Oral epithelial cell antifungal activity: approaches to evaluate a broad range of clinical conditions

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Anti-Candida activity by oral epithelial cells is considered one of several innate mucosal defense mechanisms against oropharyngeal candidiasis (OPC). OPC is the most common fungal infection in HIV disease. Previously we reported that oral epithelial cell anti-Candida activity is reduced in those with OPC, potentially representing a contributing factor to OPC. However, testing clinical epithelial cells possessing high levels of Candida has been limiting due to high background in the assay controls. HIV+ smokers often develop OPC sooner than non-smokers during progression to AIDS, suggesting additional immune aberrations. The purpose of this study was to design a means to reduce Candida associated with epithelial cells collected from saliva without affecting their in vitro growth inhibitory activity, and to employ that approach to evaluate antifungal activity in HIV+ smokers. To do so, oral epithelial cells with and without known levels of Candida were subjected to various treatments including azole, polyene, or echinocandin antifungal drugs or fixation followed by the standard growth inhibition (GI) assay. The results indicated that antifungal drugs, while effectively reducing cell-associated Candida, also affected epithelial cell function. In contrast, fixation with paraformaldehyde eliminated cell-associated Candida and had minimal effects on epithelial cell anti-Candida activity. Employing the fixation design that allowed a broad range of patients to be evaluated showed no difference in oral epithelial anti-Candida activity between HIV+ smokers and non-smokers. Therefore, oral epithelial cell antifungal activity does not appear compromised in those who smoke, reducing it as a contributing factor in susceptibility to premature OPC.

Keywords Candida, human immunodeficiency virus, oral epithelial cells, oropharyngeal candidiasis, smoking

Introduction

Oropharyngeal candidiasis (OPC) is the most common oral manifestation in persons infected with human immunodeficiency virus (HIV). Candida albicans, a dimorphic fungal commensal organism of the gastro-intestinal and reproductive tract, is the primary causative agent of OPC. OPC occurs most frequently in HIV+ persons when CD4+ T cells are reduced below 200 cells/μl. While cell-mediated immunity (CMI) by Th1-type CD4+ T cells is considered the predominant host defense mechanism against mucosal C. albicans infections [1,2], several local mechanisms also appear important. Among these are locally present cytokines that bathe the tissue and influence immune reactivity [3,4] and oral epithelial cells that have the ability to inhibit the growth of C. albicans in vitro [5]. Oral epithelial cell anti-Candida activity has been shown to
be reduced in HIV+ persons with OPC, suggesting a protective role for oral epithelial cells in vivo [5]. HIV+ smokers often acquire a premature OPC at higher CD4 cell numbers (<500 cells/μl), suggesting additional mucosal immune dysfunctions or an aberrant microenvironment [6–9]. Support for the former comes from reduced Th1-type and increased Th2-type cytokines in saliva of OPC+ smokers with >200 CD4 cells/μl compared to OPC− persons [4]. Since HIV− smokers are not at risk for OPC, there is also the possible influence of HIV itself. Still another possibility is reduced oral epithelial cell anti-Candida activity, potentially due to adverse effects of smoking on the epithelium.

Oral epithelial cells collected from saliva inhibit 60–90% of Candida growth in vitro and are active against both morphological forms of C. albicans as well as other Candida species. The mechanism includes a strict requirement for cell contact with no role for soluble factors [10]. Physical characteristics show a resistance to irradiation, partial resistance to fixation, and sensitivity to heat and detergents [10,11]. The activity is static rather than cidal [11], is non-inflammatory, and is thought to occur through a putative carbohydrate moiety on the epithelial cell [10].

A limitation of the epithelial cell assay ([3H]-glucose uptake) has been the testing of clinical samples with high numbers of Candida. Such levels of Candida result in high background levels of glucose uptake and thus interference with the interpretation of the assay. Thus, to date, clinical testing has been limited to only those with low cell-associated Candida. Alternatively, if the Candida associated with the epithelial cells could be reduced or eliminated, more intensive clinical analyses could be conducted. The purpose of this study was to investigate approaches that could reduce the effects of Candida on oral epithelial cells without effecting the antifungal activity and then to employ that approach to evaluate the epithelial cell anti-Candida activity in HIV+ smokers.

Materials and methods
Subjects

Twenty HIV− healthy laboratory volunteers and 30 HIV+ subjects were enrolled. HIV+ subjects were recruited and evaluated at the Louisiana State University Health Sciences Center (LSUHSC) HIV Outpatient Program (HOP) of the Medical Center of Louisiana, New Orleans, and the Charity Hospital Dental Clinic. Informed consent was obtained from each participant, and all procedures were conducted in accordance with the Institutional Review Board at LSUHSC, New Orleans and the research committee of the HOP. HIV status was confirmed by enzyme-linked immunosorbent assay, followed by Western blot through the Clinical Immunology Laboratory at the LSU Health Sciences Center.

Epithelial cell isolation

Unstimulated saliva (10 ml) from each participant was expectorated into a single polypropylene test tube and centrifuged at 800 g for 5 min. The cell pellet was washed twice with sterile phosphate-buffered saline (PBS), resuspended in Hanks Balanced Salt Solution (HBSS) (Gibco, Gaithersburg, MD), and passed over a 20-μm sterile nylon membrane (Spectrum, Rancho Dominguez, CA). The epithelial cell-enriched population collected from the membrane was washed, resuspended in cryopreservative solution (50% fetal bovine serum [FBS], 25% RPMI 1640 tissue culture medium, 15% dimethyl sulfoxide), and stored at −70 °C until use. At the time of use, the cells were thawed, washed twice in HBSS, once in PBS, and enumerated by trypan blue dye exclusion.

Target cells

Candida albicans 3153A from the National Collection of Pathogenic Fungi (London, UK) was grown on Sabouraud dextrose agar (Becton Dickinson, Sparks, MD) at 34 °C. One colony was used to incubate 10 ml phytone-peptone (PP) broth (Becton Dickinson) supplemented with 0.1% glucose for 18 h at 25 °C in a shaking water bath. The blastoconidia were collected, washed with PBS, and enumerated on a hemacytometer using trypan blue dye exclusion.

Growth inhibition assay

A [3H]-glucose uptake assay [5,10,12] was employed. In brief, stationary-phase blastoconidia were added to individual wells of a 96-well microtiter plate (Costar, Cambridge, MA) at 1 × 10^5 cells/ml in a volume of 100 μl PP broth (Becton Dickinson) supplemented with 10% fetal bovine serum and 1% penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco). Epithelial cells were added to triplicate wells in a volume of 100 μl PP broth at an effector to target ratio of 5:1. Controls included effector cells and target cells cultured alone. The cultures were incubated for 9 h at 37 °C in 5% CO2 in the presence of 1 μCi [3H]-glucose (PerkinElmer Life Sciences, Boston, MA). Following the incubation, 100 μl sodium hypochlorite solution (bleach) was added to all wells and left for 5 min, and the cell
Extracts were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technologies, Watertown, MA). The incorporated $[^3]$H-glucose was measured by liquid scintillation. The incorporation of glucose by Candida during the 9-h assay ranged from 15,000 to 30,000 counts per minute (c.p.m.). Incorporation of glucose by epithelial cells with low-level colonization generally ranged from 1000 and 4000 c.p.m., whereas, those with high level colonization ranged from 6000 to 21,000 c.p.m. Any epithelial cell samples with >4000 c.p.m. were excluded due to background levels too high to instill confidence in the assay. The percent growth inhibition was calculated as follows: % growth inhibition = I - (mean effector cell + Candida c.p.m.) - (mean effector cell c.p.m.) + mean Candida c.p.m. × 100.

### Approaches to test a broader range of clinical samples

#### Antifungal drug approach

To assess the effects of antifungal drugs on Candida alone, Candida ($10^3$, $10^4$, $10^5$ blastoconidia per ml) was treated with 1 ml drug: Fluconazole (Pfizer, New York, NY; 200, 500, 1000 µg/ml), amphotericin B (Sigma, St Louis, MO; 20, 50, 100 µg/ml), Caspofungin (Merck, Whitehouse Station, NJ; 500, 1000 µg/ml) diluted in PBS for 1 h, washed three times in PBS, incubated for 9 h in PP medium supplemented with 10% FBS and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml) 1µ Ci $[^3]$H-glucose at 37°C, 5% CO$_2$, and harvested. $[^3]$H-glucose uptake was detected by liquid scintillation. Controls included Candida treated with PBS alone.

To determine the effects of the drug on Candida in the presence of epithelial cells, oral epithelial cells with varying degrees of constitutive Candida were collected from 10 healthy volunteers. Cells were treated with 1% paraformaldehyde, washed twice in HBSS and once in PBS, incubated for 9 h in PP medium supplemented with 10% FBS and 1% penicillin (100 µg/ml)-streptomycin (100 µg/ml) and 1µ Ci $[^3]$H-glucose at 37°C, 5% CO$_2$, and harvested. $[^3]$H-glucose uptake was detected by liquid scintillation. Controls included Candida treated with PBS alone.

To examine the effects of fixation on oral epithelial cell anti-Candida activity, oral epithelial cells from the same 10 healthy volunteers were treated with 1% paraformaldehyde, washed twice in HBSS and once in PBS, and placed in the growth inhibition assay described above. $[^3]$H-glucose uptake was detected by liquid scintillation.

#### Fixative approach

To assess the effects of fixation on Candida alone, Candida blastoconidia ($10^3$, $10^4$, $10^5$/ml) were treated with 1% paraformaldehyde (Sigma, St Louis, MO) for 15 min, washed three times in PBS, incubated for 9 h in PP medium supplemented with 10% FBS and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml) 1µ Ci $[^3]$H-glucose at 37°C, 5% CO$_2$, and harvested. $[^3]$H-glucose uptake was detected by liquid scintillation. Controls included Candida treated with PBS alone.

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To examine the effects of fixation on oral epithelial cell anti-Candida activity, oral epithelial cells from the same 10 healthy volunteers were treated with 1% paraformaldehyde, washed twice in HBSS and once in PBS, and placed in the growth inhibition assay described above. $[^3]$H-glucose uptake was detected by liquid scintillation.

#### Anti-Candida activity in HIV+ smokers and non-smokers

The cohort consisted of 15 smokers and 15 non-smokers. High colonizers were defined as those with $>200$ colony forming units (c.f.u.) from 50 µl whole saliva cultured on Sabouraud dextrose agar (Becton Dickinson) for 48 h at 34°C. Of the smokers and non-smokers, 50% and 60%, respectively, were defined as high colonizers. None of the subjects had OPC. Clinical samples were fixed with 1% paraformaldehyde for 15 min, washed twice in HBSS and once in PBS, and placed in the growth inhibition assay described above. $[^3]$H-glucose uptake was detected by liquid scintillation.

#### Statistics

Student’s t-test was applied. Significant differences were defined as $P < 0.05$ using a two-tailed test. All statistics were performed using GraphPad Prism (GraphPad Software, San Diego, CA).
Results

Approaches to test a broader range of clinical epithelial cells for antifungal activity

Effects of antifungal drugs. Antifungal drugs represented the first approach to reduce the background effects of *Candida* on epithelial cells. The first set of experiments evaluated the effects of antifungal drugs on *Candida*. Blastococci (10^3, 10^4, 10^5/ml) were treated with different concentrations of fluconazole, amphotericin B, and caspofungin (Phase 1). Results in Table 1 show the results for 10^5 *Candida* blastococci. Amphotericin B and caspofungin, but not fluconazole, were effective in reducing *Candida* growth.

The second set of experiments examined the effects of amphotericin B and caspofungin on *Candida* in the presence of epithelial cells. For this, epithelial cells collected from 10 healthy subjects were allowed to bind *Candida* to create a condition of high colonizer and then treated with various concentrations of antifungal drugs and evaluated for background [3H]-glucose incorporation (Phase 2). Results showed that amphotericin B and caspofungin were both effective in reducing cell-associated *Candida* (Table 1).

The third set of experiments evaluated the effects of antifungal drug treatment on epithelial cell anti-*Candida* activity with varying levels of cell-associated *Candida* (Phase 3). For this, epithelial cells from the same 10 healthy volunteers were treated with various concentrations of amphotericin B and caspofungin, washed, and placed in the growth inhibition assay.

While background levels of [3H]-glucose in cells alone were low (data not shown), anti-*Candida* activity was found to be increased in those cells treated with

Table 1  Effects of antifungal drugs on *Candida* and epithelial cell anti-*Candida* activity

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (µg/ml)</th>
<th>Mean [3H]-glucose uptake (cpm) ± SEM</th>
<th>Mean % Inhibition ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Phase 1) Candida</td>
<td>(Phase 2) Candida + EC</td>
<td>(Phase 3) Epithelial Cell</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0</td>
<td>27065 ± 6025</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>25595 ± 3561</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>28000 ± 9212</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>30125 ± 10625</td>
<td>ND</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0</td>
<td>32297 ± 9959</td>
<td>8068 ± 1292</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>32297 ± 1296</td>
<td>3000 ± 1202</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>870 ± 260</td>
<td>1372 ± 592</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100 ± 20</td>
<td>1113 ± 207</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>0</td>
<td>38000 ± 2372</td>
<td>12310 ± 3620</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2000 ± 360</td>
<td>2667 ± 21</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>230 ± 90</td>
<td>1211 ± 199</td>
</tr>
</tbody>
</table>

* a cpm = counts per minute; b SEM = standard error of the mean; c 10^5 blastococci/ml; d EC = Epithelial Cell;

Mean % Inhibition = \[ 1 - \left( \frac{\text{Mean Effector cell + Candida}}{\text{Mean Effector Cell}} - \text{mean cpm Effector Cell} \right) \times 100; \]

\[ ^{1}\text{ND = Not Done; } \]

\[ ^{2}\text{bold numbers reflect significant differences from controls (} P < 0.05). \]
amphotericin B or caspofungin compared to those untreated. The mean percent inhibition from the 10 healthy volunteers is shown in Table 1. Due to artificially high levels of anti-Candida activity in drug treated cells, it was deemed that antifungal drugs could not be used to test clinical samples.

**Effect of fixation.** Based on the partial resistance of the epithelial cell activity to fixation [10], we examined whether or not fixation could be used in the clinical assay. The first experiments were to examine the effect of fixation on *Candida*. For this, blastoconidia at three concentrations (10^3, 10^4, 10^5/ml) were treated with 1% Paraformaldehyde and tested for growth. Figure 1A shows that [3H]-glucose uptake was significantly less in fixed *Candida* compared to unfixed *Candida*.

The second experiment evaluated the effect of fixation on [3H]-glucose uptake of epithelial cells with varying degrees of cell-associated *Candida* from 10 healthy subjects. Results in Figure 1B show that fixation reduced [3H]-glucose uptake by epithelial cells from any subject with high [3H]-glucose uptake in an unfixed state.

The third and final experiment was to examine the effects of fixation on epithelial cell anti-*Candida* activity. For this, epithelial cells from the same 10 healthy subjects were tested in the growth inhibition assay after fixation. Figure 2A (individual) and B (cumulative) show that fixation had minimal effects on epithelial cell anti-*Candida* activity (P = 0.14). The difference in anti-*Candida* activity between fixed and unfixed cells ranged from 0 to 30% with an average reduction of 13% by fixed cells.

**Epithelial cell anti-Candida activity in HIV+ smokers**

Using the fixation protocol we evaluated the epithelial cell anti-*Candida* activity in HIV+ non-smokers (n = 15) and smokers (n = 15). Background levels averaged 3764 c.p.m. in fixed cells. Results in Figure 3 show that epithelial-cell anti-*Candida* activity was similar in both groups (P = 0.2).

**Discussion**

Smoking is associated with premature OPC in persons who are HIV+ [6–9], and HIV infection has also been shown to influence asymptomatic mucosal *Candida* colonization [13]. An evaluation of epithelial cell anti-*Candida* activity in HIV− and HIV+ persons with and without OPC revealed that epithelial cells from HIV+ OPC+ persons had reduced activity [5]. We reasoned that HIV+ smokers might have increased susceptibility to OPC due to a similar or more profound reduction in epithelial cell activity. There has been a limitation, however, to testing clinical samples. In the study on OPC [5], only a limited number of patients with low levels of *Candida* burden could be tested due to high background levels in the assay control, that after the inclusion in the equation artificially increased the antifungal activity. On the other hand, if patients with higher fungal burdens (worse OPC) could have been

![Fig. 2 Effect of fixation on epithelial cell anti-*Candida* activity. Oral epithelial cells were isolated from unstimulated saliva of healthy subjects (n = 10) and fixed with 1% paraformaldehyde for 15 min followed by coculture with fresh *Candida*. [3H]-glucose incorporation was measured after 9 h and results are expressed as the percent inhibition of *Candida*. (A) Individual subjects; (B) cumulative data. (SEM, standard error of the mean.)](https://academic.oup.com/mmy/article-abstract/43/6/517/1008611/1)

![Fig. 3 Epithelial cell anti-*Candida* activity in HIV+ smokers. Oral epithelial cells were isolated from unstimulated saliva of HIV+smokers (n = 15) and HIV+ non-smokers (n = 15) and fixed with 1% paraformaldehyde for 15 min. Fixed cells were cocultured with *Candida*. [3H]-glucose incorporation was measured after 9 h and results are expressed as the mean percent inhibition of *Candida*. (SEM, standard error of the mean.)](https://academic.oup.com/mmy/article-abstract/43/6/517/1008611/1)
tested the reduced anti-Candida activity may have been more profound. Thus, the purpose of this study was to identify a means to reduce epithelial cell-associated Candida without affecting the epithelial cell anti-Candida activity and then to use that protocol to determine if HIV+ smokers have reduced epithelial cell anti-Candida activity.

The first approach employed antifungal drugs (amphotericin B, caspofungin, fluconazole). Both amphotericin B and caspofungin were effective in inhibiting Candida growth, including Candida artificially placed bound on epithelial cells. This is consistent with their fungicidal activity. However, neither drug could be used for clinical testing as they artificially increased the growth inhibition of Candida by the epithelial cells. This was probably due to residual drug sticking to the cells, common for polyenes and echinocandins, which then inhibited fresh Candida in the assay. Washing the epithelial cells after drug treatment up to six times using both PBS and HBSS did not resolve this problem. In addition, based on a previous study showing resistance of epithelial cell anti-Candida activity to irradiation [10] and the known inactivation of polyenes by UV light, drug-treated epithelial cells were exposed to UV light. This too failed to reduce the artificially increased activity. It was interesting that fluconazole was completely ineffective against Candida. Although fluconazole is very effective in vitro against Candida, the fungistatic property of the drug may have affected the results, especially since the drug was used in a preincubation protocol and washed out prior to functional analysis. It is also possible that the optimal concentration for the amount of Candida was not achieved in the range of concentrations chosen.

The second approach employed fixation based on the partial resistance of epithelial cell anti-Candida activity to paraformaldehyde treatment [10]. Fixation effectively inhibited Candida growth and reduced high background [3H]-glucose uptake in clinical samples with high levels of cell-associated Candida. In contrast to antifungal drugs, however, fixation had only minor effects on the growth inhibitory activity. In fact, while the range of differences in antifungal activity by fixed and unfixed cells was 0–30%, the average change was an insignificant reduction of ~13%. While it is possible that the inhibition of Candida by the fixed epithelial cells was due to residual paraformaldehyde on the cells rather than the cells themselves, this is highly unlikely. Vital staining revealed that paraformaldehyde treatment of Candida kills Candida, whereas paraformaldehyde-treated epithelial cells inhibit Candida growth without killing (data not shown) similar to unfixed epithelial cells [11]. Thus, the fixation protocol was deemed acceptable to use in clinical studies.

In the formal clinical evaluation using the fixation protocol, no significant differences in growth inhibition were found between smokers and non-smokers. Interestingly, inhibitory activity by HIV+ subjects in the present study was slightly lower compared to HIV+ or HIV− subjects we have reported on in past studies [5]. This may be due to the fixation, although activity by HIV− fixed cells used to establish the assay were as high or higher than that from the HIV+ samples. Cell viability was another possibility. Indeed, viability in HIV+ patients (36%) was reduced compared to the average viability for HIV− patients in the present study (68%). However, the same number of viable cells (5 × 10^6 cells/ml) was used in each assay. Nevertheless, the viable cells from the HIV+ persons may not have been as healthy than those from HIV− persons. Reduced viability was also a possible cause in the increased susceptibility of smokers to OPC. However, this possibility was reduced since viability in smokers (34%) and non-smokers (38%) was similar.

Based on these results, it does not appear that epithelial cell antifungal activity plays a role in the development of premature OPC in HIV+ smokers. However, we recognize that the study was somewhat limited as those with OPC were not tested. Thus, results may be different in HIV + smokers with OPC. Smoking has been shown to cause thickening and increased pigmentation of the oral epithelium as well as cause oral lesions, such as leukoplakia and stomatitis, and promote oral epithelial dysplasia and squamous cell carcinoma [14–16]. Thus, we fully expected to see differences in antifungal activity as a manifestation of such effects on the epithelium. But in the presence of normal epithelial cell anti-Candida activity, other factors, such as increased Th2-type cytokines detected in saliva of OPC+ smokers [4] and significantly higher numbers of mutations in the HIV-1 protease gene in OPC+ persons [17], may instead play a more prominent role.

In summary, a protocol has been established to test oral epithelial cell anti-Candida activity in clinical samples with high levels of cell-associated Candida. This effectively opens new avenues to evaluate epithelial cell antifungal activity in a variety of clinical settings. This was emphasized by the ability to evaluate activity in HIV+ smokers and non-smokers with high levels of cell-associated Candida. While no differences were detected in antifungal activity between the groups to explain the development of premature OPC in smokers, the ability to test the specimens provided a significant advancement for the field. In this case, other factors
such as local immunity or HIV are postulated as possible contributing factors. Clearly, more studies are needed to fully elucidate the role of smoking in the development of premature OPC in HIV+ persons.

Acknowledgements
This work was supported by Public Health Service grant DE 12178 from the National Institute of Dental and Craniofacial Research (NIDCR) at the NIH.

References