NON-LETHAL *CANDIDA ALBICANS CPH1/CPH1 EFG1/EFG1* TRANSCRIPTION FACTOR MUTANT ESTABLISHING RESTRICTED ZONE OF INFECTION IN A MOUSE MODEL OF SYSTEMIC INFECTION

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The *cph1/cph1 efg1/efg1 Candida albicans* mutant cells were non-lethal in a mouse model of systemic infection. We investigated *in vivo* proliferation and invasion of *C. albicans* cells in infected mice to elucidate the interaction between the host and the pathogen. Homogenates of kidneys from the mice infected with the wild-type and the mutant *C. albicans* cells yielded a mean of 2.1 x 10^7 CFU/g and 2.2 x 10^6 CFU/g, respectively. The kidneys from the mice infected with the wild-type cells showed extensive renal cortical necrosis associated with neutrophilic infiltration. There were also wild-type hyphal cells present in abundance. Hence, tubular necrosis leading to renal failure in the mice may be the cause of death. Although the *cph1/cph1 efg1/efg1* mutant cells were not lethal, they were capable of establishing restricted zones of infection and colonization near the renal pelvis instead of simply being cleared by the immune system in mice.

In the healthy host, *Candida* species are opportunistic fungal pathogens commonly colonizing human mucosal surfaces. *Candida* species cause not only minor infections in immunocompetent individuals (such as thrush in babies and vaginal infections in women) but also systemic infections that can be lethal in immunocompromised individuals (1). How these microorganisms switch from harmless commensals to pathogens when the opportunity arises is not clear.

Candida albicans can switch from a unicellular yeast form into two distinct filamentous forms, pseudohyphae and hyphae. The ability to switch is regulated by at least two independent pathways: a conserved mitogen-activated protein kinase pathway mediated by the transcription factor Cph1p and a cyclic AMP protein kinase A pathway mediated by another transcription factor Efg1p (2-3). Under culture conditions where the wild-type cells are highly induced to form hyphae, for instance in the presence of serum, the *cph1/cph1 efg1/efg1* mutant cells fail to form either pseudohyphae or hyphae (4). Furthermore, injections of less than

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$1x10^6$ of *cph1/cph1 efg1/efg1* mutant cells into mice is not lethal (4). The mutant cells also fail to produce gastrointestinal (GI) tract lesions in a piglet model (5-6).

In order to establish an infection, cells of *C. albicans* have to evade the immune system, survive and propagate in the host environment and then spread to new tissues. Riggle et al showed that the *cph1/cph1 efg1/efg1* mutant cells formed filaments in a matrix or on rough surface. Furthermore, immunosuppressed gnotobiotic newborn piglets infected with these mutant cells developed mild thrush lesion and superficial lesions of the eyes (7). Apparently, the *cph1/cph1 efg1/efg1* mutant cells are not completely defective in the functions associated with virulence. In this study, we investigate the in vivo proliferation and invasion of *C. albicans* cells in infected kidneys in order to shed light on why the wild-type cells but not the *cph1/cph1 efg1/efg1* mutant cells are lethal to mice in the model of systemic infection.

MATERIALS AND METHODS

Strains and media

The strains used in this study were SC5314 as the wild-type strain, (8) and *HLC54, ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG-URA3-hisG* as the mutant strain (4). Yeast Peptone Dextrose (YPD, 1% yeast extract, 2% peptone, and 2% dextrose), and Synthetic Dextrose (SD, 0.67% yeast nitrogen base without amino acid and 2% dextrose) were prepared as described in Sherman et al (9).

The mouse model for virulence

The mouse model for virulence was modified from the previous report (4). BALB/c mice (white, male), from the National Laboratory Animal Center in Taiwan, weighing between 18 and 20 g, were used to test the virulence of different Candida strains. An experiment was initiated by growing the cells on YPD plates for 2 days at 30°C, suspending the cells in saline and adjusting them to the desired concentrations after measuring the OD. The actual concentration was verified by scoring the cells in a hemocytometer and by plating to determine the viable count. Each Candida strain was tested for virulence by injecting 0.5 ml of the cell suspension into the tail vein of a mouse (1×10^6 cells per mouse). Six mice were injected for each strain and were monitored daily for their survival. These studies were carried out in accordance with the NIH Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act in an AAALAC-accredited program. The protocol, NHRI-IACUC-92008, for this study was approved by the Institutional Animal Care and Utilization Committee (IACUC) at National Health Research Institutes (NHRI).

Histopathological evaluation

The left-side kidney of each mouse was homogenized and the number of *C. albicans* cells within was scored. To determine the morphology of the organisms in kidney lesions, the right-side kidney of each infected mouse was fixed in 10% formalin and embedded in paraffin for tissue section. Hematoxylin and Eosin (H&E) stain and Grimelius Methenamine Silver (GMS) stain were performed for histopathological evaluation.

RESULTS

The cph1/cph1 efg1/efg1 mutant cells proliferated in mouse kidneys but were non-lethal

Candida cells were injected into the tail veins of mice and the survival of the mice was monitored daily. All mice infected with the wild-type cells died 4 days after injection. In contrast, injection of $1x10^6$ of *cph1/cph1 efg1/efg1* mutant cells did not cause death of any mouse even 20 days after the injection. Homogenates of kidneys from the mice infected with the wild-type and the mutant cells yielded a mean of 2.1×10^7 CFU/g (wet weight) and 2.2×10^6 CFU/g of tissue, respectively. These data demonstrate that the *cph1/cph1 efg1/efg1* mutant cells were non-lethal, despite the fact that they still proliferated in the kidneys.

The wild-type cells but not the cph1/cph1 efg1/efg1 mutant cells caused renal failure associated with extensive renal cortical necrosis in mice

To investigate why the wild-type cells but not the *cph1/cph1 efg1/efg1* mutant cells were lethal, we performed histopathological evaluation. The kidneys infected with the wild-type cells showed extensive renal cortical necrosis associated with heavy inflammation after H & E staining (Fig. 1A). Abundant neutrophilic infiltrations associated with marked tubular necrosis are shown in Fig. 1A1. The GMS stain was used to illustrate the physiological distribution and the morphology of Candida cells. Hyphal cells (Fig. 1B) with extensive filamentous growth were detected in abundance in the whole kidney (Fig. 1B1). The kidneys infected by the *cph1/cph1 efg1/efg1* mutant cells showed only mild and focal inflammation in the cortical region (Fig.

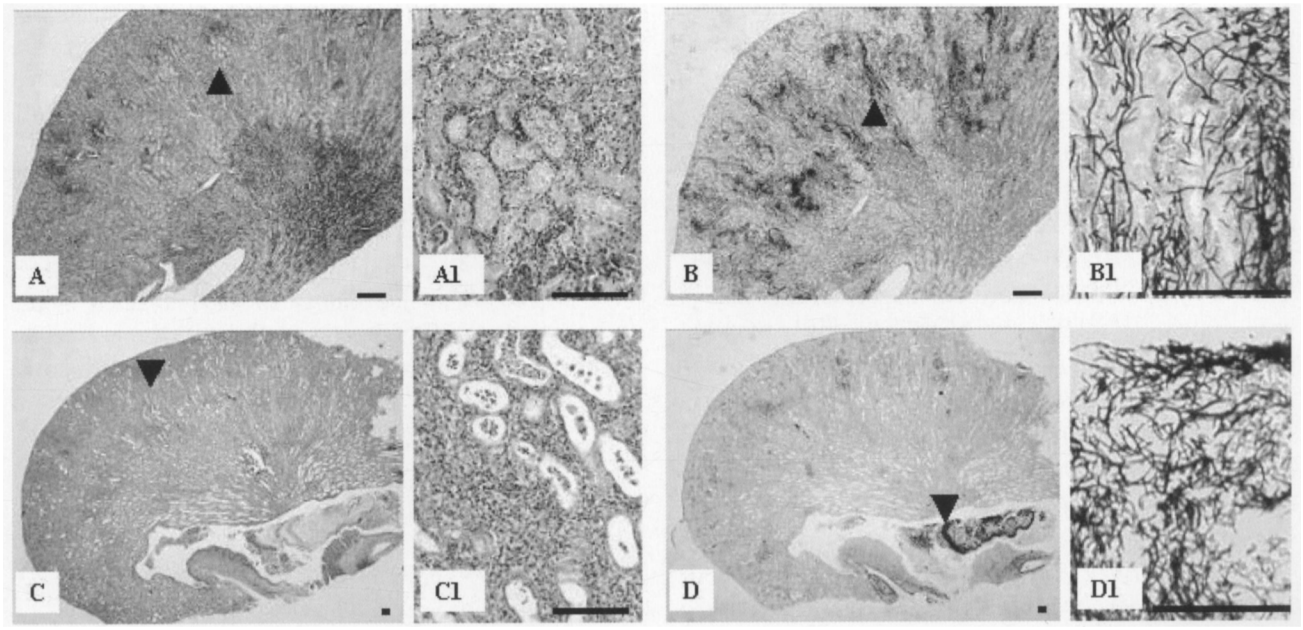

Fig. 1. *The histopathology of mouse kidneys after C. albicans infections. Each BALB/c mouse was injected with 1 x 10^6 C. albicans cells. A total of 6 mice were infected with each strain. The sections of kidneys were fixed and processed with the Hematoxylin and Eosin stain (A, A1, C and C1) and the Grimelius Methenamine Silver stain (B, B1, D, and D1). (A) and (B) were kidneys infected with the CPH1/CPH1 EFG1/ EFG1 wild-type strain (SC5314); (C) and (D) were kidneys infected with the cph1/cph1 efg1/efg1 mutant strain (HLC54). (A1)-(D1) were magnifications of (A)-(D). Arrows in A-D refer the areas that had magnification shown in A1 to D1. Scale bars of B1 and D1 refer to 125 μm and the remaining ones refer to 250 μm.*

1C). The renal tubules were still intact, though they were infiltrated by lymphocytes and neutrophils (Fig. 1C1). No mutant cell was detected in the cortical region, despite this region showing inflammatory cell infiltration (Fig. 1D). Interestingly, the mutants were present mainly in the pelvic cavity (Fig. 1D) and showed filamentous growth (Fig. 1D1).

DISCUSSION

The observation that in the BALB/c mouse model, the wild-type cells are lethal, whereas the *cph1/cph1 efg1/efg1* mutant cells are not, is consistent with the previous report with the CF mouse model (4). Though the *cph1/cph1 efg1/efg1*mutant cells fail to form filaments under standard conditions in culture media (4), they do form filaments under other conditions, such as in the tongues of immunosuppressed gnotobictic piglets, and when embedded in agar (7). Filamentous growth of the *cph1/cph1 efg1/efg1* mutant cells in the kidneys of immunocompetent mice was clearly observed in this study.

It has been reported that for the wild-type, the general pattern of infection after intravenous *C. albicans* challenge leads to rapid clearance of fungi from the bloodstream, primarily into the lungs and liver followed by an increasing population of fungi in the kidneys, which leads ultimately to the death of the animal from renal failure (10). This seems to be the cause in the mice infected with the wild-type cells in this study. One major difference observed in this study between the two *C. albicans* strains was that the wild-type cells invaded the whole kidney (Fig. 1B), whereas the mutant cells were restricted to the pelvic cavity (Fig. 1D). Interestingly, even though the mutant cells were non-lethal, they still caused focal inflammation of the kidneys. Furthermore, the neutrophiles were seen filing the renal tubules of the mice injected with the mutant cells (Fig. 1C1), although the mutant cells were not detected in the area around those tubules. It is very likely that the defense system was able to prevent mutants from invading and/or occupying those areas.

It has been reported that mutations on the *CPH1* gene do not affect the virulence of cells, whereas mutations on the *EFG1* gene reduce the virulence

of cells. Nevertheless, only when both CPH1 and EFG1 are mutated, will the virulence of Candida cells in the mouse model of systemic infection be abolished (4). Previous reports have also shown that more wild-type cells survive the host defense systems, such as macrophages or endothelial cells, than the *cph1/cph1 efg1/efg1* mutant cells do (4, 11). Though the *cph1/cph1 efg1/efg1* mutant cells still proliferated in the kidneys in this study, the amount of cells was 10-fold less than that of the wild-type cells. Furthermore, the *cph1/cph1 efg1/efg1* mutant cells were restricted in pelvic cavity despite those cells still forming filaments in the tissue. These observations suggest that the *cph1/cph1 efg1/efg1* mutant cells are more likely defective in invasion. In addition to filamentous growth, the Cph1p and Efg1p transcription factors also regulate genes such as *INT1, HWP1,* and other hyphal specific molecules, which may be involved in the process of virulence such as the adhesion of the hyphal cells (12-16). This is consistent with the observation that the adhesion ability of the *cph1/cph1 efg1/efg1* mutant cells is less than that of the wild-type cells (12, 14). The reduced invasion ability in the mutant cells can be explained by the fact that several aspartic proteinases are also regulated by Efg1p (17-18). In conclusion, both the adhesion and the aspartic proteinases assisting the invasion do not function properly in the mutant cells (19-21), resulting in the non-lethality phenotype.

Although the *cph1/cph1 efg1/efg1* mutant has been studied previously, this is the first report illustrating that the non-lethal mutant cells can establish restricted zones of infection or colonization near the renal pelvis instead of simply being cleared by the immune system in a mouse model of systemic infection. Hence, the *cph1/cph1 efg1/efg1* mutant cells still possess certain features to interfere with the host innate immune system, resulting in an alternation of the host response, such as switching from Th2 to Th1 (22). Thus, this phenomenon may explain the colonization of opportunistic pathogens in immunocompetent hosts. Our findings provide new insights that will help future studies aimed at dissecting the immune response to *C. albicans* infections.

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