molecular oral microbiology

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Induction of apoptosis in oral epithelial cells by *Candida albicans*

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SUMMARY

During infection, interactions between *Candida albicans* and oral epithelial cells result in oral epithelial cell death. This is clinically manifested by the development of oral mucosal ulcerations generally associated with discomfort. *In vitro* studies have shown that *C. albicans* induces early apoptotic alterations in oral epithelial cells; however, these studies have also shown that treatment of infected cells with caspase inhibitors does not prevent their death. The reasons for these contradictory results are unknown and it is still not clear if *C. albicans* stimulates oral epithelial signaling pathways that promote apoptotic cell death. Activation of specific death pathways in response to microbial organisms plays an essential role in modulating the pathogenesis of a variety of infectious diseases. The aim of this study was to (i) characterize *C. albicans*-induced apoptotic morphological alterations in oral epithelial cells, and (ii) investigate the activation of apoptotic signaling pathways and expression of apoptotic genes during infection. *Candida albicans* induced early apoptotic changes in over 50% of oral epithelial cells. However, only 15% of those showed mid-late apoptotic alterations. At the molecular level, *C. albicans* caused a loss of the mitochondrial transmembrane potential and translocation of mitochondrial cytochrome *c*. Caspase-3/9 activities increased only during the first hours of infection. Moreover, poly[ADP ribose] polymerase 1 was cleaved into apoptotic and necrotic-like fragments. Finally, five anti-apoptotic genes were significantly upregulated and two pro-apoptotic genes were downregulated during infection. Altogether, these findings indicate that epithelial apoptotic pathways are activated in response to *C. albicans*, but fail to progress and promote apoptotic cell death.

INTRODUCTION

Candida albicans is a commensal fungal organism commonly found as part of the normal microflora on the skin and in mucous membranes of the oral cavity, vagina and rectum (Soll, 2002). Although *C. albicans* is a commensal organism, it is capable of producing opportunistic infections when the balance between the host and this fungal organism is broken. As a result of complex interactions between host cells and fungus, various clinical manifestations of infection with *C. albicans* have been reported, ranging from mild superficial mucous membrane involvements to life-threatening disseminated and deep-seated organ infections with multisystem organ failure (Lynch, 1994).

Among the diseases associated with *C. albicans* is oropharyngeal candidiasis, also referred to as 'oral thrush'. Oropharyngeal candidiasis is the most common opportunistic fungal infection in immunosuppressed patients (i.e. bone, stem cell and solid organ transplant patients), HIV-infected populations, patients on prolonged use of broad-spectrum antibiotics and in infants, ostensibly because of their immature immune system (Barrett, 1984; Hoppe, 1997; Darouiche, 1998). During oropharyngeal candidiasis, interactions between *C. albicans* and oral epithelial cells result in massive oral epithelial cell damage (Villar *et al.*, 2005). This aberrant process of cell death in response to candidal infection leads to tissue dysfunction (Jones & Gores, 1997). This is clinically manifested by the development of erosion and ulceration in the oral mucosa, generally associated with local discomfort and dysphagia, resulting in poor nutrition and further impacting the quality of life of patients.

Apoptosis is a physiological component of the development and hemostasis of multicellular organisms, but also occurs in response to stress conditions such as infections (Norbury & Hickson, 2001). This specific type of cell death is initiated by diverse signals that originate either extracellularly or intracellularly (Popov *et al.*, 2002; Yu *et al.*, 2006). Extrinsic apoptotic signaling pathways initiate when extracellular signal ligands bind to cell death receptors located on the cell surface (Locksley *et al.*, 2001). Apoptosis can also be induced by the activation of the intrinsic apoptotic pathway. The intrinsic pathway initiates through signals that promote disruption of the mitochondrial integrity (Saelens *et al.*, 2004). This involves the participation of the Bcl-2 family of proteins (Mihara *et al.*, 2003), which directly control the mitochondrial membrane permeability and the release of pro-apoptotic factors from the mitochondrial intermembrane space into the cytosol (Dejean *et al.*, 2006; Garrido *et al.*, 2006). Translocation of cytochrome *c* into the cytosol catalyses the formation of the apoptosome complex. This leads to activation of the intrinsic activator caspase-9, which in turn cleaves and activates executioner caspase-3 (Chinnaiyan, 1999; Hill *et al.*, 2004; Lavrik *et al.*, 2005). Activated caspase-3 promotes proteolytic cleavage of multiple downstream target proteins, many of which have significant functions in the propagation of apoptosis (Cohen, 1997; Jänicke *et al.*, 1998; Slee *et al.*, 2001).

Studies *in vitro* have shown that *C. albicans* is able to inflict extensive oral epithelial cell death. Activation of specific death pathways in response to microbial organisms plays an essential role in modulating the pathogenesis of a variety of infectious diseases and the host immune response. Accordingly, microorganisms have developed mechanisms to manipulate cell death pathways in their favour. Some microbial organisms, such as *Staphylococcus aureus*, *Toxoplasma gondii* and *Leishmania amazonensis*, induce necrotic lysis of the infected cell to ensure their release from the host cell cytoplasm into the extracellular environment (Noronha *et al.*, 1996; Black & Boothroyd, 2000; Kobayashi *et al.*, 2010). In contrast, others, like *Francisella tularensis* and influenza A virus, induce low immunogenic types of cell death, and so avoid development of a robust host immune response (Brydon *et al.*, 2003; Lai & Sjostedt, 2003). In view of the possible repercussions of cell death pathways in the pathogenesis of microbial diseases, it is important to define the mechanisms by which *C. albicans* induces oral epithelial cell death. Previous studies suggested that *C. albicans* induces early apoptotic alterations in oral epithelial cells; however, these studies have shown that treatment of infected oral epithelial cells with caspase inhibitors does not prevent their death (Villar & Zhao, 2010). The reason for these contradictory results is still unknown. Recent studies have demonstrated that apoptotic cell death can occur independently of caspase activation (Ojcius *et al.*, 1998; Perfettini *et al.*, 2002; Chipuk & Green, 2005; Kroemer & Martin, 2005), suggesting that *C. albicans* may induce epithelial apoptosis through alternative pathways. Hence, the aim of this study is to (i) characterize *C. albicans*-induced apoptotic morphological alterations in oral epithelial cells using assays that detect early and late apoptotic alterations and (ii) investigate the activation of apoptotic signaling pathways and expression of anti-apoptotic and pro-apoptotic genes in *C. albicans*-infected oral epithelial cells.

METHODS

Organisms

Candida albicans strain SC5314, originally isolated from a patient with disseminated candidiasis, was

obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The organisms were routinely propagated using yeast–peptone–dextrose agar (Difco Laboratories, Detroit, MI) at 25°C.

Cell culture

Human oral keratinocyte cell line OKF6/TERT2 was used in this study. The OKF6/TERT2 are normal human oral mucosal epithelial cells that have been immortalized by forced expression of telomerase 2 via retroviral transduction and deletion of p16INK4a regulatory protein (Dickson *et al.*, 2000). Cells were maintained in keratinocyte serum-free medium 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).

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

Stationary-phase yeast cells were prepared by growth for 18 h at room temperature in yeast–peptone–dextrose broth 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 keratinocyte serum-free medium before the fungal cells were added to oral epithelial cells. Epithelial cells were seeded at or near confluence in 96-well plates, six-well polystyrene plates, chamber slides or culture dishes (3×10^4, 4×10^5, 8×10^5 or 2×10^6 cells per well, respectively), and were incubated overnight in complete keratinocyte serum-free 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 or 1 : 5 epithelial cell to fungal cell ratio (multiplicities of infection of 1 and 5, respectively) for up to 16 h. Negative controls for these experiments included uninfected epithelial 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).

Annexin V

Oral epithelial cell– *C. albicans* co-cultures were washed in PBS and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Biosciences, San Diego, CA) diluted 1 : 10 in annexin V binding buffer (BD Biosciences) for 15 min at room temperature. Live stained cultures were viewed using a laser scanning confocal microscope (Zeiss Axiovert 200 microscope; Carl Zeiss, Göttingen, Germany). The FITC-annexin-related fluorescence was recorded using excitation and emission wavelengths of 494 and 525 nm, respectively. The percentage of annexin V-positive cells was scored by analysis of 150 oral epithelial cells per time-point. Experiments were performed three times in triplicate.

Terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL)

Oral epithelial cell– *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 treated with 50 μl cytonin for 30 min and then incubated in 50 μl TUNEL reaction solution (R&D Systems, Minneapolis, MN) (0.3 μM FITC-dUTP, 3 nM dATP, 25 mM $CaCl_2$ and 25 units TdT enzyme) for 1 h at 37°C. Cultures were then treated with TdT stop solution for 15 min, and incubated in 50 μl Strep-Fluor solution for 20 min. The TUNEL staining was viewed under a confocal laser microscope using a 488-nm argon laser and a 520- to 560-nm bandpass filter. The percentage of TUNEL-positive cells was scored by analysis of 150 oral epithelial cells per time-point. Experiments were performed three times in triplicate.

Cytoplasmic histone-associated DNA fragments

Quantification of histone-associated DNA fragments was performed using a commercially available photometric enzyme immunoassay detection system (Cell Death Detection ELISAplus; Roche Molecular Biochemical, Indianapolis, IN), according to the manufacturer's instructions. Briefly, oral epithelial cell– *C. albicans* co-cultures were washed with PBS and solubilized in lysis buffer. Then, 50 μg cell lysates was added in duplicate to wells of a microplate containing 80 μl Immunoreagent (histone-biotin-

conjugated antibody and DNA-peroxidase-conjugated antibody) and incubated for 2 h at room temperature. Subsequently the color was developed by adding 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) substrate. Absorbance was measured at 405 nm. The enrichment factor (indicative of apoptosis) was calculated by dividing the absorbance of the test samples by the absorbance of the control uninfected oral epithelial cell samples. Experiments were performed three times in triplicate.

Mitochondrial membrane potential ($\Delta\Psi$m)

Mitochondrial membrane potential was measured using the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (MitoProbe™ JC-1 assay; Molecular Probes, Eugene, OR). JC-1 is rapidly taken up by healthy mitochondria with a polarized $\Delta\Psi$, leading to an increase in the concentration gradient of JC-1 and formation of red fluorescent JC-1 aggregates. Conversely, JC-1 does not accumulate in mitochondria with depolarized $\Delta\Psi$ and remains dispersed in the cytoplasm as green fluorescent monomers. Consequently, mitochondrial depolarization can be indicated by a decrease in the red-to-green fluorescence intensity ratio. Briefly, oral epithelial cells were detached from plates with trypsin-ethylenediaminetetraacetic acid (EDTA) and resuspended in PBS. Cell suspensions were then centrifuged (10 min, 450 ***g***), resuspended in PBS at 1×10^6 cells ml^{-1} and incubated with 2 μM JC-1 for 20 min at 37°C in the dark. Subsequently, epithelial cells were washed, and resuspended in 0.5 ml PBS. Flow cytometric analysis was accomplished on a flow cytometer equipped with a 488-nm argon laser using emission filters appropriate for AlexaFluor 488 dye and R-phycoerythrin. A total of 5000 events were acquired from each sample. Results are expressed as the ratio of red-to-green fluorescence. Experiments were performed five times in triplicate.

Cytochrome *c* release

To analyse whether cytochrome *c* was released from mitochondria to the cytosol during infection of oral epithelial cells by *C. albicans*, cytosolic and mitochondria-enriched fractions were prepared using a Mitochondrial Isolation Kit according to the manufacturer's instructions (Pierce, Rockford, IL). In brief, oral epithelial cells were harvested using trypsin-EDTA, washed in PBS, and resuspended in 800 μl reagent A containing a protease inhibitor cocktail (Roche). Then, 10 μl reagent buffer B was added to the cell suspension. After 5 min of incubation on ice, the suspension was mixed with 800 μl reagent C. Cells were then centrifuged at 700 ***g*** for 10 min at 4°C. The clarified supernatant was transferred to a new tube and centrifuged at 3000 ***g*** for 15 min at 4°C for a more purified preparation of mitochondria. The resulting pellets were resuspended in 500 μl reagent C, and the suspension was centrifuged at 12,000 ***g*** for 5 min at 4°C. The resultant supernatant containing the cytosolic fraction was collected and transferred to new tubes. Mitochondrial pellet fractions were then resuspended in 1× Calibrator Diluent RD5P (cytochrome *c* Immunoassay Kit; R&D Systems, Inc.). Both cytosolic and mitochondrial pellet fractions were stored at −80°C until used.

Cytochrome *c* quantification was performed using the enzyme-linked immunosorbent assay-based Quantikine Human Cytochrome C Immunoassay Kit (DCTC0 from R&D Systems Inc.), according to the manufacturer's instructions. Briefly, 100 μl cytosolic or mitochondrial cell fractions were added in duplicate to wells of a microplate coated with monoclonal antibody against cytochrome *c*, and incubated for 2 h at room temperature. Subsequently the wells were washed four times using 400 μl wash buffer, and 200 μl cytochrome *c* conjugate was added and incubated for 2 h at room temperature. Next, the wells were washed four times using 400 μl wash buffer, and the reaction color was developed by the addition of 200 μl substrate solution for 30 min at room temperature, protected from light. The reaction was stopped with 50 μl stop solution. Absorbance was measured at 450 nm and the relative amount of cytochrome *c* in the mitochondria compared with the cytoplasm was calculated from the ratio of absorbances of mitochondrial to cytoplasmic samples. Experiments were performed three times in triplicate.

Caspase activity

Activities of caspases 3, 8 and 9 were measured using APOPCYTO Caspase-fluorometric assay kits (MBL, Nagoya, Japan). Oral epithelial cell– *C. albicans* co-cultures were washed with PBS, solubilized

in lysis buffer and centrifuged at 10,000 ***g*** for 5 min at 4°C. Next, 50-μl aliquots of cell lysates were placed in 96-well microplates and incubated with 50 μl of 2× reaction buffer [10 mM dithiothreitol (DTT)] and 5 μl caspase substrates [*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC)-caspase-3 substrate, *N*-acetyl-Ile-Glu-Thr-Asp-AMC (IETD-AMC)-caspase-8 substrate or *N*-acetyl-Leu-Glu-His-Asp-AMC (LEHD-AMC)-caspase-9 substrate] for 1 h at 37°C. Hydrolysis of caspase-specific substrates was quantified by spectrofluorometry with 380-nm excitation and 460-nm emission filters. Experiments were performed four times in triplicate.

Poly[ADP-ribose] polymerase-1 (PARP-1) degradation

Oral epithelial cells were lysed in a buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 1% nonidet P-40, and a protease inhibitor cocktail (Roche). Equal amounts of soluble proteins (4–25 μg) were then subjected to sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis and transferred to hybond-P polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked overnight at 4°C with Tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween-20 (block buffer). The following day the membranes were probed with anti-PARP-1 mouse monoclonal antibody (1 : 200; clone 194C1439; BD Biosciences) or anti-β-actin monoclonal antibody (1 : 200 dilution; clone AC-15; Sigma, St Louis, MO) for 2 h. The membranes were then washed three times (20 min each time) with Tris-buffered saline containing 0.1% Tween-20 (wash solution) and probed with horseradish peroxidase-labeled goat anti-mouse antibody (1 : 20,000; Jackson Immunoresearch Laboratories, West Grove, PA) for 60 min. Signals were developed by addition of an enhanced chemiluminescence-type solution (Pierce) and exposure of membranes to X-ray film (Pierce). Signals in the film were scanned and stored as .tif images, and their intensities were determined by densitometry analysis using the Chemiimager 5500 software (Alpha Innotech Corporation, San Leandro, CA). The β-actin was used as a loading control in all experiments. Experiments were performed three times.

Real-time polymerase chain reaction (PCR) gene array expression profiling

Human Apoptosis RT^2 Profiler PCR Arrays (human apoptosis PCR array; SuperArray Biosciences, Frederick, MD) were used to assess the effect of *C. albicans* infection on the expression of key genes related to apoptosis in oral epithelial cell cultures. Each array consisted of 84 genes in duplicate, as well as housekeeping genes (*RPLP1*, *HPRT1*, *RPl13A*, *LDHA* and *ACTB*). The gene arrays were used according to the manufacturer's instructions. Briefly, total RNAs were isolated and treated with DNase using an RT^2 qPCR-Grade RNA Isolation Kit according to the manufacturer's protocol. RNA concentrations were then determined and 2 μg RNA was reverse transcribed into cDNA using the SuperArray's RT^2 PCR array first-strand kit (Super Array Bioscience), which includes a genomic DNA elimination step. The resulting cDNA was used for quantitative PCR using SuperArray RT^2 qPCR master mix. Thermal cycling and real-time detection were performed with thermocycler parameters set at step 1: 95°C for 10 min, step 2: 40 cycles of 95°C for 15 s followed by 40 cycles of 60°C for 60 s. Melt-curve analysis was completed after each PCR. Analysis was conducted using templates provided by SuperArray Biosciences. Threshold cycle (C_t) values obtained from a set threshold were analysed using the C_t Method in an Excel-based PCR Array Data Analysis Template. C_t values were normalized using the average of at least three of five housekeeping genes (*RPLP1*, *HPRT1*, *RPl13A*, *LDHA* and *ACTB*) to get a ΔC_t value and fold-changes were calculated using the equation $(2^{-\Delta C_t}\text{test}) \times (2^{-\Delta C_t}\text{control})^{-1}$. Student's *t*-test was used to compare gene transcription levels in experimental samples with those in uninfected control samples. Genes were considered differentially expressed when they were at least two fold upregulated or downregulated and $P < 0.05$. Experiments were performed twice in triplicate.

Statistical analyses

Results were expressed as means ± standard deviations of at least three independent experiments. Data between uninfected and infected oral epithelial cells were compared by a two-tailed Student's *t*-test,

assuming equal variances. The level of significance chosen in all the statistical tests was set at $P < 0.05$.

RESULTS

Apoptotic cellular alterations

Phosphatidylserine exposure

Loss of phospholipid asymmetry with phosphatidylserine exposure represents one of the earliest measurable cellular change events in apoptosis: 30.29 ± 16.24% of the camptothecin treated-cells exhibited positive staining with annexin V (Fig. 1). After 8 h of infection with strain SC5314, 57.29 ± 13.11% of OKF6/TERT2 cells showed positive annexin V staining (Fig. 1). In contrast, only 9.02 ± 4.43% of the control uninfected OKF6/TERT2 cells showed positive annexin V staining (Fig. 1). The difference in the staining levels between infected and uninfected cells was statistically significant ($P < 0.0001$).

DNA fragmentation

Incubation of OKF6/TERT2 cells with *C. albicans* strain SC5314 resulted in DNA fragmentation (Fig. 2). The percentage of TUNEL-positive OKF6/TERT2 cells during infection varied between 11.23 ± 4.76% and 13.15 ± 5.74% at lower and higher multiplicity of infection, respectively (MOI 1 and 5, respectively)

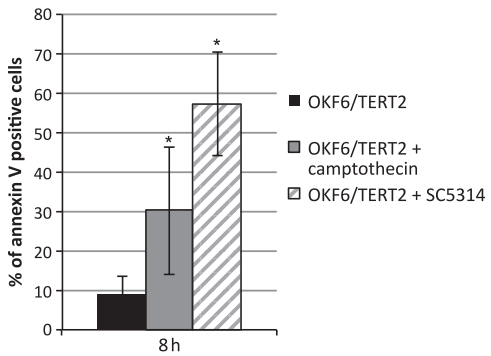

Figure 1 Effect of infection on the loss of plasma membrane asymmetry and externalization of phosphatidylserine on oral epithelial cells. OKF6/TERT2 cells were exposed to *Candida albicans* strain SC5314 for 8 h, and externalization of phosphatidylserine was detected by binding of FITC-conjugated annexin V. Stained cells were analysed by confocal microscopy. Data are shown as mean percentage of annexin V-positive cells ($n = 150$ corresponding to 100%); error bars indicate one standard deviation of the mean of three independent experiments. *$P < 0.05$ for a comparison with untreated OKF6/TERT2 cells.

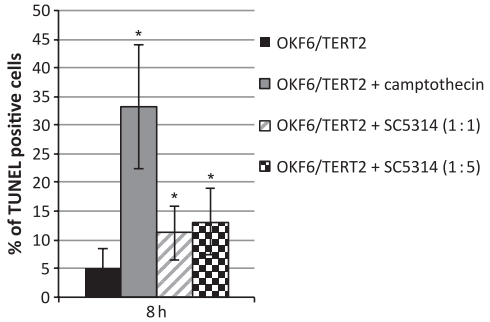

Figure 2 Effects of infection on oral epithelial DNA fragmentation. OKF6/TERT2 cells were exposed to *Candida albicans* strain SC5314 for 8 h at multiplicity of infection (MOI) of 1 and 5, and DNA degradation was detected by the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. Stained cells were analysed by confocal microscopy. Data are shown as mean percentage of TUNEL-positive cells ($n = 150$ corresponding to 100%); error bars indicate one standard deviation of the mean of three independent experiments. *$P < 0.05$ for a comparison with untreated cells.

(Fig. 2). In contrast, only 5.01 ± 3.33% of non-infected OKF6/TERT2 cells stained positive for TUNEL (Fig. 2). The difference in the numbers of TUNEL-positive cells in infected vs. uninfected cultures was statistically significant ($P < 0.0001$). In the presence of apoptotic inducer camptothecin, 33.21 ± 10.83% of OKF6/TERT2 cells stained positive for TUNEL (Fig. 2).

Caspase-activated DNases activated during apoptosis cleave genomic DNA at specific sites between nucleosomes. The ability of *C. albicans* to trigger apoptotic DNA fragmentation was assessed by the quantification of cytoplasmic histone-associated DNA fragments (nucleosomal fragments). Incubation of OKF6/TERT2 cells with *C. albicans* strain SC5314 at low and high MOI promoted a 2.21-fold (MOI 1) and 1.84-fold (MOI 5) increase in the number of histone-associated DNA fragments (Fig. 3). Camptothecin induced a 6.76-fold increase in the number of histone-associated DNA fragments above basal levels (uninfected cells) (Fig. 3). Collectively, these results suggest that *C. albicans* induces apoptotic DNA fragmentation in a subset of oral epithelial cells.

Apoptotic signaling pathways

Mitochondrial alterations

To define the signaling pathways involved in *C. albicans*-induced epithelial apoptotic alterations, the

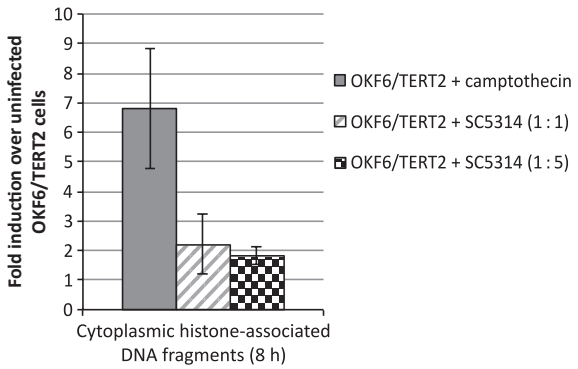

Figure 3 Effects of infection on oral epithelial internucleosomal DNA fragmentation. Enzyme-linked immunosorbent assay detection of histone-associated DNA fragments (cytoplasmic nucleosomal fragments) from extracts of OKF6/TERT2 cells infected with strain SC5314 for 8 h at a multiplicity of infection (MOI) of 1 and 5. Data are shown as relative fold induction over uninfected cells; error bars indicate one standard deviation of the mean of three independent experiments. **P* < 0.05 for a comparison with untreated cells.

mitochondrial membrane potential ($\Delta\Psi$) and the release of pro-apoptotic protein cytochrome *c* from the mitochondria into the cytosol were measured during infection of OKF6/TERT2 cells with *C. albicans*. Mitochondrial depolarization was measured experimentally by the decrease in the red-to-green fluorescence intensity ratio of JC-1 staining. In uninfected OKF6/TERT2 cells, the red-to-green JC-1 fluorescence ratio was 20.98 ± 4.77 (Fig. 4). However, *C. albicans* infection caused a marked decrease in red JC-1 fluorescence with a corresponding increase in green JC-1 fluorescence (not shown), resulting in a significant decrease of the red-to-green fluorescence ratio to 9.84 ± 5.30 at 2 h of infection (Fig. 4). This depolarization was sustained over time during infection. More specifically, the red-to-green JC-1 fluorescence ratio was 9.70 ± 3.89 and 9.96 ± 3.56, at 4 and 8 h of infection, respectively (Fig. 4).

To further assess the impact of *C. albicans* infection on epithelial mitochondrial integrity, levels of cytochrome *c* were measured in epithelial mitochondrial and cytosolic fractions. A time-dependent release of cytochrome *c* into the cytoplasm was noticed during *C. albicans* infection (Fig. 5). The reduced mitochondrial membrane potential and the release of cytochrome *c* in infected oral epithelial cells demonstrate that the mitochondrial integrity is lost during *C. albicans* infection.

Caspase activation

To gain further insight into the molecular mechanisms involved in *C. albicans*-induced epithelial apoptotic alterations, the ability of *C. albicans* to induce activation of caspase-3, -8 and -9 was evaluated over time. A significant increase in caspase-9 activity was noticed at early stages of infection (1.56- and 1.27-fold induction over uninfected cells at 6 and 8 h postinfection, respectively) (Fig. 6). Likewise, slight but significant increases in caspase-3 activities were

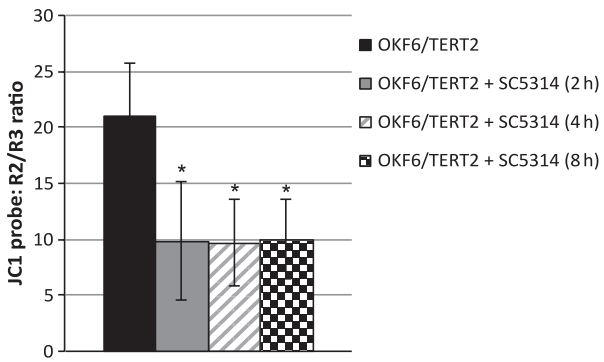

Figure 4 Changes in mitochondrial membrane potential in OKF6/TERT2 cells following *Candida albicans* infection. Mitochondrial potential ($\Delta\Psi$) was measured at 2, 4 and 8 h of infection by flow cytometry using the JC-1 probe. Data are shown as a ratio of red-to-green JC-1 staining fluorescence intensity. Mitochondrial depolarization is detected by a relative decrease in the red-to-green fluorescence intensity ratio. Bars indicate one standard deviation of the mean of five independent experiments. **P* < 0.05 for a comparison with untreated cells.

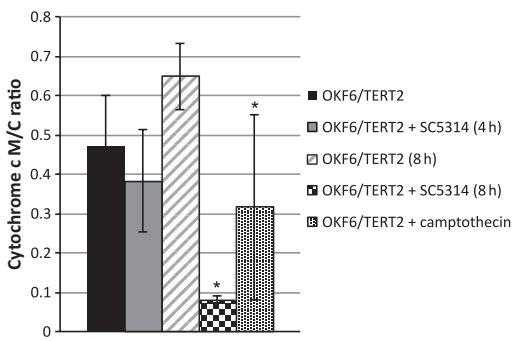

Figure 5 *Candida albicans* infection induces cytochrome *c* release into the cytosol. OKF6/TERT2 cells were exposed to *C. albicans* strain SC5314 for up to 8 h, and cytochrome *c* quantification was performed in cytosolic and mitochondria-enriched fractions using an enzyme-linked immunosorbent assay-based Quantikine Human Cytochrome C Immunoassay Kit. Data are shown as a ratio of mitochondrial-to-cytoplasmic cytochrome *c*. Error bars indicate one standard deviation of the mean of three independent experiments. **P* < 0.05 for a comparison with untreated cells.

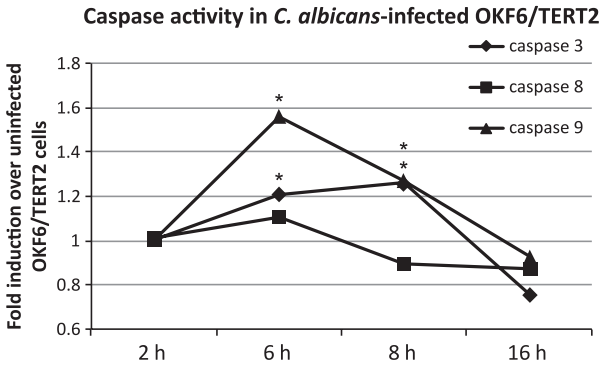

Figure 6 *Candida albicans* induced caspase activation in OKF6/TERT2 cells. OKF6/TERT2 cells were exposed to *C. albicans* strain SC5314 for 2, 6, 8 and 16 h, and caspase-3, caspase-8 and caspase-9 activities were monitored using fluorogenic caspase-specific substrates. Data are shown as relative fold induction over uninfected cells and are representative of four experiments. *$P < 0.05$ for a comparison with untreated cells.

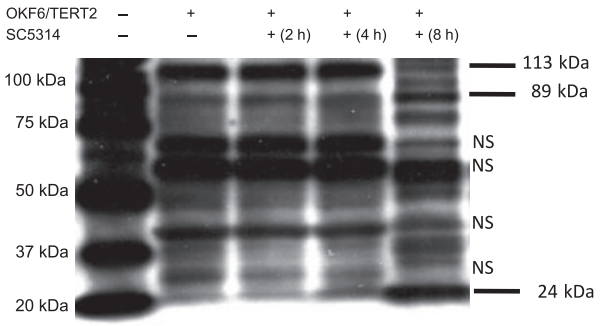

Figure 7 Time-dependent degradation of poly[ADP-ribose] polymerase-1 (PARP-1) during incubation of oral epithelial cells with *Candida albicans*. OKF6/TERT2 cells were exposed to *C. albicans* strain SC5314 for 2, 4 and 8 h, and PARP-1 integrity was analysed by Western blotting. Cell lysates of OKF6/TERT2 cells alone were analysed to determine PARP-1 content. The positions of the molecular mass markers are indicated on the left. The results of one of three independent experiments are shown in each panel. NS, non-specific bands.

noticed after 6 and 8 h of infection (1.21- and 1.26-fold induction over uninfected cells, respectively) (Fig. 6). However, after 16 h of infection, caspase-3 and caspase-9 activities decreased and reached levels similar to those observed in uninfected cells. Caspase-8 (extrinsic initiator) enzymatic activity was also measured in OKF6/TERT2 cells infected with *C. albicans*. In contrast to caspase-3 and caspase-9, the caspase-8 activity remained unchanged during *C. albicans* infection (Fig. 6).

PARP-1 integrity

The integrity and functional activity of PARP-1 was evaluated over time during *C. albicans* infection. Kinetic experiments showed that the level of intact PARP-1 (113 kDa) was maintained during the first 4 h of infection (Fig. 7). However, a significant reduction in the amount of full length PARP-1 signal compared with the signal obtained with uninfected cells was noted 8 h after fungal challenge (Fig. 7). At this time, the full-length PARP-1 protein was found to be cleaved into multiple fragments with apparent molecular weights of 24, 37, 45, 75, 89 and 101 kDa (Fig. 7). β-Actin levels remained unaltered during infection (not shown). Moreover, no bands corresponding to PARP-1 or β-actin were detected in cell lysates of *C. albicans* alone (not shown).

Expression of anti-apoptotic and pro-apoptotic genes

Expression of apoptosis-related genes was not affected by *C. albicans* during the first 4 h of infection (not shown). Eight apoptosis-related genes were significantly upregulated or downregulated at least two fold at 8 h of infection (Table 1), whereas the remaining 76 apoptosis-related genes showed no altered expression during *C. albicans* infection. More specifically, five anti-apoptotic genes, including baculoviral IAP repeat-containing-3 (*BIRC3*), Bcl2-related protein

Table 1 Differential expression of apoptosis-related genes in *Candida albicans*-infected OKF6/TERT2 cells

Gene name	Description	Function	Fold change
BIRC2	Baculoviral IAP repeat-containing protein 2	Anti-apoptotic	–2.37
BIRC3	Baculoviral IAP repeat-containing protein 3	Anti-apoptotic	+6.68
BCL2A1	Bcl-2-related protein A1	Anti-apoptotic	+2.4
BCL2L2	Bcl-2-like protein 2	Anti-apoptotic	+2.03
CARD6	Caspase recruitment domain, member 6	Pro-apoptotic	–2.01
CARD8	Caspase recruitment domain, member 8	Pro-apoptotic	–4.97
IGFLR	Insulin-like growth factor receptor	Anti-apoptotic	+2.48
MCL-1	Induced myeloid leukemia cell diff. protein	Anti-apoptotic	+2.04

The results shown are representative of two experiments performed in triplicate. Only genes showing a two fold or greater upregulation or downregulation over uninfected OKF6/TERT2 cultures and $P < 0.05$ are included. A total of 84 apoptotic-related genes were evaluated during infection.
IAP, inhibitors of apoptosis proteins.

A1 (*BCL2A1*), Bcl2 related protein A2 (*BCL2L2*), insulin-like growth factor-1 receptor (*IGF1R*), and induced myeloid leukemia cell differentiation protein (*MCL-1*) were upregulated at 8 h of infection (Table 1). On the other hand, expression of three apoptosis-related genes was downregulated at 8 h of infection. The downregulated genes included baculoviral IAP repeat-containing-2 (*BIRC2*), caspase recruitment domain-6 (*CARD6*) and *CARD8* (Table 1). These results suggest that *C. albicans* inhibits apoptosis at later phases of infection by upregulating a set of anti-apoptotic genes and downregulating a few pro-apoptotic transcripts.

DISCUSSION

The study of molecular signaling pathways involved in microbially induced host cell death is currently receiving increased attention. A recent study using an *in vitro* oropharyngeal candidiasis model demonstrated that *C. albicans* induces apoptotic alterations on oral epithelial cells (Villar & Zhao, 2010). However, activation of specific epithelial apoptotic signaling pathways in response to *C. albicans* infection has not yet been demonstrated and the mechanisms by which *C. albicans* induces oral epithelial cell death are unknown. In this study, molecular approaches were used to investigate the activation of epithelial apoptotic signaling pathways during *C. albicans* infection.

The extrinsic apoptotic signaling pathway is initiated at the cell plasma membrane upon ligation of transmembrane death receptors (Locksley *et al.*, 2001). Expression of death receptors in the oral epithelium is almost exclusively restricted to the basal cell layer (Loro *et al.*, 1999); however *C. albicans* rarely invade past the spinous epithelial cell layer. As a result of the non-coincidental spatial relationship between epithelial death receptors and invading fungal organisms, the extrinsic apoptotic signaling pathway was not examined in detail in this study. In contrast, the intrinsic mitochondrial apoptotic signaling pathway initiates from a varied array of stimuli that generates intracellular signs that act on the mitochondria (Saelens *et al.*, 2004). This pathway is defined by alterations in the mitochondrial membrane permeability that result in the release of mitochondrial pro-apoptotic proteins like cytochrome *c* and Smac/DIABLO into the cytosol (Garrido *et al.*, 2006). Once in the cytosol, these proteins activate the catalytic activity of caspases and neutralize the inhibitory effect of inhibitors of apoptosis proteins (IAP) on caspases. Our results demonstrated that *C. albicans* causes a decrease in the mitochondrial transmembrane potential ($\Delta\Phi m$) and translocation of cytochrome *c* from the mitochondria into the cytosol of oral epithelial cells, suggesting that the epithelial intrinsic apoptotic pathway is activated in response to *C. albicans*. Our findings are in agreement with previous studies showing that other infectious organisms, such as hepatitis C virus and *Staphylococcus aureus*, also induce disruption of the mitochondrial transmembrane potential with subsequent translocation of cytochrome *c* into the cytosol of infected cells (Esen *et al.*, 2001; Deng *et al.*, 2008).

Translocation of cytochrome *c* into the cytosol is followed by the binding of this pro-apoptotic protein with apoptotic protease activating factor 1 (APAF1), dATP and pro-caspase-9, leading to the formation of the apoptosome complex (Chinnaiyan, 1999; Hill *et al.*, 2004; Lavrik *et al.*, 2005). Formation of this complex leads to activation of the intrinsic initiator caspase-9 and subsequent activation of the executioner caspase-3 (Chinnaiyan, 1999; Hill *et al.*, 2004; Lavrik *et al.*, 2005). Cysteine proteases of the caspase family play a central role in the initiation and progression of cellular apoptosis. A marginal but significant increase in caspase-9 and caspase-3 activities was detected in the first 8 h of *C. albicans* infection, suggesting either a slight overall increase in caspase-3 and caspase-9 activation in the total epithelial population or a more pronounced activation of these enzymes in a small subset of infected cells. However, at later phases of infection, caspase-3 and caspase-9 activities returned to levels similar to those observed in uninfected cells. Activation of the extrinsic initiator caspase-8 was not increased in infected cells, further suggesting that the extrinsic apoptotic pathway is not activated in response to *C. albicans*.

Caspase-3 catalyses the cleavage of multiple downstream targets essential for the progression of apoptotic pathways (Jänicke *et al.*, 1998). Among these target proteins, degradation of PARP-1 (into two apoptotic fragments of 24 and 89 kDa) is expected to promote the irreversible progression of apoptotic cell death pathways (Oliver *et al.*, 1998). A significant reduction in the amount of intact PARP-1 was noticed after 8 h of infection. However, PARP-1 was cleaved both into non-apoptotic, and necrotic-like

fragments. It is likely that the slight and transient caspase-3 activation induced by *C. albicans* was not enough to promote PARP-1 degradation exclusively into apoptotic fragments and commitment of oral epithelial cells to apoptosis. Necrotic cleavage of PARP-1 is caused in part or entirely by lysosomal proteases (Gobeil *et al.*, 2001). Therefore, our data suggest that PARP-1 was also degraded by proteases other than caspase-3 during *C. albicans* infection. These data are in line with the findings from other studies showing that *Chlamydia trachomatis* induces necrotic fragmentation of PARP-1 in human cervix epithelial cells (Yu *et al.*, 2010).

Candida albicans induced early apoptotic changes in infected oral epithelial cells. These early apoptotic changes characterized by loss of phospholipid asymmetry were found in over 50% of the oral epithelial cells. However, only a small percentage (10–15%) of the infected oral epithelial cells underwent mid-late apoptotic changes, as measured by DNA degradation. These results suggest that only a small percentage of the infected oral epithelial cells undergoing early apoptotic cellular changes have progressed towards the late stages of apoptosis. It remains unclear why only a small number of epithelial cells showed late apoptotic changes during *C. albicans* infection. We propose that through their fungistatic activity, oral epithelial cells have restricted the cytopathic activity of *C. albicans* (Steele *et al.*, 2000; Barousse *et al.*, 2001; Yano *et al.*, 2005), hence halting the progression of apoptotic pathways. Alternatively, it can also be speculated that as *C. albicans* undergoes morphological transition from yeast to hyphae, it loses the virulence factors that activate epithelial apoptotic pathways. It is also conceivable that while early apoptotic changes are induced by adhesion of *C. albicans* to epithelial surfaces, late apoptotic changes only occur following host cell invasion. In support of this hypothesis, the small percentage (10–15%) of infected oral epithelial cells displaying late apoptotic morphological changes correlates well with the percentage of oral epithelial cells that internalize fungal cells during infection (Villar & Zhao, 2010). Based on the findings of the molecular analysis of oral epithelial cells infected with *C. albicans*, it is also plausible that *C. albicans* induces apoptotic alterations early during infection, but stimulates the activation of anti-apoptotic signaling pathways at later stages. The epithelium of the oral mucosa must be physically and functionally intact to provide innate and adaptive responses against *C. albicans*, so it is also plausible that activation of epithelial anti-apoptotic pathways in response to *C. albicans* might be a protective mechanism by which the host attempts to prevent epithelial damage, and fungal local tissue invasion followed by widespread bloodstream dissemination.

Apoptotic pathways initiate primarily through activation of pro-enzymes and release of sequestered pro-apoptotic proteins rather than through altered expression of anti-apoptotic and pro-apoptotic genes. In this context, the expression levels of genes encoding for proteins involved in apoptosis was investigated merely because it was noticed that apoptotic pathways were initiated but failed to progress in oral epithelial cells infected with *C. albicans.* The results from gene array experiments provided a plausible explanation for this initial observation. In support of the last two hypotheses described above, gene array expression profiling of oral epithelial cells showed for the first time that while the expression of genes involved in apoptotic pathways remained unchanged early during infection, expression of pro-apoptotic and anti-apoptotic genes was significantly inhibited and induced, respectively, at 8 h postinfection and may have prevented the progression of apoptotic pathways in *C. albicans*-infected oral epithelial cells. Among the anti-apoptotic genes, the protein encoded by *BIRC3* inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF-1 and TRAF-2 (Herbert *et al.*, 2010), and thereby promote activation of anti-apoptotic tumor necrosis factors/NK-κB/IAP pathways (Wong *et al.*, 1998). A 6.68-fold increase in the expression level of *BIRC3* was observed at 8 h of infection. *BCL2A1* and *BCL2A2* encode pro-survival proteins that prevent apoptosis by inhibiting the release of cytochrome *c* from mitochondria, and thereby caspase activation (Kluck *et al.*, 1997). Both *BCL2A21* and *BCL2A2* were also upregulated at 8 h of infection. *Igf1r* enhances cell survival by negatively interfering with TRAIL-induced apoptosis (Hilmi *et al.*, 2008). The expression level of this gene was increased 2.48-fold at 8 h. Finally, *MCL1*, which is believed to enhance cell survival through interactions with pro-apoptotic *Bcl2* family members, was upregulated by a 2.04-fold.

Also supporting the last two hypotheses were the findings that the expression levels of *CARD6*

and *CARD8* were downregulated in response to *C. albicans*. *CARD6* and *CARD8* suppress the induction of nuclear factor-κB (Stilo *et al.*, 2002; Stehlik *et al.*, 2003), a transcription factor that stimulates the expression of anti-apoptotic factors (Gillis *et al.*, 2009). Therefore lower levels of *CARD6* and *CARD8* may allow for increased activation of nuclear factor-κB and subsequent expression of anti-apoptotic factors. The mechanisms underlying upregulation of anti-apoptotic genes and downregulation of pro-apoptotic genes in response to *C. albicans* remain unknown. Nonetheless, *in vitro* data previously reported by our group (Villar *et al.*, 2004) and others (Park *et al.*, 2005; Chiang *et al.*, 2007) have shown that extensive oral epithelial cell death occurs at late phases of *C. albicans* infection. Taken together, it is likely that although epithelial apoptotic pathways are inhibited at late phases of *C. albicans* infection, at this point microbial insults have overloaded epithelial cells and massive epithelial cell necrosis occurs.

Although other studies have used cell lines derived from oral carcinomas, e.g. SCC15, TR146 and FaDu, to study *C. albicans* interactions with epithelial cells (Villar *et al.*, 2004; Park *et al.*, 2005; Wächtler *et al.*, 2011), OKF6/TERT2 cells were used in this study. OKF6/TERT2 is a non-cancerous cell line that is believed to be more representative of primary oral epithelial cells. However, it is important to point out that OKF6/TERT2 cells are created by forced expression of telomerase 2, which *per se* may affect signaling systems involved in apoptosis. Nonetheless, the results from this study are in line with previous studies demonstrating that *C. albicans* induces early apoptotic alterations in cancerous oral epithelial cell lines and primary oral epithelial cells (Villar & Zhao, 2010). Finally, preliminary kinetic confocal analysis of epithelial cultures infected with *C. albicans* at a low infectivity ratio demonstrated that only epithelial cells in direct contact with fungal cells showed annexin V externalization and DNA degradation (not shown). This finding suggests that a physical contact between oral epithelial cells and *C. albicans* is required for induction of oral epithelial apoptosis. The percentage of epithelial cell in direct contact with fungal cells is small in cultures infected at low infectivity ratios. On the other hand, most assays that aim to investigate activation of apoptotic pathways use cell lysates derived from entire culture cell populations. Therefore, analysis of epithelial cultures infected with low doses of *C. albicans* may fail to properly detect activation of apoptotic signaling pathways in a small group of infected cells. To avoid this significant limitation inherent in the use of low infectivity ratios, experiments were performed only at high infectivity ratios (MOI of 1 and 5), which may not mimic the level of infectivity found in oropharyngeal candidiasis.

Based on the results from this and other studies, we hypothesize that *C. albicans* induces early apoptotic cellular alterations following its initial adherence to oral epithelial cells. This is then followed by the activation of nuclear factor-κB signaling pathway (Moyes *et al.*, 2010), which in turn triggers the activation of anti-apoptotic pathways in the infected cells. Nonetheless, it is likely that at this point, yeast cells have already undergone morphological transformation into highly invasive and virulent hyphal forms. Hyphal cells are known to secrete soluble hydrolytic enzymes (Naglik *et al.*, 2003). The proteolytic activity of these enzymes is expected to disturb the physical integrity and promote necrotic oral epithelial cell death.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

REFERENCES

Barousse, M.M., Steele, C., Dunlap, K. *et al.* (2001) Growth inhibition of *Candida albicans* by human vaginal epithelial cells. *J Infect Dis* **184**: 1489–1493.

Barrett, A.P. (1984) Evaluation of nystatin in prevention and elimination of oropharyngeal *Candida* in immunosuppressed patients. *Oral Surg Oral Med Oral Pathol* **58**: 148–151.

Black, M.W. and Boothroyd, J.C. (2000) Lytic cycle of *Toxoplasma gondii*. *Microbiol Mol Biol Rev* **64**: 607–623.

Brydon, E.W., Smith, H. and Sweet, C. (2003) Influenza A virus-induced apoptosis in bronchiolar epithelial (NCI-

H292) cells limits pro-inflammatory cytokine release. *J Gen Virol* **84**: 2389–400.

Chiang, L.Y., Sheppard, D.C., Bruno, V.M., Mitchell, A.P., Edwards, J.E. Jr and Filler, S.G. (2007) *Candida albicans* protein kinase CK2 governs virulence during oropharyngeal candidiasis. *Cell Microbiol* **9**: 233–245.

Chinnaiyan, A.M. (1999) The apoptosome: heart and soul of the cell death machine. *Neoplasia* **1**: 5–15.

Chipuk, J.E. and Green, D.R. (2005) Do inducers of apoptosis trigger caspase-independent cell death? *Nat Rev Mol Cell Biol* **6**: 268–275.

Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem J* **326**: 1–16.

Darouiche, R.O. (1998) Oropharyngeal and esophageal candidiasis in immunocompromised patients: treatment issues. *Clin Infect Dis* **26**: 259–272.

Dejean, L.M., Martinez-Caballero, S. and Kinnally, K.W. (2006) Is MAC the knife that cuts cytochrome c from mitochondria during apoptosis? *Cell Death Differ* **13**: 1387–1395.

Deng, L., Adachi, T., Kitayama, K. *et al.* (2008) Hepatitis C Virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, Caspase 3-dependent pathway. *J Virol* **82**: 10375–10385.

Dickson, M.A., Hahn, W.C., Ino, Y. *et al.* (2000) Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* **20**: 1436–1447.

Esen, M., Schreiner, B., Jendrossek, V. *et al.* (2001) Mechanisms of *Staphylococcus aureus* induced apoptosis of human endothelial cells. *Apoptosis* **6**: 431–439.

Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C. and Kroemer, G. (2006) Mechanisms of cytochrome c release from mitochondria. *Cell Death Differ* **13**: 1423–1433.

Gillis, P.A., Okagaki, L.H. and Rice, S.A. (2009) Herpes simplex virus type 1 ICP27 induces p38 mitogen-activated protein kinase signaling and apoptosis in HeLa cells. *J Virol* **83**: 1767–1777.

Gobeil, S., Boucher, C.C., Nadeau, D. and Poirier, G.G. (2001) Characterization of the necrotic cleavage of poly (ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. *Cell Death Differ* **8**: 588–594.

Herbert, B.S., Chanoux, R.A., Liu, Y. *et al.* (2010) A molecular signature of normal breast epithelial and stromal cells from Li-Fraumeni syndrome mutation carriers. *Oncotarget* **1**: 405–422.

Hill, M.M., Adrain, C., Duriez, P.J., Creagh, E.M. and Martin, S. J. (2004) Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *EMBO J* **23**: 2134–2145.

Hilmi, C., Larribere, L., Giuliano, S. *et al.* (2008) IGF1 promotes resistance to apoptosis in melanoma cells through an increased expression of BCL2, BCL-X(L), and survivin. *J Invest Dermatol* **128**: 1499–1505.

Hoppe, J.E. (1997) Treatment of oropharyngeal candidiasis and candidal diaper dermatitis in neonates and infants: review and reappraisal. *Pediatr Infect Dis* **16**: 885–894.

Jänicke, R.U., Sprengart, M.L., Wati, M.R. and Porter, A.G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* **273**: 9357–9360.

Jones, B.A. and Gores, G.J. (1997) Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pancreas, and intestine. *Am J Physiol* **273**: 1174–1188.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**: 1132–1136.

Kobayashi, S.D., Braughton, K.R., Palazzolo-Balance, A.M. *et al.* (2010) Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun* **2**: 560–575.

Kroemer, G. and Martin, S.J. (2005) Caspase-independent cell death. *Nat Med* **11**: 725–730.

Lai, X.H. and Sjostedt, A. (2003) Delineation of the molecular mechanisms of *Francisella tularensis*-induced apoptosis in murine macrophages. *Infect Immun* **71**: 4642–4646.

Lavrik, I.N., Golks, A. and Krammer, P.H. (2005) Caspases: pharmacological manipulation of cell death. *J Clin Invest* **115**: 2665–2672.

Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**: 487–501.

Loro, L.L., Vintermyr, O.K., Liavaag, P.G., Jonsson, R. and Johannessen, A.C. (1999) Oral squamous cell carcinoma is associated with decreased bcl-2/bax expression ratio and increased apoptosis. *Hum Pathol* **9**: 1097–1105.

Lynch, D.P. (1994) Oral candidiasis. History, classification and clinical presentation. *Oral Surg Oral Med Oral Pathol* **78**: 189–193.

Mihara, M., Erster, S., Zaika, A. *et al.* (2003) p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* **11**: 577–590.

Moyes, D.L., Runglall, M., Murciano, C. *et al.* (2010) A biphasic innate immune MAPK response discriminates

between the yeast and hyphal forms of *Candida albicans* in epithelial cells. *Cell Host Microbe* **8**: 225–235.

Naglik, J.R., Challacombe, S.J. and Hube, B. (2003) Candida albicans secreted aspartyl proteinases in virulence and pathogenesis.. *Microbiol Mol Biol Rev* **67**: 400–428.

Norbury, C.J. and Hickson, I.D. (2001) Cellular responses to DNA damage. *Annu Rev Pharmacol Toxicol* **41**: 367–401.

Noronha, F.S., Ramalho-Pinto, F.J. and Horta, M.F. (1996) Cytolytic activity in the genus *Leishmania*: involvement of a putative pore-forming protein. *Infect Immun* **64**: 3975–3982.

Ojcius, D.M., Souque, P., Perfettini, J.L. and Dautry-Varsat, A. (1998) Apoptosis of epithelial cells and macrophages due to infection with the obligate intracellular pathogen *Chlamydia psittaci*. *J Immunol* **161**: 4220–4226.

Oliver, F.J., De la Rubia, G., Rolli, V., Ruiz-Ruiz, M.C., De Murcia, G. and Murcia, J.M. (1998) Importance of poly (ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. *J Biol Chem* **273**: 33533–33539.

Park, H., Myers, C.L., Sheppard, D.C. *et al.* (2005) Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. *Cell Microbiol* **7**: 499–510.

Perfettini, J.L., Reed, J.C., Israël, N., Martinou, J.C., Dautry-Varsat, A. and Ojcius, D.M. (2002) Role of Bcl-2 family members in caspase-independent apoptosis during *Chlamydia* infection. *Infect Immun* **70**: 55–61.

Popov, S.G., Villasmil, R. and Bernardi, J. (2002) Lethal toxin of *Bacillus anthracis* causes apoptosis of macrophages. *Biochem Biophys Res Commun* **293**: 349–355.

Saelens, X., Festjens, N., Vande Walle, L., Van Gurp, M., Van Loo, G. and Vandenabeele, P. (2004) Toxic proteins released from mitochondria in cell death. *Oncogene* **23**: 2861–2874.

Slee, E.A., Adrain, C. and Martin, S.J. (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* **276**: 7320–7326.

Soll, D.R. (2002) *Candida* commensalisms and virulence: the evolution of phenotypic plasticity. *Acta Trop* **81**: 101–110.

Steele, C., Leigh, J., Swoboda, R. and Fidel, P.L. Jr (2000) Growth inhibition of *Candida* by human oral epithelial cells. *J Infect Dis* **182**: 1479–1485.

Stehlik, C., Hayashi, H., Pio, F., Godzik, A.A. and Reed, J.C. (2003) CARD6 is a modulator of NF-κB activation by Nod1- and Cardiak-mediated pathways. *J Biol Chem* **278**: 31941–31949.

Stilo, R., Leonardi, A., Formisano, L., Di Jeso, B., Vito, P. and Liguoro, D. (2002) TUCAN/CARDINAL and DRAL participate in a common pathway for modulation of NF-κB activation. *FEBS Lett* **521**: 165–169.

Villar, C.C. and Zhao, X.R. (2010) *Candida albicans* induces early apoptosis followed by secondary necrosis in oral epithelial cells. *Mol Oral Microbiol* **25**: 215–225.

Villar, C.C., Kashleva, H. and Dongari-Bagtzoglou, A. (2004) Role of *Candida albicans* polymorphism in interactions with oral epithelial cells. *Oral Microbiol Immunol* **19**: 262–269.

Villar, C.C., Kashleva, H., Mitchell, A.P. and Dongari-Bagtzoglou, A. (2005) Invasive phenotype of *Candida albicans* affects the host proinflammatory response to infection. *Infect Immun* **8**: 4588–4595.

Wächtler, B., Wilson, D., Haedicke, K., Dalle, F. and Hube, B. (2011) From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage during interaction with oral epithelial cells. *PLoS ONE* **6**: e17046.

Wong, B.R., Josien, R., Lee, S.Y., Vologodskaia, M., Steinman, R.M. and Cho, Y. (1998) The TRAF family of signal transducers mediates NF-κB activation by the TRANCE receptor. *J Biol Chem* **273**: 28355–28359.

Yano, J., Lilly, E.A., Steele, C., Fortenberry, D. and Fidel, P.L. Jr (2005) Oral and vaginal epithelial cell anti-*Candida* activity is acid labile and does not require live epithelial cells. *Oral Microbiol Immunol* **20**: 199–205.

Yu, S.W., Andrabi, S.A., Wang, H. *et al.* (2006) Apoptosis-inducing factor mediates poly (ADP-ribose) (PAR) polymer-induced cell death. *Proc Natl Acad Sci U S A* **103**: 18314–18319.

Yu, H., Schwarzer, K., Förster, M. *et al.* (2010) Role of high-mobility group box 1 protein and poly (ADP-ribose) polymerase 1 degradation in *Chlamydia trachomatis*-induced cytopathicity. *Infect Immun* **78**: 3288–3297.