Original Article

Inhibitory Effect of Extracellular Polysaccharide EPS-II from *Pseudoalteromonas* on *Candida* adhesion to Cornea *in vitro**

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Abstract

Objective Fungal keratitis (FK) is a vision-threatening infection, whose treatment requires more effective and safer anti-fungal agent exploitation urgently. With this aim, we focused on the effect of an extracellular polysaccharide on fungal adhesion to human corneal epithelial cells.

Methods We performed the cytotoxicity assays of the extracellular polysaccharide EPS-II from an antarctic bacterium *Pseudoaltermonas* and evaluated its inhibitory effect on *Candida albicans* cells' adherence to human corneal epithelial cells (HCECs).

Results EPS-II, which displayed minor cytotoxicity but also promoted proliferation of HCECs, could inhibit the adherence of yeast cells to HCECs in a dose-dependent manner. EPS-II could also suppress the subsequent PI3K/AKT signaling pathway, and thereby decrease the expression of early inflammatory cytokines.

Conclusions Extracellular polysaccharide EPS-II was suggested as a new natural agent for attenuating FK.

Key words: Extracellular polysaccharide EPS-II; Candida albicans; Cornea; Adhesion; Inhibitory effect

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INTRODUCTION

Fungal infection, especially fungal keratitis (FK), is a common clinical disease and can lead to corneal blindness or even loss of the infected eye if not treated properly[1]. The main pathogens of FK that are predominant worldwide include *Fusarium*, *Aspergillus*, and *Candida*, although the geographical distribution of each fungus varies widely. The most common risk factor associated with infection by these pathogens is trauma[2-3]. Fungal yeast cells can adhere to the injured corneal epithelial cells, triggering an infection. It has been shown that the early response to fungal invasion is triggered by fungal yeast cells adhering to the extracellular matrix of epithelial cells, which activates integrins and impacts cell motility and migration through signal transduction[4]. Fungal adherence to epithelial cells is an important part of the initial invasion and the prerequisite for the pathogenesis of FK. Interruption of this adhesion might be an effective way to inhibit further fungal invasion and thus the development of FK.

Extracellular polysaccharides, with multiple bioactivities including immune regulation and anti-tumor properties, display potential inhibitory properties against pathogens[5-7]. In recent years, there has been an increase in the number of reports concerning the anti-infection properties of these polysaccharides, including anti-viral[8-9] and anti-

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bacterial[10-11] activities. However, there are few reports regarding the anti-fungal activity of polysaccharides.

Antarctic bacteria are considered to be an important resource for new drug exploitation due to their ability to live in such an extreme and specific environment. These bacteria may be a source of polysaccharides with novel structural and functional characteristics. EPS-II, an extracellular polysaccharide extracted from the antarctic bacterium *Pseudoaltermonas* sp. S-15-13[12], is a homogenous polysaccharide with a molecular weight of 62 kD. The bioactivity of EPS-II has not been studied previously. In this study, we investigated the effects of EPS-II on *Candida* keratitis in vitro and found that the polysaccharide could partly inhibit the adherence of *Candida albicans* cells to corneal epithelial cells. These results suggest the possibility of a novel strategy for development of safe and effective drugs for FK clinical prevention and therapy. Additionally, EPS-II may be useful in treatment of other infectious diseases as well.

MATERIALS AND METHODS

Preparation of Polysaccharide, Fungus, and Cells

The extracellular polysaccharide EPS-II, extracted from the antarctic bacterium *Pseudoaltermonas* sp. S-15-13 as described previously[12], was provided by the First Institute of Oceanography, State Oceanic Administration. In brief, the exopolysaccharide fractions were extracted and purified by DEAE-Sephadex A-50 ion-exchange and Sephadex G-100 gel chromatography to give EPS-II. The EPS-II was eluted as a single peak in HPLC analysis, indicating the homogeneity and purity of EPS-II. Before use, the EPS-II preparation was dissolved in normal saline and filtered through a 0.22 μm pore filtration membrane. The concentration of the stock was measured by using the anthrone-sulfuric acid method, and then the concentration was adjusted. The *Candida albicans* strain (ATCC 90 029, American Type Culture Collection) was cultured on Sabouraud's agar (Haibo, Qingdao, China) at 37 °C for 1 day. For use, *Candida albicans* cells were suspended in medium and adjusted to the proper concentration. The human corneal epithelial cell (HCEC) line (ATCC CRL-11 135) was cultured in DMEM/F12 medium with 10% FBS (Invitrogen) at 37 °C , in a humidified atmosphere of 5% CO_2/95% air.

Proliferation Assay

Proliferation of HCECs in response to EPS-II was measured by using 3-(4, 5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assays. HCECs were incubated in medium with 0, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1 600, 3 200, or 6 400 μg/mL of EPS-II for 72 hrs, followed by a 4 hr incubation with MTT. The MTT-transformed crystals were dissolved in dimethyl sulfoxide, and the absorbance at 570 nm was measured by using a microplate reader (Molecular Devices, Sunnyvale, USA). Each concentration of EPS-II was tested in triplicate.

Inhibitory Effects of EPS-II on Candida Albicans Adherence to HCECs

HCECs were placed in 24-well plates with 1×10^5 cells per well and cultured at 37 °C. After cells grew to form a confluent monolayer, 1 mL aliquots of medium with varying concentrations of EPS-II (0, 25, 50, 100, 200, or 400 μg/mL) were added for 1 hr, and then 10^6 CFU *Candida albicans* cells were added. After being incubated with fungal yeast cells for 1 hr at 37 °C, the yeast cells were washed 3 times with normal saline. Then 1 mL of lysis buffer (0.25% trypsin, 0.02% EDTA, 0.01% Triton X-100) was added to each well and kept for 10 min. The samples were gradiently diluted 10-fold, evenly spread on Sabouraud's agar plate and cultured at 37 °C for 48 hrs. The resulting number of colonies was counted. With a linear relationship between the EPS-II concentration and the percentage of inhibition, the 50% inhibitory concentration (IC50) was calculated.

Western Blotting

The serine threonine kinase (AKT) level in HCECs was analyzed by using western blots with antibodies against phosphorylated proteins. Briefly, monolayer HCECs in 35 mm plates were incubated with 10^8 CFU *Candida albicans* cells for 30 min, in either the presence or the absence of 200 μg/mL of EPS-II. Cells were then harvested in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) buffer and were sonicated. Protein components were separated on 10% SDS–PAGE gels for 2 hrs at 140 V and then transferred to PVDF membrane. The blots were blocked in 5% non-fat dry milk for 1 hr and incubated with primary antibodies against AKT and phosphorylated AKT (Cell Signaling Technology, Danvers, USA) for 1 hr at room temperature. After three washes with 10 mL of Tris-buffered saline Tween-20 (TBST), blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, USA), and proteins were visualized via enzyme-linked chemiluminescence by using the enhanced chemiluminescence (ECL) kit (Chemicon, Temecula, USA).

Real-time Polymerase Chain Reaction

Total RNA was extracted from HCECs (EPS-II-treated or EPS-II and *Candida albicans* co-treated) by using Trizol (Invitrogen). cDNA was synthesized from total RNA with an AMV first-strand cDNA synthesis kit (BBI, Toronto, Canada), according to the manufacturer's instructions. Quantitative Real-time PCR was performed by using Taqman reagents and the Applied Biosystems 7 500 Real-Time PCR System (Applied Biosystems, Foster City, USA) according to the instructions of the manufacturer. The specific primers and probes used in this assay are listed in Table 1. Cycling conditions were 10 min at 95 °C, followed by 40 two-step cycles (15 s at 95 °C and 1 min at 60 °C). Data were analyzed with the SDS System Software (Applied Biosystems) by using GAPDH as an internal control. Gene-specific C_t values were standardized based on GAPDH C_t values obtained for each cDNA.

Table 1. Primers Used in Real-time PCR

Primer	Sequence
hGAPDH	F，5′-ATGCTGGCGCTGAGTACGT-3′
	R，5′-AGCCCCAGCCTTCTCCAT-3′
	probe，5′-TGGAGTCCACTGGCGTCTTCA-3′
hIL-6	F，5′-CCCCCAGGAGAAGATTCCAA-3′
	R，5′-TCAATTCGTTCTGAAGAGGTGAGT-3′
	probe，5′-ATGTAGCCGCCCCACACAGACAG-3′
hMMP14	F，5′-GACCTCCAGTTTGCAGAGCG-3′
	R，5′-TCATCAAACACCCAATGCTT-3′
	probe，5′-CCTTTGAAGAAGACGAATTTGCCATCC-3′

Statistical Analysis

Each assay was conducted at least in triplicate. The data are presented as means±SD. SPSS (SPSS software, 11.5 version) was used for data processing. The statistical significance of the therapeutic effect was determined with a Multiple Comparison one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

RESULTS

EPS-II Displayed Minor Cytotoxicity but Promoted Proliferation of HCECs

A cell proliferation assay was carried out to examine the toxicity of different concentrations of EPS-II to corneal epithelial cells. The results indicated that EPS-II did not inhibit HCECs growth (Figure 1). After the addition of EPS-II at concentrations ranging from 6.25 to 6 400 μg/mL, the proliferative efficacy of HCECs was significantly increased with 12.5 and 25 μg/mL of EPS-II compared with the control group. The proliferative efficacy of HCECs was highest when they were treated with 12.5 μg/mL EPS-II. When the concentration of EPS-II was increased to 50 μg/mL, the proliferative efficacy was decreased, but HCECs were still stimulated to proliferate compared with the control group. Under the microscope, HCECs appeared to have good growth status. When the concentration of EPS-II ranged from 50 to 200 μg/mL, the proliferative efficacy of HCECs was decreased with no significant difference compared to controls. When the concentration was above 200 μg/mL, EPS-II showed cytotoxicity to HCECs. This cytotoxicity might be caused by the fact that the minor viscosity of EPS-II decreased the proliferative efficacy. To avoid this viscosity and thus possible cytotoxicity, the concentration of EPS-II in the following experiments did not exceed 400 μg/mL.

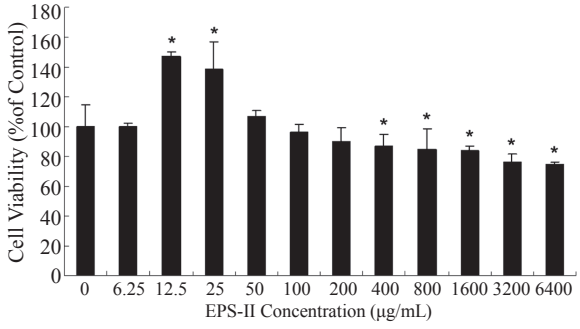

Figure 1. Cell proliferation assay. When compared with the control group, the proliferative efficacy of HCECs was significantly ($^*P<0.05$) increased after incubation with 12.5 and 25 μg/mL of EPS-II, and decreased without any significant difference after incubation with 50 to 200 μg/mL of EPS-Ⅱ. When the concentration was above 200 μg/mL, EPS-II showed a little cytotoxicity to HCECs, but still not significantly. (n=4).

EPS-II Inhibited Candida Albicans Adherence to Corneal Epithelial Cells

Since adherence to host cells is the initial step for pathogenic infection and polysaccharide may play a role during this course, we wondered if the extracellular polysaccharide EPS-II could alter fungal adherence to cells. The inhibition of *Candida albicans* adherence to corneal epithelial cells treated with various concentrations of EPS-II was examined by counting colonies of the adhered yeast cells.

Adherence inhibition by various concentrations of EPS-II is shown in Figure 2. Compared with the control group, the number of colonies formed by *Candida albicans* treated with EPS-II was significantly decreased at different extents at all concentrations of EPS-II except for 25 μg/mL. Moreover, the inhibition of yeast cell adherence was increased with the increasing concentrations of EPS-II, but there was no significant difference between the inhibition by 200 μg/mL and 400 μg/mL EPS-II. It was evident that EPS-II could inhibit *Candida albicans* yeast cell adherence to corneal epithelial cells, and the IC50 was calculated to be 91.14 μg/mL.

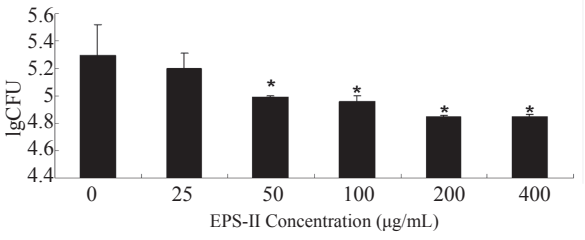

Figure 2. Inhibition of EPS-II on fungal adhesion to corneal epithelial cells. Corneal epithelial cells were inoculated in 24-well plate until confluence and pre-treated with EPS-II at the concentrations of 25 to 400 μg/mL, and then 10^6 CFU Candida albicans spores were inoculated and incubated for 30 min at 37 °C. Compared with the controlled group, the adhered spores were significantly decreased at different extents besides 25 μg/mL EPS-II, and the inhibition was increased with the up-regulated concentration of EPS-II (n=4).

EPS-II inhibited AKT Phosphorylation in Corneal Epithelial Cells

Corneal cell survival in viral infection requires phosphoinositide 3-kinase (PI3K)/AKT activation[13], to determine whether (PI3K)/AKT pathway plays a role in yeast infection. Since yeast infection also leads to some changes to cell, we assayed the AKT phosphorylation level in *Candida* infection. HCECs were co-cultured with *Candida albicans* cells in either the presence or the absence of EPS-II (200 μg/mL). After incubation for 30 min, western blotting assays and immunocytochemistry were performed. As shown in Figure 3, phosphorylated AKT was expressed in HCECs co-cultured with *Candida albicans* cells but was not expressed in HCECs in the absence of *Candida albicans*. EPS-II apparently reduced the phosphorylation level of AKT in HCECs stimulated by *Candida albicans* cells, although it had no effect on the total amount of AKT protein produced.

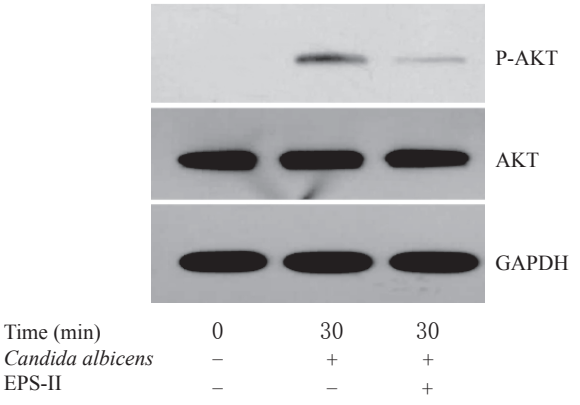

Figure 3. The effects of EPS-II on AKT signaling by Western blotting.

The protein expression of phosphorylation AKT was assessed by using the antibody of AKT and phosphorylation AKT in total cellular protein lysates. EPS-II significantly reduced the level of phosphorylation of AKT in HCECs stimulated by Candida albicans spores for 30 min at 37 °C, although it had no effect on the total amount of AKT protein.

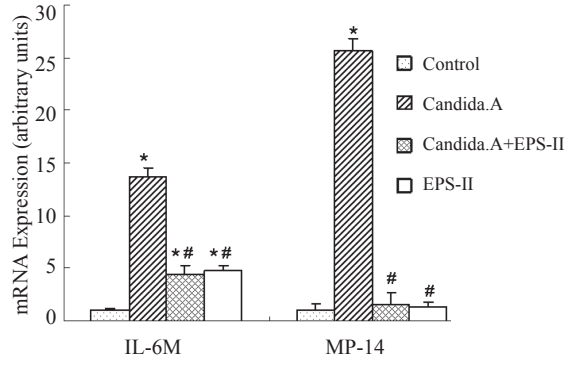

Figure 4. Changes in inflammation-related genes in HCECs treated with or without *Candida albicans* spores and EPS-II. Compared with the control, IL-6 and MMP-14 were significantly expressed in HCECs stimulated by *Candida albicans* spores for 30 min ($^*P$<0.05), while in HCECs co-treated with EPS-II and *Candida albicans* spores instantaneously, the expression of IL-6 and MMP-14 was significantly reduced compared with HCECs treated only with spores ($^\#P$<0.05).

mRNA Expression of Related Inflammatory Cytokines

Interleukin-6 (IL-6) is considered to play an

important protective role in the infected keratitis[14], and membrane type-1 matrix metalloproteinase (MMP14) is up-regulated during early wound formation[15]. In this study, we evaluated if the expression of these two inflammatory cytokines was stimulated by fungal adherence to HCECs. As shown in Figure 4, IL-6 was not constitutively expressed by uninfected HCECs. After HCECs were incubated with *Candida albicans* cells for 30 min at 37 °C, IL-6 was expressed at levels significantly greater than controls, whereas in HCECs co-incubated with EPS-Ⅱ instantaneously, IL-6 expression was significantly reduced. Likewise, MMP14 was expressed in HCECs stimulated by *Candida albicans* cells for 30 min, and its expression was significantly suppressed by the addition of EPS-II.

DISCUSSION

In the complex interactions between pathogenic fungi and host cells, the initial adhesion of fungal yeast cells to host tissues is the prerequisite for accomplishing the pathogenesis of fungal keratitis and consequently has been the topic of many scientific investigations[16-17]. Blocking the initial adhesion should be a good approach to suppressing fungal invasion. In this study, we evaluated the effect of extracellular polysaccharide EPS-II on the adherence of *candida* to corneal epithelial cells and found that EPS-II could partially inhibit *Candida albicans* cells from adhering to corneal epithelial cells. Moreover, the inhibition occurred in a dose-dependent manner. This inhibitory effect must be attributed to the fact that EPS-II blocked the adhesion of the yeast cells to HCECs by specific or nonspecific interactions. Following the adherence evaluation experiment, we examined the changes of some proteins downstream of adhesion to further confirm the inhibitory effect of EPS-II.

The adherence of fungal yeast cells to corneal epithelial cells could activate the integrins of the extracellular matrix[4]. Several adhesins have been reported to be involved in fungal adherence to epithelial or endothelial cells[18-20]. Additionally, it is well known that integrin can activate the downstream signal transduction pathway including FAK, PI3K/AKT or SHC and further activate the mechanisms of cellular reparation, including cell adhesion, motility and migration[21]. In the study, we chose the PI3K/AKT pathway for investigation because we thought it highly probable that PI3K/AKT pathway plays roles during corneal epithelial cells stimulated by *Candida albicans* cells since PI3K-AKT pathway is an important mechanism through which infection influences various cell functions[22-23]. We examined the phosphorylation of AKT and found that fungal adherence could significantly promote AKT phosphorylation in HCECs, and EPS-II could partly inhibit AKT phosphorylation in HCECs stimulated by fungal yeast cells. This observation demonstrated that the PI3K/AKT signal pathway could be activated in the early stages of fungal invasion, and EPS-II could suppress this pathway by inhibiting fungal adherence to HCECs.

During fungal invasion, some inflammatory cytokines such as IL-1β, MIP-2, and IL-6 are expressed[24]. IL-6 is a multifunctional cytokine known to up-regulate the immune response, thus generating humoral or cellular immune reactions and stimulating cells to synthesize acute phase proteins to form an acute phase inflammatory response[25]. In addition, matrix metalloproteinases (MMPs) play important roles during fungal invasion; they can degrade extracellular matrix and regulate cellular adhesion. It had been reported that MMP-2 and MMP-9 expression could be detected in rabbit cornea infected with *Fusarium solani* and *Aspergillus flavus*[26]. Additionally, MMP-14 was reported to be up-regulated during early wound formation as a means of activating integrins and regulating cellular adhesion[15]. Accordingly, we detected the expression of IL-6 and MMP-14 using Real-time PCR assay in HCECs stimulated by *Candida albicans* cells for 30 min. We found that both IL-6 and MMP-14 were up-regulated during early fungal invasion. Additionally, the assay demonstrated that EPS-II could significantly suppress the expression of IL-6 and MMP-14 as a result of the inhibition of fungal adherence to HCECs. IL-6 expression was also up-regulated in HCECs treated with pure EPS-II, the reason might be that EPS-II contains similar ligand to *Candida albicans,* which makes it not only competitively inhibit *Candida albicans* adherence to HCECs, but also activate the following cell response though binding to its corresponding receptor on cell surface. However, the expression of MMP-14 consisted with mock treated HCECs, and the reason might be that the similar ligand in EPS-II could not bind to the receptor activating MMP-14.

In conclusion, EPS-II can partially inhibit the adherence of *Candida albicans* cells to corneal epithelial cells. It can also affect the subsequent PI3K/AKT signaling pathway and thereby influence the expression of early inflammatory cytokines. These data suggest that the extracellular polysaccharide EPS-II may be a novel natural active agent useful in attenuating FK.

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