Original Article

Novel \textit{in vivo} observations on double acting points of luliconazole on \textit{Trichophyton rubrum}: an ultrastructural study

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Abstract

Scales from lesional skin of 12 patients with tinea pedis were investigated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to gain an insight into the spatial and morphological changes of dermatophytes after application of a clinical dosage of topical luliconazole 1% cream (Lulicon\textsuperscript{®} cream 1%). In all cases, \textit{Tri-chophyton rubrum} was identified. The scales from the lesions collected before and after topical luliconazole application were fixed with glutaraldehyde and subjected to SEM and TEM. For SEM, fixed specimens were first placed in 1N-KOH and then post-fixed and observed. SEM showed a swollen appearance of fungal hyphae as an early change, and then shrinkage of them showing a flattened and twisted appearance as a later change. TEM showed cell wall alterations with initial development of and accumulation of a granular structure in the outermost layer and subsequent amorphous and electron-lucent change of the thickened inner part of the cell wall. This is the first report of dramatic morphological changes of \textit{T. rubrum} before and after topical luliconazole application \textit{in vivo} demonstrated by SEM and TEM. We hypothesize that luliconazole has double acting points, on the plasma membrane and cell wall, of dermatophyte hyphae.

Key words: luliconazole, \textit{T. rubrum}, \textit{in vivo} effect, scanning electron microscopy, transmission electron microscopy.

Introduction

Luliconazole, part of a new class of imidazole derivatives, shows strong antifungal activity against \textit{Trichophyton} species both \textit{in vitro} and \textit{in vivo} \cite{1–4}. Luliconazole functions as an inhibitor of ergosterol biosynthesis and ergosterol is essential to membrane integrity and cell growth in fungi \cite{3,4}.

To assess the efficacy of an antifungal drug, specimens of dermatophyte hyphae have been cultured in a medium containing antifungal drug \cite{1–14}. Morphological changes have also been observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) \textit{in vitro} \cite{5–14}. Other authors \cite{15} used SEM to observe \textit{in vitro} structural changes in the presence of terbinafine.
utilizing a living skin equivalent model. However, no in vivo studies have been conducted to clarify the effect of topical antifungal agents on hyphae of dermatophytes infecting in the scale of a skin lesion of tinea at the ultrastructural level. Therefore, we decided to investigate morphological changes of T. rubrum inside lesional scales before and after treatment with clinical dosage of luliconazole by SEM and TEM.

**Materials and methods**

The Institutional Review Board (IRB) at the Faculty of Medicine, Tottori University, and the IRB of Taniguchi Hospital approved this study.

We randomly chose 12 patients with typical clinical symptoms and positive findings on conventional mycological examinations for tinea pedis in Tottori University Hospital and Taniguchi Hospital. The patients had not been treated with any topical or oral antifungal medication before specimens were obtained. Clinical photographs of each patient’s skin lesion were taken, and lesional scales were used in this study. Some of the scales were used for fungal culture and the others for SEM and TEM. All of the patients were treated with topical luliconazole 1% cream (Lulicon® cream 1%, Pola Chemical Industry Co., Ltd, Yokohama, Japan) once a day, in accordance with prescriptive use and dose after diagnosis. Observations were carried out before topical luliconazole treatment and 1 day and 7 days after topical exposure.

**Fungal Culture**

Scales from skin lesions before topical luliconazole treatment were subjected to fungal culture on Sabouraud dextrose agar (SDA, Nissui Pharmaceutical, Tokyo, Japan) in our mycology laboratory.
Figure 3. A SEM image of fungal hyphae after the application for 7 days. They show shrinkage of fungal bodies in a flattened (white arrows) and twisted appearance. Hyphae are 0.8 to 1.2 μm in diameter in the short axis. Double-headed arrow: 0.8 μm. Scale bar: 5 μm.

SEM

The specimens were fixed in 2.5% glutaraldehyde (GA) in 0.1 M phosphate buffered saline (PBS). These specimens were washed three times at 5-min intervals with 0.1M PBS and were then placed in 1N-KOH solution at room temperature for 6 h. After rinsing with PBS, the specimens were fixed in 1% osmium tetroxide (OsO₄) and conductive-stained with 1% tannic acid and then postfixed in 1% OsO₄ for 2 h. After dehydration through a graded ethanol series, the specimens were finally dried with t-butyl alcohol. Dried samples were examined with a S4500 field emission scanning electron microscope (Hitachi Ltd, Tokyo, Japan).

TEM

For TEM, fixed specimens were rinsed and postfixed in 1% OsO₄ for 1 h. After being dehydrated, the specimens were embedded in epoxy resin (TAAB 812 Resin kit, TAAB Laboratories Equipment Ltd, Aldermaston, Berks, UK). Ultrathin sections stained with uranyl acetate and lead citrate were examined with a JEM-1400 transmission electron microscope (JEOL Ltd, Tokyo, Japan).

RESULTS

Fungal Isolates

The cultured organisms were identified as *T. rubrum* by their colony characteristics and microscopic examination in lactophenol cotton blue wet mount in all cases.

SEM

Fungal hyphae were detected by SEM in scales from all 12 patients before topical luliconazole treatment. SEM images of intact *T. rubrum* in the scales showed straight fungal hyphae sometimes running along the surface of horny cells (Fig. 1). The fungal hyphae were approximately 2 to 3 μm in diameter and they showed a smooth surface with an equal width and without marked constriction.

Figure 4. TEM images of intact fungal hyphae in the horny layer before treatment. A hypha of *T. rubrum* is 2.5 to 3.0 μm in diameter of short axis with a cell wall of 0.4 μm in thickness (A). Double-headed arrow: 2.5 μm. Scale bar: 2 μm. The cell wall (black closing brace) shows a regular lamination [B]. C: fungal cytoplasm. H: horny cell. Arrows: fungal plasma membrane. Scale bar: 0.5 μm.
One day after application, some fungal hyphae showed a more bulky appearance (Fig. 2A), remarkably up to 4.5 μm in diameter (Fig. 2B). They showed septal partitions of relatively short intervals and extended digitate-like short branches with swollen tips (Fig. 2A). The surface of the swollen hypha was rough in appearance (Fig. 2B).

In addition to the swelling of hyphae, the most striking changes after 7 days of application were shrinkage of the fungal bodies, 0.8 to 1.2 μm in diameter in the short axis, with flattened and twisted appearance (Fig. 3).

TEM

Before topical luliconazole treatment, intact hyphae of *T. rubrum* with normal septal partitions were observed in and among horny cells with a regular keratin pattern (Fig. 4A). The cell walls of the hyphae were 0.4 μm in average thickness and showed well-organized laminated structure pattern (Fig. 4A, B).

The following morphological changes were observed after application of topical luliconazole for 1 day and 7 days. Remarkable initial and sequential alterations were observed in the fungal cell wall. The first step of the change was the appearance of a granular structure in the outermost layer of the cell wall (Fig. 5A). The size of these granules was 10–20 nm in diameter. At this point, lamination of the cell wall seemed to be well preserved. Accumulation of a granular structure resulted in reticulation in its pattern followed by release from the outermost layer (Fig. 5B). Simultaneously, the cell wall developed electron-lucent degeneration...
in the outer half layer (Fig. 6A). The outer degeneration was caused by loss of regular laminated structure, and the straggle disentanglement of lamination resulted in increased volume of the outer half layer (Fig. 6B). Subsequently, the inner half layer of the cell wall showed electron-lucent change giving rise to obscure lamination (Fig. 7). Total thickening of the cell wall corresponding to the swelling of hyphal bodies seen by SEM.

Apparent morphological changes in the plasma membrane of hyphae were not observed until the appearance of changes in the inner half layer of the cell wall. The initial change in the plasma membrane was multiple focal thinning. Some hyphae showed partial disruption of the plasma membrane that was sometimes associated with degeneration of the cytoplasm (Fig. 8). After application for 7 days, besides the findings mentioned above, completely collapsed cytoplasm of hypha was observed (Fig. 9), corresponding to shrinkage of hyphal bodies seen in SEM.

**Discussion**

Previous studies in which the *in vitro* effect of an antifungal drug was investigated by using SEM and TEM revealed several ultrastructural changes of dermatophytes grown in
showed the retracted plasma membrane and in vitro Trichophyton mentagrophytes shows a summary of TEM studies are also important for assessing the practical data obtained in vitro are useful for clinical applications, in vivo studies are also important for assessing the practical efficacy of an antifungal drug against hyphae parasitizing the superficial layer of human skin. Our SEM results were almost identical to those of previous in vitro studies [6,12]. Interestingly, the time-dependent morphological changes of hyphae (1 day, 7 days) were comparable to the changes evaluated by drug concentration in vitro. That is, bulky appearance of hyphae observed after application of luliconazole for 1 day in our study has been observed in hyphae treated at lower concentrations of several imidazoles in vitro [6,12]. The shrinkage, flattening, bending and twisting of the hyphae with wrinkled surface after application for 7 days were also identical to the morphological changes in hyphae cultured with higher concentrations of imidazoles [6,12].

Our TEM study further demonstrated new and important findings regarding morphological changes induced by luliconazole. Previous TEM study by Ohmi et al. [11] using otherazole drugs with cultured Trichophyton mentagrophytes showed the retracted plasma membrane and coarse electron-dense granules localized within the cell wall. Tameike et al. [12] demonstrated structural changes of the hyphae of T. rubrum depending concentration of lanocona -zole: an exfoliation of the outer layer of the cell wall at 0.078 ng/ml, coarse electron-dense granules within the thickened cell wall at 0.31 ng/ml, electron-dense granules in the space between cell wall and cytomembrane produced by their separation at 2.5 ng/ml, and discontinuity of cell membrane at 20 ng/ml.

We first clarified that fine granular materials were initially produced in the periphery of the cell wall, and then total cell wall degeneration occurred with electron-lucent and amorphous change causing its total thickening. It is noteworthy that these cell wall alterations preceded the plasma membrane changes. Table 1 shows a summary of TEM images of the changes in dermatophytes seen in vitro exposed to other azoles and those seen in vivo system in the present study. The fine granular structures in the cell wall periphery are of interest but their functional significance is unclear in our study. They seemed to be different from the coarse dense granules observed in the previous reports [11,12] in size and location. The cell wall of dermatophytes is composed of two layers, an outermost electron-dense and thin layer and an inner electron-lucent and thick layer [16–18]. The outermost layer is biochemically composed of peptides [19–21] and has a rodlet structure interwoven into a basket-weave-like fascicle [17,18,22]. The inner layer consists of chitin microfibrils embedded in an amorphous matrix [16,23,24]. Judging from the morphological findings, the initial degenerated granular material in the periphery of the cell wall might be derived from the outermost rodlet layer. Subsequent degeneration of the cell wall seems to occur in the inner chitin microfibril-matrix complex layer. The straggle disentanglement of lamination may reflect disintegration of the microfibrillar network in the inner layer. It is of interest that lucent degeneration of the cell wall appeared initially in the outer half of the inner cell wall. This is probably related to the structural difference between inner and outer components of the inner cell wall, the presence of which was suggested by Kitajima et al. [16].

It is not known why the cell wall degeneration precedes morphological change of plasma membrane, the main target of luliconazole. Enzymes involved in cell wall synthesis including chitin synthase(s) , 1,3-β-D-glucan synthase(s) and 1,6-β-D-glucan synthase(s) have been identified or implicated [25,26]. These are though to be located on the fungal plasma membrane [25–28]. Luliconazole may act on the cell wall synthetic system on the plasma membrane indirectly through inhibition of ergosterol biosynthesis. Another possibility is direct inhibition of cell wall biosynthesis by an unknown mechanism of luliconazole. In any case, our study suggests that luliconazole has a strong antifungal activity due to double acting points on
the plasma membrane and cell wall that have not been reported.

The novel contributions of the present study include the establishment of a non-invasive in vivo evaluation system for the topical antifungal drugs and a demonstration of the cell wall degeneration of hyphae treated with topical luliconazole using this system. The limitation of our study is that our system cannot elucidate the detailed biological mechanisms of serial morphological changes of the hyphae shown by TEM and SEM. Further biochemical and pharmacological investigation of acting points is required.

Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.
References


