Role of lung iron in determining the bacterial and host struggle in cystic fibrosis

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Reid DW, Anderson GJ, Lamont IL. Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 297: L795–L802, 2009. First published August 21, 2009; doi:10.1152/ajplung.00132.2009.—Cystic fibrosis (CF) is the most common lethal genetic disorder in Caucasian populations. It is a multiorgan system disease that affects the lungs, gastrointestinal tract, liver, and pancreas. The majority of morbidity and mortality in CF relates to chronic airway infection with a variety of bacterial species, commencing in very early infancy, which results in lung destruction and ultimately organ failure (41, 43). This review focuses on iron homeostasis in the CF lung and its role in determining the success and chronicity of Pseudomonas aeruginosa infection. There have been previous excellent reviews regarding iron metabolism in the lower respiratory tract and mechanisms of P. aeruginosa iron acquisition, and we direct readers to these articles for further background reading (31, 53, 58, 77, 96). In this review, we have brought the “two sides of the coin” together to provide a holistic overview of the relationship between host and bacterial iron homeostasis and put this information into the context of current understanding on infection in the CF lung.

Pseudomonas aeruginosa; iron homeostasis

NORMAL LUNG IRON HOMEOSTASIS

Iron is a redox active metal that is essential for the normal function of a wide range of cellular proteins (see Fig. 1). Thus, almost all forms of life, and certainly all vertebrates, have an absolute requirement for iron. Iron shuttles between two thermodynamically stable oxidation states, Fe(II) (Fe2⁺; ferrous iron) and Fe(III) (Fe³⁺; ferric iron), and it is this capacity that underlies its biological utility. Ferrous iron is very soluble in aqueous solution, and it is this form of iron that is transported across the membranes of mammalian cells. However, in biological solutions within living organisms, ferrous iron is readily oxidized to the ferric form, which is highly insoluble at physiological pH. Many proteins, such as the iron transport protein transferrin and the iron storage protein ferritin bind iron in its ferric form. Whereas ferric iron is relatively non-toxic, ferrous iron is able to catalyze reactions (such as the Fenton reaction), leading to the production of highly reactive oxygen radicals, and thus ferrous iron is quite toxic.

Iron homeostasis in the normal lung is quite complex and remains poorly understood. The lung consists of a range of cell types, and each of these must be able to acquire iron to meet their metabolic requirements. They must also be able to either store or export any excess iron to prevent it from increasing to toxic levels within the cell. As with most body cells, cells in the lung obtain their basic iron supplies from the plasma. However, the lung can also be exposed to iron from the external environment in the form of inhaled iron-rich particulate matter. Ferrous ions that are not tightly bound to proteins have the potential to catalyze reactions leading to the production of toxic oxygen radicals (as noted above) and are also available for uptake by inhaled pathogens. Thus the eukaryotic lung has evolved highly efficient means of limiting the amount of extracellular iron available for oxidative toxicity and pathogen growth.

Iron is moved around the body bound to the plasma protein transferrin (Tf). Tf has an exceptionally high affinity for iron and ensures that the level of potentially toxic “free” iron in solution is maintained at very low levels. Tf-bound iron is the major iron source for most cells, including those in the lung. Much of this iron is delivered to cells after diferric-Tf interacts with transferrin receptor 1 (TfR1) on the plasma membrane, but many cells can also utilize Tf-bound iron by nonreceptor-mediated mechanisms (29, 89). After diferric-Tf is internalized, it presents its iron to endosomes, and the metal is transported across the endosomal membrane into the cytoplasm via the membrane iron transporter divalent metal-ion transporter 1 (DMT1). This iron can then be incorporated into many proteins that are required for basic cellular functions. Intracellular iron in excess of metabolic requirements is incorporated into the cytoplasmic protein ferritin where it can be stored in a non-toxic form (8).

Under certain pathological conditions, for example, in subjects with iron loading disorders, plasma Tf can become saturated with iron, and measureable amounts of non-transferrin (and non-lactoferrin) bound iron (NTBI) can appear in the circulation, which can be efficiently taken up by many cells, including those of the lung. In addition to being found on
splice variants are important for iron uptake, and both may be expressed on the plasma membrane of cells that need to respond to iron demand quickly, such as intestinal epithelial cells. However, the proteins generated from both splice variants are important for iron uptake, and both may be particularly highly expressed on the plasma membrane of cells where the protein encoded by the IRE-containing mRNA is expressed when iron levels are low. When iron levels are high, IRP1 exists in its non-RNA binding form, whereas IRP2 is degraded. The effect of IRP binding on cellular iron homeostasis is to decrease TfR1 expression, and, therefore, iron uptake and to increase ferritin expression when iron levels are high and to raise TfR1 levels and reduce ferritin expression when iron levels are low.

endosome membranes, DMT1 is found on the plasma membrane of lung epithelial cells, and it is likely to be important in the uptake of NTBI. Other proteins that have been proposed to play a role in NTBI uptake in various tissues include the anion exchange (AE) proteins (93), the zinc transporter ZIP14 (Zrt-Irt-like protein 14) (56), and L-type voltage-dependent calcium channels (72). Since NTBI, by definition, is a form of iron not tightly sequestered by proteins, it has the potential to contribute to the generation of oxygen radicals and is considered quite a cytotoxic form of iron (72). Since DMT1 plays a central role in the cellular acquisition of transferrin-bound iron, and likely NTBI as well, its role in the lung has been investigated in more detail. The DMT1 gene encodes two types of transcripts distinguished by the presence or absence of an iron responsive element (IRE) in their 3'-untranslated region. The non-IRE variant is widely expressed in body tissues, and it is likely to encode the protein involved in endosomal iron transport, whereas the protein encoded by the IRE-containing mRNA is particularly highly expressed on the plasma membrane of cells that need to respond to iron demand quickly, such as intestinal epithelial cells. However, the proteins generated from both splice variants are important for iron uptake, and both may be expressed on the plasma membrane. The levels of both DMT1 splice variants also increase when cellular iron demands increase. Interestingly, cultured airway epithelial cells exposed to iron exhibit increased expression of the non-IRE isoform of DMT1, whereas the IRE-containing DMT1 isoform remains unchanged (32, 99). It has been proposed that lung epithelial cells (and hepatocytes) need a mechanism whereby NTBI is taken up and detoxified, and thus it makes physiological sense for a major NTBI uptake pathway to be stimulated. It has been known for many years (well before DMT1 was identified) that NTBI uptake by the liver is enhanced when iron levels are high (105). As the lung is exposed to significant amounts of catalytically active iron on a daily basis, DMT1-mediated iron uptake in conjunction with stimulated ferritin synthesis are protective mechanisms likely to reduce oxidative stress (30, 94). Importantly, with respect to cystic fibrosis (CF), DMT1 expression by airway epithelial cells is increased by bacterial endotoxin and proinflammatory mediators such as IL-1β and TNFα (60, 98), and this is likely a further adaptive response to limit iron-associated damage.

Apart from taking up iron for metabolic requirements, cells can export stored iron if needed. The only known iron export
protein is ferroportin (FPN). Cells that export large quantities of iron, such as macrophages, including alveolar macrophages, express high levels of FPN, but most cells, including airway epithelial cells, show some expression. Airway epithelial cells upregulate FPN expression in the presence of iron, and FPN is thought to play a role in lung iron detoxification, but the mechanisms remain poorly characterized at present (47, 108). This highlights the complexity of iron homeostasis in the lung, with increased intra- and extracellular iron levels having the potential to modulate cellular iron uptake (through DMT1) and export (through FPN). It is the fine balance between these processes that maintains normal cellular physiology in the lung, but when pathological changes occur (e.g., in CF with or without bacterial infection), disruption in this delicate iron balance can lead to cellular iron accumulation and exacerbate tissue damage. Alveolar macrophages (AM) express all of the necessary proteins for iron uptake, storage, and release, but they also express a specific DMT1 homolog, Nramp1, in the membrane of their phagosomes. This iron transporter clearly plays a role in pathogen killing, but whether it does so by specifically preventing bacterial access to iron, or by providing iron to the phagosome to fuel oxygen radical generation and kill bacteria, remains unresolved (106).

There are few published data on pulmonary iron metabolism in disease states. The presence of increased concentrations of ferritin and numbers of hemosiderin-laden macrophages (HLM; hemosiderin is a derivative of ferritin) in bronchoalveolar lavage fluid and induced sputum samples is well described in tobacco smokers, and HLM are prominent following pulmonary hemorrhage (25, 34, 35, 45). AM are not the only host immune cells within the lung that have the capacity to take up iron. Human polymorphonuclear cells (PMNs) cultured ex vivo are capable of acquiring iron from the P. aeruginosa siderophores pyoverdine and pyochelin (9), although the mechanism of iron acquisition has not been determined. An interesting finding was that premixing PMNs with polyvalent cationic metal ions such as gallium appeared to enhance PMN iron acquisition from siderophores, which may complement the separately described effects of gallium on P. aeruginosa iron homeostasis. PMNs may also affect bacterial access to iron by secreting lactoferrin, which chelates ferric ions but can be degraded by bacterial proteases. They may also make neutrophil gelatinase-associated lipocalin, which binds many bacterial siderophores, making them unavailable to the bacteria (5). Whether these components of PMN function are impaired in CF is unknown but worthy of further investigation.

LUNG IRON IN CF

Appreciable amounts of iron have been found within the CF airway, and this has previously been attributed to occult hemorrhage, inflammation, or a consequence of bacterial acquisition (80). There is evidence that transferrin and lactoferrin undergo proteolysis in CF (8), and this may increase the amount of iron that is present. However, the possibility that this increased iron might instead represent an inherent problem with iron handling has been considered. This was recently confirmed by Moreau-Marquis et al. (66), who found that cultured CF airway epithelial cells accumulated excessive amounts of intracellular iron, which was then lost to the extracellular space with resultant promotion of P. aeruginosa biofilm formation.

IRON AND INFECTION

Like their hosts, pathogenic bacteria require iron as a cofactor for numerous enzymes essential for metabolism, including those involved in normal respiration. Free iron in living organisms is usually present at concentrations of $10^{-9}$ M or less, which is too low to be sufficient for bacterial growth (11, 79), although higher concentrations may be present in the CF lung, as described above. In mammals, most extracellular iron is incorporated into proteins of the transferrin family, which have particularly high affinities for iron ($K_d \approx 10^{-20}$ for transferrin and lactoferrin). This “iron withholding” substantially reduces the bioavailability of iron to infecting bacteria and constitutes an important component of innate immunity against bacterial infection (79, 101). Saturating host iron-binding proteins with added iron exponentially increases bacterial pathogenicity, allowing uncontrolled bacterial replication (79). For example, addition of iron reduced the lethal dose (LD50) of P. aeruginosa by up to 1,000-fold in a mouse model (28). Iron withholding is also a key component of the systemic inflammatory response. A usual accompanying feature of systemic inflammation is sequestration of iron in the tissues, and this serves to reduce the level of saturation of circulating host iron-binding proteins (101).

While host iron limitation has evolved to combat infection, microorganisms have fought back. Bacteria have evolved multiple pathways for iron acquisition, and many of these are employed to good effect in the disease setting. One of the most effective mechanisms utilized by many bacterial species is the secretion of siderophores. These are iron-scavenging molecules with high affinities for Fe$^{3+}$ ions, typically with formation constants in the range of $10^{22}$ to $10^{35}$. Pathogenic bacteria produce multiple siderophores that are required for successful infection (12, 59, 79). In this article, the focus is on iron acquisition by P. aeruginosa through the production of the siderophores pyoverdine and pyochelin.

P. AERUGINOSA IRON HOMEOSTASIS

P. aeruginosa has evolved a highly effective armamentarium to acquire iron from its environment and successfully compete with other organisms, including the CF host, for access to this essential nutrient (see Fig. 2). P. aeruginosa has been a major focus of research because it is found in the majority of CF patients’ lungs, often at high density (over $10^8$ cfu per ml of sputum) (1, 80). Infection with P. aeruginosa is correlated with a progressive and relentless decline in patient health (13, 27, 51). There is considerable evidence that in CF, P. aeruginosa exists in biofilms rather than in a free-floating planktonic state (54, 84, 109). Biofilms are highly structured bacterial communities that are encased in a biopolymer matrix, and gene expression in biofilm-dwelling bacteria is very different from that of planktonic bacteria (18, 39, 90). Biofilms confer significant antibiotic resistance and are difficult to eradicate (95). Oxygen availability in biofilms in the CF lung may be low (104, 110), which would be expected to significantly affect the physiology of the bacteria. Iron acquisition drives much of P. aeruginosa’s behavior, and ~6% of transcribed genes in P. aeruginosa are iron responsive (96). Im-
shown in CF that pyoverdine-mediated iron transport is important for biofilm development (4, 75). Mutant strains of *P. aeruginosa* that do not synthesize pyoverdine are unable to develop normal biofilms under conditions of iron starvation, but addition of exogenous pyoverdine restores robust biofilm formation. In contrast, pyochelin appears less important for biofilm development (4).

Pyoverdine and to a lesser extent pyochelin can acquire iron from host proteins, including members of the transferrin family (88, 107). Furthermore, proteases secreted by *P. aeruginosa* can degrade lactoferrin and transferrin and thus increase the ability of pyoverdine to acquire iron from these molecules (24, 103). The availability of iron for *P. aeruginosa* in CF is complicated by the presence of other infecting organisms such as *Staphylococcus aureus* or *Burkholderia* species that may either compete for available iron or increase its bioavailability. *P. aeruginosa* has the capacity to use a wide range of siderophores synthesized by other organisms (17, 77). Microbial interspecies iron transactions in disease have been little studied, but competition for iron may have a major influence on bacterial growth. In one in vitro study that is relevant to CF, the presence of *S. aureus* caused reduced expression of iron uptake genes of *P. aeruginosa*, implying that *P. aeruginosa* was able to obtain iron from *S. aureus* (57). Conversely, coculture of *P. aeruginosa* with *Burkholderia* spp. caused increased expression of iron-responsive genes in *P. aeruginosa*, including those involved in pyoverdine and pyochelin synthesis, because of iron sequestration by the competing *Burkholderia* siderophore ornibactin (100). *Burkholderia* species from patients with CF can also make pyochelin (21, 86), which adds another mechanism whereby iron may be delivered to *P. aeruginosa*. Consistent with these potential interactions between *B. cepacia* and *P. aeruginosa*, these bacteria can exist together in mixed
biofilms (26), providing intimate interactions that offer the opportunity for exchange of siderophores and iron cross-feeding, or conversely provide a high potential for competition for iron. Whether iron sharing or iron competition predominates in this coinfection in the CF lung has not been investigated. However, superinfection with B. cepacia in the setting of preexisting chronic P. aeruginosa infection can either precipitate a life-threatening illness or be relatively innocuous (44, 61, 102). The outcome depends on the B. cepacia (and P. aeruginosa) strain, as well as host factors, but the role of iron availability in affecting bacterial behavior and virulence in this setting is worth considering (44).

EVIDENCE FOR P. AERUGINOSA IRON ACQUISITION IN THE CF LUNG

The majority of the data available on P. aeruginosa iron acquisition mechanisms has come from in vitro studies using laboratory strains, and relatively little is known about iron scavenging in the disease state. P. aeruginosa utilizes a wide range of iron-scavenging mechanisms, which have been previously reviewed (77, 96). The focus of this review is on the role of the two iron-scavenging siderophores, pyoverdine and pyochelin, in P. aeruginosa iron homeostasis in CF. Most CF strains of P. aeruginosa cultured ex vivo synthesize and utilize either type I or type II pyoverdine (23, 63), although one epidemic strain has been shown to exclusively use type III pyoverdine (52). An approach to understanding iron acquisition by P. aeruginosa in CF has been to expose P. aeruginosa cultured in vitro to CF sputum and assess the effects on bacterial gene expression (73, 74). Growing P. aeruginosa in the presence of CF sputum has been shown to upregulate the expression of a wide range of iron-acquisition genes, including those involved in pyoverdine and pyochelin synthesis and uptake, as well as heme uptake (hasA) (74). In contrast, a microarray assessment using bacterial mRNA extracted directly from CF sputum (a single patient) has demonstrated increased expression of only those genes required for pyochelin, but not pyoverdine synthesis (87). A potential explanation for the variable results obtained when using such microarray techniques comes from the observation that isolates from patients chronically infected with P. aeruginosa over many years may lose their ability to make pyoverdine, although they retain the ability to take up ferripyoverdine (23, 85). This is consistent with the fact that the concentrations of iron in CF sputa (generally greater than 10 μM) are sufficient to suppress pyoverdine-mediated transport in vitro (62). Exposure of P. aeruginosa to such iron concentrations over time in CF may allow mutations in the pyoverdine system, which would not be biologically disadvantageous (71). Thus, complex microarray analyses performed on solitary isolates run the risk of missing the true in vivo situation, especially if the resident P. aeruginosa population in the CF lung in some patients may have dispensed with the ability to produce pyoverdin (23).

An alternative approach to dissecting out P. aeruginosa iron acquisition mechanisms in CF is to look directly for the presence of siderophores in sputum. Haas et al. (37) demonstrated the presence of pyoverdine in 6/12 CF sputum samples, in amounts corresponding to a mean concentration of just under 1 μmol. The majority of the pyoverdine (54–88%) was ferrated. Spectral analysis indicated that pyoverdine was present in the remaining six samples, but the amounts were too low for purification. We have recently refined these methods and detected pyoverdine in sputum from 25 of 28 CF patients chronically infected with P. aeruginosa in amounts ranging from 0.7 to 51 μmol (unpublished observation). The presence of pyoverdine in most CF sputa implies a continued role in iron acquisition by the bacteria. The situation with pyochelin production in the CF lung is far less clear, and its much lower affinity of pyochelin for iron compared with pyoverdine and the host iron-binding proteins lactoferrin and transferrin questions the significance of pyochelin as an iron-scavenging agent in CF. However, P. aeruginosa isolates from CF patients have been found to produce pyochelin in vitro, which is consistent with the microarray work discussed earlier (38). So far as we are aware, pyochelin has not been identified in CF sputum using direct assay methods.

P. aeruginosa may also acquire iron from the CF host via siderophore-independent pathways. The presence of heme in CF sputum as a consequence of airway hemorrhage is relatively common clinically, especially during pulmonary exacerbations. P. aeruginosa takes up heme-bound iron through at least two specific pathways (70). In addition, under low-oxygen conditions such as may be experienced by bacteria living in biofilms in occluded CF airways, iron may be in the ferrous form. Genome analysis suggests that P. aeruginosa has the capacity to acquire ferrous ions through a FeoAB transport system that uses energy from GTP to import Fe^{2+} ions into cells of gram-negative bacteria (14, 15, 36), but this system has not been studied experimentally in P. aeruginosa. There are also suggestions that the airways in CF are abnormally acidified, potentially stabilizing Fe^{2+} ions and making them more available to the bacteria (92). However, there are no available data on any of these siderophore-independent bacterial iron uptake mechanisms in CF (92).

CLINICAL AND THERAPEUTIC IMPLICATIONS

The concept of targeting the “Achilles heel” of P. aeruginosa’s iron requirement is not a new one. There are several ways in which the iron homeostatic systems of P. aeruginosa may be exploited therapeutically. Conjugating antibiotics to siderophores to allow delivery direct to P. aeruginosa has been considered as part of a “Trojan horse” approach, but studies remain in their infancy (6, 7, 42, 49, 50). Interfering with P. aeruginosa iron uptake by blocking cell-surface receptors with cationic metals such as gallium or silver are promising approaches, which have been shown to inhibit P. aeruginosa biofilm formation and enhance antibiotic sensitivity (3, 40, 46, 48, 76). Alternatively, siderophore iron acquisition may be countered by the use of biological or synthetic chelators with higher binding affinities. Lactoferrin and synthetic iron chelators have been shown in vitro to inhibit P. aeruginosa growth and biofilm development (83). Very recently, currently available Food and Drug Administration-approved iron chelators deferoxamine and deferasirox were shown to enhance killing of biofilm-dwelling P. aeruginosa by tobramycin (67). Paradoxically, iron salts have also been proposed as potential anti-biofilm agents (68, 69), but such an approach would need to be monitored closely, given the ability of P. aeruginosa to scavenge iron from very many sources and the inherent risk of
oxidative damage to the lung following exposure to high levels of exogenous iron.

In summary, increased basic understanding of how iron availability and sequestration impacts on the outcome of the CF host and bacterial interaction is driving the development of new therapies that may alter the natural history of disease. The questions that remain to be addressed relate to the potential adverse effects of such treatments on *P. aeruginosa*, such as upregulated virulence in response to nutrient starvation, as well as confirmation that the CF host immune system’s capacity to eradicate *P. aeruginosa* will be enhanced.

**FUTURE RESEARCH DIRECTIONS**

The increased accumulation of iron in airway epithelial cells suggests either abnormal regulation of Tf-mediated iron uptake, altered iron storage within ferritin, or reduced iron export through FPN. Paradoxically, excessive loss of iron from CF epithelial cells (65) suggests that mechanisms involved in controlling airway iron homeostasis during CF are complex. The close functional roles of AE proteins and CFTR in chloride controlling airway iron homeostasis during CF are complex. FPN. Paradoxically, excessive loss of iron from CF epithelial cells (65) suggests that mechanisms involved in controlling airway iron homeostasis during CF are complex. The close functional roles of AE proteins and CFTR in chloride homeostasis during CF are complex. The close functional roles of AE proteins and CFTR in chloride homeostasis during CF are complex.

ROLE OF LUNG IRON IN BACTERIAL AND HOST STRUGGLE IN CYSTIC FIBROSIS

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