LABORATORY STUDY

Amelioration of cisplatin-induced acute kidney injury by recombinant neutrophil gelatinase-associated lipocalin

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ABSTRACT

We investigated the protective effect and mechanism of neutrophil gelatinase-associated lipocalin (NGAL) in a murine model of cisplatin-induced nephrotoxicity. Male Swiss-Webster mice were assigned to four groups (n = 10 in each group). Control mice received vehicle only. Mice in the experimental group were given a single intraperitoneal injection of cisplatin (20 mg/kg) to induce nephrotoxicity, and were divided into three groups. The first group received 100 μL of saline only via tail vein at the time of cisplatin administration. The second group was given biologically active recombinant NGAL via tail vein (250 μg/100 μL solution). The third group was injected with a 250 μg/100 μL solution of inactivated NGAL. After 4 days, we measured serum creatinine and urinary N-acetyl-β-D-glucosaminidase (NAG), and performed histologic studies. Biologically active NGAL significantly blunted the rise in serum creatinine (NGAL plus cisplatin 1.33 ± 0.31 versus cisplatin alone 2.43 ± 0.31 mg/dL, p < .001) as well as the increase in urine NAG (NGAL plus cisplatin 60.7 ± 14.2 versus cisplatin alone 120.5 ± 22.5 units/gm creatinine, p < .005). In addition, NGAL conferred a marked reduction in tubule cell necrosis and apoptosis (NGAL plus cisplatin 6.9 ± 1.2 versus cisplatin alone 15.1 ± 3.4 TUNEL positive nuclei per 100 cells, p < .001). These beneficial effects were completely abolished when heat-inactivated NGAL was administered instead of the biologically active form. Since induction of NGAL in kidney tubules is a known physiologic response to cisplatin, the pharmacologic use of NGAL to prevent cisplatin nephrotoxicity is likely to be safe and effective.

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Introduction

Cisplatin remains one of the most widely used chemotherapeutic agents for the treatment for many solid organ malignancies since its introduction more than 30 years ago. The anti-neoplastic efficacy of cisplatin is dose dependent, but the risk of nephrotoxicity frequently hinders the use of higher doses to maximize its therapeutic effects. Cisplatin nephrotoxicity presents most commonly with electrolyte abnormalities. However, the most serious manifestation is acute kidney injury (AKI), which occurs in about 30% of adults1–3 and up to 70% of children who receive cisplatin4 despite nephro-protective approaches such as vigorous hydration. Administered cisplatin is freely filtered at the glomerulus and rapidly accumulates in the kidney, mediated by specific transporters that are highly expressed in proximal tubule cells such as the organic cation transporters OCT1 and OCT2.7 Mechanisms leading to subsequent AKI are complex and involve several cellular processes, including cell death by apoptosis and necrosis, oxidative stress, and inflammation.6

Attempts at unraveling the molecular mechanisms of AKI have been vastly accelerated by innovative technologies such as functional genomics, bioinformatics, and proteomics.7 Genes and gene products that are differentially regulated have uncovered novel noninvasive biomarkers of AKI and illuminated pathways for targeted therapies.8 Transcriptome profiling data have consistently identified neutrophil gelatinase-associated lipocalin (NGAL) as one of the most commonly upregulated genes in the kidney in a variety of AKI models.9,10 Downstream proteomic analyses also revealed NGAL to be one of the most highly induced proteins in the kidney after ischemic AKI in animal models.11–13 The consistent finding that NGAL protein is easily detected in the urine and plasma soon after AKI has inspired a number of translational studies to evaluate NGAL as a noninvasive biomarker in human ischemic AKI.14–17 In addition, the distribution of NGAL protein in tubule epithelial cells that are undergoing proliferation in animal11–13 and human18 models of ischemic AKI has prompted the investigation of NGAL as a novel...
therapeutic agent in ischemic AKI. Indeed, several preclinical studies have now established NGAL for the amelioration of ischemic AKI.12,19–21

Gene expression profiling has also identified NGAL to be consistently upregulated in animal models of cisplatin nephrotoxicity.22,23 NGAL protein is dramatically upregulated in proximal tubule cells within 3 h of cisplatin injection in animals that develop AKI as evidenced by an increase in serum creatinine 4 days later.24 Importantly, urine NGAL is now also emerging as an early, sensitive and specific noninvasive biomarker for cisplatin-induced AKI in humans.25 However, the possible role of NGAL in ameliorating cisplatin nephrotoxicity has not been investigated. Establishing the biologic role of NGAL will add plausibility towards its proposed utility as a biomarker of cisplatin nephrotoxicity. We therefore tested the hypothesis that NGAL plays a nephro-protective role in cisplatin-induced nephrotoxicity. We examined the ability of recombinant NGAL to confer structural and functional protection in an established model of cisplatin-induced AKI.

Materials and methods

Recombinant murine NGAL

Full-length mouse NGAL cDNA was cloned into the pGEX expression vector, expressed as a fusion protein with glutathione S-transferase (GST) in Escherichia coli, and purified using glutathione-Sepharose columns (GE Healthcare Biosciences, Pittsburgh, PA) followed by thrombin cleavage as previously described.24 Purified NGAL was made endotoxin-free using the Detoxi-Gel endotoxin removing column (Thermo Scientific, Rockford, IL). Protein concentrations were determined using the Bradford assay.

Mouse model of cisplatin nephrotoxicity

The study was approved by the Institutional Animal Care and Use Committee (IACUC). We employed a well-established murine model in which the structural and functional consequences of cisplatin-induced nephrotoxicity have been previously documented.11,12,19,24 Briefly, male Swiss-Webster mice (Taconic Farms, Germantown, NY) weighing 25 g were housed with 12:12 light:dark cycle with free access to food and water. Control mice (n = 10) were injected with vehicle only. Mice in the experimental group were given a single intraperitoneal injection of cisplatin (Sigma-Aldrich, catalog #P4394, St. Louis, MO), in the dose of 20 mg/kg body weight. In preliminary studies, a lower dose (5 mg/kg body weight) did not result in structural evidence for nephrotoxicity, and the higher dose of 20 mg/kg was used for all subsequent experiments. Mice receiving cisplatin were divided into three groups (n = 10 in each group). The first group received 100 µL of saline only via tail vein injection at the time of cisplatin administration. The second group was given biologically active NGAL via tail vein in the dose of 250 µg/100 µL solution. In the preliminary studies, smaller doses of recombinant NGAL (50–100 µg/100 µL solutions) were not effective in the cisplatin nephrotoxicity model. We have also previously shown that intravenously administered NGAL is rapidly taken up by both the proximal and distal tubule cells, and rapidly appears in the urine (within 1 h of injection).19 The third group was injected with a 250 µg/100 µL solution of inactivated NGAL (inactivation achieved by boiling the solution for 15 minutes). At the end of 4 days, animals were placed in metabolic cages for urine collection. Mice were then anesthetized with triple sedative (ketamine, xylazine, and acepromazine), the abdominal cavity opened, and blood obtained via inferior vena cava puncture for measurement of serum creatinine using a quantitative colorimetric assay (Sigma-Aldrich, St. Louis, MO). The mice were sacrificed, the kidneys perfusion fixed in situ with 4% paraformaldehyde in PBS, and both kidneys harvested. Kidneys were then fixed in formalin, paraffin-embedded, and subjected to microscopy.

Microscopy

Paraffin-embedded kidneys from each of the three groups (control, cisplatin, cisplatin plus NGAL, n = 10 from each group) were sectioned at 4 µm, and stained with hematoxylin and eosin. Slides were scored for histopathologic damage in a blinded manner by one investigator (PD), as previously described.19 For each slide, we assessed three parameters (tubule dilatation, tubule cast formation, and tubule cell necrosis) in five high power fields (×40), and an average of the five fields determined for each section. Each parameter was scored on a scale of 0-4, ranging from non (0), mild (1), moderate (2), severe (3), and extensive/very severe (4). Comparisons were made to the control group, which showed none or only very mild degrees of tubule cell necrosis, tubular dilatation, and cast formation. Kidney sections were also subjected to the TUNEL assay to detect apoptotic cells using the ApoAlert DNA Fragmentation Assay Kit (Clontech, La Jolla, CA) as previously described.19 Slides were visualized with a fluorescence microscope in a blinded manner. Only cells that displayed the characteristic morphology of apoptosis (nuclear fragmentation, nuclear condensation, fluorescent nuclei) were counted as apoptotic.
Apoptosis was quantified by counting the number of apoptotic nuclei per 100 cells counted in an average of five high-power fields (×40) in each section.

**Urine measurements**

Urine samples were centrifuged at 1500 g to remove debris, and the supernatant was analyzed. We employed a colorimetric assay for the measurement of N-acetyl-β-D-glucosaminidase (NAG) (Roche Applied Science, catalog # 10875406001, Mannheim, Germany). Urine NAG has previously been shown to represent an early sensitive noninvasive assay for cisplatin nephrotoxicity that precedes the increase in serum creatinine.11,24

Urinary creatinine was measured by quantitative colorimetric assay (Sigma-Aldrich, St. Louis, MO).

**Statistical analysis**

The SPSS software was used to generate univariate statistics, including means, SD, distributions, range, and skewness. Unpaired t-test and one-way ANOVA were used as appropriate to compare means ± SD of continuous variables among different groups. The Kruskal–Wallis ANOVA on ranks was used for non-normally distributed data. To identify differences between groups, we used a multiple comparison procedure (Tukey test or Dunn’s method, depending on the normality of distribution). p < .05 was considered statistically significant.

**Results**

**Cisplatin results in structural and functional kidney injury**

We reproduced a well-established murine model in which the structural and functional consequences of cisplatin-induced nephrotoxicity have been previously documented.11,12,19,24 When compared with untreated controls, mice treated with intraperitoneal cisplatin in the dose of 20 mg/kg body weight displayed a significant increase in serum creatinine (cisplatin 2.43 ± 0.32 versus controls 0.54 ± 0.11 mg/dL, p < .001, Figure 1(A)) and urine NAG (cisplatin 120.5 ± 22.5 versus controls 41.7 ± 4.3 units/gm creatinine, p < .001, Figure 1(B)) at the end of 4 days. Histologic examination of the kidneys following hematoxylin–eosin staining showed clear evidence for nephrotoxic injury (Figure 2, top panel), including tubule cell necrosis (N), tubular dilatation (D), and intra-tubular casts (C), in the cisplatin-treated kidneys, as previously described.24 Only minimal changes were recognized in control kidneys.

**NGAL ameliorates the functional and structural consequences of cisplatin**

The administration of intravenous biologically active NGAL (250 µg/100 µL solution) at the time of cisplatin administration significantly blunted the rise in serum creatinine (NGAL plus cisplatin 1.33 ± 0.31 versus cisplatin alone 2.43 ± 0.31 mg/dL, p < .001, Figure 1(A)) as well as the increase in urine NAG (NGAL plus cisplatin 60.7 ± 14.2 versus cisplatin alone 120.5 ± 22.5 units/gm creatinine, p < .005, Figure 1(B)). In addition, NGAL conferred significant structural protection (Figure 2, top panel), as shown by the marked reduction in tubule cell necrosis (Figure 3(A)), tubule dilatation (Figure 3(B)), and intra-tubular cast formation (Figure 3(C)). These beneficial effects were completely abolished when heat-inactivated NGAL was administered instead of the biologically active form.

**Cisplatin results in tubule cell apoptosis**

Histologic examination of the kidneys following hematoxylin–eosin staining showed evidence for tubular

![Figure 1](image-url)

**Figure 1.** Serum creatinine (mg/dL), (A) and urinary NAG (units/gm creatinine, (B)). Cis, cisplatin (20 mg/kg). Cis/NGAL, cisplatin + active NGAL (250 µg/100 µL). Cis/Vehicle, cisplatin + inactive NGAL. N = 10 in each of the four groups. *p < .001 versus control. #p < .001 versus cisplatin alone.
Figure 2. Kidney sections stained with hematoxylin and eosin (H & E) or Tunel. Cis, cisplatin (20 mg/kg). Cis/NGAL, cisplatin + active NGAL (250 μg/100 μL). “N” refers to necrotic debris, “D” shows tubular dilatation, “C” represents tubule cell casts, and “A” denotes condensed nuclei characteristic of apoptosis. Representative pictures from $n=10$ in each of the four groups.

Figure 3. Necrosis score (A), tubule dilatation score (B) and tubule cast score (C) were assessed in hematoxylin–eosin stained sections and scored on a scale of 0 to 4, ranging from none (0) to severe. Apoptosis was quantified by counting the number of tunel-positive nuclei per 100 cells in an average of five high-power fields (D). Cis, cisplatin (20 mg/kg). Cis/NGAL, cisplatin + active NGAL (250 μg/100 μL). Cis/Vehicle, cisplatin + inactive NGAL. $N=10$ in each of the four groups. *$p<.001$ versus control. #$p<.001$ versus cisplatin alone.
epithelial cells undergoing programmed cell death, indicated by condensed intensely-stained nuclei (Figure 2, top panel, label A). This was confirmed and quantified by TUNEL assay, which showed the condensed and fragmented nuclei characteristic of apoptosis (Figure 2 bottom panel). Only minimal amounts of apoptosis were detectable in the untreated control kidneys (controls 1.3 ± 1.1 versus cisplatin 15.1 ± 3.4 TUNEL positive nuclei per 100 cells counted, p < .001).

**NGAL ameliorates the tubule cell apoptosis induced by cisplatin**

The administration of intravenous biologically active NGAL (250 μg/100 μL solution) at the time of cisplatin administration significantly blunted the appearance of TUNEL-positive apoptotic tubule cells (NGAL plus cisplatin 6.9 ± 1.2 versus cisplatin alone 15.1 ± 3.4 TUNEL positive nuclei per 100 cells counted, p < .001, Figure 2 and 3D). This effect was lost when heat-inactivated NGAL was administered instead of the biologically active form.

**Discussion**

In this study, recombinant NGAL partially ameliorated the functional and structural nephrotoxic consequences of cisplatin administration in a murine model. NGAL prevented the increase in serum creatinine and urine NAG, and reduced the occurrence of tubule cell necrosis and apoptosis. The salutary effect of NGAL was abolished by heat inactivation, attesting to the specificity of the biologically active form in mediating the protective effect.

The major mechanisms proposed in cisplatin nephrotoxicity include tubule cell death by apoptosis and necrosis. The present study indicates that the mechanism underlying NGAL’s protective effect is based at least in part on its ability to ameliorate tubule cell apoptosis and necrosis. NGAL’s well-known iron chelating properties may bestow protection by removing toxic iron from the extracellular space, and delivering iron to viable cells and thereby limiting apoptosis, promoting proliferation, and enhancing recovery.

NGAL has also been shown to possess anti-inflammatory and immunomodulatory properties. It is therefore likely that NGAL has a protective role in cisplatin nephrotoxicity via its anti-oxidative and anti-inflammatory properties. Support for this notion derives from *in vitro* studies that have demonstrated that oxidative stress can induce NGAL gene expression, and that NGAL confers a cyto-protective role against cisplatin toxicity via free radical scavenging.

Molecular support for the proposed role of NGAL may derive from the fact that the transcription factor NF-κB is one of the primary positive regulators of NGAL expression. Multiple studies have demonstrated the early activation (phosphorylation) of NF-κB in kidneys in cisplatin nephrotoxicity, with resultant induction of renal epithelial cell apoptosis. However, contrary to the findings reported herein with NGAL administration, inhibition of apoptosis alone by other maneuvers such as pan-caspase inhibition did not confer functional protection against cisplatin-induced nephrotoxicity.

Given the myriad roles of NF-κB, it is likely that the physiological induction of NGAL expression driven by NF-κB alone may be insufficient. Indeed, a large pharmacologic dose of NGAL was required to confer structural and functional protection (refer to the work by Mishra et al. and this study).

One limitation of this study is the fact that only a preventive role for NGAL was examined, since it was administered concomitant with cisplatin. It is not known whether NGAL given after cisplatin-induced nephrotoxicity is established would also be effective. However, in clinical practice, initiation of cisplatin therapy is usually a planned and predictable event, and the potential availability of a preventive strategy is pertinent. A second limitation is that the detection and quantitation of apoptosis reported herein was limited to microscopy and Tunel assay. The underlying mechanisms, and the apoptotic pathways involved, were not explored. A third limitation is that the possible role of NGAL in modulating processes other than apoptosis and necrosis that are germane to cisplatin-induced kidney injury (such as necroptosis and autophagy) were not examined.

In summary, pharmacologic doses of recombinant NGAL partially prevented the functional and structural nephrotoxic consequences of cisplatin administration in a murine model. NGAL prevented the increase in serum creatinine and urine NAG, and reduced tubule cell necrosis and apoptosis. Since induction of NGAL in kidney tubules is a known physiologic response to cisplatin, the pharmacologic use of NGAL to prevent cisplatin nephrotoxicity is likely to be safe and effective.

**Disclosure statement**

PD is a co-inventor on submitted patents for the use of NGAL as a biomarker of kidney injury. The other authors report no conflicts of interest.

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