Human β-Defensin-2 Expression by Keratinocytes is Induced by Co-Culture with *Trycophyton rubrum* Through Toll-Like Receptors 2 and 4

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Abstract: To determine a role of human β-Defensin-2 (HBD-2) in defense against cutaneous dermatophyte infection, we investigated the induction of HBD-2 mRNA expression by a nontumorigenic human keratinocyte-derived cell line, HaCaT cells, after co-culture with microconidia collected from the colonies of *Trichophyton rubrum*. Co-culture of *T. rubrum* or *Candida albicans* with HaCaT cells significantly induced HBD-2 mRNA expression. Furthermore, treatment with anti-TLR2 or TLR4 antibodies inhibited *T. rubrum*-induced HBD-2 upregulation, while it did not affect *C. albicans*-induced HBD-2 upregulation. Pretreatment of *T. rubrum* with Concanavalin A, which blocks mannosyl and glucosyl residues, inhibited HBD-2 expression by HaCaT cells, suggesting that mannosylated and glycosylated residues are important for HBD-2 induction. Collectively, these results suggest that *T. rubrum* induces HBD-2 expression through TLR2 and/or TLR4 pathway in keratinocytes and that HBD-2 may play a defense role in cutaneous dermatophyte infection.

Keywords: Human β-defensin-2, *Trichophyton rubrum, Candida albicans,* keratinocyte, toll-like receptor.

INTRODUCTION

The dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissues, including skin, hair, and nails, to produce chronic infection called dermatophytosis [1]. This infection is generally cutaneous and is confined to the nonliving cornified layers because of the inability of the fungi to penetrate the deeper tissues [1]. About 90% of dermatophytosis is caused by *Trichophyton mentagrophytes* and *T. rubrum* [2]. The epidermis plays an important role in the front line host defense against fungal infection [3]. Keratinocytes participate in the host defense against pathogens as well as a physical barrier. Keratinocytes stimulated with microbial organisms secrete not only various cytokines and chemokines, but also antimicrobial peptides that are integral components of the innate immune system found in mammalian [4, 5]. The fact that dermatophytes multiply within the stratum corneum, but are unable to penetrate the deeper layers of the epidermis is due in part to the presence of the epidermal antimicrobial substances, such as lysozymes and defensins, which play a critical role in protecting the skin against dermtophytic invasion [6].

One of the best-characterized families of antimicrobial peptides is the defensins, which are small cationic, amphiphilic, and cysteine-rich peptides with broad antimicrobial activity against both bacteria and fungi [7, 8]. The details of mechanisms of antimicrobial action by defensins are unclear; however, it involves targeting membranes whose composition includes negatively charged phopsholipids in contrast to mammalian cells that are made up of largely neutral zwitterionic phospholipids [9]. Human β-defensins (HBD) contain around 35 amino acid residues, including 6 cysteine residues. Four HBD1-4 have been identified and are expressed predominantly in epithelial tissues, which provide the first line of defense between an organism and the environment. HBD-2, which was originally purified from psoriatic skin lesions [10], is expressed in keratinocytes, the gingival mucosa, and the tracheal epithelium [8]. HBD-2 expression is induced by interleukin-1β, tumor necrosis factor (TNF)-α, and bacterial lipopolysaccharide, and by contact with gram-negative and gram-positive bacteria [8]. HBD-2 expression is also induced by fungi, including *Candida albicans* and *Malassezia furfur* [11, 12]. However, it remained unknown whether dermatophytes induced HBD-2 expression by keratinocytes. A recent study has shown that HBD-3 expression is induced by bacterial lipopeptides through toll-like receptors (TLR) 2 [3]. TLRs are innate immune-pattern recognition receptors that recognize a wide range of microbes and their products [13]. A recent study has revealed that TLR2 is involved in the *M. furfur*-induced expression of HBD-2 [11]. Therefore, in this study, we investigated HBD-2 expression by keratinocytes stimulated with *T. rubrum*, one of major dermatophytes, and involvement of TLRs for HBD-2 upregulation.

MATERIALS AND METHODS

Fungal Culture

The strains *C. albicans* (ATCC 26555) and *T. rubrum* (ATCC 40051) were maintained by culturing at 25°C on 1/10 Sabouraud's dextrose agar containing 0.1% peptone, 0.2% dextrose, 0.1% KH_2PO_4, 0.1% MgSO4, 2% agar, and subcloned every 2 weeks more than 2 times before use [14].

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HaCaT Cell Cultures

The HaCaT cell, a nontumorigenic human keratinocyte-derived cell line [15], which was kindly provided by Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany), was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2/ 95% air at 37°C. Its identity was confirmed by morphological features as described previously [15].

Co-Culture of HaCaT Cells with Fungi

Co-culture of HaCaT cells with microconidia of *T. rubrum* and *C. albicans* was performed according to a previous method [14]. Microconidia were collected by scraping from the colonies of *T. rubrum* and *C. albicans* grown on Sabouraud's dextrose agar and were suspended with phosphate-buffered saline (PBS) containing 0.04% Tween 80 in a glass homogenizer. The suspension was filtered with a Whatman filter model 1 (pore size, 11 μm) to collect microconidia. These microconidia were washed twice in PBS, and were resuspended in basal medium. HaCaT cells (1×10^5 cells/well) were incubated on a microplate with 6 wells at 37°C, 5% CO_2 for 24 hours, and then the microconidia (final concentration, 2×10^5 cells/well) were added to the well. HaCaT cells were collected after indicated time of co-culture.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNAs were extracted from the HaCaT cells using RNeasy Mini Kit (QIAGEN, Crawley, UK), according to the manufacturer's protocols. RNA yield and purity were determined by spectrophotometry. Total RNAs were subsequently reverse transcribed to cDNA using Superscript II (Gibco, GRAND island, NY, USA). Amplification was performed in a PCR thermal cycler MP (TaKaRa, Kyoto, Japan) for 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 70°C for 45 seconds. The final extension was performed for 10 minutes, and then for 5 minutes at 5°C. We used 5' and 3' primer sets for HBD-1, HBD-2, and β-actin (BD Clontech, Palo Alto, CA, USA). The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The density of the scanned product was determined with NIH image 1.62 software and compared with that of β-actin to semi-quantitate the PCR product.

Anti-TLR Antibody (Ab) Blocking Study

For anti-TLR Ab blocking study, we added anti-TLR2 (R&D systems, Minneapolis, MN, USA) or TLR4 (Abcam, Cambridge, UK) Ab to the wells at the same time when co-culture of HaCaT cells with microconidia was started. After 48 hours of co-culture, HBD-2 mRNA expression by HaCaT cells was evaluated by RT-PCR. Isotype-matched Abs (Beckman Coulter, Inc., Miami, FL, USA) were served as a negative control.

Pretreatment of Microconidia

To investigate which components of fungi were involved in HBD-2 expression by HaCaT cells, the microconidia were boiled for 10 minutes at 100°C or treated with 70% ethanol or Concanavalin A (Con A; Sigma-Aldrich Co., St. Louis, MO, USA) for 10 minutes, and were then co-cultured with HaCaT cells. After 48 hours of co-culture, HBD-2 mRNA expression by HaCaT cells was evaluated by RT-PCR.

Statistical Analysis

Statistical analysis was performed using Mann-Whitney U test for comparison of fold-intensity. A p value less than 0.05 was considered statistically significant.

RESULTS

HBD-2 mRNA Expression by HaCaT Cells Co-Cultured with *T. rubrum* or *C. albicans*

We investigated mRNA expression of HBD-2 by HaCaT cells after co-culture with *T. rubrum* or *C. albicans*. Co-culture of HaCaT cells with *T. rubrum* significantly induced HBD-2 mRNA expression by 18 hours of culture compared with baseline expression ($p<0.05$; Fig. **1**). This HBD-2 mRNA expression was increased until 120 hours of co-culture. By contrast, HBD-1 mRNA was not expressed until 120 hours' co-culture with *T. rubrum* (data not shown). Co-culture of HaCaT cells with *C. albicans* also significantly induced HBD-2 mRNA expression by 12 hours of culture relative to baseline expression ($p<0.01$; Fig. **1**). This HBD-2 mRNA expression was maintained at similar level until 120 hours of co-culture. Thus, co-culture with *T. rubrum* or *C. albicans* induced HBD-2, although the kinetics was different.

Effect of Anti-TLR2 or TLR4 Ab Administration on HBD-2 mRNA Expression by HaCaT Cells

It has been reported that TLR2 and/or TLR4 play a role in the recognition of several fungi, such as *C. albicans, M. furfur, Cryptococcus neoformans,* and *Aspergillus fumigatus* [16]. Furthermore, TLR2 is involved in *M. furfur*-induced HBD-2 expression [11]. We investigated the effect of anti-TLR2 or TLR4 Ab on HBD-2 mRNA expression by HaCaT cells co-cultured with *T. rubrum* or *C. albicans* for 48 hours. Treatment with anti-TLR2 Ab significantly reduced HBD-2 mRNA expression induced by *T. rubrum* compared to control Ab ($p<0.01$, Fig. **2A**). Similarly, anti-TLR4 Ab administration significantly decreased HBD-2 mRNA expression relative to control Ab ($p<0.01$). There was no significant difference in inhibitory effect between anti-TLR2 Ab and anti-TLR4 Ab treatment. By contrast, treatment with anti-TLR2 or TLR4 Ab did not affect HBD-2 mRNA expression induced by *C. albicans* (Fig. **2B**). Thus, TLR2 and TLR4 appeared to be involved in HBD-2 mRNA expression by HaCaT cells co-cultured with *T. rubrum* but not *C. albicans.*

Effect of Boiling, Ethanol Treatment, and Con A Treatment on HBD-2 mRNA Expression by HaCaT Cells

To investigate which components of fungi were involved in HBD-2 mRNA expression by HaCaT cells co-cultured with *T. rubrum* for 48 hours, the microconidia were boiled at 100 °C (to denature protein) or treated with either 70% ethanol (to destruct lipids) or Con A (to block mannosyl and glucosyl residues) before co-culture. HBD-2 mRNA expression was significantly but partially inhibited by Con A

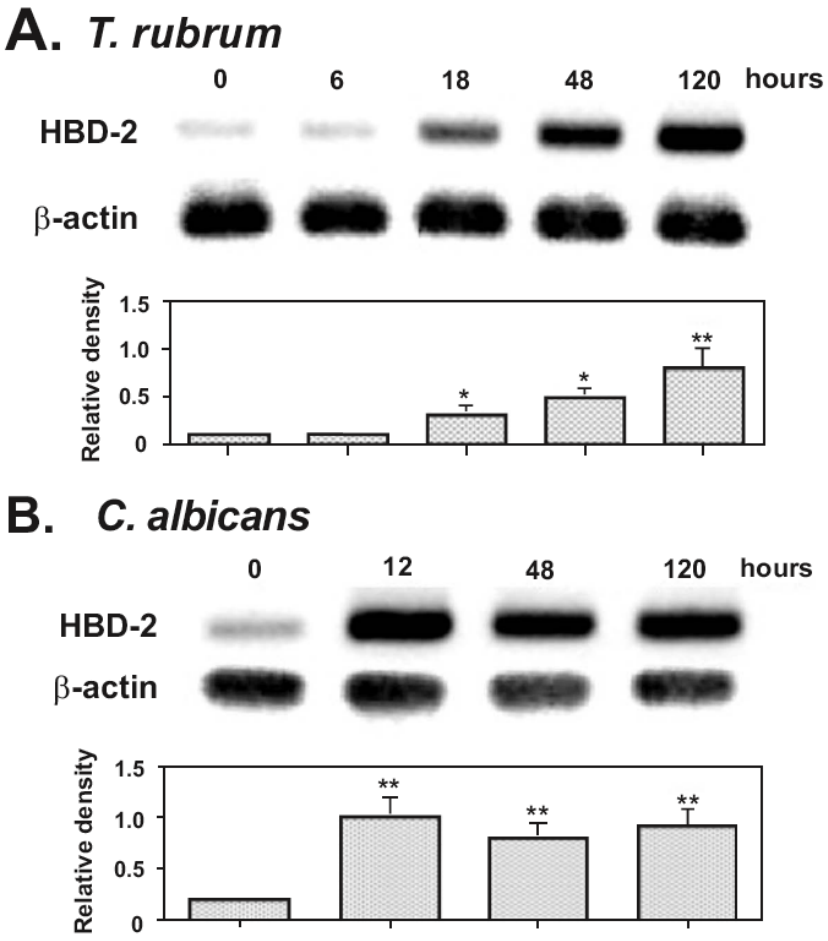

Fig. (1). The expression of HBD-2 mRNA by HaCaT cells co-cultured with *T. rubrum* (**A**) or *C. albicans* (**B**). Microconidia collected from the colonies of *T. rubrum* or *C. albicans* were co-cultured with HaCaT cells. HaCaT cells were collected after indicated time of co-culture. The PCR products were electrophoresed and stained with ethidium bromide. Representative mRNA expression of HBD-2 and β-actin is shown in the upper panel. The amount of HBD-2 mRNA was semi-quantified and normalized to the level of β-actin expression in the lower panel. All values represent the mean ± SEM of results obtained with 5 separate experiments. *$p<0.05$ and **$p<0.01$ *vs* baseline expression.

treatment relative to untreatment ($p<0.05$), while boiling and ethanol treatment did not affect HBD-2 mRNA expression (Fig. **3**). Con A treatment did not affect HBD-2 mRNA expression in the absence of *T. rubrum* (data not shown), which excluded the possibility that Con A directly reduced keratinocyte HBD-2 expression. Thus, mannosyl and glucosyl residues appeared to be partially involved in *T. rubrum*-induced HBD-2 mRNA expression.

DISCUSSION

In this study, co-culture of *T. rubrum* with HaCaT cells significantly induced HBD-2 expression. Consistent with this finding, a recent study has revealed that HBD-2 expression is elevated in the lesional skin of patients with dermatophytosis [6]. Furthermore, treatment of anti-TLR2 or TLR4 Abs inhibited *T. rubrum*-induced HBD-2 upregulation. Collectively, these results suggest that *T. rubrum* induces HBD-2 expression through TLR2 and/or TLR4 pathway in keratinocytes and that HBD-2 may play a defense role in cutaneous dermatophyte infection.

Many studies have shown that TLRs are involved in the recognition of various fungi as well as bacteria [16]. A role of TLR2 and/or TLR4 has been implicated in response to *C. albicans*: TNF-α production by macrophages from TLR2-deficient mice in response to *C. albicans* is lower than in wild-type mice [17], while macrophages from TLR4-mutant mice have impaired chemokine expression [18]. The major capsular polysaccharide of *C. neoformans*, glucuronoxylomannan, is recognized by TLR2 and TLR4 [19]. TLR2 is also required in response to *A. fumigatus*: macrophages from TLR2-deficient mice produce less amount of TNF-α following *A. fumigatus* stimulation, whereas macrophages from TLR4-deficient mice exhibited no defects in TNF-α release [20]. Similarly, TLR2 is involved in the recognition of *M. furfur* [11]. In this study, although we did not formally show that *T. rubrum* bound to TLR2 and/or TLR4, our finding that anti-TLR2 or TLR4 Ab treatment suppressed HBD-2 upregulation by *T. rubrum* suggests that *T. rubrum* is recognized by TLR2 and/or TLR4.

A recent study has revealed that mouse β-defensin-3, a homolog of HBD-2, is induced by bacterial lipopeptides through TLR2 pathway in keratinocytes [3]. Like bacteria, TLR2 is involved in the *M. furfur*-induced expression of

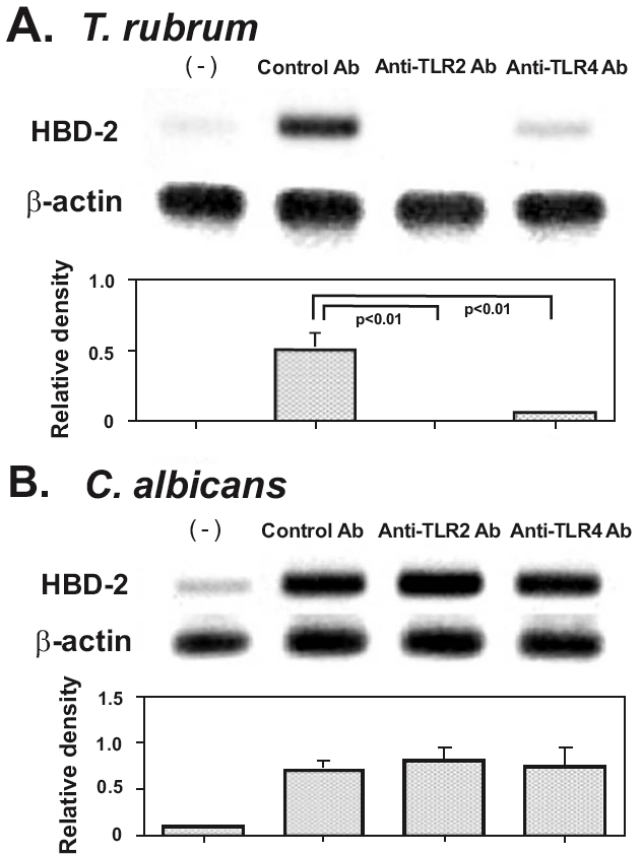

Fig. (2). Effect of anti-TLR2 or TLR4 Ab administration on HBD-2 mRNA expression by HaCaT cells co-cultured with *T. rubrum* (**A**) or *C. albicans* (**B**). Anti-TLR2 or TLR4 Ab was added to the wells at the same time when co-culture of HaCaT cells with microconidia was started. After 48 hours of co-culture, HBD-2 mRNA expression by HaCaT cells was evaluated by RT-PCR. Isotype-matched Ab was served as a negative control. The PCR products were electrophoresed and stained with ethidium bromide. Representative mRNA expression of HBD-2 and β-actin is shown in the upper panel. The amount of HBD-2 mRNA was semi-quantified and normalized to the level of β-actin expression in the lower panel. All values represent the mean ± SEM of results obtained with 5 separate experiments.

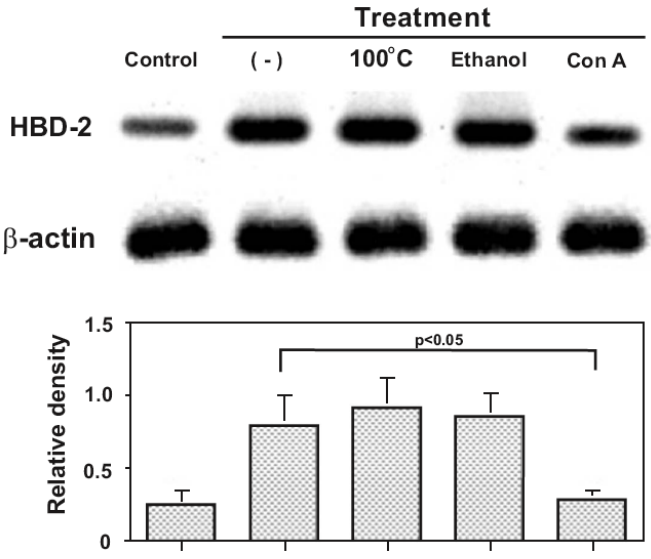

Fig. (3). Effect of boiling, ethanol treatment, and Con A treatment on HBD-2 mRNA expression by HaCaT cells. The microconidia collected from the colonies of *T. rubrum* were boiled for 10 minutes at 100 °C or treated with either 70% ethanol or Con A for 10 minutes, and were then co-cultured with the HaCaT cells. After 48 hours of co-culture, HBD-2 mRNA expression by HaCaT cells was evaluated by RT-PCR. The PCR products were electrophoresed and stained with ethidium bromide. Representative mRNA expression of HBD-2 and β-actin is shown in the upper panel. The amount of HBD-2 mRNA was semi-quantified and normalized to the level of β-actin expression in the lower panel. All values represent the mean ± SEM of results obtained with 5 separate experiments.

HBD-2 by keratinocytes [11]. However, the relationship between TLR signaling and HBD-2 expression remained unknown for *T. rubrum*. In this study, administration of anti-TLR2 or TLR4 Ab inhibited *T. rubrum*-induced HBD-2 upregulation in keratinocytes, suggesting that *T. rubrum* simulates HBD-2 expression through TLR2 and/or TLR4 pathway. Like *T. rubrum*, HBD-2 expression was also induced by co-culture with *C. albicans*. In addition, TLR2 and/or TLR4 are involved in the recognition of *C. albicans* [17, 18]. However, in this study, anti-TLR2 or TLR4 Ab could not suppress HBD-2 upregulation induced by *C. albicans*, suggesting that, unlike *T. rubrum*, TLR2 and TLR4 are not required for *C. albicans*-induced HBD-2 upregulation. Thus, mechanisms for HBD-2 expression by keratinocytes in response to fungal infection might be different between fungi.

The finding that pretreatment of *T. rubrum* with Con A inhibited HBD-2 expression by HaCaT cells suggests that mannosylated and glycosylated residues are important for HBD-2 induction. So far, 3 fungal ligands, β-glucan, glucuronoxylomannan, and phospholipomannan that stimulate TLR response, have been identified for *C. neofromans* and *C. albicans* [16]. All these molecules contain mannosylated and glycosylated residues, and β-glucan and mannans are major cell-wall components of *T. rubrum* [6, 21]. Therefore, β-glucan and mannans of *T. rubrum* may be the main stimuli that induce HBD-2 upregulation through TLRs by keratinocytes.

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