Manipulation of host angioneogenesis
A critical link for understanding the pathogenesis of invasive mold infections?

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Despite progress over the last decade, opportunistic mold infections continue to be associated with high rates of morbidity and mortality in immunocompromised patients. Given the propensity of molds to invade blood vessels, vasculopathy may be a barrier to effective delivery of antifungal drugs to infected tissue. In a recent study (Ben-Ami R, et al.10), we found that Aspergillus fumigatus suppresses endothelial cell migration, differentiation and capillary tube formation both in vitro and in an animal model system. This effect is mediated by secreted secondary metabolites such as gliotoxin. Herein, I discuss the potential implications of how invasive molds modulate host angiogenesis in experimental and clinical mold infections. Strategies that employ reversal of vasculopathy, neutralization of metabolites that inhibit endothelial function, exploration of pro-angiogenic factors as diagnostic or prognostic markers affected patients will likely be the focus of future studies. This complex, yet emerging field might add another level of knowledge and therapeutic choices in the management of these devastated infections.

Invasive aspergillosis (IA) remains a significant cause of morbidity and mortality among patients with hematological malignancies.1,2 Specifically for patients with leukemia, Aspergillus remains the most common cause of invasive fungal infection with a reported incidence of 6 to 12% and an attributable mortality of 30%.1,2 The pathogenesis of IA remains poorly understood.3 Prolonged granulocytopenia was very early identified as a major risk factor.3 More recently, data derived from animal models of infection complement these early observations, suggesting that the host pulmonary responses to Aspergillus differ according to the host immunosuppressive background.4 Chemotherapy treated mice lacking granulocytes had a rapid lung tissue invasion by fungal hyphae and dissemination to other organs, in the absence of significant pulmonary lesions and respiratory distress.4 Hyphal invasion of pulmonary blood vessels appears to have an important role in the pathogenesis of IA,5 triggering the release of pro-inflammatory cytokines and activation of coagulation cascade. The resulting intravascular thrombosis and tissue infarction leads to sequestration of the infected tissue, therefore hindering delivery of antifungal drugs and immune cells at the site of infection.5,6 The proinflammatory cytokines released by endothelial cells during invasion by Aspergillus and its resulting hypoxia are potent inducers of vascular endothelial growth factors.7 Perhaps one of the best studied of these factors is vascular endothelial growth factor (VEGF), which has a pivotal role in the regulation of both normal and abnormal angiogenesis.8,9 VEGF promotes survival of endothelial cells in vivo and in vitro. VEGF transcription is regulated by hypoxia and inflammation.8,9 Low oxygen tension induces VEGF expression by multiple mechanisms, including the transcriptional factor hypoxia inducible factor-1 (HIF-1), accumulation of adenosine in hypoxic tissues and increased stability of VEGF mRNA during hypoxia.8,9 In addition, expression of the VEGF gene is also induced by proinflammatory cytokines, including IL-1α, IL-1β, IL-6 and prostaglandin E2.8,9 The role of VEGF in
maintaining pulmonary architecture may be deduced from disease states associated with VEGF deficiency.\textsuperscript{8,9} For example, patients with pulmonary emphysema have low pulmonary VEGF levels and increased levels of endostatin, a specific VEGF antagonist. Reduced VEGF activity in emphysema is associated with endothelial and subsequently epithelial cell apoptosis, and may be a contributing factor to the extensive destruction of lung parenchyma that is characteristic of this condition. VEGF induces a strong angiogenic response in a variety of in vivo models.\textsuperscript{8,9} For example, intra-arterial or intramuscular administration of recombinant human (rh) VEGF\textsubscript{165} to rabbits with experimental chronic limb ischemia is associated with the development of collateral vessels and improved limb perfusion.\textsuperscript{8,9} Similar results were obtained in dogs and pigs with experimental coronary insufficiency. In addition, enhanced tissue perfusion has been demonstrated with the combined administration of VEGF and a pro-angiogenic factor.\textsuperscript{8,9} The rational for combining VEGF with a second growth factor is that, while VEGF stimulates angiogenesis, i.e., the formation of new blood vessels, it does not induce arteriogenesis per se, i.e., the sprouting of collateral vessels from existing arteries. In vitro, VEGF and basic fibroblast growth factor (bFGF) demonstrate potent synergistic activity in stimulating the formation of capillary tubes from confluent endothelial cells.\textsuperscript{8,9} Furthermore, the combination of VEGF and angiopoietin or bFGF increases perfusion in a rabbit model of limb ischemia more than does either agent alone.\textsuperscript{8,9}

We recently hypothesized that the combination of regional hypoxia and inflammation in the lungs of animals infected with \textit{A. fumigatus} or \textit{R. oryzae} promotes VEGF gene expression.\textsuperscript{10} To that end, we studied the expression of angiogenesis-associated genes in murine models of invasive pulmonary aspergillosis (IPA) and invasive pulmonary zygomycosis (IPZ). Balb/c mice were immunosuppressed with either cyclophosphamide (neutropenic model) or cortisone acetate (non-neutropenic), and inoculated intranasally with a concentrated suspension of spores of \textit{A. fumigatus} or \textit{R. oryzae}. Mice were sacrificed at 1 h and 24 h post infection. The expression of 84 genes associated with angiogenesis and inflammatory response was analyzed by quantitative real-time PCR from infected lungs. We found divergent patterns of expression of angiogenesis mediator-encoding genes, depending both on the infecting mold and the type of immunosuppression. Specifically, VEGF expression was decreased in neutropenic mice with IPA, despite overexpression of genes encoding for inflammatory cytokines as well as increase in HIF-1 expression.\textsuperscript{10} In contrast, in neutropenic mice, IPZ was associated with a 2.3-fold increase in VEGF expression while HIF-1 expression was increased (Ben Ami and Kontoyiannis DP, unpublished). IPA in non-neutropenic, corticosteroid treated mice was associated with less hyphal proliferation and angioinvasion and a more pronounced host inflammatory response compared with IPA in neutropenic mice. Accordingly, we found increases in pro-inflammatory cytokine gene expression in corticosteroid-treated mice with IPA, accompanied by increased VEGF and HIF-1 gene expression.\textsuperscript{10} Conversely, IPZ in this setting was characterized by the lack of cytokine and VEGF upregulation (Ben Ami and Kontoyiannis DP, unpublished).

These findings led us to postulate that, in the setting of neutopenia, \textit{A. fumigatus} suppresses host VEGF production. Relative VEGF deficiency may promote endothelial apoptosis and facilitate fungal angioinvasion. Furthermore, VEGF downregulation may interfere with collateral vessel formation (Fig. 1). Whether IPZ preferentially affects other angiogenic factors (e.g., HIF-1), or lacks specific anti-angiogenic activity, is unclear from these early results. Repletion of pro-angiogenic factors may bypass the angiogenic effect of invasive mold infections, thus promoting tissue repair and an effective immune response. These data raise interesting questions in both the delivery of translational and clinical research in the pathogenesis of invasive aspergillosis. In addition, such questions could be extrapolated in other medically important angioinvasive opportunistic molds such as the Mucorales.

**Translational Research Questions**

- Could pro-angiogenic agents e.g., Vascular Endothelial Growth Factor (VEGF) or basic Fibroblast Growth Factor (b-FGF), alone and in combination, can slow disease progression and improve survival in experimental murine aspergillosis and zygomycosis? Given the angioinvasive nature of opportunistic mold infections and the associated tissue ischemia,\textsuperscript{3} impaired perfusion is a barrier to the delivery of immune-effector cells and antifungal drugs to infected tissue. VEGF and bFGF are key mediators of angiogenesis and arteriogenesis, respectively, which have shown synergistic activity in animal models.

  - Conversely, as proof of concept, does treatment with anti-angiogenic agents (e.g., the receptor tyrosine kinase inhibitor sunitinib) enhance the vasculopathy associated with these infections, disease progression and worse survival in experimental murine aspergillosis and zygomycosis? The production of an anti-angiogenic factor by an angioinvasive mold suggests that inhibiting VEGF synthesis may be beneficial for the survival of \textit{A. fumigatus} in its host.

- What is the effect of the regulation of angiogenesis factor from hypoxia in the setting of an invasive mold? What are the implications with the cross-talk with local and systemic immune responses? There is evidence that the local hypoxic tissue microenvironment influences directly effects in Aspergillus cell polarity, resistance and virulence,\textsuperscript{11} or indirectly regulation of immune activity of innate effector immune cells.\textsuperscript{12}

- What is the influence of the type of immunosuppression (corticosteroids vs. neutropenia)\textsuperscript{13} on the intricate balance between angiogenesis/inflammation/Aspergillus infection and production of secondary metabolites? As the role of gliotoxin depends on the type of immune-suppression,\textsuperscript{13,14} it is possible that these mycotoxins play different roles in different immunosuppression scenarios (Fig. 2).

- Do antifungals add another level of complexity by having independent immunomodulatory effects\textsuperscript{15} on the angiogenicity of lungs in the setting of Aspergillus infection?

- What mycotoxins produced by molds modulate angiogenesis homeostasis? Our work suggests that gliotoxin is one of the many potential players. For example, \textit{A. fumigatus} produces an array of secondary metabolites with potent antiangiogenic properties.
activity, the best studied of which is fumagillin.\textsuperscript{16} Fumagillin was shown to attenuating production of vascular endothelial growth factor (VEGF).\textsuperscript{16}

**Clinical Research Questions**

- Because vascular tissue invasion by Aspergillus is a preeminent feature of IA in neutropenic hosts, could serum levels of a proangiogenic factor (e.g., VEGF) be a useful marker for risk stratification, early diagnosis or even prognosis of invasive mold infections in highly immunosuppressed patients? One could hypothesize that such patients (e.g., high risk leukemic patients with IA) will have an elevated serum level of VEGF compared to controls. Conversely, given the potential antiangiogenic effects of \textit{A. fumigatus}, it is possible that these patients will have a downregulation of VEGF synthesis compared with controls. The net effect of these opposing stimuli in vivo is unknown. Measurement of baseline VEGF levels (by ELISA) and serial monitoring in conjunction with well validated host, laboratory (e.g., Aspergillus galactomannan and/or Aspergillus PCR) and radiology (e.g., computerized tomography) data could allow calculation of sensitivity, specificity, negative predictive values, positive predictive values, likelihood ratios, and diagnostic odds ratios,
phenomenon would be indicated by a rapid increase in mortality in the final days of follow-up and neutrophilic infiltration of lungs on histopathology.

• What is the best method to study in vivo angiogenesis in our infection models? In our prior work, we used the matrigel plug assay (reviewed in ref. 20, Fig. 3).

The fact that this assay, is devoid of any other tissue other than capillaries might be problematic as it does not address the influence of other tissue or cell type (e.g., type-2 pneumocyte-derived pro-and anti-angiogenic factors) in neovascularization and tissue regeneration. As almost all of the work has been done in oncology background, adaptation of the model to other disease states associated with opportunistic mold infections is required.

• What the control groups will be in human studies of measurements of VEGF in leukemia patients with IA? In other words, what do we know on the circulating anti- or pro-angiogenic factors in similar patients with bacterial pneumonia or even in high risk patients without documented infections? Perhaps multiple controls will need to be employed, in order to address issues of specificity.

• Could stimulation of the VEGF axis in an attempt to battle angioinvasive molds increase the risk for cancer relapse? How should one strike the right balance in order to have the optimal therapeutic effect?

along with their associated 95% confidence intervals (CIs), of serum VEGF testing as diagnostic or prognostic parameter.

• Are VEGF inhibitors (e.g., thalidomide) an unrecognized risk factor for acquisition of invasive mold infections? Interestingly, invasive pulmonary aspergillosis was reported in a clinical trial of the VEGF inhibitor bevacizumab (Avastin) for the treatment of non-small cell lung cancer, a low-risk malignancy for IPA. As these agents are increasingly used in a variety of malignancies, prospective surveillance might be needed.

• Finally, what are they prospects of in vivo imaging (and monitoring) of an angioinvasive infection based on selective imaging using angiogenesis (e.g., VEGF) probes?

Research Pitfalls and Alternatives

One needs to be cognizant of the profound complexity of such system and the artificially of the animal models employed to study these complex questions. The cross-talk between different signalling pathways of endothelial cells in response to Aspergillus invasion is now started to be deciphered.

• In contrast with naturally occurring pulmonary aspergillosis, which can develop gradually over weeks, the acute pulmonary infection model allows a rather short follow-up period of up to 7 days. The rapid course of infection in this model may not allow adequate study of neovascularization. Furthermore, these effects might be tissue-specific (confined to lungs) as there might be important heterogeneity in type and density of specific receptors for vascular growth factors. For these reasons, we recently have devised a complementary subacute thigh infection model (Ben-Ami R, et al. AAC, in press) that could allow us the parallel study of the angiogenesis effects in two models that have different infection tissue and pace of evolution. If histopathology fails to show differences in angiogenesis in the treatment groups in the lung model, these outcomes may still be evident in the thigh model. In addition, rapid effects of VEGF on endothelial survival might be evident in the pulmonary model. Perhaps the interplay between tissue repair/inflammation/vasculature homeostasis and hypoxia is more important in infected lung tissue because of its unique blood-air interface for oxygen delivery.

• Pro-angiogenic treatment might worsen mouse survival by increasing the accessibility of blood vessels to fungal hyphae and metabolites. In addition, mice treated with VEGF and bFGF might be more susceptible to pulmonary inflammation when circulating neutrophil counts recover. Such an “immune reconstitution” phenomenon would be indicated by a rapid increase in mortality in the final days of follow-up and neutrophilic infiltration of lungs on histopathology.

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• Could stimulation of the VEGF axis in an attempt to battle angioinvasive molds increase the risk for cancer relapse? How should one strike the right balance in order to have the optimal therapeutic effect?
As the population of patients at risk for these devastating opportunistic mold infections continues to expand, there is clearly an urgent need for novel therapeutic approaches. Our initial work suggests that, Aspergillus growth and inflammation in the lung evoke neovascularization and angiogenesis. Further work is required to decipher the implications for pathogenesis and drug delivery. There is a strong amount of information in biology, pharmacology of the angiogenesis system, especially of the VEGF pathway and the development of relevant agents from the oncology field. Hopefully, such knowledge can be transported and exploited for adjunct, novel strategies for the treatment of angioinvasive molds.

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