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Identification of Fibronectin Adhesins on *Candida albicans*

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Abstract: The pattern of distribution of fibronectin adhesins on yeast and hyphal-form *Candida albicans* was studied with gold-labelled fibronectin and transmission electron microscopy. The germ-tube parts of the hyphal-form cells were the major binding sites for gold-labelled fibronectin, and the attached yeast-cell parts and yeast-form cell remained unlabelled. Therefore, the walls of the germ tube parts of hyphal-form cells might be sites of important interactions between *C. albicans* and host tissues containing fibronectin.

Key Words: *Candida abicans*, adhesin, germ tube, fibronectin

Introduction

Interest in the adherence of *C. albicans* to various surfaces has increased in recent decades. The adherence of microorganisms to various surfaces throws light on the mechanisms of pathogenesis and suggests means of controlling infection at an early stage. In the opportunistic dimorphic fungal pathogen *C. albicans,* the wall not only maintains the structural shape which characterizes each growth form, but is also the site of the initial interaction between the organism and its environment. *C. albicans* have been reported to have adhesins for extracellular matrix elements of human tissues such as fibrinogen, laminin and vitronectin (1-3).

Fibronectin binds to a variety of bacteria, including *Staphylococci* and *Streptococci Escherichia coli* and *Treponema pallidum* and also to *Saccharomyces cerevisiae* and *C. albicans* (3-11). In one study, radiolabelled yeast-form cells of *C. albicans* were deposited in fibronectin-coated microtiter wells. By counting the radioactivity in the washes of the wells, the authors determined that approximately 30 to 40% of yeast-form *C. albicans* cells adhered in a calcium-dependent fashion (8). It was shown that the adhesion of yeast-form cells to fibronectin on epithelial surfaces is mediated by surface proteins of *C. albicans* and *C. tropicalis* (8). The adhesion of *C. albicans* and *C. tropicalis* to subendothelial matrix proteins was reported by Klotz (12). These species adhered more avidly to the contracted monolayer, which exposed the subendothelial extracellular matrix, than to a confluent monolayer of bovine aortic endothelial cells (13). The binding of fibronectin by one of four clinical isolates of *C. albicans* was shown to be saturable, specific and reversible. Purified fibronectin possesses two peptide-form RGD sites in the fibronectin cell-binding domain, RGD and GRGDTP. All of these inhibited the binding of fluid-phase fibronectin to *C. albicans,* whereas carbohydrates, including D-glucose, α-methly-D-mannopyrannoside and D-mannose, did not (14). A glycoprotein on *C. albicans* was purified from a fibronectin-agarose affinity column using high-performance liquid chromatography. The adhesin consisted of approximately 75 to 80% carbohydrate and 20-25% protein by weight and existed in the form of an aggregate or multimer *in vitro* (15).

The initial proposition that cellular interactions of fibronectin with bacteria are beneficial, promoting phagocytosis and clearance through its adherent properties, has largely been supplanted by the idea that the binding of microoganisms to fibronectin is a step in their pathogenesis and is, in fact, detrimental to the host. *C. albicans* has been found to adhere better to epithelial cells under conditions that enhanced germ tube formation.

In this study, the pattern of distribution of fibronectin adhesins on yeast and hyphal-form *Candida albicans* was investigated using fibronectin-coated colloidal gold beads (Au_{18}) and transmission electron microscopy. This technique was used to show fibronectin receptors on human fibroblasts (16).

Material and Medhods

Organisms

The *C. albicans* strain GDH 2346 (NCYC 1467) used in this study was isolated at Glasgow Dental Hospital from a patient with denture stomatitis (17). The strain was supplied in the form of freeze-dried samples, from which further freeze-dried ampoules were prepared. The organism was maintained on a slope of Sabouraud dextrose agar (Difco) and subcultured monthly. Every two months, the cultures were replaced by new ones freshly grown from freeze-dried stocks.

Production of yeast and hyphal-form cells

C. albicans was inoculated from stock cultures into Yeast Nitrogen Base Medium (Difco) supplemented with 500 mM galactose (YNBGal) and incubated at 37˚C for 18 h in an orbital shaker at 150 rpm. This culture (5 ml) was used to inoculate a second flask of YNBGal medium (45 ml) and incubated at 37˚C for 24 h in an orbital shaker at 150 rpm (18). The cells in stationary phase were harvested by centrifugation (2300 g, 5 min) and were used immediately after preparation. For germ tube production, starved yeast-form cells were prepared by subculturing in modified Lee medium (19) without amino acids (MLMwAA), as modified by Tronchin et al. (20), containing (in grams per liter): $(NH_4)_2SO_4$, 5.0; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 2.5; NaCl, 5.0; glucose, 10.0; and biotin, 0.04 at pH 6.8. After incubation for 36 h at 25˚C with orbital shaking at 150 rpm, the cells in stationary phase were harvested by centrifugation and washed three times in distilled water. To obtain germ tubes, the starved yeast cells were inoculated into IX Medium 199 (Modified) with Hank's salts and 20 mM HEPES buffer, but without glutamine and sodium bicarbonate (Flow Laboratories, Irvine Scotland) at pH 6.7, as described by Dabrowa et al. (20-22) at a final concentration of 10^7 cells ml^{-1}, and then incubated at 37˚C for 1 h.

Preparation of colloidal gold

Collodial gold particles of 20 nm diameter were prepared through the controlled reduction of an aqueous solution of chloroauric acid with sodium citrate as the reducing agent (23). All solutions were prepared in deionized, double-distilled water and were passed through a 0.2 µm microfilter. Two ml of 1% (w/v) chloroauric acid was added to 198 ml of deionized, double-distilled water to give a final concentration of 0.02% (w/v) in a clean siliconized Ehrlenmeyer flask. A fresh solution of 1% (w/v) aqueous sodium citrate (6 ml) was rapidly poured into a boiling solution of the choloroauric acid with vigorous mixing. As the solution boiled, it turned dark blue and was then heated through refluxing until the typical wine-red colour of colloidal gold was reached, after which it was cooled to 4˚C. Before use, the colloid was centrifuged at 800 g for 10 min in order to remove aggregates and the supernate kept at 4˚C. The number of particles per unit volume was calculated spectrophotometrically at A_{520}, as described below.

Fibronectin-coated colloidal gold

Studies of the adsorption of macromolecules to colloidal gold particles have shown that it is pH and concentration dependent (23). The gold probe concentration was adjusted to A_{520}=1.0, equivalent to approximately $7x10^{11}$ particles ml^{-1} (24, 25). For protein adsorption, the pH of the gold particle preparation was adjusted to 7.0 with 0.2 M potassium carbonate. Two drops of 1% (w/v) polyethylene glycol (PEG, Sigma), M.W. 20,000, were mixed with the gold particle suspension (2 ml) in a conical plastic centrifuge tube for stabilization before the pH electrode was inserted.

Fibronectin-coated gold colloid was prepared according to the method of Pescioata Peters and Mosher by incubating with plasma fibronectin (16). In order to determine the appropriate amount of fibronectin for coating the colloidal gold, a saturation test devised by Horisberger and Rosset (26) and modified by Dr. L. Tetley, IBLS, Division of Infection and Immunity, University of Glasgow, was performed. First, the human plasma fibronectin was checked for purity by SDS polyacrylamide gel electrophoresis, as described by Laemmli (27).

To determine the minimum stabilizing fibronectin concentration, dissolved fibronectin, 2 mg ml^{-1} in PBS, was dialyzed against 2 mM tris-HCl containing 15 mM NaCl (pH 7.4) at 4˚C. The solution was then diluted 1: 8 in the same buffer. Serial dilutions of this diluted solution were performed in a multiwell plate (25). Fibronectin, the buffer and gold colloid were mixed and left for 2 min to complex fully, after which 50 µl of 10% (w/v) NaCl was added. After 30 sec of agitation, flocculation was observed as a change from the wine-red colour to purple-blue. Fibronectin solution (20 µl) stabilized the gold against salt-induced flocculation. From the results, an increased volume of fibronectin solution was calculated to prepare the final fibronectin gold probe (25). Fibronectin solution was added to gold colloid whilst the latter was stirred rapidly. There was a slight colour shift during the almost instantaneous complex formation. After 2 min stirring, unbound areas on the gold particles were stabilized with 1% (w/v) polyethylene glycol (PEG) at pH

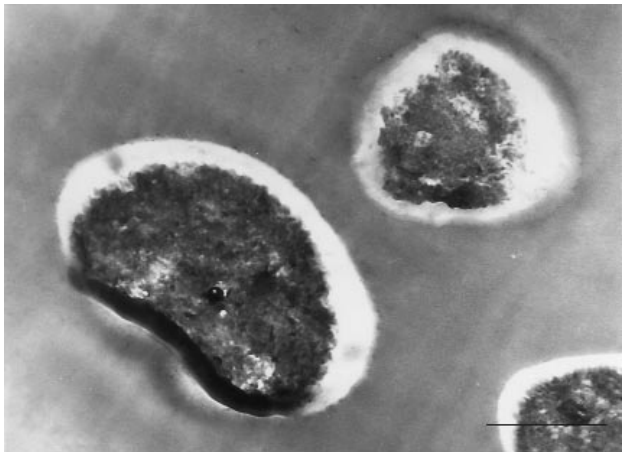

Figure 1. Micrograph of yeast-form *C.albicans* in YNBGal labelled with Au_{20}-Fn particles, 12000x. Bar represents 1 mm.

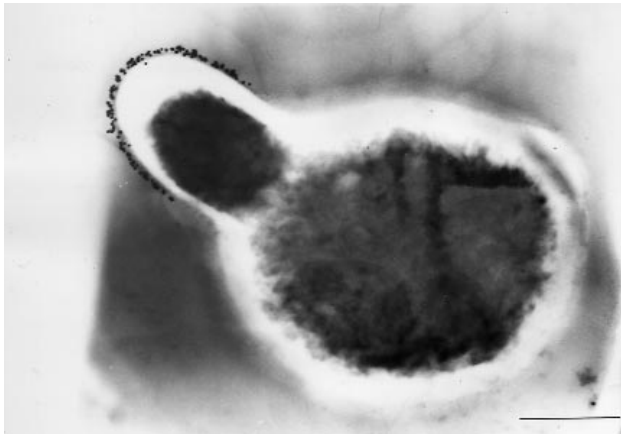

Figure 2. Micrograph of hyphal-form *C.albicans* in 199 Medium labelled with Au_{20}-Fn particles, 12000x. Bar represents 1 mm.

6.0 in deionized, double-distilled water (final concentration 0.05%). Colloidal gold coated with fibronectin (Au_{20}-Fn) was centrifuged at 28000 g for 30 min at 10˚C. At the end of centrifugation, three phases were obtained; a clear supernate containing free fibronectin; a dark red sediment at the bottom, which corresponded to the fibronectin-gold complex; and a black spot that remained along the side near the bottom of the tube - gold not stabilized by the fibronectin and PEG. The supernate was carefully aspirated as completely as possible and discarded. Fibronectin-coated gold particles were recovered and resuspended in 2 ml of 2 mM tris-HCl (pH 7.4) buffer containing 0.005% (w/v) sodium azide and kept at 4˚C overnight. The particles were centrifuged once at 28000 g for 30 min at 10˚C on the following day. After resuspension with 2 mM tris-HCl containing 15 mM NaCl at pH 7.4, fibronectin-coated gold particles were adjusted using a spectrophotometer to A_{520}=1.0. The same labelling method was used for preparing PEG-coated particles for the control.

Electron microscopy

A transmission electron microscope (EM 902, Zeiss, Germany) was used. *C. albicans* grown in YNBGal and the hyphal-form cells produced in the 199 Medium were labelled with fibronectin-coated gold particles (Au_{20}-Fn). The yeast-form cells ($2x10^8$ cells ml^{-1}) were resuspended in 2 mM tris-HCl (pH 7.4) buffer containing Ca^{++} and Mg^{++} (2 mM) and washed twice in the same buffer. The cells were fixed with 1 ml of 2.5% (w/v) paraformaldehyde (BDH), which was prepared fresh in PBS at pH 7.2. The cells were incubated at 4˚C for 30 min, centrifuged for 5 min at 3500 rpm and then mixed with 1 ml of 0.2 M glycine (Sigma) in PBS at pH 7.2 at 4˚C for 10 min. The cell pellet was spun for 5 min at 3500 rpm and washed once in 2 mM tris-HCl (pH 7.4) buffer containing Ca^{++}, Mg^{++} (2 mM) and 1% (w/v) BSA. The pellet was resuspended in 0.1 ml of the same buffer and mixed with 0.1 ml Au_{20}-Fn. After incubation of the mixture at 22˚C for 30 min, the cells were centrifuged and washed twice in 1 ml of the same buffer. The cells labelled with fibronectin-coated gold particles were fixed in 0.1 M PBS for 2 h at 20˚C and rinsed twice in tris-HCl buffer (pH 7.4) for 10 min in preparation for Transmission Electron Microscopy. The specimens were suspended in 1% osmium tetroxide (OSO_4) for 1 h. After the cells were washed three times in distilled water for 10 min each time, they were stained with 1% uranyl acetate for 30 min and then rinsed once in distilled water for 2 min. Dehydration was carried out through an alcohol series, 30, 50, 70, 90% for 5 min each, followed by 2x10 min changes in absolute alcohol. The last change was in dried absolute alcohol. After the cells were washed three times in epoxypropane (propylene oxide) for 5 min, the specimens were suspended in 1:1 epoxypropane/araldite, left on a rotator in a fume cupboard and polymerized in a fume hood oven at 60˚C for 48 h.

Results

The binding of fibronectin to the yeast and hyphal-form *C. albicans* was examined using 20 nm colloidal gold particles coated with fibronectin (Au_{20}-Fn). Au_{20}-Fn was added to yeast cells of *C. albicans* ($2x10^8$ cells ml^{-1}), which had been grown in Yeast Nitrogen Base Medium containing galactose (YNBGal). Figure 1 shows the transmission electron micrograph of the yeast-forms cells

Figure 3. Micrograph of hyphal-form *C.albicans* in 199 Medium labelled with Au_{20}-Fn particles, 7000x. Bar represents 1 mm.

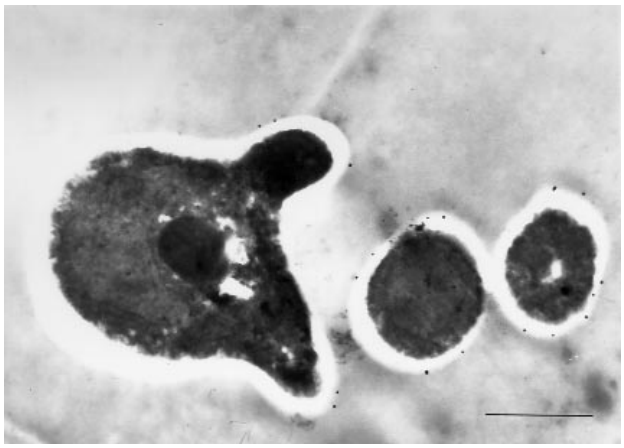

Figure 4. Micrograph of hyphal-form *C.albicans* in 199 Medium labelled with PEG-coated particles, 7000x. Bar represents 1 mm.

grown in YNBGal which do not exhibit any fibronectin-coated gold particles on their surface.

Similarly, *C. albicans* grown in 199 Medium (pH 6.7) at 37°C for 1 h under conditions enabling the production of germ tubes were labelled with Au_{20}-Fn. There were Au_{20}-Fn particles clearly visible on the germ-tube parts of hyphal-form cells but few if any on the yeast-cell parts (Figure 2). The particles are shown by arrows in the figure. Another example of the binding of fibronectin-coated gold particles to the germ-tube parts of the hyphal-form cells is shown in Figure 3. Control experiments showed that PEG-coated gold particles, Au_{20}-PEG, had little affinity for the germ-tube parts of the hyphal-form cells (Fİgure 4).

Discussion

In this study, the binding of fibronectin to the yeast and hyphal-form of *C. albicans* was monitored by transsmission electron microscopy using 20 nm gold particles bound to fibronectin (Au_{20}-Fn), (16). The preparation of the collidal gold and the labelling of fibronectin are quite simple procedures, and the use of gold particles as electron-dense markers allows for easy identification of the fibronectin-labelled cells. The reliability of this technique and its wide range of applications in transmission electron microscopy have been clearly demonstrated (23, 26). In this study, Au_{20}-Fn, when added to yeast cells grown in Yeast Nitrogen Base Medium containing galactose (YNBGal), did not exhibit any fibronectin-coated gold particles on the surface. The yeast cells grown in 199 Medium (pH 6.7) at 37°C for 1 h under conditions enabling the production of germ tubes had Au_{20}-Fn particles on the germ-tube parts of the hyphal-form cells. Control experiments were done with PEG-coated gold particles, which showed little affinity for the germ tube parts of the hyphal-form cells. One of the most significant observations in this study was the highly specific labelling of the germ-tube parts of hyphal-form *C. albicans* by the Au_{20}-Fn particles. In contrast, there were few Au_{20}-Fn particles on the yeast-cell parts of the hyphal-form *C. albicans.*

Klotz et al. have recently observed that calcium is required for the process of adherence of *C. albicans* yeast cells to various extracellular matrix proteins (28). They also obtained two proteins, 60 and 105 Kd, from yeast extracts by passing them over a fibronectin or gelatin column and eluting with EDTA. The same proteins were recovered from germ-tube forms of *C. albicans.* This showed that calcium is an important divalent cation for the binding of fibronectin to *C. albicans. C. albicans* strains adhered in higher numbers to heparinized polyethylene surfaces preadsorbed with fibronectin than to non-modified heparinized polyethylene surfaces, supporting the proposition that fibronectin mediates adhesion (29).

Different domains of fibronectin molecules may be bound to the yeast cells. Fibronectin and other extracellular matrix proteins are degraded by *C. albicans,* most likely by secreted aspartyl proteinase (30). The 120 kDa fragment of fibronectin containing the cell-binding domain and the aminoacid sequence, RGD, bind avidly to the surfaces of yeast cells (31). However, other researchers have observed that the gelatin/collagen-binding domain of fibronectin is a potent inhibitor of fibronectin binding to *C. albicans.* In addition, the fibrin I- and heparin II- binding domains also inhibit fibronectin

binding, but are less active (32). Their results also indicated that interactions with the cell-binding domain are not mediated by Arg-Gly-Asp or any other know recognition sequences. Santoni et al. showed that *C. albicans* expresses a fibronectin receptor antigenically related to $\alpha5\beta1$ integrin (33). A monoclonal antibody directed against the human $\alpha5\beta1$ subunit positively bound to the yeast and hyphal-form *C. albicans* by immunofluorescence and flow cytometric analysis. This immunoreactivity increased upon germ-tube production. Both forms bound to the Arg-Gly-Asp (RGD)-containing 120-kDa fragment of fibronectin and adhesion to intact fibronectin was markedly inhibited by the Gly-Arg-Gly-containing peptide.

Definition of the mechanisms of attachment of *C. albicans* may have important therapeutic implications. New therapeutic strategies for the treatment of candidosis may involve inhibiton of the attachment of the organism to the host cell. The apparent correlation of fibronectin with clinical invasiveness supports the idea that the adherence of *C. albicans* to fibronectin (present in blood clots and the subendothelial matrix, and coating artifical surfaces such as catheters, cardiac shunts, and valves) could play an important role in the pathogenicity of the organism (29-31). Finally, numerous human proteins such as fibrinogen, fibronectin and laminin that contain the RGD sequence may be involved in the interactions of *C. albicans* with intert surfaces such as plastic. The study of such interactions would have implications in the practical management of patients with prostheses made of these materials.

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