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The role of morphological transitions of *Candida albicans* in its survival and host invasion

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INTRODUCTION

Candida albicans is the most commonly isolated opportunistic pathogen yeast in the human body. This diploid, polymorphic fungus has adapted to warm-blooded host organisms, and, thus, it colonizes the oral, gastrointestinal and female genital mucosa of 30 to 70% of healthy individuals as part of the normal flora. However, the rise in the number and extension of predisposing factors have led over the last decades to an increase in the incidence of superficial and invasive diseases caused by *C. albicans*. The major risk factors of *Candida* infections include immune suppression (e.g. corticosteroid therapy, neutropenia, HIV infection, and diabetes), long-term hospital care, central venous catheters, use of broad-spectrum antibiotics, hormone therapies, heart and gastrointestinal surgery, age, malignant tumors, and hematologic diseases.

C. albicans has a number of virulence factors which help spread the yeast in the host. Morphological transition is considered one of the most significant virulence factors of *C. albicans*. The shift of *C. albicans* from yeast to filamentous form is regarded as one of the fast responses to changes in its environment. The transition from one morphology to another is triggered and regulated by a number of environmental and internal factors, including the pH value or the change in the CO_2 tension, the availability of various trace elements, e.g. iron, as well as various stress factors affecting the fungus at the time of colonization (such as oxidative, osmotic, cell wall, cell membrane or pH stress) or immune suppressants administered to the host.

The *C. albicans* yeast→hyphal transition appears to be commonly cited in the literature as an explanation for proliferation and the invasive infections caused in the host organization. This is because the yeast and (pseudo)hyphal morphologies along with the transition from one form to another provide the fungus with significant advantages in the various stages of the infection. The yeast form plays a role in spreading in the bloodstream and adherence to the epithelial cells. The filamentous forms cause damage to the host cells and tissues, provide a mechanism of escape from the macrophage, and, thus, participate in phagocyte lysis and colonization of various organs such as the kidneys.

The elimination of *C. albicans* from the bloodstream and blocking tissue invasion require a normal and functional innate and adaptive immune system. Therefore, patients requiring glucocorticoid therapy represent a high-risk group for *Candida* infections. Corticoids used for the prevention of transplant rejection or to treat autoimmune, vascular, dermatological or gastrointestinal diseases decrease the immune response, and significantly increase susceptibility to *C. albicans* infections.

Moreover, corticoids not only have an impact on the human body, but also affect the physiology and virulence of *C. albicans* in the normal flora. Certain corticosteroids such as methylprednisolone and hydrocortisone used in human medicine enhance *C. albicans* proliferation, germination, hypha formation, extracellular aspartyl proteinase and phospholipase production, adherence to epithelial cells and increase the colonization rates of the oral mucosa and the gastrointestinal tract.

Betamethasone is a moderately potent synthetic steroid with well-known anti-inflammatory and immunosuppressive effect used in ~1-2 mM concentrations to treat rheumatic disorders, skin conditions (itching and eczema) as well as allergies and eye infections. Betamethasone is commonly used in eye drops and ear drops as well as in gels paralelly with antimicrobial agents to prevent post-surgical infections. No literature is available on the impact of this corticoid on *C. albicans* virulence, however, a certain relationship may be suspected between betamethasone therapy and *C. albicans* infection.

To better understand the role of the typical yeast-to-hypha transition of *C. albicans* in infections, not only the study of certain environmental factors/stress types triggering the transition, but the follow-up of infections developing after immunosuppressive therapies is needed. We hope that the deeper understanding of the morphological transition of *C. albicans* will allow for the development of a novel and more efficient antifungal treatment strategy.

AIM

Our study aimed at the characterization of the morphological transition of *C. albicans* which is causing an increasing health issue. As invasive fungal infections and the yeast-to-hypha morphological transition are strongly related, the authors were looking to answer the following questions:

(i) Are the changes in the typical *C. albicans* habitat environmental factors responsible for the development of candidiases?
(ii) Does the adaptation to a certain stressor during an infection provide protection to *Candida* cells against other stressors as well?
(iii) Is the lack of immune response caused by an immunosupressive therapy the only reason for developing fungal infections?

The following research has been carried out to answer the questions raised:

1. Characterization of the impact of the environmental parameters typical for various anatomic sites of the human body to determine the branching frequency of *C. albicans* filamentous forms.
2. Study of response to environmental stress conditions of *C. albicans* mutant strains which have an increased tolerance to oxidative stress.
3. Characterizing the impact of betamethasone on fungal physiology related to *C. albicans* virulence, interaction with certain antifungal agents, susceptibility to oxidative stress, and *C. albicans* infection dynamics on human epithelial cell models.

MATERIAL AND METHODS

C. albicans strains

C. albicans strains used in this study are as follows: SC5314 (ATCC-MYA 2876), ATCC 14053 type strain, 5 clinical isolates (4774, 8387, 10934, 19890, 20072). Tert-butylhydroperoxide tolerant mutants

(*t*BOOH; AF06, 4774T, 8387T, 10934T, 19890T, 20072T) were generated from ATCC 14053 and clinical strains. Oxidative stress tolerant mutants were developed by continuous cultivation of parental strains in the presence of stepwise increasing concentration *t*BOOH (Fekete *et al*., 2007; 2008). All clinical isolates originated from the Department of Medical Microbiology, University of Debrecen.

The effect of environmental factors on the branching time of *C. albicans* filamentous forms

Effects of hemin and CO_2 on morphological transitions of *C. albicans* were investigated at pH values corresponding to that of the oral cavity (pH 4.5-8.0), gastrointestinal tract (pH 2.0-8.0) and vaginal lumen (pH 4.2-5.3). The CO_2 values chosen for the study correspond to the gastrointestinal tract (5.1-29 (v/v) %) and the vaginal lumen (6.1- 8.3 %) in the human body. *Candida* cells were inoculated in bicarbonate-free RPMI 1640 medium supplemented with 10 % fetal bovine serum. The pH of the medium was set to 4.2, 5.3, 7.0 or 8.0 depending on the experiments, and in a set of separate experiments the medium contained an additional 50 μg/ml hemin (iron source). Cultures were incubated at 37 °C with 5 % (v/v) or 10 % (v/v) CO_2 supplementation. Using a video microscopy technique, effects of hemin, CO_2 and pH on the type of filamentous morphological forms, the germination time (the time elapsed to germination), branching time (the time elapsed from germination to the first branching), and hypha lengths (germinating cells to the first branches)

in *C. albicans* cultures were examined. For each set of experiments, 8 to 23 cells were subjected to statistical analysis.

Stress cross- protection is examined on the oxidative stress tolerant *C. albicans* mutants.

In this study effects of environmental stress-generating agents on the proliferation and morphological transitions of *C. albicans* cells were investigated. The parental and mutant strains were exposed to oxidative, hyperosmotic, heavy metal, cell wall, cell membrane, unfolded protein response, pH and thermal stress.

The microbial growth was tracked in liquid medium supplemented with stress-generating agents using spectrophotometry. Growth inhibition caused by various stress conditions was calculated for the mutants and parental strains. The yeast, hypha and pseudohypha morphological forms were visualized by phase-contrast microscopy and fluorescence microscopy after calcofluor white staining.

Determination of the effect of betamethasone on the virulence of *C. albicans*

The *C. albicans* SC5314 cells were incubated in yeast extract peptone dextrose medium supplemented with 1-4 mM betamethasone at 37 °C, 3 Hz shaking frequency and at 6 h incubation time to assess the effect of betamethasone on the physiological characteristics of *C. albicans.* The proliferation of yeast cells and the growth of antimycotic (menadione sodium bisulfite, amphotericin B, fluconazole, nystatin) treated cultures

were determined by changes in optical density. Survival rates of cells were estimated by the time-kill test recommended by Klepser *et al.*

The colony size was compared on yeast extract peptone dextrose agar.

Virulence attributes including the germ tube formation in sheep serum, extracellular phospholipase activity on egg yolk medium, pseudohypha and chlamydospora formation on corn meal agar and hypha formation on Spider medium were determined.

Catalase, glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase and superoxide dismutase specific enzyme activities were determined by photometric methods.

Accumulation of reactive species productions was always detected by the formation of 2',7'-dichlorofluorescin from 2',7'-dichlorofluorescein diacetate by a fluorimetric method.

The reduced glutathione and oxidized glutathione content of the cells were estimated by rate assay of Anderson (1985).

The effect of betamethasone was determined on the dynamics of *C. albicans* infection in the epithelial cell line TR146 derived from a squamous carcinoma of buccal mucosa, C2BBe1 (clone of Caco-2) colon carcinoma cell line and A-431 vaginal epithelial cell line. *C. albicans* SC5314 cultures were pre-cultured in the absence or presence of 1 or 2 mM betamethasone (betamethasone pre-treatment). Infection of epithelial cells with *C. albicans* was performed in the absence or presence of 1 or 2 mM betamethasone (betamethasone treatment).

To characterize adhesion, *C. albicans* cells were co-incubated with epithelial cells for 1 h. The number of adherent yeast cells (stained by calcofluor white) was determined using fluorescence microscopy.
Effects of betamethasone treatments on the *C. albicans* invasion rate were determined after 3h co-incubation.

Staining with green-fluorescent Alexa Fluor 488 conjugate of succinylated concanavalin A and calcofluor white allowed for determination of the filament length, the invading (only stained by calcofluor white) and non-invading (stained by both calcofluor white and Alexa-ConA) hypha by fluorescence microscopy.

Epithelial cell damage was estimated by measuring the release of lactate dehydrogenase using the cytotoxicity detection kit (Roche, Germany).

After 24 h co-incubation of epithelial cells with *C. albicans* cells, the interleukin-6 and interleukin-8 concentrations were determined from the culture supernatants by enzyme-linked immunosorbent assays. (eBIoscience).

Data analysis

All experiments were performed in at least three independent sets. Statistical significance was calculated using Student's t test, Welch's t-test or Tukey's test (GraphPad Prism 7). P value <0.05 was considered to be statistically significant.

The pairwise comparisons of the branching and germination time of *C. albicans* filament forms were performed with two-tailed Welch's t-tests.

The resulting p values were adjusted for multiple comparisons with the Holm's procedure. In the cross- protection experiments, the inhibition of growth was calculated by subtracting microbial growths recorded in stress-exposed cultures from mean microbial growths recorded in the control cultures. The significance of the differences between mean growth inhibitions recorded for mutants and their appropriate parental strains was estimated. The p values in multiple comparisons were adjusted according to Holm (1979). Statistical analysis in both experiments were performed using the 3.1.2 version of the R statistical environment.

When monitoring the *C. albicans* and epithelial cells interaction, statistical significance was calculated using two-way ANOVA and the Tukey's test for multiple comparisons.

THE SUMMARY OF NEW SCIENTIFIC RESULTS

1. The effect of environmental factors on branching of *C. albicans* filament forms

1.1. The branching time, an easily, rapidly accessible and accurately determinable parameter in terms of video microscopy technique, was chosen to characterize the physiological state of *C. albicans* cells. Among the examined parameters, changes in the germination times of *C. albicans* cells were strain specific, in contrast to the branching time measurements that did not show dependence on culture conditions employed.

1.2. Taking into consideration the correlation between branching times and hyphal lengths (to grow paralell to the culture vessel) measured at the time

of branching, we concluded that the growth rates of hyphae were not influenced by the changes in the culture parameters.

1.3. Culturing *C. albicans* strains supported hypha (in neutral and alkaline media) or pseudohypha (in both acidic media) formations independently in the presence of hemin or 5 %–10 % CO_2 supplementation.

1.4. Culturing *C. albicans* in the presence of hemin at pH 7.0 was characterized by the shortest branching times, and increased branching times were observed at alkaline pH (pH 8.0) and at neutral pH (pH 7.0) with 5 % CO_2 supplementation. The branching of hyphae is regulated by fungal physiology, availability of nutrients and changes in some metabolite concentration. The decreased branching frequency is likely to indicate the limitation of essential nutrients which also promotes fungal invasion of host tissues.

1.5. Upon reaching deeper tissues and blood vessels by the hyphal form of *C. albicans*, the pH and the CO_2 concentration are likely to rise, which should result in a higher branching time and less branched mycelium and supporting the deeper tissues candidiases.

1.6. Increasing pH or CO_2 concentration in the presence of hemin resulted in longer branching time. These environmental conditions facilitate the invasion of the intestine by *C. albicans* in the case of bleeding/ulceration.

1.7. The acidic pH values and the presence of 5 %–10 % CO_2 can be considered a simplified model of the conditions in the vaginal lumen. The acidic pH values inhibit yeast→hyphal morphological transitions which may protect the vaginal mucosa against invasive *Candida* infection

between 4th- 14th days of the menstruation cycle. On the other hand, the increasing pH, the relatively high CO_2 levels under menstruation as well as the haemoglobin content of menstruation fluid may facilitate hypha formation and even invasion by *C. albicans*.

1.8. A new parameter, namely branching time, was successfully introduced to characterize the morphological, physiological, and virulence status of *C. albican*s under various environmental conditions which can be observed in different human anatomical niches.

2. Stress cross- protection in the oxidative stress tolerant *C. albicans* mutant strains

2.1. The oxidative stress tolerance of *C. albicans* mutants were characterized by continuous elevation of antioxidative defence that resulted in the increased tolerance against oxidative, hyperosmotic, heavy metal, cell wall, cell membrane, unfolded protein, pH and thermal stress which occur in the human body.

2.2. Analysing of the correlation between the morphological transition and general stress tolerance demonstrated that morphological transitions of *C. albicans* cells were dependent on specific stress conditions and intraspecific variations and not on the innate oxidative stress tolerance of the *Candida* cells. *C. albicans* wild type and mutant strains grew only in yeast form under no-stress conditions while yeast→hypha/pseudohypha morphological transitions were elicited sporadically in stress-exposed cultures (heat stress at 42 °C and H_2O_2 treatments). Although *C. albicans* cells may respond to environmental stress by yeast→hyphae morphological

transition this ability was not related to stress cross- protection phenomena of oxidative stress-adapted cells.

3. Effect of betamethasone treatment on the virulence of *C. albicans*

3.1. Betamethasone, which is widely used in medicine, stimulated the extracellular phospholipase production and hypha formation of *C. albicans* significantly, thus it may influence the virulence of *C. albicans.*

3.2. The betamethasone treatments stimulated the adherence of *C. albicans* to the vaginal epithelial cells and also promoted the invasion of the fungus into the intestinal and vaginal cells without correlating to the growth rate of invading *C. albicans* hyphae.

3.3. Betamethasone enhanced the *C. albicans*-induced cell damages observed for oral and intestinal epithelial cells and decreased the expression of the proinflammatory interleukin-6 and interleukin-8 in the case of oral and vaginal epithelium cells. Betamethasone thus may decrease the recruitment of immune cells to the site of infection.

3.4. Betamethasone decreased the efficiency of the antimycotics amphotericin B and nystatin against *C. albicans*. In the light of these observations the corticosteroid-polyene drug interactions may be of clinical importance and impede the efficiency of drugs employed in combination.

3.5. The immunosuppressive properties of glucocorticoids can frequently lead to *C. albicans* infections. However, these observations suggest that the exposure of microorganisms to glucocorticoids may promote quicker proliferation of the human pathogen within the human body.

3.6. The high-dose topical application of glucocorticoids may predispose patients to various epithelial *Candida* infections in the oral cavity, the gut and in the vagina.

3.7. The betamethasone glucocorticoid decreased the specific activities of catalase and superoxide dismutase, increased the level of intracellular reactive species, all these results suggested that betamethasone treatments led to oxidative stress in *C. albicans* cells.

3.8. Betamethasone treatments modified the oxidative stress sensitivity of *C. albicans* cells. When the superoxide- generating agent menadione sodium bisulfite was combinated with 2 or 4 mM BM, these drug combinations resulted in either fungistatic (yeast extract peptone dextrose medium) or fungicidal (RPMI-1640 medium) effects depending on the composition of cultural media.

SUMMARY

The invasive capability of *C. albicans,* which is an opportunistic pathogen dimorphic fungus, is frequently correlated to the yeast→ hypha morphological transition. Within the human body the rapidly changing and diverse stress condition promote the yeast→ hypha transitions, therefore can result in various types of candidiases.

According to our experimental data the physiological changes such as the elevation of pH, increasing CO_2 concentration or bleeding in the oral cavity and in the intestinal and vaginal lumens are likely to facilitate the morphological transitions and even the invasion of the human body by *C. albicans.*

According to our suggestions, anti-*C. albicans* therapies may include the reconstruction of physiologically relevant pH values and CO_2 levels at the sites of candidiases to prevent or hinder the spread of *Candida* cells toward deeper tissues, as well as the disturbance of the hemoglobin and hemin uptake by *C. albicans* through hemoglobin/heme-binding receptors on the surface of *Candida* cells.

In the human body, the peroxide-producer phagocytic cells play a crucial role in the destruction of the invading microorganisms. Our data indicates that the increased tolerance of *C. albicans* to oxidative stress provided the fungus with a defence system against other stress conditions in the host. In addition, the morphological transitions of *C. albicans* were not related to stress cross- protection phenomena.

Glucocorticoids are widely used in the medicine for their anti-inflammatory and immunosuppressive effects. Our observations suggest that the use of applications of high dose glucocorticoid betamethasone may predispose patients to *C. albicans* infections by stimulating the hypha formation of *C. albicans* and extracellular phospholipase production, affects the efficiency of antimycotics as well as the interactions between *C. albicans* and the epithelium (adhesion, invasion, cell damage, interleukin release). High dose betamethasone may make yeast cells more vulnerable to oxidants like the superoxide- generating agent menadione. The combination of glucocorticoid medications for topical use with oxidants (menadione or menadione derivatives) may be a viable alternative in the development of a new and efficient antifungal strategies for preventing or even curing fungal infections *e.g.* in the field of dermatology.

In summary, any disadvantageous environment changes *e.g.* absence or suppression of immune recognition/immune response can lead to candidiases. Therefore, a better understanding of the morphological transitions during *C. albicans* infection seems to have a primary importance in order to prevent, diagnose and alleviate fungal infections in the future.

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List of publications related to the dissertation

1. **Jakab, Á.**, Antal, K., Emri, T., Boczonádi, I., Imre, A., Gebri, E., Majoros, L., Pfliegler, V. P., Szarka, M., Balla, G., Balla, J., Pócsi, I.: Effects of hemin, CO2, and pH on the branching of Candida albicans filamentous forms.
Acta Microbiol. Immunol. Hung. *63* (4), 387-403, 2016.
DOI: http://dx.doi.org/10.1556/030.63.2016.023
IF: 0.568 (2015)

2. **Jakab, Á.**, Mogavero, S., Förster, T. M., Pekmezovic, M., Jablonowski, N., Dombrádi, V., Pócsi, I., Hube, B.: Effects of the glucocorticoid betamethasone on the interaction of Candida albicans with human epithelial cells.
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IF: 2.268 (2015)

3. **Jakab, Á.**, Emri, T., Sipos, L., Kiss, Á., Kovács, R. L., Dombrádi, V., Kemény-Beke, Á., Balla, J., Majoros, L., Pócsi, I.: Betamethasone augments the antifungal effect of menadione-towards a novel anti-combination therapy.
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Folia Microbiol. *59* (4), 333-340, 2014.
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List of other publications

5. Bertóti, R., Vasas, G., Gonda, S., Nguyen, M. N., Szőke, É., **Jakab, Á.**, Pócsi, I., Emri, T.:
Glutathione protects Candida albicans against horseradish volatile oil.
J. Basic Microbiol. 56 (10), 1071-1079, 2016.
DOI: http://dx.doi.org/10.1002/jobm.201600082
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Total IF of journals (all publications): 7,006
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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List of other submitted publications

Pfliegler WP, Boros E, Pázmándi K, **Jakab Á**, Zsuga I, Kovács R, Urbán E, Bácsi A, Majoros L, Pócsi I. Commercial strain-derived clinical *Saccharomyces cerevisiae* isolates evolve new phenotypes, but not higher pathogenicity.
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Boros E, Pfliegler WP, Kovács R, **Jakab Á**, Majoros L, Barta Z, Pócsi I. Diverse and dynamic: physiological traits of *Candida albicans* isolates from a single hospital show low niche specialization.
J. Basic Microbiol. 2017.

List of presentations and posters

Jakab, Á., Mogavero, S., Emri, T., Hube, B., Pócsi, I. (2016) Effect of glucocorticosteroid betamethasone on the virulence of *Candida albicans*. Congress of Hungarian Society for Microbiology, 2016, Keszthely, Hungary.

Jakab Á., Sipos, Kiss Á., Emri T., Pócsi I. Glucocorticosteroid influences the virulence and oxidative stress sensitivity of the opportunistic pathogen *Candida albicans*.
Spring Wind Conference, 2014, Debrecen, Hungary.

Jakab Á., Sipos L., Kiss Á., Emri T., Pócsi I. Glucocorticosteroid effects the virulence of major fungal pathogen *Candida albicans*.
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Jakab Á. The core environmental stress response and morphological transition of *Candida albicans*.
Biotechnology symposium, 2012, Debrecen, Hungary.

Jakab Á., Karányi Zs., Kiss Á., Pócsi I., Pócsi I. Morphological transitions represent no alternative to environmental stress response in *Candida albicans*.
5th Hungarian Mycological Conference, 2012, Budapest, Hungary.