Blood–brain barrier invasion by *Cryptococcus neoformans* is enhanced by functional interactions with plasmin

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Cryptococcus neoformans can invade the central nervous system through diverse mechanisms. We examined a possible role for host plasma proteases in the neurotropic behaviour of this blood-borne fungal pathogen. Plasminogen is a plasma-enriched zymogen that can passively coat the surface of blood-borne pathogens and, upon conversion to the serine protease plasmin, facilitate pathogen dissemination by degrading vascular barriers. In this study, plasminogen-to-plasmin conversion on killed and viable hypoencapsulated strains of *C. neoformans* required the addition of plasminogen activator (PA), but this conversion occurred in the absence of supplemented PA when viable strains were cultured with brain microvascular endothelial cells (BMEC). Plasmin-coated *C. neoformans* showed an enhanced invasive ability in Matrigel invasion assays that was significantly augmented in the presence of BMEC. The invasive effect of plasmin required viable pathogen and correlated with rapid declines in BMEC barrier function. Plasmin-enhanced invasion was inhibited by aprotinin, carboxypeptidase B, the lysine analogue epsilon-aminocaproic acid, and by capsule development. *C. neoformans* caused plasminogen-independent declines in BMEC barrier function that were associated with pathogen-induced host damage; however, such declines were significantly delayed and less extensive than those observed with plasmin-coated pathogen. BMEC adhesion and damage by hypoencapsulated *C. neoformans* were diminished by capsule induction but unaltered by plasminogen and/or PA. We conclude that hypoencapsulated *C. neoformans* can invade BMEC by a plasmin-dependent mechanism, *in vitro*, and that small, or minimal, surface capsule expression during the blood-borne phase of cryptococcosis may promote virulence by means of plasmin(ogen) acquisition.

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INTRODUCTION

Cryptococcus neoformans is an encapsulated fungus with a global distribution and is recognized as a major opportunistic pathogen (Bennett *et al.*, 1977; Kwon-Chung & Bennett, 1984). *C. neoformans* has been grouped into two distinct serotypes (A and D) on the basis of capsule structure/antigenicity, with serotype A responsible for 95 % of the cryptococcal infections reported worldwide (Franzot *et al.*, 1999; Idnurm *et al.*, 2005). The onset of cryptococcosis is believed to occur following inhalation of either basidiospores or desiccated yeast (Kwon-Chung, 1992; Velagapudi

et al., 2009). Both forms are optimally sized for alveolar deposition and pulmonary infection, which, if not controlled, can lead to the haematogenous spread of *C. neoformans* to the brain and other target organs.

Blood-borne *C. neoformans* demonstrates a unique tropism for the central nervous system (CNS) that typically results in cryptococcal meningitis (Kovacs *et al.*, 1985; Lee *et al.*, 1996). The ability of *C. neoformans* to penetrate the blood–brain barrier (BBB) by diverse and mutually independent mechanisms likely contributes to its tropism for the CNS. For example, both transcytosis (Chang *et al.*, 2004; Chen *et al.*, 2003) and a phagocytosis-mediated (Trojan horse) mechanism (Charlier *et al.*, 2009) have been demonstrated as routes of BBB traversal for this pathogen. Transcytosis is an energy-dependent process of internalization and transcellular transfer initiated by receptor–ligand interaction (Tuma & Hubbard, 2003), while Trojan horse entry involves the passive transfer of pathogen into the CNS

Abbreviations: εACA, epsilon-aminocaproic acid; BBB, blood–brain barrier; BMEC, brain microvascular endothelial cells; CB, carboxypeptidase B; CNS, central nervous system; HUVEC, human umbilical vascular endothelial cells; LDH, lactate dehydrogenase; PA, plasminogen activator; TEER, transendothelial electrical resistance.

Four supplementary figures are available with the online version of this paper.

within emigrating monocytes (Kim, 2008). Evidence from murine and rat models suggests that BBB damage may also contribute to CNS invasion by *C. neoformans* (Charlier *et al.*, 2005; Pai *et al.*, 2009), and others have reported damage to human umbilical vascular endothelial cells (HUVEC) during pathogen co-culture, *in vitro* (Ibrahim *et al.*, 1995a). Additionally, a possible role for the plasminogen fibrinolytic network in cryptococcosis is suggested by the plasminogen-binding activity of *C. neoformans*, *in vitro* (Stie *et al.*, 2009).

The survival and pathogenicity of *C. neoformans* within host tissues require fungal cell encapsulation. Capsular polysaccharides: (1) shield fungal cells from damage by activated leukocytes and diffusible antimicrobials (Bolaños & Mitchell, 1989; Chang & Kwon-Chung, 1994; Kozel & Gotschlich, 1982; Zaragoza *et al.*, 2008); (2) modulate host immune function (Dong & Murphy, 1995; Lipovsky *et al.*, 1998; Vecchiarelli *et al.*, 1996); and (3) regulate the transcytosis of *C. neoformans* across brain microvascular endothelial cells (BMEC), *in vitro* (Chang *et al.*, 2004). Paradoxically, however, the virulence of clinically isolated strains can vary independently of capsule size. Studies examining a molecular basis for cryptococcosis in immunocompetent patients found that more than 20% of serotype A isolates from patient cerebrospinal fluid (CSF) exhibited a cAMP-unresponsive, hypocapsular phenotype *in vitro* (D'Souza *et al.*, 2004). Other studies found that serotype A mutants lacking the transcriptional regulator Rim101 exhibited a hypocapsular phenotype that was hypervirulent in a mouse model (O'Meara *et al.*, 2010).

We have shown that capsule expression abrogates functional associations between *C. neoformans* and plasminogen (Stie *et al.*, 2009). The plasminogen fibrinolytic network is involved in a range of host functions, including tissue repair, angiogenesis, cell migration, inflammation and neuronal cell signalling (Del Rosso *et al.*, 2008; Melchor & Strickland, 2005; Plow *et al.*, 1995; Romer *et al.*, 1996; Tkachuk *et al.*, 2009). The plasminogen pro-enzyme must first be deposited on fibrin matrices or cell surfaces before it can be activated. This deposition process is mediated by five conserved triple-loop amino-terminal Kringle domains that recognize surface-accessible carboxyl-terminal lysine residues (Plow *et al.*, 1995) and/or exposed internal lysine residues (Cork *et al.*, 2009). The surface-bound plasminogen is targeted by one of two central activators, either tissue-type plasminogen activator (PA) or urokinase-type plasminogen activator (uPA), that mediate plasminogen conversion to the disulphide-linked, two-chained serine protease plasmin (Miles *et al.*, 2005). Plasmin serves as a primary effector of fibrinolysis and other critical proteolytic cascades that can potentially be used to promote or exacerbate disease when plasmin function becomes subverted by microbial pathogens (Lähteenmäki *et al.*, 2001; Plow *et al.*, 1991).

While the low-stringency binding requirements of plasminogen on host cells facilitate its involvement in diverse

physiological processes, they also permit microbial subversion of plasmin function (Ellis, 2003). Animal modelling experiments implicate microbial-directed plasmin proteolysis in the dissemination of several invasive bacterial pathogens, such as group A streptococci (Sun *et al.*, 2004), *Yersinia pestis* (Goguen *et al.*, 2000) and *Borrelia* species (Coleman *et al.*, 1997; Gebbia *et al.*, 1999). Matrix metalloproteinases (MMPs) and procollagenases (PCs) serve as primary targets of plasmin, and are implicated as mediators of plasmin-facilitated microbial dissemination across vascular and cerebrovascular barriers (Lähteenmäki *et al.*, 2001). The plasmin-activated MMPs and PCs compromise microvascular endothelium by disrupting the inter-endothelial cell adhesion junctions and associated extracellular protein networks essential for vascular barrier formation, thus promoting vascular leakage and the dispersal of plasmin-coated pathogen (Lähteenmäki *et al.*, 2001). Alternatively, the pro-enzyme, plasminogen, has itself been shown to facilitate invasion by increasing pathogen adhesion to host tissues (Attali *et al.*, 2008).

A potential role for the plasminogen fibrinolytic network in the virulence of invasive fungal pathogens is underscored by the increasing number of medically important fungi that bind plasminogen, including *Candida albicans* (Crowe *et al.*, 2003; Jong *et al.*, 2003), *Paracoccidioides brasiliensis* (Nogueira *et al.*, 2010), *Aspergillus fumigatus* (Zaas *et al.*, 2008), *Pneumocystis jirovecii* (carinii) (Fox & Smulian, 2001) and *C. neoformans* (Stie *et al.*, 2009). This interaction confers or supplements the ability of fungi to degrade physiological substrate (Nogueira *et al.*, 2010; Stie *et al.*, 2009) and enhances *Candida albicans* invasion of BMEC, *in vitro* (Jong *et al.*, 2003). In light of the strong affinity of blood-borne *C. neoformans* for the CNS, an important but unresolved question is whether plasminogen deposition contributes to cryptococcal invasion of the highly restrictive BMEC component of the BBB. In this report, we examine the effects of the plasminogen fibrinolytic network on the ability of *C. neoformans* to interact with and invade BMEC using an established *in vitro* model of the BBB.

METHODS

Strains. The fungal organisms used in this study include the *C. neoformans* serotype D strains JEC21 (*MAT α*) and B3501A (*MAT α*) and the serotype A strain C23 (Litvintseva *et al.*, 2006; Litvintseva & Mitchell, 2009). *Saccharomyces cerevisiae* strain YPH499 was included as a control. The acapsular strain FCH78 (*cap59::nat*) was generated from strain JEC21 as previously described (Stie *et al.*, 2009). The genetic identities of the JEC21 and B3501A strains were confirmed by allele-specific PCR based on strain-specific polymorphisms of the *CNB1* locus (Loftus *et al.*, 2005).

Fungal cell culture methods. Strains were cultured to mid-exponential phase in liquid yeast extract-peptone-dextrose (YPD) medium at 25 °C prior to analysis. Aggregation of exponential phase FCH78 cultures was reduced by suspension in PBS with 2.5% BSA or conditioned medium from 24 h mono-cultured BMEC. All experiments were performed using exponential phase yeast forms bearing a small, or minimal, surface capsule (defined by the absence of visible

capsule upon India ink analysis), unless otherwise indicated. Cell counts were determined by haemocytometer. Killed strains were prepared from exponential phase cultures by treatment with 3.5 % formaldehyde (Sigma) in PBS for 30 min at 25 °C or by exposure to 10 mM sodium azide, and were verified as nonviable by YPD plating or as described elsewhere (Rodrigues *et al.*, 2007).

Capsule induction, transfer and quantification. Capsule growth was induced by culturing strains in Sabouraud's broth diluted 1:10 in PBS for 96–120 h at 30 °C and confirmed by India ink analysis (Zaragoza & Casadevall, 2004). For some experiments, capsule was induced by 12 h pre-exposure to the incubation conditions used for invasion assays. Capsule diameters were calculated from micrographs of India ink-stained cells by quantitative image analysis (Carnoy 2.0). Capsule transfer experiments were performed according to a reported method (Reese & Doering, 2003). For these experiments, strain JEC21 and the isogenic *cap59* mutant strain FCH78 were used as donor and acceptor strains, respectively. Medium supernatants from capsule induction cultures were isolated by centrifugation at 1000 g for 1 min and subsequently filtered to remove residual donor cells. Medium supernatants were verified to be free of donor encapsulated cells by microscopy and plating on YPD agar. Acceptor cells were washed twice in PBS and incubated with filtered conditioned medium in PBS at a final concentration of 10^8 cells ml⁻¹ at 25 °C for 1 h under gentle agitation. After incubation, cells were washed and suspended in PBS with 1 % BSA. Capsule transfer was verified microscopically by India ink analysis at a magnification of $\times 400$ and quantified at $\times 1000$.

Plasminogen labelling and activation. Fungal cells (10^8) were incubated with 50 µg purified human plasminogen (Glu-plasminogen, Fitzgerald Industries) in 500 µl PBS with 1.5 % BSA (PBS-BSA) at 37 °C for 1 h. The plasminogen-coated cells were recovered by 2000 g sedimentation, washed twice in cold PBS-BSA, and enumerated. Plasmin-coated cells were prepared by incubating the plasminogen-coated cells with 5 µg tissue-type PA (Calbiochem) as above for 4 h, before counting.

Plasmin activity assays. Fungal strains were analysed for cell-surface plasmin activity using either the synthetic plasmin-specific substrate Chromogenix (Glu-Phe-Lys-pNA; S-2403, Fisher Scientific), or fibrinogen (Sigma), a physiological substrate of plasmin. For Chromogenix assays, plasminogen-coated fungal cells were incubated with BMEC and/or 1 µg PA, washed twice in PBS-BSA, and once in reaction buffer A [20 mM HEPES (Sigma), 120 mM NaCl, pH 7.5] without substrate. Cells were suspended in reaction buffer containing 1 mg Chromogenix ml⁻¹ and distributed into microtitre plate wells at a concentration of 10^5 in a 150 µl volume and incubated for 4 h at 37 °C, and substrate cleavage was assessed via A_{405} . Alternatively, fungal cells (10^5) were suspended in reaction buffer B (20 mM Tris/HCl, pH 7.6, 150 mM NaCl) containing 500 µg fibrinogen ml⁻¹ (Sigma). Reaction volumes of 100 µl in 1.5 ml tubes were incubated for 6 h at 37 °C. Fungal cells were removed by sedimentation and the assay supernatants fractionated by SDS-PAGE, with fibrinogen proteolysis examined on polyacrylamide gels stained with Simply Blue (Invitrogen) or by Western analysis after transfer to PVDF. Plasmin activity was inhibited by addition of the protease inhibitor aprotinin (Sigma), at 100 µg ml⁻¹.

Inhibition of plasminogen binding. Plasminogen binding sites were removed by pretreatment of strain B3501A (10^8 ml⁻¹) with carboxypeptidase B (CB) at concentrations of up to 10 U in 500 µl reaction buffer (25 mM Tris/HCl, pH 7.65, 100 mM NaCl) (Wolff *et al.*, 1962). Mock-treated controls of B3501A were incubated in the same buffer without CB. After 30 min at 37 °C, reactions were quenched by the addition of a 10-fold volume of cold reaction buffer without enzyme, sedimented for 5 min at 2000 g, washed twice in PBS and suspended in pre-warmed assay medium for use in invasion

assays. For Western blot analysis, CB-treated cells were incubated with 50 µg plasminogen in PBS-BSA for 1 h at 37 °C, washed as above, and further processed as described below. The inhibition of plasminogen binding with epsilon-aminocaproic acid (εACA; Sigma) was performed by the addition of εACA to the assay medium at the concentrations indicated in Results, immediately prior to the start of the assays.

SDS-PAGE and Western blotting. SDS-PAGE was performed as previously described (Stie *et al.*, 2009) using NuPAGE precast 10 % Bistris gels (Invitrogen). Fractionated proteins were transferred onto PVDF membranes using a Novex XCell II blot module (Invitrogen). PVDF membranes were blocked with 3 % BSA in PBS also containing 0.05 % Tween 20 for 14–18 h at 4 °C, and incubated for 1 h at 25 °C with rabbit anti-plasminogen polyclonal antibody (Fitzgerald Industries) or rabbit anti-fibrinogen polyclonal antibody (Calbiochem) diluted 1:5000 in PBS with 2 % BSA. Blots were washed four times for 5 min in wash buffer (PBS with 2 % BSA) and incubated for 1 h at 25 °C with secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma, 1:25000). Blots were washed as above and exposed for 5 min at 25 °C to peroxide and luminal/enhancer buffers (Bio-Rad) prior to chemiluminescence analysis by automated imager (AFP Imaging). Equal loading of protein samples in polyacrylamide gels was verified by densitometry analysis (ImageJ) of the total protein per lane, which varied by less than 0.2 %. The protein content of PVDF membranes was visualized using the India ink staining procedure (Hancock & Tsang, 1983), after antibody removal with Restore PLUS reagent (Pierce). Equivalent protein loading is represented by the inclusion of a dominant 50 kDa band from the total protein blots of the fungal strains examined in this study.

BMEC cell culture. Primary bovine BMEC (Cell Applications, Inc.) were acquired at second passage in cryopreserved ampules or at third passage as confluent cells in culture medium and maintained by culture at 37 °C with 5 % CO₂ in medium with 10 % fetal calf serum (FCS), amphotericin B and antibiotics. Cultured cells were passaged and subcultured using filtered flasks (Corning) pre-coated with an attachment factor solution (Cell Applications) to facilitate BMEC adherence and growth. BMEC were used for functional assays between passages 3 and 6. No discernible defects in cell morphology and growth, or decreases in viability (Trypan blue), could be detected in cultured cells up to passage 6. Cultures were routinely examined by light microscopy during growth by cell fixation in 1.5 % glutaraldehyde with 0.5 % formaldehyde for 15 min at 25 °C. Fixed cells were stained in a solution of 0.03 % R250 Coomassie with 3 % acetic acid and 50 % methanol for 10 min at 25 °C, and evaluated after gentle rinsing with Hanks' balanced salt solution (HBSS).

Measurement of BMEC barrier resistance by transendothelial electrical resistance (TEER). TEER analysis was performed using the Millicell electrical resistance system (Millipore) to examine BMEC monolayer integrity and to confirm confluent growth. TEER readings of BMEC cultured in 24-well transwell tissue culture plates (described below) were taken at several time points after seeding BMEC in transwell inserts. Maximum resistance typically occurred after 4 days of culture and persisted for at least an additional 7 days. Background TEER values included resistance across Matrigel inserts without BMEC, and were subtracted from the values reported. For some experiments, hourly TEER measurements were taken over the 12 h time-course of *C. neoformans*-BMEC invasion assays, with electrodes cleaned and resterilized before and after each reading.

BMEC paracellular permeability. BMEC paracellular permeability measurements were performed by tracer analysis using FITC-dextran with an average molecular mass of 40 kDa (Sigma). BMEC were cultivated in transwells using 500 and 700 µl of culture medium in

upper (insert) and lower chambers, respectively. Transwells were removed from incubation at 37 °C with 5 % CO₂ at various times after BMEC seeding and prepared for tracer studies by replacing the culture medium in the upper and lower chambers with 150 and 700 µl volumes of HBSS, respectively, to minimize the influence of hydrostatic pressure on the free diffusion of tracer molecules (Quan & Godfrey, 1998). Changes in BMEC monolayer permeability were examined based on a method described elsewhere (Wong & Gumbiner, 1997).

Invasion assays. Assays were performed using 24-well transwell tissue culture plates, with individual plate wells (lower chambers) fitted with inserts (upper chambers) containing 8 µm pore-size polyethylene terephthalate (PET) membranes coated with a uniform layer of Matrigel (BD Biosciences). BMEC were grown in upper chambers with the addition of attachment factor (Cell Applications) for 30 min at 37 °C with 5 % CO₂, followed by seeding with 10⁵ BMEC per insert. Fungal invasion was examined in Matrigel inserts, with or without BMEC, using different methods of plasmin(ogen) addition: (1) soluble plasminogen (15 µg), with or without 1 µg PA, in assay medium, or (2) fungal cells were pre-coated with plasmin prior to invasion assays and assayed without soluble plasminogen and PA in the assay medium.

Transwell invasion assays contained 500 and 700 µl assay medium (amphotericin B-free BMEC culture medium supplemented with 20 % plasminogen-depleted FCS) in upper and lower chambers, respectively. Plasminogen was removed from the FCS by immunoprecipitation with plasminogen-specific antiserum (Fitzgerald Industries) conjugated to CNBr-activated Sepharose beads (Amersham Biosciences) according to the manufacturer's protocol. Beads with or without antibody were incubated with concentrated FCS (pH adjusted to 7.3) for 1 h at 25 °C and washed four times for 5 min with PBS-BSA, with plasminogen depletion confirmed by SDS-PAGE/Western analysis after immunoprecipitation (Supplementary Fig. S1).

Invasion assays were initiated by the addition of 10⁵ yeast forms per insert to give an m.o.i. of 3 : 1 (yeast to BMEC ratio), followed by 12 h incubation of yeast with BMEC at 37 °C with 5 % CO₂. Fungal invasion was quantified based on the number of c.f.u. recovered by serial dilution and plating on YPD agar from 100 µl aliquots withdrawn from the lower transwell chambers. For some experiments, fungal recovery was assessed hourly, in parallel with TEER measurements. In these experiments, the lower chambers were replenished with 100 µl fresh, pre-warmed (37 °C) medium after each aliquot withdrawal. For experiments examining the invasion ability of killed fungal cells, the medium collected from the lower chambers was centrifuged at 5000 g and the resulting pellet examined by light microscopy at ×400 and ×1000 magnification for the presence of cryptococcal cells.

Experiments comparing the ability of *C. neoformans* strains bearing small versus large surface capsule to penetrate 8 µm pores by syringe filtration used 13 mm diameter, circular polycarbonate membranes with an 8 µm pore size. The polycarbonate membranes were pre-coated with 6 % BSA (Santa Cruz Biotechnology), 0.01 % Tween 20 (Sigma) in PBS for 18 h at 4 °C prior to use. The pre-coated membranes were fitted into cartridges that were Lauer-locked onto 5 ml syringes (BD Biosciences). Fungal cells were pre-washed twice in the same coating solution and added to syringes at a concentration of 10³ cells ml⁻¹.

BMEC adherence assays. Fungal–host adherence was measured in 24-well plates containing confluent BMEC. Assays were initiated by the addition of 500 yeast-form cells from *C. neoformans* serotype A strain C23, serotype D strains B3501A and JEC21, or the acapsular mutant FCH78. Assays were performed in the presence or absence of

plasminogen (15 µg) and/or PA (1 µg), and incubated at 37 °C with 5 % CO₂ for 15 min up to 108 min in the same medium used for invasion assays described above. Non-adherent cells were removed by four 5 min washes in HBSS under mild agitation. The washed endothelial cell monolayers were lysed with 500 µl per well of ice-cold sterile distilled water containing 0.05 % SDS, and the lysates plated on YPD agar for colony count determination (Whittington & Wang, 2011).

BMEC damage. Fungal cell damage to BMEC was measured using a lactate dehydrogenase (LDH) detection kit (Clontech) according to manufacturer's guidelines in 96-well plates containing BMEC monolayers, and was initiated by the addition of *C. neoformans* serotype A strain C23, serotype D strains B3501A and JEC21, or the acapsular strain FCH78. For some experiments, strains were grown in capsule induction medium and verified for capsule expression by India ink staining before addition to damage assays. Plasminogen (30 µg ml⁻¹) and/or PA (2 µg ml⁻¹) were included in the assay medium, as indicated. Damage detection assays were performed in invasion assay medium without phenol red. Reactions quantifying LDH activity in supernatants from fungal cell–BMEC co-cultures were initiated by the synchronous addition of LDH substrate to plate wells by a method described elsewhere (Henkel *et al.*, 1988). Activity was quantified as the percentage of the total LDH activity available per well after solubilization in assay buffer with 2 % Triton X-100 and subtraction of the baseline activity present in supernatants from BMEC mono-cultured controls.

Calculations. In experiments where the invasion ability of plasmin pre-coated *Cryptococcus* was compared for BMEC–Matrigel (B) versus Matrigel alone (M), the following formula was used: (number of transmigrated plasmin pre-coated cells)/(number of transmigrated non-plasmin-coated control cells)=fold increase→(fold increase across BMEC–Matrigel)/(fold increase across Matrigel)=fold increase B/M.

The following calculation is used in Table 2 to determine the relative enhancement of fungal invasion due to the presence of plasminogen and PA: (percentage invasion – Plg) – (percentage invasion + Plg). Accordingly, 'percentage invasion + Plg' represents total (100 %) invasion, where 'percentage invasion' is defined as (no. of fungal transmigrants/total fungi added per well) in the presence (+Plg) or absence (–Plg) of plasminogen and PA in the assay medium, as indicated.

Statistical analysis. Statistical comparisons were made using GraphPad Prism software, version 4.0. The *t* test and analysis of variance (ANOVA) were used, as indicated in Results. ANOVA was used in association with Dunnett's test, where indicated. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Plasminogen to plasmin conversion on *C. neoformans*

Plasminogen binds to serotypes A and D of *C. neoformans*, which, in the presence of PA, results in plasmin formation on the fungal cell surface (Stie *et al.*, 2009). To further characterize this process, viable and killed *C. neoformans* were coated with plasminogen and examined for their ability to undergo PA-dependent conversion of plasminogen to plasmin, as demonstrated by the cleavage of fungal-bound plasminogen to the plasmin heavy chain on

Fig. 1. Fungal viability influences surface plasminogen activation in the presence of BMEC. Killed or viable *C. neoformans* strains B3501A, C23 and JEC21, and *S. cerevisiae* strain YPH499, were coated with plasminogen and incubated for 4 h with (+) or without (-) soluble PA or BMEC, as indicated. (a) The activation of surface-bound plasminogen to plasmin was demonstrated by the conversion of plasminogen (Plg) to plasmin heavy chain (Pla_H). Twenty micrograms were loaded per lane, with protein loading controls located below each blot. Representative results from three separate experiments are shown. (b) Surface plasmin activity, with the plasmin substrate Chromogenix, on plasminogen-coated viable (left) and nonviable (right) strains after 4 h exposure to PA in the presence (+) or absence (-) of BMEC, as indicated. Background activity from

fungal–BMEC control co-cultures without plasminogen and PA was subtracted from the results shown. Bars represent mean and SEM from four separate experiments. * $P < 0.05$ by *t* test for same-strain comparisons under the indicated incubation conditions. (c) Upper two panels: cleavage of the alpha (α), beta (β) and gamma (γ) bands of fibrinogen by plasminogen-coated viable and nonviable fungal strains after incubation with (+) or without (–) PA or BMEC, as indicated. The proteolysis of fibrinogen resulted in the concomitant appearance of a 40 kDa degradation product (*). Control lanes (–) include fibrinogen standard incubated in assay buffer with or without BMEC, as indicated. Lower panel: the fibrinogen cleavage activity of plasminogen-coated viable strains is shown after their co-incubation with BMEC, in the presence (+) or absence (–) of PA supplementation, as indicated. After incubation with BMEC and/or PA, fungal cells were suspended in a fibrinogen solution that was subsequently evaluated for fibrinogen proteolysis from 20 μ g of total protein loaded per lane. The results shown are representative of four separate experiments.

Western blots (Fig. 1a). Robust PA-mediated plasminogen cleavage was observed on both killed and viable strains of *C. neoformans* and *S. cerevisiae*, and typically resulted in the depletion of intact plasminogen on cell surfaces (Fig. 1a). Plasminogen cleavage in these experiments resulted in the generation of functional plasmin on fungal cells, as evidenced by fungal ability to degrade the plasmin-specific synthetic substrate Chromogenix (Fig. 1b) and fibrinogen, a physiological substrate of plasmin (Fig. 1c). Fibrinogen proteolysis by fungal-bound plasmin in these studies resulted in the complete cleavage of the fibrinogen- α , - β and - γ chains with the concomitant appearance of a prominent 40 kDa band, corresponding to the principal degradation product resulting from exposure of fibrinogen to soluble plasmin (Monaco *et al.*, 2007).

Because BMEC can express regulators of plasminogen activation (Pepper *et al.*, 1996; Pepper, 2001), we determined whether the co-culture of fungal cells with BMEC influenced the PA-mediated plasminogen to plasmin conversion process on fungal cells. Incubation with BMEC supplemented with PA resulted in diminished plasminogen activation on killed *C. neoformans* and both killed and viable *S. cerevisiae* (Fig. 1a). This reduced plasminogen activation was evident from the persistence of intact plasminogen on cells, the correspondingly low levels of plasminogen cleavage on cell surfaces, and the reduced ability of fungi to degrade plasmin-specific substrates in functional assays (Fig. 1a–c). Conversely, viable strains of *C. neoformans* exhibited no compromise in plasminogen to plasmin conversion when cultured with BMEC and PA, as evidenced by the robust cleavage of fungal-bound plasminogen to plasmin on Western blots and by the strong surface-bound plasmin activity on viable *C. neoformans* in functional assays (Fig. 1a–c). Interestingly, plasminogen activation also occurred on viable (Fig. 1c, lower panel), but not killed (data not shown), strains of *C. neoformans* cultured with BMEC in the absence of PA addition, indicating the possible induction of a BMEC-derived PA activity by viable *C. neoformans*. These results led us to suggest a regulatory role for BMEC in plasmin formation occurring on the fungal cell surface. The potential importance of these findings to *C. neoformans* pathogenesis, *in vivo*, is the subject of ongoing investigations (J. Stie & D. Fox, unpublished results).

***In vitro* modelling of the BBB**

BMEC cultures were grown on 8 μ m pore transwell inserts pre-layered with Matrigel (BMEC–Matrigel) to model the effects of plasmin on CNS invasion by *C. neoformans*. We first characterized the ability of BMEC to form restrictive barriers, a defining attribute of the BBB, *in vivo*. BMEC confluency was measured over time by TEER and paracellular permeability, and further evaluated by microscopic analysis. BMEC exhibited a normal spindle-shaped morphology by Coomassie staining on day 4 of culture, with adjacent cells tightly packed into uniform monolayers (Supplementary Fig. S2a). Electrical resistance and Dextran impermeability increased gradually from day 1 of cell seeding, consistent with the formation of tight junctions between cells and monolayer development. Maximum values of resistance and Dextran impermeability were observed after 4 days of BMEC culture in transwell inserts and persisted for a minimum of 6 days thereafter (Supplementary Fig. S2b). We examined whether the highly resistant barriers formed by BMEC in transwells adversely affected the invasive ability of *C. neoformans* by comparing the ability of strains bearing a small, or minimal, capsule to traverse BMEC–Matrigel with their ability to traverse Matrigel alone. We found that cryptococcal invasion was markedly reduced across BMEC–Matrigel, relative to Matrigel alone, indicating that the BMEC cultivated in this model system form stable barriers that are highly restrictive towards cryptococcal transwell passage (Supplementary Fig. S2c).

Plasmin-dependent cryptococcal invasion is enhanced by BMEC

Plasminogen-dependent fungal invasion of BMEC–Matrigel was examined under three different conditions. Strains of *C. neoformans* expressing small, or minimal, surface capsule were either pre-coated with plasmin prior to invasion assays or actively coated with plasmin during invasion assays by the addition of plasminogen, with or without PA, to assay medium. This approach allowed us to compare the invasive ability of plasmin pre-coated *C. neoformans* with that of fungal cells that became plasmin coated as a result of plasminogen activation during co-culture with BMEC, as shown in Fig. 1(c).

C. neoformans strains pre-coated with plasmin prior to invasion assays showed significantly greater invasion of

BMEC–Matrigel or Matrigel alone relative to strains not exposed to plasminogen or plasmin (data not shown). Interestingly, this plasmin-enhanced invasion ability was greater when invasion assays were performed in the presence of BMEC. For example, plasmin pre-coated *C. neoformans* demonstrated an up to eightfold greater invasion of BMEC–Matrigel than strains not exposed to plasmin(ogen), while exhibiting a less than twofold plasmin-dependent increase in invasion of Matrigel alone. This indicated that the invasion ability of plasmin-coated *C. neoformans* was several fold greater in the presence of BMEC (Fig. 2). The significance of this finding is emphasized by the intact monolayers formed by BMEC in this study (Supplementary Fig. S2b) and the sharp decline in cryptococcal invasion in the presence of BMEC relative to Matrigel alone when assayed in the absence of plasmin(ogen) (Supplementary Fig. S2c). The dependence of plasmin-enhanced cryptococcal invasion on the serine protease activity of plasmin was indicated by the abrogation of this enhancement when the serine protease inhibitor aprotinin was included in assays (Fig. 2). By comparison, the *S. cerevisiae* strain YPH499 exhibited minimal invasion of BMEC–Matrigel, irrespective of the inclusion of plasmin or its inhibitor, aprotinin (Fig. 2). The

Fig. 2. The plasmin-enhanced invasion ability of *C. neoformans* strains is potentiated in the presence of BMEC. The effect of BMEC on the plasmin-enhanced invasion ability of *C. neoformans* was determined by comparing the invasion of plasmin-coated and non-coated *C. neoformans* strains B3501A, C23 and JEC21, and *S. cerevisiae* strain YPH499, in inserts layered with either BMEC–Matrigel or Matrigel alone after a 12 h incubation period. Invasion assays using plasmin-coated strains were conducted in the absence (Pln) or presence (Pln/Aprotinin) of the plasmin protease inhibitor aprotinin, as indicated, with fungal invasion quantified by YPD counts of fungal cells isolated from the lower chamber of transwell plates. The results were calculated using the formula presented in Methods and show the relative mean fold increases in plasmin-dependent invasion and SEM, with the greater effect of plasmin on fungal invasion of BMEC–Matrigel (B) versus Matrigel alone (M) shown as the fold increase B/M (y axis). * $P < 0.05$ by *t* test for plasmin pre-coated *C. neoformans* assayed in the presence or absence of aprotinin.

additional importance of strain viability to plasmin-enhanced invasion by *C. neoformans* was indicated by the failure of killed strains to invade BMEC–Matrigel in assays with plasminogen and PA (Supplementary Fig. S3). The inability of killed *C. neoformans* strains to traverse BMEC–Matrigel in the presence of plasminogen and PA is likely related to the reduced levels of plasminogen activation on fungal surfaces during culture in the presence, but not absence, of BMEC (Fig. 1a–c) and suggests that plasmin-enhanced cryptococcal invasion is not a passive process.

The exposure of viable *C. neoformans* strains B3501A, C23 and JEC21 to soluble plasminogen, with or without added PA, during invasion assays conferred an enhanced BMEC–Matrigel invasion ability equivalent to that exhibited by plasmin pre-coated strains (Table 1). These results provided additional evidence that *C. neoformans* could become plasmin-coated in the absence of PA supplementation during co-incubation with BMEC when plasminogen is provided in the assay medium, and are also consistent with an induction of plasminogen activation during the co-culture of viable *C. neoformans* strains with BMEC (Fig. 1c). However, there were notable variations in the invasion ability of the *C. neoformans* strains. For example, strains B3501A and C23 showed greater invasive abilities than strain JEC21 under all conditions (Table 1), while strain JEC21 exhibited greater invasion than the *S. cerevisiae* strain YPH499 (Table 1). Although strain YPH499 showed a trend toward higher transmigration activity across BMEC–Matrigel in the presence of plasmin, the increases observed did not significantly differ from plasmin-free controls (data not shown). The negligible effect of soluble plasminogen, with or without PA, on YPH499 invasion of BMEC–Matrigel correlated with the reduced plasminogen activation occurring on YPH499 when cultured in the presence of BMEC, and the apparent inability of this fungus to elicit BMEC-dependent plasminogen activation (Fig. 1a–c).

Inhibition of cryptococcal–plasminogen interactions reduces BMEC–Matrigel invasion

Endothelial cells, including the BMEC used in our studies, can strongly interact with plasminogen (Miles *et al.*, 1988), such that plasminogen activation may also occur on BMEC when soluble plasminogen is provided in the assay medium. We therefore examined the influence of inhibitors of plasmin(ogen) deposition on the ability of *C. neoformans* to invade BMEC. The surface-exposed carboxyl-terminal lysine residues expressed on *C. neoformans* serve as key binding sites for plasminogen and can be removed by treatment with CB (Stie *et al.*, 2009). Treatment of strain B3501A with increasing concentrations of CB progressively inhibited plasminogen binding, with only residual binding activity remaining after fungal exposure to 10 U CB (Fig. 3c, lower panel). The pre-treatment of strain B3501A with 10 U CB prior to invasion assays significantly reduced its plasmin-enhanced invasion

Table 1. Relative effect of plasmin(ogen) products on fungal invasion of BMEC–Matrigel

Values represent the mean fold increase of the indicated plasmin(ogen) products on fungal invasion of BMEC–Matrigel relative to Matrigel alone for strains of *C. neoformans* expressing small or minimal surface capsule. The mean fold increases shown for cryptococcal strains are based on a mean invasion without plasminogen of 572 ± 106 c.f.u. across BMEC–Matrigel and $18\,595 \pm 4406$ c.f.u. across Matrigel alone per 12 h assay. The mean recovery of *S. cerevisiae* strain YPH499 under similar conditions was 40 ± 18 and 1215 ± 221 c.f.u. across Matrigel in the presence and absence of BMEC, respectively, per 12 h assay. The values shown are mean $\pm$ SEM. Plg, plasminogen.

Treatment	B3501A	C23	JEC21	FCH78	YPH499
Non-coated (Plg only)*	3.31 ± 0.37	3.50 ± 0.84	2.50 ± 1.14	3.50 ± 0.73	0.01 ± 0.007
Non-coated (Plg + PA)*	4.30 ± 1.10	4.10 ± 0.89	2.75 ± 0.60	3.66 ± 0.80	0.04 ± 0.018
Plasmin pre-coated	5.0 ± 0.62	5.60 ± 0.93	2.8 ± 0.34	3.86 ± 0.60	0.13 ± 0.012
P value ($n > 3$)†	0.42	0.58	0.95	0.93	0.63

*Strains not pre-coated with plasmin but exposed to plasminogen or plasminogen and PA during assays, as indicated.

†Statistical comparison of the values shown under each strain using ANOVA.

of BMEC–Matrigel relative to untreated controls (Fig. 3a). As this inhibition was not complete, we next determined whether the partial effect of CB pre-treatment on plasmin-enhanced invasion was due to the regeneration of carboxyl-terminal lysine residues on *C. neoformans* during invasion assays. We found that CB-pre-treated B3501A reacquired plasminogen-binding activity as early as 4 h after the start of assays and demonstrated progressive increases in plasminogen binding thereafter, suggesting a possible basis for the lack of complete inhibition obtained with CB (Fig. 3c, upper panel).

Significant declines in the plasmin-dependent invasion ability of strain B3501A also resulted when fungal interactions with soluble plasminogen were blocked by the lysine analogue ϵ ACA (Fig. 3b). Together, these results underscore the functional significance of cryptococcal–plasminogen interactions during BMEC–Matrigel invasion.

Capsule induction effects on plasmin-enhanced invasion of BMEC–Matrigel

Carbon dioxide can serve as a stimulus for the induction of capsule growth on *C. neoformans* (Granger *et al.*, 1985). Because invasion assays are conducted in a 5% CO₂ atmosphere, we examined whether the initially small capsule of the exponential-phase cryptococcal strains used for invasion assays underwent capsule growth in response to incubation conditions and whether fungal encapsulation affected plasmin-enhanced cryptococcal invasion of BMEC–Matrigel.

The effect of capsule development on plasmin-enhanced invasion was determined using the *cap59* strain FCH78, generated by site-specific mutagenesis of strain JEC21 (Stie *et al.*, 2009). We compared the invasive ability of FCH78, with or without pre-absorbed capsule, with that of the parental strain JEC21. When fungi were coated with plasmin during invasion assays by the addition of plasminogen and PA to the assay medium, the acapsular FCH78 showed threefold greater invasion ability than

capsule pre-absorbed FCH78, indicating that encapsulation inhibits plasmin-enhanced cryptococcal invasion (Fig. 4a). Comparable enhancements in the invasive ability of acapsular FCH78 occurred when plasminogen was provided in the assay medium without PA or when cells were pre-coated with plasmin prior to invasion assays (Table 1). The plasmin-dependent increases in fungal invasion exhibited by the acapsular strain were nullified when assays were performed with the serine protease inhibitor aprotinin (data not shown). No differences were detected in the plasmin-enhanced invasion ability of the capsule-absorbed FCH78 and its parent strain, suggesting that capsule development on JEC21 during invasion assays may contribute to its low invasive ability in response to plasmin. These results are consistent with the significantly reduced plasminogen deposition on capsule-absorbed FCH78, relative to non-capsule-absorbed, and our previous findings of reduced plasminogen deposition on wild-type strains of *C. neoformans* expressing a large surface capsule (Stie *et al.*, 2009, and data not shown).

In the absence of plasmin(ogen), strains JEC21 and FCH78 showed no difference in relative BMEC invasion, nor did capsule pre-absorption measurably affect the plasmin-independent invasion ability of FCH78 (Fig. 4a). These results suggested that the differences in invasion observed between the acapsular and parent strains are due to their relative ability to functionally interact with plasmin(ogen). Findings similar to those presented in Fig. 4(a) were obtained in experiments examining the plasmin-dependent invasion ability of capsule-induced versus non-induced strains JEC21, B3501A and C23, in which capsule induction led to significantly lower BMEC invasion (data not shown). When considered collectively, the above results suggest that the presence of exogenous capsule modulates plasmin(ogen)-dependent BMEC invasion ability.

To determine whether capsule growth was induced on strains of *C. neoformans* in response to the incubation conditions used for BMEC–Matrigel invasion assays, capsule development was assessed by India ink analysis

Fig. 3. Inhibition of cryptococcal-plasminogen interactions reduces BMEC-Matrigel invasion. (a, b) BMEC-Matrigel invasion by strain B3501A was examined in the presence or absence of plasminogen and PA (Plg/PA) after: (a) removal of plasminogen-binding sites by pre-treatment with 10 U CB, or (b) the addition of a competitive inhibitor of plasminogen, the lysine analogue ϵ ACA, to the assay medium. The results shown for ϵ ACA treatment represent the mean inhibition with applied concentrations of 0.7–1.6 mM ϵ ACA. Bars represent the mean and SEM from four separate experiments. * $P < 0.05$ by t test for the datasets included under each horizontal line. (c) Upper panel: Western blot showing the re-expression of plasminogen-binding sites on strain B3501A under the assay conditions, after pretreatment with 10 U CB. Numbers represent the time (h) after the introduction of CB-pretreated fungi into the assay medium (0 h). Purified plasminogen is shown in the right-hand lane (Std). Lower panel: Western blot

showing the concentration-dependent effect of CB on the plasminogen-binding activity of strain B3501A, with the number above each lane indicating the activity units (U) of CB applied. Twenty micrograms were loaded per lane, with protein loading controls located below each blot. The results shown are representative of three separate experiments.

over a 24 h period. All strains except FCH78 developed capsule in response to our assay conditions, but varied in the extent of their capsule growth. Strain JEC21 was especially active in early capsule production, more so than either B3501A or C23, and became encapsulated after 4–6 h (Fig. 4b and Supplementary Fig. S4). By contrast, strains B3501A and C23 did not become encapsulated until 8 h of incubation, with both strains showing a lesser degree of encapsulation than strain JEC21 at the end of 24 h (Fig. 4b and Supplementary Fig. S4). Capsule induction on strains JEC21 and C23 was followed by the linear accumulation of capsule over time and resulted in two- to fourfold increases in cell size by 12 h (Fig. 4b and Supplementary Fig. S4). By contrast, B3501A, once induced to form capsule, showed little or no capsule growth over the remaining incubation time (Fig. 4b and Supplementary Fig. S4).

We next determined whether the increases in cell diameter conferred by the linear growth of capsule on strains C23 and JEC21 could impede fungal penetration of the 8 μ m-filtered inserts used for transwell invasion assays. JEC21 was selected for this analysis because of its rapid and extensive encapsulation under the assay incubation conditions (Fig. 4b and Supplementary Fig. S4). Encapsulation was induced by 12 h exposure of JEC21 to the conditions outlined in Fig. 4(b); then, the traversal of induced (large-capsule) and non-induced (small-capsule) populations was compared across 8 μ m filter membranes by two different methods. In the first method, invasion was examined across Matrigel without BMEC or plasmin(ogen). The results obtained from these experiments showed no difference in the relative invasive ability of JEC21 expressing large or small amounts of surface capsule (data not shown). In the second approach, JEC21 cultures expressing a small or large surface capsule showed equivalent penetration of 8 μ m filter membranes when subjected to syringe filtration (data not shown). The equivalent ability of JEC21 yeast forms bearing large or small capsules to penetrate the 8 μ m filter pore, either by syringe filtration or during Matrigel invasion assays, indicates that capsule size does not physically interfere with fungal ability to penetrate the filter membranes of the transwell system used for our *in vitro* model.

Cryptococcal-BMEC interactions during co-culture

The ability of *C. neoformans* to adhere to or damage endothelial cells likely contributes to its invasive ability, *in*

Fig. 4. Cryptococcal capsule induction during invasion assays and the effects of capsule development on plasmin-enhanced cryptococcal invasion of BMEC–Matrigel. (a) Invasive ability of the *cap59* mutant FCH78, relative to the parental strain JEC21, before (–) or after (+) capsule transfer to FCH78. Plasminogen (Plg) and PA were either included (Plg/PA) or omitted (Control), as indicated. Values shown are mean $\pm$ SEM from five separate experiments. * $P < 0.05$ by *t* test for comparisons between Plg/PA-treated strains. (b) Capsule growth on strains with a small, or minimal, initial capsule (0 h) was measured at various times over 24 h incubation in invasion assay medium at 37 °C with 5 % CO₂. The radial thickness of capsules is shown in μ m. Values shown are averaged values from two separate experiments.

vitro (Chang *et al.*, 2004; Ibrahim *et al.*, 1995a). We therefore examined the ability of *C. neoformans* to adhere to and damage BMEC, and the effect of plasminogen components and fungal encapsulation on cryptococcal–BMEC interactions. Fig. 5(a) shows that *C. neoformans* began to adhere to BMEC after 15 min of culture. A peak adherence of 9 % or greater was detected for strains B3501A, C23 and FCH78 after 45 to 60 min, while strain JEC21 exhibited a significantly reduced adherence of 4 % during the same period. These latter findings are consistent with the reduced invasion activity of strain JEC21 shown in Fig. 2 and Table 1, and suggest that encapsulation contributes to the impaired ability of this strain to productively interact with BMEC.

Fungal-induced BMEC cytotoxicity was determined by the presence of cytosolic LDH in the assay medium, which became detectable at 4 h of *C. neoformans*–BMEC co-culture and progressively increased up to 8 h (Fig. 5b). Strains B3501A, C23 and acapsular FCH78 demonstrated a similar cytotoxicity of 17 % after 8 h of co-culture, compared with 8 % for strain JEC21 (Fig. 5b). Fungal-induced BMEC damage required viable *C. neoformans* and was not observed after BMEC exposure to conditioned medium, killed fungi or fungal lysates (data not shown).

Although fibrinolytic components such as plasminogen (Pancholi *et al.*, 2003) and PA (Brooks *et al.*, 2006) are known to alter the adhesion properties of microbial and mammalian cells, neither component measurably altered the ability of *C. neoformans* to adhere to or damage BMEC (Fig. 5c, d). These results contrasted with the marked effects of fungal encapsulation on cryptococcal interactions with BMEC. Strains were examined for their ability to

adhere to and damage BMEC after the induction of capsule development for 4–5 days at 30 °C in 1/10-diluted Sabouraud's broth/PBS. Under these conditions, strains exhibited comparable capsule thicknesses by India ink analysis, but less extensive capsule growth than when incubated in invasion assay medium at 37 °C with 5 % CO₂ (Fig. 4b and Supplementary Fig. S4; data not shown). Encapsulated B3501A, C23 and JEC21 typically exhibited a greater than 50 % decrease in BMEC adherence and damage relative to non-induced strains with minimal capsule expression, while the adhesion of acapsular FCH78 was unaffected after growth under induction conditions (Fig. 5e, f).

Effects of plasmin and cryptococcal host damage on BMEC barrier resistance

The maximum barrier function shown for the 4–10 day cultures of BMEC in Supplementary Fig. S2 would likely be compromised as a result of either the direct cytotoxic effect of *C. neoformans* or the proteolytic activity of plasmin. We therefore used TEER to follow the relative changes in BMEC barrier resistance associated with cytotoxic events or plasmin activity during invasion assays.

BMEC that were exposed to *C. neoformans* in the absence of plasmin(ogen) exhibited decreases in resistance, but not until at least 4 h of co-culture (Fig. 6). This delay in the decline of BMEC barrier resistance corresponded with the delayed onset of fungal-induced host damage shown in Fig. 5(b). BMEC exposure to strains B3501A, C23 or FCH78 resulted in decreases of 40–50 % in barrier resistance by 6 h, with minimal declines occurring thereafter (Fig. 6b–d).

Fig. 5. Cryptococcal-BMEC adhesion and cytotoxicity. (a) Comparative adherence of *C. neoformans* strains B3501A, C23, JEC21 and FCH78 (*cap59*) to BMEC over time, measured as the percentage of adherent cells in relation to the total added fungi percentage adherence. (b) BMEC damage over time during exposure to *C. neoformans*, with percentage damage representing the amount of LDH activity detected in assay supernatants relative to the total BMEC LDH activity per well. (c, d) Fungal-BMEC adherence (c) and cytotoxicity (d) assays performed with plasminogen (Plg), plasminogen activator (PA), or both Plg and PA (Plg/PA) relative to Plg- and PA-negative controls (No Plg/PA) after 45 min (c) or 6 h (d) incubation. (e, f) Relative BMEC adherence (e) and damage (f) by fungal strains with a small capsule (Non-induced) or large capsule (Induced) after incubation times of 45 min (e) and 6 h (f). Bars represent mean and SEM from a minimum of three separate experiments. * $P < 0.05$ by *t* test for comparisons between strains in (a, b) or for same-strain comparisons in (e, f) under induced and non-induced conditions.

By comparison, strain JEC21 generated significantly smaller changes in BMEC barrier resistance over time, though the trend of observed decreases was similar for all strains of *C. neoformans* (Fig. 6a). The comparatively smaller changes in BMEC barrier resistance induced by JEC21 are consistent with the lower amount of BMEC damage detected with this strain (Fig. 5b), and with capsule-related decreases in fungal-host cell interactions anticipated from the rapid

encapsulation of this strain under the assay conditions (Figs 4b and 5e, f, and Supplementary Fig. S4).

The addition of plasminogen and PA to the assay medium allowed for both the coating of *C. neoformans* with plasmin during invasion assays and the analysis of plasmin-dependent effects on BMEC barrier resistance. The deposition of plasmin on fungi significantly altered both

Fig. 6. Effects of plasmin and cryptococcal-host damage on BMEC barrier resistance. BMEC barrier resistance was measured hourly by TEER during 12 h invasion assays. Strains JEC21, FCH78, B3501A and C23 were incubated with BMEC (a–d) with (+) or without (–) plasminogen (Plg) and PA. Controls showing the effect of plasminogen and PA on BMEC resistance in the absence of *C. neoformans* are indicated (Plg-poor/deficient medium + Plg/PA). BMEC were additionally examined in assay medium lacking both *C. neoformans* and plasminogen products (Plg-poor medium) or in medium lacking FCS (Serum-free medium). The 100 % TEER values measured in these experiments were $246.3 \pm 35.5 \Omega \text{ cm}^2$ and within the range of the maximal TEER values shown for confluent BMEC in Supplementary Fig. S2(b). Values shown are the mean $\pm$ SEM from at least three separate experiments. $P < 0.05$ by ANOVA with Dunnett's post-test for ^xsame-strain comparisons (strain $\pm$ Plg/PA), or *comparisons between the indicated datasets and Plg-poor medium controls.

the kinetics and the extent of declines in BMEC barrier resistance, as compared with non-plasmin-coated strains (Fig. 6). Plasmin-associated changes in BMEC barrier resistance became evident by 2–3 h of co-culture and sharply contrasted with the prolonged exposure times required for non-plasmin-coated fungi to initiate declines in barrier resistance (Fig. 6). For strains B3501A, C23 and FCH78, these early plasmin-mediated declines were characterized by a pronounced 20–50 % loss in BMEC barrier resistance within the first 3 h of co-culture, which doubled to 70–80 % by 6 h and represented a nearly twofold increase over those generated in the absence of

plasmin (Fig. 6b–d). By contrast, plasmin-exposed JEC21 generated 20 % declines in BMEC barrier resistance after 6 h, compared with decreases of less than 10 % in the absence of plasmin (Fig. 6a). Despite the marked changes in BMEC barrier resistance that resulted when plasmin was present in assays, the concomitant influences of plasmin versus cryptococcal-induced host damage are evident from the trajectories of barrier decline in the presence versus the absence of plasmin (Fig. 6). Collectively, these results indicate that both plasmin-dependent and plasmin-independent effects on BMEC function are discernible over time.

Plasminogen and PA did not alter BMEC barrier resistance when added to invasion assays in the absence of *C. neoformans*, indicating that the ability of plasmin(ogen) to affect changes in BMEC barrier function is dependent on the presence of *C. neoformans* (Fig. 6). BMEC cultures exposed to invasion assay medium in the absence of pathogen or plasminogen products also exhibited no changes in barrier resistance (Fig. 6). However, mono-cultured BMEC exposed to assay medium without FCS began to exhibit declines in barrier activity after 8 h, consistent with the functional importance of serum protein to microvascular endothelial cells and other *in vitro* cultured primary cell lines (Ambesi *et al.*, 2005; Schattelman *et al.*, 2007).

Effects of plasmin and cryptococcal–host damage on the recovery of fungal transmigrants

TEER measurements cannot be taken as absolute indicators of *C. neoformans*–BMEC invasion (Chang *et al.*, 2004). Therefore, to examine how plasmin- and *C. neoformans*-dependent decreases in BMEC barrier resistance affected fungal invasion of BMEC–Matrigel, the traversal of *C. neoformans* strains was evaluated hourly, in parallel with the TEER measurements shown in Fig. 6.

The recovery of non-plasmin-coated strains JEC21, FCH78, B3501A and C23 from the lower chamber of the transwells corresponded with the pathogen-induced declines in BMEC barrier resistance shown in Fig. 6. All strains showed similar patterns of recovery that began at 4 h and increased up to 6 h (Table 2). Over the same period, BMEC TEER values rapidly declined, suggesting that declines in BMEC integrity facilitate fungal invasion.

Plasmin-associated effects on fungal recovery were calculated by subtracting the recoveries of fungal transmigrants obtained in the absence of plasmin. Plasmin accelerated the time-course of fungal recovery from invasion chambers and also significantly increased the total number of fungal transmigrants recovered during 12 h invasion assays (Table 2). BMEC–Matrigel invasion by plasmin-coated *C. neoformans* became detectable after 2 h, as opposed to 4 h or later for non-plasmin-coated fungi, with strains C23 and B3501A exhibiting the most rapid and robust plasmin-dependent invasive ability (Table 2). Plasmin-enhanced invasion thus accounted for 87 % or more of the fungi recovered during the first 4 h of invasion assays and a minimum of 63 % of the fungi recovered over the remaining assay period (Table 2). The relatively low invasion ability of non-plasmin-coated *C. neoformans* suggests the comparatively minor contribution of cryptococcal–host damage to fungal invasion of

Table 2. Effects of plasmin and cryptococcal-induced host damage on fungal invasion

Results shown are from parallel invasion assays performed in the presence or absence of plasminogen and PA, which together are referred to as 'Plg'.

Time (h)	JEC21			B3501A		
	Total invasion*		Percentage invasion† (+)Plg	Total invasion*		Percentage invasion† (+)Plg
	(-)Plg	(+)Plg		(-)Plg	(+)Plg	
2	0	13 (9)‡	100	0	32 (18)‡	100
4	14 (8)	210 (13)‡	93.30	0	615 (68)‡	100
6	53 (5)	160 (22)‡	66.88	83 (5)	555 (57)‡	85.00
8	26 (3)	93 (7)‡	72.00	88 (11)	467 (40)‡	81.20
10	14 (5)	87 (8)‡	83.90	64 (12)	369 (35)‡	82.70
12	8 (2)	73 (18)‡	89.04	35 (9)	315 (29)‡	88.90
	FCH78			C23		
	Total invasion*		(+)Plg	Total invasion*		(+)Plg
	(-)Plg	(+)Plg		(-)Plg	(+)Plg	
2	0	30 (7)‡	100	0	366 (101)‡	100
4	16 (16)	400 (65)‡	96.00	71 (37)	540 (88)‡	86.90
6	111 (12)	300 (40)‡	63.00	164 (40)	503 (73)‡	67.40
8	74 (28)	320 (46)‡	76.90	141 (41)	500 (77)‡	71.80
10	69 (8)	305 (79)‡	77.40	32 (12)	280 (64)‡	88.60
12	83 (25)	334 (27)‡	75.20	29 (7)	268 (48)‡	89.20

*Mean recovery and SEM (parentheses) for three separate experiments. Total invasion is defined as the total number of transmigrated fungal cells recovered from lower transwell chambers by the procedures described in Methods.

†Calculated from the mean values of total invasion activity. Percentage invasion is defined as the percentage of fungal transmigrants obtained in the presence [(+)Plg], relative to absence [(-)Plg], of plasminogen and PA, and was calculated as described in Methods.

‡ $P < 0.05$ by ANOVA comparing plasmin-dependent versus -independent recoveries at the indicated times.

BMEC–Matrigel (Table 2). The peak recovery of plasmin-coated *C. neoformans* typically occurred within the first 4–8 h of invasion assays (Table 2) and corresponded to sharp declines in TEER values over the same period (Fig. 6). While this could suggest that declines in BMEC integrity facilitate the plasmin-dependent cryptococcal invasion of BMEC–Matrigel, fungal recovery typically declined over the final 4–6 h of the assay, even as TEER values continued to decline (Table 2, Fig. 6). Interestingly, *C. neoformans* recovery began to decline after 6–8 h of BMEC co-culture, independent of plasmin, and could thus be related to the gradual obstruction of filter membranes with cellular debris from dead or damaged cells over the course of the assays (Table 2).

DISCUSSION

Plasmin can facilitate microbial dissemination by compromising the integrity of vascular and cerebrovascular barriers, degrading extravascular fibrin nets intended to contain and neutralize the pathogen during local infection, and by activating other protease systems that degrade host tissues. The plasminogen system is known to interact with microbial surfaces through diverse lysine-containing receptor proteins, some of which additionally mediate plasminogen to plasmin conversion. Streptokinase, staphylokinase and the plasmid-encoded PA (Pla) of *Y. pestis* are examples of bacterial surface proteases that directly activate plasminogen (Bergmann & Hammerschmidt, 2007). Conversely, we find that *C. neoformans*, like many other pathogens capable of binding plasminogen (Potempa & Pike, 2009), requires host-derived PAs for plasminogen activation. Cell surface-associated enolase has been implicated as a major receptor involved in the lysine-dependent binding of plasminogen to the fungal pathogens *Candida albicans* (Jong *et al.*, 2003) and *P. brasiliensis* (Nogueira *et al.*, 2010). Cell wall-associated lysine residues are also of central importance to plasminogen binding by *C. neoformans*, and we have previously identified 10 ‘non-classical’ cell wall-associated proteins that serve as plasminogen receptors on *C. neoformans*, providing evidence for the central role of carboxyl-terminal or exposed, internal lysine residues in plasminogen–cryptococcal interactions (Stie *et al.*, 2009).

Consistent with this lysine requirement, the pre-treatment of *C. neoformans* with CB prior to assays, or addition of the lysine analogue ϵ ACA to assays, partially, but significantly, inhibited plasmin-enhanced fungal invasion. The partial inhibition by CB resulted from the regeneration of plasminogen-binding sites over the course of the assay, which could not be avoided due to the sensitivity of endothelial cells to CB (Guimarães *et al.*, 2007; Hajjar *et al.*, 1994; Reijerkerk *et al.*, 2003). The incomplete inhibitory effect of ϵ ACA on plasmin-enhanced invasion likely resulted from BMEC outcompeting fungi for soluble ϵ ACA, as suggested by the five- to 10-fold greater surface area of BMEC, which would favour ϵ ACA interactions with BMEC rather than *C. neoformans*. The short half-life (3 h) of ϵ ACA may further limit ϵ ACA availability to fungal cells

during invasion assays, given the physiological conditions used in our study (Chauhan *et al.*, 2000; Verstraete, 1985).

The C23 (serotype A) and B3501A (serotype D) strains examined in this study exhibited similar patterns of BMEC adhesion and damage. However, we found that culture conditions significantly affected the adhesion properties of these strains. For example, fungal cultures grown to mid-exponential phase yielded comparable patterns of BMEC adhesion, whereas cells from stationary-phase cultures gave significantly reduced and variable adhesion results, consistent with previous studies of changes in the cell-surface biochemistry between the exponential and stationary phases of some cryptococcal strains (Foster *et al.*, 2004), and age-related phenotypic changes that affect cryptococcal adherence properties, including proteinase secretion (Chen *et al.*, 1996). The positive correlation between cryptococcal-induced BMEC adherence and damage in this study is noteworthy. Specifically, cryptococcal-induced BMEC damage over time was proportional to fungal adherence, suggesting a causal relationship. The relatively low adherence of JEC21 to BMEC resulted in less BMEC damage than observed with fungal strains exhibiting greater adherence. Similar correlations between adherence and damage have been reported in studies with HUVEC (Ibrahim *et al.*, 1995a). The ability of *C. neoformans* to damage host cells in this latter study required the actin-dependent phagocytosis of pathogen by host cells. We are currently investigating the mechanism(s) involved in the cryptococcal-induced BMEC damage observed in our study, as well as a molecular basis for the diminished ability of strain JEC21 to engage BMEC (J. Stie and D. Fox, unpublished results).

Our findings show that plasminogen to plasmin conversion significantly enhances the invasion potential of *C. neoformans*, *in vitro*. Similar results have been described for other major fungal pathogens, including *C. albicans* (Jong *et al.*, 2003) and *P. brasiliensis* (Nogueira *et al.*, 2010), and suggest that the plasminogen fibrinolytic network may be co-opted by fungal pathogens. Certain bacterial pathogens appear to have evolved specialized strategies for plasminogen subversion that in turn regulate disease progression. For example, plasminogen to plasmin conversion by Pla mediates the rapid lymphatic and haematogenous dissemination of *Y. pestis* in infection models of bubonic plague (Sebbane *et al.*, 2006; Sodeinde *et al.*, 1992), and also plays a central role in pneumonic plague (Lathem *et al.*, 2007). The critical importance of the plasminogen system in plague pathogenesis has been further corroborated by findings in plasminogen-deficient mice, which demonstrate a 100-fold increase in LD₅₀ over wild-type (Goguen *et al.*, 2000). An interesting contrast to the rapid progress of bubonic plague is the slower disease progression associated with the limited utilization of plasmin by group A streptococci (Cole *et al.*, 2006). This alternative strategy of plasminogen subversion results from the restricted expression of streptokinase among phenotypically defined subpopulations of the pathogen during infection, and may

coordinate pathogen spread with the continued maintenance of established infection sites. While it is not known whether the plasminogen fibrinolytic network is of similar importance to the pathogenesis of *C. neoformans*, we are currently developing animal models that will allow us to address this question.

The dissemination of *C. neoformans* into the CNS has been shown to occur by two mutually independent processes that involve direct fungal cell invasion of brain microvasculature and/or pathogen dispersal by recirculating, infected phagocytes (Chang *et al.*, 2004; Charlier *et al.*, 2009). Encapsulation is essential for the survival and growth of *C. neoformans* within phagocytic vectors that facilitate cryptococcal entry into the brain by a Trojan horse mechanism. Additionally, interactions between cryptococcal-expressed hyaluronic acid and the BMEC surface receptor CD44 result in the protein kinase C α -dependent internalization of *C. neoformans* and fungal cell invasion of the brain microvasculature, *in vitro* (Chen *et al.*, 2003; Jong *et al.*, 2007, 2008a, b). Fungal urease expression also promotes cryptococcal invasion of the brain microvasculature in mouse models of cryptococcosis (Olszewski *et al.*, 2004; Shi *et al.*, 2010), by a mechanism that requires fungal cell viability, but not fungal encapsulation or host CD44 expression (Shi *et al.*, 2010). Evidence for the transcytosis of *C. neoformans* across brain microvasculature has been obtained both *in vitro* and in animal models, and has been proposed to require fungal cell encapsulation (Chang *et al.*, 2004). Other studies, however, indicate that capsule development on *C. neoformans* impairs its ability to invade endothelial cells, *in vitro* (Goldman *et al.*, 1994; Ibrahim *et al.*, 1995a; Levitz & DiBenedetto, 1989). Therefore, although capsule development is an essential virulence property of *C. neoformans*, our study and other published work provide evidence that fungal modulation of capsule size during specific stages of disease may promote pathogenesis. Acapsular *C. neoformans*, for instance, demonstrated significantly greater invasion of endothelial cells than an isogenic encapsulated strain, suggesting that the spread of blood-borne *C. neoformans* into the CNS is facilitated by attenuated capsule expression (Ibrahim *et al.*, 1995a). We similarly find that the acapsular strain of an isogenic pair exhibits a greater ability to bind to, damage and invade BMEC and, furthermore, that both acapsular strains of *C. neoformans* and those expressing a small surface capsule exhibit comparable invasive abilities. These results, together with the selective ability of cryptococcal strains having a small surface capsule to exploit the plasma-based plasminogen fibrinolytic network, could indicate that limited capsule development during the invasive, blood-borne phase of cryptococcosis promotes fungal pathogenesis by a plasmin(ogen)-dependent mechanism.

Capsule-transfer experiments indicated that fungal encapsulation inhibited invasion in the presence, but not the absence, of plasmin. A similar effect has been shown for the O-polysaccharide component of the Gram-negative

bacterial capsule. While the smooth LPS expressed by *Salmonella enterica* sterically inhibits plasminogen to plasmin conversion by the surface protease PgtE, the rough LPS capsule of *Y. pestis*, which lacks O-polysaccharide, does not obstruct plasminogen activation by Pla protease (Kukkonen *et al.*, 2004). The selective loss of O-polysaccharide by *Y. pestis* has thus been suggested to be an adaptation for high virulence (Potempa & Pike, 2009). By analogy, minimal expression of surface capsule by *C. neoformans* during the dissemination phase of cryptococcosis may facilitate virulence via fungal subversion of plasmin(ogen) function. Our findings are of additional interest given that a direct regulatory role for capsule expression has been proposed during cryptococcal invasion of BMEC by a process of fungal transcytosis (Chang *et al.*, 2004). These findings, together with our results demonstrating that capsule expression prevents cryptococcal subversion of plasmin(ogen) function, could indicate that encapsulation alternatively induces or inhibits the potentially competing pathways or mechanisms governing *C. neoformans* invasive activity.

While the low invasion activity observed for non-plasmin-coated *C. neoformans* may suggest a limited role for transcytosis in our system, it should also be considered that our experiments were designed to examine the effect(s) of plasmin(ogen) on *C. neoformans*-BMEC invasion. Furthermore, our study, unlike previous reports, distinguishes between the invasion activity of hypoencapsulated and encapsulated states of *C. neoformans*. The rapid encapsulation of strain JEC21 at least partially accounts for its reduced ability to interact with and invade BMEC in this study, given: (a) the interference of the capsule with plasminogen deposition on *C. neoformans* (Stie *et al.*, 2009), and (b) the enhanced BMEC adherence, damage and plasminogen-dependent invasion of the isogenic *cap59* strain FCH78. However, another potential consideration is intra-strain variation, which has been implicated as a possible factor underlying experimental differences between different laboratories (Franzot *et al.*, 1998), and may also contribute to the reported variability in the ability of *C. neoformans* strains to damage endothelium, *in vitro* (Chang *et al.*, 2004; Chen *et al.*, 2003; Ibrahim *et al.*, 1995a).

Host cell damage is an established property of several major fungal pathogens and is mediated by secreted phospholipases, proteinases and other hydrolytic enzymes (Mukherjee, 2001). In *Candida albicans*, the expression of phospholipase B1 (Leidich *et al.*, 1998; Naglik *et al.*, 2003), unidentified phospholipases (Ibrahim *et al.*, 1995b) or aspartyl proteases (Naglik *et al.*, 2003) correlates with invasive activity and/or mortality in clinical studies and mouse models of candidiasis. Although a role for hydrolytic enzymes in cryptococcal pathogenesis has not been widely investigated, hydrolase activity has been detected in supernatants from *in vitro*-cultured *C. neoformans* (Chen *et al.*, 1996). Serotype A and D isolates from patients with AIDS-associated cryptococcosis secrete a

range of enzymes, including lipases, esterases, amidases and glycosidases (Vidotto *et al.*, 2005, 2006). In other studies, strains of *C. neoformans* identified as high producers of extracellular phospholipase B showed two- to fivefold greater invasion of the CNS than low producers (Chen *et al.*, 1997). In addition, *C. neoformans* expresses urease activity that, although not directly cytotoxic, generates ammonia, a toxic byproduct that may account for the aberrant and potentially damage-related changes in brain microvasculature during urease-dependent fungal CNS invasion (Shi *et al.*, 2010). These observations, combined with the ability of *C. neoformans* to hijack host proteases such as plasmin, indicate that this pathogen may use both host-derived and secreted fungal enzymes to promote CNS invasion. Although a possible role for secreted fungal hydrolases during cryptococcal-induced BMEC damage in our studies cannot be dismissed, BMEC exposure to *C. neoformans*-conditioned medium did not result in detectable damage. Notably, other investigators examining cryptococcal-BMEC interactions have found that *C. neoformans* is not cytotoxic towards BMEC (Chang *et al.*, 2004; Chen *et al.*, 2003), suggesting that fungal-induced BMEC damage could differentially contribute to strain virulence.

We conclude that fungal subversion of the plasminogen fibrinolytic network is a potential factor in cryptococcosis and that capsule expression regulates the binding and conversion of plasminogen to plasmin on the surface of *C. neoformans*. Our findings provide continued insight into the well-described ability of this pathogen to interact with and manipulate host processes at the cellular and molecular levels.

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