# Amelioration of Ischemic Acute Renal Injury by Neutrophil Gelatinase–Associated Lipocalin

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*Abstract.* Acute renal failure secondary to ischemic injury remains a common problem, with limited and unsatisfactory therapeutic options. Neutrophil gelatinase–associated lipocalin (NGAL) was recently shown to be one of the maximally induced genes early in the postischemic kidney. In this study, the role of NGAL in ischemic renal injury was explored. Intravenous administration of purified recombinant NGAL in mice resulted in a rapid uptake of the protein predominantly by proximal tubule cells. In an established murine model of renal ischemia-reperfusion injury, intravenous NGAL administered before, during, or after ischemia resulted in marked amelioration of the morphologic and functional consequences, as evidenced by a significant decrease in the histopathologic damage to tubules and in serum creatinine measurements. NGAL-treated animals also displayed a reduction in the number of apoptotic tubule cells and an increase in proliferating proximal tubule cells after ischemic injury. The results indicate that NGAL may represent a novel therapeutic intervention in ischemic acute renal failure, based at least in part on its ability to tilt the balance of tubule cell fate toward survival.

Acute renal failure (ARF) secondary to ischemic injury remains a common and potentially devastating problem in clinical nephrology, with a persistently high rate of mortality despite significant advances in supportive care (1–4). Pioneering studies over several decades have illuminated the roles of persistent vasoconstriction, tubular obstruction, cellular structural and metabolic alterations, and the inflammatory response in the pathogenesis of ARF (4–7). Although these studies have paved the way for successful therapeutic approaches in animal models, translational research efforts in humans have yielded disappointing results (2–4). The reasons for this may include the multifaceted response of the kidney to ischemia and a lack of early markers for ARF (4–8). Recent advances in cellular and molecular biology of ischemic renal injury have revealed that proximal tubule cells undergo a complex temporal sequence of events. These include loss of cell polarity, cell death as a result of apoptosis and necrosis, dedifferentiation and proliferation of viable cells, and reestablishment of the epithelial phenotype (6,7). An improved understanding of the early cell injury and repair mechanisms is critical for innovative and effective therapy. Identification of interventions that may oppose tubule cell death and/or enhance the recovery phase therefore is of considerable interest.

Attempts to unravel the molecular basis of the myriad early renal responses have been facilitated by recent advances in functional genomics that have yielded new tools for genome-wide analysis of complex biologic processes such as ischemic ARF (8–11). Using cDNA microarray techniques, we recently identified neutrophil gelatinase–associated lipocalin (NGAL) as one of the most dramatically induced transcripts in the kidney early after ischemic injury (11,12). Although previous studies have indicated that NGAL may represent a novel early urinary biomarker for ischemic renal injury (12), the role of NGAL in the kidney has remained puzzling. We showed previously that in the postischemic kidney, NGAL is upregulated in tubular epithelial cells that are undergoing proliferation (12). Other recent studies have suggested that NGAL can enhance the epithelial phenotype. We therefore tested the hypothesis that NGAL may play a renoprotective role in ischemic ARF. In this study, we examined the ability of intravenously administered recombinant NGAL to modify the structural and functional consequences of ischemic acute renal injury in an established murine model. Our results indicate that NGAL may represent a novel therapeutic intervention in ischemic ARF, based at least in part on its ability to ameliorate tubule cell apoptosis and enhance tubule cell proliferation.

## Materials and Methods

### *Expression and Purification of 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* (XL1-Blue), and purified using

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glutathione-Sepharose columns (Amersham Biosciences) followed by thrombin cleavage as described previously (13–15). Purified NGAL was made endotoxin-free using the Detoxi-Gel endotoxin removing column (Pierce) as recommended by the manufacturer. Because of the remote possibility of low levels of endotoxin contamination, an "ultra-pure" batch of NGAL was prepared using an additional gel filtration column (Superdex-75, SMART system; Amersham, Arlington Heights, IL). Proteins were analyzed by SDS-PAGE followed by Coomassie blue staining or by Western blotting with a polyclonal antibody to NGAL as described (12). Protein concentrations were determined using the Bradford assay.

### *Mouse Models of Renal Ischemia-Reperfusion Injury*

We used well-established murine models in which the structural and functional consequences of brief periods of renal ischemia have been previously documented (11,12,15). Briefly, male Swiss-Webster mice (Taconic Farms, Germantown, NY) that weighed 25 to 30 g were housed with 12:12-h light:dark cycle and were allowed free access to food and water. The animals were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and placed on a warming table to maintain a rectal temperature of 37°C. Both renal pedicles were occluded with a nontraumatic vascular clamp for 30 min, during which time the kidney was kept warm and moist. The clamps were then removed, the kidney was observed for return of blood flow, and the incision was sutured. The mice were allowed to recover in a warmed cage, and timed urine collections were obtained. After various reperfusion periods, the animals were re-anesthetized, the abdominal cavity was opened, and blood was obtained *via* puncture of the inferior vena cava for measurement of serum creatinine by quantitative colorimetric assay kit (Sigma, St. Louis, MO). The mice were killed, the kidneys were perfusion fixed *in situ* with 4% paraformaldehyde in PBS, and both kidneys were harvested. One half of each kidney was snap-frozen in liquid nitrogen and stored at −70°C until further processing; a sample was fixed in formalin, paraffin-embedded, and sectioned (4 $\mu$m). Paraffin sections were stained with hematoxylin-eosin and examined histologically. The other half of each kidney was embedded in OCT compound (Tissue-Tek), and frozen sections (4 $\mu$m) were obtained for immunohistochemistry.

### *NGAL Injections*

Purified endotoxin-free NGAL was administered intravenously into mice *via* tail-vein injections. In preliminary studies, animals were treated with three different concentrations of NGAL (50, 100, or 250 $\mu$g of a 250 $\mu$g/100 $\mu$l solution), subjected to 30 min of bilateral renal artery clamping 1 h later, and examined after 24 h of reflow. When compared with animals that were pretreated with an equal volume (100 $\mu$l) of saline, the group that was given 250 $\mu$g of NGAL exhibited the best protection from the tubular damage and azotemia. In addition, when NGAL (250 $\mu$g) that was inactivated by boiling the preparation for 10 min was infused, no renoprotective effect was noted. Also, no differences in renoprotection were encountered in the renal response to the NGAL batch rendered endotoxin-free using Detoxi-gel in comparison with the "ultra-pure" batch of NGAL that was further purified by Superdex gel filtration. Furthermore, other unrelated proteins prepared in this manner were devoid of a renoprotective effect (data not shown), ruling out the remote possibility of contaminating endotoxins' mediating the response observed to injected NGAL. All subsequent studies as reported here were carried out using the 250-$\mu$g dose of endotoxin-free biologically active NGAL.

Comparisons were made between five different animal groups: nonischemic controls ($n = 8$), ischemic controls that were pretreated with 100 $\mu$l of saline alone ($n = 8$), NGAL pretreated 1 h before renal artery clamping ($n = 6$), NGAL treated during renal artery clamping ($n = 6$), and NGAL treated 1 h after renal artery clamping ($n = 6$).

### *NGAL Immunohistochemistry*

For NGAL detection, frozen kidney sections were permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with goat serum for 1 h, and incubated with primary antibody to NGAL (1:500 dilution) for 1 h. Slides were then exposed for 30 min in the dark to secondary antibodies conjugated with Cy5 (Amersham) and visualized with a fluorescence microscope (Zeiss Axiophot) equipped with rhodamine filters.

### *Histopathology Scoring*

Kidney sections of 4 $\mu$ were stained with hematoxylin-eosin and scored for histopathologic damage to the tubules in a blinded manner, as described previously (16,17). Each parameter was assessed in five high-power fields (×40) in the inner cortex and outer medullary regions (where the tubular damage was most evident), and an average was determined for each section. The parameters included tubule dilation, tubule cast formation, and tubule cell necrosis. Each parameter was scored on a scale of 0 to 4, ranging from none (0), mild (1), moderate (2), severe (3), to very severe/extensive (4).

### *Apoptosis Assays*

For the transferase-mediated dUTP nick-end labeling (TUNEL) assay to detect apoptotic nuclei, we used the ApoAlert DNA Fragmentation Assay Kit (Clontech). Paraffin sections were deparaffinized through xylene and descending grades of ethanol, fixed with 4% formaldehyde/PBS for 30 min at 4°C, permeabilized with proteinase K at room temperature for 15 min and 0.2% triton X-100/PBS for 15 min at 4°C, and incubated with a mixture of nucleotides and TdT enzyme for 60 min at 37°C. The reaction was terminated with 2× SSC, and the sections were washed with PBS and mounted with Crystal/mount (Biomeda, Foster City, CA). TUNEL-positive apoptotic nuclei were detected by visualization with a fluorescence microscope. Only cells that displayed the characteristic morphology of apoptosis, including nuclear fragmentation, nuclear condensation, and intensely fluorescence nuclei by TUNEL assay, were counted as apoptotic. Merely TUNEL-positive cells, in the absence of morphologic criteria, were not considered apoptotic. Slides were examined in a blinded manner, and apoptosis was quantified by counting the number of TUNEL-positive nuclei per 100 cells counted in an average of five high-power fields (×40) in each section.

### *Proliferation Assays*

For detection of proliferating cells, sections were incubated with an mAb to proliferating cell nuclear antigen (PCNA; 1:500 dilution; Upstate Biotechnology), and detection was accomplished by immunoperoxidase staining as recommended by the manufacturer (ImmunoCruz Staining System, Santa Cruz Biotechnology). Slides were examined in a blinded manner, and proliferation was quantified by counting the number of PCNA-positive cells per 100 cells counted in an average of five high-power fields (×40) in each section.

### *Statistical Analyses*

The SPSS software (version 8.0) was used to generate univariate statistics for each continuous variable, including means, SD, distributions, range, and skewness. The data were examined for normality and equality of distribution. One-way ANOVA was used to compare

means ± SD of continuous variables among different treatment groups. The Kruskal-Wallis ANOVA on ranks was used for nonnormally distributed data. To identify the group or groups that differed from the others, we used a multiple comparison procedure (Tukey test or Dunn's method, depending on the normality of distribution). $P < 0.05$ was considered statistically significant.

## Results

### *Expression and Purification of Recombinant Murine NGAL*

To begin exploring the role of NGAL, the peptide was expressed as a GST fusion protein in *Escherichia coli* (XL1-Blue), purified using a bioaffinity column, and cleaved with thrombin to yield the recombinant protein as described previously (13–15). Proteins were analyzed by Coomassie blue staining and by Western blotting with a polyclonal antibody to NGAL as described (12). A single clean polypeptide of the predicted size was detected by both techniques, as shown in Figure 1.

### *Intravenous NGAL Is Rapidly Taken up by Tubule Epithelial Cells In Vivo*

It was next of importance to ascertain whether purified NGAL can be delivered to its putative site of action, namely the tubular epithelial cells. Mice received intravenous NGAL (250 μg in 100 μl of saline) or an equal volume of saline alone, and the kidneys and urine were examined at various time periods. Animals that received saline were devoid of kidney or urinary NGAL. In contrast, within 1 h of NGAL injection, it was easily detected in a punctate cytoplasmic distribution predominantly in the proximal tubules but also to a lesser extent in the distal tubules, as shown in Figure 2. Identification of proximal *versus* distal tubules in these sections was based on location and morphology. In addition, NGAL was detected in the urine within 1 h of injection, as shown in Figure 3. These results confirm that exogenously administered NGAL is very rapidly concentrated in the kidney and taken up by tubule cells.

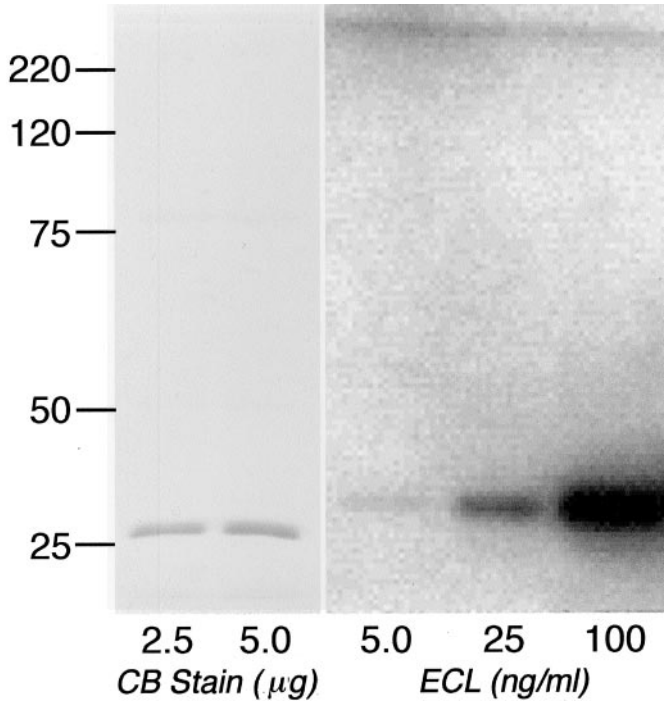


*Figure 1.* Expression and purification of recombinant murine neutrophil gelatinase–associated lipocalin (NGAL). Coomassie Blue (CB) and enhanced chemiluminescence (ECL; with polyclonal NGAL antibody) analysis of defined quantities (as shown) of recombinant purified NGAL. A single clean polypeptide of the predicted size was detected.

### *Intravenous NGAL Is Rapidly Taken up by Tubule Epithelial Cells after Ischemic Injury*

It was next of interest to determine whether purified NGAL can be delivered to the tubular epithelial cells after ischemic injury. Mice received intravenous NGAL (250 μg in 100 μl of saline) or an equal volume of saline alone and were subjected to ischemia-reperfusion injury, and the kidneys and urine were examined at various time periods. Animals that received saline were devoid of kidney or urinary NGAL at the 1-h reflow period, and NGAL was just detectable at the 3-h reflow period, as shown in Figures 2 and 3, respectively. The 3-h data represent the endogenous response of kidney tubule cells to ischemic injury, as previously reported (12). In contrast, in animals that received an injection of NGAL and were simultaneously subjected to ischemia-reperfusion injury, NGAL was easily detected in the kidney and urine with 1 h of reflow, as shown in Figures 2 and 3. This represents the rapid uptake of injected NGAL after ischemic injury, because NGAL was not detected at the 1-h reflow period in animals that received saline.

### *NGAL Ameliorates the Histopathologic Damage to Tubules Induced by Ischemia-Reperfusion Injury*

Having established that exogenously administered NGAL can be delivered to the ischemic kidney, we next wished to determine the structural consequences of this intervention. In an established murine model of renal ischemia-reperfusion injury, NGAL administered 1 h before, during, or even 1 h after ischemia resulted in a significant decrease in the histopathologic damage to tubules. Representative kidney sections obtained at 24 h of reflow and stained with hematoxylin-eosin are shown in Figure 4. Whereas the nonischemic controls displayed normal histology, animals that were pretreated with saline alone (100 μl, volume of diluent) displayed extensive features of acute tubular necrosis as described previously (11,12,15), including tubular dilation, tubular cast formation, and necrotic cells. In contrast, NGAL-treated kidneys displayed an attenuated histopathologic response. This was most evident in animals that were pretreated with NGAL but was also evident when the NGAL was administered during or even 1 h after the ischemic injury. To quantify this response, we scored kidney sections for histopathologic damage to the tubules in a blinded manner, as described previously (16,17). The results are illustrated in Figure 5. In all three parameters examined (dilation, casts, and cell necrosis), all three modalities of

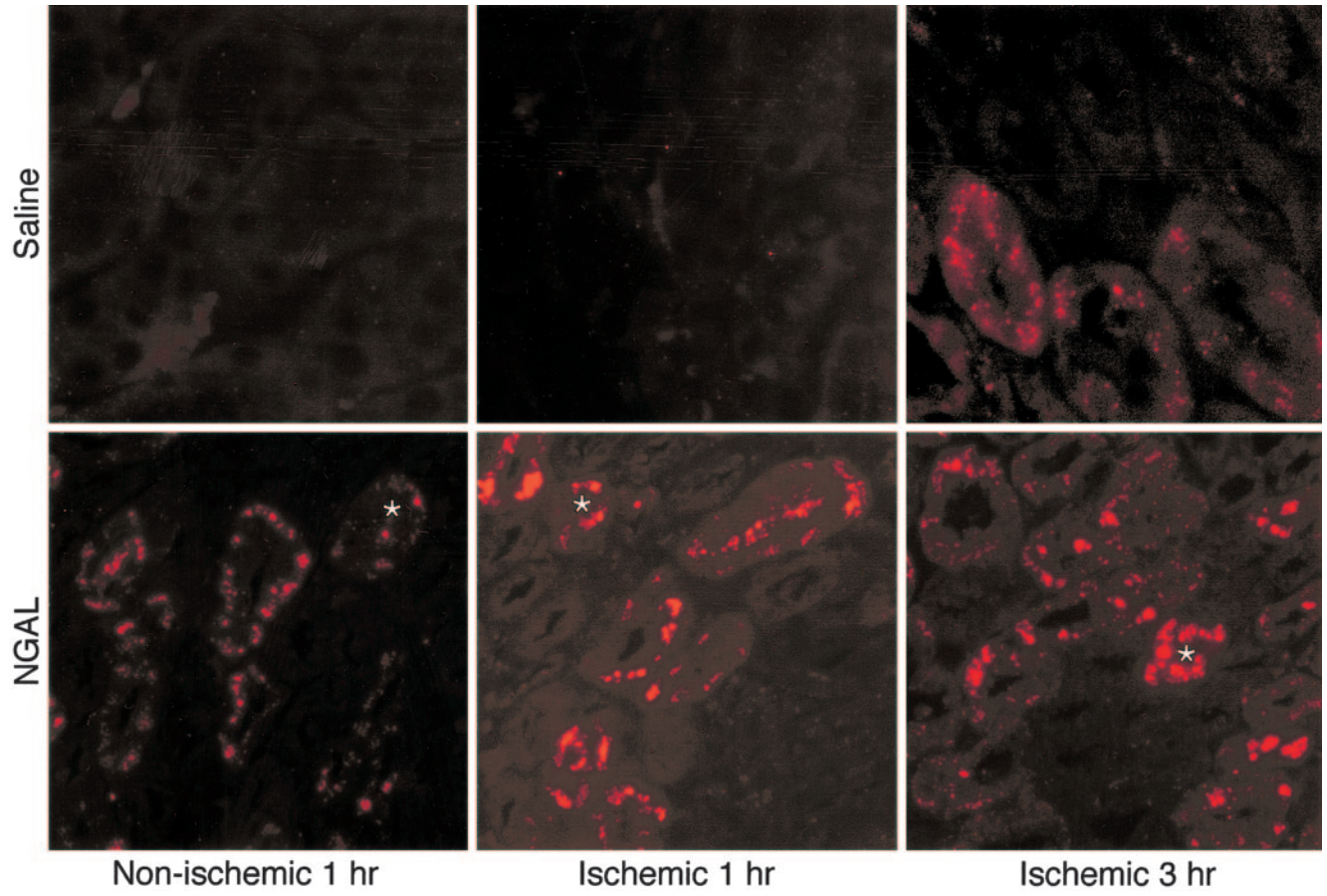


*Figure 2.* Intravenous NGAL is rapidly taken up by proximal tubule cells *in vivo*. Mice were given either NGAL (250 $\mu$g/100 $\mu$l) or saline (100 $\mu$l) by tail vein. Kidneys from control nonischemic animals 1 h after injection or ischemic kidneys 1 or 3 h after either injection were analyzed by immunofluorescence with polyclonal NGAL antibody. NGAL is taken up primarily by proximal tubule cells but also to a lesser extent by distal nephron segments (*), within 1 h in both control and ischemic animals.

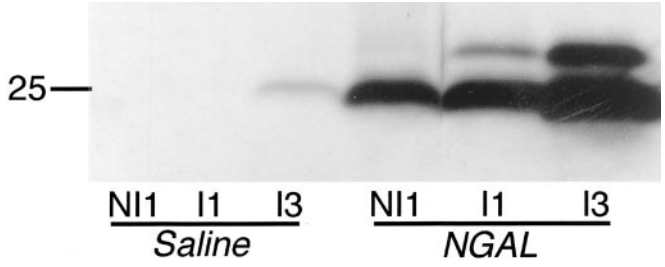


*Figure 3.* Intravenous NGAL is rapidly excreted in the urine. Mice were given either NGAL (250 $\mu$g/100 $\mu$l) or saline (100 $\mu$l) by tail vein. Timed urine collections were analyzed by Western blot with polyclonal NGAL antibody. NGAL was detected primarily as a 25-kD immunoreactive peptide. A larger peptide of ~30 kD was also detected inconsistently, as previously reported (12), which may represent a posttranslational modification. NGAL is excreted in the urine within 1 h in both nonischemic (NI) and ischemic (I) animals within 1 h of administration. Mice that received saline begin to excrete (endogenously produced) NGAL only at 3 h after ischemia.

NGAL treatment (before, during, or after ischemia) resulted in a significantly improved score when compared with controls. This difference was most striking in animals that were pretreated with NGAL, followed in a graded manner by findings in animals that were treated with NGAL during ischemia or after the ischemic insult. However, the structural protection was not complete, and even animals that were pretreated with NGAL displayed some degree of histopathologic damage (primarily some residual tubule casts), which was completely absent from nonischemic controls.

### *NGAL Ameliorates the Reduction in Kidney Function Induced by Ischemia-Reperfusion Injury*

It was next of interest to identify functional correlates to the protection from ischemia-induced structural damage afforded by NGAL injection. In our model of ischemia-reperfusion injury, NGAL administered 1 h before, during, or even 1 h after ischemia resulted in a significant decrease in the serum creatinine measured at 24 h of reflow, as shown in Figure 6. Whereas the nonischemic controls displayed normal serum creatinine (0.65 $\pm$ 0.13 mg/dl), animals that were pretreated with saline alone (100 $\mu$l, volume of diluent) displayed a significant increase in serum creatinine (2.6 $\pm$ 0.28 mg/dl). In contrast, NGAL-treated kidneys displayed an attenuated functional response. This was most evident in animals that were pretreated with NGAL (1.25 $\pm$ 0.3 mg/dl) but was also evident

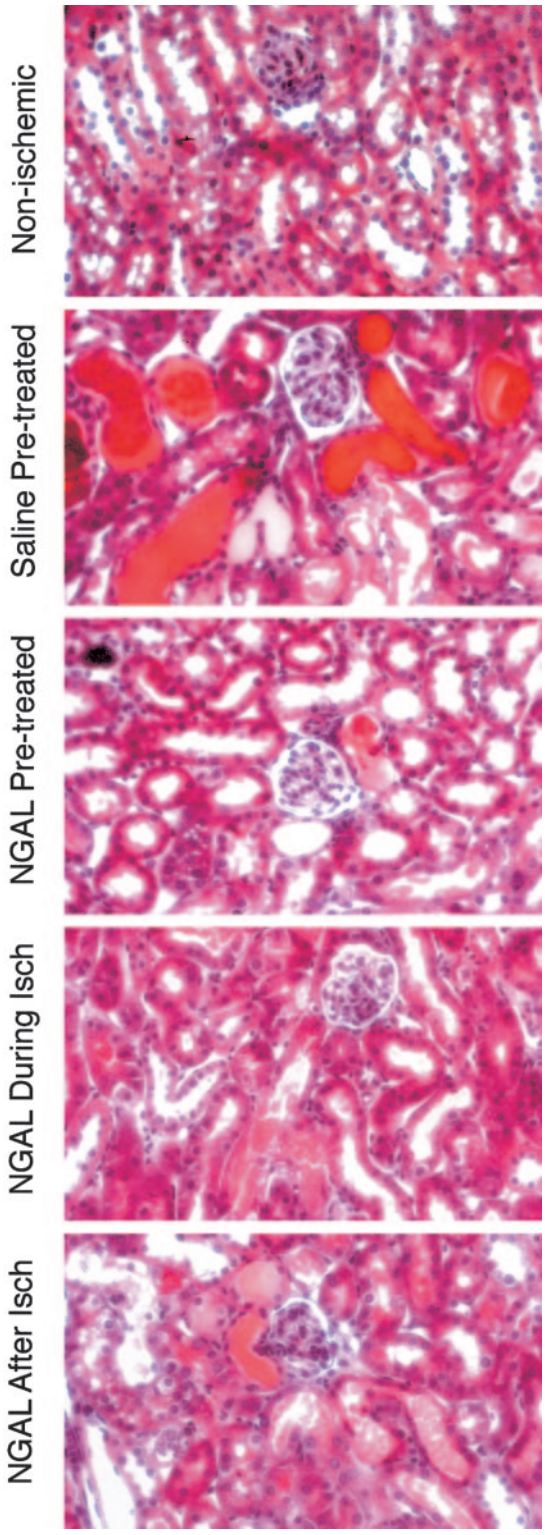


*Figure 4.* NGAL ameliorates the histopathologic damage to tubules induced by ischemia-reperfusion injury. Representative sections stained with hematoxylin-eosin of kidneys from control nonischemic mice, saline pretreated ischemic mice, or ischemic mice that were treated with NGAL 1 h before, during, or 1 h after ischemia. The saline-pretreated ischemic mice displayed extensive features of acute tubular necrosis, including tubular dilation, tubular cast formation, and necrotic cells. In contrast, NGAL-treated kidneys displayed an attenuated histopathologic response. Figure represents five animals in each group.

when the NGAL was administered during (1.5 ± 0.2 mg/dl) or even 1 h after (1.95 ± 0.2 mg/dl) the ischemic injury. However, the functional protection was not complete, and even animals that were pretreated with NGAL displayed a small but significant increase in serum creatinine when compared with nonischemic controls.

### *NGAL Ameliorates the Apoptotic Tubule Cell Death Induced by Ischemia-Reperfusion Injury*

Because apoptosis has been implicated in the tubule cell damage after ischemia-reperfusion injury (11,12,15), we next tested the hypothesis that the structural and functional protection observed with exogenous NGAL administration is a result of decreased apoptosis. Representative kidney sections that were obtained at 24 h of reflow and subjected to TUNEL assay are shown in Figure 7. Whereas the nonischemic controls displayed a minimal incidence of apoptosis (2.2 ± 0.5 cells per 100 cells examined), animals that were pretreated with saline alone (100 μl, volume of diluent) displayed a significantly greater number of apoptotic tubule epithelial cells (12.6 ± 2.2%), as shown in Figure 8. Although apoptosis was more prominent in the distal nephron, it was present in the proximal tubules as well, as described previously (11,12,15). In contrast, NGAL-treated kidneys displayed an attenuated apoptotic response. This was most evident in animals that were pretreated with NGAL (6.7 ± 1.6%) but was also evident when the NGAL was administered during (7.6 ± 0.8%) or even 1 h after (8.5 ± 0.8%) the ischemic injury. However, the protection from apoptotic cell death was not complete, and even animals that were pretreated with NGAL displayed a significantly greater degree of apoptotic damage when compared with nonischemic controls.

### *NGAL Enhances Tubule Cell Proliferation after Ischemic Injury*

We next tested the hypothesis that the structural and functional protection observed with exogenous NGAL administration is a result of enhanced tubule cell proliferation. Representative kidney sections that were obtained at 24 h of reflow and stained with an antibody to PCNA are shown in Figure 7. Whereas the nonischemic controls displayed a minimal incidence of proliferating cells (1.9 ± 0.4 cells per 100 cells examined), animals that were pretreated with saline alone (100 μl, volume of diluent) displayed a small but significant increase in the number of PCNA-positive proximal tubule epithelial cells (4.4 ± 1.2%), as shown in Figure 8. In contrast, NGAL-treated kidneys displayed a marked increase in proliferating proximal tubule cells. This was most evident in animals that were pretreated with NGAL (19.1 ± 2.1%) but was also evident when the NGAL was administered during (14.9 ± 1.2%) or even 1 h after (14.5 ± 1.2%) the ischemic injury.

### *NGAL Tilts the Balance of Proximal Tubule Cell Fate Toward Survival after Ischemic Injury*

We next estimated the overall proximal tubule cell fate after ischemic injury using a one-way ANOVA to compare means ± SD of proliferation and cell death among the various treatment

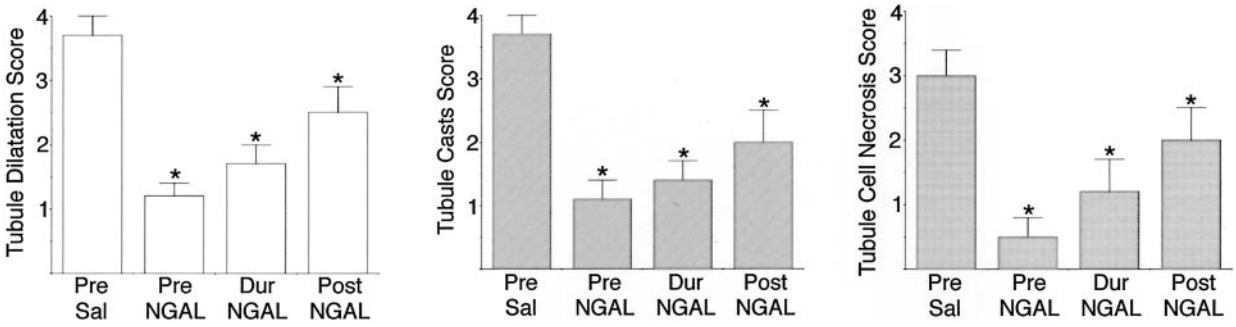


*Figure 5.* Histopathologic scoring. Sections of kidneys from ischemic mice that were pretreated with saline or treated with NGAL 1 h before, during, or 1 h after ischemia were analyzed for tubule dilation, tubule casts, and tubule cell necrosis using an arbitrary scale of 0 to 4. Values are means ± SD of five animals in each treatment group. *$P < 0.05$ *versus* saline.

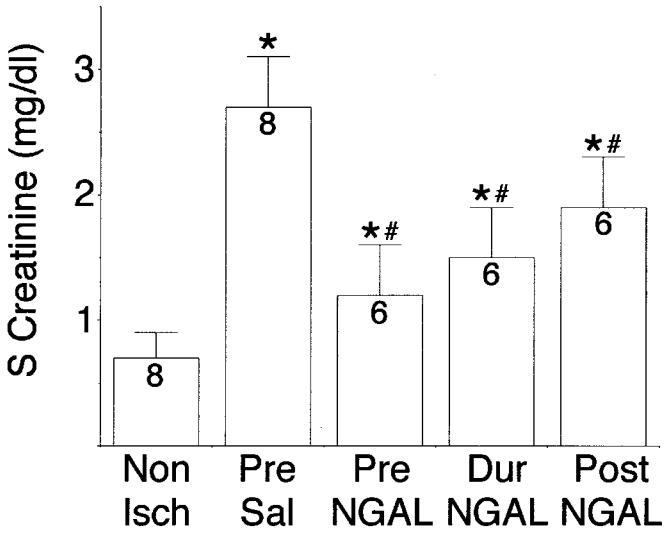


*Figure 6.* NGAL ameliorates the reduction in kidney function induced by ischemia-reperfusion injury. Serum creatinine was measured in nonischemic (Non Isch) control mice or 24 h after ischemia in mice that were pretreated with saline (Pre Sal) or treated with NGAL 1 h before (Pre NGAL), during (Dur NGAL), or 1 h after (Post NGAL) ischemic injury. Values are means ± SD of six to eight animals (as shown within the bars) in each treatment group. *$P < 0.05$ *versus* nonischemic controls; #$P < 0.05$ *versus* saline. NGAL treatments partially but not completely prevented the rise in serum creatinine after ischemic injury.

groups at 24 h of reflow. We restricted this analysis to the proximal tubule, because NGAL-induced proliferation was detected predominantly in this nephron segment. For quantifying cell death, both necrosis and apoptosis were included. A proliferation/death ratio of unity may be assumed to indicate equal rates of cell survival and death, as would be expected in the mature kidney at rest. The results are illustrated in Figure 9. Nonischemic control kidneys displayed a proximal tubule proliferation/death ratio of 0.9 ± 0.2, close to the value of unity. As expected, animals that were pretreated with saline alone (100 μl, volume of diluent) displayed a significant decrease in the proliferation/death ratio (0.34 ± 0.2), indicating that cell death is the predominant feature at the 24-h reflow time point. In contrast, NGAL-treated kidneys displayed a marked increase in the ratio of proliferating *versus* apoptotic/necrotic proximal tubule cells. This was most evident in animals that were pretreated with NGAL (3.4 ± 0.5) but was also evident when the NGAL was administered during (2.5 ± 0.4) or even 1 h after (1.7 ± 0.4%) the ischemic injury. This analysis indicates that NGAL tilts the overall balance of proximal tubule cell fate toward cell survival after ischemic injury.

## Discussion

Human NGAL was originally identified as a 25-kD protein covalently bound to gelatinase from human neutrophils (18) and was subsequently shown to be similar to the mouse 24p3 gene first identified in primary cultures of mouse kidneys that were induced to proliferate (19). NGAL is expressed at very low levels in several human tissues, including kidney, trachea, lungs, stomach, and colon (20). NGAL expression is markedly induced in stimulated epithelia. For example, NGAL concentrations are elevated in the serum of patients with acute bacterial infections, the sputum of patients with asthma or chronic obstructive pulmonary disease, and the bronchial fluid from the emphysematous lung (21). NGAL is also one of the maximally induced genes in the kidney after early ischemic injury (12). In all of these instances, the role of NGAL remains unclear (22). In some cell types, NGAL has been shown to possess a proapoptotic property. For example, in the mouse pro-B lymphocytic cell line, cytokine withdrawal resulted in a marked induction of NGAL as well as onset of apoptosis (23,24). NGAL has also been linked to apoptosis in reproductive tissues. Epithelial cells of the involuting mammary gland and uterus express high levels of NGAL, temporally coinciding with a period of maximal apoptosis (25). Thus, it is likely that a subset of epithelial cells may utilize this mechanism to regulate their own demise.

Accumulating evidence, however, suggests that NGAL can enhance the epithelial phenotype. During kidney development, NGAL is expressed by the penetrating ureteric bud and triggers nephrogenesis by stimulating the conversion of mesenchymal cells into kidney epithelia (14). In the postischemic mature kidney, NGAL is markedly upregulated predominantly in proximal tubules but also in distal nephron segments. In the

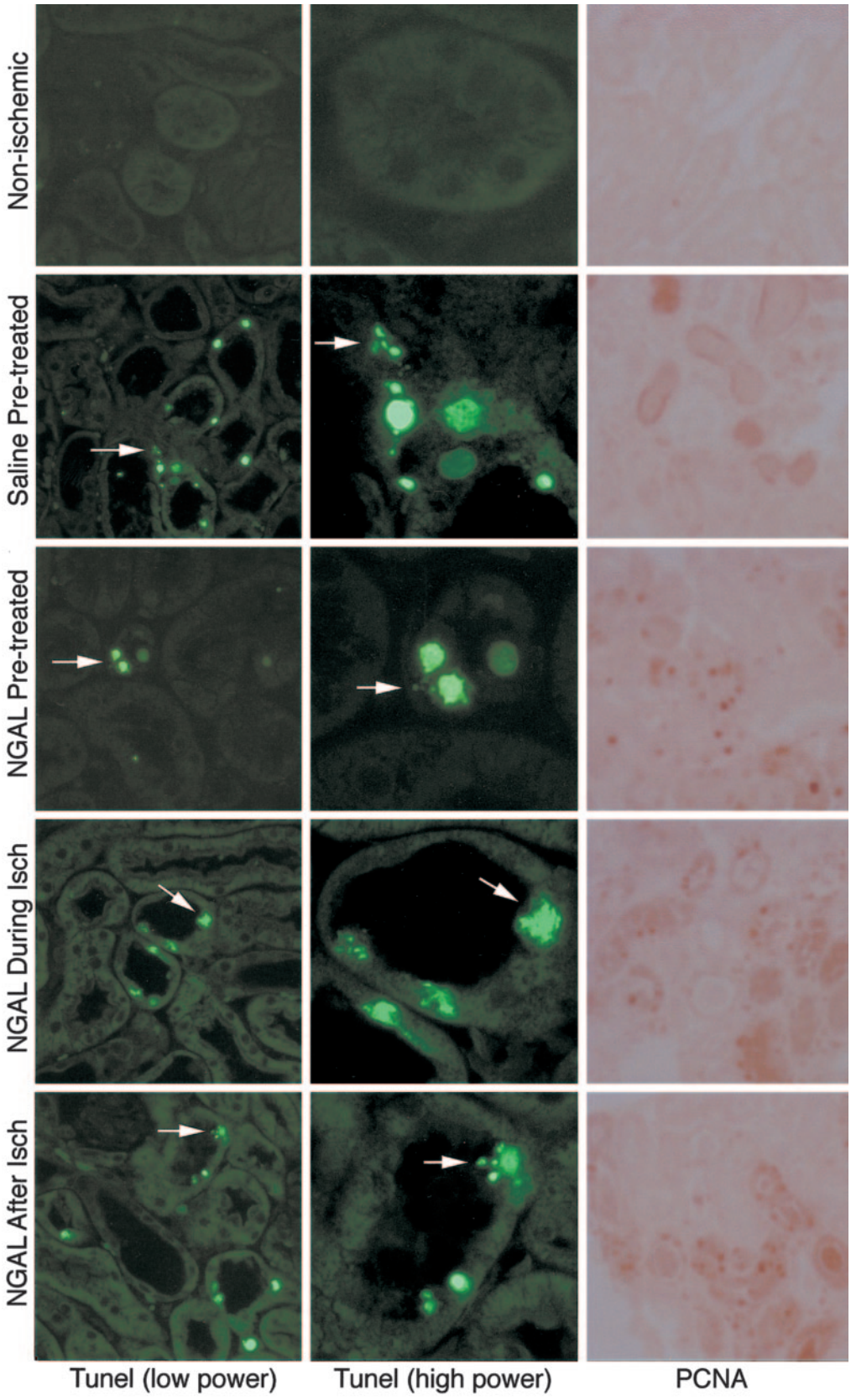


*Figure 7.* NGAL inhibits apoptosis and enhances proliferation induced by ischemia-reperfusion injury. Representative sections from nonischemic control mice or 24 h after ischemia in mice that were pretreated with saline or treated with NGAL 1 h before, during, or 1 h after ischemic injury. Shown are results of transferase-mediated dUTP nick-end labeling staining at low and high power and PCNA staining at low power. Arrows point to the condensed, fragmented, intensely staining nuclei characteristic of apoptosis. Figure represents five animals in each group.

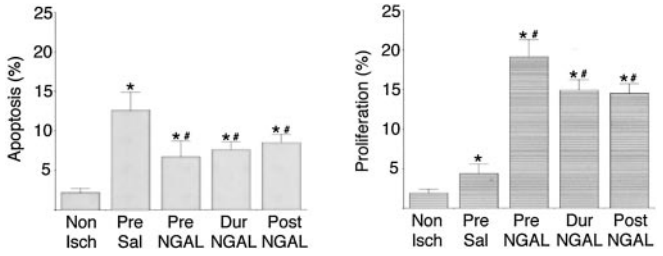


*Figure 8.* NGAL inhibits apoptosis and enhances proliferation induced by ischemia-reperfusion injury. Quantification of apoptosis (left) and proliferation (right) in kidneys from nonischemic control mice or 24 h after ischemia in mice that were pretreated with saline or treated with NGAL 1 h before, during, or 1 h after ischemic injury. Values are means ± SD of five animals in each treatment group. $*P < 0.05$ *versus* nonischemic controls; $\#P < 0.05$ *versus* saline. NGAL treatments partially but not completely prevented the increase in apoptosis after ischemic injury. NGAL treatments significantly enhanced the early proliferative response to ischemic injury.

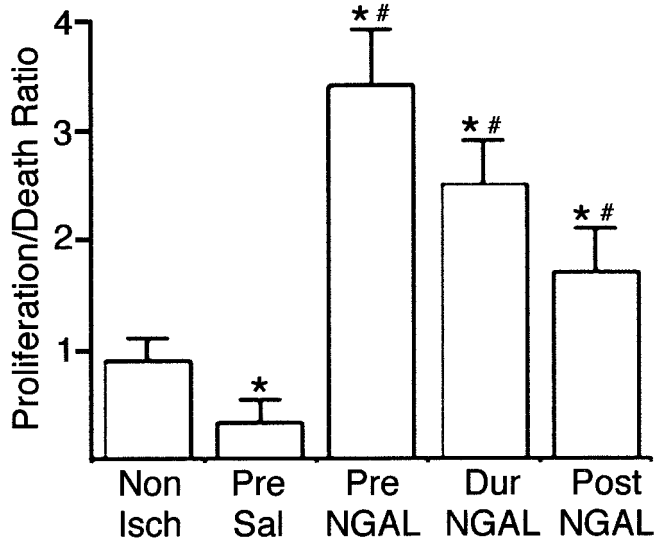


*Figure 9.* NGAL tilts the balance of proximal tubular cell fate toward survival after ischemic injury. A ratio of proliferation/death was calculated in proximal tubules from nonischemic control mice or 24 h after ischemia in mice that were pretreated with saline or treated with NGAL 1 h before, during, or 1 h after ischemic injury. Both apoptosis and necrosis were included in the estimation of cell death. Values are means ± SD of five animals in each treatment group. $*P < 0.05$ *versus* nonischemic controls; $\#P < 0.05$ *versus* saline. NGAL treatments significantly enhanced the proliferation/death ratio, thereby tilting the balance of proximal tubule cell fate toward survival after ischemic injury.

proximal tubule, NGAL co-localizes at least in part with proliferating epithelial cells (12). These findings suggest that NGAL may be expressed by the damaged tubule to induce re-epithelialization. In support of this hypothesis is the recent identification of NGAL as an iron-transporting protein during nephrogenesis (22). It is well known that the delivery of iron into cells is crucial for cell growth and development, and this is presumably also critical to renal regeneration after nephrotoxic injury. Because NGAL can be endocytosed by the proximal tubule (22), the protein could potentially recycle iron into viable cells, thereby stimulating regeneration of renal epithelial cells after ischemic injury. An alternative hypothesis is that NGAL may serve as a reservoir for iron that is released from tubule cells that are damaged by nephrotoxic injury. This might remove iron, a reactive molecule, from the site of tissue injury, thereby limiting iron-mediated cytotoxicity. It is indeed possible that both mechanisms are operative in the postischemic kidney.

In this study, exogenous administration of NGAL strikingly ameliorated the structural damage inflicted by ischemia-reperfusion injury. Both apoptosis and necrosis were significantly blunted. The mechanism by which NGAL inhibits apoptosis in this situation may be analogous to the well-documented anti-apoptotic effects of heme oxygenase 1 (HO-1). It is known that HO-1 facilitates the extracellular transport of iron, thereby limiting iron-driven oxidant stress in the intracellular compartment (26). It is likely that NGAL may also facilitate the removal of excess intracellular iron, thereby limiting oxidant-mediated apoptosis of renal tubule cell death after ischemia-reperfusion injury (27). With respect to necrosis, it was proposed recently that necrotic cell death after oxidant injury occurs by a two-stage process, namely initiation of apoptosis followed by a necrotic cell death (28). Our results suggest that a similar situation may pertain to the response of the kidney after ischemia-reperfusion injury and that apoptosis inhibition by NGAL may be effective in also preventing this "secondary" necrosis.

The results of our study indicate that NGAL tilts the overall balance of proximal tubule cell fate toward cell survival after ischemic injury. This is based on an analysis of the proliferation/death ratio in proximal tubule cells, with the assumption that a ratio of unity indicates equal rates of cell survival and death, as would be expected in the mature kidney at rest. However, the specific assays that we used (PCNA for proliferation and TUNEL for apoptosis), although standard in the field, provide only a snapshot measurement of each of these dynamic processes (*i.e.*, S-phase for proliferation and the execution phase of apoptosis). A more complete confirmation of NGAL's cellular homeostatic effects will require additional measures of proliferation (*e.g.*, Ki67 and cell cycle proteins) and apoptosis (*e.g.*, death receptor activation, induction of mitochondrial pathways, caspase cleavage).

The studies reported herein focused primarily on the ability of NGAL to counter the morphologic response of renal tubular epithelial cells to ischemia. The potential effect of NGAL infusion on several other extracellular factors involved in the pathogenesis of ischemic ARF have not been explored. For example, an additional salutary effect of NGAL on the persistent vasoconstriction, tubular obstruction, or the inflammatory response typical of ARF (4–7) cannot be ruled out.

Nevertheless, the findings reported here may have far-reaching clinical implications. ARF secondary to ischemic injury remains a common problem, with limited and unsatisfactory therapeutic options (1–4). Although previous studies suggested therapeutic approaches in animal models, translational research efforts in humans have yielded disappointing results. The rea-

sons for this likely include a lack of early markers for ARF and the multifactorial nature of the disease. Recent advances in cellular and molecular biology of ischemic renal injury have revealed that proximal tubule cells undergo a complex temporal sequence of events. These include loss of cell polarity, cell death as a result of apoptosis and necrosis, dedifferentiation and proliferation of viable cells, and re-establishment of the epithelial phenotype (6,7). Therefore, identification of factors that oppose tubule cell death and/or enhance the recovery phase may provide critical clues toward novel therapeutic options. We propose NGAL as a potential candidate that possesses both of these desirable properties. Exogenously administered NGAL seems to limit the morphologic and functional consequences of ischemia-reperfusion injury in a mouse model, by a combination of limiting tubule cell death and enhancing re-epithelialization. It will be important in future translational research to examine these cytoprotective roles of NGAL in human conditions that are known to predispose to ischemic renal injury. One good example pertains to cadaveric renal transplantation, in which oxidant-mediated apoptosis is an important contributor to tubule cell death (29). In addition to the usual complications of ARF, ischemia-reperfusion injury in the transplanted kidney is known to result in delayed graft function (30), which significantly increases the risk of graft loss and acute rejection (31). It will be intriguing to explore the morphologic effects and clinical outcomes of adding NGAL to the organ preservation solutions used during cold storage. It is hoped that such maneuvers will be successful in ameliorating the delayed graft function characteristic of cadaveric kidney transplantation. On the basis of our present findings that NGAL is at least partially effective even when administered after the ischemic insult, it is also hoped that NGAL may offer promising diagnostic and therapeutic possibilities in ischemic ARF, a common clinical condition that is still associated with a dismal prognosis and for which novel therapies are desperately needed.

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