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Actin Gene-targeted RT-PCR Could Be a Useful Method for Evaluating *In vitro* Fungicidal Activity against Dermatophytes

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This study examined the effects of the antifungal agents amorolfine hydrochloride (AMF) and bifonazole (BFZ) on actin mRNA expression determined by reverse transcription-polymerase chain reaction (RT-PCR) amplification, and the morphology of *Trichophyton mentagrophytes*. In AMF-treated cultures, the hyphal cells of *T. mentagrophytes* exhibited concentration- and/or time-dependent progressively degenerative morphological damage. Those cultures exhibiting severe and necrotic morphological changes (such as crushed, bent and flattened hyphal cells) did not express actin mRNA. In BFZ-treated cultures, similar morphological changes were seen in the hyphae of *T. mentagrophytes,* but these changes were milder than observed in the AMF-treated samples, and actin gene fragments were amplified in all of these samples. These results indicate that actin gene-targeted RT-PCR could be a useful tool for testing the susceptibility of dermatophytes to antifungal agents *in vitro*.

KEY WORDS: Amorolfine hydrochloride; Bifonazole; Actin RT-PCR; Scanning electron microscopy; Dermatophyte

Introduction

In vitro susceptibility of fungi to antifungal agents is routinely determined by measuring minimum inhibitory concentrations (MICs) or minimum fungicidal concentrations (MFCs). These tests are performed primarily to help predict the effect of antifungal agents on specific pathogens, but are also carried out for purposes such as patient care, drug discovery and development, and epidemiological studies.[1] In recent years, new methods have been developed, aimed at evaluating fungal viability while avoiding the discrepancies (caused by the presence of non-viable fungal elements or antifungal agents) that can occur between direct microscopic examination and subsequent tissue culture analysis.[2,3] Such methods include neutral red staining[3] and actin-targeted reverse transcription-polymerase chain reaction (RT-PCR).[4] The latter method is of interest to us in terms of its application to *in vitro* susceptibility testing of dermatophytes. We examined, therefore, the effects of two antifungal agents, amorolfine

hydrochloride (AMF, a morpholine derivative) and bifonazole (BFZ, an imidazole derivative) on actin mRNA expression and the morphology of *Trichophyton mentagrophytes*, one of the major causes of dermatophytosis. AMF is known to inhibit Δ^{14} sterol reductase and $\Delta^{8}\rightarrow\Delta^{7}$ sterol isomerase, and BFZ inhibits CYP51 A1 (sterol 14α-demethylase) in the ergosterol synthesis pathway.[5,6]

Materials and methods

TEST MATERIALS

The *T. mentagrophytes* strain (TIMM 3841) used in this study was available from Teikyo University Institute of Medical Mycology (Tokyo, Japan). AMF was supplied by Kyorin Pharmaceutical Co. (Tokyo, Japan), and BFZ was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

CULTURE PREPARATION

A conidial suspension of the test strain was prepared in sterile physiological saline containing 0.05% (v/v) Tween 80, from cultures grown on Sabouraud dextrose agar slants at 27 °C for 3 weeks. Following filtration through a sterile cell strainer (pore size 40 µm; Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove hyphal fragments and agar blocks, the final conidial suspension was adjusted to 10^6 conidia/ml. One hundred microlitres of this final suspension were added to 9.9 ml of Sabouraud dextrose broth, and incubated at 27 °C for 3 days to obtain mycelial growth.

DETERMINING ANTIFUNGAL ACTIVITY

Both antifungal agents were dissolved in dimethyl sulfoxide (DMSO) to prepare serial fourfold dilutions at concentrations ranging from 200 µg/ml to 3 µg/ml, and 100 µl was added to each culture of *T. mentagrophytes* mycelia and incubated for 6 h, 18 h and 48 h. Six cultures were prepared for a given concentration and incubation time, and fungal mycelia harvested from three cultures were subjected to measurement of actin gene-targeted RT-PCR or scanning electron microscopy (SEM), respectively. At the highest concentration, fungal dry weight was also measured in triplicate at a given time.

Measurement of fungal dry weight

Cultured *T. mentagrophytes* mycelia were harvested by filtration with glass microfibre filters (GF/A; Whatman International, Maidstone, England), dried and weighed.

Actin gene-targeted RT-PCR

Mycelia were harvested by centrifugation (1000 ***g***, for 15 min) and resuspended in 1 ml of 0.05 M phosphate buffer (pH 7.4) containing 5 mg/ml of lysing enzymes (from *Trichoderma harzianum*; Sigma Chemical Co.), 1 mg/ml of lysozyme (from egg white; Wako Pure Chemical Industries, Osaka, Japan), 0.7 M KCl and 40 IU/ml of RNase inhibitor (Takara Bio Inc., Shiga, Japan). The mixture was incubated at 37 °C for 1 h to partially digest the cell walls, and then harvested by centrifugation (12,000 ***g*** at 4 °C for 5 min). Total RNA was extracted according to the guanidine thiocyanate method using Isogen (Wako Pure Chemical Industries). To remove contaminating genomic DNA, 1 ml of a mix containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, 2 mM dithiothreitol, 0.2 mg/ml acetylated BSA and 2 IU/ml DNase (RT grade, Wako Pure Chemical Industries) was added to the precipitated total RNA. This mixture was incubated at 37 °C for 10 min and then centrifuged (12,000 ***g*** at 4 °C for 5 min). The supernatant was subjected to the same guanidine thiocyanate method using Isogen, and the resulting purified total RNA dissolved in 50 µl of diethylpyrocarbonate-treated water.

Reverse transcription-polymerase chain reaction was conducted using the TaKaRa RNA PCR Kit (AMV) Ver. 2.1 (Takara Bio Inc.). Total RNA (1 µl) was reverse transcribed in a 20 µl reaction mixture containing RNA PCR buffer, 5 mM $MgCl_2$, 1 mM dNTP mixture, 20 IU RNase inhibitor, 5 IU avian myeloblastosis virus-derived reverse transcriptase XL and 2.5 µM random 9-mers. The reaction was performed using an iCycler™ (Bio-Rad, Hercules, CA, USA), with one cycle time of 30 °C for 10 min, 45 °C for 30 min, 99 °C for 5 min and 5 °C for 5 min. To determine whether the RNA samples were contaminated with genomic DNA, RT-PCR using total RNA from the control cultures was conducted in the same way, but without avian myeloblastosis virus-derived reverse transcriptase XL.

The subsequent PCR reaction was performed in the same tube, using 100 µl of reaction mixture containing RNA PCR buffer, 2.5 mM $MgCl_2$, 0.25 IU/ml of TaKaRa Taq™ (Takara Bio Inc.), and 0.2 µM each of the actin-specific primers. The sequences of the oligonucleotide primers were determined from the published sequence of a *T. mentagrophytes* actin gene (Gen-Bank accession number AF152229), and were GCCTTCTACGTCTCCATCCA and CAGGAGGAGATCCAGACTGC. The reaction was initiated with denaturation at 94 °C for 2 min, followed by amplification of the cDNA fragment using 40 cycles of 94 °C for 0.5 min, 60 °C for 0.5 min and 72 °C for 1.5 min. A single terminal extension was performed at 72 °C for 7 min. An aliquot of amplification product (8 µl) was mixed with 2 µl of loading buffer (0.02% bromophenol blue, 0.02% xylene cyanol FF, 50% glycerol and 1% sodium dodecyl sulphate; Wako Pure Chemical Industries) and loaded onto a 3% agarose gel for electrophoretic separation. The gel was stained with ethidium bromide, and the separated products were visualized by ultraviolet transillumination.

Scanning electron microscopy

Mycelia were harvested by filtration with glass microfibre filters (GF/A; Whatman International) and fixed with 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) at 4 °C for 2 h. After washing twice with 0.05 M phosphate buffer (pH 7.4), they were post-fixed with 2% OsO_4 solution at 4 °C for 2 h or 1% OsO_4 solution at 4 °C overnight. Fixed mycelia were dehydrated through an ethanol series, substituted with isoamyl acetate, and dried by the critical point drying method with liquid CO_2, using a JCPD-5 critical point dryer (JEOL, Tokyo, Japan). Each dried specimen was coated with gold using the JFC-1100 ion sputter (JEOL, Tokyo, Japan), and analysed using a JSM-T2000 scanning electron microscope (JEOL, Tokyo, Japan) at 10 kV.

Results

Table 1 summarizes the growth of *T. mentagrophytes* mycelia exposed to DMSO alone or to the highest concentration of each antifungal agent.

Representative SEM images of each culture and RT-PCR products visualized in ethidium bromide-stained agarose gel are summarized in Fig. 1. Following RT-PCR, a single band of 299 bp (the expected size) was observed in each of the control cultures, indicating that the actin gene fragment was specifically amplified. After 6 h, 18 h and 48 h of incubation, almost all of the hyphae in the control cultures (DMSO only) exhibited a smooth surface, straight extensions and consistent width. No amplification bands were detected after RT-PCR and electrophoresis of the samples reverse transcribed without reverse transcriptase, indicating there was no genomic DNA contamination (data not shown).

TABLE 1:
Growth of *Trichophyton mentagrophytes* mycelia exposed to dimethyl sulfoxide alone (control), 2 µg/ml amorolfine hydrochloride (AMF) or bifonazole (BFZ) for 6 h, 18 h and 48 h. Growth is measured as dry weight, and each value represents the mean ± SE of the triplicate cultures

	Dry weight of *Trichophyton mentagrophytes* mycelia (mg/tube)			
	0 h	**6 h**	**18 h**	**48 h**
Control	2.03 ± 0.25	1.73 ± 0.36	1.85 ± 0.18	1.72 ± 0.18
BFZ	–	2.91 ± 0.14	1.90 ± 0.36	1.72 ± 0.05
AMF	–	1.67 ± 0.43	1.70 ± 0.16	2.20 ± 0.24

In the AMF-treated cultures, after 6 h of incubation, some of the hyphal cells had a wavy appearance and were curled at concentrations greater than 0.13 µg/ml of AMF. Eighteen hours' AMF incubation produced hyphae with fairly remarkable morphological changes compared with those seen after 6 h of incubation. At concentrations greater than 0.5 µg/ml of AMF, some of the hyphal cells varied in width, had wrinkled surfaces, and were somewhat entangled, in addition to having wavy and curled forms. After 48 h of incubation, the morphological changes were more severe again, and at concentrations greater than 0.5 µg/ml of AMF, most of the hyphal cells exhibited a crushed, bent and flattened (or sheet-like) appearance. Actin gene mRNA was amplified from all samples, irrespective of AMF concentration, following 6 h and 18 h of incubation, but after incubation for 48 h was only detected in the cultures exposed to the weakest concentration of AMF.

In the BFZ-treated cultures, similar morphological changes were observed in the hyphae of *T. mentagrophytes,* but these changes were milder than those observed in the AMF-treated samples, and actin gene fragments were amplified from all of these cultures.

Discussion

This study examined actin mRNA expression in the hyphae of *T. mentagrophytes* exposed to antifungal agents in relation to their morphological changes. We aimed to obtain information regarding how well actin gene-targeted RT-PCR reflects *in vitro* susceptibility of dermatophytes to antifungal agents. In eukaryotes, actin is a conserved and ubiquitous cytoskeletal protein,[7] and should be expressed constantly in living cells. Loss of actin gene expression can lead to loss of viability, as reported previously.[4] This is supported by our findings that no actin mRNA was detected in those hyphal cells exhibiting severe degenerative morphological damage induced by AMF (Fig. 1).

Sufficient fungal biomass was obtained from each of the differently treated cultures to indicate that the attenuated actin mRNA levels did not reflect a decreased amount of fungal biomass (Table 1). The results therefore suggest that actin gene-targeted RT-PCR could be a useful tool for *in vitro* susceptibility testing of dermatophytes, especially in terms of examining the fungicidal action of antifungal agents. RT-PCR does not, however, detect activity if the antifungal action is fungistatic rather than fungicidal, as seen with BFZ, which has been reported to

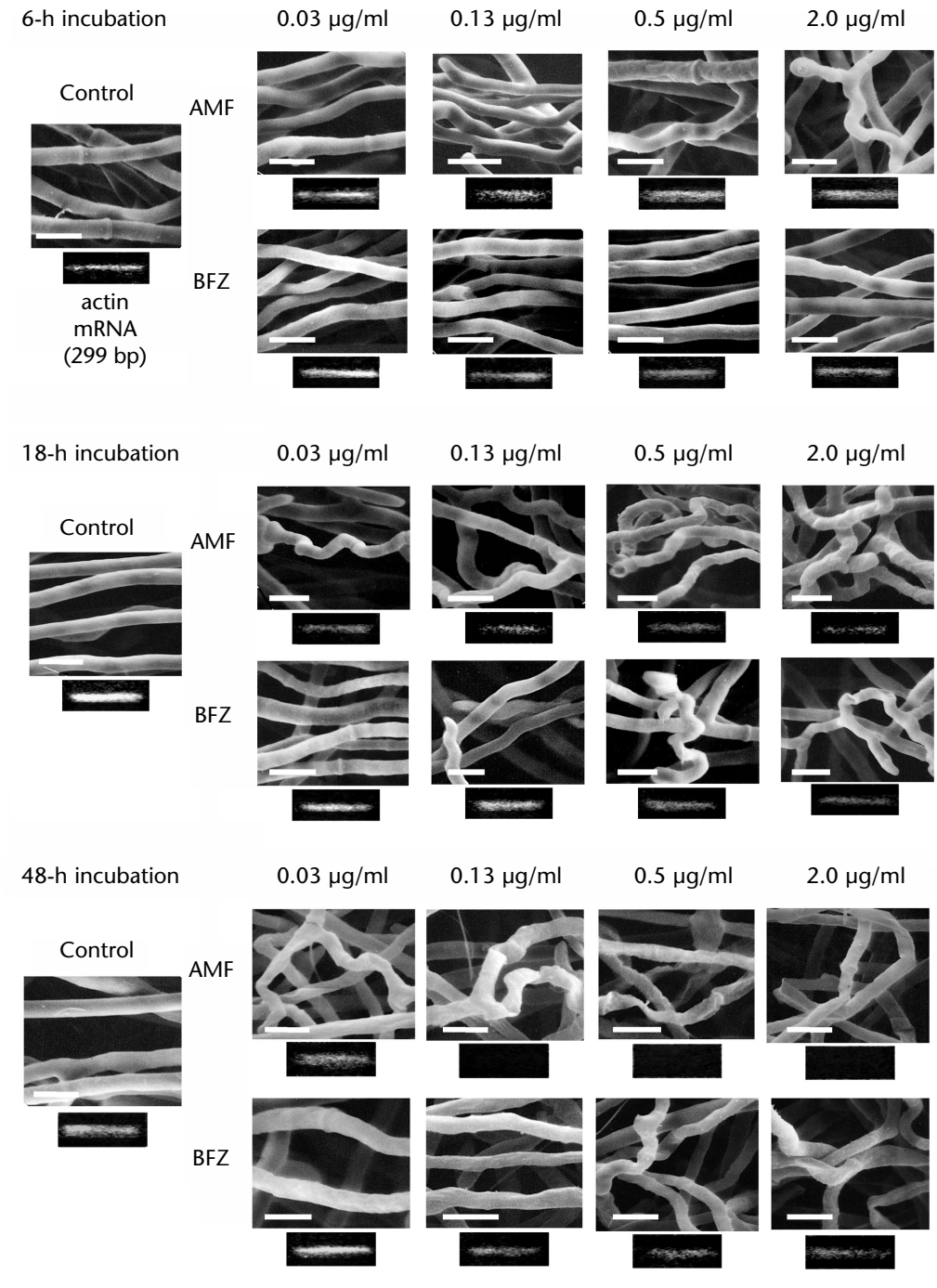

FIGURE 1: Scanning electron microscopy images of hyphae and actin mRNA expression in *Trichophyton mentagrophytes* mycelia exposed to different concentrations of amorolfine hydrochloride or bifonazole for 6 h, 18 h and 48 h. Scale bar indicates 5 μm. AMF, amorolphine hydrochloride; BFZ, bifonazole

be fungistatic.[8] If actin mRNA expression can be quantified, with high detection sensitivity, the method could be a tool for assessing *in vitro* susceptibility. Okeke *et al.*[9] have recently successfully assessed the viability of *Candida albicans* cells by RT-PCR of actin mRNA using fluorescent hybridization probes for detection. Techniques such as these, therefore, appear worthy of application in the *in vitro* susceptibility testing of fungi.

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References

1 Pfaller MA, Rex JH, Rinaldi MG: Antifungal susceptibility testing: Technical advances and potential clinical applications. *Clin Infect Dis* 1996; **24:** 776 – 784.
2 Lefler E, Haim S, Merzbach D: Evaluation of direct microscopic examination versus culture in the diagnosis of superficial fungal infections. *Mykosen* 1981; **24:** 102 – 106.
3 Naka W, Hanyaku H, Tajima S, Harada T, Nishikawa T: Application of neutral red staining for evaluation of the viability of dermatophytes and *Candida albicans* in human skin scales. *J Med Vet Mycol* 1994; **32:** 31 – 35.
4 Okeke CN, Tsuboi R, Kawai M, Yamazaki M, Reangchainam S, Ogawa H: Reverse transcription-3' rapid amplification of cDNA ends-nested PCR for ACT1 and SAP2 mRNA as a means of detecting viable *Candida albicans* in an *in vitro* cutaneous candidiasis model. *J Invest Dermatol* 2000; **114:** 95 – 100.
5 Polak-Wyss A, Lengsfeld H, Oesterhelt: Effect of oxiconazole and Ro 14-4767/002 on sterol pattern in *Candida albicans*. *Sabouraudia* 1985; **23:** 433 – 441.
6 Berg D, Regel E, Harenberg HE, Plempel M: Bifonazole and clotrimazole. *Arzneim Forsch/ Drug Res* 1984; **34:** 139 – 146.
7 Reisler E: Actin molecular structure and function. *Curr Opin Cell Biol* 1993; **5:** 41 – 47.
8 Gupta AK, Ahmad I, Summerbell RC: Comparative efficacies of commonly used disinfectants and antifungal pharmaceutical spray preparations against dermatophytic fungi. *Med Mycol* 2001; **39:** 321 – 328.
9 Okeke CN, Tsuboi R, Ogawa H: Quantification of *Candida albicans* actin mRNA by the LightCycler system as a means of assessing viability in a model of cutaneous candidiasis. *J Clin Microbiol* 2001; **39:** 3491 – 3494.

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