Original Articles

Evaluation of Pathogenicity of *Candida albicans* in Germination-ready States Using a Silkworm Infection Model

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ABSTRACT

We previously developed an *N*-acetyl-D-glucosamine (GlcNAc) medium which induces *Candida albicans* to undergo a yeast-to-hyphal transition through a cAMP-PKA pathway. Microarray analysis demonstrated that 18 genes, including *ALS3* that encodes a cell wall adhesion, were upregulated by 30-min incubation of yeast cells at 37°C in the GlcNAc medium. To investigate the differences between morphological transition and morphotype in *C. albicans* as a consequence of infection, this study utilized a silkworm infection model as an invertebrate mini-host. We prepared 3 different conditions of *C. albicans* cells *in vitro* by changing the incubation times in the GlcNAc medium: yeast-form cells at 0 min (Y0 cells), yeast-form cells in germination-ready state at 60 min (Y60 cells), and hyphal cells at 120 min (H120 cells), and compared their pathogenicities. We performed the infection study at various temperatures to find temperature-dependent virulence factors *in vivo*. Y60 cells in germination-ready state in the GlcNAc medium showed higher pathogenicity *in vivo* compared to Y0 and H120 cells at 30°C. Y60 cells proliferated in silkworms 24 h post-injection at 30°C, whereas the other 2 cell types did not. *In vitro* analysis demonstrated that Y60 cells, but not Y0 cells, germinated in the silkworm hemolymph at 30°C. However, Y0 and Y60 cells showed a similar degree of germination in the silkworm hemolymph at 37°C, although no significant difference in silkworm survival after infection with each cell type was observed at 37°C. These results suggested that the germination-ready state induced by the GlcNAc medium contributed to virulence in the silkworm.

Key words : *Candida albicans*, morphogenesis, morphotype, *N*-acetyl-D-glucosamine, pathogenicity, virulence factor, silkworm

Introduction

Candida albicans is a human commensal colonizer of mucosal tissues (i.e., oral, vaginal, and gastrointestinal) and skin. *C. albicans* can become pathogenic, often causing an opportunistic infection in individuals with immune defects, such as oral candidiasis and vaginal candidiasis. The predominance of this fungus in the gastrointestinal tract often leads to a systemic dissemination. The increase in the number of reports on the emergence of resistance strains against frontline antifungal agents is currently considered as a potential health issue¹⁾. Therefore, identification

of virulence factors that can be targeted by novel antifungal agents is warranted.

To understand the virulence mechanism of *C. albicans*, a number of invertebrate mini-host systems using *Drosophila melanogaster*^{2,3)}, *Caenorhabditis elegans*^{4,5)}, and *Galleria mellonella*^{6–8)} have been developed. These invertebrate models offer a number of advantages over mammalian vertebrate models because of their small size and minimal associated ethical issues, and thus these models are considered useful in investigating *C. albicans* pathogenicity in mucosal infections^{9,10)}. *C. albicans* undergoes a morphological transition from yeast to hyphal form in response to various environmental stimuli, includ-

ing temperature, pH, and nutrients. Some theories suggest that the loss of morphological switching is accompanied by a diminished virulence^{11–13}. Some studies using invertebrate mini-host systems commonly focused on the morphological switching of *C. albicans*. Fuchs et al.¹⁴ recently evaluated the roles of genes involved in hyphal morphogenesis in *C. albicans* pathogenicity using the *G. mellonella* model maintained at 37°C. They demonstrated that filamentation is important, but not sufficient to kill *G. mellonella* and that other virulence factors may be associated with genes that regulate filamentation. Earliev, Hamamoto et al.¹⁵ developed a silkworm model for *C. albicans* infection to evaluate the effectiveness of antifungal agents and obtained results equivalent to those using a mouse model. Hanaoka et al.¹⁶ also used a silkworm model to evaluate the role of the protein phosphatase gene in *C. albicans* pathogenicity. Interestingly, all pathogenicity studies using silkworm infection models were performed specifically at 27°C.

In this study, we evaluated the consequence of morphological transition and morphotype in *C. albicans* infections using the silkworm infection model. Several reports indicated that the positional effects of *URA3* influenced the phenotypes of morphological switching and virulence in *C. albicans* mutants^{17–20}. In addition, it has also been recently shown that genetic manipulation of *C. albicans* can lead to aneuploidy, which in turn may occasionally influence the degree of virulence²¹. Therefore, the results obtained from these molecular biotechnological analyses could potentially lead to incorrect interpretations of the potential contributions of specific genes in pathogenesis. To reduce problems related to genetic manipulation of the *C. albicans* genome, we developed a strategy that does not require the use of genetic manipulation. We previously developed a hyphal-inducing *N*-acetyl-D-glucosamine (GlcNAc) medium²², in which 3 different cell stages of *C. albicans* were prepared by altering the incubation times at 37°C: yeast-form cells at 0 min, yeast-form cells in germination-ready state at 60 min, and hyphal cells at 120 min. Morphological events in *C. albicans* maintained *in vitro* are strongly influenced by temperature. The capability of maintaining silkworms at various temperatures serves as an advantage for this invertebrate animal model, allowing us to evaluate temperature-dependent pathogenicities in *C. albi-*

cans.

Materials and Methods

Strain and growth conditions

C. albicans strain SC5314 was maintained as frozen glycerol stocks at –80°C before streaking on YPD (1% yeast extract, 2% Bacto-peptone, and 2% glucose) agar medium at 37°C. Germ tubes were induced in a GlcNAc medium (L-thiazolidine-4-carboxylic acid, L-proline, sodium bicarbonate, sodium phosphate monobasic, sodium acetate, and *N*-acetyl-D-glucosamine, pH 6.65) according to the method described by Cho et al.²². The logarithmic phase cells (10^6 cells/ml) cultured in YPD medium at 37°C with constant agitation were treated with 20 mM hydroxyurea (HU; Wako) for 1 cell cycle to synchronize the cell cycle. HU-treated cells were then inoculated into the GlcNAc medium at a final concentration of 1×10^5 cells/ml and maintained at 37°C. The GlcNAc medium promoted the transition from yeast to hyphae without cellular growth after incubation for 60 min, reaching around 100% transition at 120 min. Cells were collected at 0 min (cells in yeast form), 60 min (cells in yeast form but ready to germinate and enter hyphal growth), and 120 min (cells in a hyphal form), and washed with phosphate buffer. The hyphal cells incubated in the GlcNAc medium for over 120 min aggregated during sample preparation.

Silkworm infection experiments

Silkworms were infected with *C. albicans* following the protocol of Hamamoto et al.¹⁵ Fifth instar larvae of the o211 silkworm strain were provided by the Institute of Genetic Resources Faculty of Agriculture, Kyushu University, which is a participant in the National Bio-Resource Project of the MEXT, Japan. On the first day, the larvae were fed for the entire day with an adequate amount of mulberry leaves at room temperature (25°C), those reaching an approximate body weight of 1.2 g were collected the next day. *C. albicans* cells ($50 \mu\text{L}$) were injected into the hemolymph through the dorsal surface using a 26-gauge needle ($1\text{--}3 \times 10^4\text{--}10^6$ cells/g-silkworm). After injection, the silkworms were placed at 20°C, 30°C, and 37°C without food and their survival monitored at 10, 21, 24, 32, 48, 58 h post-injection. More than 15 silkworms were used for each condition, with the experiments replicated, making a total of more than 30

silkworms.

The morphology of *C. albicans* these are known to thrive within their hosts and was observed by injecting silkworms ($n = 3$) with the fungus (10^5 cells/g-silkworm), incubating at 30°C for 24 h, fixing in 15% formalin neutral buffer solution (Sigma), and embedding in paraffin. Sections were transversely cut and stained with periodic acid Schiff (PAS). To count the number of *C. albicans* cells in silkworms 24 h after injecting 10^5 cells/g silkworm at 30°C, the silkworms ($n = 5$) were wiped with swabs, homogenized, and diluted with phosphate buffer. The samples were then plated on a YPD agar medium, incubated at 37°C for 24 h, and colonies emerging on the plate were counted.

Morphogenesis of *C. albicans* in hemolymph *in vitro*

Hemolymph was collected from larvae by cutting off the rear legs according to the method described by Kaito et al.²³⁾. The hemolymph was diluted with insect physiological saline (IPS) buffer (150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris-HCl pH 6.9, 10 mM EDTA, and 30 mM sodium citrate). The suspension was centrifuged at $700 \times g$ for 5 min at 4°C to remove insect-cell components such as hemocytes. The supernatant was then passed through a $0.45\text{-}\mu\text{m}$ filter and retained as germ-free hemolymph. *C. albicans* cells were inoculated in 10% germ-free hemolymph in IPS for 2 h at 30°C or 37°C. The morphology of *C. albicans* was examined under a microscope, and the percentage of hyphal cells was determined by screening approximately 200 *C. albicans* cells.

Microarray analysis

Microarray analysis was conducted according to the method described by Cho et al.²⁴⁾. Briefly, *C. albicans* strain JCM9061 cells at logarithmic phase were adjusted to 3×10^4 cells/mL and incubated in the GlcNAc medium at 37°C for 30 min. In this condition, the cells underwent a morphological transition from yeast to hyphae as efficiently as that observed in *C. albicans* strain SC5314. Total RNA was extracted from the cells and microarray analysis was performed. Experiments were replicated, with the quality of the arrays examined during each assay. Genes that were shown to be upregulated by more than 2-fold relative to that of logarithmic phase cells were selected. Replicate experiments showed correlation coefficients of > 0.9979 .

Statistical analysis

Data are shown as means $\pm$ standard deviations obtained from at least 2 independent experiments. Statistical differences between groups presented in Fig. 2 were evaluated using a two-tailed student's *t* test. A *P* value of < 0.05 was considered significant.

Results

GlcNAc medium induced morphological transitions in *C. albicans*

We previously developed a GlcNAc medium that can induce *C. albicans* to morphologically transform from yeast to hyphae at 37°C²²⁾. In this medium, germ tubes started to emerge after 60 min, and almost 100% of the cells possessed germ tubes at 120 min of culture (Figs. 1A and 1B). We also confirmed by counting colony-forming units (CFU) that no yeast-form cells were growing in this medium (data not shown).

Cho et al.²⁴⁾ previously reported that the intracellular cAMP level of *C. albicans* increased after 180 min of incubation in the GlcNAc medium. We performed DNA microarray analysis to identify the genes affected by incubating *C. albicans* in this medium. Eighteen genes showing at least a two-fold upregulation after incubation in the GlcNAc medium for 30 min are listed in Table 1. These genes have been associated with GlcNAc catabolism, amino acid biosynthesis/permease, carbon metabolism including gluconeogenesis, glyoxylate cycle, and β -oxidation. In addition, the genes *ALS3*, which encodes an adhesin and is a cyclic AMP protein kinase A (cAMP-PKA) pathway dependent; *FRP1*, which encodes ferric reductase; *HSP12*, which encodes a heat-shock protein; and *DQD1*, which encodes a putative 3-dehydroquinase dehydratase, were found to be upregulated. Several genes downregulated at least two-fold were also observed, with the majority of these genes involved in protein synthesis (data not shown).

Effect of *C. albicans* morphotype on silkworm mortality

To examine whether the morphotype of *C. albicans* influenced the pathogenicity in silkworms, we prepared 3 types of *C. albicans* cells by changing their incubation time in the GlcNAc medium, namely 0 min, 60 min, and 120 min for the generation of Y0, Y60, and H120 cells, respectively (Fig 1B).

Fig. 1. *C. albicans* transition from yeast to hyphal form in the GlcNAc medium. *C. albicans* at logarithmic phase (10^5 cells/ml) was inoculated in the GlcNAc medium and incubated at 37°C. (A) Time-dependent transition of *C. albicans* from yeast to hyphae. The percentage of germ tube formation at various time points was determined through microscopic examination of at least 100 cells. (B) Morphology of *C. albicans* at various incubation times in the GlcNAc medium. Bar represents 10 μm.

In order to examine whether *C. albicans*-induced silkworm mortality follows a dose-dependent manner, larvae were injected with 10^6 , 10^5 , and 10^4 *C. albicans* Y0 cells and their mortality at 30°C was recorded. As shown in Fig. 2A, over 60% of the larvae injected with *C. albicans* (1×10^6 cells) were killed within 24 h and 100% within 40 h. Larvae injected with 10^5 cells showed 100% survival at 24 h, whereas a 50% larvae mortality was observed within 45 h. Over 70% of the larvae injected with 10^4 cells were alive even at 50 h post-injection. As shown in Fig. 2B, the larvae infected with *C. albicans* turned black in color, possibly because of the melanization process described by Hamamoto et al.¹⁵⁾. In contrast, all of the larvae injected with heat-inactivated cells (10^6 cells) or phosphate buffer survived 58 h post-injection (Fig. 2A). These results indicated that whole, living cells of *C. albicans* killed silkworms in a dose-dependent manner, which corresponds to the results of Hanaoka et al.¹⁶⁾. Examination of the morphology of *C. albicans* in the larval tissues also showed that 24 h after inoculation of 10^5 cells

of the fungus Y0 cells showed the presence of both yeast and hyphal forms adhering to the tissues (Fig. 2C).

We compared the effect of *C. albicans* morphotype on its pathogenicity in silkworms by injecting each cell type, Y0, Y60, and H120 cells. The survival rate of those incubated at 30°C is shown in Fig. 3A; no apparent differences were observed between the Y0 cells and H120 cells. However, Y60 cells showed higher pathogenicity than the other 2 cell types, indicating that a morphological transition from yeast to hyphae, but not of yeast-form or hyphal cells, is involved in the pathogenesis of our silkworm model. We also addressed the question whether mortality of silkworms is associated with the proliferation of *C. albicans* in infected larvae. We counted the CFU from injected silkworms 24 h post-injection. The groups injected with 1×10^5 Y0 cells or H120 cells showed a lower number of CFU than that injected with Y60 cells (Fig. 3B). The group injected with Y60 cells showed a proliferation of *C. albicans* at 24 h post-injection. These results correlated well

(a)

(b)

(c)

Fig. 2. (A) Survival rates of larvae infected with *Candida albicans* Y0 cells. Yeast-form cells at 0 min after incubation in the GlcNAc medium (Y0 cells) were prepared as described in Materials and Methods and then injected into larvae (10^4 , 10^5 , and 10^6 cells/g-silkworm). The silkworms were kept at 30°C and those surviving were counted at various time points. *C. albicans* suspensions in phosphate buffer and heat-treated cells (10^6 cells/g-silkworm) injected into the larvae were used as control. (B) Living larvae before injection (left) and dead larvae at 48 h post-injection of *C. albicans* (10^6 cells/g-silkworm). (C) PAS staining of fungal cells in silkworm tissues. Y0 cells (10^5 cells/g-silkworm) were injected into larvae. Larval tissue sections were obtained 24 h post-injection and at 30°C . Bar represents $10\mu\text{m}$.

with the higher pathogenicity of Y60 cells. The infection model at 37°C enables the study of temperature-related virulence of *C. albicans*, especially when the normal human body temperature is also 37°C . An advantage of silkworms and *G. mellonella* over other invertebrate mini-hosts is

their survival at 37°C^{10} . We also observed a slightly higher pathogenicity using Y60 cells compared to the other 2 cell types at 37°C (Fig. 3A). Comparison of 32-h survival rates between Y0 and Y60 cells at 30°C and 37°C showed a small 1.9-fold increase in mortality at 37°C ($P = 0.096$), whereas a

Fig. 3. Effect of *C. albicans* morphotype on its pathogenicity in silkworms. Yeast-form cells at 0 min after incubation in the GlcNAc medium (Y0 cells), yeast-form cells in germination-ready state at 60 min (Y60 cells), and hyphal cells at 120 min (H120 cells) were prepared as described in Materials and Methods. (A) Silkworms were injected with each cell type (10^5 cells/g-silkworm) and incubated at various temperatures, and their survival rates were determined at various time points. (B) Comparison of *C. albicans* burden in silkworms based on cell type. Silkworms ($n = 5$) injected with Y0, Y60, or H120 cells were homogenized 24 h post-injection. The samples were diluted, plated onto a YPD agar medium, and incubated at 37°C for 24 h. Colonies that emerged on the agar plates after 24 h were counted. Each dot indicates the number of *C. albicans* in 1 silkworm. The horizontal line represents the value of the group. (C) Comparison of survival rates in Y0 and Y60 cells 32 h post-injection at 30°C and 37°C.

significant 3.2-fold increase ($P = 0.0014$) was observed at 30°C (Fig. 3C). The infection assay of injecting the 3 types of the cells (10^5 cells) into the larvae at 20°C resulted in a 100% survival rate at 58 h post-injection (Fig. 3A), similar to the results

obtained after injecting heat-inactivated cells or phosphate buffer.

Morphogenesis in hemolymph *in vitro*

Inoculation of *C. albicans* in 10% hemolymph *in*

vitro showed that it remained in its yeast form in Y0 cells after 2 h of incubation at 30°C. In contrast, approximately 70% of the Y60 cells germinated 2 h post-injection (Figs. 4A and 4C). At 37°C, no differences were observed in either cell type; approximately 90% of the cells germinated at 2 h (Figs. 4B and 4C), indicating that Y60 cells have a higher capacity to undergo a morphological transition than Y0 cells at 30°C. In addition, these results also suggest that silkworm hemolymph may contain hyphal-inducing factors.

Discussion

Animal models have been used to investigate virulence factors in *C. albicans* applying various research techniques. Mutant strains of *C. albicans* have also been commonly used for infection studies. We developed a new approach for evaluating the pathogenicity of 3 different GlcNAc-induced *C. albicans* cell conditions in a silkworm infection model.

Our results showed that silkworm mortality was dependent on the concentration of injected cells (Fig. 2). Y60 cells proliferated to an average of 10⁶ cells CFU in the silkworms and induced higher mortality than the other 2 cell types (Fig. 3), thereby indicating higher pathogenicity. Y60 cells showed a yeast form, but its cellular condition was different from that of Y0 cells. Our microarray analysis demonstrated that the GlcNAc medium induced the upregulation of some genes including cAMP-PKA dependent genes by 30-min incubation (Table 1). We propose that at least upregulation of cAMP-PKA dependent genes would be maintained in Y60 cells because Cho et al.²⁴⁾ reported a time-dependent increase of intracellular cAMP level during 180-min incubation in the GlcNAc medium. Therefore, Y60 cells were ready for germination and could transition from yeast to hyphae when cultured in 10% silkworm hemolymph for 2 h at 30°C, whereas Y0 cells could not. H120 cells were hyphal in form but showed the same level of pathogenicity as that observed in Y0 cells, suggesting that the transition from yeast to hyphae, but not the morphotypes of yeast-form and hyphal cells, is involved in *C. albicans* virulence in the silkworm. The morphological switch is a complex transition process involving changes in cellular form and properties, including adhesion and invasion into host cells. We found a marked upregulation of *ALS3* by 30-min incubation

in the GlcNAc medium, which facilitates mucosal invasion through the epithelial receptor E-cadherin and is a cAMP-PKA pathway dependent gene^{25, 26)}. Y60 cells may thus possess higher ability to adhere to the host cells than Y0 cells. Although H120 cells in hyphal form should highly express Als3p on the cell surface and thus adhere to the tissues, the cells showed reduced virulence compared to Y60 cells, which supports that the transition from yeast to hyphae is important for *C. albicans* virulence in the silkworm. Silkworm possesses several defense mechanisms in its innate immune system. Hemocytes are responsible for a number of defense responses in insects, including phagocytosis, nodulation, and melanization²⁷⁾. We propose that Y60 cells are capable of undergoing rapid transition from yeast to hyphal form *in vivo*, thus evading the host's immune responses, and resulting in their proliferation and tissue colonization.

On the basis of the capacity of silkworms to survive at the human physiological temperature of 37°C, we thus performed an infection study at this temperature (Fig. 3A). We observed a slightly higher pathogenicity of Y60 cells compared to other cell types. However, the difference in mortality between the Y0 and Y60 cells at 32 h post-injection was less apparent at 37°C (Fig. 3C). We observed no differences in cell morphology between Y0 and Y60 cells cultured in 10% hemolymph and maintained at 37°C, with both cells showing the same extent of germination (Fig. 4C). This result may explain no significant difference in mortality rate between the Y0 and Y60 cells observed at 37°C as shown in Fig. 3C. In addition, these data suggest that other virulence factors may be involved in silkworm infection at 37°C. We also found a marked upregulation of the genes involved in the GlcNAc metabolism and amino acid biosynthesis by 30-min incubation in the GlcNAc medium (Table 1). However, the virulence of these genes has not yet been clarified and we plan to investigate the role of these genes in silkworm infection in the future.

To date, invertebrate mini-host systems including *D. melanogaster*^{2, 3)}, *C. elegans*^{4, 5)}, *G. mellonella*^{6–8, 14)} and, more recently, Zebra fish²⁸⁾ have been developed to study the virulence factors of *C. albicans* in epithelial infections. In this study, we used the silkworm model because of its low cost, the association of minimal ethical

(a)

(b)

(c)

Fig. 4. Morphogenesis of *C. albicans* in hemolymph cultures. *C. albicans* Y0 and Y60 cells were inoculated in 10% germ-free hemolymph for 2 h at 30°C (A) or 37°C (B). The morphology of the cells was determined by microscopic examination. Bar represents 10 μm. (C) The percentage of hyphal cells was determined by observing 200 cells.

Table 1. Genes upregulated by incubation in the GlcNAc medium^a

Category	Gene	Function	Fold change
GlcNAc utilization	<i>NAG1</i>	Glucosamine-6-phosphate deaminase	83.8
	<i>DAC1</i>	GlcNAcN-6-phosphate deacetylase	76
	<i>NGT1</i>	GlcNAc-specific transporter	66.9
	<i>HXK1</i>	GlcNAc kinase	33.8
Amino acid biosynthesis/permease	<i>ILV5</i>	Valine biosynthesis	4
	<i>ARO8</i>	Aromatic amino acid aminotransferase	4.6
	<i>LYS2</i>	Lysin biosynthesis	4.4
	<i>ARG1</i>	Arginine biosynthesis	22.1
	<i>GAP1</i>	Amino acid permease	13.2
Carbon metabolism	<i>CIT1</i>	Citrate synthase	2
	<i>FAA23</i>	Fatty acyl-CoA synthetase	6.3
	<i>FBP1</i>	Fructose-1,6-bisphosphatase	4.3
Others	<i>ALS3</i>	Hypha specific ALS family cell wall adhesin	23.6
	<i>FRP1</i>	Ferric reductase	4
	<i>HSP12</i>	Heat-shock protein	4.6
	<i>DQD1</i>	Putative 3-dehydroquinate dehydratase	4.7
	IPF4119.5	Unknown function	4.7
	IPF6617.repeat1	Unknown function	3.7

a. Genes upregulating more than 2-fold relative to that of logarithmic phase cells before

issues, and its ease of manipulation. Our findings suggested that the yeast to hyphal transition induced by the GlcNAc medium contributed to the virulence of *C. albicans* in the silkworm. Our findings provide a convenient approach to investigating the relationship between pathogenicity and morphological events in *C. albicans*. To validate the experimental approach and findings in this study, a mouse/rat mucosal infection model will be utilized in our future research studies.

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