The Role of Hepcidin in Iron Sequestration during Infections and in the Pathogenesis of Anemia of Chronic Disease

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Abstract

Systemic infection or inflammation causes a decrease in intestinal iron absorption and impairs the release of recycled iron from macrophages. Decreased availability of iron may deny this essential element to invading pathogens and may inhibit their multiplication and other metabolic processes but also results in anemia of chronic disease. This article reviews recent discoveries that shed light on the regulation of iron metabolism during infection and iron overload, and point to the central role of a newly discovered peptide, hepcidin. Evidence to date indicates that hepcidin is a negative regulator of intestinal iron absorption, placental iron transport, and the release of iron from macrophages that recycle iron from senescent red cells. It may also be the central mediator of iron sequestration during infections and inflammatory states and the mediator of anemia of chronic disease. Rapid progress in this area is a good example of the beneficial effects of improvements in peptide analysis and chemistry, advances in genomics, and the increasing use of transgenic mice to determine the function of newly discovered genes and proteins.

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This is the kind of story that I would have told Yaacov Matzner on an airplane or bus to a hematology meeting. It is a short story about good luck and good science, with many participants all over the globe. My involvement began during our survey of antimicrobial peptides and proteins in various body fluids. In 1995, I read a report from Bensch and his colleagues in Germany that human plasma contained a new β-defensin (named human β-defensin-1) produced in the kidney [1]. We confirmed and expanded on this discovery, characterized the peptides and their activity, and located the site of synthesis in the epithelial cells of the distal tubule and collecting ducts [2]. The urine also yielded a surprise in the form of several peptide peaks that were not defensins. We collected many liters of urine and developed a procedure for purifying the new peptides. On amino acid sequencing and mass spectrometry, these turned out to be very unusual, containing 8 disulfide linked cysteines in the space of 20 amino acid residues. The other peaks also belonged to this peptide but contained slightly longer versions, 22 and 25 amino acids in size. The peptides were cationic but were distinct from any previously characterized peptide family. Within one hour of receiving the peptide sequences, a computer search of the genomic databases showed that the genomic sequence had been determined as part of a large-scale sequencing project but the gene encoding our small peptide was not recognized as such. The collection of human expressed sequence tags also contained an mRNA fragment that encoded our peptide.

We obtained the EST clone and used collections of mRNA from various human organs (organ-blot) to establish that the mRNA was predominantly expressed in the liver. We then recloned the full-length mRNA, extended the mRNA sequence, and matched it to the genomic sequence in the database. The full-length peptide precursor was 84 amino acid long and contained a characteristic endoplasmic reticulum-targeting signal sequence, and a stretch of cationic amino acids characteristic of a prohormone convertase (furin)-type cleavage site that would generate the 25 amino acid peptide. We produced both the 20 and 25 amino acid forms of hepcidin by chemical synthesis, then successfully refolded the peptides and allowed them to form the requisite disulfide bonds. In reverse-phase high performance liquid chromatography and electrophoretic analysis, the synthetic peptides behaved identically to the native forms. Because of their resemblance to other antimicrobial peptides we tested them for antimicrobial activity. The peptides turned out to be broadly antimicrobial but were somewhat less potent than many other previously characterized antimicrobial peptides. Based on this activity and their origin in the liver we named them "hepcidin" 20 and 25 respectively.

By its size and cysteine content, hepcidin also resembled the antimicrobial peptides of certain insects whose blood (hemolymph) becomes antimicrobial after they are infected or injured [3]. Like hepcidin, the insect peptides are produced in the insect equivalent of the liver, the fat body. The response of human hepcidin during a systemic infection was unexpectedly tested sooner than had been planned: I developed prostatitis, and because of a busy travel schedule neglected the early symptoms. Several days later, the infection extended into the epididymis and I became clinically septic. Indeed, the urine serially collected during and immediately after the septic episode initially contained very high concentrations of hepcidin that declined as the infection resolved.

Fortunately for further progress in this project, we had entered our early peptide sequences and mRNA sequences into the Genbank database and provisionally labeled the product as an antimicrobial peptide. We were very pleased when we received an e-mail from a French group (Drs. Loreal and Pigeon) who cloned and sequenced differentially expressed mRNAs in the livers of iron-overloaded mice. One of the transcripts that was highly expressed in the iron overloaded livers was very similar to the human hepcidin mRNA. Their group had also gone on to sequence the human mRNA and gene. Importantly, they could also test the induction of hepcidin by infection in the mouse by injecting lipopolysaccharide,

EST = expressed sequence tags

and their experiments directly confirmed our human observation in a much better controlled system. We decided to coordinate our efforts and submit our papers jointly to the *Journal of Biological Chemistry* [4,5]. The antimicrobial activity and inducibility of hepcidin by infection were later confirmed in a fish model (hybrid striped bass) [6]. Remarkably, the bass hepcidin mRNA was increased 4,500-fold by bacterial infection.

A few months before the papers were to appear back-to-back in preliminary form in the *Journal of Biological Chemistry*, the group of Forssmann et al., that had previously found human β-defensin-1, now reported isolating hepcidin (which they named liver-associated antimicrobial peptide or LEAP) from human plasma [7] as a part of a large and remarkably successful effort to enumerate and characterize its peptide components. To find the plasma peptides, these investigators analyzed huge volumes of human hemodialysate obtained as a waste by-product of the treatment of patients with renal failure. The pores in the dialysis membrane allow peptides to cross into the bath from where they can be recovered and characterized. Their independent discovery confirmed our suspicion that the peptide was secreted by the liver into plasma and filtered into the urine essentially unchanged.

Eventually, additional insight into the unusual structure of hepcidin came from our collaboration with structural biochemists [8]. I mentioned the hepcidin story to a colleague at the University of Iowa, Brian Tack, and he put me in touch with two groups that had recent experience in the characterization of disulfide-rich peptide structures. After several months of intense work the structure finally came together. Although we had predicted a simple "bent beta-turn" structure for hepcidin in our initial paper, the disulfide linkage we proposed was incorrect. In considering possible structures for hepcidin we prematurely dismissed the rare possibility that two immediately adjacent cysteines could form a disulfide bond. The NMR studies indicated just that.

In the meantime, the French group of Nicolas, Vaulont, et al. ingeniously exploited a lucky break. A knockout mouse constructed to study the role of a transcription factor USF2 in glucose metabolism developed spontaneous hemochromatosis with iron overload in the liver and other organs, but in the macrophage-rich spleen iron accumulation was less than in normal controls [9]. The two murine hepcidin genes were located immediately downstream of the knockout region and these mice did not express any hepcidin transcripts. The hepcidin promoter region did not contain any USF2 binding motifs, and the authors correctly surmised that the phenotype was the result of an inhibitory effect of the knockout construct on nearby gene expression. The accompanying editorial by Fleming and Sly in the *Proceedings of the National Academy of Sciences* went on to predict that hepcidin is the long-sought iron regulatory hormone made in the liver in response to inflammation and iron overload and acts on the small intestine to inhibit iron absorption and on macrophages to inhibit iron release. They speculated that hepcidin induction by inflammatory stimuli is responsible for anemia of chronic disease [10]. From my point of view as an innate immunologist, hepcidin could be the signal that connects the response to systemic infection to iron metabolism, restricting the availability of iron to invading pathogens.

Two other transgenic mice then strengthened this hypothesis [11]. The first mouse was made to overexpress hepcidin in the liver using a liver-specific promoter. These mice were born severely anemic, presumably as a result of the inhibition of iron transport in the placenta. Mosaic mice that survived had severe iron deficiency anemia despite adequate dietary iron intake. The other mouse, a differently constructed USF2 knockout, had entirely normal iron metabolism, indicating that USF2 was not directly involved in the regulation of hepcidin expression. These experiments solidified the role of hepcidin as a central regulator of iron absorption.

Clinical studies with representative patient populations will be necessary to confirm the suspected role of hepcidin as the mediator of anemia of chronic disease in chronic infections, inflammatory conditions, and possibly in cancer. Are there other diseases in which hepcidin plays a pathogenic role? Early human genetic analyses indicate that hepcidin mutations or deletions are not a common cause of human hemochromatosis [12]. However, a recent study provides evidence that dysregulated hepcidin production is the cause of refractory anemia seen in patients with large hepatic adenomas [13].

In summary, a picture has emerged in which hepcidin acts as a signal between innate immunity and iron metabolism [Figure 1]. We still do not know the mechanism by which hepcidin regulates iron transport. It remains to be determined what the hepcidin receptor is and how its signal is transduced. Current data provide strong evidence for hepcidin as a regulator of intestinal absorption of iron and the transport of iron across the placenta, but the evidence for the role of hepcidin in the regulation of iron recycling by macrophages is somewhat weaker. Clearly, much remains to be done. Nevertheless, the rapid progress made in this new area is a tribute to the hard work of a number of research groups around the globe. Yaakov Matzner would have enjoyed hearing about it.

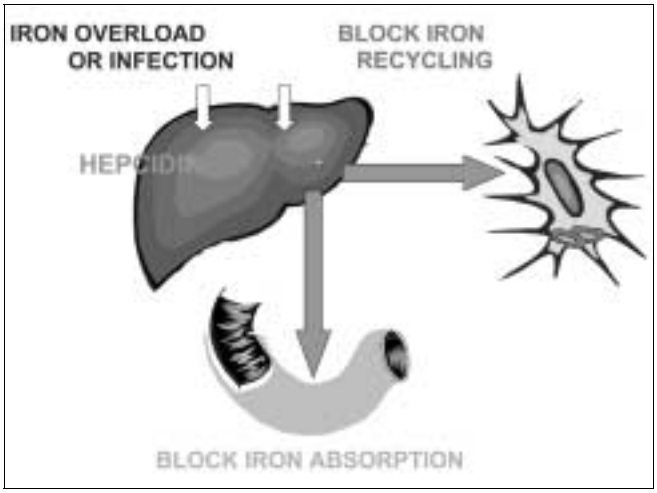

Figure 1. The proposed role of hepcidin during infection or iron overload. Hepcidin released from the liver inhibits iron uptake in the small intestine and blocks the recycling of iron from senescent red cells in macrophages of the spleen.

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Capsule

Targeted vaccination

Policy-makers have been debating how to respond to an intentional release of smallpox in the United States. Halloran et al. generated model communities of 2,000 people, interacting within schools and neighborhoods. They looked at the effects of targeted vaccination of those in close contact with smallpox cases relative to mass vaccination carried out before or after the release event. The presence of residual immunity from prior vaccination increased the effectiveness of the targeted strategy more than mass vaccination. Under all strategies, targeted vaccination prevented more cases per dose of vaccine than did mass vaccination.

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Capsule

High risk of colorectal cancer among Ashkenazi Jews

A genetic mutation, often found in the offspring of Ashkenazi Jews (origin Eastern Europe), can double or even treble the risk of colorectal cancer (CRC), reports *Science*. Two independent teams that analyzed the DNA from over 3,000 Ashkenazi Jews in northern Israel and New York City found an association between mutations in the gene *BLM* and increased susceptibility to the disease. This mutation is present in about 1% of this ethnic group. Dr. Gruber, director of clinical cancer genetics at the University of Michigan's Cancer Center, explained: "When this mutation is inherited from both parents, it increases an individual's predisposition to cancer." However, he stressed that although the mutation can be detected with DNA analysis it is too soon for DNA tests to be used as a screening tool.

Colorectal cancer is the leading cause of deaths from cancer in Israel and the second leading cause in the U.S. Over 2,000 people are diagnosed with CRC in Israel every year, but incidence rates vary widely among different ethnic groups. Close to 150,000 Americans were diagnosed with the disease in 2002. In a companion paper in the same issue of *Science*, scientists at the University of Cincinnati report that transgenic mice designed to carry the human BLM mutation have the same increased risk. The results show the value of combining molecular genetics with traditional epidemiology in cancer research.

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