Short Report

Resistance of Aspergillus fumigatus to Micafungin is Increased by Exogenous β-glucan

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ABSTRACT

Aspergillus species are ubiquitous in the environment and Aspergillus fumigatus can cause life-threatening infections in immunocompromised patients. β-1,3-/1,6-glucan is a major fungal cell wall polysaccharide that has various biological effects on the infected host, but little is known about the influence of β-glucan on the fungus itself. In a previous study, we demonstrated that the cell wall β-glucan content could be increased in Aspergillus spp. by addition of β-glucan to the culture medium. In this study, we investigated the influence of β-glucan on the susceptibility of A. fumigatus to antifungal agents. A. fumigatus was cultured in the presence or absence of β-glucan for antifungal susceptibility testing based on changes of the growth rate and morphology. Susceptibility to micafungin, a β-glucan synthase inhibitor, was about 10-fold lower when β-glucan was added to the culture medium. On the other hand, susceptibility to amphotericin B and voriconazole was similar in either the presence or absence of β-glucan. These results strongly suggest that β-glucan has an important physiological role in Aspergillus spp.

Key words: Aspergillus fumigatus, cell wall, micafungin, β-glucan

Introduction

Aspergillus species are common fungi in the environment, being found in the soil, air, and dust. While some Aspergillus spp. are beneficial, others are potentially harmful. Aspergillus fumigatus is a representative cause of various fungal diseases, such as allergic bronchopulmonary aspergillosis (ABPA), chronic necrotizing Aspergillus pneumonia, aspergilloma, and invasive aspergillosis. These diseases can be life-threatening in immunocompromised patients.

With progress in medicine, the elderly population and the number of immunocompromised patients have gradually increased, which has in turn led to the increase in the number of patients with mycoses. Among patients with mycoses, Aspergillus spp. were most frequently detected according to the Annual of Pathological Autopsy Cases in Japan.

Treatment of aspergillosis involves surgical resection and/or chemotherapy, with micafungin, voriconazole, and amphotericin B being the representative antifungal agents used. Micafungin is a cell wall synthase (Fks) inhibitor that is often used in combination therapy, while voriconazole is a cell membrane synthesis inhibitor. Amphotericin B binds to ergosterol in the cell membrane and destabilizes the membrane. Voriconazole is recommended as the primary treatment for invasive aspergillosis, with liposomal amphotericin B as an alternative to voriconazole, while echinocandins such as micafungin are second-line agents for invasive aspergillosis.
β-glucan is a major component of the fungal cell wall and is also found in plants, algae, and bacteria. Thus, β-glucan not only exists in the environment, but can also be detected in the serum of patients with deep mycosis. β-Glucan is a well-known pathogen-associated molecular pattern (PAMP) and is recognized by the C-type lectin receptor, dectin-1, which is expressed on the surface of antigen-presenting cells such as macrophages. Stimulation of dectin-1 by β-glucan leads to production of various cytokines, including TNF-α and IL-6.

In our previous study, we examined the influence of β-1,3-glucan on fungal growth and cell wall structure. A. fumigatus was cultured in C-limiting medium with or without β-1,3-glucan (0.5% of laminarin, curdlan, or curdlan-oligo). Hyphal growth was promoted in liquid and solid cultures by the addition of β-1,3-glucan, but glucose and dextran did not enhance growth. The influence of exogenous β-glucan on cell wall structure was examined by enzymolysis and NMR spectroscopy, which showed a change in the cell wall content of β-1,3-glucan.

In the present study, the susceptibility of A. fumigatus to antifungal agents was examined in the presence or absence of laminarin in the culture medium.

**Materials and methods**

Chemicals: Laminarin (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) was purchased for use as β-glucan.

Fungal strains: The strains used in this study (A. fumigatus NBRC33022, 30870, 31952, and 7080) were purchased from NITE Biological Resource Center and maintained on potato dextrose agar at 25°C.

**Culture media and conditions**

C-limiting medium was originally described by Shepherd and Sullivan and is composed of (NH₄)₂SO₄, 2 g; KH₂PO₄, 2 g; CaCl₂ · 2H₂O, 0.05 g; MgSO₄ · 7H₂O, 0.05 g; ZnSO₄ · 7H₂O, 1 mg; CuSO₄ · 5H₂O, 1 mg; FeSO₄ · 7H₂O, 0.01 g; biotin, 25 μg; and carbon source, 10 g/l; with final pH at 5.2. Aspergillus was cultured at 37°C in C-limiting medium with modification of carbon source from 1.0% sucrose to either, 0.5% sucrose + 0.5% glucose, or 0.5% sucrose + 0.5% β-glucan (laminarin) for antifungal susceptibility testing.

**Antifungal susceptibility test**

Metabolic analysis – The susceptibility test was conducted by microdilution method. Aspergillus (2 × 10⁴ conidia/ml) was incubated at 37°C in an antifungal dilution series for 24h with micafungin (Astellas Pharma Inc., Osaka, Japan) or voriconazole (Sigma-Aldrich Co. LLC., St. Louis, USA). CellTiter-Blue (Promega Co., Madison, USA) was then added according to the manufacturer’s protocol, and Abs600 was measured in the supernatant.

Microscopy – The morphological changes caused by antifungals were observed in each well using a microscope (20 × magnification).

Inhibition ratio – The inhibition ratio was calculated using the following formula:

\[ \text{Inhibition ratio} = \frac{\text{Abs600, sample}}{\text{Abs600, control}} \times 100 \]

Each experiment was performed in duplicate and repeated at least three times.

IC₅₀ – The antifungal concentration causing 50% inhibition of growth was determined as the IC₅₀ from the inhibition ratio graph.

**Results**

**Effect of exogenous laminarin on susceptibility of A. fumigatus to micafungin**

In our previous study, we examined the effects of carbon source on the synthesis of β-glucan and found that the growth of A. fumigatus was enhanced in the presence of β-glucan (laminarin and curdlan), and the β-glucan content in the cell wall was increased. We also found that a minimum concentration of low molecular weight carbon source, such as sucrose or glucose, was necessary for optimum growth of Aspergillus, thus, 0.5% sucrose was included in all the experiments in this study. It was assumed that exogenous β-glucan stimulated the signaling mechanism that promoted cell wall synthesis. To determine the effect of exogenous β-glucan on A. fumigatus, we investigated the susceptibility of this fungus to micafungin (a cell wall synthase inhibitor). When A. fumigatus was cultured with laminarin, growth inhibition by micafungin was significantly decreased at concentrations higher than 6.25 μg/ml, in contrast to the effects of sucrose and glucose (Fig. 1).

Microscopy did not reveal any hyphae in cultures with sucrose or glucose at micafungin
concentrations of 50 and 200 µg/ml. However, hyphae were recognized in cultures with laminarin at micafungin concentrations of both 50 and 200 µg/ml (Fig. 2a). Moreover, growth of hyphae was seen after 72 h in the laminarin group at a micafungin concentration of 100 µg/ml, whereas almost no hyphal growth was observed in the sucrose group (Fig. 2b).

**Fig. 1.** Influence of carbon source on micafungin susceptibility of *A. fumigatus* NBRC 33022.

Spores (2 × 10⁴ conidia/ml) of *A. fumigatus* were inoculated into C-limited medium and incubated at 37°C with 5% CO₂ for 24 h. CellTiter-Blue solution was added, and incubation was continued for an additional 4 h. After centrifugation, reduced dye was measured in the supernatant at 600 nm. Suc: C-limiting medium + 0.5% sucrose. Glc: C-limiting medium + 0.5% glucose. Lam: C-limiting medium + 0.5% laminarin.

Values represent the mean ± standard deviation, n = 4. Significant difference from sucrose: *p < 0.01

**Fig. 2.** Microscopic observation of *A. fumigatus* NBRC 33022 in the presence or absence of micafungin in each medium.

Suc: C-limiting medium + 0.5% sucrose. Glc: C-limiting medium + 0.5% glucose. Lam: C-limiting medium + 0.5% laminarin.

(a) Mycelium after 24 h of culture at 37°C with 5% CO₂.

(b) Growth kinetics of *A. fumigatus* NBRC 33022 treated with micafungin (100 µg/ml).
Thus, laminarin decreased the susceptibility of the standard strain (NBRC33022) to micafungin.

Next, we evaluated the susceptibility of other *A. fumigatus* strains (NBRC30870, NBRC31952 and NBRC7080) by determining IC₅₀ values. For all strains tested, resistance to micafungin increased when laminarin was added to the culture medium in comparison with addition of sucrose or glucose (Table 1).

### Table 1. Effect of carbon source on susceptibility of *A. fumigatus* to micafungin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fumigatus</em> NBRC33022</td>
<td>Sucrose</td>
<td>25</td>
</tr>
<tr>
<td><em>A. fumigatus</em> NBRC30870</td>
<td>Glucose</td>
<td>25</td>
</tr>
<tr>
<td><em>A. fumigatus</em> NBRC31952</td>
<td>Laminarin</td>
<td>&gt;200</td>
</tr>
<tr>
<td><em>A. fumigatus</em> NBRC7080</td>
<td>Sucrose</td>
<td>25</td>
</tr>
</tbody>
</table>

*IC₅₀ (µg/ml) of micafungin for *A. fumigatus* strains in different culture media. Susceptibility was tested using the resazurin assay.*

Influence on antifungal agents that target the cell membrane

The main functions of the microbial cell wall are to protect and maintain the correct cellular architecture. During growth, biosynthesis and degradation of the cell wall occur concomitantly, especially around the nascent region, with cell membrane construction occurring in a progressive manner. We assumed that exogenous β-glucan would not only influence cell wall synthase inhibitors but also cell membrane synthesis inhibitors. Amphotericin B and voriconazole are representative antifungal agents acting on the cell membrane. We evaluated the susceptibility of *A. fumigatus* to these two agents by assessing metabolic activity. However, no significant differences were recognized among the different carbon sources in cultures with either amphotericin B or voriconazole (Fig. 3). These findings showed that exogenous β-glucan only reduced the sensitivity of *A. fumigatus* to echinocandins.

### Discussion

Mechanisms of drug resistance include target mutations, target overexpression, and increased efflux pump activity. There have been many reports about mutation of *cyp51* in relation to azole resistance²⁰, while echinocandin resist-

ance is known to occur by mutation of *fks*²¹. The mechanism of echinocandin resistance has been discussed in detail for *Candida* spp. According to Perlin, amino acid substitutions associated with
resistance occur at two highly conserved hot-spot regions of Fks encompassing residues Phe641-Pro649 and Arg1361 (or its equivalent) in C. albicans and most other Candida spp. However, the mechanism of echinocandin resistance employed by Aspergillus spp. is not well known. Echinocandins have a fungicidal effect on Candida spp., whereas these agents inhibit germination and hyphal growth in the case of A. fumigatus\textsuperscript{20}. This might make it relatively difficult to investigate the effects of echinocandins on Aspergillus. Recently, it was reported that formation of biofilms from serum components reduced the susceptibility of fungal pathogens to antifungal agents.\textsuperscript{21} This might make it relatively difficult to investigate the effects of echinocandins on Aspergillus. Moreover, resistance occur at two highly conserved hot-spot regions of Fks encompassing residues Phe641-Pro649 and Arg1361 (or its equivalent) in C. albicans and most other Candida spp.\textsuperscript{20} However, the mechanism of echinocandin resistance employed by Aspergillus spp. is not well known. Echinocandins have a fungicidal effect on Candida spp., whereas these agents inhibit germination and hyphal growth in the case of A. fumigatus.\textsuperscript{20} This might make it relatively difficult to investigate the effects of echinocandins on Aspergillus. Recently, it was reported that formation of biofilms from serum components reduced the susceptibility of fungal pathogens to antifungal agents.\textsuperscript{21} This might make it relatively difficult to investigate the effects of echinocandins on Aspergillus. Moreover, a recent study reported that the cell wall of A. fumigatus was composed of various components, and that usually synthesis of each component was coupled and coordinated with each other. The uncoupling, however, of the mechanism of echinocandin resistance in A. fumigatus was coupled and coordinated with each other. The uncoupling, however, of the mechanism of echinocandin resistance in A. fumigatus was coupled and coordinated with each other. The uncoupling, however, of the mechanism of echinocandin resistance in A. fumigatus was coupled and coordinated with each other. The uncoupling, however, of the mechanism of echinocandin resistance in A. fumigatus was coupled and coordinated with each other. The uncoupling, however, of the mechanism of echinocandin resistance in A. fumigatus was coupled and coordinated with each other. The uncoupling, however, of the mechanism of echinocandin resistance in A. fumigatus was coupled and coordinated with each other. The uncoupling, however, of the mechanism of echinocandin resistance in A. fumigatus was coupled and coordinated with each other.

Ishibashi et al. reported that the α / β ratio of glucan in the presence of laminarin was altered in the precursor region of Fks encompassing residues Phe641-Pro649 and Arg1361 (or its equivalent) in A. fumigatus.\textsuperscript{19} Thus, the increased cell wall content of β-glucan enhanced micafungin resistance. This suggests that exogenous β-glucan may promote the overexpression of Fks (β-glucan synthase) in A. fumigatus, thus increasing the drug target. Micafungin binds to β-glucan synthase and inhibits β-glucan synthesis\textsuperscript{20}, so an increase of the drug target could reduce sensitivity to micafungin. Ishibashi et al. also reported that the α / β ratio of glucan was altered in the presence of exogenous β-glucan.\textsuperscript{19} In recent years, studies on cell wall integrity, including the role of the MAPK pathway, have progressed, and the mechanisms of the cell wall stress response have been clarified.\textsuperscript{24, 27} Exogenous β-glucan may influence the cell wall integrity of A. fumigatus and thus reduce susceptibility to micafungin.

**Conflict of interest**

All authors declare no conflict of interest.

**References**


