect of Mg status on protein expression of the uptake and efflux transporters using the A2780 cell line in vitro. Untreated A2780 cells maintained in 100% Mg-containing media had significantly higher CTR1 (Fig. 6, A and B) and MRP4 (Fig. 6, A and E) expression than untreated A2780 cells maintained in Mg-deficient media or cisplatin-treated A2780 cells maintained in 100% Mg-containing media. Overall, among cisplatin-treated A2780 tumor cells grown in vitro, no differences were observed in the protein expression of CTR1, MRP2, MRP4, and MRP6, irrespective of Mg levels in the media [control = 100% Mg, Mg deficiency = 10% Mg (±Mg supplementation)] (Fig. 6, A—E). Similarly, we did not observe any significant differences in the Pt accumulation/levels in A2780 human ovarian cancer cells grown in vitro, irrespective of their maintenance in either 100% Mg, Mg-deficient (10% Mg), or Mg-supplemented (10%/100% Mg) media (Fig. 7C). Together, these results suggest that Mg status regulates Pt accumulation in renal epithelial cells in vivo (Fig. 7A) and cisplatin-treated LLC-PK1 renal epithelial cells in vitro (Fig. 7B). However, A2780 human ovarian cancer cells are less sensitive to changes in extracellular Mg concentrations, as shown by unaltered uptake and efflux transporter expression and similar cellular platinum accumulation in Mg-deficient (±Mg supplemented) cisplatin-treated cells (Fig. 7C).

In summary, our results show for the first time enhanced kidney injury following cisplatin in A2780 tumor-bearing Mg-deficient mice (vs. cisplatin-treated controls) and significant renal benefits of Mg supplementation (following Mg deficiency) in preventing the renal injury, inflammation, and apoptosis caused by cisplatin in tumor-bearing mice (Fig. 8). We also demonstrate that while renal uptake and efflux transporter expression is altered by Mg status, the transporter expression in the A2780 tumors grown in nude mice is more tightly regulated and unaltered by Mg deficiency and/or supplementation (Fig. 8). Similarly, Mg status did not affect Pt accumulation in A2780 cells in vitro, nor did it affect uptake or efflux transporter expression in cisplatin-treated A2780 cells. Most importantly, these data indicate that Mg supplementation does not promote tumor growth in vivo and/or compromise cisplatin efficacy in the ovarian tumor-bearing mouse model (Fig. 8) and thus warrant additional studies in other tumor-bearing mouse models. Finally, future-large scale, long-term studies are required to better understand the renoprotective role of Mg in cancer patients receiving cisplatin and to monitor the effects of Mg deficiency vs. the long-term effects of Mg supplementation on tumor-related outcomes, including metastases and survival.

ACKNOWLEDGMENTS
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GRANTS
This work was supported by The Feinstein Institute for Medical Research. I. Rosales was supported by a Fellowship provided by the International Society of Nephrology.

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

AUTHOR CONTRIBUTIONS

REFERENCES
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