Adhesion and invasion of *Candida albicans* from periodontal pockets of patients with chronic periodontitis and diabetes to gingival human fibroblasts

JANAINA C. O. SARDI*, CRISTIANE DUQUE*,†, FLÁVIA S. MARIANO*, MARCELO R. MARQUES‡, JOSÉ F HÖFLING* & REGINALDO B. GONÇALVES§

*Department of Oral Diagnosis, Microbiology and Immunology Division, Piracicaba Dental School, University of Campinas, Piracicaba, Brazil, †Department of Dentistry, Federal Fluminense University, Nova Friburgo, Rio de Janeiro, Brazil, ‡Department of Morphology, Histology Division, Piracicaba Dental School, University of Campinas, Piracicaba, Brazil, & §Faculty of Dentistry, Periodontology Division, University Laval, Québec, Province Québec, Canada

The objectives of this study were to evaluate clinical isolates of *Candida albicans*, particularly their adhesion to and invasion of gingival human fibroblasts in culture and to measure nitric oxide concentration (NO) produced by fibroblasts in the presence of these yeasts. Sixteen strains of *C. albicans* isolated from patients with chronic periodontitis and diabetes mellitus type II were divided on the basis of phenotypic tests into two groups, i.e., highly or weakly hydrophobic. Primary cultures of human fibroblasts were isolated from gingival biopsies and after subsequent subcultures, the cells were seeded into culture plates and incubated for 24 h. *C. albicans* strains were inoculated into these plates and maintained for 2 and 4 h to assess their adhesion and invasion, respectively. The number of adherent or invasive yeasts was evaluated by assessing colony-forming units (CFU). The production of NO by fibroblasts was also quantified. The results showed that strains with high hydrophobicity had a greater ability to adhere and invade fibroblasts (*p* < 0.05, ANOVA and Tukey). The production of NO was higher for the most hydrophobic strains, but did not reach statistical difference with the weakly hydrophobic isolates. These data indicated that the hydrophobicity may play a role in the adhesion and invasion of *C. albicans* in fibroblast cultures.

**Keywords** *Candida albicans*, periodontal disease, diabetes mellitus type II, hydrophobicity, human fibroblasts

**Introduction**

*Candida albicans* is a regular inhabitant of the human mucosal surfaces and part of its microbiota, but can also cause infections ranging from those of the superficial mucosal to haematogenously disseminated disease [1]. Oral mucosal surfaces are the primary oral reservoirs of these yeasts, followed by both supra and subgingival biofilm [2]. *C. albicans* has been isolated from periodontal pockets in different forms of periodontitis, especially in HIV-positive and diabetic patients [3–5]. This fungus has a range of virulence factors that can be potentially relevant to the pathology of periodontal disease, such as the ability to adhere and invade gingival connective tissue [6]. One important virulence characteristic of *C. albicans* is its cell surface hydrophobicity (CSH), due to the mannosylated surface proteins that cover the fungal cells. Some of these proteins allow the yeast to adhere to the host cells through the increase in hyphal forms and resistance against macrophages, both of which are essential for the establishment of chronic lesions. *C. albicans* cells with high cell surface hydrophobicity rates have a greater capability of adherence to different host tissues when compared to cells with low rates of hydrophobicity [7].

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Correspondence: Janaina de Cássia Orlando Sardi, Piracicaba Dental School, University of Campinas (UNICAMP), Av. Limeira, 901, CEP 13414-903, Piracicaba, São Paulo, Brazil. Tel.:+55 19 2106 5321; Fax: +55 19 2106 5322; E-mail: janaina-sardi@uol.com.br

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The analysis of the adherence capacity of *Candida* spp. to both oral epithelial and connective tissue cells would be of interest to obtain a better understanding of the dissemination, infection and/or persistence of these yeasts in the oral environment. Most previous studies have used exfoliated epithelial cells derived from host tissues, such as buccal and vaginal cells or established epithelial cell lines to determine fungal adherence [8]. However, cultures of fibroblasts from either primary or lineage cells, have provided specific and reproducible models of the adherence and invasion of yeasts [9]. Additionally, this system is useful for studying the production of several products of fibroblasts, e.g., cytokines and nitric oxide, which are related to the progression of inflammation and tissue destruction [10]. These molecules are key to understanding the pathogenesis of many diseases, such as periodontitis. The objectives of this study were to evaluate the ability of *C. albicans* isolates from periodontal pockets of patients with chronic periodontitis and diabetes to adhere to and invade gingival human fibroblasts in culture, to compare these same capabilities relative to the hydrophobicity of the isolates, and to measure the nitric oxide concentration (NO) produced by fibroblast cells in the presence of these yeasts.

**Methods**

This study protocol was approved (protocol number 062/2008) by the Ethical Committee of Research, Piracicaba Dental School, UNICAMP.

**Gingival fibroblast isolation**

The procedure for establishing the primary cultures of gingival fibroblasts was based on that described by Somerman et al. [11]. Briefly, biopsies of healthy gingiva were obtained from the retromolar area of a single individual, i.e., a 23-year-old, non-smoker, without systemic alterations, who was undergoing third molar extractions for orthodontic reasons. The gingival samples were placed in a 15 ml centrifuge tube (BD - Labware) containing ‘biopsy’ medium; Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 250 μg/ml gentamicin sulfate, 5 μg/ml amphotericin B, 100 μg/ml streptomycin and 100U/ml penicillin (Gibco BRL, Life Technologies, Rockville, MD, USA). Each gingival fragment was then rinsed five times in the biopsy medium and cut into small pieces in a sterile Petri dish (35 × 100 mm) using a sterile scalpel. The microfragments were then removed, and the cells trypsinized and inoculated into new wells for orthodontic reasons. The gingival samples were confluent. The microfragments were then removed, and the cells trypsinized and inoculated into new wells for the experiment.

**Study population**

Eight subjects, ranging in age from 31–68 years, from the Graduate Clinic of the Piracicaba Dental School, State University of Campinas (UNICAMP) who had generalized chronic periodontitis and diabetes mellitus type 2 were included in the studies. Fasting glucose and HbA1c levels were obtained from all patients to confirm the diagnosis of diabetes. The glycemic control was performed by each patient using insulin supplementation. Exclusion criteria were: use of antibiotics and periodontal treatment during the previous 6 months, pregnancy, smoking, systemic disease, immunosuppression, clinical manifestation of oral candidosis, the use of partial and/or total prosthesis, the use of an orthodontic apparatus or treatment with any medication that could interfere with the periodontium or the response to periodontal therapy. Clinical diagnosis of chronic periodontitis were defined by probing depths of ≥5 mm (periodontal pockets) in ≥10 teeth and radiographic bone loss, ranging from 30–50%, in ≥20 teeth. After clinical measurements, the supragingival biofilm was removed with sterile gauze and subgingival samples were taken from periodontal pockets in each subject using a sterile periodontal curette. The samples from each site were immediately diluted and plated onto a Sabouraud dextrose agar (SDA) with cloramphenicol and onto chromogenic medium (CHROMagar Candida, Biomerieux, Paris, France), with both media incubated at 37°C for 48 h in a reduced oxygen atmosphere (10% CO₂ and 90% air). The green colonies grown on the chromogenic agar plates were randomly selected and cultures stored in glycerol stock at −20°C for laboratory analysis.

**Identification of Candida**

*Candida* spp. strains for the investigation were identified by PCR (Polymerase Chain Reaction) as *C. albicans*. The primers were designed especially for this study using the following free softwares, i.e., Primer 3 version 4.0 and Net primer Free Primer Analysis. The primers correspond to a portion of the gene AAT1a (ID 3643468) F: 5’ ACTGCTCAACACCATCTCTGG 3’ and R: 5’ CACAGGCAATGAAAGGAAT 3’ with fragment size of 472bp. Thermal conditions used for the PCR were 38 cycles at 94°C for 1 min, 53°C for 1 min and 72°C for 30 s.
**Cellular superficial hydrophobicity assay (CSH)**

*C. albicans* isolates were inoculated separately into 15 ml of Sabouraud dextrose (SD) broth (Difco Laboratories Detroit, MI, USA) and incubated overnight at 37°C in 5% CO₂. Hydrophobicity assays were conducted according to those as described by Rodrigues et al. [12]. Briefly, yeast pellets were harvested and washed twice in a phosphate buffer at a pH of 8.0. A cell suspension was prepared in the same buffer to obtain an optical density (A0) of 0.4–0.6 (at 600 nm). Then, 150 μl of hexadecane was added to tubes containing 3 ml of the cell suspension, incubated at 30°C and mixed twice for 30 s every 10 min. After 30 min, phase separation was observed and the optical density of the lower aqueous phase (A1) was measured and compared to that obtained prior to the mixing procedure (A0). The percentage of cells in the hexadecane layer (adhered cells) was used to estimate the hydrophobicity (H) using the following formula, % H = A0 – A1/ A0 × 100%. This assay was performed in duplicate. Strains were identified as HH (High Hydrophobicity) and LH (Low Hydrophobicity).

Sixteen strains were included in this study, i.e., eight HH and eight LH. Two strains (one HH and one LH) were obtained from each patient.

**Adhesion and invasion assays**

These tests were carried out according to the methods, with modifications, previously described by Cogo et al. [13]. Firstly, the adhesion and invasion of *C. albicans* were assessed in fibroblasts obtained from primary cultures of human gingival fibroblasts as described above. The GF cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 mg/ml L-glutamin), MEM, essential amino acids (Gibco Laboratories) and 2.20 g/l sodium bicarbonate. For the experimental studies, GF cells were cultured in 25 cm² tissue culture flasks (Corning Inc., Corning, NY, USA) at 37°C in an atmosphere of 5% CO₂. Cells from these culture flasks, at approximately 1 × 10⁵ cells/well, were used to inoculate 24-well tissue culture plates containing supplemented RPMI and incubated at 37°C in 5% CO₂ for 24 h to reach a confluent monolayer. Before the experiments, *Candida* spp. strains were grown in SD broth for 18 h and centrifuged at 4000 g for 10 min to obtain pellets for the subsequent cell counts in a hemocytometer. GF cells previously cultured for 24 h were washed three times with phosphate buffered solution (PBS; pH 7.5) and a total of 1 × 10³ yeasts/cm² was inoculated into these plates, to obtain a ratio of 1:100 GF:yeasts.

For the adhesion assays, *C. albicans* cells were maintained in contact with fibroblasts for 2 h. After this, the wells were washed three times with PBS to remove unattached yeasts and the GF cells detached at 37°C for 2 min using 1 ml trypsin-EDTA (Gibco) diluted in PBS. An aliquot of 20 μl was removed, serially diluted, inoculated onto SDA and incubated for 48 h. The number of adhered yeasts in the fibroblasts was assessed by counting the colony-forming units/ml (CFU/ml). In the control group, cultures were neither washed with PBS nor treated with trypsin-EDTA in order to determine total CFU/mL correspondent to all unattached and attached yeasts.

For the invasion assays, the experiments were repeated but after 4 h of contact fibroblasts–yeasts the unattached yeasts were removed by washing the wells three times with PBS and killed by incubation in RPMI containing 0.2 μg/ml nystatin (Sigma) for 30 min. The wells were then washed three times with PBS and GF cells were disrupted by means of incubation in 1 ml of cold sterile water for 20 min. Cell lysates were serially diluted and inoculated onto SDA plates that were incubated at 37°C for 2 days. Invasive yeasts were analyzed to determine their CFU/ml. For the control group, the cells were not submitted to washing with PBS and incubation with nystatin. Therefore all unattached and attached yeasts were collected to obtain the total number of CFU/ml. All assays were performed in duplicate, in two independent experiments. For adhesion and invasion, the percentage was calculated based on each control group, e.g., adhered cells × 100/control adhesion.

**Measurement of nitrite concentration (NO)**

The concentration of NO produced by gingival fibroblasts after 2 (adhesion) and 4 h (invasion) of incubation with *C. albicans* was measured by the quantification of nitrite, a stable conversion product of NO. A standard procedure using Griess reagent (1:1 mixture v/v) consisting of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H3PO4 was employed. The medium (100 μl) and Griess reagents (100 μl) were mixed and left for 30 min.

<table>
<thead>
<tr>
<th>Hydrophobicity</th>
<th>Adhesion %**</th>
<th>Invasion %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH</td>
<td>68.89*</td>
<td>53.91*</td>
</tr>
<tr>
<td></td>
<td>(4.66)</td>
<td>(9.13)</td>
</tr>
<tr>
<td>LH</td>
<td>3.00</td>
<td>22.55</td>
</tr>
<tr>
<td></td>
<td>(2.45)</td>
<td>(8.27)</td>
</tr>
</tbody>
</table>

HH, High Hydrophobicity; LH, Low Hydrophobicity.

*For each parameter (hydrophobicity, adhesion and invasion %), there was statistical difference between the groups HH and LH, according to ANOVA and Tukey test (p < 0.05).

**For adhesion and invasion assays, the percentage was calculated based on each control group. For example: adhered cells × 100/control. See methods."
at room temperature. The samples were read at 550nm [14], with the assay performed in duplicate.

**Statistical analyses**

Data concerning adhered and invasive *C. albicans* were analyzed using ANOVA and statistical differences among the groups, according to hydrophobicity, and were determined using Tukey’s post hoc. All statistical analyses were conducted at a significance level of 5% (SPSS, version 17.0).

**Results**

**Cellular superficial hydrophobicity assay**

Among the 16 *C. albicans* strains, eight were considered highly hydrophobic (HH) and eight had low hydrophobicity (LH). The mean percentage for hydrophobicity was statistically different between the HH (68%) and LH (3%) groups (Table 1). The CSH results ranged from 0.36–6.20% for the LH and 62.40–74.93% for the HH strains (Fig. 1).

**Adhesion and invasion assays**

All strains were able to adhere and invade gingival fibroblasts but the HH strains had a greater capacity to adhere and invade cells (Table 1) than LH isolates. The mean adhesion was 53.91% and 22.55% and for invasion, 24.4% and 9.8% with HH and LH strains, respectively. The number of adhered cells was always higher than those that invaded with both HH and LH isolates, except for strains 5LH and 8HH (Fig. 1). There was no difference between the HH and LH strains in the adhesion and invasion assays of the control groups (without treatments, trypsin for adhesion test or nistatin for invasion test) (*p* > 0.05). However, when controls were compared to the adhesion and invasion groups, this difference was remarkable (*p* < 0.05).

**Measurement of nitrite concentration**

NO production by fibroblasts was observed for all strains and there was an increase in concentration with the time of exposure. The concentration of NO was higher in HH strains, but there was no statistical difference between the HH and LH groups (Fig. 2A). NO production by fibroblasts was very high especially in contact with strains 2HH and 4HH, in both the adhesion and invasion assays (Fig. 2B).

**Discussion**

Microbial adherence to biological materials is an essential initial step in the infectious process. In addition to the presence of microbial adhesins and receptors on host cells, microbial surface hydrophobicity has been described as an important factor that influences adhesion of microorganisms to biological or inert surfaces [12]. Cell surface hydrophobicity plays an important role in mediating the adhesion of yeasts to epithelial, splenic, kidney, fibroblast and lymph node cells [15]. An increase of CSH also enhances the virulence of *C. albicans* in an animal model [16]. Experiments have suggested that high CSH impairs phagocytosis, increasing resistance to blood clearance and as a consequence, the virulence of *C. albicans* [12]. To understand the pathogenesis of candidosis, in vivo and *in vitro* studies have been developed to characterize and quantify the adhesion of *C. albicans* to cell surfaces [17,18].

The binding of *C. albicans* to mucosal surfaces has been demonstrated as an important step in the infectious
process, particularly in the oral cavity [17]. The ability of Candida spp. to adhere to different cells is a determinant factor for infection, persistence, and dissemination of these species in oral tissues. Nikawa et al. [19] quantitatively evaluated the adhesion of oral strains of C. albicans, C. tropicalis, and C. glabrata to human gingival epithelial cells, gingival fibroblasts, and pulmonary fibroblasts. These authors observed that most of the tested Candida strains
had significantly more adherence to gingival epithelial cells than other types of fibroblasts. However, fibroblast cultures, whether primary or lineage cells, were found to be a specific and reproducible model for the investigation of adherence and invasion by yeasts [9]. In the present study, both processes were successfully assessed using fibroblasts. When compared to the control groups, there was a significant reduction in *C. albicans* counts, showing that after 2 or 4 h, adhesion and invasion, respectfully, may be observed for this species when brought into contact with human gingival fibroblasts.

Environmental factors, such as diet, composition of body fluids and presence of antifungal agents may have an effect on cell surfaces, thereby modulating *Candida* adhesion [20]. Although many studies have demonstrated that the adhesion process is related to bindings between host adhesins and mannoproteins of *C. albicans*, CSH seems to also play an important role in this process [17]. Epithelial cells and fibroblasts are the primary components of the oral mucosa and any injury to these cells may lead to the development of oral diseases, such as candidiasis or periodontal disease [21].

The current results show that CSH is an important, non-specific factor for *C. albicans* adhesion to and invasion of oral fibroblasts. Highly hydrophobic cells were found to be more invasive than cells of low hydrophobicity in this present study. Changes in the environmental conditions can trigger the formation of hyphae. Thewes et al. [1] assessed the *in vitro* invasion of *C. albicans* in human tissue (fibroblasts and epithelial cells) and found that reduction in the formation of hyphae (a characteristic of the less hydrophobic cells) decreases the invasive properties and reduces the ability of the yeast to invade tissue cells. The gingival pocket and gingival crevicular fluid were found to be favorable environments for germination and hyphal growth of *C. albicans*. *Candida* hyphae have a greater ability to adhere to host surfaces and penetrate tissues when compared with yeast cells [6]. Therefore, *C. albicans* could have a role in the infrastructure of periodontal microbial plaque and participate in the adherence to periodontal tissue [6]. In a previous study conducted by the present authors, CSH in reduced oxygen and anaerobiosis was studied (data unpublished) and the results indicated that strains were more hydrophobic when grown in anaerobic conditions. In anaerobiosis, *C. albicans* appears in the form of hyphae, which is much more common than in other atmospheric conditions. Hydrophobic interactions may be of importance in promoting tissue invasion by the filamentous form of yeasts [22].

Another virulence factor of *Candida* spp. is their resistance to innate and cell-mediated immunity that may help in the progression of inflammation in the surrounding tissues [6]. In addition to the presence of several cytokines, NO production in the microenvironment of periodontal disease, has been observed and acts directly on the maintenance or progression of inflammation and tissue destruction [10]. When NO is locally produced in high concentrations, it has a cytotoxic or cytostatic effect against cells infected by fungi, bacteria, protozoa, tumor cells, and adjacent cells resulting in extensive tissue destruction [10]. The current authors investigated NO production by gingival fibroblasts in the presence of *C. albicans*. The results demonstrated that gingival fibroblasts were able to produce NO in the presence of this yeast after 2 and 4 h of exposure. Interestingly, after 4 h, NO production was detected in greater quantity than at 2 h, suggesting that a longer interaction between cells and the pathogen produced higher levels of NO by cells. Highly hydrophobic strains have a tendency to stimulate an increase in the production of NO, although a statistical difference was not found when these strains were compared to those of lesser hydrophobicity. The presence of this molecule in periodontal disease may reflect the involvement of an additional mediator in the regulation of bone resorption related to the progression of the disease [23]. The role of *C. albicans* in periodontal disease is not clear and further studies are needed to demonstrate the clinical significance of the findings. Only a few species of microorganisms normally found in diseased gingival pockets can penetrate the epithelia surface and provoke inflammation. In this current study, the ability of *C. albicans* to adhere and invade human gingival fibroblasts was demonstrated, as well as their ability to stimulate the production of NO by these host cells.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**References**


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