行政院國家科學委員會專題研究計畫　成果報告

巨噬細胞與白色念珠菌交互作用中分泌性 aspartyl proteinases 所扮演的角色(2/2)

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The Role of Secreted Aspartyl Proteinases In the Macrophage - *Candida albicans* Interaction (2/2)

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行政院國家科學委員會補助專題研究計畫成果報告

巨噬細胞與白色念珠菌交互作用中分泌性 aspartyl proteinase 所扮演的角色

The Role of Secreted Aspartyl Proteinases In the Macrophage - *Candida albicans* Interaction

(2/2)

The Protective Role of Antibody against *Candida albicans* Secreted Aspartyl Proteinase 5 (Sap5)

抗 Sap5 抗血清對白色念珠菌增殖的抑制作用之評估

計畫編號：NSC　91－2314－B－002－130－
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一.中文摘要

愈來愈多的証據顯示白色念珠菌（*Candida albicans*）分泌性 aspartyl proteinase 4-6 基因（*SAP4-6*）在散佈性念珠菌感染扮演重要角色，但是該基因的蛋白質產物的研究很少。我們之前的研究已証明 Sap4-6 蛋白會在白色念珠菌誘發形成 hyphae 時表現並分泌出來。利用前述研究中所製備的抗 Sap5 抗血清，我們在此研究中進一步証明，罹患白色念珠菌菌血症的 13 名病人血清中有 Sap5 或 Sap6 抗體的存在，而其他重症病人或健康人的血清中並沒有此抗體存在。比較抗 Sap5 抗血清與免疫前對照血清對白色念珠菌在 RPMI 培養基中的增殖的影響，抗血清有較強的抑制念珠菌野生株增殖的作用。此抑制作用之差異，在下列情況下不存在，如①野生株的培養基中加 aspartyl proteinase 的抑制劑，pepstatin A（10μg/ML）；②白色念珠菌Δ*sap4-6* 變異株；③非白色念珠菌的念珠菌，如 *Candida tropicalis*，*C. glabrata* 及 *C. parapsilosis*。而且，白色念珠菌血液培養菌株分泌 Sap 能力不同，抗血清抑菌效果也不同。因此，抗 Sap5 抗血清的保護作用是藉由影響 Sap4-6 蛋白的功能而達到的。此外，抗血清的抑制作用與抗黴菌藥，fluconazole，的抑菌效果有相加的作用，而不是拮抗作用。我們的研究結果提供了初步証據顯示，以 Sap5 為標的的免疫療法有其前瞻性。

關鍵字：白色念珠菌，抑菌作用，抗 Sap5 抗血清

Summary

Growing evidences suggest that *Candida albicans* secreted aspartyl proteinase 4,5,6 genes (*SAP4-6*) contribute to the development of disseminated infections. Our previous study demonstrated that Sap4-6 proteins are secreted during hyphae formation.

In this study, the inhibitory effect of anti-Sap4-6 antibodies in the multiplication of *C. albicans* and the interaction with fluconazole were studies. This study demonstrated that anti-Sap4-6 antibodies exists in serum collected from patients recovered from disseminated candidiasis, but not from patients with *C. glabrata* fungemia, or other critically ill patients. Anti-Sap5 antiserum provided stronger inhibitory effect on multiplication of *C. albicans* wild type strain than pre-immune control serum. This protective effect was not significant for *C. albicans* wild type strain in the presence of aspartic proteinase, pepstatin A, for a Δ*sap4-6* triple mutant, or for non-*albicans Candida* species. Thus, this inhibitory effect was at least in part through modulation of the function of Sap4-6 proteins. Besides, anti-Sap5 antibodies enhanced fluconazole activity, a fungistatic agent. Our data provided a rationale in development of vaccination to immunocompromised patients with high risk of disseminated candidiasis or passive immunization for treatment in combination with antifungal agent.

Key words: *Candida albicans*, inhibitory activity, anti-Sap5 antiserum

二.緣由與目的

The incidence of nosocomial fungal infection has increased substantially during the last two decades [1,2]. *Candida* species are responsible for 10% of all nosocomial bloodstream infections [1] and became the leading pathogens of nosocomial bloodstream infection at National Taiwan University Hospital (NTUH) since 1993 [2]. Nosocomial *Candida* infection was an independent poor prognostic factor for critically ill patients [3] and nosocomial candidemia is associated with a high mortality rate [4]. The leading cause of candidiasis, *Candida albicans*, is an imperfect diploid dimorphic fungus that resides as a commensal of the mucosae and the gastrointestinal tract. In facing the emerging fungal treat and limited strategies of antifungal therapy, explore for virulence factor against *C. albicans*, as a new antifungal target is paramount important in recent years [5].

Among several potential virulence factors of pathogenic *Candida* species, *C. albicans* secreted aspartyl proteinases (Saps) under the control of a multi-gene family (*SAP1-9*) are regulated differentially at the mRNA level in vitro, and are implicated in the breakdown of several host substrates [6]. *SAP4, SAP5 and SAP6* subfamily mRNAs were first detected during hyphae formation [7]. Cumulated evidences suggest that Sap4-6 might play an important role in development of hematogenously disseminated infections [8-12]. For examples, Sap4-6 antigens have been found on phagocytosed *Candida* blastoconidia by immunofluoresence staining [11]. A Δ*sap4-6* triple mutant resulted in attenuated mortality of guinea pigs and mice compared with macrophages than the wild-type 53% more effectively after contact with macrophages than the wild-type strain [11]. Furthermore, among different *sap* mutant strains, only a *Δsap4-6* mutant induced significant less organ invasion and reduced tissue damage in comparison to the wild-type strain [12]. These results suggest that Sap4-6 might play an important role in development of hematogenously disseminated infections.

Our previous study confirmed in the protein level that Sap4-6 proteins were expressed and secreted in *C. albicans* during hyphae formation induced by temperature and pH shift in modified Lee's medium [13]. Sap5 secreted earlier than Sap4/6 and these was only minimal amount of Sap4/6 secreted in the absence of Sap5. Our previous findings suggest that Sap5 play a central role of secretion of Sap4-6 subfamily. Recently, assessment of expression of *SAP* genes by in vivo expression technology revealed that only *SAP5* was strongly activated throughout the course of infection [14,15]. Therefore, using these antibodies against Sap5 and Sap4/6, this study demonstrated the presence of anti-Sap4-6 antibodies in the sera of patients recovered from disseminated candidiasis. Furthermore, antibody against Sap5 inhibited proliferation of *C. albicans* in RPMI-1640 medium, but not *C. tropicalis, C. papapsilosis,* or *C. glabrata*. The inhibitory effect against *C. albicans* was strain-dependent. This effect was insignificant for *C. albicans* wild type strain in the presence of Sap inhibitor, pepstatin A, or for a Δ*sap4-6* triple mutant.

三.研究方法與過程

Yeast strains and growth conditions. The *C. albicans* wild type strain SC5314 [16] and the *sap4,5,6* null mutant strains *Δsap4-6* [9] were used in this study. C. *albicans* SC5314, a blood culture isolate from a patient with disseminated

candidiasis, was used as the DNA template for cloning individual *Sap* genes and expression of His-tagged fusion proteins. The following liquid media were used: modified Lee's medium (pH 4.5 and pH 6.5) for induction of Sap4-6 proteins as described previously [13], and RPMI-1640 for determination of viability of *Candida* following the treatment of sera or antifungal agent (fluconazole). Bovine serum albumin (Sigma) was added as indicated. *C. albicans* was grown in an orbital incubator at the indicated temperatures. For hyphae formation and expression of Sap4,5,6 proteins, *C. albicans* was incubated in modified Lee's medium (pH 4.5) at 25°C for 48 h (stationary phase), transferred to pre-warmed PRMI-1640 (final cell density approximately $5x10^6$ cells/ml) with or without sera, then incubated at 37°C for 24 h. Hyphae production of different strains during different time points was checked by microscopy. Aspartic protease inhibitor, pepstatin (P 4265, Sigma Chemical Co., Mo., USA) was dissolved in 95% ethanol, diluted 1:50 in sterile H_2O, and added to culture medium at a final concentration of 10 ug/mL as indicated.

Western blot analysis. Recombinant Sap2-6 proteins (100 ng each) prepared as described previously [13] were separated by SDS-PAGE, transferred onto Immobilon-P membrane (Millipore Corp.), and incubated with either specific anti-Sap4-6 antibodies or patient sera at room temperature for 60 min, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:5000). Bound antibodies were detected with the ECL system (Amersham) according to the manufacturer's instructions. To determined the presence of Sap4-6 in the culture medium, supernatants of 2 ml of Candida culture were collected and concentrated for Western blot analysis as described [13].

Sera collected from patients who recovered from systemic candidiasis. Sera were collected from 6 patients who recovered from systemic candidiasis (blood culture grew *C. albicans,* and there were symptoms and signs suggestive of systemic infection). Control sera were collected from three healthy volunteers and from three hospitalized patients without invasive candidiasis due to *C. albicans* (one with coronary artery disease and idiopathic pulmonary hypertension, one with acute myelogenous leukemia and pulmonary tuberculosis, one with fungemia due to *Candida glabrata*). For SDS-PAGE, 100 ng each of recombinant Sap protein (Sap2-6) and proteins precipitated from equal amounts of the culture supernatants of *C. albicans* after induction for Sap4-6 were loaded. Patient sera (1:500) were used as first antibody and HRP goat anti-human IgG (1:5000) (Cappel) as secondary antibody for immunoblotting analysis. Antimannan antibodies were determined as well (Sentori, France).

Antifungal susceptibility testing. Antifungal stock solutions were prepared from standard powders and susceptibility testing were performed according to NCCLS M27-A guidelines [17] and as previously described [18].

Time-kill curve determinations. C. albicans wild type strain SC5314 was selected for 24-h kill curves for fluconazole, anti-Sap5 antiserum, or both. Growth control was determined in RPMI-1640 alone. Experiments were performed at least in triplicate. Each culture was incubated at 37°C and samples were collected at indicated times and was CFU was determined by plating on Sabouraud dextrose agar. CFU were counted after 18-24 h of incubation at 35°C.

Inhibitory and fungicidal activity. The percentage of inhibition of multiplication (at time x) was calculated by the formula [1 - (experimental CFU/control CFU)] x 100. "Control" refers to growth at time x without active components (control serum, anti-Sap antibodies, or fluconazole). When the number of experimental CFU is less than the inoculum, this is referred to as fungicidal activity, i.e., reduction of inoculum (time zero) CFU, was determined by the formula [1 - (experimental CFU/inoculum)] x 100.

Statistical analysis. Student's *t* test was used to compare statistical differences between groups. Bonferroni's adjustment to the *t* test was used for multiple comparisons against a single control group [19]. Time-kill curves were analyzed at 0, 6 and 24 h by analysis of variance. Statistical significance was $P < 0.05$ (two-tail). Time-kill curves were analyzed at indicated time points by analysis of variance.

四.研究結果

Presence of antibodies against Sap4-6 and mannan in convelescent-phase sera of patients with candidiasis. Table 1 shows that serum from 4 patients with *C. albicans* fungemia reacted with either recombinant Sap5 or Sap6 proteins. However, there were no detectable anti-Sap4-6 antibodies in the sera collected

from 2 patients with recurrent fungemia. All these 6 patients had anti-manna antibody. The sera from three hospitalized patients without invasive candidiasis due to *C. albicans* and 3 healthy volunteers had no anti-Sap4-6 and anti-mannan antibodies. All 12 sera evaluated did not react with recombinant Sap2, Sap3 or Sap4.

Inhibition of C. albicans *multiplication by most, but not all, of human serum.* Heat-inactivated human serum (10%) in RPMI significantly inhibited multiplication of *C. albicans* compared with multiplication in RPMI alone in a 24-h assay (98.3% to 99.4%, $P < 0.05$; figure 1, sample 1-3). The inhibitory effect of serum collected from healthy volunteer was as strong as those collected from patients treated with antifungal agents (96.6% to 100%; figure 1, sample 4-7). However, serum collected from a patient with acute leukemia status post chemotherapy and from a patient with Wagnar sarcoma did not have significant inhibitory effect on multiplication of *C. albicans* (figure 1, sample 8,9).

Time-kill curves for C. albicans *in RPMI alone and RPMI containing anti-Sap5 antiserum*. Twenty-four-hour kill curves for *C. albicans* wild type strain SC5314 for RPMI with or without anti-Sap5 antiserum (10%) were shown in figure 2. Multiplication of *C. albicans* in antiserum became stasis over 6-24-h duration as compared with the exponential phase of growth control curve in RPMI alone ($P < 0.06$).

Inhibition of C. albicans *multiplication by anti-Sap4-6 antibodies.* Anti-Sap5 antiserum significantly inhibited multiplication of the *C. albicans* wild type strain and a Δ*sap4-6* triple mutant compared with multiplication in RPMI alone in a 2-h, 6-h, or 24-h incubation after induction of hyphae formation (figure 3). The inhibitory activity of anti-Sap5 antiserum for wild type strain was stronger than that of control serum (82.8% ± 0.8% vs. 89.9% ± 1.9%, $P = 0.004$) and the difference existed at 6-h or 24-h incubation. In the following studies, inhibitory effect of antiserum was determined at 24-h incubation. Anti-Sap4 and anti-Sap6 antisera had similar inhibitory effect on multiplication of *C. albicans* as anti-Sap5 antiserum did (data not shown). A Δ*sap4-6* triple mutant was more susceptible to inhibitory effect of either antiserum or control serum than wild type strain did. The difference of inhibitory effect on multiplication of *C. albicans* between antiserum and control serum existed when serum was added to culture medium either at 6-h or 24-h after induction for hyphae formation (figure 4). When aspartic proteiase inhibitor, peptatin A (10 ug/mL), was added to culture medium, the difference of inhibitory effect of multiplication on *C. albicans* wild type strain between antiserum and control serum was insignificant (figure 5A). In the presence of peptatin A, inhibitory effect of antiserum for wild type strain was similar to that of either control serum or antiserum for the Δ*sap4-6* triple mutant (81.2% to 84.7%; figure 5A,B). Therefore, the protective effect provided by anti-Sap5 antiserum compared with control serum targeted on the function of Sap proteins, particularly Sap4,5,6 proteins.

Combined effect of anti-Sap5 antiserum and fluconazole. When fluconazole was added to culture medium at 24-h after induction of hyphae formation in RPMI containing 10% of serum, inhibitory effect of antiserum and fluconazole was stronger than control serum and fluconazole (figure 6A, right). Besides, inhibitory effect of antiserum and fluconazole was stronger than antiserum alone (74.1% ± 2.4% vs. 41.9% ± 6.1%, $P = 0.001$). The protective effect of antiserum also existed when fluconazole was added to culture medium (RPMI containing 10% serum) before induction of hyphae formation (figure 6B). Besides, inhibitory effect of combination of serum and fluconazole was stronger when treatment given before hyphae formation (figure 6A,B).

Strain variation of inhibitory effects of anti-Sap5 antiserum. Inhibitory effects of control serum and anti-Sap5 antiserum on multiplication of six bloodstream isolates of *C. albicans* were determined (figure 7). Inhibitory effect of control serum for different strains ranged from -3.3% to 78.4%. However, except for strain 2001K46, inhibitory effects of anti-Sap5 antiserum were relatively similar (66.7% to 79.1%). Western blot analysis demonstrated that the expression levels of Sap5 and Sap4/6 proteins in RPMI containing 10% fetal calf serum varied among different isolates (data not shown). Thus, variation in inhibitory effect of control serum for different bloodstream isolates might be compensated by anti-Sap5 antiserum through modulation of Sap4,5,6 activity. Inhibitory effect of antiserum was significant higher than control serum for 6 or 7 bloodstream isolates of *C. albicans* evaluated.

*Inhibition of anti-Sap5 antibody on multiplication of non-*albicans Candida *species*. Comparing to the significant inhibitory effect of anti-Sap5 antiserum for C. albicans bloodstream isolates, there was no inhibitory effect for *C. tropicalis, C. papapsilosis* and *C. glabrata* (figure 7). Besides, either control serum or antiserum did not demonstrate inhibitory effect as they were for *C. albicans*. Western blot analysis using anti-Sap5 antibody did not demonstrate any

cross reactivity of Sap4,5,6 proteins in the culture supernatants of these three *Candida* species under the same induction condition (data not shown).

五.研究討論

This study demonstrated that anti-Sap4-6 antibodies exists in serum collected from patients recovered from disseminated candidiasis, but not from patients with *C. glabrata* fungemia, or other critically ill patients. Anti-Sap5 antiserum provided stronger inhibitory effect on multiplication of *C. albicans* wild type strain than pre-immune control serum. This protective effect was not significant for *C. albicans* wild type strain in the presence of aspartic proteinase, pepstatin A, for a Δ*sap4-6* triple mutant, or for other *Candida* species. Thus, this inhibitory effect was at least in part through modulation of the function of Sap4-6 proteins. Besides, anti-Sap5 antibodies enhanced fluconazole activity, a fungistatic agent. Our data provided a rationale in development of vaccination or passive immunization to immunocompromised patients with high risk of disseminated candidiasis to replace widespread use of antifungal prophylaxis and resulting emergence of antifungal resistance.

六.計畫成果自評

Goal to be completed

1. Compare the inhibitory effects of anti-Sap5 antiserum and control serum in the presence of momocyte or macrophage.

2. Evaluate the expression of Sap4-6 during macrophage-*Candida* interaction

七.參考文獻 (References)

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