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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論 文 内 容 の 要 旨

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.

論文審査及び最終試験又は学力の確認の結果の要旨

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[論文審査の結果の要旨]

皮膚の微生物に対する感染防御機構に関して，これまでブドウ球菌や緑膿菌，大腸菌などに対する抗菌ペプチドがいくつか同定されてきた。しかし，真菌に対する防御システムは未だ明らかにされていない。一方，慢性炎症性皮膚疾患のひとつである乾癬では，白癬菌の感染頻度が低いことが知られている。

申請者は，乾癬病変の角層抽出物中の抗白癬因子を検索した。その結果，強い抗白癬活性をもつ分子量11,368 Da のタンパク質を単離し，N 末端アミノ酸配列決定および質量分析からこのタンパク質がPsoriasin (S100A7)であると同定した。さらに，分子内に2カ所存在するシステインが還元されたS100A7が活性を示すこと，白癬菌以外に，アスペルギルス，マラセチア，クモノスカビ，ミクロスポルムにも抗真菌作用を示すこと，共焦点レーザー顕微鏡および電子顕微鏡を用いて，白癬菌やアスペルギルスでは，細胞質や胞子に還元型 S100A7 が取り込まれ，細胞内小器官が破壊されること等を明らかにした。皮膚細胞内に存在するチオレドキシンやグルタチオンがS100A7を還元型に変換し，還元型S100A7が真菌細胞内で亜鉛イオンをキレートして活性酸素を蓄積させるため，真菌細胞はアポトーシスに陥ると考えた。ヒト白癬病変部の角層中に還元型 S100A7 が増加していることを示し，モルモット白癬モデルやマウス肺アスペルギルスモデルにおいて還元型 S100A7 が真菌の増殖抑制を示すことを確認した。本研究によって，生体の真菌防御システムの一端が明らかにされたと考えられ，博士(医学)の学位授与に値すると判断した。

[最終試験又は学力の確認の結果の要旨]

申請者は，乾癬病変に白癬菌の感染頻度が低いことから，乾癬病変の角層抽出物より抗真菌ペプチドを単離した。抗真菌活性が真菌細胞のアポトーシス誘導と考えられること，動物実験での有効性を明らかにした。関連分野の知識も豊富で，学位授与に値すると判断した。（主査：吉山　裕規）

申請者は，乾癬病変から抗真菌作用をもつタンパク質　Psoriasin (S100A7)を単離・同定した。さらにS100A7の抗真菌作用とそのメカニズムを，動物モデルや分子生化学的手法を駆使して明らかにした。真菌感染症治療など幅広い分野への応用が期待できること，関連分野の知識も豊富であることなどから学位授与に値すると判断した。（副査：和田　孝一郎）

申請者は，乾癬患者から抗真菌タンパク質 Psoriasin (S100A7)を同定し，その抗真菌メカニズムを様々な実験系を駆使し解明した。また，動物実験においてその効力を確認し，臨床応用可能な重要な知見を得た。審査時における関連知識も豊富で，質疑応答も的確なため，学位授与に十分値するものと判断した。（副査：松本　健一）