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Interaction of *Candida albicans* with periodontal ligament fibroblasts limits biofilm formation over elastomer silicone disks

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ABSTRACT

Objective: Candida albicans is the most numerous commensal and potentially pathological yeast in the human oral cavity. The purpose herein is to investigate the ability of *C. albicans* to form a biofilm in the presence of periodontal ligament (PDL) fibroblasts.
Material and methods: Silicone elastomer disks (SE) were transferred to wells containing PDL cells. *C. albicans* suspension was added to each well. The whole mixed culture was then allowed to form a biofilm for 48 h. Biofilms were quantified by tetrazolium-salt-based (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]- 2H-tetrazolium hydroxide (XTT). Furthermore, biofilm was visualized by confocal scanning laser and scanning electron microscopy. Migration of *C. albicans* and its ability to form biofilms in presence of PDL cells was determined by using a transwell system. Last, elutes obtained from co-culturing *C. albicans* and PDL cells were added to SE disks and covered with *C. albicans*. The culture plate was then incubated to allow biofilm formation. Biofilms formed over SE disks were quantified using XTT.
Results: PDL cells significantly limited the biofilm formation at incubation interval of 48 h. PDL cells induced less biofilm compared to mature and thick hyphae in the absence of PDL cells as seen in confocal scanning laser and scanning electron microscopy. The presence of PDL cells limited the migration and formation of biofilm by *C. albicans*. Elutes obtained from co-culturing PDL cells with C. *albicans* for one hour induced significantly less biofilm.
Conclusions: This is the first study to report that PDL cells exhibit antifungal activity. While the exact mechanism of how PDL cells limited biofilm formation is yet unknown, it was clear that competent PDL cells promote resistance to *C. albicans* biofilm formation.

1. Introduction

Candida albicans, being the most frequent commensal and periodically pathogenic yeast in the oral cavity (Conti & Gaffen, 2010). Like many oral microbes, *C. albicans* form and live within a biofilm matrix composed of exopolysaccharides, proteins, and nucleic acids that protect them from the environment and immune system (Siqueira & Sen, 2004; Gomes, Fidel, Fidel, & de Moura Sarquis, 2010). Biofilm formation, leading to immune-evasion and immune-modulation of the host defense, is considered a key virulence factor of *C. albicans*. Formation of a biofilm can provide the *C. albicans* community protection against antimicrobial agents as compared with those in a nomadic state (e.g. planktonic cells). *C. albicans* in biofilm can be 100-fold more resistant to antifungal fluconazole and 20- to 30-fold more resistant to antifungal amphotericin B than planktonic cells (Kumamoto, 2002). Several studies and *in vitro* models have been established to characterize *C. albicans* biofilm formation on common bio-prosthetic materials such as polymethylmethacrylate, which is used in the construction of dentures as well as silicone elastomer, a model material used for indwelling devices including catheters. Previous studies have indicated that biofilm development occurs in three distinct phases. The first phase begins with the adherence of *C. albicans; yeast forms,* to its substrate (≈0 to 11 h). Intermediate developmental phase features attached cells proliferation to form microcolonies and begin to deposit an extracellular matrix (≈12 to 14 h). Finally, the maturation phase (≈24 to 72 h) characterized by forming a dense network of filamentous forms (pseudohyphae and hyphae), and become encased in the exopolymeric matrix (Ramage, Mowat, Jones, Williams, & Lopez-Ribot, 2009; Chandra et al., 2001).

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Periodontitis is a chronic inflammatory disease affecting the integrity of tooth supporting tissues induced by variety of microorganisms, including yeast (Slots, Rams, & Listgarten, 1988). More recently, 30% of patients with chronic periodontitis compared to 15% with healthy subjects had *C. albicans* in periodontal pockets (Canabarro et al., 2013). *C. albicans* can evade the host defense and induce a complex immune response that ultimately determines the clinical outcome of the infection. Polymorphonuclear neutrophils and macrophages are known to be the most important inflammatory cells involved in the defense against *C. albicans* (Ashman & Papadimitriou, 1995). Periodontal ligament fibroblasts (PDL cells) are involved in the formation and maintenance of periodontal fibrous tissue connecting teeth to the alveolar bone (Beertsen, McCulloch, & Sodek, 1997). PDL cells play a crucial role in the early infection as well as resolution stage of infection at root surfaces (Jonsson, Nebel, Bratthall, & Nilsson, 2011). Thus, studying the interaction between *C. albicans* infection and periodontal cells should advance current understanding of the mechanism involved in the etiology of chronic periodontitis related to fungal infections.

The central hypothesis of this investigation was that PDL cells may influence the biofilm formation by *C. albicans*. There were no data published on the interaction of PDL cells with *C. albicans*. It is unknown whether PDL cells have any antifungal activity during *C. albicans* colonization. Using co-culture model system, the current study sought to determine whether PDL cells results in less biofilm formation by *C. albicans* on silicone disks (SE). The flat sheeting of SE disks was chosen to facilitate biofilm formation and produce undistorted images of biofilm formed by microscopic techniques as described previously (Kuhn, Chandra, Mukherjee, & Ghannoum, 2002). It also allows to design future studies aimed to understand the pathogenicity in other *ex/in vivo* oral models.

2. Material and methods

2.1. Cell culture, C. albicans, and growth conditions

Primary human PDL fibroblasts isolated from human periodontal tissues were obtained from ScienCell (Carlsbad, CA) and grown in complete culture medium; Dulbecco's modified Eagle's medium (Life Technologies, CA) supplemented with antibiotics and 10% fetal bovine serum (FBS), at 37 °C in a humidified 5% CO_2 atmosphere. Cells between the 4th and 6th passages were used in the present study.

The *C. albicans* wild strain CA42 (formerly known as SC5314 (Fonzi & Irwin, 1993) was used. A non-filamentous; unable to form biofilms, cph1Δ/efg1Δ double mutant strain CA80 (Lo et al., 1997), was used to validate model presented. Both strains were a courtesy of Dr. Audrey Atkin, University of Nebraska, Lincoln and were grown in yeast extract-peptone-dextrose (YPD) medium (Difco Laboratories, Detroit, MI.) from fresh Sabouraud dextrose agar plates (Difco) and incubated for 24 h at 37 °C in a shaker at 250 rpm. Cells were harvested and washed twice with phosphate buffered saline (PBS). Cells were then re-suspended in 10 mL of PBS, counted following serial dilution, standardized, and used immediately.

2.2. Silicone disks preparation

Silicone elastomer (SE) was purchased (Invotec International Incorporated, Jacksonville, FL). This material was shown to promote *C. albicans* biofilm and often used in indwelling devices (Hawser & Douglas, 1994). The SE is supplied as a flat sheet that facilitates quantifications and imaging the biofilm formed. They were cut with a carpenter's hole punch to produce standardized samples of 1.5 mm thick, and 3 mm diameter. SE disks were washed, autoclaved and incubated in fetal bovine serum (FBS) for 24 h at 37 °C. The pretreatment with FBS is known to promote hyphal formation (Chandra et al., 2001).

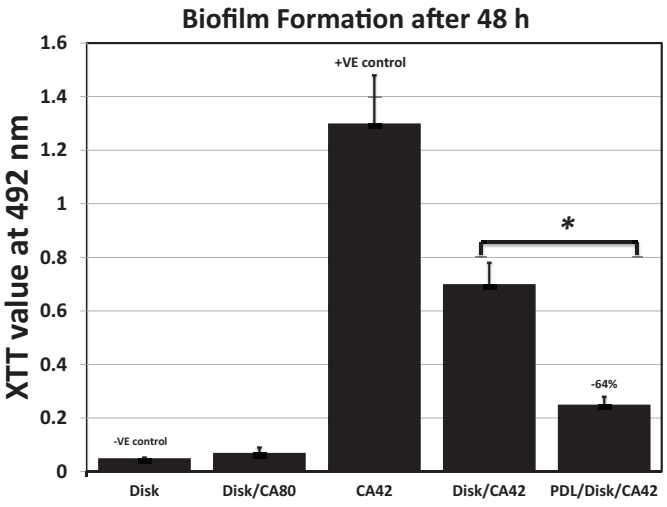

Fig. 1. Metabolic activity (XTT) of *C. albicans* biofilms formed for 48 h on SE disks in the presence (PDL/Disk/CA42) or absence (Disk/CA42) of PDL cells. Disks without inoculation of *C. albicans* was used as negative control. *C. albicans* (CA42) grown onto wells was used as positive control. Disks inoculated with *C. albicans* (CA80) was used to validate model used. All groups had $n = 6$. Metabolic activity is presented as an optical density at 492 nm. *P value of groups compared was significantly different at $\alpha = 0.05$.

2.3. Quantitative measurement of biofilms

PDL cells that reached confluence in culture medium were collected, washed, and counted with a haemocytometer. A total of 10^5 cells/well were plated in complete culture medium onto 24-well plates and grown overnight in 5% CO_2 at 37 °C to allow adherence to the surface. The SE disks were transferred to wells according to following groups ($n = 6$, repeated three times): (Group 1) no PDL cells or *C. albicans*, (Group 2) CA80 strain, (Group 3) CA42 strain, and (Group 4) CA42 strain and PDL cells. Group 1 and 2 served as negative controls. Additional wells pretreated with FBS with no SE disks received CA42 strain served as positive control. *C. albicans* suspension containing 10^5 cells was used. The whole mixed culture, in complete culture medium; was then allowed to form a biofilm at 37 °C in a humidified 5% CO_2 atmosphere. Following maturation for 48 h, SE disks were transferred to new culture plates and biofilms were quantified by tetrazolium-salt-based (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide (XTT) as described previously (Chandra et al., 2001). The reduction of XTT to formazan crystals can only take place in the presence of viable cells and the necessary reductase enzymes.

2.4. Confocal microscopy analysis of biofilms

Biofilm formation by *C. albicans* on SE disks in the presence of PDL cells was investigated using confocal scanning laser microscopy(CSLM) as described previously (Chandra, Mukherjee, & Ghannoum, 2008). New experiments were designed as described above. After co-culturing SE disks with *C. albicans* in the presence of PDL cells, SE disks were stained with FUN-1 (L7009, Molecular Probes, Eugene, Oreg.). Following biofilm formation, disks were removed and transferred to new 24-well plates. Wells containing biofilm disks were submerged in FUN-1 at a final concentration of 10 μM (from 10 mM stock). The plates were then incubated for 30 min at 37 °C. The disks were removed from the wells, placed in 35-mm glass-bottom microwell dishes (MatTek Corp., Ashland,