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Rhizopus oryzae Adheres to, Is Phagocytosed by, and Damages Endothelial Cells In Vitro

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Rhizopus oryzae is the most common cause of zygomycosis, a life-threatening infection that usually occurs in immunocompromised patients. A characteristic hallmark of zygomycosis is angioinvasion by the fungus, resulting in thrombosis and subsequent tissue necrosis. Interactions between R. oryzae and vascular endothelial cells are therefore likely of central importance to the organism’s pathogenetic strategy. We studied the ability of R. oryzae to adhere to and damage human umbilical vein endothelial cells (HUVECs) in vitro. We report that R. oryzae spores and germ tubes adhere to HUVECs, whereas only spores adhere to subendothelial matrix proteins. Additionally, R. oryzae damages endothelial cells. This endothelial cell damage requires direct contact and subsequent phagocytosis of the fungus. Surprisingly, R. oryzae viability was not required for damage, but phagocytosis was required for dead R. oryzae to cause damage. These results elucidate the nature of R. oryzae-endothelial cell interactions, which are likely central to the angioinvasion and tissue necrosis seen during zygomycotic infections. The fact that dead R. oryzae damage human endothelial cells may, in part, explain the lack of efficacy of fungicidal agents during clinical disease.

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cytosis by endothelial cells. Surprisingly, R. oryzae does not need to be viable to cause endothelial cell damage. These results have important implications in the pathogenesis of mucormycosis and may ultimately impact antifungal therapy of this extremely lethal infection.

MATERIALS AND METHODS

Organisms and culture conditions. Clinical isolates of Mucorales were obtained from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio. These organisms included R. oryzae 99–880 (a brain isolate), 99–892 (a lung isolate), and 99–133 (a bone marrow isolate) and Macor sp. strain 97–1083 (a blood isolate). R. oryzae HUMC02 was obtained from a patient with rhinocerebral mucormycosis treated at Harbor-UCLA Medical Center. The organisms were grown on potato dextrose agar (PDA) for 3 days at 37°C. The sporangiospores were collected in endotin-free phosphate-buffered saline (PBS), pH 7.4 (Irvine Scientific, Irvine, Calif.) containing 0.01% Tween 80 and washed once with PBS without Tween 80. Spores were sonicated for 5 s, using a Branson Sonifier 450 (output level 3; Branson Ultrasonics, Danbury, Conn.), counted with a hemacytometer, and adjusted to the desired concentration in RPMI 1640 with glucose (Gibco, Grand Island, N.Y.).

To obtain germ tubes of R. oryzae, spores were harvested from PDA plates and processed as described above. An inoculum of 5 × 10^6 spores per ml was added to YPD medium (1% yeast extract [Difco Laboratories, Detroit, Mich.], 2% Bacto-peptone [Difco], 2% d-glucose) and shaken at 37°C for 4 h until small germ tubes (3 to 5 μm) protruded from the cells as determined by microscopic examination. Because of their small size, pregerninated hyphae could be accurately quantified by counting on a hemacytometer, just like spores.

To examine the effect of viability on endothelial cell interactions, R. oryzae spores or germ tubes (5 × 10^6/ml) were killed by heating in a water bath at 60°C for 1 h or suspension in 70% ethanol or 2.5% glutaraldehyde for 30 min. Cells were washed with PBS, and an aliquot of the organisms was inoculated onto PDA for overnight culture at 37°C to confirm that the fungi had been killed.

Saccharomyces cerevisiae CRY2a (4) was a generous gift from Y. Wang (Institute of Molecular and Cellular Biology, Singapore). This strain was maintained on YPD medium until used in the adherence assay.

Preparation of endothelial cells. Human umbilical vein endothelial cells (HUVECs) were obtained by a modification of the method of Jaffe et al. (8). The cells were grown in M-199 medium containing 2 mM L-glutamine, penicillin, and streptomycin (all from Gibco) and supplemented with 10% fetal bovine serum and 1% bovine calf serum (Gibco Bio-Products, Woodland, Calif.). Second-
third-passage cells were grown to confluency in 96-well or 24-well tissue culture plates (BD Biosciences, Bedford, Mass.) coated with 0.2% gelatin matrix (Vitrogen; Celtrix, Palo Alto, Calif.). All incubations were in 5% CO2 at 37°C. To investigate whether HUVEC damage requires direct contact between endothelial cells and R. oryzae, organisms were added to cell culture membrane inserts (pore size, 0.45 μm; Falcon, Lincoln Park, N.J.) suspended above HUVECs, and specific 51Cr release was determined as described above.

**Statistical analysis.** Statistical comparisons were performed by using the non-parametric Steel test for multiple comparisons. Correlations were calculated by the nonparametric Spearman rank sum test. P values of <0.05 were considered significant.

**RESULTS**

*R. oryzae* specifically adheres to HUVECs in vitro. To discern if *R. oryzae* could adhere to endothelial cells, we cocultured *R. oryzae* with HUVECs, subendothelial matrix proteins, or plastic. As a negative control, *S. cerevisiae* was also incubated with endothelial cells, and indeed the organism demonstrated no adherence to the endothelial cells (Fig. 1). Because differences in adherence of *R. oryzae* spores and germ tubes to subendothelial matrix proteins had been previously described (1), we tested the adherence of both spores and germ tubes. *R.
oryzae spores and germ tubes both adhered to endothelial cells in an inoculum-dependent manner (Fig. 1) (inoculum adherence correlation \[r_h/\] = 0.94 for spores and 0.74 for germ tubes; \(P < 0.001\) for both by Spearman rank sum test). The adherence of spores to endothelial cells was greater than their adherence to both fibronectin and gelatin for all inocula (\(P < 0.05\)), although adherence to both fibronectin and gelatin was detectable with the larger inocula. In contrast, germ tubes adhered to endothelial cells for all inocula and demonstrated no adherence to fibronectin, gelatin, or plastic (\(P < 0.05\) for endothelial cell adherence versus all other substrates at all inocula).

In separate experiments we tested the ability of pooled human serum to enhance or inhibit \(R.\) oryzae adherence to HUVECs. Serum slightly inhibited the adherence of larger inocula of \(R.\) oryzae spores to endothelial cells (Table 1). In contrast, serum almost doubled the adherence of pregerminated \(R.\) oryzae spores with the small inoculum and had no effect on adherence with the larger inocula.

**FIG. 2.** Interactions of \(R.\) oryzae spores and germ tubes with endothelial cells. Photomicrographs of endothelial cell monolayers infected with \(R.\) oryzae spores (A to C) and germ tubes (D to F) after 1 h (A and D), 4 h (B and E), and 8 h (C and F). Original magnification for A, B, D, and E is ×20 and for C and F is ×40. Arrows indicate organisms.


**R. oryzae** damages endothelial cells in vitro. To define the ability of **R. oryzae** to damage endothelial cells, we cocultured **R. oryzae** with $^{51}$Cr-labeled HUVECs. Morphologically, phase-contrast microscopy revealed that spores inoculated onto HUVECs began to swell and initiate germination by 4 h and by 8 h had formed mature hyphae (Fig. 2A to C). Pregeneration of the spores in YPD for 4 to 5 h prior to inoculation onto HUVECs resulted in small germ tubes that were readily quantifiable by counting, as with spores. This is made evident by the small size of the germ tubes even following an hour of incubation on endothelial cells (Fig. 2D). Following 4 h of incubation of pregerminated **R. oryzae** with HUVECs, full hyphal formation was seen, and extensive hyphal mats were seen by 8 h of incubation (Fig. 2E to F). With an inoculum of $5 \times 10^5$, both **R. oryzae** spores and germ tubes damaged HUVECs after a 5-h culture (median damage = 21% for both). Subsequent damage studies were all carried out at 5 h and focused on **R. oryzae** germ tubes because they are likely more reflective of in vivo interactions. The damage to HUVECs mediated by **R. oryzae** germ tubes was inoculum and time dependent (Fig. 3, $p = 0.94$ for inocula and 0.76 for time; $P < 0.0001$ for both). Culture in the presence of serum had no effect on the damage to HUVECs (median $^{51}$Cr release = 7% versus 8%, 21% versus 22%, and 35% versus 34% for $1 \times 10^5$, $5 \times 10^5$, and $1 \times 10^6$ inocula in the absence or presence of serum). Subsequent damage experiments were carried out in the presence of 10% pooled human serum to more closely mimic in vivo conditions and to preserve endothelial cell integrity during prolonged incubation. We also tested the abilities of multiple clinical isolates of **R. oryzae** to damage HUVECs. All **R. oryzae** strains tested germinated equivalently by visual inspection and damaged HUVECs to a similar degree and significantly more than a strain of **Mucor** (Fig. 4).

**Damage to endothelial cells from **R. oryzae** requires phagocytosis of the organism but not viability.** We have previously found that phagocytosis of **Candida albicans** and **Cryptococcus neoformans** is required for these fungi to damage HUVECs (3, 7). To determine if **R. oryzae**-mediated damage requires a similar interaction with endothelial cells, we tested the ability of **R. oryzae** to damage HUVECs in the presence or absence of membrane inserts. Inserts were added above the HUVECs, and **R. oryzae** was added to the top of the inserts. The presence of the membrane insert completely abolished the ability of **R. oryzae** to damage HUVECs (Fig. 5A), indicating that direct contact of the mycelium is necessary for injury to HUVECs to occur.

We then tested the ability of cytochalasin D, a known inhibitor of HUVEC-mediated phagocytosis (3, 7), to block **R. oryzae**-mediated damage. Increasing concentrations of cytochalasin D increasingly blocked **R. oryzae**-mediated damage (Fig. 5B), demonstrating that HUVEC phagocytosis of the fungus was likely required for the organism to damage HUVECs.

Pilot studies utilizing dead **R. oryzae** as negative controls for damage assays suggested that fungal viability was not required for HUVEC damage. We therefore tested the ability of **R. oryzae** killed by several different techniques to damage HUVECs. Heat-killed, glutaraldehyde-fixed, and ethanol-killed **R. oryzae** mediated damage to HUVECs equivalent to that with viable **R. oryzae** (Fig. 6). To confirm that phagocytosis was required for dead **R. oryzae** to damage HUVECs, we tested the ability of cytochalasin D to abolish damage mediated by dead organisms. Just as for live **R. oryzae**, cytochalasin D significantly blocked damage mediated by heat-killed **R. oryzae** (Fig. 7).
DISCUSSION

Because angioinvasion is a hallmark of zygomycotic infections, R. oryzae interaction with endothelial cells lining blood vessels is likely an integral component of the organism’s pathogenetic strategy. We find that R. oryzae spores and hyphae adhere to and damage HUVECs. This adherence phenomenon is specific, since R. oryzae did not adhere to plastic in our assay. In contrast, Bouchara et al. reported that R. oryzae spores, but not germ tubes, adhere to plastic (1). The discrepancy between our finding and that of Bouchara et al. (1) is likely due to methodological differences in adherence quantification. Of note, we found that R. oryzae spores adhere to subendothelial matrix proteins significantly better than do R. oryzae hyphae; however, spores and hyphae adhere equivalently to HUVECs. The disparity of spore and germ tube adherence to subendothelial matrix proteins, but equivalent adherence to HUVECs, indicates that R. oryzae adhesins to endothelial cells are likely distinct from the adhesins used to bind to subendothelial matrix proteins.

We also found that R. oryzae has the ability to damage HUVECs irrespective of the organism’s morphology. Endothelial cell damage mediated by R. oryzae did not require serum, akin to our previous findings with C. albicans (3) but distinct from Cryptococcus neoformans, which required serum to cause endothelial cell damage (7). As with both C. albicans and C. neoformans (3, 7), R. oryzae damage to HUVECs required direct contact to and subsequent phagocytosis of the organism by HUVECs. This is evident by the abolishment of damage in the presence of membrane inserts and cytochalasin D. However, in clear distinction from C. albicans and C. neoformans (3, 7), R. oryzae does not need to be viable to damage HUVECs. This damage caused by R. oryzae is also distinct from that from Aspergillus fumigatus in that nonviable hyphae, but not spores, of A. fumigatus have been reported to cause endothelial cell damage (10). By contrast, in our study both nonviable spores and hyphae of R. oryzae caused damage to HUVECs, possibly indicative of the presence of a toxin in R. oryzae.

Adherence to, internalization by, and subsequent injury to HUVECs by R. oryzae likely occur in vivo during mucormycosis. These processes may contribute to the ischemic necrosis often seen with mucormycosis infections. The capability of
dead *R. oryzae* to cause damage to human cells indicates that administration of fungicidal antibiotics that result in *R. oryzae* death may fail to prevent surrounding tissue necrosis in vivo. This may be one reason why *R. oryzae* infections are so refractory in patients.

In conclusion, we show that *R. oryzae* interacts with endothelial cells, likely via specific receptors that induce its own phagocytosis. *R. oryzae* damage to endothelial cells appears to require phagocytosis but does not require organism viability. These results elucidate the crucial interaction between *R. oryzae* and endothelial cells and may have implications for treatment of this refractory pathogen.

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