学 位 論 文 の 要 旨

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学 位 論 文 名　Disulphide-Reduced Psoriasin is a Human Apoptosis-Inducing Broad-Spectrum Fungicide

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INTRODUCTION

It is amazing that the permanent exposure and colonization of our body surfaces by various fungi does not usually cause infections in healthy individuals. Surprisingly, it is largely unknown how and why human body surfaces resist fungal pathogens. Healthy human lungs are highly efficient at clearing airborne fungal spores without causing lung inflammation, suggesting that innate defense strategies to control fungal pathogens do exist in the epithelium. Epithelial antimicrobial peptides are the candidate effector molecules that could play a role in defending the body against fungal infections. Although the disulphide reduced form of human **β**-defensin-1 shows—apart from its bactericidal activity—strong activity against *Candida albicans*, there is no systematic study investigating antifungals with human epithelial origin that might control the growth of filamentous fungi at body surfaces. To address this important question, we analyzed lesional skin from patients with psoriasis—a skin disease with an unexpected resistance to fungal infections —in an attempt to identify human antifungals.

MATERIALS AND METHODS

Aspergillus fumigatus (*A. fumigatus*), *Candida albicans* (*C. albicans*), *Malassezia furfur* (*M. furfur*), *Microsporum canis* (*M. canis*), *Rhizopus oryzae* (*R. oryzae*), *Trichophyton mentagrophytes* (*T. mentafrophytes*), and *Trichophyton rubrum* (*T. rubrum*) were cultured in a Sabouraud liquid medium for assaying antifungal proteins. Fungal growth was photometrically measured at 595 nm. Purification of the antifungal protein from lesional psoriatic scale extracts was performed by high

performance-liquid chromatography (HPLC). Its structural identification as reduced psoriasin (redS100A7) was performed using matrix assisted Laser desorption/ionization-mass spectrometry (MALDI-MS), electrospray ionization-mass spectrometry (ESI-MS), amino acid sequencing, SDS/PAGE and Western blot analyses. Psoriasin mutants were generated as SUMO-fusion proteins, which were cleaved by SUMO-protease and further purified by HPLC. Morphological studies of redS100A7-treated and N, N, N′, N′-Tetrakis (2-pyridylmethyl)-ethylenediamine (TPEN)-treated fungi were performed with transmission electron microscope (TEM) and scanning electron microscope. For immunogold TEM, S100A7 locations in treated *T. rubrum* were identified with antibodies. Apoptosis induction was tested with the TdT-mediated dUTP nick end labeling (TUNEL)-assay and SYTOX-green staining. *In vivo* activities of redS100A7 and TPEN as antifungal agents were investigated in guinea pig tinea pedis and mouse Aspergillus lung infection models. The study protocol was approved by the Ethics Committee of Shimane University and written informed consent was obtained from all subjects. All experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Shimane University and they were handled according to our institutional guidelines.

RESULTS AND DISCUSSION

An antifungal agent was purified by several HPLC steps to homogeneity. SDS/PAGE, Western blot analyses, amino acid sequencing, and MALDI-MS analyses revealed that the antifungal protein was the reduced form of psoriasin, S100A7. redS100A7 inhibits various filamentous fungi, including the mold *A. fumigatus*, *M. furfur*, *M. canis*, *R. oryzae*, *T. mentagrophytes*, and *T. rubrum,* but not *C. albicans*. Antifungal activity was inhibited by Zn^{2+}, suggesting that redS100A7 interferes with fungal zinc homeostasis. Because S100A7-mutants lacking a single cysteine are no longer antifungals, we hypothesized that redS100A7 is acting as a Zn^{2+}-chelator. To elucidate whether opening the disulphide bond of oxidized psoriasin (oxS100A7) causes secondary structure changes, we performed circular dichroism (CD) spectroscopy. Zn^{2+}-exposure causes a shift of the CD spectrum, supporting the hypothesis that Zn^{2+} produces a conformation change. This hypothesis is also supported by the findings of a mass of 22,958 Da, corresponding to a redS100A7 dimer plus Zn^{2+} ions by ESI-MS. Immunogold TEM studies revealed that it penetrates fungal cells, implicating possible intracellular actions. In support with our hypothesis, the cell-penetrating Zn^{2+}-chelator TPEN was found to function as a broad-spectrum antifungal. Ultrastructural analyses of redS100A7-treated *T. rubrum* revealed marked signs of apoptosis, suggesting that its mode of action is induction of programmed cell death. TUNEL, SYTOX-green analyses, and caspase-inhibition studies supported this for both *T. rubrum* and *A. fumigatus*. Whereas redS100A7 can be generated from oxS100A7 by action of thioredoxin or glutathione, elevated redS100A7 levels in fungal skin infection indicate induction of both S100A7 and its reducing agent *in vivo*.

To investigate whether redS100A7 and TPEN are antifungals *in vivo*, we used a guinea pig tinea pedis model for fungal skin infections and a lethal mouse Aspergillus infection model for lung infection. An ablated guinea pig foot was treated with redS100A7, TPEN, or the vehicle. The foot was then infected with 1.5 x 10^7 *T. mentagrophytes* conidia. After 3 days of infection, the infected areas were analyzed microscopically, and with Periodic acid Schiff (PAS) reagent and Fungiflora Y staining. Fungal invasion into the stratum corneum was recorded. Both redS100A7 and TPEN showed a significant protective effect in the guinea pig model of *T. mentagrophytes* infection and found antifungal activity in both *in vivo* animal systems. Immunocompromised mice were infected with 2 x 10^7 *A. fumigatus* conidia for 2 consecutive days. Whereas all mice in the control group did not survive the infection, mice in the redS100A7-or TPEN-treatment survived the invasive fungal infection throughout the 7-day observation period. Numerous *A. fumigatus* conidia and hyphae, as well as massive neutrophil infiltrates, were observed in the untreated control lungs after 3 days. In contrast, fungal burdens were hardly found in redS100A7- or TPEN-treated mice. However, lung histology showed massive inflammatory infiltrates, possibly as a result of dead and degenerating hyphae.

The finding that cystein-thiol-lacking S100A7 derivatives show less antifungal activity and S100A7 mutants lacking cystein are not fungicidal suggests that free-thiol groups are essential for the antifungal activity of S100A7 and points toward a unique mode of action of redS100A7. Interestingly, the growth of *Escherichia coli (E. coli)* was similarly inhibited by S100A7 mutants lacking cystein, as seen with oxS100A7, corroborating that both free thiols are essential for antimycotic, but not bactericidal activity. Antibacterial activity of oxS100A7 is mainly restricted toward *E. coli*; far lower activity was seen against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and is inhibited by Zn^{2+} at pH 7.4. This finding suggests that histidine-based Zn^{2+}-binding sites in the oxS100A7 and in the S100A7 mutants lacking cystein are involved in *E. coli*-cidal activity at pH 7.4. However, at pH 5.5, the normal skin pH, where histidine-based Zn^{2+}-binding sites are inactive, oxS100A7 is bactericidal with a different mode of action, targeting the bacterial membrane by forming pores.

CONCLUSION

Our data represent a previously undescribed mechanism of action for an antimicrobial protein. We propose that the redS100A7 acts as a principal human antifungal protein that induces apoptosis in fungi by penetrating the fungal cell membrane and sequestrating Zn^{2+} from an intracellular target via a newly formed thiol-based metal-binding site, which is similar to that seen with the antimicrobial peptide human defensin 5. We therefore suggest that fungus-cell–penetrating Zn^{2+}-chelators, like redS100A7 and TPEN, could become useful and important therapeutic agents against opportunistic, superficial, or invasive fungal infections.