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Candida albicans Structural and Secreted Proteins Modulate CD4/CD8 Ratio in Tumor Infiltrating Lymphocytes of Spontaneous Adenocarcinoma Bearing Mice

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ABSTRACT

Background: *Candida albicans* is one of the most important opportunistic pathogens that suppress immunologic mechanisms of the host. It is speculated that structural and secretory proteins of *C. albicans* have immunomodulatory effects in cancer. **Objective:** To evaluate the effects of *C. albicans* structural and secreted proteins on intratumoral CD4/CD8 ratio as well as the survival rate in BALB/c tumor model. **Methods:** Structural and secretory proteins from *C. albicans* were isolated and examined for their effects on tumor growth and survival of adenocarcinoma bearing mice. **Results:** The results indicated that in mice treated with *C. albicans* structural protein, the survival rate significantly decreased compared with the control groups. Also, mice treated with secretory proteins showed a decrease in survival rate but it was not statistically significant ($p>0.05$). Investigating the frequency of tumor infiltrated $CD4^+$ and $CD8^+$ T lymphocytes indicated that the percentages of tumor infiltrated $CD4^+$ T lymphocytes in response to structural and secreted proteins were higher compared to the control groups. **Conclusion:** Our study suggests that *C. albicans* structural and secreted proteins modulate intratumor T lymphocyte infiltration.

Keywords: ***Candida albicans*, Lymphocytes, Spontaneous Adenocarcinoma, Structural Proteins, Secreted Proteins**

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INTRODUCTION

Candida albicans is one of the most important opportunistic pathogenic fungi that suppresses immunologic mechanisms of the host. In the establishment of the pathogenic process the cell wall of *C. albicans* plays an important role. It contains important antigens and some other compounds that affect the homeostatic equilibrium of the host in favor of the parasite and develops several virulence traits causing the invasion of host tissues and the avoidance of host defense mechanisms (1-3). Some of the antigens of *C. albicans* supposedly contribute to immune dysfunction associated with chronic infections (4,5). A complex assortment of hydrolytic enzymes such as proteinases (secreted aspartyl proteinase), phospholipases, acid phosphatase, chitinases, esterase and glucoamylase can be found in culture filtrates of *C.albicans* cells. These enzymes are putative virulence factors of *C. albicans* (6,7). It is believed that extracellular hydrolytic enzymes are most relevant for systemic infections (8). *C. albicans* is a highly prevalent cause of disease, especially in malignant patients. Extensive experimental evidence demonstrates that this fungus has immunomodulatory effects (9). Invasive fungal infections are among the leading causes of morbidity and mortality in cancer patients (10). The number of patients affected by malignancies who develop fungal infections has increased dramatically during recent years (11,12). This increase is due to factors such as host defense impairment due to intensive cytotoxic chemotherapies including transplantation procedures, ablative radiation therapy, and use of corticosteroids or cyclosporine, barrier disruption following cytotoxic chemotherapy, prolonged use of a number of broad-spectrum antibiotics and finally use of central venous catheters (CVC). Candida species are the most frequent cause of invasive fungal infections in these patients, as demonstrated in various clinical and postmortem studies (13,14). The most common outcome of candidemia is an increase in the mortality rate and there are few reports about immunomodulatory effects of this pathogen in the background of breast cancer. We hypothesized that there might be structural and secretory proteins of *C. albicans* with immunomodulatory effects in cancer. Therefore, our study was focused on the effects of structural and secretory proteins of *C. albicans* on the tumor mass status. Furthermore, the pattern of tumor infiltrated CD4+ and CD8+ T lymphocyte were also analyzed.

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MATERIALS AND METHODS

Animals. Eight to ten weeks old inbred BALB/c mice were purchased from the Pasteur Institute of Iran (Tehran, Iran). Spontaneously occurring tumor (mammary tumor) was obtained from a female Balb/c mouse purchased from the same Institute.

Preparation of Structural Proteins of *C.albicans*. *C. albicans* (ATCC10231) was cultured on SDA (Sabourauds Dextrose Agar) for 48 hours. Yeast cells were washed twice with PBS (Phosphate buffer saline) and collected by centrifugation (3000 x g, 10 min), suspended in a small volume of 1 mM PMSF (Phenylmethyl sulfonyle fluoride) and then lysed by vortexing with glass beads (5 mg beads per mg cells). Finally, a complete cell breakage was obtained. The cell lysis was assessed by examining the preparation with an invert microscope. Then the samples were dialyzed in PBS for 48 h. Protein as-

say was carried out by Bradford method (15). Isolated proteins were freeze-dried and stored at -70°C for further use.

Preparation of the Supernatants from the Culture of *C.albicans*.

C. albicans (ATCC10231) cells were maintained in Sabourauds liquid medium after shaking at 27°C. 5×10^7 cells of *C. albicans* were counted and cultured in 50 ml of RPMI 1640 supplemented with 20 mM HEPES (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Life Technologies, Paisley, U.K.), and 10% FBS (Fetal Bovine Serum) (HyClone Laboratories, Logan, UT) and incubated at 37°C and 5% CO_2 for two weeks (5). The culture supernatant was collected by centrifugation (3000 g, 15min) and concentrated using a freeze drier. Concentrated supernatant from the culture of *C. albicans* was fractionated by gel filtration chromatography (Sephadex G-100). Bradford method (15) was used for protein assay and the fractions containing protein were stored at -70°C for further use.

Tumor Transplantation. Spontaneously occurring tumors (mammary tumors) were obtained from a female Balb/c mouse purchased from the Pasteur Institute of Iran (Tehran, Iran). Tumor tissue was removed from the mouse body, put in sterile PBS and fragmented into 0.5 cm³ pieces. Then each piece was implanted subcutaneously in syngenic BALB/c mice. In each group, tumors developed and appeared on the transplanted region after about a week.

Administration of Cell Wall and Secretary Proteins of *C. albicans* to Experimental Groups. After 3-7 days of tumor transplantation, the mice were categorized into four groups. The first group received 200 µg of structural proteins in 100 µl PBS (n = 5), the second group received 200 µg fraction of the supernatant from the culture of *C. albicans* in 100 µl PBS (n = 5), the third group (control group) received 100 µl of culture medium (n = 5) and the fourth group (control group) received 100 µl PBS (n = 5) daily by I.V. injection. Injections in all four groups were continued for eight days. Tumor volume was measured during this period in all four groups using a Caliper in two perpendicular (width and length) directions. The mortality rate was followed for one month.

Evaluation of Intra-Tumor TCell Subpopulations. After tumor transplantation and various treatments, the animals were sacrificed and the solid tumors were cut into small pieces with forceps and scalpels. The pieces were rinsed twice with PBS. The suspensions were passed through 150 µm stainless steel mesh and then the cells were washed twice and used for flowcytometry analysis.

Immunofluorescent Staining of the Cells. For the staining of the cells obtained from the tumors, fluorescent anti-CD4 and anti-CD8 antibodies (Serotec, UK) were used. We established the reference immunophenotypic pattern using standard procedures. In this study, 100µl of intra-tumor cells were immunostained with 10 µl mAbs conjugated with fluorscein isothiocyanate (FITC) in Q-Prep apparatus. Then three immunopreps were added to them automatically; 0.7 ml Immunopreps A (formic acid 1.2 ml), 0.32 ml Immunoprep B (sodium carbonate 6.0 g/l , sodium chloride 14.5 g/l, sodium sulfate 31.3 g/l) and 0.14 ml Immunoprop C (paraformaldehyde 10.0 g/l, phosphate buffer 9 Coulter). All the samples were then kept at 2-8 ºC and in the dark for further analysis.

Flowcytometry Analysis. The cell samples were counted on an EPICS Coulter flowcytometer with serial filter configuration. The analysis was focused on total cells (total gate) by using Coulter software.

Statistical Analysis. All the experiments were carried out twice and the results were depicted as the mean ± SD of triplicate determinations. Statistical analysis was per-

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formed using log-rank test for survival analysis. Tumor volume was estimated using the formula $V= 1/2 \times L \times W^2$, where, V is the volume, L is the length and W is the width. Relative tumor volumes (day 8) were calculated using tumor volume (day 8)/ tumor volume (day 0) ×100 and statistical analysis on tumors were performed using the Mann-Whitney test on Relative Tumor Volume. In all of the cases, p values <0.05 were considered to be statistically significant.

RESULTS

Measurement of Tumor Volume and Survival Rates Following Treatment. The tumor volume changes were assessed 8 days after treatment with *C. albicans* proteins (Figure 1). The results indicated that the tumors in the *C. albicans* protein treated groups grew more rapidly than the control groups. The cancer bearing mice treated with *C. albicans* cell wall and secreted proteins for 8 days significantly enhanced the tumor growth compared to the control groups ($p<0.05$, Figure 2). Moreover, the mice in each group were observed for their survival periods. The data in Figure 3 shows that in the mice treated with *C. albicans* cell wall protein, survival rate decreased significantly compared to the control groups ($p<0.05$) and in the mice treated with secretory proteins from *C. albicans*, there was no significant decrease in survival rate compared to the control groups ($p>0.05$).

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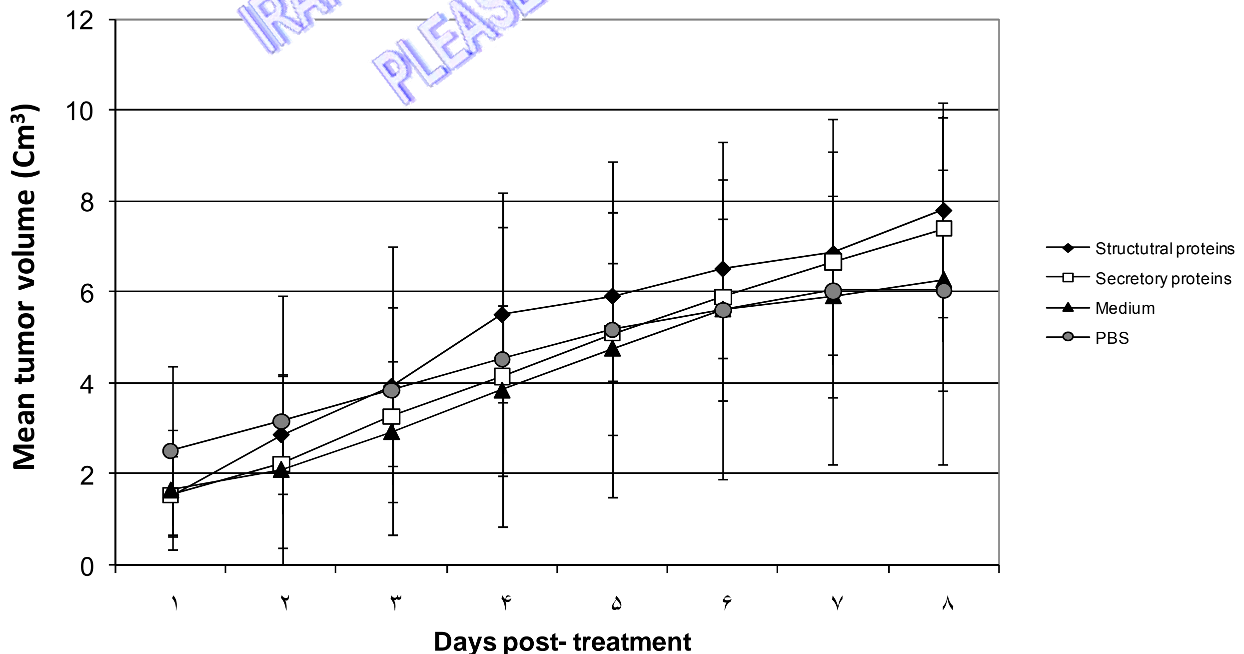

Figure 1. Change of tumor volumes after treatment with *C. albicans* structural or secretory proteins. 200 µg of *C. albicans* structural or secretory proteins in100 µl PBS were injected to mice for 8 days and the controls received an equal volume of the medium or PBS. The values are mean ± SD of tumor volumes (n = 5 mice per group).

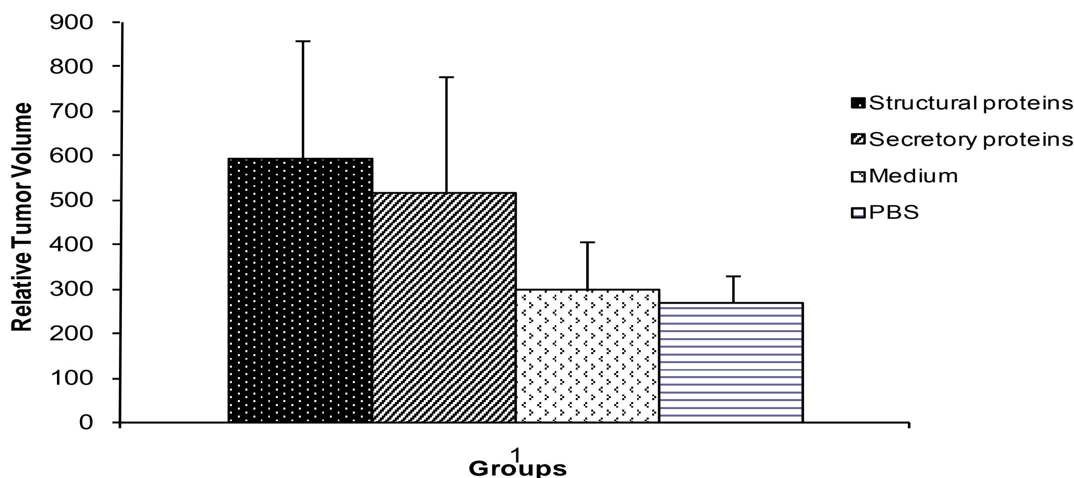

Figure 2. Relative tumor volumes after treatment with *C. albicans* structural or secretory proteins. 200 µg of *C.albicans* structural or secretory proteins in100 µl PBS were injected to mice for 8 days and the controls received an equal volume of the medium or PBS. The values are mean ± SD of relative tumor volumes. (n = 5 mice per group).

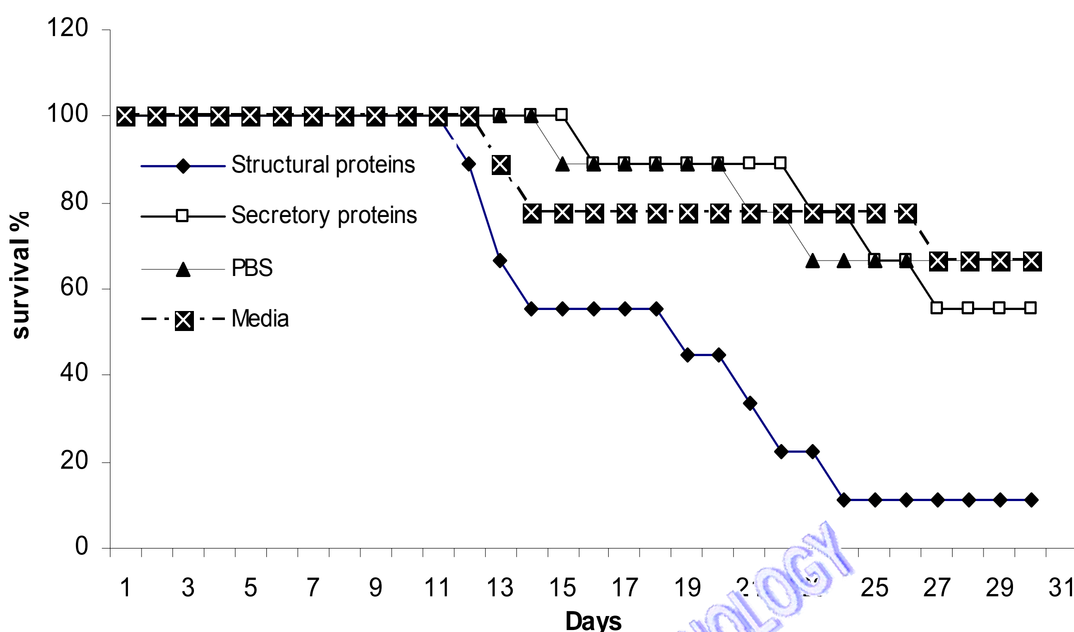

Figure 3. Survival curve of the mice in different groups. Mice were transplanted with tumor cells. After 3-7 days, the mice were treated with 200 µg of *C. albicans* structural or secretory proteins in 100 µl PBS for 8 days and the controls received an equal volume of the medium or PBS. The survival rates were recorded daily for 30 days.

Measurement of Tumor Infiltrating lymphocyte Subpopulations. Analysis of tumor infiltrating lymphocytes was carried out using flowcytometry method. Tumor infiltrating cells were stained with anti-mouse CD4 and CD8 and their percentages were analyzed in the total gate format. Table 1, showed that treatment with these proteins

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significantly increased the percent age of the tumor infiltrated $CD4^+$ T lymphocytes compared to the control groups ($p<0.05$) and that *C. albicans* cell wall proteins significantly decreased the CD4/CD8 ratio compared to the control group, however, the decrease of CD4/CD8 ratio in the mice treated with the secretory proteins was not significant.

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Table 1. Percentage of $CD4^+$ and $CD8^+$ tumor-infiltrated lymphocytes and the CD4/CD8 ratio.

Groups	T cell subpopulation		$CD4^+/CD8^+$ ratio
	$CD4^+$ Mean ± SD	$CD8^+$ Mean ± SD	Mean ± SD
Structural proteins	82.6 ± 8.09[a]	4.01 ± 1.73	23.82 ± 10.52 [c]
Secretory proteins	87.1 ± 5.45[b]	3.03 ± 1.58	39.61 ± 26.29
Media (control)	63.8 ± 8.70	0.92 ± 0.27	68.76 ± 16.80
PBS (control)	57.38 ± 9.78	0.94 ± 0.32	60.51 ± 21.37

Tumor bearing mice were treated with 200 µg of *C. albicans* cell wall or secretory proteins in 100 µl PBS for 8 days and the controls received an equal volume of the medium or PBS. The values are mean ± SD (n=5 mice per group).
[a] Significantly different from the control groups.
[b] Significantly different from the control groups.
[c] Significantly different from the control groups.

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DISCUSSION

Infections due to *Candida spp.* remain the most frequent ones in cancer patients and are characterized by a high mortality rate, ranging from 18% to 30% in different series (16-18). Pathogenicity of *C. albicans* also depends on a variety array of microorganism-related putative virulence factors (19,20) including antigenic variability, phenotypic switching, adhesion to host cells and tissues, cell surface hydrophobicity and production of extracellular enzymes (21–23). Fungal cell wall contains most of the biological functions related to pathogenicity and virulence. It also demonstrated that *C. albicans* cell wall and secreted proteins modulate some immunologic parameters in murine breast cancer model. Results of the present study indicated that *C. albicans* cell wall and secreted proteins modulate CD4/CD8 ratio in tumor infiltrating lymphocytes and increase tumor mass of spontaneous adenocarcinoma bearing mice. *C. albicans* cell wall and secreted proteins are two major components of *C. albicans* that have a variety of immunologic functions. Our findings also showed that treatment of cancer bearing mice with cell wall and secreted proteins of *C. albicans* resulted in a significant enhancement in tumor size and a decrease in survival rate. Our previous study had shown that cell wall and secreted proteins of *C. albicans* have antiproliferative effects on lymphocyte proliferation (15). Lymphocyte proliferation is generally related to cell mediated immunity, so it can be deduced that these proteins could potentially suppress cellular immunity.

A variety of studies have shown that components of *C. albicans* are able to suppress immune response in different ways (24-27). Murciano et al. showed that killed *C. albicans* inhibits gamma interferon secretion by murine NK cells (24). Chinen et al. showed that secretory proteins from *C. albicans* suppress IL-1β and nitric oxide production by macrophages (5). Also various studies have confirmed that *C. albicans* proteins suppress immune responses (28-30). Our results showed that both *C. albicans* cell wall and secretory proteins significantly increased the tumor size. Accordingly, it may be the immunosuppression induced in the tumor microenvironment by *C. albicans* cell wall and secretory proteins which had caused tumor enhancement. In fact, under these conditions, the tumor will contact a microenvironment with a lower immunologic response, enhancing its progression.

The nature of infiltrating lymphocytes in the tumor microenvironment correlates with disease prognosis (31). Analysis of intratumor infiltrating lymphocytes following treatment with *C. albicans* cell wall and secreted proteins revealed that these proteins significantly increased the percentage of the $CD4^+$ subpopulation and decreased the CD4/CD8 ratio of intratumor infiltrating T lymphocytes. Considering the enhancement of tumor size after treatment with *C. albicans* cell wall and secreted proteins, one asks whether these $CD4^+$ cells, belong to T helper or T regulatory subtypes. The increase in the intratumor $CD4^+$ lymphocytes may be correlated with immunologic suppression in this model. Increase of tumor size in the test group revealed that these cells might be the regulatory T cells. Recent evidence has shown that regulatory T cells play an important role in cancer and in suppression of immune responses against cancers (32-36). Therefore, the mechanism involved in our study might be the induction of the regulatory T cells. Further studies may reveal the mechanisms implicated. Reports about CD4/CD8 ratios show that an increase in this ratio is related to a better prognosis (37), but our study showed a significant decrease in CD4/CD8 ratio of the intratumor infiltrating T lymphocytes. This finding is in agreement with the tumor mass enhancement and an increase in the mortality rate of the test groups. Further study on the role of *C. albicans* cell wall and secretory proteins will be useful to clarify the effects of *C. albicans* components on tumor models and the role of regulatory T cells in this model. In conclusion, our study has demonstrated that some protein components of *C. albicans* have immunomodulatory effects in tumor models and may help tumor progression but further studies in this field will be useful to clarify the effects on other components of the immune system.

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