Cell damage caused by vaginal Candida albicans isolates from women with different symptomatologies

Daniella Renata Faria, Karina Mayumi Sakita, Luciene Setsuko Akimoto-Gunther, Érika Seki Kioshima, Terezinha Inez Estivalet Svidzinski and Patrícia de Souza Bonfim-Mendonça*

Abstract
The present study aimed to characterize cell damage caused by vaginal Candida albicans isolates from women with different symptomatologies. It was evaluated 12 clinical isolates of C. albicans from vaginal samples: 4 from asymptomatic women (AS), 4 from women with a single episode of vulvovaginal candidiasis (VVC) and 4 from women with recurrent vulvovaginal candidiasis (RVVC). We evaluated the ability of C. albicans to adhere to human cervical cancer cells (SiHa), the yeast–SiHa cell interactions and cell damage. All of the clinical isolates presented a high adhesion capacity on SiHa cells. However, clinical isolates from symptomatic women (VVC and RVVC) had higher filamentation after contact (24 h) with SiHa cells and a greater capacity to cause cell damage (>80 %). Clinical isolates from symptomatic women had greater potential to invade SiHa cells, suggesting that they are more pathogenic than AS isolates.

Vulvovaginal candidiasis (VVC) is an infection of the vulva and vagina that is characterized by the abnormal growth of yeasts [1]. VVC affects millions of women each year, generating severe discomfort and interfering with sexual relationships and work performance [1]. Candida spp. are commensal fungi, but conditions of imbalance between yeast and the host (e.g. immunodeficiency) provide the conditions in which opportunistic fungal infection, such as VVC, can develop [2]. Approximately 22% of women have asymptomatic vaginal yeast colonization by Candida spp., and 5% of women have four or more episodes per year, which characterizes recurrent VVC (RVVC) [3, 4]. Candida albicans is the most frequently isolated species (50–80% of cases) [3, 5, 6]. The pathogenesis of VVC begins with adherence of the yeast to the vaginal mucosa, followed by asymptomatic colonization; the response to certain stresses by either the host or yeast leads to the onset of disease [7, 8]. The transition from commensalism to parasitism is associated with the symptomatology of VVC [2]. Little research has been reported for differences in potential virulence among clinical isolates of VVC. Recent studies from our laboratory found that vaginal C. albicans isolates from women with different symptomatologies have different behaviours that cause alterations in immunological defence mechanisms [9]. Nonetheless, the damage that this yeast can cause in host cells is not fully understood. The present study sought to characterize the cell damage caused by vaginal C. albicans isolates from women with different symptomatologies. Thus, to the best of our knowledge, this is the first study to describe cell damage caused by different clinical isolates of VVC.

The present study was performed with yeasts isolated from vaginal samples from women from our previous study and who were grouped according to symptomatology [10]. The symptoms considered for the VVC and RVVC groups as follows: vaginal discharge, vulvovaginal itching, vulvovaginal burning sensation, dysuria and dyspareunia, in addition to the presence of yeasts in vaginal secretion. Altogether, 12 clinical isolates of C. albicans were evaluated: 4 from asymptomatic women (AS), 4 from women with a single episode of vulvovaginal candidiasis (VVC) and 4 from women with 2 or more symptoms in 4 or more episodes within a 12-month period (RVVC). Vaginal sample collection, culturing and yeast identification were performed according to our previous study [10]. The yeasts samples were maintained at −80 °C in the collection of the Medical Mycology Laboratory of the State University of Maringá, Paraná, Brazil. The study was approved by the Ethics Committee on Human Research (COPEP; CAAE no. 02200093000-09; report no. 435/2009).
To evaluate the adhesion of C. albicans, human cervical cancer cells (SiHa) were used. The cells were cultured at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) that contained 10% fetal bovine serum (Gibco/Invitrogen) and 1% penicillin/streptomycin (Gibco/Invitrogen). After 24 h, they were subcultured in a 24-well plate (Techno Plastic Products) at a concentration of 2.0×10⁵ cells ml⁻¹. The yeasts were suspended in DMEM and the inoculum was adjusted to 1.0×10⁶ yeasts ml⁻¹. Then they were added to each well, which contained a monolayer of SiHa cells. After 2 and 24 h incubation, the cells were removed using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco/Invitrogen) solution, plated on Sabouraud dextrose agar (SDA; Difco) and incubated at 37°C for 24 h. The number of viable yeast cells was determined as the number of colony-forming units (c.f.u. ml⁻¹).

After adhesion to SiHa cells as described above, the bottom of each well was detached and scanning electron microscopy (SEM) was performed. The wells were washed three times with phosphate-buffered saline (PBS), fixed with 2.5% glutaraldehyde diluted in 0.1 M cacodylate buffer (Sigma; pH 7.2), and dehydrated in an ascending series of alcohol solutions. The samples were critical-point dried with CO₂ (CPD-030; Balzers Instruments), coated with gold (Shimadzu; IC-50 ion coater) and photographed with a scanning electron microscope (FEI; Quanta 200) at ×1000 and ×3000 magnification.

Trypan blue (Gibco) was used to measure cell damage. After the adhesion of C. albicans to SiHa cells for 24 h, the wells were washed twice with PBS, and the cells were detached with trypsin-EDTA. Trypan blue was prepared according to the manufacturer’s protocol and added to Eppendorf tubes (Techno Plastic Products) with the cells. Cell damage was determined as the percentage of cells that were stained blue (dead cells) compared with control SiHa cells that were grown in the absence of C. albicans.

The results were compared using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple-comparison post hoc test and Student’s t-test. The data were analysed using Prism 6.0 software (GraphPad). Values of P≤0.05 were considered to be statistically significant. The experiments were performed in triplicate and repeated in three separate assays.

In general, all of the isolates, independent of group, were able to adhere to SiHa cells at different times (Table 1). After 2 h contact with SiHa cells, the number of yeast cells that adhered was 1.35×10⁶, 2.31×10⁶ and 1.60×10⁶ c.f.u. ml⁻¹ in the AS, VVC and RVVC groups, respectively. Although it was not significantly different, an increase in the number of yeast cells that adhered was observed after 24 h: 1.87×10⁶, 3.56×10⁶ and 1.92×10⁶ c.f.u. ml⁻¹, respectively (P>0.05).

Cell adhesion is a complex and multifactorial phenomenon that depends on initial contact between the yeast cell wall and surface [11, 12]. In the present study, SiHa cells were used as the model, and interactions with selected clinical isolates of C. albicans were observed at two different times. Interestingly, no difference was found in yeast adherence rates, independent of group. Nevertheless, it is possible to observe a trend for greater adhesion potential for the VVC and RVVC groups on SiHa cells, as shown in Table 1. In VVC episodes, the adhesion capacity of C. albicans is considered to be a prerequisite for colonization and possibly the development of an infectious process [1, 7]. It is important to highlight that although the adherence rates were not significantly different, the yeasts were able to remain adhered to SiHa cells up to 24 h (Table 1).

SEM was used to understand the interaction of AS, VVC and RVVC isolates with SiHa cells at 2 and 24 h. The images revealed that up to 2 h of interaction between C. albicans and cells, the yeast remained predominantly of the blastoconidia form for all of the clinical isolates tested, [Fig. 1a(i), b(i), c(i), a(ii), b(ii), c(ii)], (white arrows). At 24 h of yeast–SiHa cell contact, different behaviours were observed among the groups compared with the initial time of observation. The AS isolates continued to be predominantly in the blastoconidia form, [Fig. 1d(i), d(ii)]. By contrast, the VVC and RVVC isolates were predominantly in the pseudohyphae form, [Fig. 1e(i), f(i), e(ii), f(ii)] (black arrows). This pattern persisted in 80% of the images analysed in more than 50 microscopic fields.

We strongly believe that adhesion is important for the maintenance of yeast in colonization, but filamentation is in fact the main factor responsible for the symptoms of VVC and RVVC. Phenotypic changes in C. albicans are important for the host invasion process, which can promote the virulence of yeast during the infectious process [13]. Previous studies have shown that micro-organisms are nearly nonexistent as free planktonic forms in host tissues, but are grouped together to form a multicellular community and cause tissue damage [14, 15]. Thus, we investigated whether the prolonged permanence of clinical isolates to SiHa cells is able to cause tissue damage.

Cell damage that was caused by the interaction between C. albicans and SiHa cells was evaluated using trypan blue. The percentage of cell damage was higher in the RVVC isolate compared with the AS and VVC isolates.

**Table 1. Adhesion (yeast cell ml⁻¹) of Candida albicans (AS, VVC and RVVC) to human cervical cancer cells (SiHa)**

<table>
<thead>
<tr>
<th></th>
<th>2 h (yeast cell ml⁻¹)</th>
<th>±SD</th>
<th>24 h (yeast cell ml⁻¹)</th>
<th>±SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>1.35×10⁶</td>
<td>1.01×10⁶</td>
<td>1.87×10⁶</td>
<td>1.14×10⁶</td>
<td>0.5179</td>
</tr>
<tr>
<td>VVC</td>
<td>2.31×10⁶</td>
<td>2.31×10⁶</td>
<td>3.56×10⁶</td>
<td>1.15×10⁶</td>
<td>0.0764</td>
</tr>
<tr>
<td>RVVC</td>
<td>1.60×10⁶</td>
<td>1.04×10⁶</td>
<td>1.92×10⁶</td>
<td>1.07×10⁶</td>
<td>0.6816</td>
</tr>
</tbody>
</table>

The data are expressed as the mean of three separate experiments. No significant difference was found among groups (P>0.05). AS, isolates from asymptomatic women; VVC, isolates from women with a single episode of vulvovaginal candidiasis; RVVC, isolates from women with recurrent vulvovaginal candidiasis.
symptomatic groups (VVC, 89.5%; RVVC, 80.0%) and lower in the asymptomatic group (AS, 54.8%), as shown in Fig. 2. Cell damage in SiHa cells was statistically significant in the AS, VVC and RVVC groups compared with the controls, and significantly different between the symptomatic and asymptomatic groups ($P<0.05$). The assay showed that the VVC and RVVC groups of isolates had differential ability to cause cell damage. *C. albicans* in these groups had greater adhesion potential and were able to cause considerable cell damage (cell death $>80\%$). Previous studies have reported that after yeast adhesion to the epithelium, there is active penetration of the fungus or endocytosis by the host cells. Following this internalization process, some enzymes are released, causing lysis of the epithelium and consequent tissue damage [16, 17].

In conclusion, understanding the pathogenesis of different clinical manifestations of VVC may contribute to the development of preventive measures and future therapies for VVC and RVVC. The clinical isolates of *C. albicans* from symptomatic women (VVC and RVVC) had greater invasion potential into epithelial cells, which was demonstrated in particular by filamentation and the induction of cell death, as observed by SEM and in the trypan blue assay, which suggests that these isolates were more pathogenic than the AS isolates. The cell damage shown in this study is relevant because women with VVC or RVVC present clinical symptoms that are different from those of women who have only been colonized by *C. albicans* (the AS group). In fact, *C. albicans* filamentation is an important virulence factor in the pathogenesis of VVC. Based on these results, the pathogenicity of *C. albicans* in patients
with RVVC may be strongly related to lysis of the vaginal epithelium.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

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