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Candida albicans induces early apoptosis followed by secondary necrosis in oral epithelial cells

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SUMMARY

The capacity of *Candida albicans* to invade and damage oral epithelial cells is critical for its ability to establish and maintain symptomatic oropharyngeal infection. Although oral epithelial cells are reported dead after 18 h of candidal infection, activation of specific epithelial cell-death pathways in response to *C. albicans* infection has not yet been demonstrated. Considering the key role of oral epithelial cell damage in the pathogenesis of oropharyngeal candidiasis, the aim of this study was to characterize this event during infection. Using an oral epithelial–*C. albicans* co-culture system, we examined the ability of *C. albicans* to induce classic necrotic, pyroptotic and apoptotic cellular alterations in oral epithelial cells such as osmotic lysis, exposure of phosphatidylserine on the epithelial cell plasma membrane and internucleosomal DNA fragmentation. It was found that the ability of *C. albicans* to kill oral epithelial cells depends on its capacity to physically interact with and invade these cells. Caspase-dependent apoptotic pathways were activated early during *C. albicans* infection and contributed to *C. albicans*-induced oral epithelial cell death. Earlier apoptotic events were followed by necrotic death of infected oral epithelial cells. Hence, *C. albicans* stimulates oral epithelial signaling pathways that promote early apoptotic cell death through the activation of cellular caspases, followed by late necrosis.

INTRODUCTION

Oropharyngeal candidiasis is the most common opportunistic fungal infection in bone marrow transplant, hematopoietic stem cell transplant, solid organ transplant and human immunodeficiency virus-infected populations, occurring in as many as 90% of these patients (Feigal *et al.*, 1991; Penk & Pittrow, 1999; Redding *et al.*, 1999; Ohmit *et al.*, 2003; Hermann *et al.*, 2005). In these oral lesions, fungal invasion into the superficial layers of the oral epithelium is accompanied by the destruction of oral epithelial cells (Montes & Wilborn, 1968; Cawson & Rajasingham, 1972; Eversole *et al.*, 1997; Farah *et al.*, 2000). This is clinically manifested by the development of erosions and ulcerations on the oral mucosa generally associated with local discomfort, altered taste sensation and dysphagia, resulting in poor nutrition and further impacting the quality of life of immunocompromised patients (Neville *et al.*, 2009). The capability of *Candida albicans* to invade and damage oral epithelial cells is critical for its ability to establish and maintain symptomatic oropharyngeal infection (Park *et al.*, 2005; Villar *et al.*, 2005; Chiang *et al.*, 2007). Experimental models using cultured human

oral epithelial cell lines have demonstrated that *C. albicans* invades these cells by inducing its own endocytosis (Park *et al.*, 2005; Chiang *et al.*, 2007) and that extensive host cell death occurs following intracellular fungal invasion (Park *et al.*, 2005; Chiang *et al.*, 2007).

Although oral epithelial cells are reported dead after 18 h of candidal infection (Villar *et al.*, 2004, 2005; Park *et al.*, 2005), activation of specific epithelial cell-death pathways in response to *C. albicans* infection has not yet been demonstrated. Nevertheless, it is plausible that specific cell-death pathways are activated in response to *C. albicans* infection. Apoptosis, pyroptosis and necrosis are three mechanisms by which eukaryotic cells die. Necrosis is considered to be a pathological reaction to major perturbations in the cellular environment, leading to cytoplasmic swelling, osmotic lysis and release of intracellular contents into the extracellular environment. Apoptosis is involved in multiple physiological processes including cellular homeostasis and morphogenesis, but also occurs in response to stress conditions such as intracellular infections (Thompson, 1995). Apoptotic caspases cleave cellular substrates, resulting in the characteristic features of apoptosis, which include cytoplasmic and nuclear condensation, disruption of the mitochondrial membrane, DNA fragmentation and maintenance of an intact plasma membrane. The contents of apoptotic cells are packaged into membrane-enclosed apoptotic bodies that are removed by phagocytosis, resulting in an absence of inflammation (Thompson, 1995). Pyroptosis is morphologically and mechanistically distinct from other forms of cell death. Caspase 1-dependence is a defining feature of pyroptosis, which is further characterized by rapid plasma membrane rupture and release of proinflammatory intracellular contents (Fink & Cookson, 2005). Considering the key role of oral epithelial cell damage in the pathogenesis of oropharyngeal candidiasis the aim of this study was to characterize this event during infection. The central hypothesis of this work is that *C. albicans* stimulates specific epithelial signaling pathways that promote oral epithelial cell death. To investigate our hypothesis, we examined the ability of *C. albicans* to induce classic necrotic, pyroptotic and apoptotic cellular alterations in oral epithelial cells such as osmotic lysis, exposure of phosphatidylserine on the epithelial cell plasma membrane and internucleosomal DNA fragmentation.

METHODS

Organisms

C. albicans strain SC5314, originally isolated from a patient with disseminated candidiasis, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The organisms were routinely propagated using yeast–peptone–dextrose (YPD) agar (Difco Laboratories, Detroit, MI) at 25°C.

Cell culture

Oral epithelial cell line SCC15 and primary epithelial cells were used in this study. The oral epithelial cell line SCC15 is derived from a well-differentiated squamous cell carcinoma and was obtained from the ATCC. Cells were maintained in keratinocyte serum-free medium (KSFM) supplemented with 0.1 ng ml^{-1} epidermal growth factor, 50 μg ml^{-1} pituitary bovine extract, 0.4 mM $CaCl_2$ and antibiotics (100 U ml^{-1} penicillin; 100 μg ml^{-1} streptomycin). Primary cultures were established from discarded gingival tissues obtained anonymously from systemically healthy donors undergoing periodontal surgical procedures. Briefly, to establish oral epithelial cell cultures, within 4 h after excision the tissues were washed in antibiotics and gentamicin-supplemented media and incubated overnight in 0.4% dispase at 4°C. Next, the epithelial layer was enzymatically and mechanically separated from the underlying connective tissue and then incubated in 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid. After trypsin neutralization and washing, cell suspensions were seeded in 25-cm^2 flasks at a density of 2.5×10^6 cells in complete EpiLife™ media (Cascade Biologics Inc., Portland, OR), supplemented with 0.05 mg ml^{-1} gentamicin and antibiotics (penicillin/streptomycin). Purity of cultures with respect to epithelial cells was assessed by staining with a fluorescein isothiocyanate (FITC) -conjugated mouse monoclonal anti-human cytokeratin antibody, following the manufacturer's instructions (clone MNF116; Dako, Carpinteria, CA) and subsequent fluorescence-activated cell sorting (FACS) analysis (not shown). Primary epithelial cells were used between passages 3 and 5.

Co-culture of *C. albicans* with epithelial cell monolayers

Stationary-phase yeast cells were prepared by growth for 18 h at room temperature in YPD broth supplemented with 2% (weight/volume) dextrose. The fungal cells were harvested by centrifugation and washed in phosphate-buffered saline (PBS). Subsequently, yeast cells were counted with a hemocytometer, and the final concentration was adjusted in complete KSFM or EpiLife medium before the fungal cells were added to oral epithelial cells.

Epithelial cells were seeded at or near confluence in six-well or 96-well polystyrene plates (4×10^5 or 3×10^4 cells/well, respectively; Corning Incorporated, Corning, NY), and were incubated overnight in complete KSFM or EpiLife^{TM} medium at 37°C in a 5% CO_2 atmosphere until they became confluent. The following day the medium was discarded, and the cells were challenged with suspensions of stationary-phase viable organisms at a 1 : 1 fungal cell to epithelial cell ratio for up to 16 h. Negative controls for these experiments included uninfected cultures and *C. albicans* alone. Positive controls included oral epithelial cells treated with the apoptosis-inducing agent camptothecin for 12 h (12 μM, Calbiochem, Darmstadt, Germany).

Cell-death assay

The ability of *C. albicans* to injure oral epithelial cells was assessed by the CytoTox-96 assay (Promega, Madison, WI), which measures the release of lactate dehydrogenase (LDH) from dying cells. In these experiments, oral epithelial cells were co-cultured with strain SC5314 for up to 16 h, and LDH released from the co-culture system was quantified by spectrophotometry performed according to the manufacturer's instructions. Uninfected cultures (control 1) and *C. albicans* alone (control 2) incubated under identical conditions were included as negative controls. The total amount of LDH released was estimated by treating control uninfected oral epithelial cells with 9% Triton X-100 for 1 h. The LDH released in the presence of *C. albicans* was quantified by using the following formula: [(experimental – control 1 – control 2)/(total – control 1)] ×100. The values were expressed as percentages of the total amount of LDH released.

To gain insight into the molecular mechanisms by which *C. albicans* damages oral epithelial cells, some experiments were carried out in the presence of a membrane filter separating oral epithelial cells from *C. albicans* SC5314. Likewise, to inhibit *C. albicans* internalization by oral epithelial cells, some experiments were performed in the presence of the actin inhibitor cytochalasin D (0.2 μM, Sigma, St Louis, MO).

To investigate the role of caspase activation on cell death inflicted by *C. albicans*, in some experiments, oral epithelial cells were preincubated with the irreversible pan-caspase inhibitor Z-VAD-FMK or caspase-1 inhibitor VAD-OMe-FMK (50 μM, Calbiochem) for 1 h before infection. All experiments were performed at least three times in triplicate.

Annexin V assay

Exposure of phosphatidylserine and loss of plasma membrane integrity on oral epithelial cells were detected by flow cytometry and confocal microscopy using phosphatidylserine-binding anticoagulant annexin V and propidium iodide (PI). Combination of FITC-conjugated annexin V and PI staining enabled us to discriminate between live cells (low level of fluorescence), early apoptotic cells (staining positively only for annexin V), late apoptotic/necrotic cells (dual stained for annexin V and PI), and necrotic cells (staining positively only for PI).

Flow cytometry

At the end of the experimental period, oral epithelial cells were harvested, washed with PBS, resuspended (5×10^5 cells ml^{-1}) and incubated in 100 μl of binding buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 1.8 mM $CaCl_2$; BD Biosciences, San Diego, CA) containing 2 μg FITC-annexin V (BD Biosciences, San Diego, CA) and 0.1 μg PI (BD Biosciences, San Diego, CA) for 15 min at 4°C. Cells were then further diluted in an additional 400 μl 50 μg ml^{-1} PI in HEPES buffer and assayed for fluorescence by flow cytometry. FITC-annexin V and PI-related fluorescences were analysed using a FACSCalibur flow cytometer using FL1-H (525 nm) and FL2-H (575 nm) filters, respectively.

Confocal microscopy

Oral epithelial cells were seeded on chamber slides (8×10^5 cells per slide) (Nalge Nunc International

Corp., Naperville, IL) and were incubated overnight in complete KSFM or EpiLife medium at 37°C in 5% CO_2. The following day the medium was discarded, and the cells were challenged with 1.6×10^6 stationary-phase viable organisms in complete KSFM or EpiLife medium. After 4 and 8 h of incubation at 37°C in 5% CO_2, oral epithelial–*C. albicans* co-cultures were washed twice in PBS. Subsequently, cultures were stained with annexin V-FITC (BD Biosciences, San Diego, CA) diluted 1 : 10 in annexin V binding buffer (BD Biosciences, San Diego, CA) or PI (Sigma, St. Louis, MO) for 15 min at room temperature. Live stained cultures were viewed under a confocal microscope. The percentage of annexin V-positive and PI-positive cells was scored by analysis of 150 oral epithelial cells per time-point.

Controls for these experiments included *C. albicans* alone, uninfected oral epithelial cells, and oral epithelial cells treated with camptothecin (12 μM) for 12 h. Experiments were performed at least three times in triplicate.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay

For assessment of apoptotic internucleosomal genomic DNA fragmentation, oral epithelial cells were subjected to terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. Oral epithelial cells were seeded on glass slides (4×10^5 cells per slide) contained in six-well polystyrene plates (Corning Incorporated, Corning, NY) and were incubated overnight in complete KSFM or EpiLife medium at 37°C in 5% CO_2. The following day the medium was discarded, and the cells were challenged with 8×10^5 stationary-phase viable organisms in complete KSFM or EpiLife medium. After 4 and 8 h of incubation at 37°C in 5% CO_2, oral epithelial–*C. albicans* co-cultures were washed in PBS and fixed in 4% (weight/volume) paraformaldehyde (pH 7.2), at room temperature for 10 min. Subsequently, cultures were washed twice in PBS, treated with 50 μl of cytonin for 30 min and then incubated in 50 μl of TUNEL reaction solution (R&D Systems, Minneapolis, MN) (0.3 μM FITC-dUTP, 3 nM dATP, 25 mM $CoCl_2$ and 25 units of TdT enzyme) for 1 h at 37°C. Cultures were then treated with TdT stop solution for 15 min, washed twice with PBS and incubated in 50 μl Strep-Fluor solution for 20 min. Stained cultures were mounted in fade-retarding mountant and viewed under a confocal microscope. DNA strand breaks were quantified by measuring the incorporation of dUTP to the 3-hydroxyl ends of double-stranded DNA. Controls included *C. albicans* alone, uninfected oral epithelial cells, oral epithelial cells treated with the apoptosis-inducing agent camptothecin (12 μM) for 12 h and co-cultures incubated without deoxynucleotidyl transferase. The percentage of TUNEL-positive cells was scored by analysis of 150 oral epithelial cells per time-point. Experiments were performed at least three times in triplicate.

Osmoprotection assay

Oral epithelial cells were co-cultured with strain SC5314 in the presence or in the absence of 30 mM of various osmoprotectants, including sucrose (effective hydrodynamic diameter 0.9 nm), PEG1450 (effective hydrodynamic diameter 2.4 nm) and PEG3350 (effective hydrodynamic diameter 3.8 nm). All of these chemicals were purchased from Sigma. LDH release was measured at the end of the experimental period and experiments were performed at least three times in triplicate.

Statistical analyses

Statistically significant differences in the frequency of annexin V-positive and PI-positive cells, and dUTP incorporation in infected vs. uninfected oral epithelial cells was determined by a two-tailed *t*-test, assuming equal variances between groups. The statistical significance of the differences in cell death in control, infected cultures and cultures treated with camptothecin was also determined by two-tailed *t*-test, assuming equal variances between groups. Differences were considered statistically significant at a *P*-value <0.05.

RESULTS

Requirement for host cell–fungal cell interactions on induction of oral epithelial cell death

Several lines of evidence indicate that interactions between oral epithelial cells and live and metabolically active *C. albicans* are strongly implicated in the pathogenesis of oropharyngeal candidiasis. First we confirmed that growth rates and metabolic activity of

C. albicans strain SC5314 was not affected under co-culture conditions, as determined by direct cell counts of yeast cells and by the 2,3-bis(2-methoxy-4-nitro-5-sulfopheny)-5-[(phenylamino) carbonyl]-2H-tetrazolium-hydroxide (XTT) assay (not shown). To determine whether *C. albicans* requires physical cell-to-cell contact to kill oral epithelial cells, *C. albicans* SC5314 and oral epithelial cell cultures were separated by transwell inserts and co-cultured in this manner for up to 16 h. Strain SC5314 promoted significant oral epithelial cell damage, inducing the lysis of up to 54.8 ± 7.2% of oral epithelial cells at the end of the co-culture period (Fig. 1A,B). Cytotoxicity was time dependent (Fig. 1B) and increased when the infectious challenge was amplified (not shown). Physical separation of SCC15 cells and *C. albicans* strain SC5314 resulted in complete inhibition of epithelial cell death (Fig. 1A). Similar results were observed in primary oral epithelial cells (not shown).

Internalization of *C. albicans* by oral epithelial cells is inhibited by the presence of the actin inhibitor cytochalasin D. Therefore, to investigate the role of fungal internalization in oral epithelial cell death, we performed experiments in the presence of cytochalasin D. Cytochalasin D alone had no effect on the viability of epithelial and candidal cells, as determined by quantification of LDH release and XTT assay, respectively (not shown). Quantification of the number of endocytosed fungal organisms revealed that 11.2% of the fungal cells were internalized by SCC15 and primary oral epithelial cells after 4 h of co-culture and that longer incubation of *C. albicans* strain SC5314 with epithelial cells did not result in additional internalization (not shown). Treatment of SCC15 cells with cytochalasin D reduced the internalization of strain SC5314 by 87.3 ± 1.1% (not shown) and impaired the ability of this fungal organism to kill these cells (Fig. 1A). Similar results were observed in primary oral epithelial cells (not shown). These results show that the ability of *C. albicans* to kill oral epithelial cells depends on its capacity to physically interact and invade these cells.

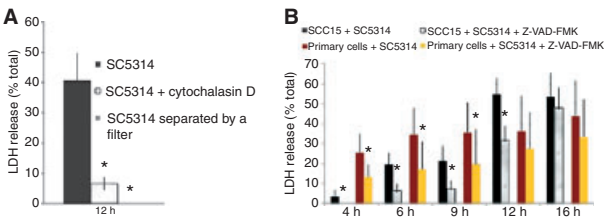

Figure 1 Ability of *Candida albicans* to induce oral epithelial cell death. SCC15 cells were pretreated with (A) cytochalasin D or (B) Z-VAD-FMK and then infected with *C. albicans* SC5314 in the presence (no contact) or absence (contact) of a separating well insert. The ability of SC5314 to injure SCC15 cells was assessed by measuring the release of lactate dehydrogenase (LDH) into the culture supernatant. The maximum amount of LDH released by oral epithelial cells was estimated by treating control uninfected oral epithelial cells with Triton X-100. Results are expressed as percentages of the total amount of LDH released. Mean values were obtained by analysis of at least three independent experiments, each performed in triplicate, and the error bars indicate one standard deviation of the mean. An asterisk indicates that the *P*-value is <0.05 for a comparison with cells infected with SC5314.

Caspase-dependent apoptotic pathways are activated during *C. albicans* infection

To gain further insight into the molecular mechanisms by which *C. albicans* damages oral epithelial cells, we investigated the ability of *C. albicans* to induce oral epithelial cell apoptosis and pyroptosis. Apoptosis is triggered by the activation of multiple caspases. Therefore, we analysed whether Z-VAD-FMK, an irreversible pan-caspase inhibitor, prevented cell death induced by *C. albicans*. Z-VAD-FMK had no noticeable effect on the viability of uninfected oral epithelial cells (not shown). Pretreatment of SCC15 and primary oral epithelial cells with VAD-FMK resulted in a significant reduction of epithelial cell death during the first 9–12 h of infection (Fig. 1B). However, VAD-FMK treatment did not reduce oral epithelial cell death after 12–16 h of infection (Fig. 1B).

The hallmark of pyroptosis is the activation of caspase-1, therefore we performed additional experiments in the presence of caspase-1 inhibitor VAD-OMe-FMK. Pretreatment of oral epithelial cells with VAD-OMe-FMK had no effect on the viability of uninfected oral epithelial cells (not shown). Moreover, VAD-OMe-FMK failed to prevent epithelial cell death during infection (not shown), indicating that pyroptosis is not activated when oral epithelial cells are infected with strain SC5314.

C. albicans induces early oral epithelial cell apoptosis

Apoptosis is characterized by activation of caspase pathways that culminate in specific changes in

cellular morphology, including alterations in the plasma membrane and DNA fragmentation. To determine whether *C. albicans* induces oral epithelial cell apoptosis, we investigated its ability to induce internucleosomal DNA fragmentation and exposure of phosphatidylserine on the outer leaflet of epithelial cell plasma membranes.

To monitor exposure of phosphatidylserine and loss of plasma membrane integrity during infection, oral epithelial cells were simultaneously stained with annexin V and PI, and analysed by flow cytometry and confocal microscopy. Non-infected SCC15 cells showed low levels (<6%) of annexin V and PI staining (Fig. 2A–C). Four hours after infection with strain SC5314, 16.54% of the SCC15 cells showed annexin V staining, 3.73% of the cells showed staining with both annexin V and PI, and 3.42% of the cells were stained with PI only (Fig. 2A–C). Prolonged incubation of SCC15 cells with strain SC5314 resulted in a relative decrease in the number of annexin V-positive cells and an increase in the percentage of PI-positive cells (Fig. 2A,C), suggesting that at later stages of infection *C. albicans* disrupt the integrity of the epithelial cell membranes. Treatment of SCC15 cells with the apoptotic inducer camptothecin led to an increase in the number of cells that stained positive for annexin V plus PI and annexin V only (Fig. 2A,B). Similar results were noticed when SCC15 and primary oral epithelial cells cultures were analysed under confocal microscopy (Fig. 2 D,E).

Apoptosis is also accompanied by the internucleosomal degradation of chromosomal DNA. Incubation of SCC15 and primary oral epithelial cells with *C. albicans* SC5314 resulted in DNA fragmentation (Fig. 3A). The percentage of TUNEL-positive SCC15 cells during infection varied between 10.71 and 14.1%, whereas only 2.88% of the non-infected SCC15 cells stained positively (Fig. 3B). Similar results were observed in primary oral epithelial cells (not shown). Collectively, these results suggest that *C. albicans* induces early apoptosis followed by necrosis of oral epithelial cells.

Lytic activity of *C. albicans*

Several microorganisms form pores on the host cell membranes, thereby leading to osmotic lysis of host cells (Fink & Cookson, 2006). Based on the incorporation of the non-permeant dye PI on infected oral epithelial cells (Fig. 2E), and on the fact that *C. albicans* induced oral epithelial cell death even in the presence of pan-caspase inhibitors (Fig. 1B), we hypothesized that *C. albicans* forms pores on oral epithelial cell membranes, thereby leading to osmotic lysis of these cells.

To estimate the size of the pores produced by *C. albicans* on oral epithelial cell membranes, we infected oral epithelial cells with *C. albicans* SC5314 in the presence of osmoprotectants of different molecular mass. Treatment of oral epithelial cells with osmoprotectants alone had no effect on the viability of these cells (not shown). Cell death was significantly reduced when SCC15 cells were infected with *C. albicans* SC5314 in the presence of PEG1450 or PEG3350, whereas sucrose had no protective effect (Fig. 4). Similar results were observed in primary oral epithelial cells (not shown). Sucrose, PEG1450 and PEG3350 have diameters of 0.9, 2.4, and 3.8 nm, respectively; indicating that the pores produced by *C. albicans* are between 1.0 and 2.4 nm.

DISCUSSION

Host cell death following microbial infection is typically classified as necrotic, apoptotic, or pyroptotic. While necrosis is characterized as accidental cell death as the result of physical damage; apoptosis and pyroptosis are strictly regulated genetic and biochemical suicide programs that are critical during development and tissue homeostasis, and in modulating the pathogenesis of a variety of infectious diseases (Thompson, 1995). Our study showed that *C. albicans* stimulates oral epithelial signaling pathways that promote early apoptotic cell death through the activation of cellular caspases, followed by late necrosis.

Although this is the first time that *C. albicans* has been shown to induce epithelial apoptotic cell death, other experimental models using cultured macrophages and neutrophils have demonstrated that *C. albicans* inactivates anti-apoptotic proteins Bcl-2 and Bcl-xL and triggers activation of cellular caspases (Rotstein *et al.*, 2000; Ibata-Ombetta *et al.*, 2003), resulting in host cell apoptosis. The relatively small number of oral epithelial cells (10–15%) displaying signs of apoptosis during infection correlates well with the low percentage of fungal cells internalized by oral epithelial cells during the early stages of infection

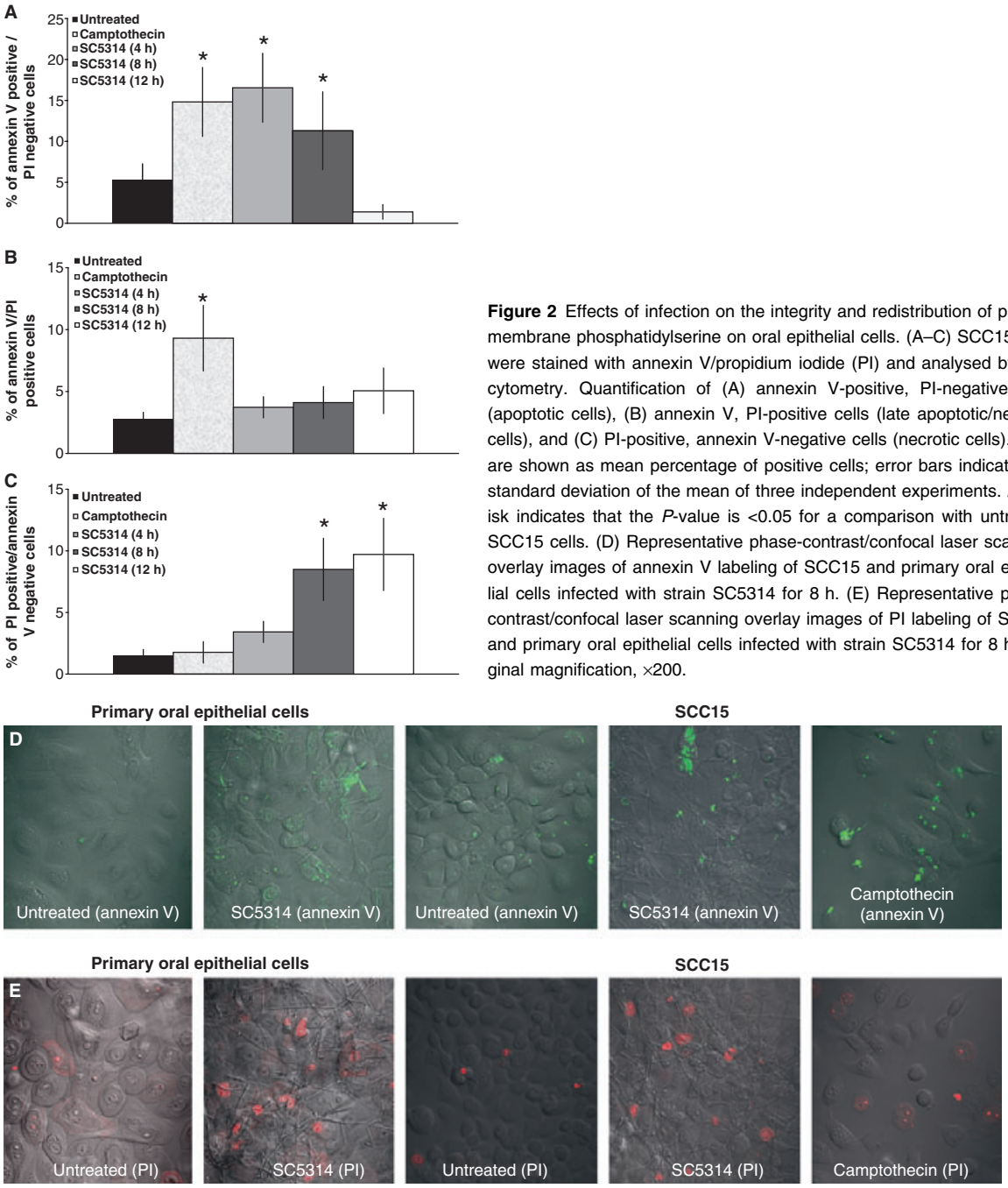

Figure 2 Effects of infection on the integrity and redistribution of plasma membrane phosphatidylserine on oral epithelial cells. (A–C) SCC15 cells were stained with annexin V/propidium iodide (PI) and analysed by flow cytometry. Quantification of (A) annexin V-positive, PI-negative cells (apoptotic cells), (B) annexin V, PI-positive cells (late apoptotic/necrotic cells), and (C) PI-positive, annexin V-negative cells (necrotic cells). Data are shown as mean percentage of positive cells; error bars indicate one standard deviation of the mean of three independent experiments. Asterisk indicates that the *P*-value is <0.05 for a comparison with untreated SCC15 cells. (D) Representative phase-contrast/confocal laser scanning overlay images of annexin V labeling of SCC15 and primary oral epithelial cells infected with strain SC5314 for 8 h. (E) Representative phase-contrast/confocal laser scanning overlay images of PI labeling of SCC15 and primary oral epithelial cells infected with strain SC5314 for 8 h. Original magnification, ×200.

(11%). Accordingly, it is very plausible that *C. albicans* is only able to trigger epithelial apoptotic pathways following host cell internalization. In support of this hypothesis, other microbial pathogens, such as *Staphylococcus aureus* and *Shigella flexneri*, also need to be within the host cell cytoplasm to cause apoptosis (Zychlinsky & Sansonetti, 1997; Menzies & Kourteva, 1998). An alternative explanation is that that the small percentage of epithelial cells undergoing apoptosis during early phases of infection is the result of fungistatic activity of these cells (Steele *et al.*, 2000, 2001; Nomanbhoy *et al.*, 2002; Yano *et al.*, 2005) or their potential to restrict expression of virulence genes in *C. albicans* (Samaranayake *et al.*, 2006). Current studies are under way to investigate these potential mechanisms.

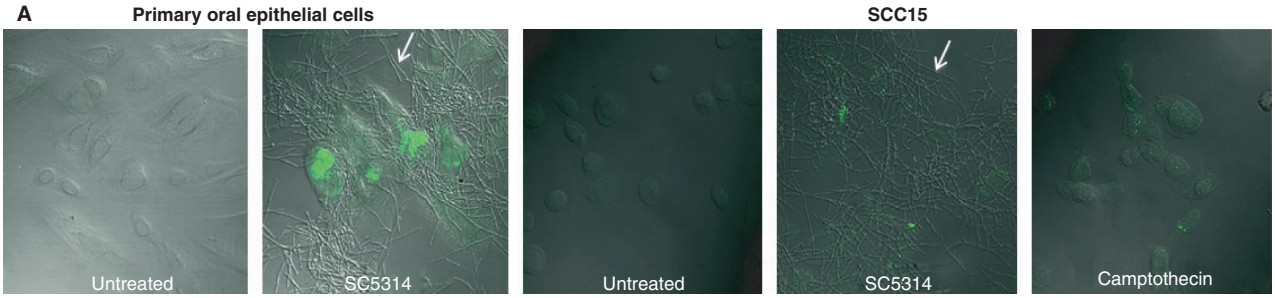

Figure 3 Effects of infection on oral epithelial DNA fragmentation. (A) Representative phase contrast/confocal laser scanning overlay images of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) labeling of SCC15 and primary oral epithelial cells infected with strain SC5314 for 8 h. Strain SC5314 filamentous and formed true hyphae over epithelial cell monolayers (white arrow). (B) Quantification of TUNEL-positive SCC15 cells. Data are shown as mean percentage of TUNEL-positive SCC15 cells ($n = 150$ corresponding to 100%); error bars indicate one standard deviation of the mean of three independent experiments. Asterisk indicates that the *P*-value is <0.05 for a comparison with untreated SCC15 cells. Original magnification, ×200.

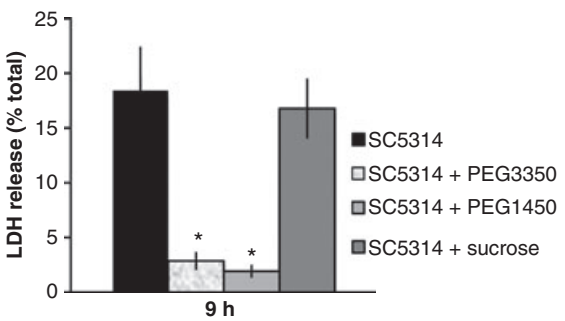

Figure 4 Osmoprotectants inhibit the ability of *Candida albicans* to induce oral epithelial cell death. Lactate dehydrogenase (LDH) release from SCC15 cells infected with *C. albicans* SC5314 in the presence or in the absence of PEG3350, PEG1450 and sucrose. Maximum amount of LDH released by SCC15 cells was estimated by treating uninfected SCC15 cells with Triton X-100. Results are expressed as percentages of the total amount of LDH released. Mean values were obtained by analysis of at least three independent experiments, each performed in triplicate, and the error bars indicate one standard deviation of the mean. Asterisk indicates that the *P* value is <0.05 for a comparison with cells infected with SC5314.

Recent advances in identification of virulence factors responsible for *C. albicans* induction of apoptosis in host cells indicate the participation of multiple factors in this process. *C. albicans* continuously secretes farnesol as a quorum-sensing molecule (Hornby *et al.*, 2001; Ramage *et al.*, 2002; Weber *et al.*, 2008) which, when accumulated above a threshold level, inhibits morphological transition from yeast to hyphae and biofilm formation, without influencing cellular growth rates in *C. albicans* (Hornby *et al.*, 2001; Ramage *et al.*, 2002; Sato *et al.*, 2004). Like other isoprenoid alcohols, farnesol induces apoptosis in a variety of mammalian cells (Burke *et al.*, 2002; Rao *et al.*, 2002; Wright & McMaster, 2002; Wiseman *et al.*, 2007) and could therefore be involved in oral epithelial cell death during *C. albicans* infection. Although physical separation of *C. albicans* and oral epithelial cells completely abolished host cell death, it is possible that only high concentrations of *C. albicans*-secreted farnesol induce oral epithelial apoptosis, and that these are solely achieved when fungal organisms are internalized by host cells. Farnesol is continuously produced *in situ* by aging planktonic cultures and biofilms (Hornby *et al.*, 2001; Ramage *et al.*, 2002). Therefore, alternatively, it is possible that farnesol production did not reach physiologically relevant levels during the experimental period evaluated in this study. Host cell apoptosis can also be induced by phospholipomannan (Ibata-Ombetta *et al.*, 2003), a phylogenetically unique glycolipid composed of -1,2-oligomannosides and phytoceramide (Poulain *et al.*, 2002; Trinel *et al.*, 2002), which is primarily expressed on the cell wall surface of *C. albicans* yeast cells (Jouault *et al.*, 1994, 1998; Poulain *et al.*, 2002; Trinel *et al.*, 2002).

We also provide evidence that a second cytopathic mechanism that differs from apoptosis is induced later during infection of oral epithelial cells

with *C. albicans* SC5314. This cytopathic effect, characterized by cellular swelling, LDH release, uptake of membrane-non-impermeable dye, and disruption of the integrity of the cell membrane, was independent of caspase activation and abrogated by osmoprotectants larger than 1.0 mm. Altogether, these results indicate that *C. albicans* induces oral epithelial cell necrosis during the late stages of infection. Secretion of hydrolytic enzymes, such as aspartyl proteinases (Korting *et al.*, 2003) and phospholipases (Barrett-Bee *et al.*, 1985; Ghannoum, 2000), is considered an important virulence trait of *C. albicans*, and could be involved in the lysis of oral epithelial cells. Accordingly, secreted aspartyl protease contributes to endothelial cell injury by *C. albicans* (Ibrahim *et al.*, 1998), and is important for epithelial cell damage in an *in vitro* model of oral candidiasis (Schaller *et al.*, 1998, 1999). Likewise, phospholipase B has been shown to target and digest host membrane phospholipids, leading to cell lysis (Leidich *et al.*, 1998). Induction of host cell necrosis by microbe secreted proteins has been documented to epithelial cells infected with *Streptococcus pneumoniae* and *Streptococcus suis* (Gottschalk *et al.*, 1995; Hirst *et al.*, 2002). Pneumolysin, a pore-forming toxin encoded by *S. pneumoniae* is required for the induction of cell lysis in *S. pneumoniae*-infected epithelial cells (Hirst *et al.*, 2002). Similarly, intracellular secretion of suilysin, a thiol-activated hemolysin, by *S. suis* is necessary for induction of epithelial cell lysis (Gottschalk *et al.*, 1995). Further experiments, using secreted aspartyl protease and phospholipase mutant strains, are needed to establish the subset of *C. albicans* hydrolytic mediators responsible for late cell damage and their mechanism of action.

Induction of apoptosis during early phases of infection, followed by late necrosis has been previously reported during infection with other pathogenic microorganisms (Gao & Abu Kwaik, 1999; Chen *et al.*, 2006). The two strategies (apoptosis followed by necrosis) used by *C. albicans* to kill oral epithelial cells may be required to ensure that this fungal organism remains inside host cells long enough to germinate into hyphal forms, more readily to secrete soluble hydrolytic enzymes that disrupt the integrity of host cells, leading to necrotic cell death. Alternatively, it is possible that *C. albicans* induces host cell apoptosis, but simultaneously activates host factors with anti-apoptotic properties. Accordingly, infection of oral epithelial cells with *C. albicans* results in activation of members of the nuclear factor-κB (NF-κB) family of transcription factors (Moyes *et al.*, 2008). NF-κB is a ubiquitous transcription factor involved in the expression of multiple early response genes, including the expression of host cell anti-apoptotic factors that block apoptotic cell death (Gillis *et al.*, 2009). It is possible that activation of NF-κB during the early phases of infection with *C. albicans* inhibits epithelial apoptotic pathways. However, as NF-κB activation takes place after a couple of hours of infection, when hyphal organisms are already formed and secrete large amounts of hydrolytic enzymes, it fails to prevent lytic epithelial cell death. Alternatively, it is plausible that the necrotic cells detected during late phases of infection represent late apoptotic cells that have not been eliminated by phagocytosis.

In conclusion, our data support the hypothesis that multiple epithelial cell death mechanisms are activated in response to *C. albicans* infection. *C. albicans* stimulates epithelial signaling pathways that promote earlier apoptotic, followed by secondary necrotic, cell death. Here we present novel information that has a direct impact on our understanding of the pathogenesis of this mucosal infection. It remains to be established whether the fungal organism or the host preferentially benefit from epithelial cell death. Host cell death may facilitate the escape of this fungal organism from epithelial cells after its internalization. On the other hand, the ability of microbial organisms to induce host cell damage may play a role in promoting proinflammatory responses that result in the recruitment and activation of professional immune cells (i.e. neutrophils and macrophages) into the infected mucosa. The exact contribution of host cell damage on each of these events needs to be further evaluated.

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