Neutrophil Gelatinase-Associated Lipocalin and Hepcidin: What Do They Have in Common and Is There a Potential Interaction?

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Key Words

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Abstract

Iron is the fourth most common element in the Earth's crust and is crucial for life. Over the last few years, our understanding of iron metabolism has dramatically increased due to the discovery of hepcidin, which is produced by hepatocytes and modulated in response to anemia, hypoxia and inflammation. It has been found that anemia upregulates lipocalin 2 (NGAL; neutrophil gelatinase-associated lipocalin) in the liver and serum. The aim of this review is to summarize the current knowledge dealing with a possible role of hepcidin and NGAL in iron metabolism and its regulation, particularly in kidney disease. Elevated NGAL a few days after insult is a possible preventive or protective mechanism limiting renal injury. NGAL is an innate antibacterial factor as well as hepcidin. NGAL binds siderophores, thereby preventing iron uptake by bacteria. Hepcidin, an antibacterial defensin, prevents iron absorption from the gut and iron release from macrophages, leading to hypoferrremia and anemia. Both proteins sequester iron, but by different mechanisms. However, these proteins involved in iron metabolism do not

seem to be independently related. Taking into account the antimicrobial moieties of NGAL, further studies are needed to address the role of NGAL in iron metabolism and inflammation in renal failure.

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Iron is the fourth most common element in the Earth's crust and the most abundant transition metal in the human body. It is a paradoxical element in that it is required by all aerobic organisms, as it is essential for growth and survival. It is essential primarily to ensure transport of oxygen or to catalyze reactions of electron transfer, nitrogen fixation or DNA synthesis. On the other hand, it is also toxic due to its capacity to react with oxygen and to catalyze the production of reactive oxygen species. Iron is virtually insoluble under conditions occurring in our cells and body fluids, and maintaining the correct iron balance is crucial for health. All living organisms have evolved sophisticated mechanisms to maintain appropriate iron levels in their cells and within their body [1]. Over the last few years, our understanding of iron metabolism has dramatically increased due to the discovery of hepcidin. The regulation of iron metabolism involves the in-

Table 1. Proteins involved in iron metabolism

Protein	Description, potential role
Transferrin	usually measured in the laboratory as the total iron-binding capacity
Transferrin receptor-sTFR	carrier protein for transferrin
Ferritin	the cellular storage protein for iron and an acute phase reactant
Iron responsive element-binding protein	an intracellular reporter of iron status, which is also called iron regulatory protein or factor
HFE	hemochromatosis gene, which has been detected, as well as mutations of which are responsible for hereditary hemochromatosis
Divalent metal transporter	also known as DMT1, Nramp2, DCT1, solute carrier family 11, member 2 (Slc11a2)
Duodenal iron transporter-SFT	a stimulator of iron transport
Ferroportin	iron export protein
Hephaestin	iron export protein
Hemojuvelin	a hepcidin regulator
Hepcidin	a negative regulator of intestinal iron absorption as well as macrophage iron release

teraction of a number of specific proteins as well as the interplay between iron absorption and iron loss [1]. Proteins involved in iron metabolism are presented in table 1 [1].

The normal iron content of the human body is about 3–4 g, predominantly in the form of hemoglobin in circulating erythrocytes (approximately 2.5 g), iron-containing proteins such as myoglobin, cytochromes, catalase (about 400 mg), bound to transferrin (3–7 mg) [1]. The remainder is storage iron in the form of ferritin or hemosiderin.

The majority of iron is bound up in hemoglobin, though several proteins bind iron directly. Normally, one third of transferrin is saturated with iron (i.e. $\text{Fe}/\text{TIBC} = 33\%$) [1]. Transferrin transports iron between cells. Transferrin receptors are present on hepatocytes and epithelial cells of the small intestine, including duodenal crypts [1]. They probably contribute to the body's iron sensing [1]. Each receptor can bind two transferrin molecules and, after their endocytosis, iron is offloaded (four Fe^{3+} atoms)

in acidified vacuoles. The complex of the apotransferrin and transferrin receptor is then recycled on the cell surface and released into circulation [1].

Ferritin is the cellular storage protein for iron. Ferritin is also an acute phase reactant and, along with transferrin and the transferrin receptor, is a member of the protein family that orchestrates the cellular defense against stress and inflammation [1]. The serum level of ferritin generally reflects overall iron storage, with 1 ng of ferritin per ml approximating 10 mg of iron stores [2].

Ferroportin has been found to be strongly expressed in enterocytes, macrophages and placental syncytiotrophoblasts [3, 4]. It is a major iron transporter out of cells. It transports iron from the mother to the fetus, transfers absorbed iron from enterocytes into the circulation and participates in the recycling of iron from senescent erythrocytes into macrophages, and from them into the circulation. Ferroportin is upregulated by the amount of available iron [5], and downregulated through its interaction with hepcidin [6].

Kidney and Iron Metabolism

It has been shown recently that the kidney is also involved in iron metabolism. There is abundant expression of Nramp2 (DCT1) in the proximal tubule and collecting ducts of the kidney [7]. This raises the possibility that there is an iron excretory pathway in the kidney, counteracted by its constitutive reabsorption. On the other hand, Nramp1 expressed in the neutrophil granules known to harbor NGAL and lactoferrin is believed to reduce the availability of iron in the phagocytic vacuoles by pumping iron into the cytosol.

Iron and Host Defence

Iron also plays an important role in host defense responses to infectious agents. There are several host defense proteins, such as lactoferrin and siderocalin (lipocalin 2, NGAL), both of which are released from neutrophils at sites of infection/inflammation, or divalent metal transporter protein (DMT-1), which is a transmembrane protein and an isoform of natural resistance-associated macrophage protein (Nramp2), which seem to function in infected tissue or cells to sequester iron from invading microorganisms [1]. The growth and metabolism of many microorganisms is exquisitely iron-sensitive [8]. Bacteria require iron for the production of the

Fig. 1. The main pathways of iron storage and exportation by macrophages, and the potential role of hepcidin and NGAL in restricting bacterial growth. Apo-NGAL = NGAL devoid of iron or siderophore. Modified from Muñoz et al. [55].

superoxide dismutase that protects them from host oxygen radicals [9]. Since the iron concentration in the environment or in the host is too low to sustain the optimal conditions for invading microorganisms, both bacteria and the host mechanisms have to tackle this problem. Bacteria-developed siderophores (high-affinity iron-binding molecules) retrieve iron from transferrin or lactoferrin of the host (fig. 1). Defense mechanisms of the host include an increased production of iron-binding proteins (i.e. transferrin), a fall in dietary iron absorption, an increase in iron storage proteins and release of apolactoferrin from neutrophils to sequester the iron at the site of invading microorganisms. Hypoferremia develops within hours of the inflammatory stimulus in order to counteract pathogens and retard tumor growth [10]. The finding that administration of iron during infection/inflammation worsens the course of the disease stresses the problem of the scarcity of free iron in the body and the efficiency of iron sequestration as an antibacterial strategy [11].

NGAL and Iron and Anemia

Lipocalins are a diverse family of over 20 small soluble proteins, which are often secreted. They have a three-dimensional structure, which comprises a single eight-stranded, continuously hydrogen-bonded antiparallel β -barrel. It forms an enclosing cavity capable of binding

several low molecular weight molecules, including iron [12, 13]. Generally, lipocalins are considered as transporters with several different functions, including immune response, cell growth, proliferation and metabolism, iron transportation, and synthesis of prostaglandins [14].

NGAL is a protein of the lipocalin family group of molecules that bind low molecular weight ligands and function in various biological processes ranging from vitamin delivery to pheromone transport [12–15]. NGAL is a 25-kDa monomer with neutrophil gelatinase, although the association appears to be functionally irrelevant [15]. NGAL is thought to be an acute-phase protein with up-regulated expression in different inflammatory conditions as well as in different cancers [15]. Recently, it has been found that NGAL binds small iron-carrying molecules (so called siderophores) and it is critical in various states, including bacterial infection and kidney injury [16–18]. Its potency as a bacteriostatic agent is due to the sequestration of iron, thus depriving bacteria of iron, leading to failure to grow and subsequent death [16]. NGAL was also reported to protect MMP-9 (matrix metalloproteinase 9) from degradation. Moreover, NGAL was demonstrated to protect against acute ischemic kidney injury [19] and urinary NGAL was found to aid in the noninvasive diagnosis of HIV-associated nephropathy and screen for HIV-associated nephropathy-related tubular damage [20].

In his elegant paper, Goetz et al. [16] revealed the mechanism of NGAL antibacterial iron depletion strategy of the innate immune system. At the site of inflammation/infection, NGAL is released by neutrophils to sequester bacterial ferric siderophores. NGAL only binds iron in complex with a nonheme compound. More precisely, it tightly binds bacterial catecholate-type ferric siderophores through a cyclically permuted hybrid electrostatic/cation- π interaction, while at the same time other components of the innate immune system, such as lactoferrin, simply bind to and sequester free iron. Further support for this finding came from the study on NGAL-deficient mice conducted by Flo et al. [21], which developed a markedly increased sensitivity to Gram-negative bacterial infections and an increased susceptibility to death from sepsis [22].

It has been suggested that NGAL comprises a critical component of innate immunity to exogenous bacterial infections. This is consistent with normal expression of NGAL in a number of human tissues typically exposed to the external environment, including the respiratory, gastrointestinal and urinary tracts [23]. Moreover, as suggested by Schmidt-Ott et al. [24], distal nephron-urinary NGAL, synthesized in acute kidney injury and chronic kidney disease, acts as a prophylactic antimicrobial agent preventing ascending infection from the lower urinary tract.

Recently, the potential role of NGAL as a biomarker of acute kidney injury was highlighted [25]. Mislocalized iron may be the underlying mechanism of the acute tubular necrosis [26]. Iron-catalyzed damage is thought to be one of the earliest events in kidney dysfunction. In post-ischemic kidney, NGAL is markedly upregulated in proximal tubules and the distal nephron segment [19]. In the former, it co-localizes at least in part with proliferating epithelial cells [19]. The similar pattern of proximal tubule NGAL expression was observed following nephrotoxic injury after cisplatin administration [26]. In the mouse model of ischemia-reperfusion kidney injury, administration of recombinant NGAL before or just after the insult resulted in marked preservation of kidney structure and function. It also attenuates apoptosis of tubule cells and enhances tubular cell proliferation [19].

NGAL could potentially recycle iron into the viable cells, thus stimulating growth and development. NGAL may also serve as a storage place for iron released from damaged tubule cells. By removing iron from the site of injury, NGAL may limit iron-mediated cytotoxicity. These protective effects were due to simultaneous transport of iron and siderophores to the proximal tubules. At

the same time, upregulation of heme-oxygenase-1 (a protective antioxidant) and preservation of N-cadherin expression in the proximal tubules were demonstrated [19]. NGAL was also identified as iron-transporting protein during nephrogenesis [18]. However, the overall repertoire of NGAL function remains to be established.

Some suggestions on a possible relationship between NGAL and anemia were proposed by Jiang et al. [27]. They found that anemia upregulated lipocalin 2 in the liver and serum in a murine model. In addition, Weizer-Stern et al. [28] studying thalassemia, an inherited anemia, reported decreased hepcidin expression together with increased NGAL expression in the liver in a thalassemia intermedia mouse model. Ho et al. [29] studied urine biomarkers of acute kidney injury (using mass spectrometry-proteomic analysis). They reported the opposite predictive effects for the development of acute kidney injury of urinary hepcidin and NGAL. Upregulation of hepcidin in the thick ascending limb of the cortex and connecting tubules after renal ischemia may lead to downregulation of the ferroportin exporter, preventing the further release of free toxic iron [29, 30]. Moreover, expression of hepcidin in the liver is regulated, not only by hypoxia, anemia and inflammation, but also by heemojuvelin (as a co-receptor) and the bone morphogenetic protein pathway. NGAL, by binding to siderophore iron, limits iron availability to possible pathogens. Thereafter, the NGAL-siderophore complex delivers iron into cells to permit growth and development [16]. While information today has implicated NGAL-mediated iron uptake only in renal development, it is possible that it functions elsewhere, particularly when normal pathways are absent.

Hepcidin and Iron

On the other hand, our understanding of the molecular control of iron metabolism has increased dramatically over the past 5 years due to the discovery of hepcidin [31, 32]. This is a circulating antimicrobial peptide mainly synthesized in the liver, which has recently been proposed as a factor regulating the uptake of dietary iron and its mobilization from macrophages and hepatic stores. Kulaksiz et al. [7] suggested that the kidneys, in addition to the liver, are involved not only in the synthesis, but may also participate in hepcidin elimination as well. Hepcidin seems to be filtered and reabsorbed in the proximal tubule, similarly to β 2-microglobulin [33].

Hepcidin connections to iron metabolism were postulated by Nicolas et al. [34] and Pigeon et al. [35]. Nicolas et al. [34] identified that the murine gene hepcidin had a

role in iron metabolism completely serendipitously as they were working on glucose metabolism-related transcription factor USF2 (upstream stimulatory factor 2) knockout mice, which unexpectedly were iron-overloaded. Pigeon et al. [35] was searching for iron upregulated genes when they discovered that murine hepcidin mRNA expression was increased by iron overload and decreased by iron depletion. Injection of hepcidin inhibited intestinal iron absorption in mice independent of their iron status and did not require the HFE gene product [36]. Rivera et al. [3] showed that hepcidin injection results in dose-dependent hypoferremia. On the other hand, iron administration in humans induced hepcidin secretion in urine [37].

These findings suggest that hepcidin plays a role as a negative regulator of intestinal iron absorption and iron release from macrophages. Evidence from transgenic mouse models indicates that hepcidin is the predominant negative regulator of iron absorption in the small intestine, iron transport across the placenta and iron release from macrophages [38]. Hepcidin controls intestinal iron absorption by regulating ferroportin expression on the basolateral membrane of enterocytes [39]. Hepcidin directly regulates the expression of ferroportin on cell membranes, binding to ferroportin and inducing its internalization and degradation, thus trapping iron in enterocytes, macrophages and hepatocytes. The net effect of hepcidin is the diminished absorption of dietary iron, sequestration of iron in macrophages and sequestration of iron in hepatic stores.

Hepcidin is an acute phase protein [40] that is synthesized to restrict the body's iron stores and prevent iron from being requisitioned by invading microorganisms, but this does not explain how it responds to the body's iron needs. It is possible that this missing link involves hypoxia.

Recently, matriptase-2, a liver transmembrane serine protease, has been found to have a major role in controlling hepcidin gene expression. In mice, defects in the *Tmprss6* gene encoding matriptase-2 result in high hepcidin expression and cause severe microcytic anemia. Similarly, mutations in the human *TMPRSS6* gene have been identified in patients with iron-refractory iron deficiency anemia-IRIDA [41]. IRIDA is associated with severe microcytosis, whereas the anemia of chronic disease is typically normocytic. In IRIDA high serum ferritin, low TSAT, even after parenteral iron treatment, is typical for findings.

Hepcidin and Iron and NGAL

Patients with chronic kidney disease have a chronic inflammatory state. This is due to many underlying factors, including an enhanced incidence of infections, the uremic milieu, elevated levels of proinflammatory cytokines, frequent presence of widespread arteriosclerosis, etc. There are additional factors with hemodialysis which may contribute to this process (impure dialysate, bio-incompatible dialysis membranes, intercurrent infections) [42–45]. In addition, iron metabolism is disturbed in chronic inflammatory diseases. Iron supplementation is often introduced in dialyzed patients to replace or to maintain iron stores, particularly in patients treated with erythropoiesis-stimulating agents.

Recently, Bolognani et al. [46] have suggested that altered NGAL levels in hemodialyzed patients were probably due to the fact that this protein was involved in iron metabolism. They suggested that elevated NGAL was probably related to its involvement in iron equilibrium. They reported that NGAL was predicted by hsCRP, TSAT and spKt/V. They also proposed that NGAL might be a new tool in the assessment of iron deficiency. In our recent study, we tested the hypothesis that NGAL might be related to hepcidin, not only to iron metabolism prevalent in hemodialyzed patients [47]. We found that in multiple regression analysis, residual renal function, hepcidin, creatinine and hsCRP were predictors of serum NGAL in hemodialyzed patients. We concluded that NGAL was highly induced in dialyzed patients. NGAL could reflect both kidney function and iron metabolism. However, these proteins involved in iron metabolism do not seem to be independently related.

It has been shown recently that the kidney is also involved in iron metabolism, as hepcidin was isolated from human urine [31]. On the other hand, NGAL, which was discovered by serendipity, is an iron-trafficking protein, a member of the non-transferrin-bound iron pool and an alternative to the transferrin-mediated iron-delivery pathway [17]. Both NGAL and hepcidin express antimicrobial properties. NGAL binds siderophores and interrupts the enterochelin-specific iron acquisition by bacteria, whereas hepcidin, an acute phase reactant, has intrinsic antibacterial activity like defensins killing on contact [48, 49] (fig. 2). Bacteria-developed siderophores retrieve iron from transferrin or lactoferrin of the host. However, while lactoferrin chelates iron directly, NGAL is unique in that it is specific for iron reserved for bacterial use as a ferric siderophore complex. The host has also developed defense mechanisms, such as increased production of iron-binding proteins (i.e. transferrin), fall in

[52] due to its role in iron metabolism. In addition, both proteins are associated with inflammation as well. Similarly, NGAL-binding bacterial catecholate-type ferric siderophores act as potent bacteriostatic agents by sequestering iron.

Elevated NGAL a few days after insult is a possible preventive or protective mechanism for limiting renal injury; however, extracorporeal circulation, such as a hemodialysis session, is a permanent insult to the body. We have to bear in mind that NGAL is an innate antibacterial factor as well as hepcidin. Microorganisms have de-

Fig. 3. Iron metabolism in the macrophage.

veloped several mechanisms to secure their iron supply (which is very scarce as a soluble molecule). The major mechanism is the ability to synthesize siderophores, the strongest known iron chelators. They are able to recover iron from transferrin and lactoferrin. NGAL binds siderophores, thereby preventing iron uptake by bacteria. Hepcidin, an antibacterial defensin highly induced in the liver as a response to IL-6 stimulation, prevents iron absorption from the gut and iron release from macrophages, leading to hypoferremia and anemia. Both proteins, therefore, sequester iron, but by different mechanisms (fig. 3).

At the present time, there is a lack of validated assays and robust data available for both hepcidin and NGAL; therefore, their possible relationship is still a matter of debate. Further research will hopefully elucidate this potential interaction. At present, there is no consensus on the best assay for hepcidin measurement, and assays for hepcidin detection and quantification in serum or urine

have not been generally available. Recently, other serum hepcidin assays based on the surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS), liquid chromatography tandem mass spectrometry (LC-MS/MS) and isotope dilution micro-HPLC-tandem mass spectrometry were developed, but they are not widely and commercially available [53]. ELISA and RIA assays for hepcidin determination are commercially available. There is also a commercially available ELISA assay for NGAL determination on both serum/plasma and urine. Moreover, using a point-of-care device (Triage, Inverness, Fla., USA), whole blood or plasma NGAL levels could be easily quantitatively available in 15 min and the device requires only a microliter sample. Urinary NGAL assay requires only 150 µl of urine, and results are available within 35 min on the Architect (Abbott) platform.

In conclusion, the subclinical inflammatory state is frequently found in chronic kidney disease. Moreover,

chronic kidney disease is associated with an impaired immune system. Both NGAL and hepcidin, elevated in chronic kidney disease, are not only involved in iron metabolism, but are also associated with inflammation and may be related to anemia. However, there is still an open question as to whether anemia in chronic kidney disease is the price to pay or an adaptive mechanism? As

reported recently, anti-hepcidin antibody treatment modulated iron metabolism and was effective in a mouse model of inflammation-induced anemia [54]. Whether this treatment would be effective in humans remains to be established, as does a possible role of NGAL in the modulation of iron metabolism and its contribution to anemia.

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