γ-Glutamyl Transpeptidase-Deficient Mice Are Resistant to the Nephrotoxic Effects of Cisplatin

Marie H. Hanigan,* Ernest D. Lykissa,† Danyelle M. Townsend,‡ Ching-Nan Ou,† Roberto Barrios,† and Michael W. Lieberman†

From the Department of Cell Biology,* University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; the Department of Pathology,† Baylor College of Medicine, Houston, Texas, and the Department of Cell Biology,‡ University of Virginia, Charlottesville, Virginia

We have proposed that the nephrotoxicity of cisplatin, a widely used chemotherapy drug, is the result of the binding of cisplatin to glutathione and the subsequent metabolism of the cisplatin-glutathione complex via a γ-glutamyl transpeptidase (GGT)-dependent pathway in the proximal tubules. To test the hypothesis that GGT activity is essential for the nephrotoxicity of cisplatin, the effects of cisplatin were examined in wild-type and GGT-deficient mice. Mice were treated with 15 mg cisplatin/kg. Five days after treatment, renal histopathology, blood urea nitrogen levels, serum creatinine, platinum excretion, and renal tubular necrosis were examined in wild-type and GGT-deficient mice. The wild-type mice, with and without N-acetylcysteine supplementation, had significantly elevated levels of blood urea nitrogen, serum creatinine, and renal tubular necrosis. There was no evidence of nephrotoxicity in the GGT-deficient mice regardless of N-acetyl cysteine supplementation. No differences in renal platinum excretion were seen comparing wild-type and GGT-deficient mice, nor was there any significant difference in renal platinum accumulation. These data suggest that renal cisplatin toxicity is dependent on GGT activity, and is not correlated with uptake. The results support our hypothesis that the nephrotoxicity of cisplatin is the result of the metabolism of the drug through a GGT-dependent pathway. (Am J Pathol 2001, 159:1889–1894)

Cisplatin is used to treat tumors of the testes, ovaries, bladder, squamous cell tumors of the head and neck, and non-small cell lung tumors.1 Dose-related and cumulative renal insufficiency is the major dose-limiting toxicity of cisplatin.2 Renal toxicity is modulated by intravenous hydration, but surveillance of renal function is essential after administration of cisplatin. The toxicity is specific to the proximal tubule cells. The mechanism by which these cells become targets of cisplatin-induced damage is not understood.

Previous studies in our laboratory have shown that acivicin blocks the nephrotoxicity of cisplatin in rats.3 Acivicin is an inhibitor of the enzyme γ-glutamyl transpeptidase (GGT).4 GGT is expressed on the luminal surface of the proximal tubule cells and hydrolyzes γ-glutamyl bonds.5–7 The activity of this enzyme has been shown to be essential for the metabolic activation of a series of nephrotoxic aromatic hydrocarbons that are conjugated to glutathione in vivo.8 Based on the data from our acivicin studies we proposed that cisplatin is metabolized to a nephrotoxin via this same metabolic pathway.9 However, in addition to inhibiting GGT, acivicin also inhibits the de novo synthesis of purine and pyrimidine and blocks L-glutamine requiring enzymes.10

To determine whether GGT activity is necessary for the renal toxicity of cisplatin we analyzed the nephrotoxicity of cisplatin in GGT-deficient mice.11 GGT is present on the luminal surface of absorptive and secretory ducts throughout the body.12 The highest level of activity is on the luminal surface of the proximal tubule cells in the kidney.11 Its most common physiological substrates are glutathione and glutathione conjugates.13 The extracellular cleavage of the γ-glutamyl bond by GGT initiates the hydrolysis of glutathione into its three constituent amino acids that are taken up by proximal tubule cells preventing their loss from the body.14 The GGT-deficient mice excreted large amounts of glutathione in their urine and as a result became cysteine-deficient.11 The cysteine deficiency caused growth retardation, cataract development, alteration in coat color, and premature death between 10 to 18 weeks of age. Addition of N-acetylcysteine (NAC) to the drinking water of GGT-deficient mice supplemented their supply of cysteine, resulting in weight gain and an amelioration of the other effects of the cysteine deficiency.

Supported by grant CA 57530 from the National Cancer Institute (to M. H. H.) and grant ES 07827 from the National Institute of Environmental Health Sciences (to M. W. L.).

Accepted for publication August 16, 2001.

Address reprint requests to Marie H. Hanigan, Biomedical Research Center, Room 264, 975 N.E. 10th St., Oklahoma City, OK 73104. E-mail: marie-hanigan@ouhsc.edu.
In this study we administered a nephrotoxic dose of cisplatin to GGT-deficient mice and their wild-type littermates to determine whether GGT activity is essential for the nephrotoxicity of cisplatin. Half of the mice in each group were supplemented with NAC in their drinking water. The body weights of the mice, rate of platinum excretion, blood urea nitrogen (BUN), serum creatinine, platinum accumulation in the kidney, and kidney histology were assessed after treatment with cisplatin.

Materials and Methods

Animals

The GGT-deficient C57BL/6/129SvEv hybrid mouse strain developed by Lieberman and co-workers was used for these studies. Mice were generated and genotyped as previously described. Female C57BL/6/129SvEv-GGT deficient (−/−) mice and female wild-type (+/+ ) littermates 8 to 12 weeks old were used. The mice were fed autoclaved Autoclavable Rodent Laboratory Chow 5010 from Purina Mills (St. Louis, MO) and water ad libitum.

NAC Treatment

One week before the cisplatin treatment NAC was added to the drinking water of six of the GGT-deficient mice and six of the wild-type mice to supplement their cysteine levels. NAC (SigmaUltra grade; Sigma Chemical Company, St. Louis, MO) was dissolved in water at 10 mg/ml and the pH adjusted to 6.7. These mice were supplemented continuously with NAC throughout the experiment.

Cisplatin Treatment

One day before injection with cisplatin 10 GGT-deficient (−/−) mice and 12 wild-type (+/+ ) littermates were transferred to individual cages. Each cage was lined with Whatman 1 qualitative filter paper for the collection of urine. The mice were placed on a mesh (Alumax co-polymer gutter guard; Home Products, Lancaster, PA) that was suspended ~1 cm above the filter paper and through which the feces and urine passed to the filter paper below. Cisplatin (Platinol-AQ) was obtained as a sterile 1 mg/ml solution in 0.9% NaCl from Bristol-Myers Laboratories (Evansville, IN). Mice were injected intraperitoneally with 15 mg of cisplatin/kg of body weight. The dose of cisplatin was selected based on data from dose-response studies of the nephrotoxicity of cisplatin in C57BL/6 and 129 Ola mice. The filter paper was changed and collected at 2, 4, and 6 hours, and every 24 hours thereafter for a total of 5 days. Five days after injection the mice were weighed and sacrificed. Blood was obtained by heart puncture. The kidneys were removed and weighed. Half of each kidney was fixed for histology and half was stored frozen for platinum analysis.

Serum Assays

Serum was prepared and stored at −20°C. BUN and serum creatinine levels were determined in the clinical laboratory at Texas Children’s Hospital (Houston, TX), with a Vitros Analyzer (Ortho-Clinical Diagnostics Division of Johnson and Johnson Company, Rochester, NY). The serum creatinine concentration was calculated using the two-point rate test. The cut-off for the normal range of BUN (≤40 mg/dL) and serum creatinine values (≤0.2 mg/dL) was based on the values obtained previously for normal untreated C57BL/6 mice and for C57BL/6/129SvEv hybrids. Samples from five untreated C57BL/6 mice were included in the BUN and serum creatinine assays. Those samples were assayed with the samples from this experiment. None of the BUN or serum creatinine values for the C57BL/6 mice exceeded the cut-off values for the normal values.

Histology

Kidneys were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Histopathological analysis of the kidneys was performed by R.B. The samples were coded before analysis and read blind to prevent observer bias. Kidneys were assigned a score based on the extent of damage to the kidney as follows: 1, normal histology; 2, mild changes including dilation of the tubules, and small areas of necrosis observed in less than half the proximal tubules; 3, moderate changes including protein casts, desquamated epithelium, and evidence of necrosis in more than half the tubules; 4, severe changes similar to 3 but more severe including extensively desquamated tubules, necrotic tubules many containing cellular debris, protein casts, and inflammation.

Platinum Analysis

The feces were discarded. Filter papers and kidneys were stored at −80°C until analyzed. The filter papers and kidneys were hydrolyzed in nitric acid. Platinum levels were quantitated with a Hewlett Packard Model 4500 ICP-MS system (Hewlett Packard, Wilmington, DE) equipped with a crossflow-type nebulizer as previously described.

Statistics

Body weight of the mice after treatment with cisplatin, platinum excretion data, and platinum levels in the kidney were analyzed by two-way analysis of variance to identify significant differences related to GGT expression or NAC supplementation. Significant differences between groups in kidney damage (histopathology), BUN, and serum creatinine were detected with the Fisher exact test.
Figure 1. Cumulative urinary excretion of platinum from GGT-deficient and wild-type mice as a percentage of administered dose. All mice were injected with 15 mg/kg of cisplatin. The data show the cumulative percentage of the administered dose that was excreted throughout the course of the experiment. Data are shown for each of the four groups: unsupplemented GGT-deficient mice (filled circles), NAC-supplemented GGT-deficient mice (filled diamonds), unsupplemented wild-type mice (open circles), and NAC-supplemented wild-type mice (open diamonds). Analysis of the data by analysis of variance did not detect any statistically significant differences between the groups.

Results

Excretion of Platinum

Platinum excretion was analyzed at 2-hour intervals for the first 6 hours after injection and then at 24-hour intervals thereafter. In all four treatment groups the platinum excretion was rapid with most of the platinum excreted in the first 2 hours (Figure 1). The GGT-deficient mice supplemented with NAC excreted a higher percentage of the injected platinum than the other three groups. However, a two-way analysis of variance showed no statistically significant difference in platinum excretion that could be correlated with either mouse strain or NAC supplementation.

Two of the smallest GGT-deficient mice, not supplemented with NAC, died during the course of the experiment. A 6g mouse died on day 2 and a 9g mouse was found dead on day 5. Previous studies have shown that the GGT-deficient mice that are not supplemented with NAC begin to die at 10 weeks of age because of the cysteine-deficiency that develops in these mice. Data from these two mice were excluded from the analysis of cisplatin nephrotoxicity. No other mice died during the experiment.

Body Weight

The average body weights of mice at the start of the experiment and 5 days after cisplatin injection are shown in Table 1. There was a 10 to 14% decrease in the weight of all four groups of mice, but there was no statistically significant correlation between body weight change and mouse strain or NAC supplementation.

BUN and Serum Creatinine

The nephrotoxicity of cisplatin in the GGT-deficient and wild-type mice was assessed by BUN and serum creatinine levels in blood collected 5 days after cisplatin treatment. The GGT-deficient mice were smaller than the wild-type mice and we were unable to obtain a sufficient amount of blood for testing from two of the GGT-deficient mice supplemented with NAC. BUN levels >40 mg/dL and serum creatinine levels >0.2 mg/dL were scored as elevated. In all mice there was a perfect concordance in scoring nephrotoxicity with BUN and serum creatinine levels. Cisplatin was nephrotoxic to wild-type mice (Table 2). BUN and serum creatinine levels were elevated in four of six wild-type mice not supplemented with NAC. Both markers were elevated in six of six wild-type mice supplemented with NAC. In contrast, cisplatin was significantly less toxic to GGT-deficient mice (P < 0.05). Neither of the two unsupplemented GGT-deficient mice had elevated levels of BUN or serum creatinine after treatment with cisplatin. Among the GGT-deficient mice supplemented with NAC, only one of four that had sufficient serum for analysis had elevated levels of BUN and serum creatinine.

Table 1. Body Weight of Wild-Type and GGT-Deficient Mice Treated with Cisplatin

<table>
<thead>
<tr>
<th></th>
<th>Start weight (g)</th>
<th>End weight (g)</th>
<th>Starting weight, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (+/-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No supplement</td>
<td>18.5 ± 2.3</td>
<td>14.2 ± 1.8</td>
<td>77†</td>
</tr>
<tr>
<td>With NAC</td>
<td>19.3 ± 1.8</td>
<td>14.6 ± 0.89</td>
<td>76</td>
</tr>
<tr>
<td>GGT-deficient (-/-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No supplement</td>
<td>9.5 ± 2.1</td>
<td>8.6 ± 3.2</td>
<td>90</td>
</tr>
<tr>
<td>With NAC</td>
<td>13.4 ± 1.2</td>
<td>10.4 ± 3.0</td>
<td>78</td>
</tr>
</tbody>
</table>

†Values are mean ± SD.

Table 2. Nephrotoxicity of Cisplatin in Wild-Type and GGT-Deficient Mice

<table>
<thead>
<tr>
<th></th>
<th>Elevated BUN</th>
<th>Elevated serum creatinine</th>
<th>Renal tubular necrosis (histology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (+/-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No supplement</td>
<td>4/6</td>
<td>4/6</td>
<td>4/6†</td>
</tr>
<tr>
<td>With NAC</td>
<td>6/6</td>
<td>6/6</td>
<td>4/6†</td>
</tr>
<tr>
<td>With and without NAC</td>
<td>10/12</td>
<td>10/12</td>
<td>8/12</td>
</tr>
<tr>
<td>GGT-deficient (-/-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No supplement</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>With NAC</td>
<td>1/4‡</td>
<td>1/4‡</td>
<td>0/6</td>
</tr>
<tr>
<td>With and without NAC</td>
<td>1/6‡</td>
<td>1/6‡</td>
<td>0/8‡</td>
</tr>
</tbody>
</table>

‡Statistically significant differences between wild-type and GGT-deficient mice were detected with a Fisher Exact Test.
creatinine. GGT-deficiency correlated with protection from cisplatin-induced nephrotoxicity in the mice supplemented with NAC ($P < 0.05$). When the data from both the NAC supplement mice and nonsupplemented mice were combined, statistical analysis again showed GGT-deficient mice had significantly less nephrotoxicity than the wild-type mice ($P < 0.05$).

**Renal Histopathology**

H&E-stained sections of the kidneys were assessed for evidence of renal damage. Five days after treatment with cisplatin, the proximal tubules in the wild-type mice showed severe damage in the corticomedullary region (Figure 2A). Many tubules were dilated and the epithelium desquamated. Cellular debris was observed in damaged tubules. Protein casts, an indicator of tubular damage, were also present. The data from the histological analysis correlated with renal damage as detected by elevated BUN and serum creatinine (Table 2). However, renal damage detected by BUN and serum creatinine in two NAC-supplemented wild-type mice was not evident by histological analysis of the kidney. The effect of GGT was apparent in comparing the renal histology of the GGT-deficient mice treated with cisplatin to the wild-type mice. The kidneys of the GGT-deficient mice appeared normal 5 days after treatment with cisplatin (Figure 2B). Tubular necrosis was not observed in any of the GGT-deficient mice, nor were protein casts present. The number of wild-type mice with histologically identified toxic renal lesions (8 of 12) was significantly greater than the number of GGT-deficient mice (0 of 8, $P < 0.01$).

**Platinum Accumulation in the Kidneys**

The renal platinum concentration for each mouse is shown in Figure 3. The platinum concentration in the kidney of the unsupplemented wild-type mice was $16.4 \pm 8.0$ $\mu$g platinum/g kidney and $12.8 \pm 6.3$ for the NAC-supplemented group. The unsupplemented GGT-deficient mice had $7.6 \pm 3.7$ $\mu$g platinum/g kidney and the NAC-supplemented GGT-deficient mice had $8.8 \pm 3.9$ $\mu$g platinum/g kidney. The higher mean platinum concent-
trations in the wild-type groups were the result of three mice with platinum concentrations >20μg/g. Analysis by two-way analysis of variance did not detect any significant difference in renal platinum concentration because of either GGT expression or NAC supplementation.

Discussion

This study was designed to test the hypothesis that GGT activity is necessary for the nephrotoxicity of cisplatin. We evaluated drug excretion, renal toxicity, platinum accumulation, and weight loss in wild-type and GGT-deficient mice treated with cisplatin. The data showed that cisplatin was more nephrotoxic in wild-type mice than in GGT-deficient mice. Significantly more wild-type mice had elevated BUN, elevated serum creatinine, and renal tubular necrosis than the GGT-deficient mice. However, GGT did not affect the excretion of cisplatin, the accumulation of platinum in the kidney, or cisplatin-induced weight loss.

GGT has been found to be an essential component of the metabolic activation of a series of halogenated alkenes and quinones to nephrotoxins. These compounds form glutathione-conjugates that are hydrolyzed to cysteinylyl-glycine-conjugates by GGT on the surface of the renal proximal tubule cells. The conjugates are further cleaved to cysteine-conjugates by aminopeptidase which is also on the surface of the cell. The cysteine-conjugates are taken up into the cell and converted to a reactive thiol by either cysteine-conjugate β-lyase or S-oxidase. Studies of the nephrotoxic halogenated alkene, trichloroethene, show that <0.01% of the drug is metabolized to the toxic form. Therefore, blocking this pathway would not result in significant differences in drug excretion or drug accumulation in the tissue.

We have proposed that the nephrotoxicity of cisplatin is the result of the metabolism of a small portion of the drug through the same pathway that activates halogenated alkenes. Two groups of investigators have identified cisplatin-glutathione-conjugates that form spontaneously in solution. Intracellular platinum-glutathione-conjugates have been isolated from tissue culture cells treated with cisplatin. In vitro studies in our laboratory have shown that cisplatin-glutathione-conjugates, cisplatin-cysteinyl-glycine-conjugates, and cisplatin-cysteine-conjugates are all more toxic to proximal tubule cells than cisplatin. We have also shown that inhibition of cysteine-conjugate β-lyase by aminooxyacetic acid (AOAA) inhibits the nephrotoxicity of cisplatin in mice.

Cisplatin is excreted in the urine. The excretion of cisplatin was similar in wild-type and GGT-deficient mice. Approximately 20% of the platinum was excreted within the first 2 hours after administration with little additional platinum excreted throughout the subsequent 5 days. These data are consistent with those reported for excretion of the nephrotoxic halogenated alkenes and quinones. Lau and Monks found that inhibiting GGT activity with acivicin blocked the nephrotoxicity of 2-bromohydroquinone but did not alter urinary excretion of the compound.

In the clinic there is considerable variation among patients in the amount of kidney damage induced by treatment with cisplatin. There is no way to predict which patients will be most susceptible to cisplatin-induced renal damage. This variability may reflect differences between patients in the activity of enzymes involved in the renal metabolism of the drug.

The levels of platinum accumulation in the kidney and weight loss in the mice did not differ significantly between the wild-type and GGT-deficient mice. Treatment with acivicin also inhibits the nephrotoxicity without altering renal platinum accumulation. Our studies in rats showed that acivicin did not prevent reduced food consumption and a significant cisplatin-induced weight loss in the 5 days after treatment.

In this study we have shown that expression of GGT is necessary for the nephrotoxicity of cisplatin, but expression of GGT alone is not sufficient. Expression of GGT in the tumor cells does not alter the response of the tumor to cisplatin. The downstream enzymes in the pathway must also be present. The renal form of cysteine-conjugate β-lyase, the last enzyme in the proposed pathway, is expressed only in the proximal tubules. The localized co-expression of these enzymes in the proximal tubules provides an explanation for the specificity of cisplatin toxicity toward these cells.

Our data may have important implications for the prevention of cisplatin-induced nephrotoxicity and for the design of new platinum-based drugs. Defining this metabolic pathway provides new strategies for inhibiting the nephrotoxicity of cisplatin. This new paradigm will also aid in the design of the next generation of platinum-based drugs by identifying the structural motifs of substrates in this pathway, and eliminating those motifs in new drugs under development.

Acknowledgments

We thank Donna Atwood and Andy Bahler for assistance with the animal studies and Chris Prater with the platinum analysis.

References