Interactions of *Aspergillus fumigatus* with endothelial cells: internalization, injury, and stimulation of tissue factor activity

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Invasive aspergillosis causes significant mortality among patients with hematologic malignancies. This infection is characterized by vascular invasion and thrombosis. To study the pathogenesis of invasive aspergillosis, we investigated the interactions of *Aspergillus fumigatus* conidia and hyphae with endothelial cells in vitro. We found that both forms of the organism induced endothelial cell microfilament rearrangement and subsequent endocytosis. Conidia were endocytosed 2-fold more avidly than hyphae, and endocytosis was independent of fungal viability. Endocytosed conidia and hyphae caused progressive endothelial cell injury after 4 hours of infection. Live conidia induced more endothelial cell injury than did live hyphae. However, endothelial cell injury caused by conidia was dependent on fungal viability, whereas injury caused by hyphae was not, indicating that conidia and hyphae injure endothelial cells by different mechanisms. Neither live nor killed conidia increased tissue factor activity of endothelial cells. In contrast, both live and killed hyphae stimulated significant endothelial cell tissue factor activity, as well as the expression of tissue factor antigen on the endothelial cell surface. These results suggest that angioinvasion and thrombosis caused by *A fumigatus* hyphae in vivo may be due in part to endothelial cell invasion, induction of injury, and stimulation of tissue factor activity. (Blood. 2004;103:2143-2149)

Introduction

Invasive aspergillosis has become one of the leading causes of death among bone marrow transplantation and leukemia patients. Even with current therapy, the mortality rate of invasive aspergillosis is 80% to 90% in immunocompromised patients. *Aspergillus fumigatus* is responsible for 90% of invasive *Aspergillus* infections. This ubiquitous mould releases numerous conidia into the atmosphere, which are small enough (2 to 3 μm in diameter) to reach the pulmonary alveoli after they are inhaled. Once the conidia reach the alveoli, they swell and germinate, producing hyphae that invade the pulmonary parenchyma. These hyphae have a marked tropism for blood vessels. A key finding in invasive aspergillosis is angioinvasion, which subsequently leads to thrombosis and tissue infarction. In immunocompromised hosts, invasion of the pulmonary vasculature can result in widespread hematogenous dissemination to organs such as the brain, kidneys, heart, and eyes.

During angioinvasion, *A fumigatus* hyphae interact with vascular endothelium. We hypothesize that this interaction plays an important role in the pathogenesis of invasive aspergillosis. Once the hyphae have entered the bloodstream, they must adhere to and penetrate the endothelial cell lining of the blood vessels to invade the deep tissues of the target organs.

In addition, the prominent thrombosis at sites of *A fumigatus* angioinvasion suggests that the organism stimulates endothelial cells to become prothrombotic. A major mechanism by which endothelial cells can promote intravascular thrombus formation is by expressing tissue factor, also known as thromboplastin or CD142. This 45-kDa transmembrane cell surface glycoprotein is expressed by endothelial cells, platelets, and leukocytes. It is also present on endothelial microparticles. Tissue factor binds to factor VII, and forms a tissue factor–factor VIIa complex, which initiates the extrinsic coagulation cascade.

To study the pathogenesis of angioinvasion and thrombosis during invasive aspergillosis, we investigated the mechanisms by which *A fumigatus* hyphae invade and injure vascular endothelial cells in vitro. We also examined whether these hyphae were able to stimulate endothelial cells to express tissue factor.

Material and methods

Reagents and antibodies

Lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4) was from List Biological Laboratories (Campbell, CA). Human factor VII and human factor X were from Enzyme Research Laboratories (South Bend, IN). Pefachrome FXa and Russel viper venom were from Centerchem (Norwalk, CT). Alexa Fluor 488 phalloidin, Alexa Fluor 568 goat anti–rabbit immunoglobulin G (IgG), and Slow Fade mounting medium were from Molecular Probes (Eugene, OR). Mouse anti–tissue factor monoclonal antibody, IgG1 (clone GMA-311) was from Green Mountain Antibodies.
A fumigatus (Georgetown University). All fungal strains used in these experiments were Soni er 450 (output level 2; Branson Ultrasonic, Danbury, CT). The suspension was gently sonicated for 10 seconds using a Branson apparatus. By microscopic examination, the majority of the organisms were single germ tubes, with a few small clumps containing 2 to 37 conidia in Sabouraud broth on gelatin-coated Petri dishes for 5 to 6 hours at 37°C. Next, the medium above the germinated conidia was aspirated and the conidia or germ tubes were obtained by incubating the medium with a cell scraper. PBS without Tween 80 in phosphate-buffered saline (PBS), pH 7.4 and then washed twice in PBS with 1% BSA. Next, the endothelial cells were permeabilized in 0.1% (vol/vol) Triton X-100 in PBS for 5 minutes, and then blocked with 1% BSA in PBS for 30 minutes. The microfilaments were stained with Alexa Fluor 488 phalloidin following the manufacturer’s instructions. To detect tissue factor expression, the endothelial cell monolayers were grown to confluence in 96-well plates containing detachable wells. The cells were incubated overnight with 1 μCi (0.037 MBq) Na251 CrO4 (ICN Biomedicals, Irvine, CA) per well. The following day, the unincorporated tracer was aspirated and the wells were rinsed twice with prewarmed Hanks balanced salt solution. Next, endothelial cells were incubated with 10^7 organisms per well in 100 μL tissue culture medium. After incubation, the upper 50% of the medium was aspirated from each well and then the wells were manually detached from one another. The amount of 51 Cr in the aspirates and in the wells was determined by gamma counting. To measure the spontaneous release of 51 Cr, uninfected endothelial cells exposed to medium alone were processed in parallel. In experiments with killed organisms, the equivalent volume of the supernatant from the last thimerosal wash was added to the endothelial cells to control for the release of any toxic material as a result of the thimerosal treatment. The results were adjusted for the volume of medium removed from the wells and the percent specific release of 51 Cr was calculated using the following formula: (experimental release - spontaneous release)/(total incorporation - spontaneous release) × 100. Each experiment was performed in triplicate at least 3 different times. After 8 hours of incubation in medium alone, the average spontaneous release of 51 Cr was 24% ± 4%. To determine the maximal release of 51 Cr by the endothelial cells, the cells were lysed by the addition of 0.5% Triton X-100 to the medium. The average specific release of 51 Cr in these cells was 85% ± 5%. This specific release of 51 Cr was essentially the same when Triton X-100 was added to either endothelial cells incubated with medium alone or infected with A fumigatus for 8 hours.
In preliminary experiments, we incubated *A. fumigatus* hyphae with $^{51}$Cr under the same labeling conditions that were used for the endothelial cells. These hyphae incorporated minimal $^{51}$Cr (data not shown). Therefore, *A. fumigatus* cells were unlikely to take up a significant amount of $^{51}$Cr released by the injured endothelial cells.

For the experiments to determine if soluble factors released by *A. fumigatus* could injure the endothelial cells, a modification of the above injury assay was used. The endothelial cells were seeded on 24-well tissue culture plates and cell culture inserts (pore size, 0.4 µm; BD Biosciences) were placed in each well, suspended approximately 1 mm above endothelial cells. Next, $6 \times 10^5$ conidia was added to the endothelial cells, the cell culture inserts, or both. At the end of the incubation, the medium was gently removed and the endothelial cells were lysed by the addition of 0.5 mL of 6 N NaOH. The lysed cells were aspirated and the wells were rinsed with 0.5 mL RadiacWash (Atomic Products, Shirley, NY). These rinses were added to the cell lysate. The $^{51}$Cr activity was determined in the medium and in the cell lysates and the specific release of $^{51}$Cr calculated as described above.

**Tissue factor assay**

The expression of tissue factor by the endothelial cells was measured by a colorimetric assay. All reagents and solutions used in these experiments were endotoxin-free. Briefly, confluent endothelial cells in 96-well plates were washed once with warm PBS and incubated with $10^5$ *A. fumigatus* or *C. albicans* cells for 2, 4, 8, and 16 hours. Control wells with *A. fumigatus* alone were run in parallel. As a positive control, endothelial cells were stimulated with 1 ng LPS per milliliter. At each time point, the medium above the cells were run in parallel. As a positive control, endothelial cells were stimulated with 1 ng LPS per milliliter. At each time point, the medium above the cells were run in parallel.

**Results**

**Interactions of *A. fumigatus* conidia, germ tubes, and hyphae with endothelial cells**

We first examined the interactions of *A. fumigatus* conidia, germ tubes, and hyphae with endothelial cells in vitro using phase contrast microscopy. When the organisms were added to the endothelial cells as resting conidia, they settled onto the cells within 30 minutes (Figure 1A) and began to swell by 3 hours (Figure 1B). Some of these swollen conidia were visible in the perinuclear position of the endothelial cells, suggesting that they had been internalized (Figure 1B). After 8 hours of infection, the swollen conidia became polarized and began to germinate. Both pear-shaped organisms and a few germ tubes were visible at this time point (Figure 1C).

During invasive aspergillosis, it is highly probable that the inhaled *A. fumigatus* conidia germinate and form hyphae before they come in contact with the vascular endothelium. Therefore, we examined the endothelial cell interactions of organisms that had been pregerminated prior to being added to the endothelial cell monolayers. These germ tubes rapidly settled onto the monolayer (Figure 1D) and progressively elongated so that a hyphal mat was formed by 3 hours (Figure 1E). After 8 hours of infection, this hyphal mat had completely covered the endothelial cells (Figure 1F).

**Conidia and hyphae induce rearrangement of endothelial cell microfilaments and subsequent endocytosis**

We compared the endothelial cell uptake of *A. fumigatus* conidia and germ tubes using a differential fluorescence assay. After 45

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**Figure 1. Interactions of *A. fumigatus* conidia and germ tubes with endothelial cells.** Photomicrographs of endothelial cell monolayers infected with *A. fumigatus* H237 conidia (A-C) and germ tubes (D-F) after 30 minutes (A,D), 3 hours (B,E), and 8 hours (C,F). Original magnification × 20. Arrows indicate the organisms.
minutes, 2-fold more conidia than germ tubes were internalized by the endothelial cells (Figure 2). Addition of cytochalasin D, a microfilament inhibitor, reduced the uptake of conidia and germ tubes by 60% and 80%, respectively. The inhibitory effect of cytochalasin D on the internalization of the organisms indicates that both forms of A fumigatus invade endothelial cells by inducing their own endocytosis.

To visualize the involvement of microfilaments in the endocytosis of A fumigatus, the infected endothelial cells were stained with Alexa Fluor 488 phalloidin and viewed by confocal microscopy. Endocytosis of both conidia and hyphae was accompanied by the polymerization of endothelial cell actin around the organisms (Figure 3). Some endothelial cells endocytosed multiple organisms, particularly conidia. In some cases, 2 endothelial cells could be observed endocytosing different portions of the same hyphaus. After endocytosis was complete, the internalized organisms were no longer surrounded by polymerized actin. A similar pattern of actin polymerization was observed when the endothelial cells were incubated with killed organisms (data not shown), indicating that the endocytosis of A fumigatus is independent of the viability of the organism.

A fumigatus conidia and germ tubes injure endothelial cells by different mechanisms

It is known that C albicans and Cryptococcus neoformans, which interact with endothelial cells during hematogenous dissemination, cause significant endothelial cell injury in vitro. We therefore evaluated the extent of endothelial cell injury caused by A fumigatus conidia and germ tubes. When the endothelial cells were infected with resting conidia, cellular injury became detectable after 4 hours (Figure 4A). At this time, the conidia had become swollen, but had not yet germinated. The extent of endothelial cell injury caused by A fumigatus increased progressively with time as the swollen conidia subsequently germinated (Figure 1 and Figure 4A).

To investigate whether different strains of A fumigatus varied in their capacity to injure endothelial cells, we tested 5 clinical isolates of A fumigatus in the endothelial cell injury assay. All 5 isolates caused a similar amount of endothelial cell injury after 8 hours of infection (Figure 4B).

Next, we compared the extent of endothelial cell injury caused by live and killed conidia and germ tubes. Live conidia of A fumigatus caused greater endothelial cell injury than did live germ tubes after 8 hours of infection (Figure 4C). Fungal viability was important for endothelial cell injury by conidia, as killed conidia induced minimal injury after 8 hours (Figure 4C) or even after 16 hours of incubation (data not shown). Surprisingly, endothelial cell injury caused by germ tubes was independent of fungal viability, because killed germ tubes induced the same extent of endothelial cell injury as did live germ tubes (Figure 4C). These results suggest that A fumigatus conidia and germ tubes injure endothelial cells by different mechanisms.

Endothelial cell injury by A fumigatus requires endocytosis of the organism

To determine if direct contact is required for A fumigatus to injure endothelial cells, we interposed cell culture inserts between the endothelial cells and live conidia. This procedure completely abolished endothelial cell injury after 8 hours (Figure 5A) and 16 hours (data not shown) of infection.

Next, we infected the endothelial cells in the presence of cytochalasin D to determine whether endocytosis of A fumigatus is required for endothelial injury to occur. We found that 70 nM cytochalasin D caused an 89% reduction in endothelial cell injury caused by A fumigatus (Figure 5B). This concentration of cytochalasin D had no visible effect on the A fumigatus cells and caused less than 5% specific release of $^{51}$Cr when added to uninfected endothelial cells (data not shown).

A fumigatus hyphae induces tissue factor expression

In invasive aspergillosis, angioinvasion is accompanied by thrombus formation. One mechanism by which A fumigatus can induce intravascular thrombosis is by inducing tissue factor expression by endothelial cells. We therefore investigated whether A fumigatus conidia and hyphae stimulate endothelial cell tissue factor activity. We found that the basal level of tissue factor activity expressed by uninfected endothelial cells was low, although this level varied with the endothelial cell donor (range, 0.78 to 2.34 factor Xa units). Incubation of endothelial cells with live or killed conidia for up to 16 hours resulted in minimal increase in tissue factor activity (Figure 6). In contrast, germ tubes stimulated a progressive increase in tissue factor activity, starting at 4 hours of

![Figure 2. Endocytosis of A fumigatus by endothelial cells. A fumigatus H237 conidia and germ tubes were incubated with endothelial cells for 45 minutes in the presence of cytochalasin D or absence of the microfilament inhibitor, cytochalasin D. The number of organisms (orgs) endocytosed by the endothelial cells was determined by a differential fluorescence assay. Results are mean ± standard deviation of 3 experiments. *$P<.001$ compared with cells without cytochalasin D; §§$P<.05$ compared with conidia.](image-url)
Endothelial cell injury was measured after 8 hours of infection and the results are the mean ± standard deviation of at least 3 independent experiments. Error bars indicate standard deviation. *P < .001 versus uninfected control wells; §P < .05 versus live organisms; †P < .001 versus conidia.

Discussion

During angioinvasion by *A. fumigatus* in both experimental animal models and humans, only hyphal phase organisms are visible in the tissues.\(^2\),\(^4\),\(^5\),\(^24\) This finding indicates that the hyphus is the principal form of the organism that interacts with endothelial cells and suggests that hyphae have a unique capacity to survive and proliferate within the host. We therefore compared the interactions of *A. fumigatus* conidia and hyphae with endothelial cells.
endothelial cells suggests that a factor associated with the cell surface of the germ tubes causes endothelial cell injury when the germ tubes are endocytosed. In support of this conclusion, we have found that cell wall extracts of *A. fumigatus* hyphae can also injure endothelial cells (L.M.L.B. and S.G.F., unpublished data, October 2001).

By interposing filter inserts between *A. fumigatus* and the endothelial cells, we determined that direct contact between the organisms and endothelial cells appears to be necessary for endothelial cell damage to be induced under these culture conditions. Moreover, the finding that inhibiting endocytosis of the organism with cytochalasin D blocked endothelial cell injury indicates that endocytosis is an additional prerequisite for the induction of endothelial cell injury. *A. fumigatus* is known to secrete a variety of lytic enzymes including proteases and phospholipases. 27,28 It is possible that these secreted enzymes are most active when they achieve high concentrations in the endocytic vacuole of the endothelial cell. As mentioned previously, it is also likely that the hyphal cell wall itself is toxic to endothelial cells.

To investigate potential mechanisms by which *A. fumigatus* causes thrombosis at sites of angioinvasion, we examined whether this organism could induce endothelial cells to express tissue factor activity. We found that *A. fumigatus* hyphae, but not conidia, stimulated endothelial cells to express tissue factor activity. This finding again indicates that *A. fumigatus* conidia and hyphae interact differently with endothelial cells. There are 2 lines of evidence that demonstrate that the increase in tissue factor activity was due to the endothelial cells rather than *A. fumigatus*. First, neither live nor killed *A. fumigatus* hyphae had any detectable tissue factor activity in the absence of endothelial cells. Furthermore, endothelial cells exposed to killed *A. fumigatus* hyphae expressed similar amounts of tissue factor activity as did endothelial cells exposed to live hyphae. Second, tissue factor antigen was detectable on the surface of the infected endothelial cells, but not on *A. fumigatus* hyphae.

*C. albicans* is another angioinvasive fungus and its interactions with endothelial cells in vitro have been investigated extensively. This organism is similar to *A. fumigatus* in that both live and killed hyphae are endocytosed by endothelial cells and this process can be inhibited by cytochalasin D. 14,16,19 However, a major difference between the 2 organisms is that *A. fumigatus* conidia are endocytosed by endothelial cells more avidly than hyphae, whereas *C. albicans* hyphae are endocytosed more avidly than blastospores. 15 There are also significant differences in endothelial cell injury caused by *A. fumigatus* and *C. albicans*. Unlike *A. fumigatus* conidia, *C. albicans* blastospores do not cause detectable endothelial cell injury. 15 Furthermore, although killed *A. fumigatus* conidia, *C. albicans* hyphae cause significant endothelial cell injury, killed *C. albicans* hyphae do not. 19 Therefore, *A. fumigatus* and *C. albicans* likely injure endothelial cells by different mechanisms.

We also found that *C. albicans* did not stimulate endothelial cell tissue factor activity. This result was particularly interesting because *C. albicans* is known to stimulate multiple endothelial cell responses including the expression of leukocyte adhesion molecules, and secretion of proinflammatory cytokines and prostaglandins. 20,29-31 The difference in the ability of *A. fumigatus* and *C. albicans* to induce a prothrombotic state in endothelial cells in vitro parallels the histopathology of infections caused by these organisms in vivo. Invasive aspergillosis is characterized by extensive intravascular thrombosis at foci of infection, 3-5,23 whereas thrombosis is not a prominent feature of disseminated candidiasis. 32,33

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**Figure 7.** Expression of tissue factor on the surface of infected endothelial cells. Endothelial cell monolayers grown on glass coverslips were incubated for 8 hours with tissue culture medium (A), LPS (B), or germ tubes of *A. fumigatus* (C-D). The expression of tissue factor on the endothelial cell surface was detected by confocal microscopy using an anti–tissue factor monoclonal antibody (A,C). Panel D shows the differential interference contrast image corresponding to the same field as panel C. Results are representative of 3 independent experiments. Original magnification × 400.
Other microorganisms, such as *Staphylococcus aureus* and *Neisseria meningitidis* are known to stimulate endothelial cells to express tissue factor in vitro. It is likely that stimulation of endothelial cell tissue factor expression contributes to formation of vegetations during staphylococcal endocarditis and to disseminated intravascular coagulation during meningococcemia. Other microbial pathogens, such as cytomegalovirus, *Chlamydia pneumoniae*, *Streptococcus sanguis*, and *Plasmodium falciparum* may also induce tissue factor expression at sites of infection. However, these organisms induce tissue factor expression by activating monocytes, which can express tissue factor by themselves, as well as stimulate endothelial cells to express tissue factor. Whether monocytes augment *A. fumigatus* induction of endothelial cell tissue factor expression has not yet been determined.

In summary, *A. fumigatus* hyphae invade endothelial cells in vitro by inducing their own endocytosis. The internalized organisms then injure the endothelial cells and stimulate them to express tissue factor. This process provides a potential mechanism for the vascular invasion and thrombosis that characterize invasive aspergillosis. Studies to examine the mechanism by which *A. fumigatus* hyphae induce tissue factor expression and to confirm tissue factor expression at foci of invasive aspergillosis in vivo are currently in progress.

**Acknowledgments**

We thank the perinatal nurses at Harbor-UCLA Medical Center for collecting the umbilical cords, Quynh Phan for help with tissue culture, and Dr Matthew J. Schibler at the Brain Research Institute at UCLA for expert assistance with confocal microscopy.

**References**

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