Medical Mycology September 2006, **44**, S115–S117

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Interactions of *Aspergillus fumigatus* with vascular endothelial cells

Y. KAMAI*, L. Y. CHIANG*, L. M. LOPES BEZERRA†, T. DOEDT*, A. S. LOSSINSKY‡ D. C. SHEPPARD§ & S. G. FILLER*#

**Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA, USA, †Laboratório de Micologia Celular e Proteômica/IBRAG, Universidade do Estado do Rio de Janeiro, Brazil, ‡Huntington Medical Research Institutes, Pasadena, CA, USA, §Department of Microbiology and Immunology, McGill University, Montreal, Canada, and #David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*

Invasive aspergillosis is characterized by two different types of angioinvasion. During pulmonary aspergillosis, hyphae are initially outside of the pulmonary vasculature and they invade the endothelial cell lining of the blood vessels by passing from the abluminal to the luminal surface. Some of these hyphal fragments can break off and circulate in the bloodstream. In severely immunocompromised hosts, these blood-borne hyphal fragments adhere to the luminal surface of the endothelial cells and they penetrate the endothelial cell lining of the vasculature by passing from the luminal to the abluminal surface. We have set up *in vitro* models of luminal and abluminal endothelial cell invasion by *Aspergillus fumigatus*. Luminal invasion by hyphae results in both endothelial cell damage and stimulation of tissue factor expression. Abluminal invasion causes less endothelial cell damage than luminal invasion, but greater induction of endothelial cells genes encoding cytokines, leukocyte adhesion molecules and tissue factor. These differences in the endothelial cell response to luminal versus abluminal infection may indicate significant differences in the pathogenesis of hematogenously disseminated versus locally invasive versus aspergillosis.

Keywords invasive, aspergillosis, endothelial cells

Introduction

A characteristic feature of invasive aspergillosis is fungal invasion of the blood vessels. This angioinvasion is seen both in the lungs during invasive pulmonary aspergillosis as well as in other organs during hematogenously disseminated aspergillosis. Angioinvasion results in intravascular thrombosis and tissue infarction, which produces an area of dead tissue that is an excellent food source for the fungus. Also, intravascular thrombosis and tissue infarction result in reduced entry of leukocytes and antifungal drugs into areas of infection. Because angioinvasion is likely important for the pathogenesis of invasive aspergillosis, our group has begun studying the interactions of *Aspergillus fumigatus* with vascular endothelial cells *in vivo* and *in vitro*.

In mice that have been immunosuppressed and then infected with *A. fumigatus* conidia via an aerosol, *A. fumigatus* hyphae can be seen invading the walls of pulmonary blood vessels after about 5 days of infection, prior to neutrophil recovery [1]. The endothelial cell lining is completely absent from the wall of the blood vessel that has been invaded by the hyphae, whereas the endothelial cell lining of the uninvolved wall of the blood vessel remains intact. Also, most of the invaded blood vessels are thrombosed. After 10 days of infection, after neutrophil recovery, one can see numerous neutrophils adherent to the endothelial cell lining of blood vessels adjacent to foci of infection. This margination is a clear evidence of endothelial cell activation because the endothelial cells must express

Correspondence: S. G. Filler, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA, USA. E-mail: sfiller@ucla.edu

DOI: 10.1080/13693780600897989

leukocyte adhesion molecules in order for the neutrophils to adhere to them. Leukocytes can also be observed in the deeper tissues. The cells have clearly penetrated through the endothelial cell lining of the blood vessel and are likely trafficking towards the area of infection.

To understand the mechanisms by which *A. fumigatus* damages and stimulates endothelial cells, we investigated the interactions of *A. fumigatus* conidia and germ tubes with human umbilical vein endothelial cells *in vitro*. Germ tubes were produced by adding conidia to petri dishes and incubating them in Sabourauds broth for 5–6 h. We then exposed endothelial cells to medium alone, conidia, or germ tubes for 8 h and measured the extent of endothelial cell damage by a chromium release assay. Live conidia caused significantly more endothelial cell damage than did germ tubes. However, the mechanism of endothelial cell damage caused by conidia and germ tubes appeared to be different. Killed conidia caused almost no damage, whereas killed germ tubes caused the same amount of damage as live germ tubes. These results suggest that damage to endothelial cells by germ tubes is caused by a toxic factor that is associated with the cell wall [2].

Next, we investigated the endothelial cell expression of tissue factor in response to *A. fumigatus*. Tissue factor is a transmembrane glycoprotein that activates the extrinsic coagulation pathway, which results in the cleavage of prothrombin to form thrombin, and induces the formation of a fibrin clot. Endothelial cells normally have anti-coagulant activity, but in response to some stimuli they can express tissue factor on their surface and become procoagulant. We incubated endothelial cells with medium, conidia or germ tubes for 4, 8 or 16 h and then measured endothelial cell tissue factor activity by a colorometric assay. Germ tubes induced significant tissue factor activity at all time points, whereas conidia did not. To confirm that infection with *A. fumigatus* hyphae up-regulated the expression of tissue factor antigen on the surface of endothelial cells, we used indirect immunofluorescence with an anti-tissue factor monoclonal antibody. Uninfected endothelial cells expressed low levels of tissue factor antigen on their surface. After the endothelial cells were infected with *A. fumigatus* hyphae for 8 h, there was strong expression of tissue factor on all endothelial cells. These results suggest that the intravascular thrombosis at sites of *A. fumigatus* angioinvasion is caused in part by up-regulation of endothelial cell tissue factor activity [2]. We have also found that *A. fumigatus* hyphae induce endothelial cells to express the leukocyte adhesion molecules, E-selectin and vascular cell adhesion molecule 1 (VCAM-1). Interestingly, conidia induce minimal expression of these leukocyte adhesion molecules.

There are two different types of angioinvasion that go on during invasive aspergillosis. During pulmonary aspergillosis, the conidia are inhaled into the lung where they form hyphae. These hyphae are initially outside of the blood vessel and they invade the endothelial cell lining of the pulmonary vasculature by passing from the abluminal to the luminal surface. Some of these hyphal fragments can break off and circulate in the bloodstream. In severely immunocompromised hosts, these blood-borne hyphal fragments can seed distant organs and cause hematogenously disseminated aspergillosis. In this disease, the hyphal fragments adhere to the luminal surface of the endothelial cells and they penetrate the endothelial cell lining of the vasculature by passing from the luminal to the abluminal surface.

Endothelial cells are polarized. For example, some cell membrane proteins such as the glucose transporter are highly enriched on the abluminal and lateral surfaces of endothelial cells, whereas other protein such as mast-cell growth factor are present mainly on the luminal surface [3,4]. Interestingly, quiescent endothelial cells constitutively secrete 3-fold more von Willebrand factor abluminally than luminally. However, when these cells are stimulated with tumor necrosis factor α (TNF-α), virtually all of the von Willebrand factor is secreted luminally, while interleukin 6 (IL-6) is released mainly abluminally [5,6]. Finally, endothelial cells respond differently to the same stimulus depending on whether it is applied to the luminal or abluminal surface. For instance, endothelial cells exhibit a greater increase in permeability when TNF-α is applied to their luminal surface compared to their abluminal surface [7].

We have set up *in vitro* models of abluminal and luminal endothelial cell infection to determine if the endothelial cell response to these two types of infection is different. When hyphae are added to the abluminal surface of endothelial cells, they cause significantly less damage than when added to the luminal surface. In fact, by scanning electron microscopy, the hyphae can be seen to penetrate completely through the endothelial cells without causing visible evidence of endothelial cell damage. Interestingly, although abluminal infection of endothelial cells causes less damage than luminal infection, it stimulates greater expression of E-selectin,

IL-8, TNF-α, and tissue factor mRNA. These differences in the endothelial cell response to abluminal versus luminal infection may indicate significant differences in the pathogenesis of invasive versus hematogenously disseminated aspergillosis. We are currently investigating whether the mechanisms of endothelial cell stimulation in response to abluminal versus luminal infection are different.

Acknowledgements

We thank Norma Solis and Q. Trang Phan for assistance with tissue culture and the perinatal nurses at the Harbor-UCLA Medical Center Pediatric Clinical Research Center for collection of umbilical cords. This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, by grants R21AI064511 and MO1RR00425, and Contract No. N01-AI-30041.

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