Mycopathologia **138:** 13–19, 1997.

Quantification of thigmotropism (contact sensing) of *Candida albicans* and *Candida tropicalis*

H. Nikawa, H. Nishimura, T. Hamada & S. Sadamori
Department of Prosthetic Dentistry, Hiroshima University School of Dentistry, 1-2-3 Kasumi Minami-ku, Hiroshima 734 Japan

Received 22 October 1996; accepted in final form 2 July 1997

Abstract

To quantify the thigmotropism, we adapted the our previous method using a chemotaxifilter system in combination with a bioluminescent adenosine triphosphate (ATP) assay based on firefly luciferase-luciferin system and analyzed the relationship between the ability of germ tube formation and thigmotropism of *C. albicans* and *C. tropicalis.*

Both the ability to form germ tube and the amount of hyphae exhibiting thigmotropism varied depending upon both the species and strains of *Candida*. *C. albicans* formed more germ tubes than *C. tropicalis.* A good correlation was observed between the ability to form a germ tube and the capacity for thigmotropism, and the results gave a level of significance ($p < 0.05$).

Further, SEM observation revealed that relatively long hyphae of *C. tropicalis* with penetrated through the pores of filter membrane. This phenomenon may be of importance in the development of pathogenesis of *C. tropicalis* as well as *C. albicans.*

Key words: *Candida albicans, Candida tropicalis,* germ tube, hypha, thigmotropism

Introduction

Candida albicans is the most common opportunistic pathogen of humans. Several factors, such as adherence, persorption, dimorphism and/or germ tube formation, phenotypic switching, interference with host defense system, synergism with bacteria, and production of hydrolases or other metabolites, have been proposed to be virulence factors of this fungus (1). Among these virulence factors, dimorphism and/or germ tube formation is thought to be one of the most important factors, since there are several ways in which germ tube or hyphae formation contributes to candidal persistence.

Reports have shown that the germ tube formation enhances the adherence of this fungi to host cells (2–5). Tronchin et al. (1988) has suggested that the germ tube possesses adhesins that enable the fungus to colonize inert surfaces, such as plastics (6). Several human proteins, such as laminin, fibrinogen and C3d have shown to bind specifically to germ tube of *C. albicans* cells (7--10). Germ tube formation has also been shown to be involved in the invasion into epithelium of the mucosa and vascular endothelia using a combination of physical and enzymatic forces (11,12). Hence, considerable effect has been focused on the characterization of germ tube formation and dimorphic regulation.

Recently, Sherwood et al. (1992) demonstrated thigmotropism (contact sensing) of hyphae of this fungus (13) and Gow et al. (1994) characterized the nature of their touch-sensitive responses (14). The phenomenon should be of importance in the invasion of the epithelial surfaces during pathogenesis. However, at present, there is no information available on the relationship between the germ tube formation and thigmotropism. In addition, it is well known that some isolates of *C. tropicalis* possess the ability to form germ tube, but it was not known whether other *Candida* species exhibit thygmotropism.

The experiments described here quantify the thigmotropic responses. We adapted the our previous methods using a chemotaxifilter system (15) in combination with a bioluminescent adenosine triphosphate (ATP) assay based on firefly luciferase-luciferin system. This

is a simple and convenient method for accurate enumeration of viable cells (16) and has been used previously to examine bacterial (17,18) and fungal biofilms (19).

Thus the purpose of the present study was to analyze the relationship between ability of germ tube formation and thigmotropism of *C. albicans* and *C. tropicalis*. Preliminary scanning electron microscopic studies were also performed to investigate the nature of the *Candida* thigmotropic response.

Materials and methods

Microorganisms and growth conditions

Candida albicans GDH 16, GDH18, GDH 19, GDH 20, *C. tropicalis* IFO 1070, GDH 1362 and GDH 0465 were used in this study. *C. tropicalis* IFO 1070 was purchased from Institute Fermentation for Osaka, and all of GDH strains were oral isolates obtained from the routine microbiology services of the Glasgow Dental Hospital and School. The isolates were identified by sugar assimilation test using the API 20C system (API Products, Biomeroux, Lyon, France) and "germ tube" test (20).

A loopful of yeast cells were inoculated into yeast nitrogen base medium (Difco, Detroit, USA) containing 250 mM glucose and grown aerobically at 37°C (21). After overnight culture, the yeast was harvested in the late exponential growth phase, washed twice with sterilized distilled water and resuspended to a final concentration of 10^7 cells/ml by use of a haemocytometer (15). Germ tube formation.

The germ tube formation of *Candida* spp. was determined by a modified method of Ibrahim et al (22). One hundred microliters of yeast suspension (10^7 cells/ml) was added to 500 μl of neat serum (human serum; Sigma Chemical Co. St Louis, MO, USA), and samples were incubated at 37°C for 2 hrs. Sample were then fixed by adding 500 μl of 4% (vol/vol) glutaraldehyde in distilled water at 4°C for 2 hrs. The number of yeast cells with germ tubes, of length at least 1 blastospore diameter, was counted microscopically by the use of haemocytometer. Each strain was tested in triplicate, and at least 600 yeast cells per strain were examined.

Thigmotropism assay

A chemotaxicell (pore size 3 μm; Kurabo, Osaka, Japan; 15) was placed on 20% (v:v) serum agar, made with human serum (Sigma Chemical Co. St Louis, MO, USA), pre-warmed to 37°C, so that the filter of Chemotaxicell was in contact with the surface of the agar (13). Subsequently, 150 μl of yeast suspension was dispensed into each Chemotaxicell and the whole assembly was incubated at 37°C for 72 hrs. The surface area of filter of Chemotaxicell for culture was 0.48 cm^2.

Afterwards, each filter with fungi was carefully removed from Chemotaxicell, washed in a ultrasonic device for 10 min and then washed vigorously by rinsing three times for a total of 60 seconds with distilled water to remove non-penetrating organisms or associated blastospores. Then the filter with remaining hyphae was immersed in 1.0 ml of the extraction-reagent (benzalkonium; 23) and allowed to react for 15 min with ultrasonication. The resultant reagent solution was then subjected to ATP- measurement to quantify the amount of ATP using a bioluminescence apparatus (ATPA-100, TOA Electronics Ltd., Tokyo, Japan) (17,19). This apparatus used the firefly-luciferase system to determine the concentration of cellular ATP and is based upon the measurement of light emission produced during the oxidation of luciferin by molecular oxygen in the presence of ATP and magnesium ions. In this system, the light intensity is directly proportional to the concentration of ATP (23).

The assays were carried out on two independent occasions in quadruplicate. Numerical data were analyzed by analysis of variance (ANOVA) and Tukey's multiple range test at 5 and 1% levels.

Ultrastructural observations

For ultrastructural studies, specimens were removed from Chemotaxicells, washed with sterile distilled water and fixed in 2.5% glutaraldehyde. Afterwards they were dehydrated through a graded series of ethyl alcohol (50–90%), immersed in *t*-butyl alcohol (three times for 10 mm) and stored at 4°C. Each specimen was then freeze dried, sputter coated with a layer of gold to a thickness of 20–25 nm and observed under a Scanning Electron Microscope (SEM, JMS-6300, Joel, Tokyo, Japan) using standard procedures (15,19).

Results

As shown in Fig. 1a, the ability to form germ tube varied depending upon the species and isolate of *Candida*. *C. albicans* had the higher ability to form germ

tube than that of *C. tropicalis*, as generally accepted, and *C. albicans* GDH 18 showed the most, decreased in the order of GDH 20, GDH16, and *C. albicans* GDH 19 and three isolates of *C. tropicalis* showed the least (ANOVA, $p < 0.01$). Similarly, the amount of hyphae conducted by thigmotropism was highest with *C. albicans* GDH 18, decreased in the order of GDH20 $\fallingdotseq$ GDH16 $\geqq$ GDH 1362, GDH 19, GDH 0465, and *C. tropicalis* IFO 1070 showed the least (Fig. 1b). A relatively good correlation was observed between the ability to form germ tube and thigmotropism, and the results gave a level of significance ($p < 0.05$; Fig. 2).

SEM observation revealed that at the initial stage of germ tube formation on the Chemotaxifilter (4-hrs incubation), both germ tubes and a small amount of blastospores were on the inner surface of the filter, and that germ tube began to penetrate the filter pore (Fig. 3a). After 24 hrs-incubation, a relatively large amount of hyphae penetrating through the filter pore was observed (Fig. 3b).

After three days incubation, a large amount of hyphae of *C. albicans* GDH 18 formed multilayered networks on the inner surface of the filter was observed (Fig. 4a). Similarly, a layered network was also observed with *C. albicans* GDH 16 (Fig. 4b). As to *C. albicans* GDH 19 and 20, relatively thin hyphal network was formed and helical hyphae was occasionally observed (Fig. 4c,d).

As shown in Fig. 4e–g, relatively long hyphae of *C. tropicalis* were mainly located on the periphery of the filter, and hyphal growth with penetrating through the filter pore which may be introduced by thigmotropism was observed.

Discussion

The significance of topographic changes in germ tube or hyphae of *C. albicans* induced by contact sensing or thigmotropism was excellently suggested by Sherwood et al. (1992) (13). They also suggested that contact guidance of hyphae of *C. albicans* should confer an advantage in the penetration of human epithelial layers. Since this topic should be of importance in considering the pathogenesis of this fungus, it seems to be important to assess the interrelation between the thigmotropism and virulence of this fungus. However, there has been no method or information to quantify this phenomenon.

To quantify the thigmotropism, firstly, we adapted the chemotaxifilter system (15). This filter enables us to culture or grow hyphal phase of *Candida* on the known area of the filter. Secondly, we used a bioluminescent adenosine triphosphate (ATP) assay based on firefly luciferase-luciferin system, which is known as a simple and convenient method for accurate enumeration of viable cells (16) and has been previously used for fungal biofilm (19). We previously confirmed that the quantitative ability of this method for *C. albicans* cells both in hyphal and blastospore phase.

As shown in Figs. 1 and 2, a relatively good correlation was observed between the ability to form germ tube (germ tube test) and thigmotropism, and the results gave a level of significance ($p < 0.05$). This finding suggested that the ability to form the hyphae by thigmotropism may be partly attributed to the potential of isolates to form germ tubes. However, we recently revealed that the thigmotropism of *Candida* should be one of the predisposing factors of biofilm formation on pellicle-coated acrylics, though no significant correlation was observed between the ability of germ tube formation and fungal biofilm formation (unpublished data). Thus, as suggested by Gow et al. (1994) (14), the (topographic) nature of the surface, on which the hyphal growth was induced, may also be involved in this phenomenon.

In addition, SEM observation revealed that a large amount of hyphae of *C. albicans*, particularly with GDH 18, formed multilayered networks on the inner surface of the filter, with small amount of blastospore cell (Fig. 4a–d), most of which were observed to be budding hyphal form from our previous study (15). In contrast, as shown in our previous study, when we used the liquid media, such as neat serum, diluted serum or TC 199, to obtain the hyphae on the same Chemotaxifilter, the fungal layer formed on the inner surface of the filter was mainly comprised of a large amount of blastospore phase, while a large amount of hyphal growth penetrated through the filter pore was observed on the outer surface of the filter at the same time (15). Although, it is difficult to offer a reason for this strange phenomena, we speculate that the phenomena may be partly attributed to the differences in the manner of supply of nutrients from liquid and agar medium, since Sherwood et al. (1992) has suggested that the hyphae grew away from the nutrients (13). Further analysis should be needed on the phenomenon.

Interestingly, as shown in Fig. 4e–g, relatively long hyphae of *C. tropicalis* were observed. It should be noted that two isolates of *C. tropicalis* which possess

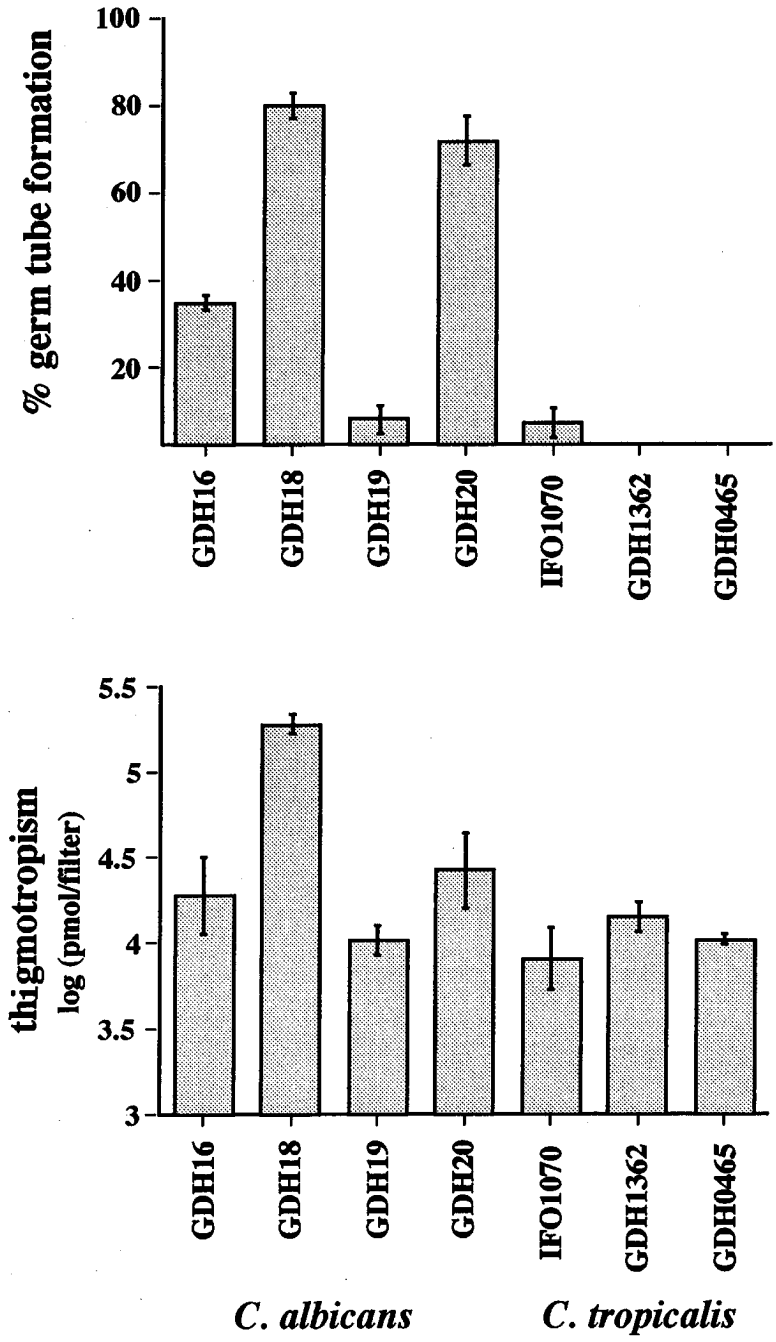

Figure 1. Ability to form germ tubes of *Candida albicans* (GDH 16, GDH 18, GDH 19 and GDH 20) and *Candida tropicalis* (IFO 1070, GDH 1362 and GDH 0465) (a) and the amount of hyphae induced by thigmotropism (b)

very low ability to form germ tube (GDH 1362 for $0.789 \pm 0.191\%$; GDH 0465 for $0.8821 \pm 0.337\%$) showed the hyphal growth with penetrating through the filter pore which may be introduced by thigmotropism. Since Sherwood et al. (1992) has suggested the importance of thigmotropism of *C. albicans* in the pathogenesis (13), thigmotropism of *C. tropicalis*, we demon-

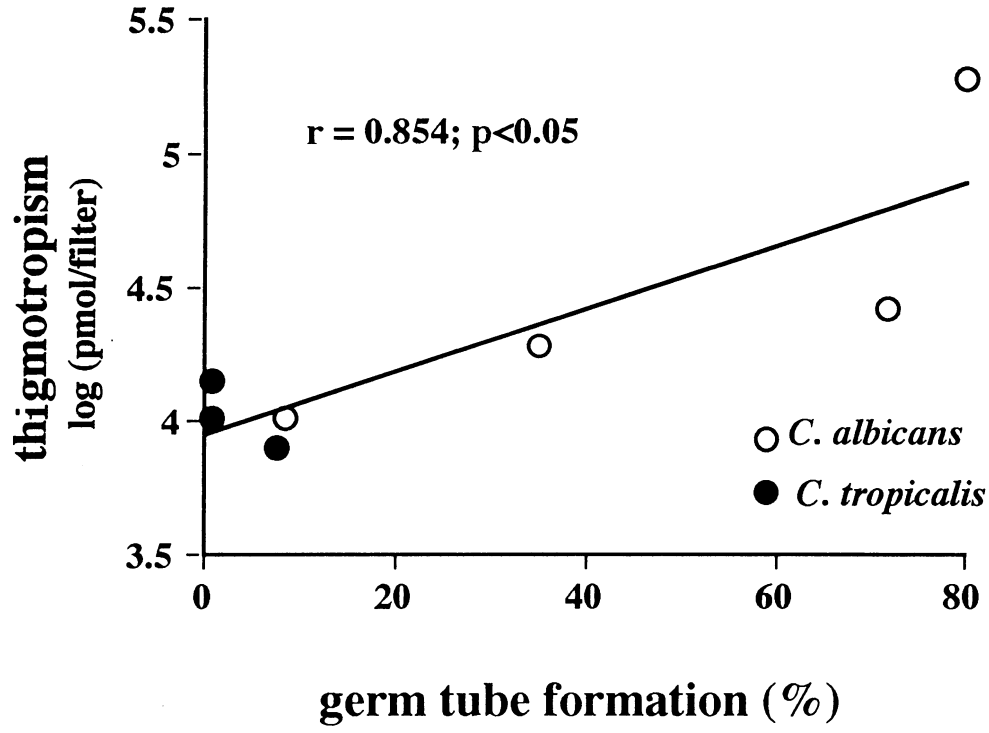

Figure 2. Relationship between ability to form germ tubes and the amount of hyphae induced by thigmotropism.

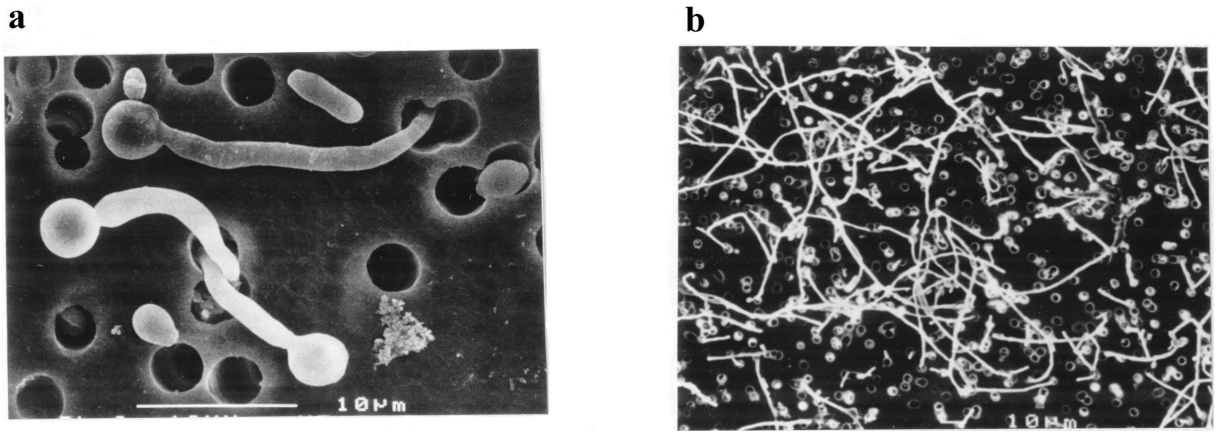

Figure 3. An example of germ tube penetration at 4-hrs incubation (a) and hyphal penetration through the filter pore at 24-hrs incubation (b).

strated here, may also be one. of the important factors in the development of pathogenesis of this species.

References

1. Cannon RD, Holmes AB, Monk BC. Oral *Candida*: Clearance, colonization, or Candidiasis? J Dent Res 1995; 74: 1152–1161.
2. Kimura LH, Pearsall NN. Adherence of *Candida albicans* to human buccal epithelial cells. Infection and Immunity 1978; 21: 64–68.
3. Kimura LH, Pearsall NN. Relationship between germination of *Candida albicans* and increased adherence to human buccal epithelial cells. Infection and Immunity 1980; 28: 464–468.
4. Sandin RL, Rogers AL, Patterson RJ, Beneke ES. Evidence for mannose-mediated adherence of *Candida albicans* to human buccal cells in vitro. Infection and Immunity 1982; 35: 79–85.

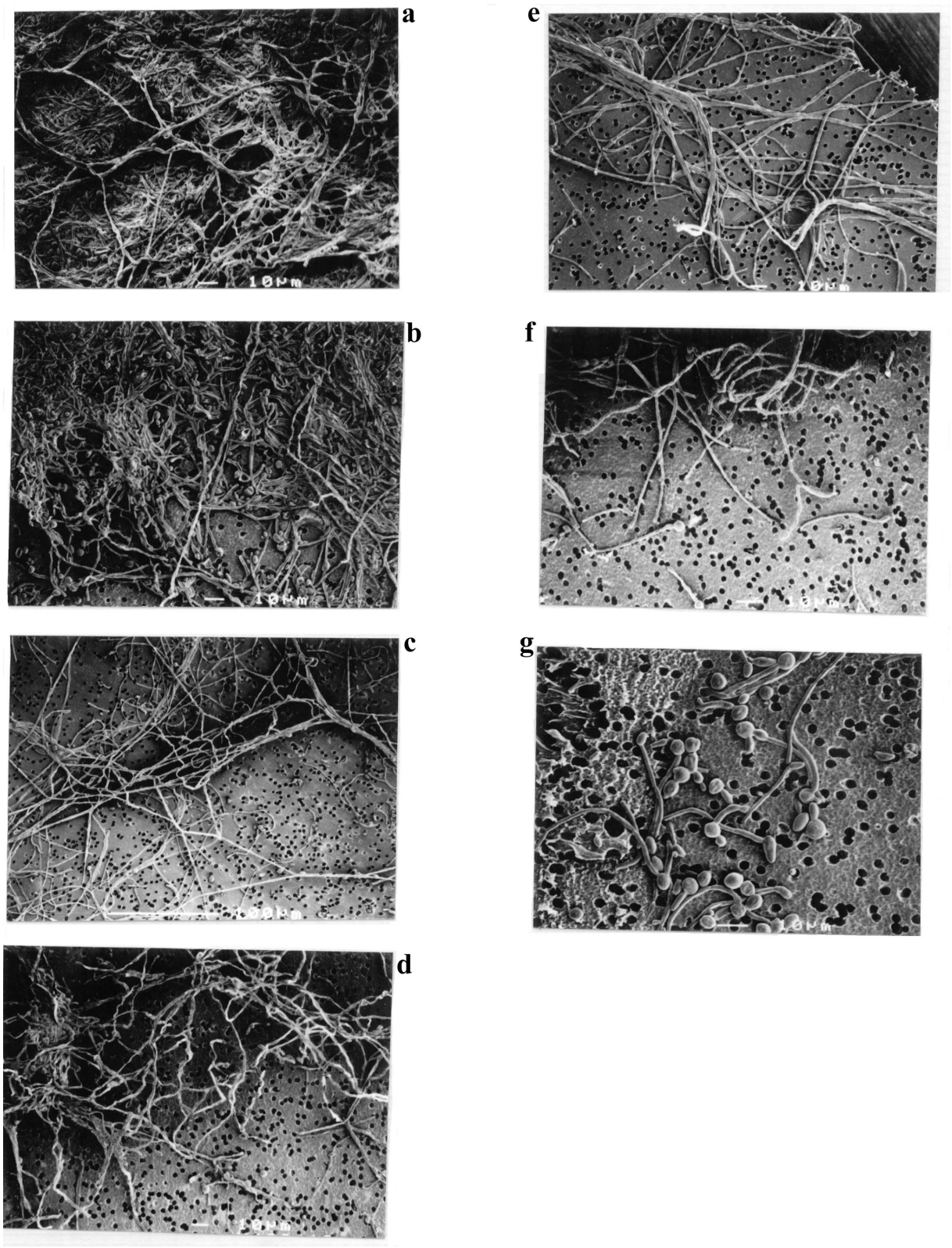

Figure 4. SEM observations on hyphal formation of *Candida* spp. induced by thigmotropism. *Candida albicans* GDH 18 (a), GDH 16 (b), GDH 19 (c), GDH 20 (d), *Candida tropicalis* IFO 1070 (e), GDH 1362 (f) and GDH 0465 (g).

5. Sobel JD, Myers P, Kaye D, Levison ME. Adherence of *Candida albicans* to human vaginal and buccal epithelial cells. J Inf Dis 1981; 143: 76–82.
6. Tronchin G, Bouchara J-P, Robert R, Senet J-M. Adherence of *Candida albicans* germ tubes to plastics: ultrastructural and molecular studies of fibrillar adhesins. Infection and Immunity 1988; 56: 1987–1993.
7. Bouali, A, Robert R, Tronchin G, Senet J-M. Binding of human fibrinogen to *Candida albicans* in vitro: A preliminary study. J Med Vet Mycolology 1986; 24: 345–348.
8. Bouchara J-P, Tronchin G, Annaix V, Robert R, Sennet J-M. Laminin receptors on *Candida albicans* germ tubes. Infection and Immunity 1990; 58: 48–54.
9. Page S, Odds FC. Binding of plasma proteins to *Candida* species in vitro. J Gen Microbiol 1988; 134: 2693–2702.
10. Tronchin G, Robert R, Bouali A, Senet J-M. Immunocytochemical localization of in vitro binding of human fibrinogen to *Candida albicans* germ tube and mycelium. Annual Inst Pasteur/Microbiology 1987; 138: 177–187.
11. Hube B, Turver CJ, Odds FC, Eiffert H, Boulnois GJ, Kochel H, Ruchel R. Sequence of *Candida albicans* gene encoding the secretory aspartate proteinase. J Med Vet Mycology 1991; 29: 129–132.
12. Kwon-Chung, KJ, Lehman D, Good C, Magee PT. Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans.* Infection and Immunity 1985; 49: 571–575.
13. Sherwood J, Gow NAR, Gooday GW, Gregory DW, Marshall D. Contact sensing in *Candida albicans*: a possible aid to epithelial penetration. J Med Vet Mycology 1992; 30: 461–469.
14. Gow NAR, Perera THS, Sherwood-Higham J, Gooday GW, Gregory DW, Marshall D. Investigation of touch-sensitive responses by hyphae of the human paththogenic fungus *Candida albicans.* Scanning Microscopy 1994; 8: 705–710.
15. Nikawa LI, Nishimura H, Yamamoto T, Samaranayake LP. A novel method to study the hyphal phase of *Candida albicans* and to evaluate its hydrophobicity. Oral Microbiol Immun 1995; 10: 110–114.
16. Kricka U. Clinical and biochemical application of luciferases and luciferins. Anal Biochem 1988; 175: 14-21.
17. Berlutti LS, Passariello C, Comodi-Ballanti MR, Thaller MC. Proteolytic enzymes: a new treatment strategy for prosthetic infections? Antimicrobal Agents and Chemotherapy 1993; 37: 2618–2621.
18. Farber BF, Wolff AG. Salicylic acid prevents the adherence of bacteria and yeast to silastic catheters. J Biomed Mat Res 1993; 27: 599–602.
19. Nikawa H, Nishimura H, Yamamoto T, Hamada T, Samaranayake LP. The role of saliva and serum in *Candida albicans* biofilm formation on denture acrylic surfaces. Microbial Ecology in Health and Disease 1996; 9: 35–48.
20. Silverman Jr S, Migliorati CA, Epstein JB, Samaranayake LP. Laboratory diagnosis of oral candidosis. In Samaranayake LP, MacFarlane TW Eds. Oral Candidosis. Wright, London, 1990: 213–237.
21. Nikawa H, Hayashi S, Nikawa Y, Hamada T, Samaranayake LP. Interactions between denture lining material, protein pellicles and *Candida albicans.* Arch Oral Biol 1993; 38: 631–634.
22. Ibraham AS, Mirbo F, Filler SG, Banno Y, Cole GT, Kitajima Y, Edwards Jr. JE, Nozawa Y, Channoum MA. Evidence implicating phospholipase as a vilurence factor of *Candida albicans.* Infection and Immunity 1995; 63: 1993–1998.
23. Siro RM, Romar H, Lovgren T. Continuous flow method for extraction and bioluminescence assay of ATP in baker's yeast. Eur J Appl Microb Biotech 1982; 15: 258–264.

Address for correspondence: Hiroki Nikawa, Department of Prosthetic Dentistry, Hiroshima University, School of Dentistry, 1-2-3 Kasumi Minamiku, Hiroshima 734 Japan.
Phone: 81 822 575681; Fax: 81 822 575684