TGF- β 1 induces transendothelial migration of the pathogenic fungus *Sporothrix schenckii* by a paracellular route involving extracellular matrix proteins

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Sporotrichosis, a mycosis caused by *Sporothrix schenckii*, is characterized by lymphocutaneous lesions. In immunocompromised hosts, this fungus may invade the bloodstream and disseminate to other tissues, such as lung and bone. Our group previously showed that *S. schenckii* yeasts adhere to endothelial monolayers and that this interaction is modulated by cytokines. Using 3.0 μ m-pore culture inserts, the present work shows that transforming growth factor (TGF)- β 1 led to a $80 \pm 26\%$ increase in fungal migration across endothelial monolayers and inhibited fungus internalization by $55 \pm 23.5\%$, when compared to untreated cells. The major surface endothelial molecules recognized by *S. schenckii* were not modulated by TGF- β 1. These data suggested that a paracellular route is preferentially used by *S. schenckii* during the transmigration of cultured endothelial cells. It was further observed that TGF- β 1 increased the subendothelial matrix exposure and that anti-fibronectin (anti-FN) and anti-laminin (anti-LM) antibodies abolished the increase in *S. schenckii* association with endothelial monolayers induced by TGF- β 1. These antibodies also inhibited ($38.2 \pm 4.29\%$ and $50.8 \pm 17.3\%$, respectively) the adhesion of *S. schenckii* to freshly prepared native endothelial matrices. Furthermore, transendothelial migration of *S. schenckii* was blocked by anti-FN and anti-LM antibodies. These data indicate that TGF- β 1-induced *S. schenckii* adhesion to endothelial monolayers results from the increased exposure of the subendothelial extracellular matrix and that this event may contribute to the enhancement of transendothelial migration.

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INTRODUCTION

Sporotrichosis, a disease caused by the thermally dimorphic fungus *Sporothrix schenckii*, is an emergent infection in immunocompromised hosts and is considered as an endemic mycosis in Mexico, Costa Rica, Guatemala, Colombia, Brazil, Uruguay, South Africa, India and Japan (Bustamante & Campos, 2001; Rosa *et al.*, 2005; Schubach *et al.*, 2005).

The infection usually begins after traumatic inoculation of the fungus into the skin, and benign lymphocutaneous lesions are the most prevalent forms of the disease. However, there is a growing incidence of disseminated forms, especially in Brazil, mainly due to zoonotic transmission of the disease (Lima Barros *et al.*, 2003; Welsh, 2003; Schubach *et al.*, 2005; Lopes-Bezerra *et al.*, 2006). Although fungaemia is rarely described in sporotrichosis, clinical

complications, such as the spread of infectious fungi to the eyes, central nervous system, bones, cartilages and lungs, may arise in immunocompromised patients or in individuals with a history of alcohol abuse (Kauffman, 1999; Kauffman *et al.*, 2000; Carvalho *et al.*, 2002; Silva-Vergara *et al.*, 2005). Reports concerning the mechanisms involved in the interaction of *S. schenckii* with mammalian cells remain scarce.

Our group previously showed that the yeast parasitic phase of *S. schenckii* adheres to endothelial cells (Figueiredo *et al.*, 2004) and to extracellular matrix (ECM) proteins found in the subendothelium (Lima *et al.*, 1999, 2001, 2004). In addition, this fungus invades endothelial cells without affecting their viability (Figueiredo *et al.*, 2004). The adherence was strongly stimulated by previous treatment of endothelial monolayers with interleukin (IL)-1 β or transforming growth factor (TGF)- β .

Whereas IL-1 β has been largely implicated in the acute phase of inflammation, inducing the expression of cell adhesion molecules on the endothelial surface (Muller,

Abbreviations: ECM, extracellular matrix; FCS, fetal calf serum; FN, fibronectin; HUVEC, human umbilical vein endothelial cell; LM, laminin; TGF, transforming growth factor.

2003), TGF- β 1 plays a central role in later stages, by inhibiting the activation of inflammatory cells and stimulating granuloma formation, angiogenesis and fibrosis (Branton & Kopp, 1999; Roberts *et al.*, 1986). Skin lesions of patients with sporotrichosis usually show a mixed granulomatous and pyogenic inflammation (Lurie, 1963; Koga *et al.*, 2002), while histopathological examination of the livers and spleens of mice inoculated with *S. schenckii* reveals a strong granulomatous reaction (Fernandes *et al.*, 1999).

In the present work, the cellular mechanisms involved in the stimulation of *S. schenckii* adherence to endothelial monolayers by TGF- β 1 were investigated.

METHODS

Reagents. TGF- β 1 was obtained from Invitrogen Life Technologies. Polyclonal antibodies against whole *S. schenckii* acetone-dried yeast cells were produced and characterized as previously described (Lopes-Alves *et al.*, 1994). Rabbit polyclonal anti-human fibronectin (anti-FN; primary antibody and HRP-conjugated) and goat anti-rabbit peroxidase conjugate were purchased from Dako. A rabbit polyclonal anti-laminin (anti-LM) serum was kindly provided by Dr Jean-Philippe Bouchara (Université d'Angers, France). A mouse monoclonal antibody against human VE-cadherin was obtained from Santa Cruz Biotech. Phalloidin-FITC conjugated and *o*-phenylenediamine (OPD) were acquired from Sigma Aldrich. AlexaFluor 568-conjugated goat anti-rabbit immunoglobulins were obtained from Molecular Probes; Uvitex was a kind gift from Dr Scott Filler (UCLA, Torrance, CA); human plasma FN was freshly prepared by gelatin-Sepharose chromatography as previously described (Vuento & Vaheri, 1979). The purity of these preparations was analysed by immunoblotting after SDS-PAGE; Igepal CA-630 (octylphenoxypolyethoxy-ethanol) was acquired from Sigma. Vecta-shield mounting medium was purchased from Vector Laboratories and the ECL protein biotinylation system was purchased from GE Healthcare.

***S. schenckii* growth conditions.** *S. schenckii* strain 1099-18, used throughout this study, was originally obtained from the Mycology Section, Department of Dermatology, Columbia University, NY, USA. This strain was grown in Sabouraud agar at room temperature and then samples were maintained in Sabouraud agar, stored at 4 °C. The yeast phase of *S. schenckii* was grown in brain heart infusion broth (BHI, Oxoid) at 37 °C on a rotary shaker (150 r.p.m.). Fungus samples from the 4 °C stocks were subcultured until complete differentiation into the yeast form and assessed by optical microscopy. The cells were then harvested and washed with sterile 50 mM PBS, pH 7.2. The cell suspension was adjusted to 10^8 cells ml⁻¹ to perform the assays. The non-pathogenic yeast *Saccharomyces cerevisiae*, used as a control in some experiments, was grown in YPD medium [1% (w/v) yeast extract, 2% (w/v) bactopectone, 2% (w/v) glucose], under constant agitation (240 r.p.m.), at 29 °C for 18 h.

Isolation of human endothelial cells. Primary human umbilical vein endothelial cells (HUVECs) were isolated, as previously described (Jaffe *et al.*, 1973), and grown in 199 medium (M-199, Sigma), supplemented with 15 mM HEPES, antibiotics (100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹), 2 mM L-glutamine, and 20% (v/v) fetal calf serum (FCS, Cultilab) (complete medium). The cells were used in first or second passages only and subcultures were obtained by treatment of confluent cultures with 0.025% (w/v) trypsin/0.2% (w/v) EDTA solution in PBS.

Yeast transendothelial migration assay. *S. schenckii* migration through the HUVEC monolayer was determined using polystyrene filters (3.0 µm-pore Falcon transparent culture inserts; 6.5 mm diameter; 0.33 cm² area; Becton Dickinson) inserted in 24-well plates. These inserts, containing a filter lined with a confluent endothelial monolayer, separate the luminal (upper) and abluminal (lower) compartments. In the standard assay, endothelial cells were seeded on the filters (80 000 cells per chamber) and grown until they reached confluence (2 days). The growth of endothelial monolayers was monitored daily by inverted light microscopy. Transmigration assays were performed with intact endothelial monolayers, selected through analysis of the flux of BSA (10 mg ml⁻¹ in M-199) across monolayers grown for 12, 24 and 48 h. After allowing protein passage for 1 h, BSA present in the lower chamber was determined by protein determination (Quick Start Bradford dye reagent, Bio-Rad). Monolayers presenting rates of protein translocation from the upper chamber to the lower chamber of less than 2% cm⁻² h⁻¹ were considered intact, as previously described (Albelda *et al.*, 1988). Under the culture conditions used, BSA translocation rates reached the desired scores for intact monolayers after 48 h cell growth. Cellular juxtaposition in confluent monolayers was further confirmed after immunofluorescence staining with a mouse anti-VE-cadherin mAb, revealed with an anti-mouse-TRITC (tetramethylrhodamine isothiocyanate) conjugate antibody. In transmigration assays, each well of the luminal compartment was inoculated with 10^6 *S. schenckii* cells in M-199 medium (100 µl) and allowed to stand at 37 °C in an atmosphere of 5% CO₂ for different periods (3, 6, 18 and 24 h). At the end of the incubation period, the abluminal medium (initially 200 µl) was collected and the concentrations of *S. schenckii* cells were determined by cell counts in a haemocytometer. Migration of the non-pathogenic fungus *Sac. cerevisiae* was also measured under the same conditions. The effect of TGF- β 1 on *S. schenckii* migration through the endothelial monolayers was investigated by adding this cytokine [10 ng ml⁻¹ in serum-free M-199, containing 0.1% (w/v) BSA], together with *S. schenckii* yeast to the luminal compartment for 6 h. The number of migrating fungi was determined as above and compared with the controls, in wells without cytokine treatment. In some transmigration experiments, endothelial monolayers were first incubated with TGF- β 1 (4 h) and then incubated for 30 min either with rabbit polyclonal anti-FN (10 µg ml⁻¹, Dako), or with a rabbit anti-LM serum (1:1000), both of them diluted in PBS. Control purified rabbit IgGs (10 µg ml⁻¹) and a pre-immune rabbit serum were used as controls. After this time, 10^6 *S. schenckii* cells in M-199 medium were allowed to stand at 37 °C in an atmosphere of 5% CO₂ for 6 h.

Yeast internalization assay. The number of *S. schenckii* yeasts internalized by endothelial cells was determined by a previously described modified differential fluorescence assay (Lopes-Bezerra & Filler, 2004), which distinguishes externally bound from internalized yeast cells. In some experiments, 0.1 µM cytochalasin D was added to selected coverslips before the addition of *S. schenckii*. Each experiment was performed in triplicate and at least 100 organisms were counted on each coverslip. The number of endocytosed organisms was determined by subtracting the number of *S. schenckii* double immunolabelled with primary anti-*S. schenckii* primary and AlexaFluor-conjugated secondary antibodies (extracellular yeasts) from the number of organisms labelled with Uvitex (total organisms). Stained samples were viewed under epifluorescence (Nikon Eclipse E400 microscope) using filters for AlexaFluor 568 (red fluorescence) and Uvitex (blue fluorescence).

Interaction of endothelial cell extracts and *S. schenckii* yeasts. Total extracts from cell surface-biotinylated endothelial intact monolayers grown in 25 cm² T-flasks, treated or not for 4 h with 10 ng ml⁻¹ TGF- β 1 [in serum-free M-199, containing 0.1% (w/v) BSA], were prepared using a commercial kit (GE Amersham). These

extracts were allowed to incubate with *S. schenckii* and proteins bound to the yeasts were analysed by Western blotting, as previously described (Figueiredo *et al.*, 2004). In order to obtain a biotinylated-enriched fraction containing only cell-surface molecules, endothelial extracts were further affinity purified using 1 ml HiTrap streptavidin columns (GE Amersham). Briefly, the same amount of total protein (as determined by the BCA micromethod, Pierce) from both TGF- β -treated and untreated cell monolayers was applied to HiTrap columns previously equilibrated in 10 ml lysis buffer, at a rate of 1 ml h⁻¹, at room temperature. After washing the columns with lysis buffer, bound proteins were eluted with 8 M guanidine chloride, pH 1.5. The eluted samples were immediately desalted in Sephadex G-25 columns previously equilibrated in lysis buffer, and determination of the protein content showed that 60 μ g total protein was obtained by this method, for each condition. These affinity-purified fractions were immobilized in 96-well ELISA plates (5 μ g ml⁻¹ in 0.2 M sodium bicarbonate buffer, pH 9.4), overnight at 4 °C. After a saturation step with 1 mg BSA ml⁻¹ in PBS, 10⁶ yeasts cells per well suspended in PBS were incubated for 1 h at 37 °C. The wells were washed three times with cold PBS containing 0.05 % (v/v) Tween-20 (PBS/T) and then incubated with either a rabbit anti-*S. schenckii* polyclonal or a pre-immune serum (1:500 in PBS), for 1 h at 37 °C. Adherent yeasts were revealed after appropriate washing steps and incubation of the wells with a goat anti-rabbit IgG peroxidase-linked conjugate (1:2000) in PBS/T containing 0.1 % (w/v) BSA. The reaction was developed with *o*-phenylenediamine (OPD) [1.0 mg ml⁻¹ in 0.01 M sodium citrate buffer, pH 4.5 containing 0.05 % (v/v) H₂O₂] for 10 min and then stopped with 3 M H₂SO₄. Absorbance at 490 nm was measured using an automated spectrophotometer reader (Bio-Rad ELISA Reader). Each condition was assessed in triplicate and the results represent the mean of at least three independent experiments.

Actin rearrangement assay and analysis of yeasts adhering on FN matrix. In order to observe the effect of TGF- β 1 on cytoskeleton organization, endothelial monolayers were grown on 13 mm diameter glass coverslips in 24-well plates, until reaching confluence, and then treated or not with TGF- β 1 [10 ng ml⁻¹ in serum-free M-199, containing 0.1 % (w/v) BSA] for 4 h. To examine the actin microfilament rearrangements, the cells were washed three times with serum-free M-199, fixed and permeabilized with absolute methanol for 10 min and then saturated with 1 % (w/v) BSA in PBS (PBS/BSA). The cells were incubated with FITC-conjugated phalloidin (0.1 μ g ml⁻¹) for 1 h at room temperature. Coverslips were washed three times with PBS and mounted on microscope glass slides with Vectashield (three slides per condition). Fluorescent image capturing was performed with a Nikon Coolpix 995 digital camera coupled to a Nikon Eclipse E400 epifluorescence microscope. Cells displaying either cortical actin or stress fibre arrangements were quantified, in at least five fields, using the highest magnification ($\times 1000$). The results were expressed as the percentual ratio of total cells adhering in each condition and the experiment was repeated twice, with comparable results. In infection experiments, 5 $\times 10^6$ yeasts were incubated with endothelial monolayers, treated or not with TGF- β 1, at 37 °C in an atmosphere of 5 % CO₂ for 90 min. After three washing steps with PBS, cell monolayers were fixed with 3.7 % (v/v) formaldehyde in PBS and then incubated with rabbit anti-human FN antibodies at 10 μ g ml⁻¹ in PBS/BSA, at room temperature for 1 h, followed by incubation with a goat anti-rabbit AlexaFluor 568-conjugated antibody (1 μ g ml⁻¹) in PBS/BSA for 1 h. Cell monolayers were then permeabilized with 0.1 % Triton X-100 in PBS for 5 min and then incubated with FITC-conjugated phalloidin (0.1 μ g ml⁻¹), as above. Next, the endothelial cells were washed three times and incubated with 1 % (v/v) Uvitex, in PBS, for 30 min. Coverslips were mounted in Vectashield solution and observed with a fluorescence microscope, as above. The number of yeasts either adhering to the FN-immunostained matrix, or associated with the endothelial cell bodies, was determined, in both TGF- β 1-treated and untreated cells.

Each experiment was performed in triplicate and the number of cell- or matrix-associated organisms was counted on each coverslip, in at least five fields, using the highest magnification ($\times 1000$).

Quantification of secreted endothelial FN by ELISA. A quantitative immunoenzymic assay for detecting FN secreted into the culture media by endothelial cells, treated or not with TGF- β 1 (10 ng ml⁻¹ in serum-free M-199, containing 0.1 % BSA), was performed as previously described (Morandi *et al.*, 1994).

***S. schenckii* association with endothelial cells in the presence of antibodies against ECM proteins.** Confluent monolayers on 13 mm diameter glass coverslips placed in 24-well tissue culture plates were pretreated or not with 10 ng TGF- β 1 ml⁻¹ in serum-free M-199, containing 0.1 % (w/v) BSA, for 4 h. The wells were then incubated with a rabbit anti-human FN antibody (10 μ g ml⁻¹), or with a rabbit anti-LM serum diluted 1:1000 in PBS. Pre-immune purified rabbit IgGs (10 μ g ml⁻¹) and a pre-immune rabbit serum were used as controls. The cells were then washed with serum-free M-199 and incubated with 5 $\times 10^6$ yeasts at 37 °C in an atmosphere of 5 % CO₂ for 90 min. After infection, the endothelial cultures were washed three times with serum-free M-199 and stained by the Panotic L.B. haematological dye system [0.1 % (v/v) triaryl methane/0.1 % (v/v) xanthenes/0.1 % (v/v) thiazines, Laborclin] as described elsewhere (Figueiredo *et al.*, 2004). HUVECs on glass coverslips were placed on microscope slides with Entellan (Merck) for observation under a light microscope (Nikon Eclipse E200). Each experiment was performed in triplicate and 100 organisms were counted on each coverslip. The results were expressed as the mean number of yeasts per high-power field.

Interaction of *S. schenckii* with native endothelial matrix. Native subendothelial ECM was obtained from endothelial monolayers grown for 48 h in 96-well ELISA microtitre plates (sterilized by UV irradiation) in M-199 supplemented with 2 % (v/v) FCS. The cells were washed with serum-free M-199 and then lysed for 3 min with 0.1 M NH₄OH in PBS containing 0.1 % (v/v) Triton X-100, 1 mM PMSE, 40 μ M leupeptin and 10 mM *N*-ethylmaleimide (Morandi *et al.*, 1994). The residual matrix fraction was rinsed three times with PBS, saturated overnight with 1 mg BSA ml⁻¹ in PBS and used in fungus adhesion assays. Immobilized matrices were incubated either with anti-human FN polyclonal antibody (10 μ g ml⁻¹) or with a rabbit anti-LM serum diluted 1:1000 in PBS for 1 h at 37 °C. Control purified rabbit IgGs (10 μ g ml⁻¹) and a pre-immune rabbit serum were used as controls. Biotinylated *S. schenckii* yeasts (10⁷ yeasts in 1 ml bicarbonate buffer), prepared using a commercial kit following the manufacturer's instructions, were layered on endothelial matrix plates (10⁶ yeasts in 100 μ l per well in PBS) for 1 h at 37 °C. Adhering yeasts were detected by an indirect ELISA using a streptavidin-peroxidase conjugate (GE Healthcare). In some experiments, the interaction of yeasts with endothelial ECM was also quantified in the presence of divalent cations (Ca²⁺ and Mg²⁺).

Statistical analysis. Unless stated otherwise in the legends, the data were compared by analysis of variance and the Tukey–Kramer test. For datasets for which normal distribution could not be assumed, analysis was done by the non-parametric Mann–Whitney *U*-test. Differences between data were considered significant when *P* < 0.05.

RESULTS

TGF- β 1 stimulates *S. schenckii* transmigration across endothelial monolayers

Sporotrichosis is an infection that is not limited to the subcutaneous tissue, since *S. schenckii* may disseminate,

mainly to the osteoarticular system, in both immunocompromised and immunocompetent hosts (Lopes-Bezerra *et al.*, 2006). Our group previously reported that *S. schenckii* yeast-like cells associate with endothelial monolayers *in vitro* (Figueiredo *et al.*, 2004) and this interaction is stimulated by TGF- β 1. As the mechanisms used by this pathogen to invade the host are still unknown, further investigation regarding whether TGF- β 1 modulates the transendothelial migration of yeasts towards the subendothelium was undertaken.

In order to verify the ability of *S. schenckii* to migrate across endothelial monolayers, an *in vitro* system was used in which *S. schenckii* yeasts (10^6 cells) were inoculated into the upper compartment of a 3.0 μ m transparent porous polystyrene insert, previously seeded with endothelial cells grown for 48 h in complete M-199 and then changed to serum-free M-199 supplemented with 0.1 % (w/v) BSA. Since non-brain endothelium is characterized by a relatively low electrical resistance coupled with relatively high resistance to macromolecules, the integrity of endothelial monolayers was verified prior to using them in transmigration assays, by measuring the BSA translocation rate. For yeast transendothelial migration experiments, only HUVEC-covered inserts presenting BSA translocation rates of less than 2 % $\text{cm}^{-2} \text{h}^{-1}$ were selected, which was the maximum acceptable translocation rate established for an intact non-brain endothelial cell monolayer (Albelda *et al.*, 1988; Haake & Lovett, 1994). The average rate found for the cell monolayers used in the experiments described was 1.12 ± 0.46 % of BSA $\text{cm}^{-2} \text{h}^{-1}$. The migration of *S. schenckii* across untreated endothelial monolayers was compared with that exhibited by the non-pathogenic fungus *Sac. cerevisiae*. Fig. 1 shows that *S. schenckii* migration across endothelial monolayers significantly increased in a time-dependent manner, whereas *Sac.*

cerevisiae yeasts seemed unable to efficiently pass across the endothelial monolayers.

Previous data from our laboratory showed that the interaction of *S. schenckii* with HUVECs was optimally induced by the treatment of endothelial monolayers with TGF- β 1 for at least 4 h (Figueiredo *et al.*, 2004). Thus, studies regarding the effect of TGF- β 1 on fungal transmigration across endothelial monolayers were undertaken after a 6 h treatment with this cytokine, an incubation time showing significant amounts of transmigrating yeasts. Treatment of endothelial cells with TGF- β 1 led to an 80 ± 26 % increase in fungal migration, as compared to untreated monolayers (data not shown). In these conditions, TGF- β 1 was present during the entire experiment, but the increase detected was exclusively due to endothelial activation, since fungal transmigration across inserts devoid of endothelial cells was not affected by TGF- β 1 treatment (data not shown).

The internalization of *S. schenckii* yeasts by endothelial cells is influenced by treatment with TGF- β 1

In the present work, a differential fluorescence assay was used in order to analyse the effect of TGF- β 1 on the internalization of *S. schenckii* by HUVECs *in vitro*. Using this method, adhering yeasts display a differential fluorescent staining and thus can be distinguished from yeasts internalized by HUVECs. Previous work by our group showed that internalized yeasts are detectable in infected endothelial monolayers after an incubation time of at least 1 h (Figueiredo *et al.*, 2004). Observation revealed that the number of yeasts associated with HUVECs reached a plateau after 3 h interaction with HUVECs. The treatment of endothelial cells with TGF- β 1 prior to fungal addition led to a 116 ± 42 % increase in *S. schenckii* adhesion to HUVECs after 3 h interaction. However, under the same conditions, the internalization of yeast cells was diminished by 55 ± 23.5 %, when compared to untreated cells (data not shown).

The interaction of *S. schenckii* yeasts with endothelial surface proteins is not affected by TGF- β 1

Previous papers by others report that TGF- β 1 induces the cellular expression of integrins (Roberts *et al.*, 1988; Heino *et al.*, 1989; Zambruno *et al.*, 1995). Our group also demonstrated that a few endothelial membrane proteins function as putative receptors for *S. schenckii* in primary adhesion events, although no investigation into whether TGF- β 1 acts by increasing the expression of such proteins on endothelial cell surface was undertaken (Figueiredo *et al.*, 2004). In order to verify whether TGF- β 1 treatment increased the expression of endothelial proteins that bind to *S. schenckii* surface molecules, 10^8 yeasts were allowed to interact with biotinylated endothelial surface extracts for

Fig. 1. Time-course of transendothelial migration of *S. schenckii*. Yeasts (10^6 cells ml^{-1}) were added to the luminal chamber in M-199 supplemented with 0.1 % (w/v) BSA: $\blacktriangle$, *S. schenckii*; $\blacksquare$, *Sac. cerevisiae*. Migration towards the abluminal chamber was measured by haemocytometer counting. Data represent typical experiments, repeated three times and showing <20 % difference among them. * $P < 0.01$.

2 h at room temperature. These extracts were prepared from intact endothelial monolayers, treated or not with TGF- β 1, which were submitted to the biotinylation procedure shortly before monolayer dissolution with a mild detergent-containing buffer. The time of 2 h was selected for this experiment, since in previous experiments maximal adherence of *S. schenckii* to HUVECs was observed at times ranging from 90 to 120 min (Figueiredo *et al.*, 2004). Fig. 2 shows that the profile of biotinylated endothelial proteins associated with the fungus surface was not significantly affected by TGF- β 1 (lanes 3 and 4), although some differences in the composition of control and TGF- β 1-treated total extracts are evident (lanes 1 and 2). In addition, 10^6 yeast cells were allowed to interact with purified biotinylated endothelial surface protein fractions, isolated from endothelial cell extracts by streptavidin-affinity chromatography, as described in Methods. Biotinylated fractions from both TGF- β 1-treated and untreated cells were immobilized in 96-well microtitre plates (5.5 μ g protein per well) and fungi adhering to the protein-coated wells were detected with a specific anti-*S.*

Fig. 2. Interaction of *S. schenckii* yeasts with endothelial cell extracts. Intact endothelial monolayers were surface-biotinylated as described in Methods. Then extracts were obtained in the presence of mild non-ionic detergent and protease inhibitors. SDS-PAGE analysis was performed of biotinylated endothelial surface proteins incubated with 10^6 *S. schenckii* yeasts for 2 h. Bound proteins were resolved in non-reducing conditions using a 10% polyacrylamide separating gel. After transferring to PVDF membranes, proteins were probed with streptavidin-peroxidase conjugate and revealed using a chemiluminescence reagent. Total endothelial extracts: lane 1, control; lane 2, TGF- β 1-treated. Yeast-bound endothelial surface proteins: lane 3, control; lane 4, TGF- β 1-treated. This is a typical experiment repeated more than five times, with similar results.

schenckii polyclonal antibody. This ELISA approach confirmed that the treatment of HUVECs with TGF- β 1 did not modify the binding of yeasts to putative receptors on the endothelial surface (data not shown).

Taken together with the results concerning fungus transendothelial migration and internalization, these data suggest that *S. schenckii* yeasts may preferentially use the paracellular route for invading TGF- β 1-activated endothelial monolayers, rather than the transcellular phagocytic route (transcytosis) used by different classes of pathogens (Adam, 2001; Sukumaran *et al.*, 2002; Chen *et al.*, 2003).

TGF- β 1 alters microfilament cytoskeleton organization in endothelial cells

Some reports have implicated TGF- β 1 in the induction of changes in endothelial cell phenotype, including loss of cell-cell contact, rearrangement in endothelial cell actin cytoskeleton and impairment of endothelial permeability (Coomber, 1991; Molony & Armstrong, 1991; Hurst *et al.*, 1999; Goldberg *et al.*, 2002). In this study, a fluorescence microscopy approach was used to analyse changes in actin distribution in HUVECs treated with TGF- β 1 for 4 h. Although the presence of stress fibre-bearing cells was observed under all conditions, the quantification of at least five high-power fields showed that TGF- β 1 treatment increased the number of endothelial cells exhibiting abundant stress fibre cells (37.5 ± 4.3 % in treated cultures compared to the 12.45 ± 2.4 % observed in untreated cells; Fig. 3a, b). TGF- β 1 also promoted the loss of cortical actin; 87.55 ± 8.7 % of total adhering cells in control compared to 62.5 ± 4.4 % in TGF- β 1-treated HUVECs (Fig. 3a, c). TGF- β 1-dependent mobilization of actin into stress fibres resulted in cell elongation (Fig. 3b and inset), compared with the control condition (Fig. 3c and inset).

TGF- β 1 increases *S. schenckii* adhesion by exposing subendothelial ECM

Since TGF- β 1 seems to increase *S. schenckii* adhesion and migration through endothelial monolayers without engaging the endocytic pathway or endothelial surface receptors, investigation regarding the participation of immobilized components present in native secreted endothelial matrices was undertaken. Our group previously reported that *S. schenckii* adheres to purified immobilized adhesive proteins, especially plasma-derived FN (Lima *et al.*, 1999, 2001, 2004). Fluorescence microscopic analysis of FN incorporated into the subendothelial matrix of cultures treated or not with TGF- β 1 showed that, in treated cultures, areas exhibiting FN-positive staining (Fig. 4b, d) were larger than those observed in control conditions (Fig. 4a, c). Adhering yeasts were observed in areas of exposed matrix under all conditions. Quantitative analysis of yeast cells associated with either matrix-exposed areas or endothelial cell bodies showed that TGF- β 1 specifically increased the number of subendothelial matrix-associated

Fig. 3. Analysis of endothelial actin arrangement by fluorescence microscopy. HUVECs (2.5×10^5) seeded on 13 mm diameter coverslips in 24-well plates, treated or not with 10 ng TGF- β 1 ml $^{-1}$ for 4 h, were processed for immunofluorescence and then incubated with phalloidin-FITC conjugate. Cells were observed with a Nikon Eclipse E-400 epifluorescence microscope. (a) Stress-fibre- and cortical actin-bearing cells were quantified as described in Methods, using a high-magnification immersion objective ($\times 1000$ magnification). The results are expressed as a percentual rate of the cells in each field. Black bars, control; grey bars, TGF- β 1 treated. * $P < 0.05$. (b) Control condition, showing endothelial cells (★, cell bodies) predominantly in close disposition and exhibiting strong cortical actin staining (arrows) (initial magnification $\times 400$; bar 50 μ m); (c) TGF- β 1-treated cultures showing longitudinal actin fibres (initial magnification $\times 400$; bar 50 μ m). The insets in (b) and (c) show the typical pattern of cortical actin-bearing or stress fibre-bearing cells at higher magnification ($\times 1000$), as used for the quantification in (a): cortical actin-bearing cells show a peripheral phalloidin distribution, with cell bodies (stars) almost devoid of FITC staining in the central region of the cell, whereas stress fibre-bearing cells display a clear bundle of actin fibres.

performed in the presence of anti-FN and anti-LM polyclonal antibodies. HUVEC monolayers were pretreated with TGF- β 1 and then further incubated with the blocking antibodies or their respective non-immune controls. The yeasts (5×10^6 cells) were then allowed to interact with endothelial cells for 2 h. As shown in Fig. 5, polyclonal antibodies against FN and LM efficiently inhibited the increase in *S. schenckii* adhesion to HUVECs treated with TGF- β 1. However, no inhibition of adhesion to untreated endothelial cells was observed (data not shown).

In addition, using a quantitative ELISA for FN detection in conditioned media, the treatment of HUVECs with TGF- β 1 for 6 h (the time also used in transmigration assays) resulted in a significant increase of 20% in FN secretion compared with untreated cells (data not shown). These results suggest that the important increase in fungal transendothelial migration across TGF- β 1-treated monolayers could be related either to enhanced exposure to subendothelial matrix proteins, such as FN and LM, or to increased incorporation of FN in the subendothelium; or it may be caused by some combination of both mechanisms.

***S. schenckii* adhesion to native subendothelial ECM is inhibited by anti-FN and anti-LM antibodies**

Previous work from our group, which demonstrated the interactions of *S. schenckii* with purified FN and LM, was performed with proteins adsorbed on plastic surfaces, or in their soluble forms (Lima *et al.*, 1999, 2001, 2004). However, data concerning the interaction of this fungus with native cell matrices are still lacking. Endothelial ECM was obtained after cell treatment with PBS containing 0.1 M NH_4OH and 0.1% (v/v) Triton X-100. Fungal

yeasts, but revealed no influence on the interaction of *S. schenckii* with endothelial cells (Fig. 4e).

Our group recently showed that LM, an important basal lamina molecule also present in endothelial matrices, binds to *S. schenckii* yeasts (Lima *et al.*, 2004). To further investigate whether the increase in *S. schenckii* adhesion to endothelial cells promoted by TGF- β 1 was due to the exposure of subendothelium, adhesion assays were

adherence to cell matrices by an immunoenzymic method was studied with a specific anti-*S. schenckii* antibody described elsewhere (Lima *et al.*, 2001). Since preliminary evidence obtained by our group was compatible with the idea that FN and LM are major ligands for *S. schenckii* in the subendothelium, we investigated whether antibodies

against these proteins were able to inhibit fungal adherence to native matrices. In order to prevent cross-reactivity between anti-rabbit HRP-conjugated immunoglobulins and rabbit antibodies against FN, LM or *S. schenckii* cells, the ELISA system was modified by biotinylating the *S. schenckii* yeasts, which were then detected with a strepta-

Fig. 4. Interaction of *S. schenckii* with HUVEC monolayers treated or not with TGF- β 1. HUVECs (2.5×10^5 cells) seeded on 13 mm diameter coverslips were treated with 10 ng TGF- β 1 ml $^{-1}$ for 4 h. The cytokine-treated (b, d) and untreated monolayers (a, c) were then incubated with *S. schenckii* yeast cells in a ratio of 20 yeasts per endothelial cell. The cultures were processed for immunofluorescence with a phalloidin-FITC conjugate for detection of cell microfilaments (green fluorescence), with an anti-FN rabbit polyclonal antibody followed by an AlexaFluor 568 conjugate (red fluorescence) and with 1 % Uvitex, which stains *S. schenckii* yeast cells (blue fluorescence) and also endothelial nuclei (N). Arrows point to yeasts adhering to FN-containing subendothelial matrix. Original magnification of (a) and (b), $\times 200$; bars 25 μ m. (c, d) Digital magnifications of the indicated areas in (a) and (b). (e) Yeasts associated with either cell bodies (black bars) or ECM (grey bars) were quantified, as described in Methods, using a high-magnification immersion objective ($\times 1000$ magnification). The results are means $\pm$ SD. Data were analysed by the non-parametric Mann–Whitney *U*-test ($n=9$, for each condition).

vidin-peroxidase conjugate. Pretreatment of endothelial ECM with anti-FN and anti-LM significantly inhibited *S. schenckii* adhesion, by $38.2 \pm 4.29\%$ and $50.8 \pm 17.3\%$, respectively (Fig. 6a). No inhibition was achieved with the respective rabbit pre-immune polyclonal antibodies used as negative controls, and this condition was considered as 100 % adhesion. However, it is important to highlight that the yeast adherence observed in the presence of these control antibodies was indistinguishable from the adherence observed with endothelial matrices not treated with antibodies (data not shown).

Since our group previously demonstrated that the interaction of *S. schenckii* with immobilized FN is Ca $^{2+}$ -dependent, but not Mg $^{2+}$ -dependent (Lima *et al.*, 2001), we investigated whether the interaction of *S. schenckii* with native endothelial matrices was also affected by divalent ions. As shown in Fig. 6(b), the presence of Ca $^{2+}$ (1 mM) during the interaction significantly increased the adherence of *S. schenckii* to the endothelial ECM and the addition of EDTA significantly reversed this effect. The presence of Mg $^{2+}$ (1 mM) had no effect on the interaction.

Antibodies against FN and LM inhibit *S. schenckii* transmigration across endothelial monolayers

The data presented so far were strongly suggestive of the fact that the interaction of *S. schenckii* with FN and LM, both of these secreted by endothelial cells towards the subendothelial matrix, could partially account for the stimulating effect of TGF- β 1 on *S. schenckii* adhesion and transendothelial migration. In an attempt to confirm this hypothesis, transmigration assays were performed in the presence of the above-described blocking polyclonal antibodies directed against FN and LM. As shown in Fig. 7, the incubation of HUVEC monolayers, treated with 10 ng TGF- β 1 ml $^{-1}$ for 4 h with anti-FN and anti-LM antibodies efficiently inhibited *S. schenckii* transmigration, whereas no effect was observed with the same antibodies when they were incubated with untreated cultures (data not shown), or when cell monolayers were incubated with control immunoglobulins.

To the best of our knowledge, this is the first report in the literature affirming the possibility that a fungal pathogen could take advantage of the paracellular route to cross endothelial barriers. Taken together, the data presented here stress the importance, in relation to

pathogenic micro-organisms, of binding ECM proteins in host tissues and also reinforce the concept of FN- and LM-recognizing cytoadhesins as major virulence factors of fungal pathogens.

DISCUSSION

TGF- β 1, a multifunctional cytokine, plays an important role in regulating tissue repair and regeneration by inducing ECM deposition (Branton & Kopp, 1999). TGF- β 1 also plays a pivotal role in the late repair phase of inflammatory response, characterized by connective tissue recruitment and intense blood vessel formation, which precede the organization of granulation tissue (Wong & Wahl, 1991). Misregulation of this inflammation phase leads to excess matrix production and to fibrotic disorders (Branton & Kopp, 1999).

The participation of TGF- β 1 in fungal infections is still poorly documented and somewhat controversial, when compared to the systemic immune responses described for infections involving protozoa and mycobacteria (Reed, 1999). Studies performed with mice suggested a role for TGF- β 1 in determining a protective response against *Candida albicans*, as demonstrated by the enhancement of TGF- β 1 production by resistant infected animals (Spaccapelo *et al.*, 1995). However, the local production of this cytokine was demonstrated in liver granulomas of human candidiasis patients (Letterio *et al.*, 2001) and in omental granulomas of mice infected with *Paracoccidioides brasiliensis* (Nishikaku & Burger, 2003).

Since our group previously reported that *S. schenckii* adheres to human endothelial cells *in vitro* (Figueiredo *et al.*, 2004), the question remained whether this interaction would favour the transmigration of infecting *S. schenckii* across endothelial barriers. The present results showed that *S. schenckii* transmigrated across cell monolayers in a time-dependent manner, whereas the non-pathogenic strain of *Sac. cerevisiae* migrated at a reduced rate.

Previous treatment of confluent endothelial sheets with 10 ng TGF- β 1 ml $^{-1}$ enhanced fungal transmigration by $80 \pm 26\%$, suggesting that active TGF- β 1 at infection sites could act as a facilitating factor for pathogenic dissemination. The next step was to determine which route (transcellular or paracellular) was preferentially used by the invading fungi in this condition. In fact, our previous

work showed that *S. schenckii* yeasts can be detected inside endothelial cells after 1 h of interaction and that infected HUVECs remained viable for as long as 48 h, but the impact of this ability on the overall process of endothelial infection was not considered (Figueiredo *et al.*, 2004). It has been suggested that actin cortical patches may be essential for the initiation of endocytosis (Engqvist-Goldstein & Drubin, 2003). Interestingly, treatment of HUVECs with TGF- β 1 induced a modification in the microfilament cytoskeleton structure, leading to an actin rearrangement from a cortical pattern to an array of perpendicular stress fibres, as already described by others (Goldblum *et al.*, 1999; Hurst *et al.*, 1999).

Although in the present work we show that the internalization of *S. schenckii* yeasts was decreased in the presence of TGF- β 1, our data do not support the idea that this cytokine directly interferes with the endocytosis of *S. schenckii* yeasts by endothelial cells. First, it became evident that the treatment of HUVECs with TGF- β 1 does not

Fig. 5. Inhibition of *S. schenckii* adherence to TGF- β 1-treated endothelial monolayers by anti-FN and anti-LM antibodies. HUVECs (2.5×10^5 cells) seeded onto 13 mm glass coverslips, treated or not with 10 ng TGF- β 1 ml^{-1} for 4 h, were pre-incubated with polyclonal anti-FN antibodies or rabbit control IgGs (both at $10 \mu\text{g ml}^{-1}$), or with an anti-LM serum or a pre-immune serum (both at 1 : 1000). Then cultures were incubated with *S. schenckii* yeasts (5×10^6 cells) for 90 min. After washing steps, coverslips were stained by the Panotic L.B. haematological dye system and observed in a light microscope. Each condition was performed in triplicate and 100 organisms were counted on each coverslip. The results are expressed as the mean number $\pm$ SD of adhering yeast cells per high-power field, obtained from three different experiments. Data were analysed by the non-parametric Mann-Whitney *U*-test; * $P < 0.01$, ** $P < 0.05$, *** $P < 0.05$ as compared to untreated cultures, and to cultures treated with anti-FN and anti-LM antibodies, respectively.

modify the profile of endothelial membrane proteins interacting with the fungal surface. Second, the present data show that the amount of yeasts associated with subendothelial matrix was strongly augmented in TGF- β 1-treated cultures, whereas the number of yeasts directly associated with endothelial cells was not significantly modulated by this cytokine. Indeed, the treatment of HUVECs with TGF- β 1 resulted in significant exposure of the subendothelial matrix. This result is corroborated by the work of others, who demonstrated that TGF- β 1 promotes a strong rearrangement of endothelial adherent junctions and actin cytoskeleton, resulting in increased areas of cell separation (Goldblum *et al.*, 1999; Hurst *et al.*,

Fig. 6. Interaction of *S. schenckii* yeasts with native endothelial matrix. Native subendothelial ECM was obtained by the NH_4OH /Triton X-100 extraction of HUVEC monolayers grown in 96-well plates. (a) Immobilized matrices were incubated either with anti-FN polyclonal antibody ($10 \mu\text{g ml}^{-1}$), or with anti-LM serum (1 : 1000), with each control running in parallel wells, used at the same dilutions (control purified IgG or pre-immune serum, respectively). Biotinylated *S. schenckii* yeasts (10^6 yeasts) were layered on endothelial matrices for 1 h at 37°C and adhering yeasts were detected by an indirect ELISA using a streptavidin-peroxidase conjugate. Adhesion obtained in the presence of control IgGs was considered as 100 %, and data are means $\pm$ SD. (b) The interaction of yeasts with endothelial ECM was also quantified in the presence of divalent cations (1 mM Ca^{2+} and Mg^{2+}). Data are expressed as A_{490} and each value is the mean $\pm$ SD of triplicates within the same experiment. Similar results were obtained in at least three experiments; * $P < 0.05$, ** $P < 0.001$, *** $P < 0.05$, as compared to control.

Fig. 7. Transendothelial migration of *S. schenckii* is inhibited by anti-FN and anti-LM antibodies. Confluent monolayers grown on 3.0 μ m porous inserts were treated with TGF- β 1 for 4 h, pre-incubated with anti-FN antibody (10 μ g ml⁻¹) and anti-LM antiserum (1:1000) for 30 min and then incubated with *S. schenckii* yeasts for 6 h, in the presence or absence of 10 ng TGF- β 1 ml⁻¹. Migration towards the abluminal chamber was measured by haemocytometer counting. Non-immune IgG or pre-immune serum were used as controls. The results are expressed as the mean number $\pm$ SD of migrating yeast cells. The experiment was repeated three times, with similar results; * $P < 0.05$, as compared to the control condition (untreated cultures).

1999). Thus, we can conclude that this cytokine has a direct effect on the interaction of *S. schenckii* with the subendothelial matrix, which may interfere with the probability of a yeast cell interacting with host cells.

Previous work by our group investigated the role of ECM components as ligands for *S. schenckii*. This fungus can bind to FN, LM and type II collagen, and also reveals differences in binding capacity according to the morphological form considered – yeast or conidia (Lima *et al.*, 1999). The interaction with FN involves different sites on this molecule and is likely mediated by glycopeptide cytoadhesins present on the fungus surface (Lima *et al.*, 2001, 2004). The present morphological analysis of TGF- β 1-treated cultures, by immunofluorescence microscopy, confirmed the predominant binding of yeasts to FN incorporated to the subendothelial matrix.

In this work, the treatment of endothelial monolayers with anti-FN and anti-LM polyclonal antibodies significantly inhibited the TGF- β 1-induced adherence of yeasts. Despite the fact that soluble FN strongly binds to endothelial surface *in vitro* (Effron *et al.*, 1983), a possible role for FN and LM associated with apical endothelial surfaces is unlikely, since use of the same antibodies was unable to inhibit the adhesion of *S. schenckii* to untreated endothelial monolayers (unpublished results). These data strongly

suggested that the major role of this cytokine in the current experimental model was to favour the exposure of ECM components to invading yeasts.

In general, TGF- β 1 stimulates the synthesis of collagens, FN, LM, tenascin and proteoglycans (Ignatz *et al.*, 1987; Bassols & Massagué, 1988; Pearson *et al.*, 1988). A number of works have described the effects of TGF- β 1 in the secretion of FN by endothelial cells of diverse origins (Sankar *et al.*, 1996; Usui *et al.*, 1998; Neubauer *et al.*, 1999; Shanker *et al.*, 1999), most of them after 24 h of TGF- β 1 treatment. The present data showed that the level of FN secreted in conditioned medium, following TGF- β 1 treatment, was 20 % higher than in control cultures, under the conditions of the transmigration assay (6 h), suggesting that TGF- β 1 promotes not only the exposure of matrix components, but also the enrichment of FN content in these matrices.

In addition, the adhesion of *S. schenckii* yeasts to native subendothelial matrices was dependent on Ca²⁺, but not on Mg²⁺. Since our group previously showed that FN binding to *S. schenckii* was enhanced in the presence of higher concentrations of Ca²⁺ (1 mM), whereas 1 mM Mg²⁺ showed no effect (Lima *et al.*, 2001), the present observation reinforces the proposal that FN bound to ECM is one of the major ligands for *S. schenckii* yeasts in TGF- β 1-activated endothelium; however, other ligands for *S. schenckii* may exist in the subendothelial matrix that are also modulated by divalent cations. In support of this idea is the fact that the same anti-FN antibody used to inhibit increased adhesion of *S. schenckii* yeasts to TGF- β 1-treated endothelial monolayers was only capable of inhibiting the adhesion of yeasts to native matrices by 37 %. Moreover, this possibility is corroborated by the fact that *S. schenckii* adhesion to native endothelial matrices is also significantly inhibited by anti-LM polyclonal antibodies.

Evidence that the ability of binding subendothelial matrix molecules is relevant for the transposition of the endothelial barrier by *S. schenckii* was clearly demonstrated here, by the fact that the same anti-FN and anti-LM antibodies capable of inhibiting fungal adhesion to the endothelium and to native matrices were also efficient at blocking *S. schenckii* transmigration through endothelial monolayers. We therefore suggest that this pathogen preferentially reaches subendothelial sites through a paracellular pathway, possibly through the engagement of FN- and LM-binding cytoadhesins expressed on its cell wall, although this remains to be verified.

It has been suggested that the enhanced expression of TGF- β 1 in fungi-infected granuloma (Spaccapelo *et al.*, 1995; Letterio *et al.*, 2001; Nishikaku & Burger, 2003) could favour residence inside host cells by inhibiting immune responses, as already described for other pathogens (Reed, 1999). However, the present work reinforces another possible contribution of this cytokine, by stimulating the association of a pathogenic fungus, such as *S. schenckii*, with host tissue stroma and, thus, providing additional clues that implicate FN- and LM-recognizing cytoadhesins as virulence factors for this pathogenic fungus.

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