Pathogenesis of Mucormycosis

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Mucormycosis is a life-threatening infection that occurs in patients who are immunocompromised because of diabetic ketoacidosis, neutropenia, organ transplantation, and/or increased serum levels of available iron. Because of the increasing prevalence of diabetes mellitus, cancer, and organ transplantation, the number of patients at risk for this deadly infection is increasing. Despite aggressive therapy, which includes disfiguring surgical debridement and frequently adjunctive toxic antifungal therapy, the overall mortality rate is high. New strategies to prevent and treat mucormycosis are urgently needed. Understanding the pathogenesis of mucormycosis and the host response to invading hyphae ultimately will provide targets for novel therapeutic interventions. In this supplement, we review the current knowledge about the virulence traits used by the most common etiologic agent of mucormycosis, *Rhizopus oryzae*. Because patients with elevated serum levels of available iron are uniquely susceptible to mucormycosis and these infections are highly angioinvasive, emphasis is placed on the ability of the organism to acquire iron from the host and on its interactions with endothelial cells lining blood vessels. Several promising therapeutic strategies in preclinical stages are identified.

Mucormycosis is an infection caused by fungi belonging to the order Mucorales [1]. *Rhizopus oryzae* is the most common organism isolated from patients with mucormycosis and is responsible for ~70% of all cases of mucormycosis [2–4]. The major risk factors for mucormycosis include uncontrolled diabetes mellitus in ketoacidosis, other forms of metabolic acidosis, treatment with corticosteroids, organ or bone marrow transplantation, neutropenia, trauma and burns, malignant hematologic disorders, and deferoxamine therapy in patients receiving hemodialysis [3, 5, 6]. Because of the increasing prevalence of diabetes mellitus, cancer, and organ transplantation in the aging US population, the number of patients at risk for this deadly infection is dramatically increasing [7]. Unfortunately, despite disfiguring surgical debridement and adjunct antifungal

therapy, the overall mortality rate for mucormycosis remains >50%, and it approaches 100% among patients with disseminated disease or those with persistent neutropenia [3, 8]. Clearly new strategies to prevent and treat mucormycosis are urgently needed, and such strategies can be facilitated by clear understanding of the pathogenesis of the disease.

HOST DEFENSE AGAINST MUCORMYCOSIS

Clinical and experimental data clearly demonstrate that individuals who lack phagocytes or have impaired phagocytic function are at higher risk of mucormycosis. For example, severely neutropenic patients are at increased risk for developing mucormycosis. In contrast, patients with AIDS do not seem to be at increased risk for developing mucormycosis [5]. These findings suggest that neutrophils, but not necessarily T lymphocytes, are critical for inhibiting fungal spore proliferation. Furthermore, both mononuclear and polymorphonuclear phagocytes of normal hosts kill Mucorales by the generation of oxidative metabolites and the cationic peptides, defensins [9–11]. A recent study showed that

exposure of neutrophils to *R. oryzae* hyphae results in up-regulation in Toll-like receptor 2 expression and in a robust proinflammatory gene expression with rapid induction of NF-κB pathway–related genes [12]. In the presence of hyperglycemia and low pH, which is found in patients with diabetic ketoacidosis (DKA), phagocytes are dysfunctional and have impaired chemotaxis and defective intracellular killing by both oxidative and nonoxidative mechanisms [13].

Concordant with these clinical observation, inhalation of Mucorales sporangiospores by immunocompetent animals does not result in the development of mucormycosis [9]. In contrast, corticosteroid-immunosuppressed or animals with DKA die of progressive pulmonary and hematogenously disseminated infection [9, 14]. Moreover, the ability of inhaled sporangiospores to germinate and form hyphae in the host is critical for establishing infection. Although pulmonary alveolar macrophages harvested from lungs of immunocompetent mice are able to ingest and inhibit germination of *R. oryzae* sporangiospores, these bronchoalveolar macrophages have limited capacity to kill the organism in vitro [9]. In contrast, pulmonary alveolar macrophages of immunosuppressed mice are unable even to prevent germination of the sporangiospores in vitro or after intranasal infection [9].

The exact mechanisms by which phagocytes are impaired by ketoacidosis, diabetes mellitus, and corticosteroids are yet to be determined. Furthermore, phagocyte dysfunction alone cannot explain the high incidence of mucormycosis among patients with DKA, because the incidence of mucormycosis among these patients is increased more than the incidence of infections caused by other pathogens [3, 5, 15]. Therefore, Mucorales must possess unique virulence traits that enable the organism to exploit the unique state of immunosuppression and physiologic impairment seen in this subset of patients (see below).

The skin barrier represents a host defense against cutaneous mucormycosis, as evidenced by the increased risk for developing mucormycosis in persons with disruption of this barrier. The agents of mucormycosis are typically incapable of penetrating intact skin. However, burns, traumatic disruption of the skin, and persistent maceration of skin enables the organism to penetrate into deeper tissues. These organisms could originate from traumatic implantation of contaminated soil or water (eg, the outbreaks after natural disasters, as was seen after the tsunami in Indonesia in 2004 and after the destructive tornadoes that occurred in Joplin, Missouri, in June 2011). Contaminated surgical dressings and nonsterile adhesive tape have been shown to be the source of primary cutaneous mucormycosis [16, 17]. Furthermore, mucormycosis can even be introduced through direct access, as was seen with the use of contaminated tongue depressors in neonates [18] or the use of contaminated wooden applicators used to mix drugs given to immunocompromised patients [19]. These cases illustrate an alarming shift in mucormycosis cases from mainly community-acquired infections to nosocomial infections in susceptible hosts.

IRON UPTAKE AND MUCORMYCOSIS PATHOGENESIS

In addition to host factors that predispose patients to mucormycosis, Mucorales possess virulence factors that enable the organism to cause disease. One such trait is the ability to acquire iron from the host. Iron is an essential element for cell growth and development, contributing to many vital processes of the cell [20]. Therefore, successful pathogens use multiple processes for obtaining iron from the host. Recent data demonstrate that the level of available, unbound iron in serum plays a critical factor in uniquely predisposing patients with DKA to mucormycosis [21, 22]. In mammalian hosts, iron is bound to host carrier proteins, such as transferrin, ferritin, and lactoferrin. This sequestration avoids toxic effect of free iron [20, 21]. This strategy of limiting iron availability is also a major universal host defense mechanism against microbes and against Mucorales in particular, because *R. oryzae* grows poorly in normal serum unless exogenous iron is added [21, 22].

The clinical observation that patients with DKA are uniquely susceptible to mucormycosis lends support to the role of iron uptake in the pathogenesis of the disease. Patients with DKA have elevated levels of free iron in their serum, and such serum supports growth of *R. oryzae* at acidic pH (7.3–6.88) but not at alkaline pH (7.78–8.38) [21]. Furthermore, adding exogenous iron to serum allowed *R. oryzae* to grow profusely at acidic conditions but not at pH ≥7.4. Finally, simulated acidic conditions decreased the iron-binding capacity of serum samples collected from healthy volunteers, suggesting that acidosis per se disrupts the capacity of transferrin to bind iron, probably by proton-mediated displacement of ferric iron from transferrin [23]. As proof of principle, animal data showed that mice with DKA were protected from *R. oryzae* infection by administration of iron chelators, such as deferiprone and deferasirox [24, 25], which are not used by Mucorales as xenosiderophores. However, not all Mucorales have the same susceptibility to effective iron chelators. For example, a study showed that *Cunninghamella bertholletiae* and *Mucor* species display higher deferasirox minimal inhibitory and fungicidal concentrations than do *Rhizopus* species [26].

Another clinical observation highlights the central role of host iron availability in predisposing patients to mucormycosis. Patients receiving dialysis who are treated with the iron chelator deferoxamine are also uniquely susceptible to a deadly form of mucormycosis [27–30]. The bacterial siderophore, deferoxamine, predisposes patients to *Rhizopus* infection by acting as a xenosiderophore [22]. Deferoxamine strips ferric iron from transferrin and attaches itself on the mold through an inducible receptor, and the iron is transported intracellularly by an active

reduction of the ferric form into the more soluble ferrous form [31]. Concordant with these results, administration of deferoxamine worsens survival among guinea pigs infected with *Rhizopus* but not among those infected with *Candida albicans* [22, 31, 32]. In addition, in vitro studies of radiolabeled iron uptake from deferoxamine in serum show that *Rhizopus* is able to incorporate 8-fold and 40-fold more iron than can *Aspergillus fumigatus* and *C. albicans,* respectively [22]. Finally, a major risk factor for mucormycosis in transplantation includes underlying myelodysplastic syndrome, which probably predisposes patients to the disease because of iron overload resulting from repeated blood transfusions [33].

Fungi can obtain iron from the host by using high-affinity iron permeases or low-molecular-weight iron chelators (siderophores) [20, 34]. The high-affinity iron permeases are present in fungi as part of a reductive system containing redundant surface reductases that reduce ferric into the more soluble ferrous form. The reduced ferrous iron generated by the surface reductase is, in turn, captured by a protein complex consisting of a multicopper oxidase and a ferrous permease [34–36]. The genome sequencing project identified 3 ferric reductases, 6 copper oxidases, and 1 high-affinity iron permease. Indeed, recent data show that the gene encoding high-affinity iron permease (*FTR1*) is expressed by *R. oryzae* during murine infection and inhibition of *FTR1* gene expression by RNA-I, or reduction of *FTR1* copy number by gene disruption reduces the virulence of the fungus in animal models of mucormycosis [37]. Of importance, passive immunization with anti-Ftr1p immune serum protected mice with DKA from infection with *R. oryzae* [37]. Thus, *FTR1* is a crucial virulence factor for *R. oryzae*, and anti-Ftr1p passive immunotherapy represents a promising strategy to improve outcomes of deadly mucormycosis.

Rhizopus is known to secrete rhizoferrin, a siderophore that belongs to the polycarboxylate family [38]. This siderophore supplies *Rhizopus* with iron through a receptor-mediated, energy-dependent process [31, 38]. In this regard, the genome-sequencing project of *R. oryzae* identified 13 possible siderophore permeases that might act as receptors for siderophores, including rhizoferrin or deferoxamine (Table 1). However, it is not currently known whether rhizoferrin transports iron by release of iron extracellularly or whether the siderophore is internalized before releasing iron in the cytoplasm. What is known is that rhizoferrin is inefficient in obtaining iron from serum [22, 31]; therefore, the contribution of the organism's endogenous siderophores to its virulence in a mammalian host is likely to be minimal. The lack of rhizoferrin ability to take iron from serum is also highlighted by the adaptation of the organism to use xenosiderophores, such as deferoxamine, which are more efficient in obtaining iron from the host.

A third mechanism by which fungi can obtain iron from the host is through use of heme [39, 40]. The *Rhizopus*

Table 1. ***Rhizopus oryzae*** **Genes Believed to Be Involved in Iron Uptake.**

Iron Metabolism-Related Genes	Open Reading Frame
Reductive system	
Ferric reductases	RO3G_05460.1
	RO3G_04617.1
	RO3G_10468.1
Copper oxidases	RO3G_03472.1
	RO3G_07290.1
	RO3G_10569.1
	RO3G_06637.1
	RO3G_15489.1
	RO3G_07572.1
High-affinity permease	RO3G_03471.1
Siderophore permeases	RO3G_16758.1
	RO3G_05990.1
	RO3G_00075.1
	RO3G_12627.1
	RO3G_11434.1
	RO3G_09431.1
	RO3G_02798.1
	RO3G_16094.1
	RO3G_02779.1
	RO3G_02337.1
	RO3G_10304.1
	RO3G_03821.1
	RO3G_00019.1
Uptake from heme (heme oxygenase)	RO3G_07326
	RO3G_13316
SreA	RO3G_07659.3
Ferritin	RO3G_08744.3
	RO3G_14254.3

These genes were identified from the Fungal Genome Sequencing Initiative at the Broad Institute (http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/MultiHome.html).

genome project revealed 2 homologues of the heme oxygenase (Table 1) [41]. These 2 *R. oryzae* homologues may enable *R. oryzae* to obtain iron from host hemoglobin and might explain the angioinvasive nature of *R. oryzae*. Of interest, we found that *R. oryzae* that had reduced copy numbers of *FTR1* also had lagging growth on media supplemented with heme [37]. Therefore, *FTR1* in *R. oryzae* may act as a cytoplasmic membrane permease that facilitates intracellular heme uptake, which is followed by release of ferric iron through degradation with heme oxygenases intracellularly. Other genes likely to be involved in the ability of *R. oryzae* to take up iron include *SreA*, a transcriptional regulator that has been described in *A. fumigatus* to be required for adaptation to the ambient iron availability [42], and 2 orthologes probably encoding ferritin required for intracellular storage of iron. Figure 1 shows the 3 mechanisms of iron uptake that are likely to be operative during mucormycosis.

HOST–PATHOGEN INTERACTIONS

Mucormycosis infections are characterized by extensive angioinvasion that results in vessel thrombosis and subsequent tissue necrosis [3, 6, 43]. Ischemic necrosis of infected tissues can prevent delivery of leukocytes and antifungal agents to the foci of infection. This angioinvasion likely contributes to the capacity of the organism to hematogenously disseminate to other target organs. Consequently, damage of and penetration through endothelial cells or the extracellular matrix proteins lining blood vessels is likely to be a critical step in the pathogenetic strategy of *R. oryzae*. Therefore, understanding the mechanisms by which these processes occur may lead to new approaches to prevent and/or treat mucormycosis.

An earlier study showed that *R. oryzae* can adhere to the extracellular matrix laminin and type IV collagen [44]. We have found that *R. oryzae* strains adhere to human umbilical vein endothelial cells in vitro and invade these cells by induced endocytosis [45]. Endocytosed *R. oryzae* damages endothelial cells, and prevention of endocytosis abrogates the ability of the

organisms to cause endothelial cell damage [45]. More recently, glucose-regulated protein (GRP78) was identified to act as a receptor that mediates penetration through and damage of endothelial cells by Mucorales [46]. GRP78 (also known as BiP/HSPA5) was discovered as a cellular protein induced by glucose starvation [47]. It is a member of the HSP70 protein family that is mainly present in the endoplasmic reticulum. It functions as a major chaperone that is involved in many cellular processes, including protein folding and assembly, marking misfolded proteins for proteosome degradation [48], regulating calcium homeostasis, and serving as a sensor for endoplasmic reticulum stress [49]. Despite its main function as a cellular chaperone protein, recent studies reported the translocation of a fraction of GRP78 to the cell surface in a variety of cells [50].

It is of interest that elevated concentrations of glucose and iron consistent with those noted during DKA-enhanced surface GRP78 expression and resulting penetration through and damage of endothelial cells by Mucorales in a receptor-dependent manner. Mice with DKA had enhanced susceptibility to mucormycosis and had increased expression of GRP78 in the
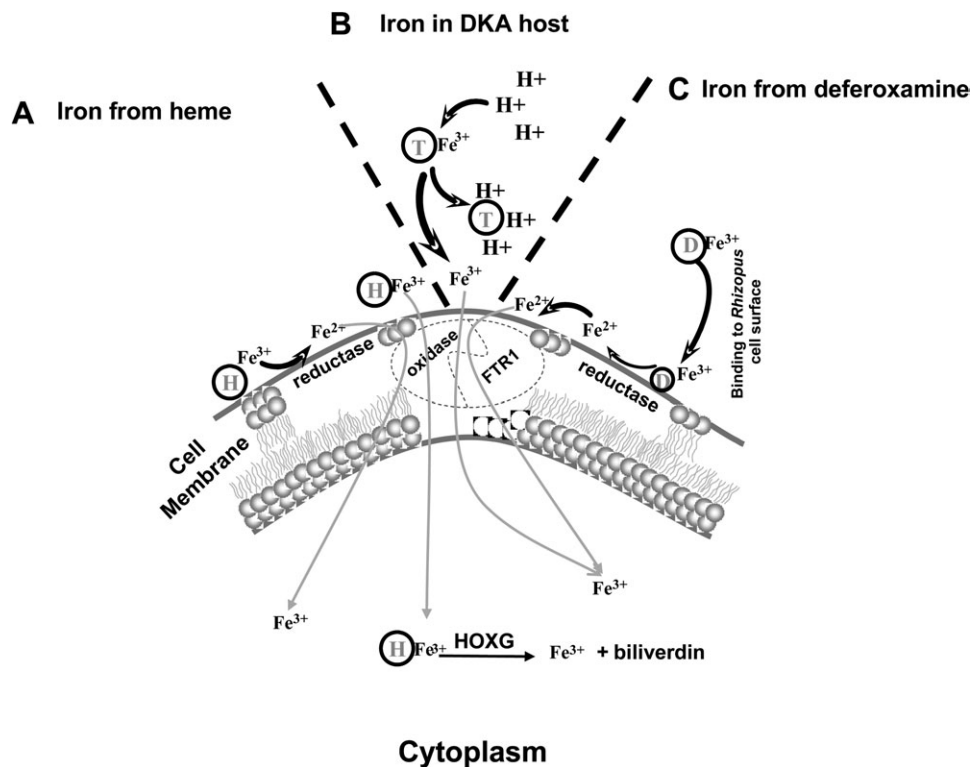

Figure 1. Proposed mechanisms of iron uptake by Mucorales during mucormycosis. *A*, Because of the angioinvasive nature of the disease, heme (H) is likely to represent a source of iron to the invading fungus, which either takes up heme intracellularly or strips ferric iron from heme by the action of the reductase-permease system. If heme is transported intracellulary, ferric iron is obtained by the action of heme oxygenase in the cytoplasm. *B*, In patients with DKA, proton (H^+)–mediated displacement of ferric iron (Fe^{3+}) from transferrin (T) increases the availability of iron, which is transported intracellularly by the reductase-permease system. *C*, Deferoxamine (D) directly chelates iron from transferrin, resulting in ferrioxamine (iron-deferoxamine complex). The fungus then liberates ferrous iron from ferrioxamine by reduction at the cell surface. In all cases, iron is transported across the cell membrane by the copper oxidase–iron permease (FTR1) complex.

sinus, lungs, and brains, compared with normal mice. Note that anti-GRP78 immune serum protected mice with DKA from mucormycosis [46]. Although it is currently unknown whether anti-GRP78 immune serum can protect the neutropenic host from mucormycosis, these observations provide novel insight into the unique susceptibility of patients with DKA to mucormycosis and could provide a foundation for novel therapeutic interventions.

Unlike most fungi (eg, *A. fumigatus*), which are non-pathogenic in *Drosophila melanogaster* flies, Mucorales rapidly infect and kill wild-type flies [51]. Whole-genome expression profiling in wild-type flies after infection with *R. oryzae,* compared with *A. fumigatus,* identified genes selectively down-regulated by *R. oryzae* that act in pathogen recognition, immune defense, stress response, detoxification, steroid metabolism, or tissue repair [51].

OTHER PUTATIVE VIRULENCE FACTORS

Some *Rhizopus* species, especially *R. microsporus* [52] and *R. chinensis* [53], are known for their ability to produce the mycotoxin rhizoxin, an antimitotic macrocyclic polyketide metabolite. Recent studies demonstrated that rhizoxin is not biosynthesized by *Rhizopus*, but rather by an intracellular, symbiotic bacterium of the genus *Burkholderia* [54]. Rhizoxin has long been known to be crucial to the plant pathogenic strategy of *Rhizopus,* but it does not seem to have a substantive role in causing mammalian disease. Organisms that have been rendered to be bacteria free by ciprofloxacin treatment or those that cannot produce rhizoxin because of the absence of *Burkholderia* are still pathogenic in mouse and fruit fly models of infection [55].

Other putative virulence factors include the ability of *Rhizopus* to secrete lytic enzymes, including aspartic proteinases [56]. In addition, *Rhizopus* species have an active ketone reductase system, which may be an additional virulence factor by enhancing growth in the acidic and glucose-rich environment seen in ketoacidotic states [57]. To date, none of these potential virulence factors have been definitively proven to be essential for the development of mucormycosis. Finally, a study demonstrated that Mucorales gain increased and transient virulence in 2 infectious models when exposed to voriconazole [14]. Although the study did not explore the mechanism by which voriconazole exposure increases virulence of Mucorales, it may provide an explanation for the emergence of mucormycosis in patients with hematologic malignancies who are receiving voriconazole prophylaxis [58–62].

Differences in virulence across species in the order Mucorales may also provide insight into the complex repertoire of virulence factors causing aggressive invasive disease in some species and infrequent mortality in others, despite environmental ubiquity. *Cunninghamella bertholettiae* carries the highest mortality rates among reported cases of infection with Mucorales. However, little is known about the properties of this organism that contribute to its pathogenesis. Differences in the aforementioned virulence factors and host evasion may contribute to enhanced infection. Other factors, such as differential thermotolerance and rate of germination at the interface of pulmonary alveolar macrophages, may also contribute to interspecies differences in virulence.

FUTURE DIRECTIONS

The logical extension of the observations of the roles of key virulence factors, such as iron use by *R. oryzae*, is to develop therapeutic strategies that will translate to interventional clinical trials. Such clinical trials require considerable time and effort in study design, implementation, and analysis. The possible benefits of interventions that would complement existing therapies would be profound for patients with mucormycosis.

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