Engineered Keratinized Oral Mucosa Decreased *C. albicans* Transition Through the Production of Keratins 10, 14, 16, and 19 by Oral Epithelial Cells

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Abstract: The aim of this study was to evaluate the link between *Candida albicans* growth and dimorphism and the production of keratins by oral epithelial cells. Various culture models (monolayer and non-keratinized and keratinized engineered human oral mucosa) were produced and used for this purpose. Cell morphology, tissue structure, and the transition of *C. albicans* were assessed following cell and tissue infections. Keratin production by epithelial cells exposed to *C. albicans* was evaluated by Western blotting. Following contact with *C. albicans*, epithelial cells in the monolayer cultures showed differentiating phenotypes. Compared to the keratinized tissue, the non-keratinized mucosa displayed visible disorganization. The transition of *C. albicans* from blastospore to hyphal form was significantly lower in the keratinized oral mucosa model. This was correlated with the high levels of differentiating (K10) and proliferating (K14, K16, and K19) keratins in the keratinized tissue, suggesting that tissue stratification contributes to controlling *C. albicans* pathogenicity *via* keratin production. Thus, the transition of *C. albicans* from blastospore to hyphal form may be linked to keratin production. This may ultimately have implications in the control of oral candidiasis as well as in denture design to prevent denture stomatitis.

Keywords: Epithelial cells, *Candida albicans*, keratins, yeast transition.

INTRODUCTION

Oral candidiasis is an opportunistic infection that is common among the elderly, particularly those who wear dentures, and remains largely undiagnosed. Denture stomatitis is the term used for the chronic inflammation of denture-bearing mucosa and is characterized by erythema of the palate and alveolar ridges [1].

The etiology of denture stomatitis is multifactorial, involving both local and systemic predisposing factors [2, 3]. Microbial plaque accumulation on the intaglio surface of removable dentures plays a critical role by promoting a switch from commensal to pathogenic oral flora [4]. Microbial communities attach themselves to different surfaces and grow in the form of biofilms located on human tissue and as a variety of biomaterials including acrylic dentures, causing morbidity, such as denture stomatitis. Numerous studies have reported the association between *C. albicans* and denture stomatitis [5, 6]. Oral candidiasis associated with bioprosthetic surfaces is by far the most common fungal infection in denture wearers [1]. The denture-palate interface offers a unique ecological niche for microorganism colonization because of the relatively anaerobic and acidic environment that favors yeast proliferation [4]. Hence, dentures act as reservoirs that harbour *Candida spp*. within a mixed-species bacterial biofilm.

Although the exact pathogenesis is still unclear, a combination of trauma and *Candida* infection has been suggested as etiologic factors [7]. Inflammatory papillary hyperplasia of the palate (IPHP) is related to ill-fitting dentures, poor denture hygiene and wearing dentures 24 h a day. Its frequency in denture-wearing elderly patients varies between 5 and 20%. Old dentures are associated more frequently with IPHP than are newer ones, independent of denture quality [8].

The histopathological appearance of IPHP exhibits papillary projections covered by keratinized stratified squamous epithelium. The underlying connective tissue varies from loose and edematous to densely collagenized and it is usually infiltrated by chronic inflammatory cells consisting mainly of lymphocytes and plasma cells [9].

Various factors are thought to contribute to the virulence of *C. albicans*. These include adhesion to host tissue, the ability to undergo reversible morphogenetic transitions between budding (yeast) and filamentous (hyphae and pseudohyphae) growth forms, the secretion of extracellular hydrolases, and the rapid switching between different phenotypic forms [10, 11]. The contribution of yeast-hyphae morphogenesis to *C. albicans* virulence has been hotly debated [12, 13]. However, it is clear that hyphal development is closely associated with tissue invasion [12, 13].

Host defenses against *Candida* infections are complex, involving both cellular and humoral factors. Cell-mediated immunity (CMI) is thought to be the predominant host defense mechanism against mucosal candidiasis [14]. In addition to their role of anti-*Candida* CMI in the oral mucosa, epithelial cells may be involved in the innate immunity against bacterial and yeast infections *via* an active inflammatory process [15, 16].

Epithelia protect the underlying tissue from environmental influences, such as physical damage and bacterial

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infection, to maintain homeostasis. Differentiation and morphology are used to classify oral epithelial tissue into two broad categories: keratinizing stratified squamous epithelia, such as the palate and the gingival tissue within the oral cavity, and non-keratinizing stratified epithelia, such as the buccal oral mucosa and the oesophagus [17, 18]. A critical aspect of keratinizing stratified epithelia is that the cells undergo a terminal differentiation program that results in the formation of a mechanically resistant surface composed of cornified cells filled with keratin filaments. This keratin envelope protects the underlying epithelial cells [19, 20].

Keratins, the predominant cytoskeletal component in keratinizing stratified epithelia, are divided into two families: Type I, containing acidic proteins (keratins K9-K20) and Type II, containing basic or neutral proteins (keratins K1-K8). Various keratins are distributed throughout the different strata of the epithelium. Within stratified epithelia, the keratin pair K5/K 14 is expressed in the basal proliferative layer. Keratin 19 is expressed in basal cells of non-keratinizing epithelia. K6 and K16 are constitutively expressed in different stratified epithelia, including the tongue, palate, gingiva, and esophagus [19, 20]. Differentiating suprabasal cells of gingival tissue express keratins K1 and K10. In buccal mucosa tissue, keratins K1 and K10 are expressed in a sporadic fashion [21]. The presence of K1 and K10 transcripts may be involved in the rapid response of buccal epithelium to various stimuli that put a heavier functional demand on the tissue [21].

Modified keratin distribution (expression) may be correlated with changes in epithelial cell differentiation and barrier protection function. Dentures, for example, cause profound alterations to the environmental conditions. Palatal keratinization is notably lower in the epithelium under and around partial and complete dentures [22, 23]. In addition, the epithelium thickens due to an increase in its constituent cell layers, while the degree of inflammation appears to be inversely proportional to the degree of keratinization [24, 25]. A reduced keratinization is then likely to promote *C. albicans* adhesion by providing the ideal conditions for it to proliferate and to switch from blastospore to hyphal form, which in turn may enable *C. albicans* to penetrate into the tissue, resulting in deep infection. The aims of this study were thus (A) to investigate the effect of keratins on *C. albicans* transition from blastospore to hyphal form and (B) to determine the effect of *C. albicans* on the production of keratins K10, K14, K16, and K19 which are known to be involved in oral epithelial cell differentiation and proliferation.

EXPERIMENTAL PROCEDURES

Oral Epithelial Cell Isolation and Culture

Small samples of palatal mucosa were collected from gingival graft patients with their informed consent. The biopsies were treated with thermolysin (500 μg/ml) to separate the epithelium from the lamina propria [26]. Epithelial cell suspensions were obtained by treating the tissue with a 0.05% trypsin-0.01 M EDTA solution. Freshly isolated epithelial cells ($9 \times 10^3/cm^2$) were cultured in a 3: 1 mixture of Dulbecco's modified Eagle's medium-Ham's F12 medium (DMEH) (Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with 24.3 μg/ml adenine, 10 μg/ml human epidermal growth factor (Chiron Corp., Emeryville, CA, USA), 0.4 μg/ml hydrocortisone (Calbiochem, La Jolla, CA, USA), 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 2×10^{-9} M 3, 3', 5', triiodo-L-thyronine, 10^{-10} M cholera toxin (Schwarz/Mann, Cleveland, OH, USA), 100 U/ml penicillin, 25 μg/ml gentamicin (Schering, Pointe-Claire, QC, Canada), and 5% fetal calf serum (NCS, fetal clone II, Hyclone, Logan, UT, USA). Following characterization [26], the oral epithelial cells were cultured, resulting in a large number of cells. Passage-two epithelial cells were used for this study. Cells isolated from the same biopsy (donor) were used to produce monolayer cultures, non-keratinized engineered human oral mucosa (nkEHOM), and keratinized engineered human oral mucosa (kEHOM).

Oral Fibroblast Isolation and Culture

Once separated from the epithelium, the lamina propria was placed in a collagenase P solution (0.125 U/ml; Boehringer Mannheim, Laval, QC, Canada) for 45 min at 37°C to extract the fibroblasts. Isolated cells (2×10^6) were seeded in 75 cm^2 flasks (Falcon, Becton Dickinson, Cockeysville, MD, USA) and grown in Dulbecco's modified Eagle's (DME) medium (Invitrogen Life Technologies) containing 5% fetal calf serum (Invitrogen Life Technologies), 100 IU/ml penicillin G, 25 μg/ml streptomycin, and 0.5 μg/ml fungizone (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Following culture, the fibroblasts were used for this study. The cells isolated from the same biopsy (donor) were used to produce nkEHOM and kEHOM.

Preparation of Engineered Human Oral Mucosa

Engineered human oral mucosa (EHOM) were produced as described previously [26]. Briefly, engineered lamina propria was produced by mixing bovine skin collagen (Sigma-Aldrich) with normal human oral fibroblasts (1.5×10^6 cells). The mixture was poured into a Petri dish (35 mm in diameter) containing an anchorage to prevent collagen contraction. Tissue was grown in culture in 5% fetal calf serum supplemented with Dulbecco's modified Eagle medium. Four days later, the engineered lamina propria was seeded with oral epithelial cells ($9 \times 10^4/cm^2$) to obtain the EHOM. Tissue was grown until the epithelial cells reached confluence. To mimic the keratinized palatal mucosa tissue, EHOM were then raised to an air-liquid interface for 5 more days to allow the epithelium to stratify [27, 28]. Following this process, as we have shown previously, the EHOM was a well-organized and stratified tissue in which epithelial cells expressed proliferating keratins such as Ki-67, K14, and K19 and also differentiating keratin K10. In this model, the epithelial cells interacted with fibroblasts in the lamina propria by secreting basement membrane proteins such as laminin and type IV collagen [26].

Candida albicans Growth

A clinical *C. albicans* (*Candida*-associated stomatitis) isolate was used in this study [29]. The yeast was grown on Sabouraud dextrose agar (SAB, Becton-Dickinson, Sparks, MD, USA) at 30°C. For the *C. albicans* suspensions, one colony was used to inoculate 10 ml of phytone-peptone (PP) medium (Becton-Dickinson) supplemented with 0.1% glucose. The culture was grown to the stationary phase for 18 h at 37°C in a shaking water bath. The blastoconidia were col-

lected, washed with PBS, and enumerated using a hemacytometer [30]. The culture was adjusted to 10^7 cells/ml and used to infect the tissue.

Histological Analysis of Tissue Structure and Epithelial Cell Morphology

Immediately following each contact period, the monolayer cultures were washed twice with PBS, observed under an optical microscope (Nikon Canada Inc, Mississoga, Ontario, Canada**)**, and photographed. The non-infected and *C. albicans*-infected EHOMs were used for histological analysis. Biopsies were taken from each tissue, fixed with Bouin's solution, and embedded in paraffin. Thin sections (4 µm) were stained with Masson's trichrome and mounted with a coverslip in 50% glycerol mounting medium, observed under an optical microscope, and photographed. The figures are representative pictures of six different experiments.

Count of *C. albicans* Hyphal Forms and Protein Extraction from Epithelial Cells

C. albicans (10^6/cm^2) was seeded onto an epithelial cell monolayer, nkEHOM and kEHOM (see Fig. (**1**)). The infected monolayer, EHOMs, and uninfected controls were cultured for 2, 4, 8, and 24 hours. Following each contact period, the culture media were collected from the monolayer and the EHOMs. The cells in the monolayer were detached, and the epithelia of the EHOMs were separated from the lamina propria and collected. The cells and epithelia were treated with lysis buffer. The transition of *C. albicans* was assessed using the culture media and the monolayer and EHOM lysates. Four aliquots were taken from each sample, rigorously mixed to separate as much as possible the clumps of fungal and basically hyphal forms, then placed in a hemacytometer and examined under an inverted microscope to count the blastospores and hyphae. We counted, as hyphae, the separate hyphal cells and cell units bounded by septa within a filament. More than two hundred blastospore and hyphal forms were counted from each sample. The percentage of *C. albicans* transition was calculated by dividing the number of hyphal forms by the total number of *C. albicans* cells. The results are expressed as the means ± SD of eight different experiments and are given as the percentage of transition. The cell and epithelial lysates were also used to assess the effect of *C. albicans* on keratin (K10, K14, K16,

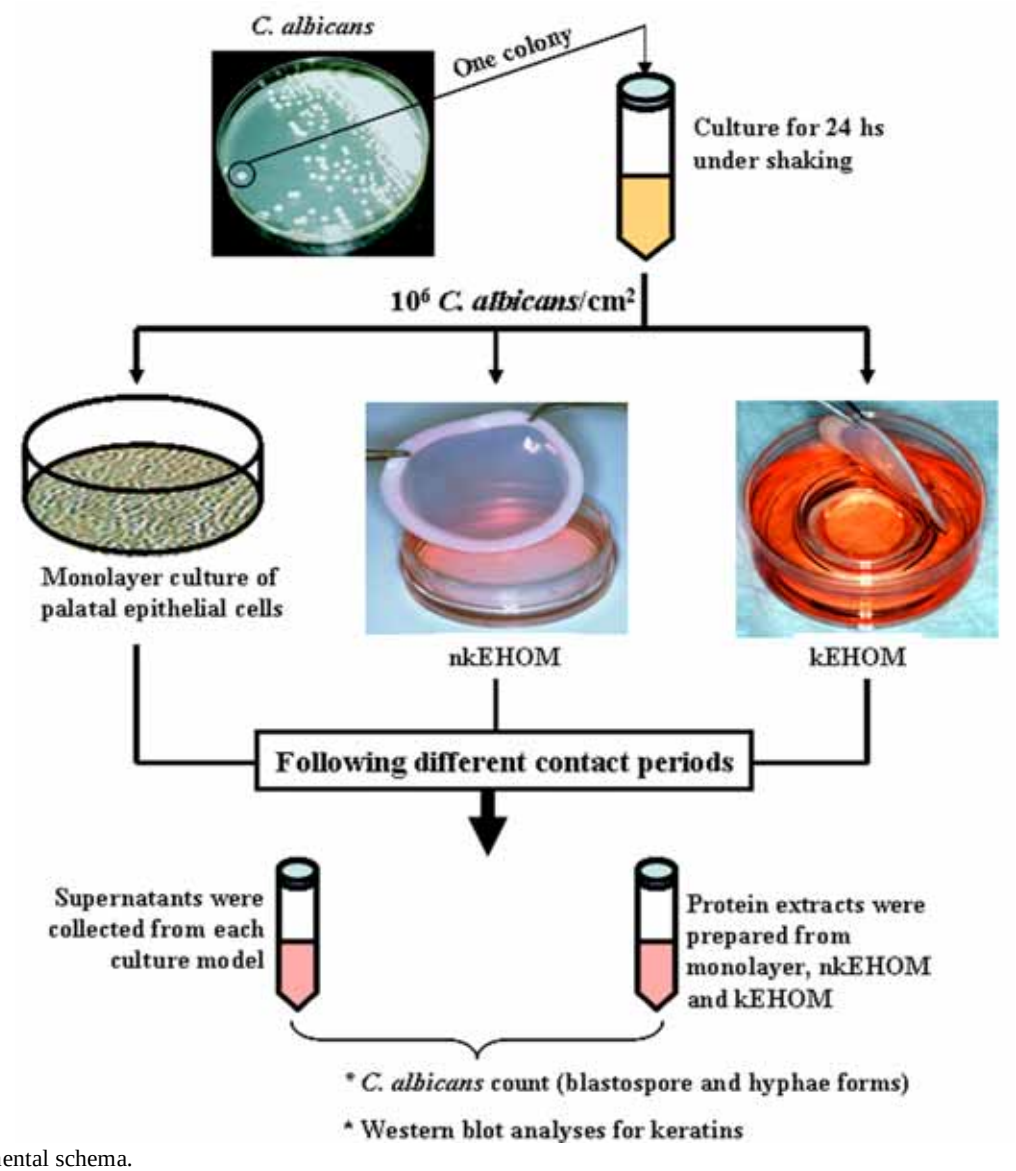

Fig. (1). Experimental schema.

and K19) production by the epithelial cells by means of Western blotting.

Western Blotting and Immunodetection of K10, K14, K1, and K19

Western blotting was performed as described previously [31]. Protein extracts were prepared by solubilizing unstimulated and C. albicans-stimulated oral epithelial tissue in lysis buffer containing 1 mM HEPES, 0.5% Nonidet P-40, 0.5 mM $MgCl_2$, 0.1% β-mercaptoethanol, 0.1% sodium dodecyl sulfate, 1 µg/mL aprotinin, 3 µg/mL pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride. Equal amounts of total protein were loaded onto an SDS-PAGE gel. Following electrophoretic separation, the proteins were transferred to a nitrocellulose membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA) and blocked for 1 h with TBS-T (100 mM Tris, pH 7.5, 0.9% NaCl and 0.05% Tween 20) containing 5% bovine serum albumin. The membrane was incubated overnight at 4ºC with mouse anti-human cytokeratin-10/13 (DE-K13), cytokeratin-14 (C-14), which is an affinity-purified goat polyclonal antibody, mouse anti-human cytokeratin 19 (A53/B/A2) monoclonal antibody (1: 200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse anti-human K16 (1: 200, MediCorp, Montréal, QC, Canada) monoclonal antibody. The membrane was washed (1 x 15 min and 3 x 5 min) in TBS-T and incubated for 30 min at room temperature with the appropriate secondary horse peroxidase-labeled antibody (1: 1000, Amersham Biosciences, Buckinghamshire, UK). After washing with TBS-T (1 x 15 min and 2 x 5 min), the blots were developed using an ECL kit (Amersham Biosciences). The relative intensity of the scanned bands was measured using image analyzing Scion Imaging Software (Scion Corporation, Frederick, MD, USA) based on the surface area and intensity of each band. Test surface area values were compared to those obtained with the control samples.

Statistical Analysis

Experimental values are given as means ± SD. The statistical significance of differences between the control values and the test values was evaluated using a one-way ANOVA. Posterior comparisons were performed using Tukey's method. Normality and variance assumptions were verified using the Shapiro-Wilk test and the Brown and Forsythe test, respectively. All of the assumptions were fulfilled. *P*-values were declared significant at 0.05. The data were analyzed using the SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA).

RESULTS

Tissue Structure and Cell Morphology

Histological analyses (Fig. **2**) revealed that *C. albicans* did cause a side effect for both cells and tissue. Although the 2 and 4 h contact produced no visible damage, and the 8 h contact produced only slight changes, significant disorganization was observed in the monolayer cultures and tissue after 24 h of contact (Fig. **2d-f**). This was characterized by the presence of a high number of differentiated cells (large cells with faint nuclei, large cytoplasm) in the monolayer cultures (Fig. **2d**) and in the upper epithelial layers of the *C. albicans*-infected tissue (Fig. **2e**,**f**). *C. albicans* infection

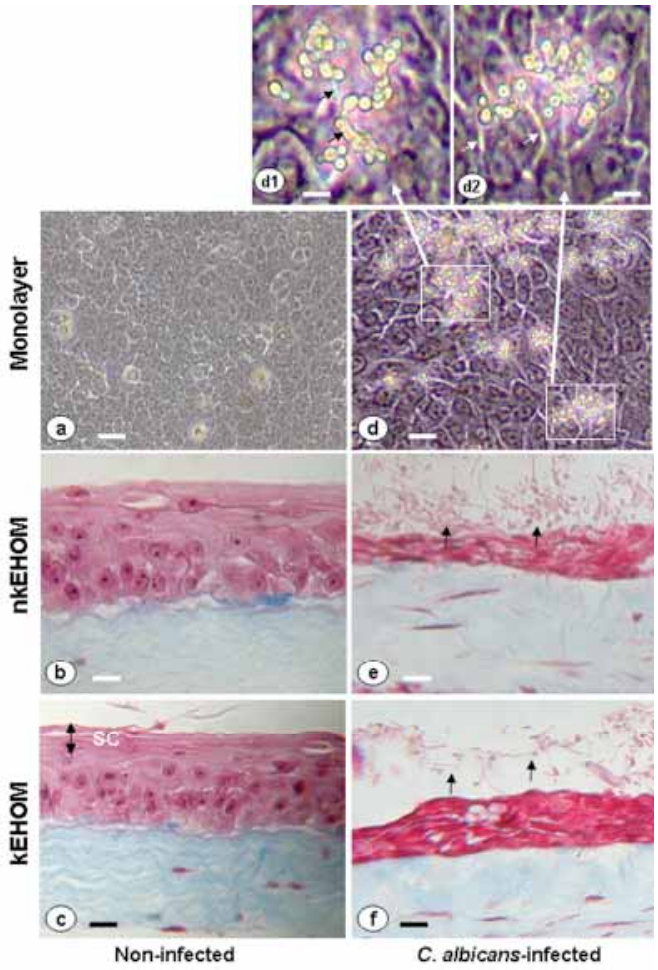

Fig. (2). Cell morphology and histological features of the tissue following infection with *C. albicans*. The cell morphology and tissue structures were assessed 24 h post-infection. Monolayer cultures (**a**,**d**,**d1**,**d2**) were observed under an optical microscope. The tissues (**b**,**c**,**e**,**f**) were stained with Masson's trichrome and observed under an optical microscope. Of interest is the high density of *C. albicans* hyphal forms in the nkEHOM (arrowhead), compared to the kEHOM (arrowhead). Transition forms are also identified in the monolayer cultures (arrowhead in (**d1**) and (**d2**)). Representative photographs are for five different experiments. Scale bars, 50 µm for photos (**a**) to (**f**), and 100 µm for photos (**d1**) and (**d2**). SC = stratum corneum.

also affected the basal layer profile (reduced cell numbers and disorganized structure) basically in the non-keratinized tissue. On the other hand, *C. albicans* adopted a hyphal form beginning at 8 h post-infection. The hyphae were in direct contact with the tissue (data not shown). At 24 h post-infection, the number of *C. albicans* hyphal forms appeared more significant on the nkEHOM than on the kEHOM (Fig. **2**). These data suggest that tissue structure may control *C. albicans* transition.

Effect of Tissue Keratinization on *C. albicans* Transition

As dentures reduce tissue keratinization [22, 23], which may promote *C. albicans* adhesion and transition from blastospore to hyphal form, we used three different models to investigate the effect of tissue stratification (organization of the epithelial tissue on a multilayer structure including the stratum corneoum) on *C. albicans* transition. As shown in

Fig. (**3**), *C. albicans* adhered to the monolayer and to the EHOMs and changed from blastospore to hyphal form. Following a growth of 2 h on the monolayer, the proportion of hyphal forms was over 70%, gradually decreasing to approximately 50% after 24 h. When *C. albicans* was cultured on the nkEHOM, the proportion of hyphal forms remained under 50% after 2 h, with no significant changes at 4, 8, and 24 h. The proportion of hyphal forms was even lower on the kEHOM, remaining under 20% even after 24 h. The degree of transition thus depended on the model. The monolayer promoted *C. albicans* transition, whereas the kEHOM reduced yeast transition. These results are reflected in the proportion of hyphal forms in the culture supernatants (data not shown). Indeed, following counts of the blastospore and hyphal forms of *C. albicans*, the hyphal state reached approximately 60% two hours post-contact with the monolayer, with no significant changes up to 24 h. The proportion was approximately 20% in the culture supernatant of the nkEHOM. In the kEHOM, very few if any hyphal forms were present in the supernatant, which supports the findings shown in Fig. (**2f**). This suggests that compared to the nkEHOM, the kEHOM significantly ($p < 0.05$) blocked the transition of *C. albicans* from blastospore to hyphal form.

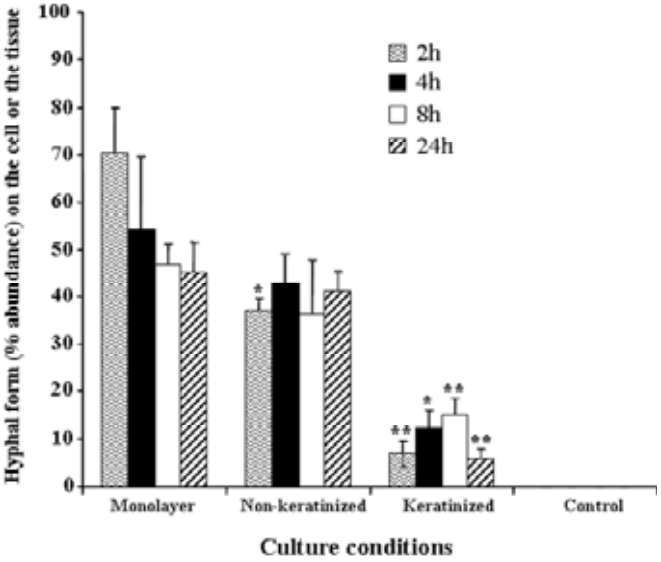

Fig. (3). Proportion of *C. albicans* hyphal forms following adhesion to epithelial cells and EHOMs. *C. albicans* was put in contact with oral epithelial cells in a monolayer culture nkEHOM and in kEHOMs for various periods of time. At each time point, epithelial cell and EHOM samples were collected, washed extensively, and lysed, and the number of blastospore and hyphal forms were counted. The percentage (%) of *C. albicans* transition was calculated by dividing the number of hyphae by the total number of *C. albicans* cells x 100. Results are the means ± SD of eight different experiments. The control refers to the percentage of *C. albicans* transition when cultured alone. The results obtained with the three models were statistically compared to the control ($*p < 0.05$, $**p < 0.01$). The results obtained with the monolayer culture model were also compared with those of the nkEHOM or kEHOMs.

Effect of *C. albicans* on Differentiating Keratin K10 Production

We also investigated the effect of *C. albicans* on K10 production. As shown in Fig. (**4**), *C. albicans* significantly ($p < 0.05$, $p < 0.01$) promoted K10 production by the epithelial cells in the monolayer. Cell differentiation (K10 production) was initiated at the onset of the infection and was maintained throughout the 24 h study period. The increase in K10 was confirmed by the presence of differentiating epithelial cells with a large cytoplasm and a faint nucleus. When the kEHOM and nkEHOM were infected with *C. albicans*, K10 production was significantly reduced ($p \leq 0.05$), compared to that of the monolayer. K10 production in the non-keratinized models was significantly ($p < 0.05$) low, contrasting that produced by the keratinized models. Overall, these results suggest that *C. albicans* promoted cell differentiation in the non-stratified model and that stratification prevented the disruption of the cell differentiation by *C. albicans*.

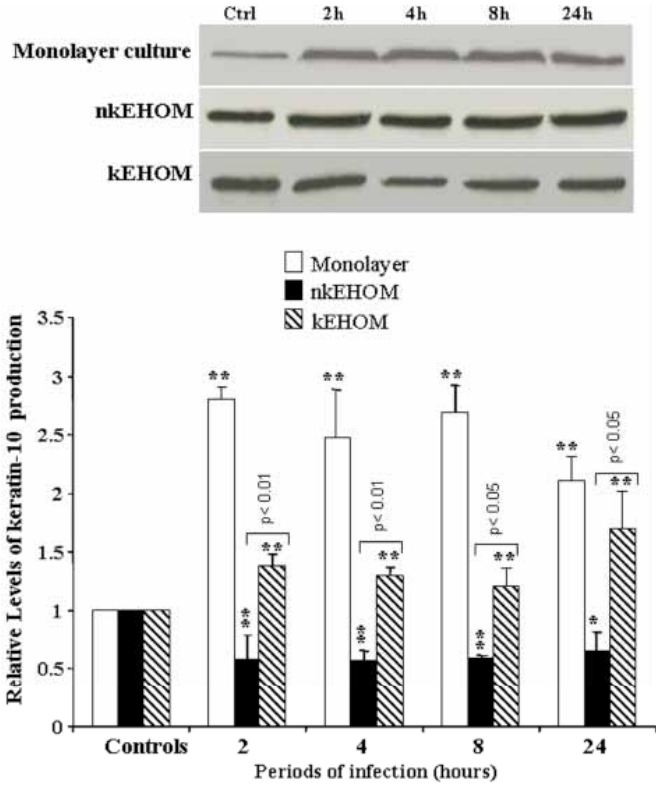

Fig. (4). *C. albicans* increased K10 production by oral epithelial cells in the monolayer culture and kEHOM models. The total protein (100 µg) from lysates of unstimulated (Ctrl) and *C. albicans* (10^6 cell/cm^2)-stimulated oral epithelial cells was analyzed by Western blotting for the presence of K10. Unstimulated epithelial cells and EHOMs were used as controls. Changes in K10 production were determined by band scanning using the Scion Image software program for Windows. Histograms are the means ± SD of four experiments. The results obtained with the three models were statistically compared to the control ($*p < 0.05$, $**p < 0.01$). The results obtained with the monolayer culture model, nkEHOM, or kEHOMs were also compared.

Effect of *C. albicans* on Basal Layer Keratin K-14 Production

The effect of *C. albicans* on basal layer K14 production by oral epithelial cells was investigated using the three models. As shown in Fig. (**5**), the K14 production by the monolayer decreased slightly at 4 h post-infection, remaining stable thereafter up to 24 h. K14 production by the epithelial cells in the nkEHOM decreased significantly ($p < 0.05$) at

2 h post-infection and also remained stable up to 24 h. K14 production by the epithelial cells in the kEHOM also decreased significantly ($p < 0.05$) at 2 h, although this decrease was not significant at 24 h. K14 production significantly ($p < 0.01$) increased in the keratinized model, compared to that in the non-keratinized model. These results suggest that *C. albicans* had a moderate effect on K14 production by the oral epithelial cells, which, however, depended on the model used.

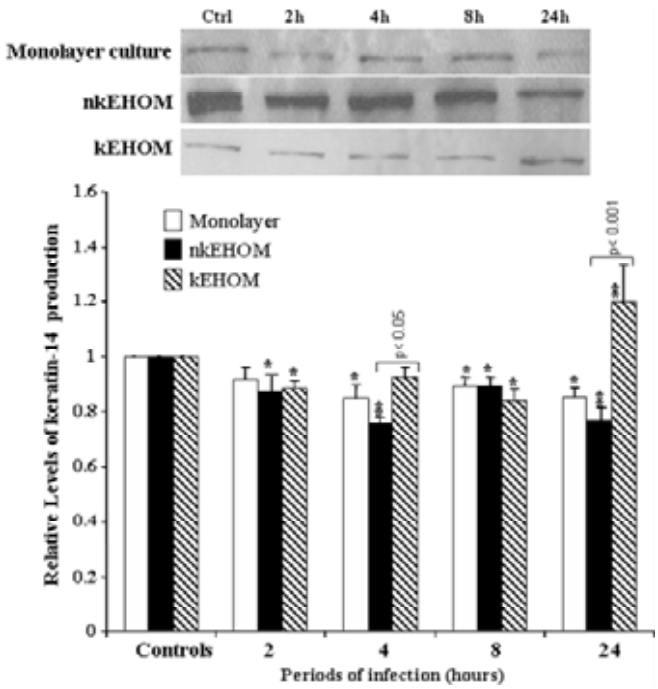

Fig. (5). *C. albicans* increased K14 production by the oral epithelial cells in the monolayer and kEHOM models. The total protein (100 µg) from lysates of unstimulated (Ctrl) and *C. albicans* (10^6 cells/cm^2)-stimulated oral epithelial cells was analyzed by Western blotting for the presence of K14. Unstimulated epithelial cells and EHOMs were used as controls. Changes in K14 production were determined by band scanning using the Scion Image software program for Windows. Histograms are the means ± SD of four experiments. The results obtained with the three models were statistically compared to the control (*$p < 0.05$, **$p < 0.01$). The results obtained with the monolayer culture model, nkEHOM, or kEHOMs were also compared.

Effect of *C. albicans* on Proliferating Keratin K16 Production

The effect of *C. albicans* on K16 production was investigated using the three models. As shown in Fig. (**6**), *C. albicans* promoted K16 production by the monolayer, with a marked increase at later periods. The effect was even more pronounced in the nkEHOM in contrast to the controls. However, K16 production decreased slightly in the kEHOM. The decease in K16 production was significantly ($p < 0.05$) more pronounced when comparing the non-keratinized to the keratinized models. These results suggest that *C. albicans* promoted cell proliferation in the nkEHOM and that keratinization prevented cell proliferation in response to the *C. albicans* infection.

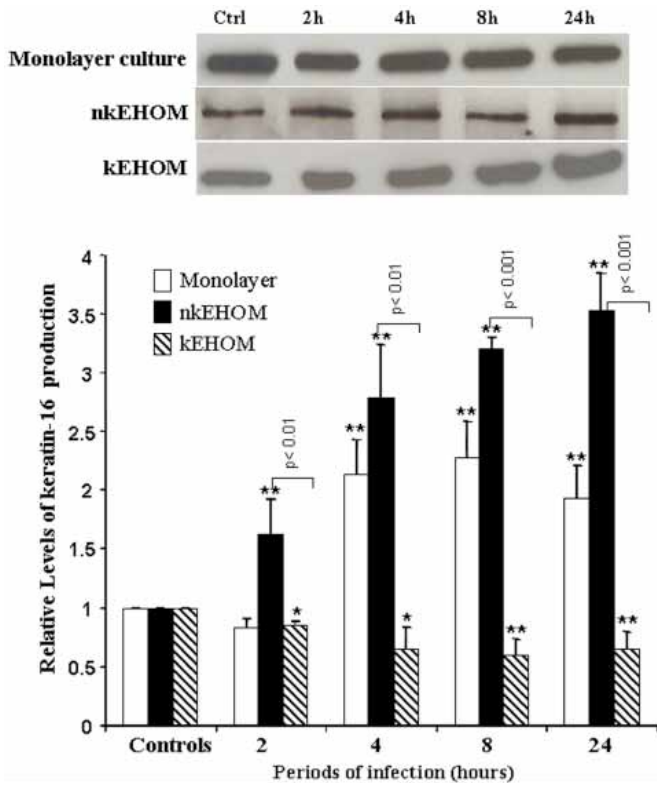

Fig. (6). *C. albicans* increased K16 production by oral epithelial cells in the monolayer and nkEHOM models. The total protein (100 µg) from lysates of unstimulated (Ctrl) and *C. albicans* (10^6 cells/cm^2)-stimulated oral epithelial cells was analyzed by Western blotting for the presence of K16. Non-stimulated epithelial cells and EHOMs were used as controls. Changes in K16 production were determined by band scanning using the Scion Image software program for Windows. Histograms are the means ± SD of four experiments. The results obtained with the three models were statistically compared to the control (*$p < 0.05$, **$p < 0.01$). The results obtained with the monolayer culture model, nkEHOM, or kEHOMs were also compared.

Effect of *C. albicans* on Fetal Keratin K-19 Production

The effect of *C. albicans* on K19 production was investigated using the three models. As shown in Fig. (**7**), K19 production significantly ($p < 0.01$) increased in the kEHOM, beginning 2 h post-contact and continued to augment to 24 h post-contact. *C. albicans* had little effect on K19 production by both the monolayer and the nkEHOM. K19 production in the non-keratinized model differed significantly ($p < 0.05$) from that obtained with the keratinized model. These results suggest that the keratinized layer protected against *C. albicans* and promoted K19 production.

DISCUSSION

The oral mucosal epithelium is a barrier to physical, microbial, and chemical agents that may cause local cell injury [32] and is divided into different layers, including the stratum corneum [33]. The disruption of oral mucosal integrity can lead to infections such as denture-associated candidiasis. The oral epithelium of denture-bearing mucosa becomes less keratinized [23], which may allow *C. albicans* to come into

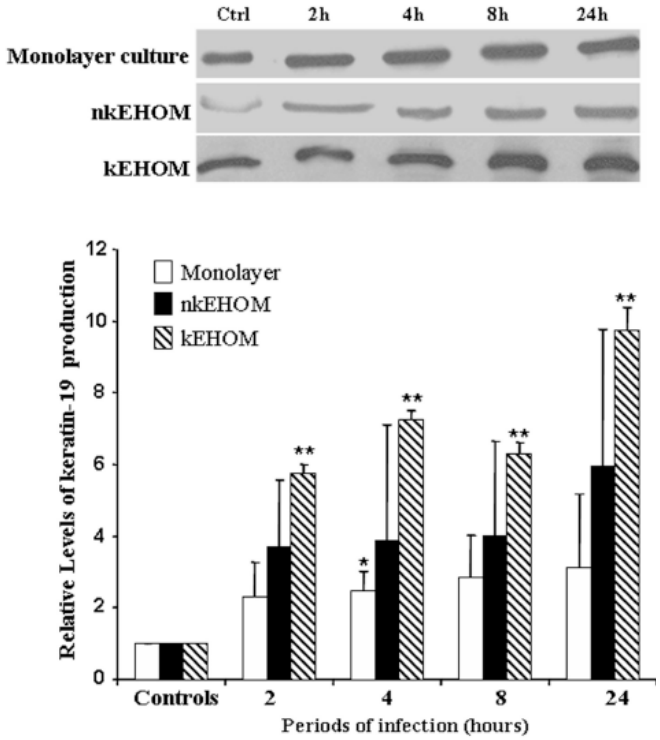

Fig. (7). *C. albicans* increased K19 production by oral epithelial cells in the monolayer and kEHOM models. The total protein (100 μg) from lysates of non-stimulated (Ctrl) and *C. albicans* (10^6 cells/cm^2)-stimulated oral epithelial cells was analyzed by Western blotting for the presence of K19. Non-stimulated epithelial cells and EHOMs were used as controls. Changes in K19 production were determined by band scanning using the Scion Image software program for Windows. Histograms are the means ± SD of four experiments. The results obtained with the three models were statistically compared to the control (*$p < 0.05$, **$p < 0.01$). The results obtained with the monolayer culture model, nkEHOM, or kEHOMs were also compared.

contact with deeper tissue layers, thereby facilitating its transition and subsequent pathogenicity. To test this hypothesis, three different oral mucosa models were used with varying degrees of tissue stratification and keratinization. We demonstrated for the first time that tissue stratification controls the transition of *C. albicans* from blastospore to hyphal form. The proportion of hyphal forms increased, from 10% in the stratified kEHOM to almost 70% in the monolayer. The tissue found under and around dentures is generally less keratinized and denture stomatitis is frequent (10-75%) among denture wearers [22, 23, 34]. This may be mimicked by the engineered non-keratinized models showing highly adherent hyphae, compared to the keratinized models.

In the absence of a denture, the oral palate is cornified [33] tissue. *C. albicans* may adhere to keratinized cells, yet is removed by desquamation which reduces its carriage and controls its pathogenicity [35]. In contrast, the oral tissue under and around dentures is less stratified and less keratinized. The fact that the area of the oral mucosa covered by a total prosthesis is greater than that covered by a partial denture may be an important factor explaining the presence of ulcerative lesions in full denture wearers. Another factor that may promote tissue hyperplasia could be denture pressure [36]. The environment between the denture and the palate may also contribute to the decreased tissue keratinization [37]. When *C. albicans* adheres to this tissue, it is in direct contact with epithelial cells that secrete certain proteins, which in turn may promote *C. albicans* growth and transition. This hypothesis is supported by the results obtained with the three models used in this study. Complete dentures may contribute to reducing palate stratification in the keratinized cell layer [37], thereby promoting *C. albicans* adherence [38], which may partly explain the high incidence of oral candidiasis in denture wearers.

To confirm the role of stratified tissue on *C. albicans* pathogenicity, we investigated the production of various keratins by oral epithelial cells following contact with *C. albicans*. The epithelial cell monolayer produced greater amounts of differentiating K10 than did the kEHOM and nkEHOM following contact with *C. albicans*. This is in agreement with previously reported results indicating that K10 expression varies with cell type [39], developmental stage [40], tissue differentiation [41], and pathological changes [42]. Given the effect of *C. albicans* on K10 production, we investigated the effect on proliferative and basal layer K14 and showed that the yeast did not affect K14 production. These results are in agreement with those reported by Kellokoski *et al.* (1991) who reported that oral mucosa biopsies harvested from women with genital human papillomavirus (HPV) infections do not express or produce K14 [43].

While *C. albicans* had little effect on K14 production, it did increase K16 production in both the monolayer and the nkEHOM model, which displayed a much higher level of organization compared to the monolayer. *C. albicans* reduced K16 production in the kEHOM, which is in agreement with previously reported results [44]. Our study as well as others [43] suggest that epithelial cell proliferation, and thus K16 production, is promoted during the inflammatory stage of *C. albicans* infection.

As our models were composed of heterogeneous epithelial cell populations (cells at various stages of proliferation and differentiation), *C. albicans* may both potentiate the differentiation of epithelial cells in the late proliferative stage as well increase the proliferation rate of proliferating epithelial cells. This may explain the hyperplasia observed in the oral mucosal tissue of patients with oral candidiasis [45]. *C. albicans* also had an effect on fetal keratin K19 production. The greatest stimulation of K19 production was observed in the kEHOM. As K19-producing cells are in the basal layer, therefore not in contact with *C. albicans*, this suggests the yeast had an indirect effect, possibly due to soluble mediators produced either by upper cell layers that were stimulated by *C. albicans*, or by the yeast itself. We know that K19 is a sensitive molecule that can be used as a marker for gingival inflammation [46]. Pritlove-Carson confirmed this by showing that K19 expression is significantly higher in inflamed sulcular epithelium and external oral epithelium than in normal tissue [47]. Our results are thus in agreement with these observations of a link between K19 production and inflammation, such as that observed with denture stomatitis.

In conclusion, this study shows that the transition of *C. albicans* from blastospore to hyphal form depends on the tissue model. The interaction of *C. albicans* with epithelial cells and tissue increases the production of proliferating keratins K14, K16, and K19 and differentiating keratin K10. These keratins down-regulate the yeast's proliferation and transition from blastospore to hyphal form.

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