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Relationship between *Candida albicans* Virulence during Experimental Hematogenously Disseminated Infection and Endothelial Cell Damage In Vitro

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Candida albicans **must penetrate the endothelial cell lining of the vasculature to invade the deep tissues during a hematogenously disseminated infection. We compared 27 *C. albicans* mutants with their wild-type parent for their capacity to damage endothelial cells in vitro and cause a lethal infection in mice following tail vein inoculation. Of 10 mutants with significantly impaired capacity to damage endothelial cells, all had attenuated virulence. Therefore, the endothelial cell damage assay can be used as a screen to identify some virulence factors relevant to hematogenously disseminated candidiasis.**

During the initiation of hematogenously disseminated candidiasis, blood-borne organisms must adhere to and penetrate the endothelial cell lining of the blood vessels to invade the deep tissues. One mechanism by which *Candida albicans* can penetrate the vascular endothelium is by damaging and eventually killing the endothelial cells. Damaged endothelial cells detach from the basement membrane, leaving gaps through which the organism can invade. Also, the exposed basement membrane can be avidly bound by additional organisms (18).

C. albicans damages human vascular endothelial cells in vivo and in vitro (5, 10, 17, 25). Maximal endothelial cell damage (ECD) occurs in vitro when *C. albicans* adheres to and invades the endothelial cells and then secretes lytic enzymes (2, 10, 11, 13, 16). Moreover, some *C. albicans* mutants with filamentation defects cause significantly less ECD than the wild-type parent strain (24). These filamentation mutants also have attenuated virulence in various experimental models of infection (reviewed in reference 23). We hypothesized that the in vitro assay for *C. albicans*-induced ECD (ECD assay) can serve as a model for certain aspects of host-pathogen interactions in vivo, such as the ability of the organism to adhere to, invade, and injure host cells. This hypothesis predicts that some mutants with virulence defects will be defective in the ECD assay. The goal of the present study was to investigate this prediction.

C. albicans **strains.** The genotypes and sources of the *C. albicans* strains used here are listed in Tables 1 and 2. Each strain was grown overnight in yeast nitrogen base broth (Difco, Detroit, Mich.) supplemented with 2% glucose (wt/vol) at 20°C on a rotating drum. The blastospores were harvested by centrifugation, washed with phosphate-buffered saline, enumerated with a hemacytometer, and suspended in RPMI 1640 medium (Irvine Scientific, Santa Ana, Calif.).

ECD assay. We used our standard ^{51}Cr release ECD assay to determine the abilities of mutants of *C. albicans* to damage endothelial cells in vitro (24). The ECD assay was performed in 96-well tissue culture plates (Corning Inc., Acton, Mass.) with endothelial cells isolated from human umbilical cord veins, as described previously (24). The inoculum was 4×10^4 organisms per well, and the organisms were incubated with the endothelial cells for 3 h in 5% CO_2 at 37°C. At the end of the incubation period, the wells were examined with an inverted microscope, and the morphology of each strain was recorded. Each strain was tested in triplicate on two separate occasions. The wild-type parent strain, SC5314, was included as a positive control in all assays, and the results were expressed as a percentage of the ECD caused by this strain.

Virulence testing. To provide a uniform assessment of the virulence of all mutants, we grew and processed them as described for the ECD assay, except that the growth medium was YPD broth (1% yeast extract, 2% peptone, and 2% glucose [wt/vol]) and the organisms were suspended in phosphate-buffered saline. We infected 10 to 12 male BALB/c mice (20 g; National Cancer Institute, Bethesda, Md.) with 5×10^5 blastospores of each strain via the tail vein. All inocula were confirmed by colony counting. The mice were monitored at least three times daily, and moribund mice were euthanized. The survival of mice infected with the different mutants was compared with that of mice infected with the wild-type parent, SC5314. All mouse experiments were carried out according to the NIH guidelines for the ethical treatment of animals.

Statistics. Differences in ECD caused by the different strains were compared by using analysis of variance, and differences in survival of the mice were analyzed with the log-rank test. The Bonferroni correction for multiple comparisons was applied to all statistical analyses. P values of ≤ 0.05 were considered significant.

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TABLE 1. Relationship between the ability of *C. albicans* mutants to damage endothelial cells in vitro and virulence

Strain	Relevant genotype	Strain background[a]	Morphology on endothelial cells[b]	ECD (%)[c]	Mouse virulence (%)[d]	Reference
AS18	*tec1*::*hisG*/*tec1*::*hisG* pVEC	CAI4	Yeast; some hyphae	0 ± 4[e]	250[f]	28
CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434*		Yeast; a few pseudohyphae	2 ± 1[e]	>420[f]	12
CAN34	*cph1*::*hisG*/*cph1*::*hisG efg1*::*hisG*/*efg1*::*hisG-URA3-hisG*	CAI4	Yeast	3 ± 4[e]	>420[f]	22
CAP1-312	*pmt1*::*hisG*/*pmt1*::*hisG-URA3-hisG*	CAI4	Short hyphae	8 ± 6[e]	>420[f]	33
CAN33	*efg1*::*hisG*/*efg1*::*hisG-URA3-hisG*	CAI4	Yeast	13 ± 18[e]	275[f]	22
DAY25	*rim101*::*ARG4*/*rim101*::*URA3*	BWP17	Pseudoyphae; short hyphae	43 ± 4[e]	>420[f]	7, 8
CAS10	*phr1*::*hisG*/*phr1*::*hisG-URA3-hisG*	CAI4	Yeast; pseudohyphae	47 ± 4[e]	>420[f]	27
CLJ1	*cla4*::*hisG*/*cla4*::*hisG-URA3-hisG*	CAI4	Short hyphae	49 ± 11[e]	>420[f]	21
VIC25	*mds3*::*UAU1*/*mds3*::*URA3*	BWP17	Short hyphae	54 ± 5[e]	200[f]	9
AS1-pRC2312	*tpk2*::*hisG*/*tpk2*::*hisG* pRC2312	CAI4	Short hyphae	66 ± 10[e]	200[f]	30
CADW3	*cat1*::*hisG*/*cat1*::*hisG*/*cat1*::*hisG-URA3-hisG*	CAI4	Hyphae	75 ± 11	343[f]	36
BH52-1-17	*sap2*::*hisG*/*sap2*::*hisG-URA3-hisG*	CAI4	Hyphae	77 ± 3	138	15
CAL3-URA	*hwp1*::*hisG*/*hwp1*::*hisG ura3*::λ*imm434*/*URA3*::*ura3*::λ*imm434*	CAI4	Hyphae	78 ± 11	120	Unpublished[g]
CAN20	*cst20*::*hisG*/*cst20*::*hisG-URA3-hisG*	CAI4	Hyphae	81 ± 17	175[f]	19
BH24-15-1	*sap1*::*hisG*/*sap1*::*hisG-URA3-hisG*	CAI4	Hyphae	82 ± 14	113	15
DAY185	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*H1S1*::*his1*::*hisG arg4*::*hisG*/*URA3*::*ARG4*::*arg4*::*hisG*	BWP17	Hyphae	86 ± 21	129	7
BH1-1-1	*sap3*::*hisG*/*sap3*::*hisG-URA3-hisG*	CAI4	Hyphae	87 ± 17	75	15
CAP2-234	*pmt6*::*hisG*/*pmt6*::*hisG-URA3-hisG*	CAI4	Slightly shorter hyphae	88 ± 9	180[f]	34
CAI4-URA3	*ura3*::λ*imm434*/*ura3*::λ*imm434* pARG4-URA3	CAI4	Hyphae	89 ± 9	114	Unpublished
CKY141	*czf1*::*hisG*/*czf1*::*hisG-URA3-hisG*	CAI4	Short hyphae	93 ± 11	171[f]	4
BCa7-4	*rbt1*::*hisG*/*rbt1*::*hisG-URA3-hisG*	CAI4	Hyphae	96 ± 17	200[f]	3
DAY286	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*URA3*::*ARG4*::*arg4*::*hisG*	BWP17	Hyphae	97 ± 12	71	Unpublished
CAI12	*ura3*::λ*imm434*/*URA3*::*ura3*::λ*imm434*	CAI4	Hyphae	97 ± 13	86	35
CAL3	*hwp1*::*hisG*/*hwp1*::*hisG-URA3-hisG*	CAI4	Hyphae	98 ± 6	320[f]	29
SC5314	Wild type		Hyphae	100 ± 8	100	12
DSY459	*sap4*::*hisG*/*sap4*::*hisG sap5*::*hisG*/*sap5*::*hisG sap6*::*hisG*/*sap6*::*hisG-URA3-hisG*	CAI4	Hyphae	101 ± 10	175[f]	26
C	*int1*::*hisG*/*int1*::*hisG-URA3-hisG*	CAI4	Hyphae	104 ± 25	>420[f]	14
CAI4-GFP	pADH1-yEGFP3	CAI4	Hyphae	108 ± 15	86	6

[a] CAI4 genotype, *ura3*::λ*imm434*/*ura3*::λ*imm434*; BWP17 genotype, λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*; auxotrophies were complemented in all mutants except DAY286 (His$^-$) and CAI4 (Ura$^-$).

[b] After 3 h of incubation.

[c] Measured by specific ^{51}Cr release. Results are averages ± standard deviations of two experiments and are expressed as the percentage of ECD caused by strain SC5314. The average specific ^{51}Cr release caused by strain SC5314 was 25% ± 3%.

[d] Results are median survival times for mice infected with each strain (10 or 11 mice per strain) and are expressed as the percentage of the median survival time for mice infected with strain SC5314. The median survival of mice infected with strain SC5314 was 4 days (interquartile range, 3 to 5 days).

[e] $P < 0.05$ compared to the amount of ECD caused by strain SC5314.

[f] $P < 0.05$ compared to the survival of mice infected with strain SC5314.

[g] Sharkey et al., Abstr. 6th ASM Conf. Candida Candidiasis.

ECD$^-$ mutants had attenuated virulence. Of the 27 *C. albicans* mutants tested in the ECD assay, 10 caused significantly less damage to endothelial cells than the wild-type control strain (Table 1). We have designated mutants that cause reduced damage to endothelial cells ECD$^-$. To verify that the ECD$^-$ phenotype was the result of disruption of the genes of interest, we measured the ECD caused by the complemented strain of each ECD$^-$ mutant. The ECD caused by all of the complemented strains was similar to that induced by the wild-type control strain (Table 2).

Next, we investigated the virulence of each of the mutants in the murine model of disseminated candidiasis. Seventeen of

TABLE 2. ECD caused by complemented strains of *C. albicans*

Strain	Relevant genotype[a]	ECD (%)[b]
AS18	*tec1*::*hisG*/*tec1*::*hisG* pCaTEC1	102 ± 22
CAI4-URA3	*ura3*::λ*imm434*/*ura3*::λ*imm434* pARG4-URA3	89 ± 9
CADS5	*cph1*::*hisG*/*cph1*::*hisG efg1*::*hisG*/*efg1*::*hisG* (*EFG1*)	120 ± 23
CAP1-3121+pCT29	*pmt1*::*hisG*/*pmt1*::*hisG* pCaPMT1	93 ± 18
CAN35	*efg1*::*hisG*/*efg1*::*hisG* (*EFG1*)	102 ± 15
DAY44	*rim101*::*ARG4*/*rim101*::*URA3* pRIM101	87 ± 27
CAS11	*phr1*::*hisG*/*phr1*::*hisG* (*PHR1*)	122 ± 22
CLJ5+pVEC-CaCLA4	*cla4*::*hisG*/*cla4*::*hisG* pVEC-CaCLA4	88 ± 29
VIC21	*mds3*::*UAU1*/*mds3*::*URA3* pMDS3	103 ± 7
AS1-pCaTPK2	*tpk2*::*hisG*/*tpk2*::*hisG* pCaTPK2	86 ± 18

[a] Auxotrophies were complemented in all mutants. All mutants formed normal hyphae on endothelial cells after 3 h of incubation.

[b] Measured by specific ^{51}Cr release. Results are the averages ± standard deviations of two experiments and expressed as the percentage of ECD caused by strain SC5314. The average specific ^{51}Cr release caused by strain SC5314 was 24% ± 10%.

the 27 mutants had significantly attenuated virulence compared to the wild-type control strain (Table 1). The virulence of the complemented strains of 15 of these 17 mutants was tested either in this study (*ura3*/*ura3*) or previously by other investigators (3, 7, 9, 19, 22, 28, 30, 33, 34, 35) (all but the *cat1*/*cat1*/*cat1* and *czf1*/*czf1* strains) and was found to be restored.

Of note, all 10 strains that were ECD$^-$ were also significantly less virulent. The genes that were disrupted in the ECD$^-$ mutants specify transcription factors, signal transduction pathway members, and enzymes, the majority of which have been postulated or shown to have a role in virulence. Many of the ECD$^-$ strains had defects in signal transduction pathways that govern hyphal formation (Cla4p, Efg1p, Cph1p, and Tec1p) (20–22). It has been found that the *cla4*/*cla4* and *efg1*/*efg1* strains are ECD$^-$ at least in part, because they have reduced capacity to invade endothelial cells (24). Mds3p, Phr1p, and Rim101p all regulate filamentation in response to pH (8, 9, 27) and are required for maximal ECD. Interestingly, when the *mds3*/*mds3* strain was incubated with endothelial cells, it formed hyphae that were only about 30% shorter than those of the wild-type strain. Therefore, Mds3p likely governs the ability of *C. albicans* to damage endothelial cells independently of filamentation. Conversely, the *czf1*/*czf1* and *pmt6*/*pmt6* strains, which formed only short hyphae on the endothelial cells, were ECD$^+$. Therefore, *C. albicans* factors other than the ability to form long hyphae are necessary for the organism to damage endothelial cells.

Two of the ECD$^-$ strains did not have mutations in signal transduction pathways. The *pmt1*/*pmt1* strain, which is defective in O-mannosylation of multiple proteins, has been reported to adhere poorly to host cells because of reduced mannosylation of adhesins such as Als1p (33). Thus, this strain is probably ECD$^-$ because it adheres poorly. CAI4 (*ura3*/*ura3*), a simple uridine auxotroph, was also ECD$^-$. Uridine, in the form of UDP, is required to transfer sugars to carbohydrate polymers and glycoproteins, such as those that make up the cell wall. As a result, Ura$^-$ strains have decreased adherence to host cells (1), and we observed that the Ura$^-$ strain was unable to form true hyphae on endothelial cells in the absence of uridine. Therefore, the Ura$^-$ strain was almost certainly ECD$^-$ because of its defects in adherence and hyphal formation, rather than growth rate.

It has been reported that the locus at which *URA3* is integrated can influence the virulence of *C. albicans* (L. L. Sharkey, W.-L. Lia, and W. A. Fonzi, Abstr. 6th ASM Conf. Candida Candidiasis, abstr. S-11, 2002) (31, 32). We measured the amount of ECD caused by two different *hwp1*/*hwp1* mutants: CAL3, in which *URA3* was integrated at the *HWP1* locus, and CAL3-URA, in which *URA3* was integrated at its native locus. The extent of ECD caused by both strains was not significantly different from that caused by the wild-type strain (Table 1). However, CAL3 had significantly attenuated virulence in the mouse model, whereas CAL3-URA did not. Therefore, the results of the ECD assay appear to be relatively independent of the locus at which URA3 is integrated in *C. albicans*.

Although 17 of the 27 mutants had reduced virulence, 7 of them were ECD$^+$. Thus, the ECD assay has a significant false-negative rate. Such a result is not unexpected, because factors other than the ability to damage endothelial cells are clearly important for the normal virulence of *C. albicans*. Some of these mutants may be defective in virulence either before or after their interactions with endothelial cells. For example, the *cat1*/*cat1*/*cat1* strain has been shown to be more susceptible to leukocyte killing than the wild-type parent strain (36). Also, some genes, such as *TPK2*, have homologs (*TPK1*) in the genome that may compensate partially for the null mutant defect. Finally, it is possible that some of the strains that were ECD$^+$ when tested under the current conditions (3-h incubation with the endothelial cells in RPMI 1640 medium) might have been ECD$^-$ when exposed to the endothelial cells for different incubation times or in different media.

In summary, the *C. albicans* ECD assay identifies some classes of virulence factors. It also provides the opportunity for detailed analysis of certain host-pathogen interactions under controlled experimental conditions. We are currently using this assay to screen random insertion mutants of *C. albicans* to identify new virulence factors and virulence regulatory pathways.

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