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Transferrin-mediated iron sequestration as a novel therapy for bacterial and fungal infections

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Pathogenic microbes must acquire essential nutrients, including iron, from the host in order to proliferate and cause infections. Iron sequestration is an ancient host antimicrobial strategy. Thus, enhancing iron sequestration is a promising, novel anti-infective strategy. Unfortunately, small molecule iron chelators have proven difficult to develop as anti-infective treatments, in part due to unacceptable toxicities. Iron sequestration in mammals is predominantly mediated by the transferrin family of iron-binding proteins. In this review, we explore the possibility of administering supraphysiological levels of exogenous transferrin as an iron sequestering therapy for infections, which could overcome some of the problems associated with small molecule chelation. Recent studies suggest that transferrin delivery may represent a promising approach to augment both natural resistance and traditional antibiotic therapy.

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Introduction

In recent years the crucial role of iron in microbial growth and pathogenesis has garnered increasing attention. Virtually all microbes must obtain iron in order to survive and propagate [1–4,5••], including disease-causing pathogens that establish infections in mammalian hosts. Many microbes have thus evolved specialized mechanisms to acquire this limited resource. Conversely, an evolutionarily conserved, common host strategy to control microbial growth is to strictly regulate levels of available free iron. The microbial requirement for iron suggests that strategies aimed at blocking iron acquisition by microbes might form the basis for promising, novel anti-infective therapies. Unfortunately, previous approaches using small molecule iron chelators have not proven safe and effective for treating clinical infections. In this review, we examine the potential of transferrin, a mammalian iron-binding protein, to be developed as a novel therapeutic for bacterial and fungal disease.

Limitations of small molecule iron chelators as therapeutics

The simplest approach to blocking iron acquisition by pathogens is the use of small molecule chelating agents that sequester iron and prevent microbial uptake. Numerous investigators over several decades have characterized a variety of iron sequestering agents that inhibit the growth of microbes *in vitro* [6,7,8,9••,10•]. However, several crucial barriers have limited development of small molecule chelators as therapies for infection. First is the production by bacteria of siderophores (e.g., *Staphylococcus aureus* staphyloferrin A and B, and *Acinetobacter baumannii* acinetobactin) that are secreted by the microbe to scavenge and acquire iron in environments where bioavailability is low [11,12••]. Siderophores are extremely strong binders to ferric (Fe^{3+}) iron, and often possess iron affinities that are 10^{10}-fold to 10^{20}-fold higher than small molecule or biological iron chelators [13,14]. Similarly, fungal species such as *Candida* also produce high affinity iron siderophores, and both fungi and bacteria (e.g., *Acinetobacter*) can uptake high affinity xeno-siderophores that are produced by other bacteria (e.g., desferoxamine) [15–21]. The extremely high iron affinities of microbial-derived siderophores, which are far higher than affinities for small molecule chelators, has led to the perception that iron acquisition by high affinity siderophores cannot be overcome *in vivo* by chelation-based therapy.

An additional problem is that small molecule chelators alter metabolic disposition of iron in ways that may be injurious to the host. For example, chelators reduce iron availability to myeloid cells, which are normally the predominant recyclers of iron in the host, and increase its excretion into renal tubules where iron is not normally found. Thus, serious toxicity to bone marrow, kidneys, and other organ systems can occur during small molecule iron chelator therapy [22,23].

As a result of these factors, *in vivo* testing of iron chelation strategies has focused on eukaryotic pathogens (e.g., malaria and molds) [24,25]. Unfortunately, the most advanced effort to develop a small molecule chelator into clinical trials for infection failed, as a recent randomized,

controlled trial of patients with mucormycosis found that small molecule iron chelation was not safe or effective [26•]. Nevertheless, the profound requirement for iron acquisition of microbes continues to spur translational efforts to develop novel therapies.

Transferrin as an innate immune mediator

Given how fundamental iron acquisition is to microbial survival, it is not surprising that in mammals, the concentration of free iron in tissue fluids is less than 10^{-24} M. This exceedingly low concentration is maintained predominantly by the iron-binding protein transferrin [27•]. Transferrin is an abundant serum glycoprotein that mediates transport and homeostasis of iron levels in the plasma and extracellular tissue fluid. The protein contains two homologous lobes, each with a single high-affinity iron-binding site. Average transferrin levels in the serum are between 1 and 4 mg/ml, and under normal physiological conditions, the protein remains approximately 30% iron-saturated [28]. Normal levels of unsaturated transferrin help to maintain the concentration of free iron in tissue fluids at levels that are restrictive for uncontrolled microbial growth. Many studies have identified transferrin as one of the major components necessary for the antimicrobial activity of serum [29,30]. Conversely, increased iron stores have been reported to correlate with increased frequency and severity of many bacterial and fungal infections, as well as sepsis [31–34].

In vitro antimicrobial effects of transferrin

These observations have led investigators to consider a biological-based strategy for iron sequestration using exogenous transferrin. Numerous studies have demonstrated the ability of transferrin to restrict microbial growth *in vitro* due to its iron sequestration capacity [35–37]. Pathogenic organisms whose growth is inhibited by transferrin include both Gram-negative and Gram-positive bacterial pathogens such as *Pseudomonas aeruginosa* [38], *Klebsiella pneumonia* [39], *Yersinia pseudotuberculosis* [40], *A. baumannii* [8], and *Bacillus anthracis* [41], as well as fungal pathogens, such as *Candida* species and *Histoplasma capsulatum* [42,43].

Our group has also assessed the *in vitro* efficacy of transferrin against diverse bacterial and fungal pathogens. We conducted time–kill curves and determined minimum inhibitory concentrations (MICs) of recombinant human transferrin (rhTransferrin) against *S. aureus* (Gram-positive bacterium), *A. baumannii* (Gram-negative bacterium) and *C. albicans* (fungus). Transferrin had an MIC of 6 μg/ml for the virulent strains *S. aureus* LAC and *A. baumannii* HUMC1, and a 60 μg/ml MIC for virulent *C. albicans* SC5314, demonstrating concentration-dependent static activity against all three pathogens [44••]. At the 60 μg/ml concentration (10-fold above the MIC), transferrin mediated a >3 log reduction in *S. aureus* CFUs at 24 h compared to growth control [44••]. For *A. baumannii*, both the 6 and 60 μg/ml concentration mediated 10-fold to 100-fold reductions in CFUs/ml at 24 h compared to growth control. For *C. albicans*, the 60 μg/ml dose mediated minor growth inhibition at 6 h, and 3-fold reductions in CFUs at 24 h. Higher concentrations (120 and 360 μg/ml) mediated substantial inhibition of growth at all time points [44••].

Because transferrin targets host iron, rather than a biochemical target on microbes, we hypothesized that it would exert minimal selective pressure driving resistance. We found that serial passage of each organism in the presence of a subinhibitory concentration of rhTransferrin for 20 generations led to no change in susceptibility. Antimicrobial activity was inhibited by the addition of exogenous iron or iron-loaded siderophores, as well as anti-transferrin antibodies. In addition, intracellular iron levels in all three pathogens were markedly reduced following exposure to rhTransferrin in a dose-dependent manner [44••]. Thus, transferrin acts as a static, not cidal, agent against a broad spectrum of human pathogens.

Iron is a crucial electron acceptor in the oxidative phosphorylation cascade that leads to ATP generation in both prokaryotes and eukaryotes [45]. Treatment of *A. baumannii*, *S. aureus*, and *C. albicans* with rhTransferrin resulted in disrupted membrane potentials in all three pathogens in a dose-dependent manner, as early as 1 h following treatment, with increased effect at 6 h [44••]. Although both *C. albicans* and *S. aureus* experienced some degree of membrane potential recovery at 24 h, this may have been due to the liberation of intracellular iron stores from dying organisms, which became available to saturate transferrin. Disrupted membrane potentials were maintained when the transferrin was separated by a filter from the microbes, and were totally reversed by the addition of exogenous iron. Thus, the effect of transferrin on microbial membrane potentials appeared to be due to iron sequestration.

Challenges to standardizing *in vitro* testing of transferrin

When testing transferrin MICs to a variety of organisms, we noted substantial variability between assays that used serum from different batches, due to variability in the concentration of iron and iron binding proteins in the serum [44••]. Thus, reproducible transferrin MIC testing requires conducting the assay in media without serum. Rich growth media that are normally used for susceptibility testing also posed challenges due to the high levels of free iron. Such media do not recapitulate the normal, exceedingly low free iron levels in human blood and tissues. When tested in RPMI in the absence of serum, human apo-transferrin MICs were highly reproducible. However, the apo-transferrin MICs were substantially lower than the physiological concentration of transferrin (a mixture of apo-transferrin and holo-transferrin) in