Adherence and Invasion Studies of Candida albicans Strains, Using In Vitro Models of Esophageal Candidiasis

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The adherence of clinical and commensal isolates and reference collection strains of Candida albicans to a human esophageal cell monolayer (HET1-A) and reconstituted human esophageal tissue was compared. Isolates from patients with a severe form of esophageal candidiasis or candidemia adhered to HET1-A cells to a significantly greater extent than did isolates from patients with mild esophageal candidiasis or commensal and reference collection strains. In addition, C. albicans strain SSK21, which lacks the ssk1 response regulator gene of a 2-component signal transduction pathway, adhered less readily to the HET1-A cells than did parental cells or a gene-reconstituted strain. In a reconstituted esophageal tissue model, all clinical strains but not commensal or reference collection strains penetrated the epithelium, albeit at different rates. Hyphal formation following yeast cell adherence to the esophageal tissue was a requirement for invasion. Scanning electron microscopy was also used to confirm the colonization of the esophageal tissues by various strains. These studies indicate that both the HET1-A and the reconstituted esophageal tissue models can be used as in vitro targets to evaluate the adherence phenotype and invasiveness of C. albicans strains.

Candida albicans is a commensal organism of humans. It is usually found on the mucous membranes of the gut, oral cavity, and vaginal canal, and changes in host immunity (e.g., depletion of the CD4+ lymphocyte cells in patients with AIDS) result in disease at these sites. However, invasive disease can occur as a result of neutropenia after cancer immunotherapy or immunosuppression in patients who have undergone allogeneic transplantation. C. albicans is an opportunist, but data from animal models indicate that it possesses virulence factors required for disease establishment [1, 2]. Included among the virulence factors are host recognition (adherence) biomolecules (adhesins), phase transition (morphogenesis or the reversible conversion of yeast cells to filamentous growth), expression of invasive enzymes (secreted aspartyl proteases and phospholipase B), and phenotypic switching [1].

During endoscopic examination of patients with esophageal candidiasis, we have observed different stages of inflammation and severity [3] that have been classified previously by Kodsi et al. [4]. These differences in host response to the organism have also been observed in a large number of patients in intensive care units (ICUs) who have, after 5–8 days of hospitalization, also had an increase in anti-Candida antibody titer without verification of a positive culture result from clinical samples [5–8]. Although interpretation of antibody results depends on the quantity and the subclass of antibody, these observations suggest variation in the host response to the fungus. It is not entirely clear why patients develop a local or a systemic C. albicans invasion; however, it is hypothesized that candidiasis appears in different forms, depending on the specific underlying host deficiency [1].

We hypothesize that the type of disease and the host response depends in part on the invasiveness of the strain of C. albicans that is causing disease. This hypothesis has also been suggested from the data of Barrett-Bee et al. [9], who showed that blood isolates of C. albicans have enhanced expression of several virulence factors, compared with commensal strains. Furthermore, Ibrahim et al. [10] demonstrated that an isolate of C. albicans (CA30) capable of infecting mice via a gastrointestinal route had increased phospholipase production, compared with another strain (CA87) that was unable to infect mice and had low phospholipase activity. In this regard, we have compared the adherence properties of 5 groups of C. albicans strains. These groups include esophageal isolates from patients exhibiting 2 different host responses (Kodsi stages 3/4 [group 1] and 1/2 [group 2]), blood isolates (group 3), commensal strains (group 4), and strains from reference collections (group 5). The hypothesis we are testing is that disease manifestations may be partly related to strain differences in the expression of virulence factors.
factors. As a measurement of virulence, we quantitated the adherence of strains to a human esophageal cell line (HET1-A) and characterized the interactions of these strains with reconstituted human esophageal tissue.

In addition, we compared the adherence of a signal transduction mutant of \textit{C. albicans} (ssk1) to the HET1-A cells with its parent and a gene-reconstituted strain. The ssk1 mutant cannot form hyphae on standard laboratory agar media and is avirulent in a systemic murine candidiasis model [2]. In addition, hyphal forms of the organism flocculate extensively when grown in medium 199 [2]. The latter phenotype indicates that the cell-surface properties are altered, compared with those of wild-type strains; hence, it seemed reasonable to measure the ability of the ssk1 mutant to adhere to host cells.

**Methods**

\textit{Candida} strains and growth conditions. Strains were isolated from patients with different types of candidiasis, including esophageal infections and candidemia. All strains are listed in table 1, along with their source (patient or reference collection) and are described below with regard to the patient populations that were surveyed. Strains SC5314 and CSSK21, in which the ssk1 response regulator gene is deleted, and strain CSSK23, which is reconstituted with ssk1, have been described elsewhere [2]. For adherence assays, the strains were propagated from frozen stocks (Microbank; Pro-Lab Diagnostics) on Sabouraud dextrose agar (Difco) at 30°C for 24 h and then for 2 successive passages on yeast-peptone agar (Difco) supplemented with 500 mM D-galactose. Cells were collected in Hanks’s balanced salt solution (HBSS; Gibco BRL) containing magnesium and calcium and were washed once with HBSS. All strains were identified by their typical color on ChromAgar (CHROMagar Co.) or CandiSelect agar (EM Science), by germ-tube formation, and by reactions in the Vitek system. In addition, thermostolerance (growth at 42°C) andpolymerase chain reaction were used to verify the strains as \textit{C. albicans}. All strains were collected over a 1-year period and were stored at −70°C.

Source of strains. For the adherence studies, clinical isolates were obtained from patients with esophageal disease (groups 1 and 2), from blood cultures (group 3), or from the oral cavity or skin of healthy individuals (group 4; commensal strains). Reference collection strains (group 5) were also used in adherence studies.

Patients with esophageal disease in groups 1 and 2 were distinguished by an index—the Kodsi stage—that reflects the type of lesions exhibited by the patient [4]. The higher the Kodsi stage number, the greater the degree of inflammation. For example, patients with Kodsi stage 1 esophagitis have some plaque (<2 mm) and hyperemia of the mucosa. Patients with Kodsi stage 2 esophagitis have plaque of ≥2 mm and edema along with hyperemia. Patients with Kodsi stage 3 esophagitis have confluent plaque with microulcerative erosions, pseudomembrane, and/or bleeding. Patients with Kodsi stage 4 esophagitis have symptoms similar to those of patients with stage 3 esophagitis but with complications, such as stenosis, fistulation, and perforations of the mucosal wall. Patients in groups 1 and 3 had underlying conditions, such as pancreatitis, polytrauma, and complications following surgery, and patients in group 2 had liver disease or cancer or were alcoholics.

Table 1. Percentage adherence of \textit{Candida albicans} from various patient groups to a human esophageal cell line, HET1-A.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of isolates$^a$</th>
<th>Description$^b$</th>
<th>Percentage adherence, mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Patient in an ICU; esophagitis, Kodsi stage 3/4</td>
<td>7.6 (2.1)</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Esophagitis, Kodsi stage 1/2</td>
<td>5.2 (1.0)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Candidemia</td>
<td>8.8 (2.2)</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Commensal strains; no obvious candidiasis</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>Reference collection strains ATCC 14053, CBS 2730, and CBS 562</td>
<td>1.5 (0.2)</td>
</tr>
</tbody>
</table>

$^a$ No. also indicates the no. of patients in groups 1–4.

$^b$ The Kodsi stage reflects the type of lesions exhibited by the patients [4]. The higher the Kodsi stage no., the greater the degree of inflammation (see Methods).

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**Adherence assays with HET1-A cells.** The procedure of Enache et al. [11] was used to measure adherence to HET1-A cells. HET1-A human esophageal cells were cultured in serum-free medium (EPM2; Biological Research Faculty and Facility [BRFF]) in 75-cm², 250-mL polystyrene flasks (Sarstedt). The cells were cultured for 5–7 days to near confluency and then were collected by treatment of the monolayer with 3 mL of disassociation medium (PET [polivinylpyrrolidone, EGTA, trypsin]; BRFF), were washed twice with HEPES buffered saline (BRFF), and were suspended in the serum-free medium. HET1-A cells were seeded on sterile glass coverslips (VWR Scientific) that had been placed in 6-well tissue culture dishes (Corning) at a density of 10⁶ cells/coverslip in a total of 2 mL serum-free medium. After 72 h, each monolayer was washed with HBSS and infected with 10⁷ yeast cells in duplicate in 2 mL of HBSS. The infected monolayers were incubated at 30°C for 90 min, washed 4 times with HBSS, fixed in methanol, and stained with crystal violet.

The percent of HET1-A cells with adhering yeast cells was determined by counting 600 HET1-A cells per strain. Adherence was expressed as the mean percentage ± SEM of HET1-A cells with adhering yeast cells. To measure differences among the groups and account for the unequal numbers of observations per group, 1-way analysis of variance was used. Multiple comparison tests were used to maintain a type I error of α = .05 for all multiple comparisons. Statistical software (SAS, version 6.12; SAS Institute) was used for all calculations.

**Adherence assays with reconstituted, human esophageal epithelium.** Reconstituted, esophageal epithelium was prepared by SkinEthic Laboratory from a human cell line originally derived from a human esophageal squamous cell carcinoma (Kyse 510; German Collection of Microorganisms and Cell Cultures). Reconstituted tissue was grown in a chemically defined medium without antibiotics (500 µL of modified complete Dulbecco’s 153 medium: 5 µg/mL insulin and 1.5 mM Ca²⁺) on inert polycarbonate filters for 12 days. The tissues were incubated in 6-well dishes at 37°C, 5% CO₂ (saturated humidity).

For adherence studies with the reconstituted esophageal tissue, we used 2 \textit{C. albicans} strains from each group described above. All strains were grown on Sabouraud agar for 24 h at 30°C, and single
colonies were used to inoculate the yeast-peptone-galactose broth medium (Gibco BRL) described above for the HET1-A assays. Strains were grown in shake culture for 20–24 h at 30°C and were centrifuged, washed 3 times in PBS (pH 7.4), and standardized to $4 \times 10^7$ cells/mL. A 50-μL aliquot of this suspension ($2 \times 10^7$ cells) was added to each tissue. At designated time intervals for a total of 4 days, duplicate tissues infected with each strain were washed 3 times with PBS and were fixed for ≥24 h in 10% formalin in PBS for periodic acid–Schiff (PAS) stain.

After being fixed and dehydrated, the tissues were placed vertically in paraffin at room temperature. Sections (4 μm) were prepared from tissues infected at designated times, transferred to glass slides coated with albumin, and stained using PAS. For scanning electron microscopy, other tissues infected for 24 h with a strain from each group were fixed in 2.5% glutaraldehyde and 2% formaldehyde, washed 3 times with PBS, and post-fixed with osmium tetroxide. After being washed 3 times with PBS, the tissues were dehydrated in a graded series of ethanol solutions and gold-coated after critical point drying. We used a Leo (Zeiss Oberkochen) scanning electron microscope. In some experiments, tissues were infected and prepared for PAS or scanning microscopy at 4 and 8 h after infection.

Results

Adherence of strains to HET1-A cells. The adherence of isolates from each of the 4 patient groups and reference strains to HET1-A cells is shown in table 1. With several of the strains, adherence assays were repeated with similar results. The data indicate significant differences ($P < .05$) in adherence between isolates from group 1 versus groups 4 and 5 and group 3 versus groups 4 and 5. Therefore, patient isolates (esophageal and blood [groups 1 and 3]) adhered to a greater extent than did the commensal (group 4) or reference strains (group 5). Of importance, isolates from patients with a higher Kodsi index (group 1) adhered to HET1-A cells at a significantly higher rate than did isolates from patients with a lower Kodsi index (group 2). We also compared the adherence of C. albicans SC5314 parental cells with a strain deleted of $ssk1$ (Δ$ssk1$, SSK21) and adherence of a gene-reconstituted strain (SSK23) with HET1-A cells. The Δ$ssk1$ strain adhered to HET1-A cells about half as well as the parent, and reintroduction of $ssk1$ restored adherence (strain SSK23) to near wild-type levels (SC5314, 6.5%; SSK21, 3.2%; SSK23, 5.2%).

Adherence to and penetration of reconstituted esophageal tissue. Human esophageal squamous carcinoma cells were seeded on polycarbonate filters, and within 12 days a tissue, composed of numerous layers of cells, formed on the upper surface of the filters. The tissues were easily visualized by light microscopy, using a PAS stain (figure 1A). The adherence and invasion by wild-type C. albicans (SC5314; figure 1B–1D) was
followed over time. *C. albicans* SC5314, a well-characterized, virulent strain, formed filaments on the tissues by 4 h, began invasion of the tissue by 8 h, and had penetrated the entire tissue by 24–48 h (figure 1). Destruction of the tissues accompanied invasion by strain SC5314, especially 4 days after infection (data not shown).

Representative strains from each patient group were also compared by histopathology, using tissues stained with PAS (figure 2). The ability of strains from each group to adhere and penetrate the tissue to the underlying filter varied. The most invasive strains were those from groups 1 and 3 (figure 2A and 2B) and, to a much lesser extent, group 2 (figure 2C). In general, all of these strains penetrated the entire epithelium within 2 days. Although the extent of tissue invasion is difficult to estimate, it appeared that the strains from group 3 were the most invasive since they penetrated the tissues to the filter within 1 day. The group 2 strains and group 4 (commensal) strains mostly grew on the surface of the tissue (figure 2C and 2D), and some invasion was noted by 48 h with these strains. The extent of invasion by the 2 reference collection strains (ATCC 14053 and CBS 2730 [group 5]) varied and seemed to be a function of the ability of each strain to convert to a filamentous growth pattern (data not shown). *C. albicans* ATCC 14053 formed hyphae and could invade the tissue by 2 days; *C. albicans* CBS 2730 was unable to form hyphae and did not penetrate the tissue within 4 days. Thus, invasion by all strains was dependent on the conversion of yeast cells to a filamentous growth.

As determined by use of scanning electron microscopy, all tested strains adhered to the surface of the esophageal tissue by 24 h. All clinical isolates demonstrated a filamentous growth pattern, whereas the commensal strains grew as mixtures of yeast and hyphae (data not shown). In contrast, reference strain CBS 2730 only formed yeast cells on the surface of the tissues.

**Discussion**

*C. albicans* is a commensal fungus of the mucous membranes of healthy individuals. Infection of the esophagointestinal mucosa [4, 5] develops under a variety of conditions (e.g., in patients with AIDS). However, candidiasis occurs in a number of clinical settings, including in patients in an ICU after general surgery [8], in immunosuppressed patients [12], and in patients with liver diseases [3, 6]. Morbidity associated with candidiasis is a common occurrence in select patient populations [7].

**Figure 2.** Histopathological sections of human esophageal tissue infected with *Candida albicans*. A. Group 1, patients in an intensive care unit who had Kodsi stage 3/4 esophagitis. B. Group 3, patients with candidemia. C. Group 2, patients with Kodsi stage 1/2 esophagitis. D. Group 4, commensal strains, no obvious candidiasis. Invasion is more extensive with group 1 and 3 strains; group 2 and 4 strains colonize the tissue but little invasion occurs. Tissues were stained with periodic acid–Schiff reagent (magnification, ×400).
Conceptually, adherence of the organism to host tissues should be one of the first steps in the disease process [1, 13]. Following selective adherence of the organism, invasion is probably accompanied by phase transition (the interconversion of yeast and filamentous growth forms) as well as the secretion of enzymes that aid in tissue penetration by the organism [1, 14]. We previously examined the adherence of *C. albicans* to HET1-A cells [11]. In that study, we observed that galactose-grown yeast cells were more adherent than cells grown in glucose when the former was used at high (500 mM) concentrations. Hence, for these studies, we used the same medium to evaluate the adherence of patient strains to HET1-A cells and to reconstituted esophageal tissue. The percent of HET1-A cells with adhering yeasts was not as high in the current study as in the study described above, but the differences among strains, with regard to adherence, are very clear. Strains from patients with certain forms of candidiasis (esophageal Kodsi stage 3/4 and blood [groups 1 and 3]) showed a higher level of adherence than the strains from the other study groups. These differences were statistically significant.

From preliminary observations, the *ssk1* mutant (strain SSK21) grew only in the yeast or pseudohyphal morphology on the esophageal tissue and adhered less readily, whereas the parental strain (SC5314) produced hyphae on the tissue and appeared to remain adherent more readily. The gene-reconstituted strain (SSK23) produced both yeast and hyphae on the tissue, and adherence was intermediate to SSK21 (data not shown). Furthermore, the adherence of the *ssk1* mutant, compared with that of the parental and gene-reconstituted strains, was impaired in its ability to adhere to HET1-A cells. Thus, it would appear that the cell surface of the mutant is altered by the deletion of this response regulator, signal transduction protein [2].

Specific cell-surface changes in this strain have not been described, although we have observed that this same strain floculates extensively when grown in media that promote hyphal growth [2]. Also, using scanning electron microscopy, we observed that the mutant strain appears to colonize reconstituted esophageal tissue less readily. This could be due in part to the growth form (a yeast/pseudohyphal morphology) of the organism. Thus, these observations indicate that the virulence of the mutant strain may be associated with its relative inability to colonize and invade host tissues. The observations with the *ssk1* mutant indicate the complexity of pathogenesis with this organism. That is, the adherence and invasion phenotypes are closely correlated with morphogenesis. Furthermore, the HET1-A and tissue models should be reliable in vitro assays for the measurement of virulence for gene-deleted mutants of *C. albicans*.

Human reconstituted epithelium was first used by Schaller et al. [15–17] to develop a model of oral epithelial and epidermal candidiasis. Our model of human esophageal tissue is intended to serve as an in vitro system to evaluate the pathogenesis of esophagitis caused by *C. albicans*. We have shown that, in contrast to the oral epithelium model [16], the invasion of the esophageal epithelium by the virulent strains occurs within the first few hours after infection. Thus, strain SC5314 begins to invade the tissue by 4 h after infection, and by 4 days an extensive destruction of the epithelium occurs. Weide et al. [18] have demonstrated that *C. albicans* can penetrate a monolayer of colonic cells, but that study did not assess the invasiveness of other strains.

In the present study, invasion of the esophageal tissue seemed to be dependent on the ability of strains to convert to a hyphal growth form, an observation also noted with the invasion of endothelial cells by *C. albicans* [19]. An invasion of the superficial, esophageal epithelium in rabbits has been demonstrated by Hoshika et al. [20–22] and in other animal models [23]. In the studies by Hoshika et al. [20–22], the relationship between *C. albicans* and the epithelial surface of a rabbit esophagus involved an initial attachment, followed by subepithelial yeast cell insertion into the tissues, cavitation, and, last, invasion of the organism by the formation of hyphae.

Our assays have advantages. First, the intact epithelium constitutes an important barrier that protects the host against local infection by microorganisms. Thus, both models focus on the primary interactions between yeast and host cells. Second, our data show that HET1-A and esophageal tissue adherence assays are useful for evaluating differences in virulence among strains that are either genetically distinct or isogenic except for a single gene difference. For example, in the case of strain SC5314 and the isogenic set of strains (*ssk1* mutants), there seems to be good correlation between their virulence in animal models and their adherence and invasion potential as demonstrated in our study. Third, both assays are reproducible. On the other hand, there are limitations with these models. For example, they are useful for studying primary reactions between pathogens but cannot mimic the contribution of host factors to protection (e.g., the role of host cells or secreted products [mucins] in the defense of *Candida* infections) [24]. However, we are now exploring the possibility of reconstituting specific immune components in the esophageal tissue assay or adding specific host components (e.g., mucin) in order to determine their contribution to *Candida*-host interactions at mucosal surfaces. Also in our model, we could not consider the role of blood flow in resistance. In this regard, Gianotti et al. [25] described an inverse linear correlation between translocated yeast cells and the blood flow in the small intestine of pigs.

In summary, these models provide a reasonable approach to studying differences in virulence among strains of *C. albicans*. Our data support the hypothesis that host responses and disease severity may be related in part to differences in the virulence of strains. Since the esophagus is a target of the organism during candidiasis (especially in patients with AIDS), these models should be relevant to studying host interactions. It is puzzling that commensal isolates should express lower levels of adherence; in fact, the same picture emerges with regard to phos-
phospholipase B activity of blood isolates versus commensal isolates (i.e., there is lower activity in the latter) [26]. It is generally regarded that most strains that cause disease are commensals from the patient [27, 28]. Perhaps regulatory events occur that switch the organism from a benign commensal to an invasive state. The nature of these regulatory events and, indeed, their very existence remain speculative.

Our observations on the virulence of C. albicans strains are similar to those of De Bernardis et al. [29] with C. parapsilosis [29]. In that study, the authors showed that skin isolates had a higher expression of secretory aspartyl proteinase and were more vaginopathic than blood isolates in a rat model. On the other hand, the skin isolates were avirulent in a murine invasive candidiasis model, whereas the blood isolates were highly pathogenic. No correlations could be established with these 2 groups of isolates when compared by colony morphotype, karyotype, or resistotype analysis.

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References