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Dual Action of Neutrophil Gelatinase–Associated Lipocalin

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Neutrophil gelatinase–associated lipocalin (NGAL) is expressed and secreted by immune cells, hepatocytes, and renal tubular cells in various pathologic states. NGAL exerts bacteriostatic effects, which are explained by its ability to capture and deplete siderophores, small iron-binding molecules that are synthesized by certain bacteria as a means of iron acquisition. Consistently, NGAL deficiency in genetically modified mice leads to an increased growth of bacteria. However, growing evidence suggests effects of the protein beyond fighting microorganisms. NGAL acts as a growth and differentiation factor in multiple cell types, including developing and mature renal epithelia, and some of this activity is enhanced in the presence of siderophore:iron complexes. This has led to the hypothesis that eukaryotes might synthesize siderophore-like molecules that bind NGAL. Accordingly, NGAL-mediated iron shuttling between the extracellular and intracellular spaces may explain some of the biologic activities of the protein. Interest in NGAL has been sparked by the observation that NGAL is massively upregulated after renal tubular injury and may participate in limiting kidney damage. This review summarizes the current knowledge about the dual effects of NGAL as a siderophore:iron-binding protein and as a growth factor and examines the role of these effects in renal injury.

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Neutrophil gelatinase–associated lipocalin (NGAL) is a 21-kD protein of the lipocalin superfamily. Lipocalins comprise a class of proteins that are characterized by eight β-strands that form a β-barrel defining a calyx. The calyx binds and transports low molecular weight molecules (1), which are thought to define the biologic activity of the lipocalin. Only to mention a few examples, retinol-binding protein binds and transports vitamin A (2), the lipocalin α 1-microglobulin scavenges heme (3), and nitrophorin-type lipocalins carry heme groups complexed with nitric oxide (4). The ligand of NGAL was discovered by Goetz *et al.* (5) on the basis of the observation that recombinant NGAL, when expressed in bacteria, appeared either colorless or light rosé, depending on the bacterial strain used for expression of the protein. This color was found to be related to the presence of iron and a small iron-binding molecule called enterochelin (or its degradation product, 2,3-dihydroxybenzoic acid), which is produced by some strains of bacteria (5). Bacteria produce siderophores to scavenge iron from the extracellular space and use specific transporters to recover the siderophore:iron complex, ensuring their iron supply. Accordingly, NGAL prevented growth of the bacterial strains that rely on the production of enterochelin to satisfy their iron demands (5). The biologic significance of this finding was demonstrated in genetically modified mice, which are deficient for both copies of the NGAL gene. These animals were more sensitive to certain Gram-negative bacteria and more readily died of sepsis than did wild-type mice (6,7). Therefore, NGAL comprises a critical component of innate immunity to bacterial infection.

NGAL seems to have more complex activities than its antimicrobial effect. The expression of NGAL rises 1000-fold in humans and rodents in response to renal tubular injury, and it appears so rapidly in the urine and serum that it is useful as an early biomarker of renal failure (recently reviewed in reference [8]). Induction of NGAL may limit tubular injury, an effect that may be independent from its bacteriostatic actions. In fact, mounting evidence points toward growth factor effects of NGAL that modulate various cellular responses, such as proliferation, apoptosis, and differentiation, but this is not well understood mechanistically. Some of these effects, however, are enhanced when NGAL is associated with siderophores and iron, raising the possibility that in the absence of bacterial infection, endogenous molecules associate with NGAL to mediate its iron-binding properties. This review summarizes current understanding of NGAL's cellular effects and their relation to its siderophore:iron-carrying properties.

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NGAL Promotes Differentiation and Structural Organization of Renal Epithelial Cells

In a search for factors that induce the differentiation of kidney progenitors in the metanephric mesenchyme into renal epithelia, we isolated NGAL from a ureteric bud cell line (9). NGAL targets a peripheral compartment of rat metanephric mesenchyme, which contains stromal cells and early epithelial progenitors (10). Upon application of NGAL, these cells proliferate and upregulate genes that are typical of the early epithelial progenitor (Cadherin11) and the renal stroma/capsule (Col6a, Col5a, Acvrl1, Nfix, Tacr3, and Tenascin; Figure 1 and unpublished data). This is followed by epithelial differentiation of the mesenchymal progenitors, which generates nephron-like structures that express markers of glomeruli, proximal tubules, loops of Henle, and distal tubules (9) (Figure 2). The importance of the peripheral compartment is illustrated by the fact that removal of these cells abolishes NGAL activity (9) but does not negate the actions of other epithelial inducers (11). These data indicate that NGAL promotes epithelial differentiation by targeting a stromal/interstitial/progenitor niche at the periphery of the developing kidney.

The differentiation-inducing properties of NGAL are not limited to the embryonic kidney. In a 4T1-Ras–transformed mesenchymal tumor cell line, NGAL induces markers of epithelial cells (12). Furthermore, in cultured collecting duct cells, NGAL is expressed downstream of hepatocyte growth factor and promotes the organization of epithelial cells into tubular structures (13). Antagonization of NGAL induction by expression of NGAL shRNA induces cystic structures rather than properly assembled tubules. Therefore, in addition to inducing epithelial characteristics in nonepithelial cells, NGAL seems to affect the structure of established epithelia. It is interesting that glycodelin, another protein of the lipocalin family, displays effects on epithelial differentiation very similar to NGAL (14). *In vivo*, NGAL protein is expressed predominantly by stimulated, growing, dysplastic, or involuting epithelial cells, pointing to a relevance of the *in vitro* observations in pathologic states (15–19). Despite these important pieces of evidence, the *in vivo* role of NGAL in modulating the phenotype of the epithelial lineage in growth and disease remains to be determined and presents an imperative challenge for future studies.

NGAL Signals *via* Cell Surface–Associated Receptors That Mediate Its Cellular Uptake

Recent studies have greatly enhanced our understanding of how NGAL signals to its target cells. Two cell surface receptors of very different molecular structure have been identified. One receptor, called 24p3R (alluding to NGAL's original name, 24p3), is a protein that originally was referred to as brain organic cation transporter, which is a membrane-associated protein with 12 predicted transmembrane helices (20). Overexpression of 24p3R in HeLa cells induces binding and uptake of NGAL, which results in specific biologic responses (see the next section) (20), yet the specific expression of this receptor in NGAL target cells and the requirement for this receptor to mediate NGAL responses *in vitro* or *in vivo* remain to be determined.

A second molecule that acts as a receptor for NGAL is the well-characterized multiprotein receptor megalin-cubulin (21). Megalin binds NGAL with high affinity, and uptake of NGAL into a yolk sac cell line is blocked by antimegalin antibodies (21). Megalin is expressed by proximal tubule cells in the kidney, which are known target cells of NGAL (22). Furthermore, mice that are genetically deficient in megalin excrete NGAL in the urine (Figure 3) (22). This would parallel the proximal tubular uptake by megalin/cubilin-type receptors of other iron-binding proteins, including α 1-microglobulin (23), free hemoglobin (24), and transferrin (25), and the urinary excretion of these ligands in mice that are genetically deficient in these receptors. However, the extent of participation of megalin in proximal tubular NGAL uptake and the role of the alternative receptor 24p3R in these cells are unknown at present. In sum, although much work remains with respect to the identification of the relevant NGAL receptors in different cells and tissues, the characterization of these receptors will greatly advance our understanding of NGAL action on specific cell types, including renal epithelial cells.

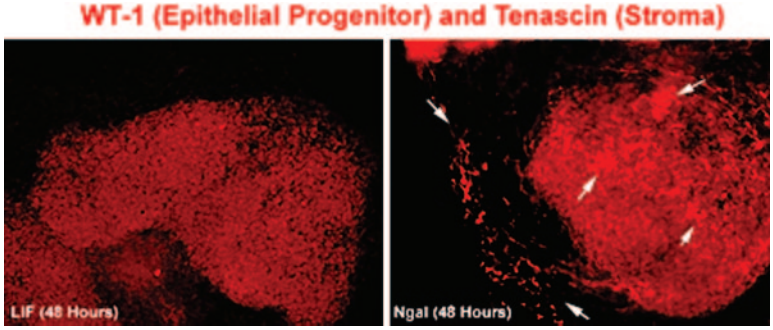

Figure 1. Neutrophil gelatinase–associated lipocalin (NGAL) targets a stromal-epithelial compartment. NGAL induces expansion of the stroma (tenascin, extracellular matrix staining, arrows) in rat metanephric mesenchyme, whereas epithelial induction by leukemia inhibitory factor (LIF) results in the loss of the stromal compartment. WT-1, nuclear stain, identifies epithelial progenitors.

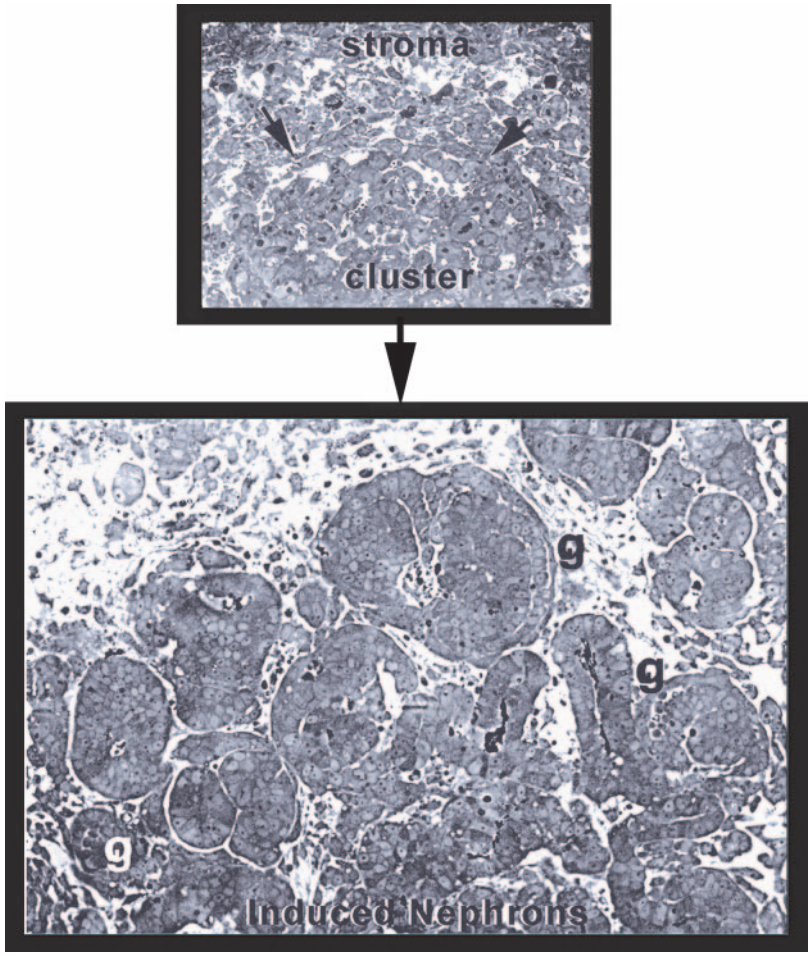

Figure 2. NGAL induces epithelial conversion in rat metanephric mesenchyme. (Top) Embryonic metanephric mesenchyme in culture with fibroblast growth factor 2 (FGF2) and TGF-α to maintain viability. Note the cluster of epithelial progenitors demarcated by arrows and surrounded by stroma and early progenitors cells. The epithelial progenitors expand and convert into fully polarized epithelia and induced nephrons when additional growth factors such as NGAL are added (bottom). g, nascent glomeruli.

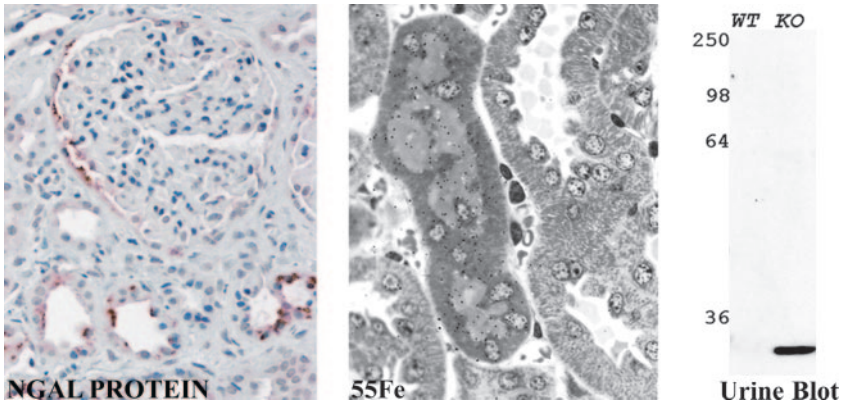

Figure 3. Filtration and capture of systemic NGAL. NGAL protein is captured by the proximal tubule (left) and delivers iron from NGAL:enterochelin:^{55}Fe injected subcutaneously (middle). Note the radioactive grains in the proximal tubule but not in other segments of the nephron. Capture of NGAL may be due to megalin-mediated uptake because NGAL (21-kD band on immunoblot) is found in the urine of megalin knockout (KO) animals but not in wild-type (WT) animals (right; courtesy of T. Willnow and E.I. Christensen).

NGAL-Mediated Iron Delivery Induces Specific Cellular Responses

On the basis of recent studies, an unusual model of intracellular signaling in response to NGAL's association with its receptors is beginning to evolve (Figure 4). In particular, the cellular events differ strikingly—at least in some biologic systems—depending on whether NGAL is associated with iron (9,20,22). Cellular uptake of NGAL is followed by distribution of the protein in endosomes (9). Different trafficking routes of endosomal NGAL have been proposed depending on the cell type and the association of NGAL with its binding partners. In kidney-derived cell lines, siderophore:iron-associated NGAL (holo-NGAL) traffics to endosomes and releases iron from the complex, which results in regulation of iron-responsive genes, such as ferritin and transferrin receptor (9,20). Similarly, in the adult mouse kidney *in vivo*, systemically applied holo-NGAL is taken up by proximal tubule cells, where it delivers ^{55}Fe (Figure 3) (22). The endosomal NGAL protein core is either degraded in lysosomes (22) or recycled to the extracellular space (20). On the basis of these observations, siderophore:iron-associated NGAL is predicted to facilitate cytoplasmic iron delivery into target cells (Figure 4A). A recent report suggested that the situation may be different when NGAL is delivered into target cells in the absence of the siderophore:iron complex. In this setting, NGAL is proposed to scavenge intracellular iron and exit the cell *via* the endosomal recycling pathway (20) (Figure 4B). Therefore, at least some of the biologic effects of NGAL may depend markedly on its association with the siderophore:iron complex.

This notion is supported by different biologic responses to NGAL depending on the ligand. For instance, siderophore:iron-associated NGAL is more effective than apo-NGAL in inducing epithelial characteristics in 4T1-Ras–transformed mesenchymal tumor cells (12). Also, NGAL:enterochelin:iron stimulates the expression of an iron-dependent reporter construct and downregulates iron-repressed genes (26), whereas NGAL:enterochelin (which can chelate iron) displays the opposite effect. Most dramatic, in MDCK cells or 24p3R-transfected HeLa cells, apo-NGAL might induce apoptosis as a result of depletion of intracellular iron pools, but this effect is essentially abolished if siderophore:iron-loaded NGAL is used (20). Importantly, the presence of iron, rather than the presence of siderophores, seems to account for the distinct biologic effects. This is demonstrated by assays on rat metanephric mesenchyme, where NGAL:enterochelin:Fe displays epithelialization-inducing activity, whereas apo-NGAL or NGAL loaded with iron-free enterochelin are not as active (9). In addition, substitution of iron with gallium in the siderophore complex decreases NGAL's differentiation-inducing properties (9). Finally, the NGAL:siderophore:iron complex partially compensates for the growth deficiency of cultured embryonic kidneys in the absence of transferrin, the predominant iron carrier *in vivo*. (27). These data indicate that the association of NGAL with siderophore and iron and delivery of the complex to cells underlies its actions in certain biologic settings.

In some instances, NGAL seems to exert cellular effects that are independent of iron transport. Most notable, the branch-promoting effects of NGAL in renal epithelial cells occur at concentrations below those that are required for iron transport and are independent of the association of the NGAL:siderophore complex with iron (13). In this setting, NGAL produces activation of extracellular signal–regulated kinase. Although it remains to be determined whether this effect is a direct downstream event or NGAL obtains a ligand during the experiment, it poses the possibility that NGAL also acts *via* classical growth factor–induced signal transduction pathways independent of ligands.

Many additional questions remain unanswered in the current models of NGAL's cellular action. Most important, the source and the exact molecular nature of the siderophore, which is essential for NGAL's iron-binding properties, is unclear in eukaryotic cells. Although siderophores clearly are produced by

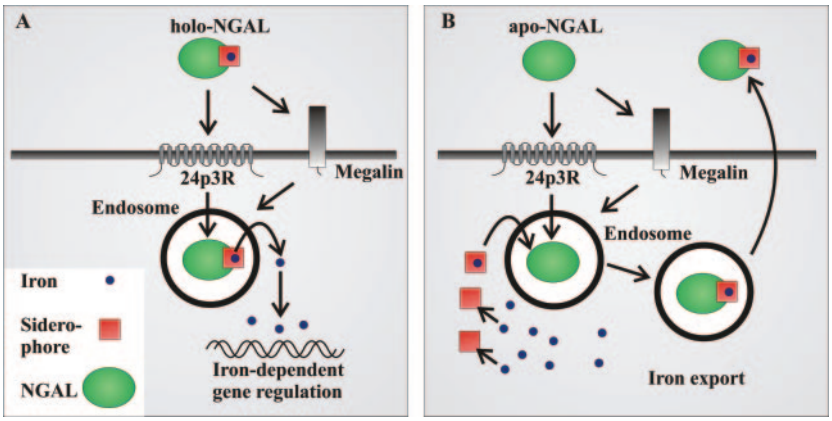

Figure 4. Schematic model of NGAL-mediated iron traffic. (A) Siderophore:iron-associated NGAL (holo-NGAL) delivers iron into the cell. After receptor-mediated uptake, NGAL traffics in acidic endosomes, which promote the release and cytoplasmic accumulation of iron, resulting in regulation of iron-dependent genes (9). (B) Siderophore:iron-free NGAL (apo-NGAL) captures intracellular iron and transports it to the extracellular space. Endosomal NGAL captures iron *via* a hypothetical intracellular siderophore, which is followed by recycling to the extracellular space as per Devireddy *et al.* (20).

bacteria, only a few studies provide evidence for the presence of eukaryotic siderophores. More than two decades ago, two groups (28,29) identified a 1500-Da iron-binding molecule from organs, blood, and urine, which was produced under iron-limiting conditions, bound to iron specifically and transferred iron into cells, indicating that it possesses the typical characteristics of a siderophore. Most interesting, one of the groups assayed the activity of this molecule with a bacterial growth assay, suggesting that the compound was highly related to a bacterial siderophore (29). In addition, recent experimental evidence indicates the presence of an NGAL binding partner in eukaryotic cells that mediates its iron-binding activity. This binding partner is present in urine from healthy individuals. Although apo-NGAL alone is not able to bind ^{55}Fe in a filter retention assay, the addition of a <3000-Da urine extract leads to iron retention by apo-NGAL, indicating that a low molecular weight urinary molecule displays siderophore-like characteristics (22). Similarly, NGAL that is produced by a IL-3–deprived pro-B-lymphocytic cell line is able to associate with ^{55}Fe (as determined by immunoprecipitation of NGAL), whereas purified apo-NGAL is not, suggesting that NGAL that is produced by this cell line associates with an endogenous siderophore (20). Despite these advances, the hypothetical eukaryotic siderophore has not been purified, and its molecular structure is unknown.

NGAL Is Induced in Systemic Disease and Renal Injury

The expression of NGAL mRNA and protein by various cell types is subject to extensive regulation. Serum levels of NGAL are elevated markedly in bacterial infections (30), which is consistent with NGAL's proposed function as an endogenous bacteriostatic protein that scavenges bacterial siderophores. However, increases of serum NGAL levels have been reported in the setting of systemic disease in the absence of overt bacterial infection, most notably during the acute-phase response (31) and in renal tubular injury (8,22,32–35). In the latter setting, human serum NGAL levels are increased on the order of seven- to 16-fold (22), and human urinary NGAL levels increase by 25- to 100-fold (22), which has led to the development of assays for NGAL for the early detection of renal tubular injury in human (8). In mouse serum, NGAL rises 300-fold, mouse NGAL mRNA is induced by nearly 1000-fold in the kidney (Figure 5) (8,22), and mouse urine NGAL rises 1000-fold. Importantly, systemic application of the NGAL:siderophore:iron complex in mice before acute ischemic tubular injury causes protection of tubular epithelial cells and blunts the decline in renal function in these animals (22,36). The NGAL complex reduces apoptosis and preserves N-cadherin expression in renal epithelia after ischemia, which may be mediated by an NGAL-induced upregulation of the renal-protective enzyme heme oxygenase-1 in the ischemic kidney (22). NGAL when associated with a siderophore (enterochelin) redirects ^{55}Fe from the liver or spleen to the kidney (22). In addition, NGAL that is associated with the siderophore:iron complex is more effective in preventing renal tubular damage than NGAL alone or NGAL that is associated with a siderophore:gallium complex. These data suggest that NGAL expression and redirection of siderophore and iron delivery to the proximal tubule (*e.g.*, in sepsis) may play a role in

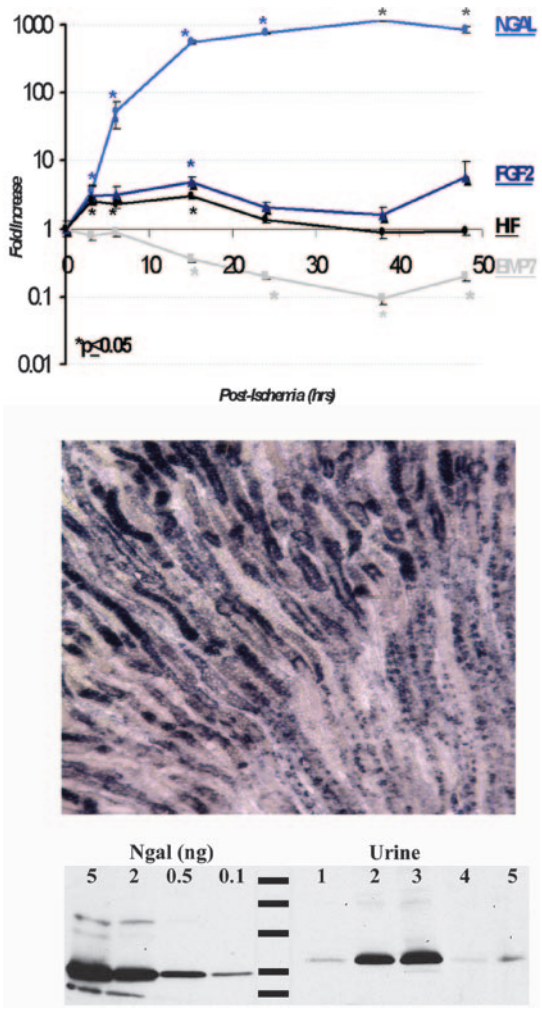

Figure 5. (Top) Ischemic kidneys synthesize NGAL for at least 50 h after reperfusion manifest by an approximately 1000-fold increase in NGAL message (measured by real-time reverse transcriptase–PCR). The mRNA levels of NGAL are compared with the smaller degrees of modulation of genes that are relevant to acute renal injury (hypoxia-inducible factor [HIF], FGF2, and bone morphogenic protein 7 [BMP7]; *significantly modulated from time zero). Note the log scale of mRNA abundance. (Middle) After renal injury, NGAL mRNA is expressed predominantly in the loop of Henle and collecting ducts as determined by *in situ* hybridization. (Bottom) Urine from patients with mild (lanes 1 and 4) and severe (lanes 2, 3, and 5) renal epithelial injury contains NGAL as analyzed by immunoblot for NGAL protein (NGAL standards are shown on the left). The molecular weight difference between standards and samples is due to glycosylation.

NGAL's tissue-protective effects (22). We speculate that the increases in NGAL levels after renal tubular injury may serve to limit injury in recurrent insults or even ameliorate the degree of damage in an ongoing insult, although this latter view was challenged recently (7).

The trafficking of NGAL in the setting of renal injury may be more complicated than initially assumed. This is suggested by the observation that NGAL mRNA in the ischemic kidney is

synthesized largely in the loop of Henle and collecting ducts (Figure 5) (8), which are not the primary sites of ischemic renal injury. A similar phenomenon was documented in a series of elegant papers that showed that the loop of Henle responds to renal ischemia despite that the site of major damage is the proximal tubule (37–40). Measurement of renal vein NGAL indicates that this locally synthesized pool of NGAL is not introduced efficiently into the circulation (K.M. and J.B., unpublished observations) but rather seems to be excreted into the urine. This is supported further by the calculated fractional excretion of NGAL (>100%) in the urine, which strongly suggests that urinary NGAL is derived at least partially from local synthesis in the kidney. In marked contrast, the bulk of NGAL protein that is detectable in the postischemic kidney is localized to the damaged proximal tubule (Figure 3) in a lysosomal compartment. Although this is in seeming contradiction to NGAL synthesis in the distal nephron, it suggests that NGAL is delivered to the proximal tubule from the circulation. This is explained most likely by glomerular filtration of circulating NGAL (as expected on the basis of its low molecular weight of 21 kD) and subsequent uptake by proximal tubular epithelia *via* endocytosis (22). This idea is supported by our observation that tagged NGAL, when injected into the circulation, is enriched in the proximal tubule but does not appear in the urine in large quantities (<0.2%) (22).

Conclusion

We have proposed a two-compartment model of NGAL trafficking in renal injury (8), where urinary NGAL is derived from local synthesis in the kidney in distal parts of the nephron within hours of an insult, whereas proximal tubule NGAL derives from the circulating NGAL pool, which may stem from extrarenal sources of NGAL. In this model, systemic NGAL that is produced in the setting of sepsis or renal disease may serve to limit proximal tubular damage, whereas NGAL that is synthesized locally in the kidney may exert bacteriostatic effects in the distal urogenital tract. Although urinary tract infections are not a typical consequence of renal injury, they are a common cause of Gram-negative sepsis, and it is tempting to speculate that NGAL that is synthesized by the failing kidney may limit growth of Gram-negative bacteria in the lower urinary tract. Although these ideas are compelling, this model certainly requires additional experimental confirmation, which may be achieved by tissue-specific deletion of renal and extrarenal NGAL pools (8).

As in the other biologic systems studied, many questions with respect to the detailed role of NGAL in renal tubular injury remain unanswered at this point. These include the precise role of NGAL *in vivo* in the setting of various types of acute renal injury (no renal phenotype has been identified yet in NGAL knockout mice in one type of ischemic model) and the identification of endogenous ligands of NGAL in the setting of renal injury in the absence of bacterial infection. The challenge of future studies is to solve these questions and establish an integrated model of NGAL actions *in vivo*.

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