Original Article

Anticandidal Activity of Polymorphonuclear Leukocytes and Serum Transferrin in Tumor-Implanted Mice

Toshihiko Watanabe, Takeshi Mikami, Tatsuji Matsumoto,
Masuko Suzuki

Department of Microbiology, Tohoku College of Pharmacy,
4-1, Komatsushima 4 Chome, Aoba-ku, Sendai, 981, Japan

[Received: 29, March 1996. Accepted: 8, October 1996]

Abstract

Meth-A fibrosarcoma-implanted mice were examined for protective activity to lethal *Candida albicans* infection. The number of peripheral blood polymorphonuclear leukocytes (PMNs) was markedly increased in the mice, and their candidacidal function was also activated. The growth of *C. albicans* *in vitro* was inhibited by the addition of serum from Meth-A-implanted mice. This activity was inhibited by the addition of an inhibitor of transferrin, ferric sulphate.

These findings indicated that *C. albicans* cells were efficiently eliminated by the activated PMNs and that the elimination was increased by the serum transferrin in Meth-A-implanted mice.

Key words: *Candida albicans*, Polymorphonuclear leukocytes, Transferrin, Meth-A

Introduction

Candida albicans is a ubiquitous pathogenic fungus associated with infections of immunocompromised hosts. *C. albicans* is a dimorphic fungus which undergoes a transition from the yeast form to the hyphal form depending on the growth conditions¹⁾.

In tumor-implanted models, a change in the immune system such as corpulence of lymphoid tissue and an increase of peripheral leukocytes is sometimes observed^{2,3)}. The function of CD4⁺ T cells is reduced in a tumor-implanted state⁴⁾, and an immunosuppressive factor, transforming growth factor- β , has been suggested as a cause of this immune dysfunctions⁵⁾. Although the T-lymphocyte-mediated immune system was inactive, the host resistance to *C. albicans* infection was augmented in a tumor-implanted state^{6,7)}.

We earlier reported that the serum transferrin was increased in Meth-A-implanted mice, and the transferrin led the bone marrow cells to adopt a GM-CSF responsive state⁸⁾. Although the number of phagocytes and transferrin is increased in Meth-A-implanted mice, the anticandidal effect of these agents was not examined *in vivo*. To identify the anticandidal mechanism in tumor-implanted

mice, we studied here the involvement of peripheral PMNs and serum transferrin.

Materials and Methods

Reagents

Catalase and ferric sulphate were purchased from Wako Pure Chemical Industries. Human transferrin was obtained from Chemicom International Inc., CA, USA.

Animal and tumor

Four- to 6-week-old male BALB/c mice were obtained from SLC, Shizuoka, Japan. Methylcholanthrene-induced Meth-A fibrosarcoma cells (Meth-A, 1×10^6) were subcutaneously transplanted into the left groin of each mouse.

Cultivation of *C. albicans*

C. albicans NIH A-207 strain was grown in a liquid medium containing 0.5% yeast extract, 1% peptone, and 2% D-glucose with shaking at 28 °C for 48hr.

Serum

Serum was collected from the Meth A-implanted mice at 21 days after the tumor transplantation. The serum samples were pooled and stored at –85°C.

Resistance to *C. albicans* infection

C. albicans viable cells (2×10^6 cells) were intravenously injected 21 days after the Meth A-implantation. The survival time was observed up

別刷請求先: 松本 達二

〒981 宮城県仙台市青葉区小松島 4-4-1
東北薬科大学微生物学教室

to 15 days after the infection.

Preparation of peripheral blood PMNs

Peripheral blood PMNs were prepared as described previously⁹⁾. Briefly, in mice they were prepared from heparinized blood by dextran sedimentation followed by Lympholyte M (Wako Pure Chemical Ind.) differential centrifugation. Purity of the PMNs was more than 90%.

Number of viable cells of *C. albicans* from the kidney

Meth A-implanted mice were infected as described above. Twenty-four hours after the infection, the number of viable *C. albicans* cells in the kidney homogenate was determined by counting the CFU on a Sabouraud agar plate.

Candidacidal activity of peripheral blood PMNs

This was conducted in accordance with the method described⁹⁾. Briefly, 2×10^5 viable cells of *C. albicans* were suspended in $10 \mu\text{l}$ of HBSS containing 10 (v/v)% normal mouse serum. The suspension was added to a suspension of blood PMNs (1×10^5) in $100 \mu\text{l}$ of the same medium, and the mixture was incubated in a 96-well plate at 37°C for 3hr in a 5% CO_2 incubator. The culture supernatant was removed and the cells were lysed by two consecutive washings with distilled water. The cell lysate was plated onto Sabouraud dextrose agar at 27°C for 48hr and the number of *C. albicans* cells was determined by counting the colonies. Killing(%) = $[(A - B)/A] \times 100$, where A and B are the number of colonies before and after a 3-hr incubation period.

Alamar blue viability assay

The serum diluted with RPMI1640 medium ($10 \mu\text{l}$) and *C. albicans* viable cells (1×10^3 cells/RPMI1640 medium $100 \mu\text{l}$) were incubated with or without the inhibitor at 37°C in 5% CO_2 for 12hr. The extent of the growth of *Candida* hyphal cells was determined by the Alamar blue viability assay¹⁰⁾. Briefly, $10 \mu\text{l}$ of Alamar blue (Alamar

Biosciences Inc., CA, USA) was added to the *C. albicans* and incubated for 3hr; then, the absorbance at 545nm-620nm was measured. Alamar blue changes in color from 620nm to 545nm in response to chemical reduction of growth medium resulting from the cell growth. Change of color was not affected by serum.

Statistical analysis

Values were shown as means $\pm$ S.E., and these data were statistically analyzed using Student's *t*-test.

Results

Number of PMNs from Meth-A-implanted mice

Peripheral blood PMNs were collected from normal and Meth-A-implanted mice. The number understood in Meth-A-implanted mice after 3 weeks (3.0×10^6 cells/ml) was about 9 fold higher than that in normal mice (3.6×10^5 cells/ml, $P < 0.01$).

Table 1. Resistance to *C. albicans* infection in Meth-A-implanted mice

	Mean survival days	D/T ^{e)}
Exp. 1		
<i>C. albicans</i> ^{a)}	3.0 ± 0.0	12/12
<i>C. albicans</i> + Meth-A ^{b)}	$7.9 \pm 1.0^d)$	10/12
Meth-A ^{c)}	$10.9 \pm 1.2^d)$	8/12
Exp. 2		
<i>C. albicans</i> ^{a)}	2.1 ± 0.1	8/8
<i>C. albicans</i> + Meth-A ^{b)}	$9.6 \pm 1.3^d)$	8/8
Meth-A ^{c)}	$10.9 \pm 1.4^d)$	5/8
Exp. 3		
<i>C. albicans</i> ^{a)}	2.1 ± 0.1	9/9
<i>C. albicans</i> + Meth-A ^{b)}	$9.5 \pm 0.9^d)$	8/9
Meth-A ^{c)}	$11.2 \pm 1.0^d)$	7/9

a) Normal mice infected with *C. albicans*.

b) Meth-A-implanted mice infected with *C. albicans*.

c) Non-infected Meth-A-implanted mice.

d) $P < 0.001$, compared with normal mice.

e) D/T, number of dead mice to total number of mice.

Fig. 1. Survival of Meth-A-implanted mice. Meth A cells (1×10^6) were transplanted s.c. into BALB/c mice. At 21 days after the tumor-implantation, *C. albicans* viable cells (2×10^6) were inoculated i.v. ○, Normal mice infected with *C. albicans*. △, Meth A-implanted mice infected with *C. albicans*. □, Non-infected Meth A-implanted mice. The figure shown is the result of Experiment 1 in Table 1.

Resistance to *C. albicans* infection in Meth-A-implanted mice

Mice were inoculated intravenously with *C. albicans* viable cells and survival of the animals was checked. As shown in Fig. 1 and Table 1, the mice in the control group died within 3 days after the infection, but the Meth-A-implanted mice showed an extended life span. To estimate the protection against *C. albicans* infection in Meth-A-implanted mice, the number of viable *Candida* cells in the kidney was counted 1 day after the infection. The number ($6.7 \pm 6.6 \times 10^3$ CFU/kidney) was significantly lower than that in the normal control ($230 \pm 40 \times 10^3$ CFU/kidney, $P < 0.001$), indicating that the delayed lethal response to *C. albicans* infection was enhanced in Meth-A-implanted mice.

Candidacidal activity of peripheral blood PMNs

Peripheral blood PMNs were collected from mice 3 weeks after Meth-A-implantation, and the candidacidal activity of the cells (1×10^5 cells) against *C. albicans* viable cells (2×10^5 cells) was $70.0 \pm 2.0\%$, significantly higher than that in the PMNs from normal mice ($48.9 \pm 2.2\%$, $P < 0.01$).

Growth-inhibitory activity in serum from Meth-A-implanted mice against *C. albicans*

The activity in serum inhibiting the growth of *C. albicans* was determined by the Alamar blue method. As shown in Fig. 2, the serum from Meth-A-implanted mice decreased the growth of *C. albicans* compared with normal serum. However, the activity was not detected in a 1/1000 dilution of serum from either Meth-A-implanted or normal mice.

Effect of catalase and ferric sulfate on the growth inhibitory activity in serum

Since catalase is a scavenger of H_2O_2 , we examined the correlation between H_2O_2 and the growth-inhibitory factor in serum by catalase. The

inhibition assay was conducted at a 1/100 dilution, because the minimum effective concentration of serum dilution from Meth A-implanted mouse was 1/100 as shown in Fig. 2. Catalase prevented the growth-inhibitory activity in serum from these mice, but not by the resolution of H_2O_2 because the heat-inactivated (100°C , 1min) catalase also inhibited this activity (Fig. 3). As catalase is an iron-containing enzyme¹¹⁾, we examined the effect of ferric sulfate to determine whether the growth-inhibitory activity in the serum is related to an iron-binding protein. The addition of ferric sulfate markedly inhibited the candidacidal activity (Fig. 3), but did not promote the growth of *C. albicans* (data not shown).

Discussion

Augmentation of resistance to *C. albicans* infection has been reported in several tumor-implanted states^{6,7)}. The role of phagocytes in the resistance in this state might be important because the cell-mediated immune system was generally suppressed in a tumor-implanted state⁴⁾.

In this study, we confirmed that the resistance to *C. albicans* infection was augmented in Meth-A-implanted mice (Fig. 1). Both the number and candidacidal activity of peripheral blood PMNs were increased. The PMN-mediated protection to *C. albicans* infection was enhanced in Meth-A-implanted mice. Moreover, we found that the serum from Meth-A-implanted mice had significantly higher activity to inhibit the growth of *C. albicans* cells than that from normal mice (Fig. 2). NO and H_2O_2 are antimicrobial agents secreted by the phagocytes^{12,13)}. The amount of NO in the serum from Meth A-implanted mice measured by Griess reagent¹⁴⁾, was below the detection level (data not shown). Catalase exists in most animal cells, plant cells and bacteria, and it catalyzes H_2O_2 to H_2O and O_2 to prevent the accumulation

Fig. 2. Growth-inhibitory activity of *C. albicans* in the serum. The serum diluted with RPMI1640 medium ($10 \mu\text{l}$) was incubated with *C. albicans* viable cells (1×10^3) at 37°C for 12hr. The number of viable cells was measured by the Alamar blue viability assay. □ Control mice. ▨ Meth A-implanted mice. * $P < 0.001$ vs. normal control.

Fig. 3. Effects of catalase and ferric sulfate on the activity in serum from Meth A-implanted mice to inhibit growth of *C. albicans*. The serum of Meth A-implanted mouse was collected at 3 weeks after the tumor-implantation. Ten microliters of the serum diluted with RPMI1640 ($\times 1/100$) or transferrin ($40 \mu\text{g/ml}$) and $100 \mu\text{l}$ of *C. albicans* (1×10^4 cells/ml in RPMI1640) were mixed and incubated with or without $10 \mu\text{l}$ of catalase ($100 \mu\text{g/ml}$) or ferric sulfate ($4 \mu\text{g/ml}$) for 12hr. After the incubation, the number of viable *C. albicans* was measured by the Alamar blue viability assay. Growth-inhibition (%) = $[(A-B)/A] \times 100$, where A and B are the absorbance corresponding to the group cultured in RPMI1640 only and the group cultured with samples, respectively.

* $P < 0.001$ vs. serum only. ** $P < 0.001$ vs. transferrin only.

of H_2O_2 ¹¹⁾. Although catalase abolished the growth-inhibitory activity in the serum, heat-inactivated catalase had the same effect (Fig. 3). These findings indicated that the serum H_2O_2 was not responsible for the inhibitory activity. Catalase is a hemoprotein which contains iron as protoporphyrin-iron. Addition of ferric sulphate inhibited the activity in the serum from Meth A-implanted mice to inhibit the growth of *C. albicans*. Iron is a universal requirement for cellular proliferation¹⁵⁾. These findings suggested that a component of the serum inhibited the uptake of iron into *C. albicans*. Transferrin is the iron transport protein in serum, and iron-free transferrin (Apo-transferrin) inhibits the growth of *C. albicans* by interfering with its iron-uptake¹⁶⁾. The above findings indicated that the antifungal apo-transferrin might be increased in serum of Meth A-implanted mouse.

Anticandidal activity was also observed in other mice implanted with tumors such as sarcoma 180 and MM46 fibrosarcoma (data not shown). Thus we concluded that the antifungal system was generally developed in the experimental mouse tumor-system.

Acknowledgment

We thank Dr. Makiko Kobayashi for helpful advice.

References

1) Casanova M, Lopez-ribot JL, Martinez JP,

Sentandreu R: Characterization of cell wall proteins from yeast and mycelial cells of *Candida albicans* by labelling with biotin: comparison with other techniques. *Infect Immun* **60**: 4898-4906, 1992.

- 2) Balducci L, Hardy C: High proliferation of granulocyte-macrophage progenitors in tumor-bearing mice. *Cancer Res* **43**: 4643-4647, 1983.
- 3) Lan S, Rettura G, Levenson SM, Seifter E: Granulopoiesis associated with the C3HBA tumor in mice. *J Natl Cancer Inst* **67**: 1135-1138, 1981.
- 4) Tada T, Sano H, Sato S, Shima J, Fujiwara H, Hamaoka T: Immune dysfunction expressed selectively on L3T4⁺ T cells in the tumor-bearing state. *J Leukocyte Biol* **47**: 149-157, 1990.
- 5) Tada T, Ohzeki S, Utsumi K, Takiuchi H, Muramatsu M, Li X, Shimizu J, Fujiwara H, Hamaoka T: Transforming growth factor- β -induced inhibition of T cell function. *J Immunol* **146**: 1077-1082, 1991.
- 6) Okawa Y, Murata Y, Kobayashi M, Suzuki M, Suzuki S: Augmentation of host resistance to *Candida albicans* infection in ascites tumor-bearing mice. *Microbiol Immunol* **36**: 517-521, 1992.
- 7) Robinette EH, Mardon DN: Delayed lethal response to *Candida albicans* infection in mice bearing the Lewis lung carcinoma. *J Natl Cancer Inst* **55**: 731-733, 1975.
- 8) Watanabe T, Okada A, Mikami T, Matsumoto T, Suzuki M: Investigation of phagocyte-inducible factor in tumor-implanted mice. *Biol Pharm Bull* **18**: 1643-1646, 1995.
- 9) Kobayashi M, Watanabe T, Suzuki S, Suzuki M: Effect of *N*-acetylchitohexaose against *Candida albicans* infection of tumor-bearing mice. *Microbiol*

- Immunol **34**:413-426, 1990.
- 10) Pfaller MA, Grant C, Morthland V, Rhine-Chalberg J: Comparative evaluation of alternative methods for broth dilution susceptibility testing of fluconazole against *Candida albicans*. J Clin Microbiol **32**: 506-509, 1994.
- 11) Ruffer M, Steipe B, Zenk MH: Evidence against specific binding of salicylic acid to plant catalase. FEBS Letters **377**: 175-180, 1995.
- 12) Rementeria A, Garcia-Tobalina R, Sevilla MJ: Nitric oxide-dependent killing of *Candida albicans* by murine peritoneal cells during an experimental infection. FEMS Immunol Med Microbiol **11**: 157-162, 1995.
- 13) Thomas EL, Milligan TW, Joyner RE, Jefferson MM: Antibacterial activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral streptococci. Infect Immun **62**: 529-535, 1994.
- 14) Modolell M, Corraliza IM, Link F, Soler G, Eichmann K: Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophage by TH1 and TH2 cytokines. Eur J Immunol **25**: 1101-1104, 1995.
- 15) Campagnari AA, Shanks KL, Dyer DW: Growth of *Moraxella catarrhalis* with human transferrin and lactoferrin: Expression of iron-repressible proteins without siderophore production. Infect Immun **62**: 4909-4914, 1994.
- 16) Shiraishi A, Arai T: Antifungal activity of transferrin. Sabouraudia **17**: 79-83, 1979.