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Candida albicans Amphotericin B-Tolerant Persister Formation is Closely Related to Surface Adhesion

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Abstract *Candida albicans* persisters have so far been observed only in biofilm environment; the biofilm element(s) that trigger(s) persister formation are still unknown. In this study, we tried to further elucidate the possible relationship between *C. albicans* persisters and the early phases of biofilm formation, especially the surface adhesion phase. Three *C. albicans* strains were surveyed for the formation of persisters. We tested *C. albicans* persister formation dynamically at different time points during the process of adhesion and biofilm formation. The number of persister cells was determined based on an assessment of cell viability after amphotericin B treatment and colony-forming unit assay. None of the planktonic cultures contained persisters. Immediately following adhesion of *C. albicans* cells to the surface, persister cells emerged and the proportion of persisters reached a peak of 0.2–0.69 % in approximately 2-h biofilm. As the biofilm matured, the proportion of persisters decreased and was only 0.01–0.02 % by 24 h, while the number of persisters remained stable with no significant change. Persisters were not detected in the absence of an attachment surface which was pre-coated. Persisters were also absent in biofilms that were scraped to disrupt surface adhesion prior to amphotericin B treatment. These results indicate that *C. albicans* antifungal-tolerant persisters are produced mainly in surface adhesion phase and surface adhesion is required for the emergence and maintenance of *C. albicans* persisters.

Keywords *Candida albicans* · Persisters · Biofilm · Adhesion

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Introduction

Candida albicans is the most prevalent opportunistic human fungal pathogen and is particularly problematic in immuno-compromised patients such as those with AIDS and cancer, or in organ transplant patients [1]. *Candida* infections are often untreatable and

sometimes can result in life-threatening diseases with a high rate of mortality approaching 40–70 % [2–9]. It is believed that biofilm formation and drug tolerance can lead to the recalcitrant and untreatable nature of some fungal infectious diseases [7, 10]. Biofilm-related drug tolerance may be due to high density and low growth rate within the biofilm, binding of large molecule antibiotics to the exopolymer matrix, and up-regulation of biofilm-associated genes such as *MDR1*, *CDR1* and *CDR2* [11–15]. Indeed, increasing evidence indicates that the mechanism of biofilm-related drug tolerance is multifactorial [7, 10].

In 2006, LaFleur and colleagues found that only a small population in the *C. albicans* biofilm could survive when challenged with lethal antifungal treatment [16]. The subpopulation of survivors is known as persisters [16–21]. Persisters can reconstitute a new biofilm with a similar proportion of persisters and an equal susceptibility to antifungal treatment when they are harvested and re-incubated [10, 16, 21–23]. Just as for their bacterial counterparts, these *C. albicans* persisters exhibit a non-hereditary, multi-drug tolerance [16, 23–25]. Therefore, persisters were implicated as the main determinants of high biofilm tolerance to antifungal and to the recurring symptoms of fungal infectious diseases [18, 24].

Intriguingly, while bacterial persisters can be detected in both planktonic and biofilm conditions [24, 26–28], *C. albicans* persisters have so far been observed only in biofilms [16, 17, 20]. Persisters are thought to be dormant phenotypic variants of the regular cell population [10, 16, 17, 21, 23, 24]. Although investigations into the mechanism of fungal persister cell formation are at an early stage, some studies have investigated this clinically important area. Biofilms seem to be essential for the formation of *Candida* persisters, but some mutants that cannot form mature biofilms, such as Δefg1/Δcph1, can still produce wild-type levels of persister cells [16]. This result indicates that the maturation of biofilms is not the essential condition for *Candida* persister formation. However, the previous studies have been focused on the persister formation in mature biofilms (48 h) and none of these studies have been conducted to investigate persister formation in the early phases of biofilms [16–20]. Thus, these previous results are unsatisfactory and further studies are still necessary.

To search for the possible determinants, we dynamically tested the formation of *C. albicans* persisters at different time points (0–24 h) during biofilm development. *C. albicans* persisters were first reported to occur following treatment with a high concentration of amphotericin B or chlorhexidine [16]. Since their identification, dose-dependent killing has been the only effective and straightforward method of isolating persisters [16–20]. In the present study, we used a similar protocol with amphotericin B to measure persisters and three *C. albicans* strains were tested. The persister levels were also tested in biofilms grown in the pre-coated wells of microtiter plates, of which the surface had been previously occupied by other strains. Additionally, we tested the persister levels in biofilms that were scraped to disrupt surface adhesion prior to amphotericin B treatment.

Materials and Methods

Strains and Growth Conditions

Candida albicans strains 3153A [16], SC5314 [29, 30] and YEM30 [31] were tested for the presence of persisters after exposure to amphotericin B, and *C. albicans* strain CAI-4 (*ura3Δ::imm434/ura3Δ::imm434*) [16] was used to pre-coat the attachment surface in this study. *C. albicans* strain CAI-4 is *URA3* null mutant, and *C. albicans* strains 3153A, SC5314 and YEM30 are wild types. Stock cultures of *C. albicans* strains were routinely propagated in yeast extract peptone dextrose [YPD; 1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose] solid medium containing 1.5 % (w/v) agar. The inocula of yeast cells were prepared by transfer of a single colony from YPD solid medium to YPD medium and incubated at 37 °C for 12 h in an incubator shaker at about 100 rpm.

Antifungal Susceptibility Assay

The minimal inhibitory concentration (MIC) of the tested *C. albicans* strains to amphotericin B (Amresco, USA) was determined by the broth microdilution method based on the CLSI M27-A2 guidelines [32]. Briefly, cells were harvested by centrifugation at 6000× *g* for 3 min from overnight culture, washed twice in sterile PBS and re-suspended in RPMI 1640 medium. Cells were cultured in 96-well microtiter plates (Corning Costar, USA) at a density of

$(1–5) \times 10^3$ cells ml^{-1}. The cell suspensions were treated with a series of twofold dilutions of amphotericin B in RPMI 1640 medium, while the negative controls were conducted with the same amount of vehicle. The MIC was determined by incubating the inoculated cultures for 24 h at 37 °C and observing inhibition of growth based on turbidity. Experiments were performed with at least four independent cultures.

Preparation and Amphotericin B Challenge of Planktonic Cultures

Planktonic cultures of *C. albicans* were prepared according to the methods described by Al-Dhaheri and Douglas [17]. Briefly, *C. albicans* cells were harvested by centrifugation, washed twice in sterile PBS and re-suspended in RPMI 1640 medium plus MOPS at 1.0×10^7 cells ml^{-1}. The planktonic cells were aliquoted into wells of microtiter plates at 100 µl per well and treated for 24 h with amphotericin B (100 µg ml^{-1}).

Candida albicans Biofilm Development on the Surfaces of Wells of Microtiter Plates

Candida albicans biofilm development on the surfaces of wells of flat-bottom 96-well microtiter plates was induced as described by Ramage et al. [33, 34]. Briefly, cells were harvested by centrifugation, washed twice in sterile PBS and re-suspended in RPMI 1640 medium plus 0.165 M MOPS. The suspension was adjusted to the desired density of 1.0×10^6 cells ml^{-1} after counting with a hemocytometer. The cell suspension was dispensed into the wells of microtiter plates at 100 µl per well, and the plates were incubated at 37 °C in an incubator shaker at approximately 100 rpm. Biofilms were formed over a series of time intervals (0.5, 1, 2, 4, 6, 8, 12 and 24 h).

Preconditioning the Surfaces of Microtiter Plates with *Candida albicans* Strain CAI-4

Candida albicans strain CAI-4 culture was incubated overnight at 37 °C in YPD medium. Cells were harvested by centrifugation, washed twice in sterile PBS and re-suspended in RPMI 1640 medium plus 0.165 M MOPS and adjusted to the desired density of $(3–5) \times 10^7$ cells ml^{-1} after counting with a hematocytometer. The cell density was chosen to ensure that the surface of microtiter plates was entirely coated after preconditioning 24 h with *C. albicans* CAI-4. The cell suspension was aliquoted into the wells of 96-well microtiter plates at 100 µl per well, and the plates were incubated at 37 °C for 24 h in an incubator shaker at approximately 100 rpm. Then, the medium was aspirated and the wells were washed twice with sterile PBS to remove nonadherent cells. Subsequently, one hundred microliters of above cell suspension at a concentration of 1.0×10^6 cells ml^{-1} was dispensed into the wells of 96-well microtiter plates and incubated at 37 °C at approximately 100 rpm over a series of time intervals (0.5, 1, 2, 4, 6, 8, 12 and 24 h).

Amphotericin B Challenge of *Candida albicans* biofilm

Amphotericin B was dissolved in RPMI 1640 medium at 100 µg ml^{-1}, which exceeded 10× MIC to minimize survival of potential spontaneous resistant mutants. After biofilm formation, the RPMI 1640 medium was discarded and nonadherent cells were removed by washing twice in sterile PBS. The biofilms were then randomly categorized into three groups: The first group was directly challenged with 100 µg ml^{-1} amphotericin B for 24 h at 37 °C; the second group was scraped to disrupt surface adhesion before challenged with 100 µg ml^{-1} amphotericin B for 24 h at 37 °C; and the third group was used as an untreated control to determined the growth levels of *C. albicans* in biofilms by colony-forming unit assay.

Candida albicans Persisters Determination

Selective SC-Ura medium (SC minus Uracil; Sigma-Aldrich, USA) was employed in this experiments because *C. albicans* CAI-4 could not grow on this medium. The quantification of persisters was performed as previously described by LaFleur et al. [16]. Briefly, the biofilms challenged with amphotericin B for 24 h were washed twice with sterile PBS, scraped from the surface of wells of microtiter plates and re-suspended in 100 µl PBS. Then, the suspensions were serially diluted in PBS, and viable cells were counted by plating 5 µl drops on the SC-Ura medium agar plates and incubated at 37 °C for 48 h. The percentage

of persisters was measured by comparing the number of viable cells with that in control biofilm at the same time point.

Live/Dead Cell Analysis and Confocal Laser Scanning Microscopy (CLSM)

LIVE/DEAD FungaLight yeast viability kit (Invitrogen, USA) employed in the live/dead cells analysis. One microliter of SYTO®9 dye and 1 µl of propidium iodide solution were added to 1 ml of 1 M Tris–HCl buffer (pH 6.8) and blended; the mixtures were then applied to stain the samples of *C. albicans* biofilms. After biofilm formation or antifungal treatment, RPMI 1640 medium and nonadherent cells were removed from the microtiter plate by thoroughly washing the biofilms twice with sterile Tris–HCl buffer. Dye mixtures (100 µl) were added to each sample and incubated at room temperature or 37 °C and protected from light for 15–30 min. After incubation with the dyes, stained biofilms were visualized with Zeiss LSM780 confocal laser scanning microscope.

Statistical Analysis

Statistical significances were determined by Student's *t* test. All statistical analyses were computed using SPSS 17.0. Data were expressed as the mean ± standard deviation (SD). The level of statistical significance for all tests was set at p values <0.05.

Results

Presence of Persisters in *Candida albicans* Biofilms and Planktonic Cultures

Biofilms and planktonic cells of three *C. albicans* strains were surveyed for the presence of persisters after amphotericin B treatment. There were no detectable survivors in any planktonic cultures of the tested *C. albicans* strains. However, persisters were found in biofilms of all tested *C. albicans* strains. For all tested *C. albicans* strains, the persister population was small, representing approximately 0.021, 0.014 and 0.012 % of the total population of mature 24-h biofilms of *C. albicans* 3153A, SC5314 and YEM30, respectively.

Formation of *Candida albicans* Persisters During Biofilm Formation

We dynamically tested persister formation during surface adhesion and biofilm development. The persister cells emerged immediately after *C. albicans* cells adhered to surface and significantly increased at 0–2 h ($p < 0.05$; Fig. 1). The proportion of persisters reached a peak (0.2–0.69 %) in approximately 2-h biofilm. The number of persisters in 2 h biofilms was 295.0 ± 11.90, 103.75 ± 11.43 and 227.5 ± 18.43 (cell/well), representing approximately 0.69, 0.25 and 0.36 % of the total population of biofilms of *C. albicans* 3153A, SC5314 and YEM30, respectively. During fungal cell growth, proliferation, aggregation, hyphae formation and biofilm maturation, the number of persister cells in the biofilm remained stable without significant change ($p > 0.05$; Fig. 1). As a result, the proportion of persisters declined, yielding a fraction of only 0.01–0.02 % in 24-h biofilm. The persister population in 24-h biofilms was 292.5 ± 8.34, 103.75 ± 8.00 and 218.75 ± 19.87 (cell/well), representing approximately 0.021, 0.014 and 0.012 % of the total population of biofilms of *C. albicans* 3153A, SC5314 and YEM30, respectively. Interestingly, persister cells were absent in biofilms that were scraped to disrupt surface adhesion prior to amphotericin B treatment.

Since killing of *C. albicans* biofilms by amphotericin B followed a biphasic pattern [16], with persisters surviving extended periods of time, exhibiting slow, steady cell death, we tested the survivors of *C. albicans* to see whether they showed this characteristic of persisters. Hence, we exposed the biofilms of *C. albicans* to amphotericin B for a prolonged period, quantifying the survivors at several time points. Our results reflect slow, steady cell death for up to 8 h of amphotericin B treatment (Fig. 2), which is characteristic of persisters. Therefore, the persisters detected in the present study are "true" persisters.

Effect of Pre-coated Surface on Persister Formation in the Biofilm

Given that *C. albicans* persisters only seemed to arise after adhesion to the attachment surface, we investigated whether pre-coated attachment surface would effect the formation of *C. albicans* persisters. *C. albicans* strain CAI-4 was used in this study, which

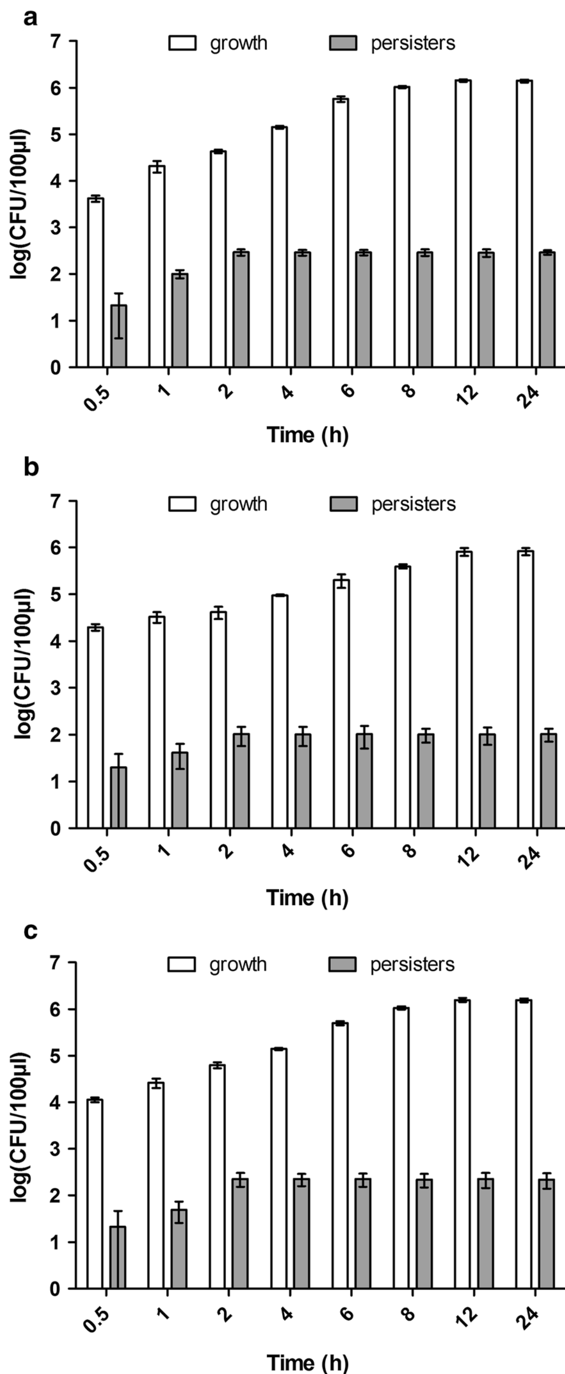

Fig. 1 Persister formation during biofilm development. Biofilms are incubated over a series of time intervals as indicated and challenged with amphotericin B. Growth levels and persister levels in biofilms of *C. albicans* 3153A (**a**), *C. albicans* sc5314 (**b**) and *C. albicans* YEM30 (**c**). *Error bars* indicate standard deviations, and results are from 16 independent experiments

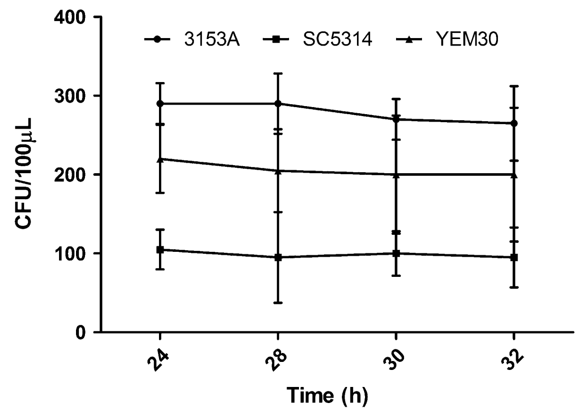

Fig. 2 Prolonged amphotericin B treatment of *C. albicans* biofilms. Persister levels in biofilms of *C. albicans* 3153A, SC5324 and YEM 30 during prolonged amphotericin B treatment. Persister levels during the prolonged amphotericin B treatment are not changed significantly ($p > 0.05$). *Error bars* indicate standard deviations, and results are from four independent experiments

does not grow on SC-Ura medium. Accordingly, we pre-coated the surfaces of wells of microtiter plates with *C. albicans* CAI-4 before incubating biofilms of the tested *C. albicans* strains. The growth levels of *C. albicans* in biofilms were significantly elevated at 0–2 h ($p < 0.05$; Fig. 3), whereas no significant differences were observed at 4–24 h ($p > 0.05$; Fig. 3). However, biofilms grown on the pre-coated surface, like the planktonic cultures of the tested *C. albicans* strains, appeared to lack persisters completely, in which there were no detectable survivors after amphotericin B treatment.

CLSM Visualization of *Candida albicans* Biofilms and Persisters

We stained the biofilms of *C. albicans* 3153A with a LIVE/DEAD FungaLight yeast viability kit, which contains two dyes, SYT0®9 and propidium iodide. Yeasts with intact cell membranes stain fluorescent green, whereas yeasts with damaged membranes stain fluorescent red.

Our data showed that *C. albicans* biofilm formation on the surface of wells of microtiter plates proceeded in four distinct developmental phases: adhesion of *C. albicans* cells to the surface; proliferation and co-aggregation; production and release of exopolymer matrix; and biofilm maturation. In the adhesion phase

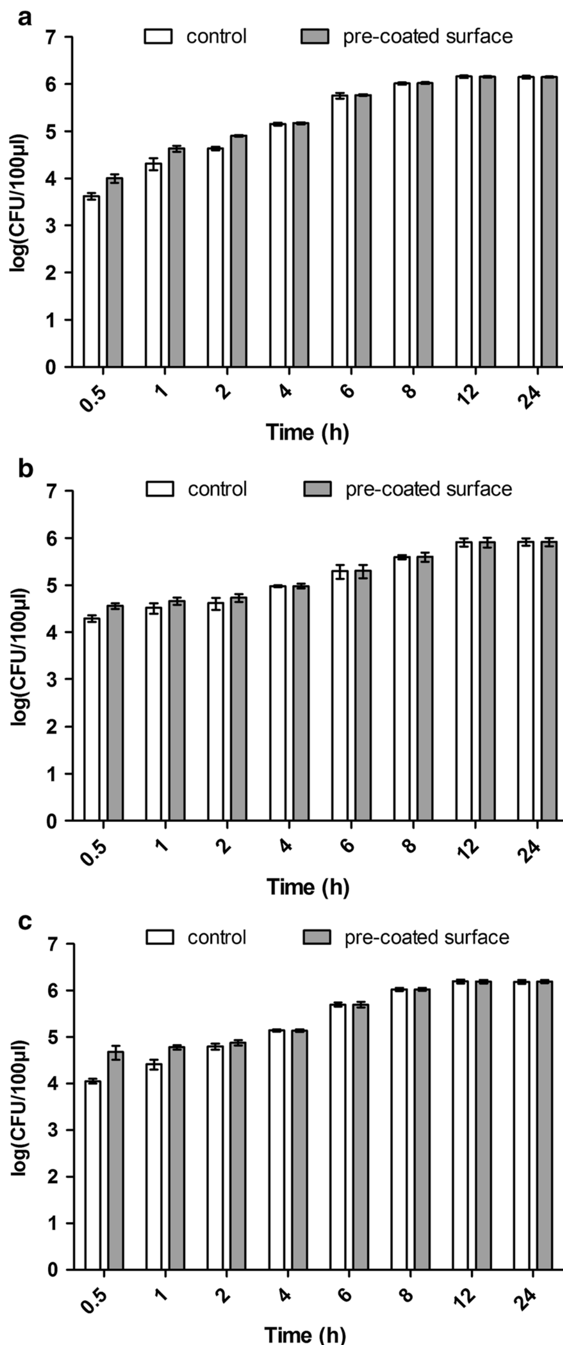

Fig. 3 Effect of pre-coated surfaces on *C. albicans* growth in the biofilm. Biofilms are incubated on the pre-coated surface of microtiter plates. Growth levels in biofilms of *C. albicans* 3153A (**a**), *C. albicans* sc5314 (**b**) and *C. albicans* YEM30 (**c**). *Error bars* indicate standard deviations, and results are from 16 independent experiments

(0–2 h), *C. albicans* cells, in the form of blastospores, adhered to the surface in a random manner (Fig. 4a). At 2–4 h, co-aggregation among *C. albicans* cells led to the emergence of microcolonies on the surface of the microtiter plates, following which the yeast cells budded and started to form hyphae (Fig. 4b). At 4–6 h, *C. albicans* cells presented as yeast cells, pseudohyphae and hyphae and tended to aggregate along the surface irregularities. By 6 h, the emergence of exopolymer matrix was apparent in the form of a "mist-like" covering of the microcolonies. At 8–12 h, cell density increased, and multilayer cells containing all fungal morphologies covered the attachment surface in an intricate network of spatially scattered, intertwined hyphae (Fig. 4c). As the biofilm matured after 24 h of growth, a dense network of yeast cells aggregating along the hyphae covered the entire well surface (Fig. 4d). Fluorescent red was rarely detected in biofilms.

After exposure to amphotericin B, the majority of biofilm cells were killed and a very small fraction of cells survived, which were persister cells (Fig. 4e–h). These rare surviving persisters were morphologically unremarkable and looked like regular cells in biofilms untreated with amphotericin B. The results showed that *C. albicans* persisters emerged immediately upon adhesion to the surface (0–2 h; Fig. 4e). As the biofilms matured, fluorescent green was still rarely detected in the treated biofilms (2–24 h; Fig. 4e–h).

Discussion

Persisters with high tolerance to antibiotics are described as dormant variants of regular cells that are produced stochastically in microbial populations [10, 16, 21, 23, 24]. These cells may be an unappreciated source for the recalcitrance of chronic infectious disease [18, 24]. Now there were some landmark studies demonstrated that the level of persister formation was closely associated with the duration of microbes in vivo and that persisters were directly linked to the clinical manifestation of disease [18, 35]. However, the mechanism underlying persister formation remains unclear.

Previous studies have reported that *C. albicans* persisters, in contrast to their bacterial counterparts,

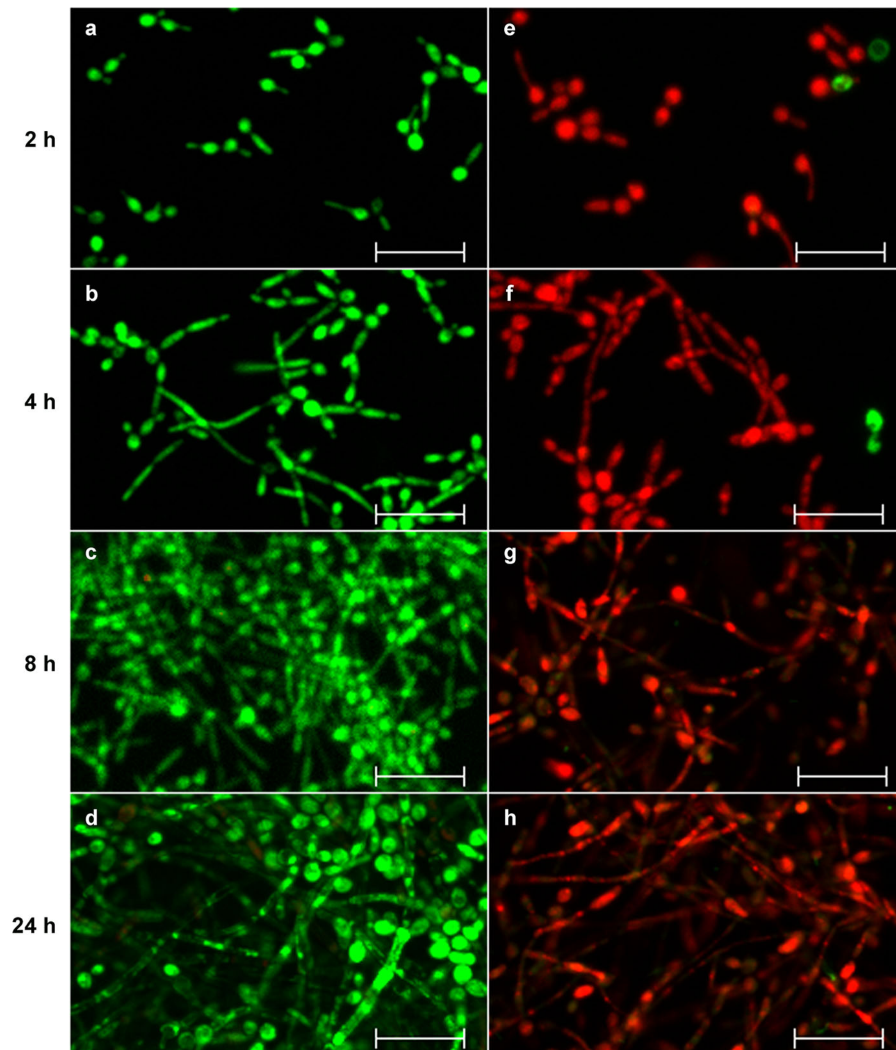

Fig. 4 Live/dead cell analysis of *C. albicans* biofilms *C. albicans* 3153A. Biofilms are incubated in the wells of microtiter plates over a series of time intervals (2, 4, 8 and 24 h). CLSM micrographs are taken at ×200 magnification and *scale bar* equals 20 μm. Images of biofilms (**a**–**d**) and biofilms after amphotericin B treatment (**e**–**h**). Live cells stain fluorescent green, whereas dead cells stain fluorescent red

could only be detected in the biofilm state [16, 17, 20]. In the present study, we again confirmed that *C. albicans* persisters were observed only in biofilms while the planktonic cultures lacked persisters completely. However, the persister population was small and accounted for only 0.01–0.02 % of the total cell population of the mature 24-h biofilms. The proportion of persisters is similar to reports by Al-Dhaheri and Douglas but much less than that detected by LaFleur et al. [16, 17]. The reason for the disparity may be due to the different *C. albicans* strains employed in the two studies.

However, it is worth noting that in the present study, persister population was discovered in biofilms of *C. albicans* strain SC5314, representing approximately 0.014 % of the total cell population of mature 24-h biofilms. The result is diametrically opposite to that of Al-Dhaheri and Douglas, who demonstrated that persisters were absent in biofilms formed by *C. albicans* SC5314 [17, 19]. But the previous studies only tested the mature 48-h biofilms, whereas our kinetic study has more discriminatory power.

Candida species biofilm formation has been reported to proceed through early, intermediate and

maturation phases [34, 36]. Similar to previous findings [34], we found that *C. albicans* biofilm formation on the surface of microtiter plates proceeded through adhesion (0–2 h), proliferation and co-aggregation (~2–6 h), matrix formation (~6 h) and maturation (~24 h) phases. During our dynamic monitoring of persister formation in biofilm, we found that once they adhered to the surface, *C. albicans* persisters emerged rapidly, and the number of persisters reached a plateau by 2 h (Fig. 1). In our study, CLSM images revealed that *C. albicans* cells were present as blastospores adhering to the surface at 0–2 h (adhesion phases) and that beyond 2 h, some of the fungal cells began to aggregate and formed microcolonies, which then merged and produced a three-dimensional structure surrounded by exopolymer matrix [4, 7, 36]. With the maturation of biofilm, the number of persisters did not significantly increase. The results indicate that *C. albicans* persisters are mainly produced during the adhesion phase.

Unexpectedly, we observed that for all tested *C. albicans* strains, persisters were completely absent in biofilms grown on the pre-coated surface with *C. albicans* strain CAI-4. We do not understand why persisters were absent from biofilms grown on the "yeast layer," but it did. The results suggested that the formation of *C. albicans* persisters was closely related to the adhesion of *C. albicans* cells to the surface, rather than the complex architecture or other steps of biofilm formation, such as aggregation, morphogenetic transition and secretion of exopolymer matrix. This finding is consistent with a previous study that suggested some mutant strains defective in both hyphal and biofilm formation were able to produce wild-type levels of persisters [16]. Considering these findings, we speculate that surface adhesion, rather than other steps of biofilm formation, is closely related to the formation of *C. albicans* persisters.

In the present study, we also found persister cells were completely absent in biofilms that were scraped to disrupt surface adhesion prior to amphotericin B treatment. This finding suggests that persisters are revived from a dormant state and lose their antifungal tolerance when surface adhesion is disrupted. The revival of persisters has been mentioned previously, although the underlying mechanisms are unknown [24]. The above results demonstrate that surface adhesion is required not only for the formation of *C. albicans* persisters but also for their maintenance.

In summary, we speculate that fungal adhesion to surface triggers cell signal transduction systems prior to biofilm formation. As a result, a subpopulation of cells transfers to persister cells, which is controlled by unknown mechanism. This special state protects cells from lethal interactions between antifungal and their targets. Further studies are required to determine differential gene expression profiles from persisters and regular cells in order to elucidate the molecular basis required for persisters.

Conclusion

This study demonstrates that *C. albicans* antifungal-tolerant persister cells are produced mainly in the surface adhesion phase of biofilm formation. Furthermore, our findings indicate that that surface adhesion, rather than other steps of biofilm formation, is required for the formation and maintenance of *C. albicans* persisters.

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