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Host Cell Invasion in Mucormycosis: Role of Iron

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Abstract

Clinical hallmarks of mucormycosis infections include the unique susceptibility of patients with increased available serum iron, the propensity of the organism to invade blood vessels, and defective phagocytic function. These hallmarks underscore the critical roles of iron metabolism, phagocyte function, and interactions with endothelial cells lining blood vessels, in the organism's virulence strategy. In an attempt to understand how *Mucorales* invade the host, we will review the current knowledge about interactions between *Mucorales* and the host while evading phagocyte-mediated killing. Additionally, since iron is an important determinant of the disease, we will focus on the role of iron on these interactions. Ultimately, a superior understanding of the pathogenesis of mucormycosis will enable development of novel therapies for this disease.

Introduction

Mucormycosis (zygomycosis) is caused by fungi belong to the order *Mucorales*.[1–3] This life-threatening infection occurs in patients with: 1) increased available serum iron (e.g. from diabetic ketoacidosis [DKA]); 2) weakened immune system due to neutropenia or steroid treatment; 3) and/or trauma.[2] Recent data have demonstrated a striking increase in the number of reported cases of mucormycosis, possibly due to the rising prevalence of risk factors including diabetes, cancer, and organ transplantation in the ageing population of developed countries.[4], [5] For example, there has been an alarming rise in the incidence of mucormycosis at major transplant centers and the number of cases over a 15 year period has more than doubled.[5], [6] In fact, the prevalence of mucormycosis is up to 8% in autopsied patients with leukemia.[7] Additionally, a recently published population based study demonstrated a 70% increase in mucormycosis cases between 1997 and 2006.[8] Further, data from a tertiary care center demonstrated ≥ 400% increase in the incidence of mucormycosis, mainly among DKA patients between 1991 and 2007 (Fig. 1).[9,10] These studies suggest that the incidence of mucormycosis is increasing in both immunocompromised and DKA patients alike.

Despite disfiguring surgical debridement and adjunctive antifungal therapy, the overall mortality of mucormycosis remains approximately ≥40%, and it approaches 100% in

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patients with disseminated disease, or persistent neutropenia.[2,4–6,11,12] 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.

Mucorales infect the host either through inhalation, ingestion and/or through direct inoculation of fungal spores through an abraded skin due to trauma.[13] Therefore, during early steps of the infection *Mucorales* interact directly with epithelial cells and basement membranes which separate the host cells from the underlying stroma. Further, clinical hallmarks of *R. oryzae* infection include its remarkable angiotropism and the susceptibility of patients with increased available serum iron and/or altered phagocytic function. The angioinvasion and subsequent hematogenous dissemination during mucormycosis indicate that the organism interacts in vivo with: 1) endothelial cells lining the vasculature; 2) the subendothelial membrane which is made accessible to the fungus upon damaging endothelial cells. The hypersusceptibility of patients with increased available serum iron to infection by *Mucorales*, but not other pathogenic fungi, highlights the central role of iron metabolism in the organisms' virulence strategy. For example, patients in DKA are uniquely susceptible to mucormycosis. These patients are known to have elevated free iron generated by proton-mediated liberation from transferrin due to the acidic pH of their blood.[14, 15] Finally, the susceptibility of patients lacking functional phagocytes underscores the vital role of these cells in the host defense against *Mucorales*. Consequently, for *Mucorales* to be successful pathogens, they must adhere to and invade host tissues while evading host defense mechanisms. Therefore, understanding the mechanisms by which these processes occur may lead to new approaches to prevent and/or treat mucormycosis.

Currently no information is available on how the fungus invades lung, sinus or intestinal epithelial cells. Therefore, this review will focus on the interaction between *R. oryzae*, and phagocytes as well as its interactions with extracellular matrix proteins and endothelial cells. Further, because increased free serum iron represents a major risk for mucormycosis, [2,14,15] the effect of iron on these interactions is discussed.

A. Interactions between *R. oryzae* and extracellular matrix components

Basement membranes are extracellular protein matrices that separate epithelial and endothelial cells from underlying stroma.[16] They provide structural support for these cells and serve as barriers to the passage of macromolecules and invading pathogens. The majority of basement membrane proteins consist of laminin and collagen IV. Epithelial cell damage has been reported in patients who are susceptible to mucormycosis, such as diabetics or patients receiving chemotherapy. This damage in turn exposes the extracellular matrix proteins so that they can directly interact with the pathogen. In this respect, an early study showed that *R. oryzae* can adhere to laminin and type IV collagen, but not to fibronectin [17]. This attachment occurs with spores prior to germination and decreases dramatically when the spores germinate. Furthermore, adherence of *R. oryzae* to laminin and collagen is specific as determined by anti-laminin and anti-collagen antibodies blocking studies as well as receptor competition experiments.[17]

R. oryzae is known to harbor an expanded family of genes encoding for the proteolytic enzymes, including secreted aspartic proteinase (SAP) and subtilases gene families[18,19] and multiple studies demonstrate the production of these enzymes by *R. oryzae*.[20–23] Genes specifying these lytic enzymes, which have been shown to contribute to the virulence of other organisms,[24,25] are present in larger numbers in *R. oryzae* compared to other fungi (28 SAP genes and 23 subtilases genes).[18] These genes are expressed in patients with mucormycosis[26,27] and their products likely facilitate the penetration of the organism through extracellular matrix proteins and invasion of host cells.

B. Host Defense against mucormycosis

Phagocytes are the major line of defense against *Mucorales*.[28,29] Inhalation of *Mucorales* spores by immunocompetent animals does not result in the development of mucormycosis (unpublished data).[29] In contrast, neutropenic hosts are at increased risk for developing mucormycosis. Furthermore, corticosteroids and diabetes, both of which are known to suppress phagocyte functions, cause animals inhaling *R. oryzae* spores to die from progressive pulmonary and hematogenously disseminated infection.[29–31]

Both mononuclear and polymorphonuclear phagocytes of normal hosts kill *Mucorales* by the generation of oxidative metabolites and the cationic peptides, defensins.[29,32,33] However, during DKA where there is hyperglycemia and acidosis, phagocytes display dysfunctional chemotaxis and intracellular killing of *R. oryzae* by both oxidative and non-oxidative mechanisms.[34] The dysfunction in phagocyte anti-*Rhizopus* activities during DKA is likely due to the direct effects of hyperglycemia and acidosis. Also the elevated free iron found in DKA patients[14, 15] might be toxic to phagocytes.[35–37] Additionally, spleen cells of mice fed excess levels of iron secrete less IFN-γ, [38] a cytokine that upregulates killing of several *Mucorales* family members (including *R. oryzae*) by human polymorphonuclear leukocytes (PMNLs).[39] Therefore, it is likely that during DKA excess levels of iron lead to impaired phagocytic function. This hypothesis is supported by impaired chemotaxis of neutrophils in response to *R. oryzae* infection in mice given excessive amount of iron compared to normal mice.[40] This impaired chemotaxis is reversed upon treating mice with the *Mucorales* cidal iron chelator, deferasirox.[40]

It is also likely that members of the *Mucorales* order suppress the immune recognition during infection. In this respect, a study using whole genome expression profiling in *Drosophila melanogaster* after infection with *R. oryzae* identified host genes that was selectively down-regulated, act in pathogen recognition, innate immune defense mechanisms and tissue repair mechanisms.[41] These findings might further explain the success of *Mucorales* in evading host defenses and in causing extensive tissue necrosis.

C. Endothelial cell-*R. oryzae* interactions

Damage of and penetration through the endothelial cell lining of the blood vessels is likely a critical step in the pathogenetic strategy of *Mucorales* because angioinvasion is a hallmark of mucormycosis.[1,2,13,42] This angioinvasion often results in vessel thrombosis and subsequent tissue necrosis which can prevent delivery of leukocytes and antifungal agents to the foci of infection, thereby further exacerbating the disease.[2,43,44] A focus of our research is how *Mucorales* adhere to and invade the endothelium. These studies mainly utilize germinated *R. oryzae* (germlings) since this is the form that is likely to interact with endothelial cells during tissue invasion.

1. Adherence and invasion of human umbilical vein endothelial cells by *R. oryzae*.—

R. oryzae germlings adhere avidly to endothelial cells but not to bare plastic.[45] Furthermore, *R. oryzae* germlings are able to cause endothelial cell injury *in vitro* independent of any serum factors.[45] This process requires direct contact between the organism and endothelial cells because membrane inserts separating *R. oryzae* from endothelial cells completely abrogates injury.[45] Endothelial cell injury requires internalization of *R. oryzae* (i.e. invasion of the endothelium) because the use of the endothelial cell microfilament inhibitor, cytochalasin D, blocks internalization of and *R. oryzae*-induced endothelial cell injury.[45] In addition, chelation of endothelial cell iron prevents *R. oryzae* from invading and damaging endothelial cells, which suggests that host iron can modulate the ability of *Mucorales* to cause disease.[46] Of note, mice treated with

the deferiprone and deferasirox (two iron chelators that deprive *R. oryzae* from acquiring external iron) are protected from hematogenously disseminated mucormycosis.[40,47]

Unlike other fungi (e.g. *C. albicans*,[48] *Cryptococcus neoformans*,[49] etc.), we have determined that fungal-induced endothelial cell injury does not require fungal viability since dead germlings (heat-, ethanol-, or gluteraldehyde-killed) are able to cause a similar degree of injury to endothelial cells as do live organisms.[45] This ability of dead germlings to cause injury is also dependent on their internalization by endothelial cells. Injury mediated by dead germlings is induced by cell-associated rather than soluble factors since cell debris, but not the supernatant, from broken germlings as well as cell wall material from regenerating protoplasts of *R. oryzae* germlings cause equivalent injury to endothelial cells as live organisms do (unpublished data). Similar results are obtained when cell wall materials are collected from other members of the *Mucorales* order such as *R. microsporus*, *Mucor*, *Cunninghamella*, and *Absidia* (unpublished data). These results indicate that endothelial cell injury caused by *Mucorales* is dependent, at least in part, on a toxin like substance(s) that is associated with the cell wall.

2. GRP78 is a novel endothelial cell receptor for Mucorales—To identify the host receptor(s) that are utilized by *Mucorales* to invade endothelial cells, we used the affinity purification process developed by Isberg and Leong,[50] in which extracts of endothelial cell membrane proteins were incubated with intact *R. oryzae* germlings. A 78 kDa endothelial cell protein found to bind to *R. oryzae* but not *S. cerevisiae* (which does not adhere to or invade endothelial cells).[45] The major band at 78 kDa was identified as Glucose Regulated Protein 78 (GRP78). This protein is a novel host receptor which mediates invasion and subsequent injury of endothelial cells by *Mucorales*, but not *C. albicans* or *A. fumigatus*.[46] Additionally, GRP78 is a specific and universal receptor for germlings and not spores of several *Mucorales* members.[46] Although GRP78 is utilized by *R. oryzae* to invade endothelial cells, it does not play a role in initial fungal adherence to host cells.[46] These results provide support to a model by which the fungus invades endothelial cells through a two step approach that initially involves adherence of the fungus to a receptor followed by binding to GRP78, which triggers invasion.

GRP78 (also known as BiP/HSPA5) was discovered as a cellular protein induced by glucose starvation [51]. 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, [52] regulating Ca^{2+} homeostasis, and serving as a sensor for endoplasmic reticulum stress.[53] 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.[54]

Of interest, we found that elevated concentrations of glucose and iron, consistent with those seen during DKA, enhance GRP78 surface expression and resulting invasion and injury of endothelial cells in a receptor-dependent manner. These results are concordant with our finding that chelation of endothelial cell iron protects these cells from *R. oryzae*-induced injury in vitro.[46] We also found that mice in DKA, which have enhanced susceptibility to mucormycosis, have increased expression of GRP78 in their sinus, lungs, and brain versus normal mice and that anti-Grp78 immune serum protects these mice from mucormycosis. [46] Collectively, these data offer an explanation for the longstanding mystery as to why hosts in DKA are uniquely predisposed to mucormycosis infection and provide a foundation for novel therapeutic interventions against this deadly infection.

Conclusions

Mucormycosis has a remarkably high morbidity and mortality and its incidence is on the rise. This disease represents the third most common fungal infection in hematologic malignancy patients.[55–57] The availability of iron in the host environment likely plays a critical role in predisposing the host to mucormycosis. Therefore, strategies focused on depriving the fungus from host iron can be beneficial in preventing or treating the disease.

Mucorales enter the host through inhalation, ingestion, or through direct inoculation via abraded skin. The organism invades the host through attachment to extracellular matrix proteins and/or possibly via adherence to epithelial cells while evading killing by resident phagocytes. Further, blood vessel invasion and subsequent hematogenous dissemination happens through interacting with extracellular matrix proteins and endothelial cells via attachment to GRP78 which is upregulated in elevated iron and glucose concentrations seen in susceptible hosts such as DKA. Lack of vasculature phagocytes (e.g. neutropenia) or presence of dysfunctional PMNL (e.g. DKA) further exacerbates the infection. Figure 2 summaries our hypothesis of how *R. oryzae* causes disease in a susceptible host. However, many questions remain unanswered. For example, it is still unknown what is the fungal ligand(s) that mediates attachment/invasion of the organisms to host cells? Furthermore, since adherence and invasion are two independent processes and GRP78 is a receptor for *Mucorales* during invasion but not adherence, it is still to be determined what other host cell receptor(s) mediates the initial steps of *Mucorales* adherence to endothelial cells. Finally, studies focused on investigating the interactions of *Mucorales* with endothelial cells only address mucormycosis pathogenesis during hematogenous dissemination and organ seeding but not during the early stages of infection. Investigations focused on studying interactions between *Mucorales* and lung, sinus, or intestinal epithelial cells will better enhance our understanding of how the infection occurs and likely to provide promising targets for immuno-prophylactic or therapeutic strategies against mucormycosis.

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Highlights

We review the how Mucorales invade host constituents. Elevated available serum iron compromises phagocyte killing of Mucorales. Mucorales binds to extracellular matrix proteins and invade endothelial cells. Iron enhances host cell invasion by increasing expression of the host receptor GRP78. Iron chelation therapy in animals is protective against mucormycosis.

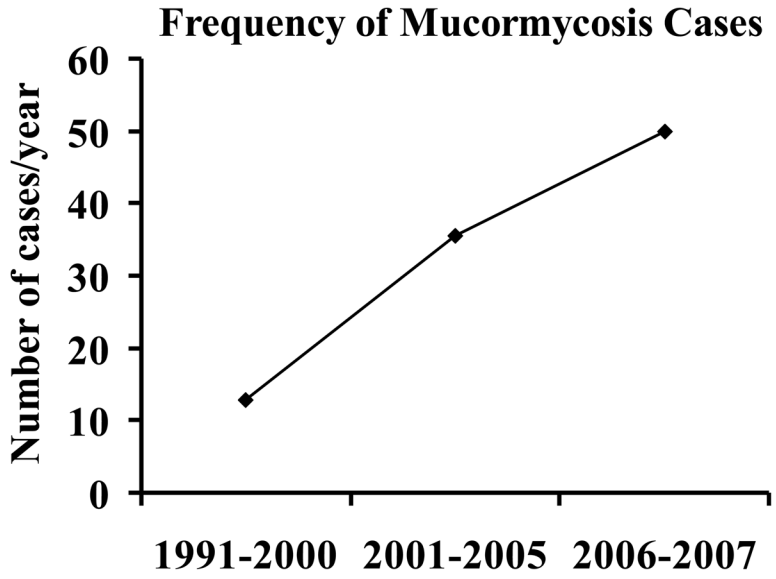

Figure 1.
Increasing incidence of mucormycosis at a tertiary care center compiled from Chakrabarti et al. 2008 and 2009.[9,10]

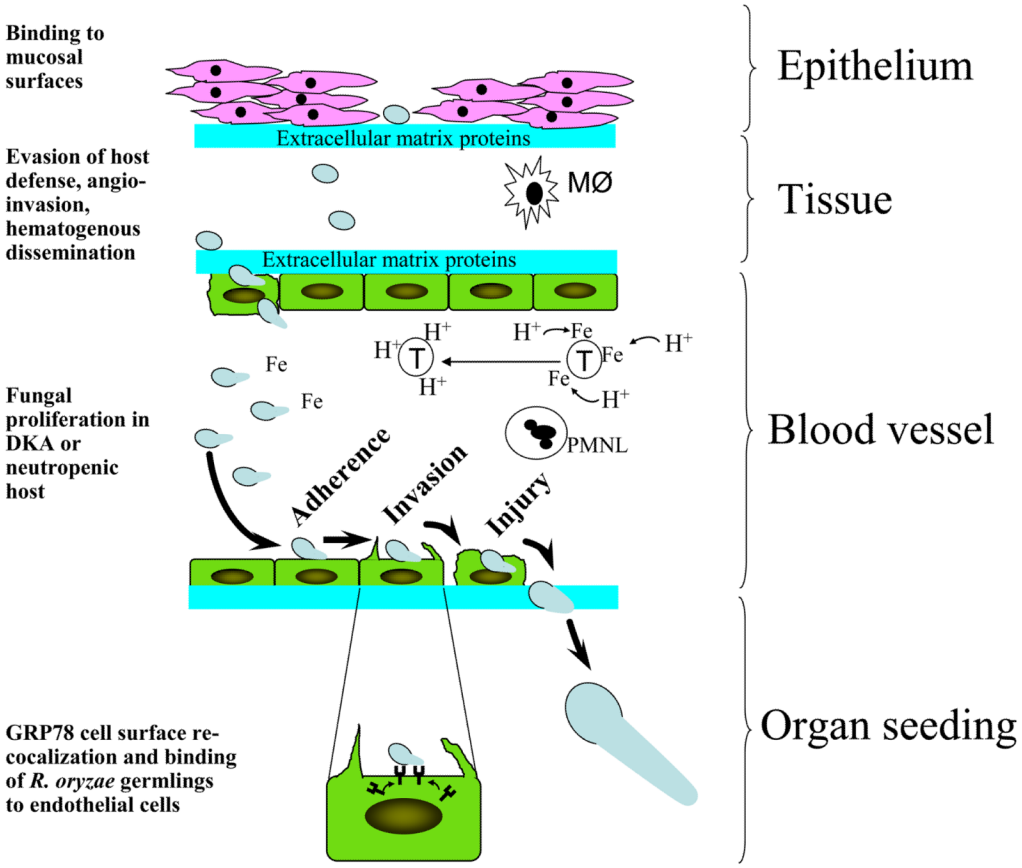

Figure 2.

Proposed mechanisms of host invasion by *Mucorales* during mucormycosis. Mucormycosis is contracted through inhalation, ingestion, or direct inoculation into an abraded skin of *Mucorales* spores. These spores enter tissues by binding to exposed extracellular matrix proteins due to epithelial cell damage in a susceptible host. It is also possible that *Mucorales* first invade epithelial cells then penetrate the extracellular matrix protein via the action of secreted proteases. Once inside the tissues and due to immunosuppressive predisposing factors, *Mucorales* evade tissue macrophage (MØ)-mediated killing and invade blood vessels by binding to extracellular matrix proteins followed by invading endothelial cells. In a susceptible host, *Mucorales* thrive in an invaded blood vessel due to the abundance of free iron (e.g. release of iron from transferrin (T) via a proton-mediated mechanism in DKA patients) and the lack of or the presence of ineffective PMNL. Germlings adhere to endothelial cell through a receptor that is yet to be determined. Next, *Mucorales* invade endothelial cells by binding to GRP78 which is overexpressed and re-localized to the endothelial cell surface in high concentrations of glucose and iron often seen in DKA patients. Finally, fungal hyphae penetrate the blood vessel and seed target organs.