We evaluated the immunomodulatory effects of three different classes of antifungal agents on the human monocytic cell line, THP-1, which had been stimulated in vitro with Aspergillus fumigatus conidia. Cells treated with amphotericin B (AmB), micafungin (MF), and voriconazole (VCZ), at concentrations not affecting cell viability, reduced production of tumor necrosis factor (TNF-α) in response to conidia, with the greatest reduction noted with VCZ. The reduction of TNF-α production correlated with TNF-α gene expression assessed by PCR and nuclear factor κB (NFκB) levels. Co-stimulation with granulocyte-macrophage colony stimulating factor abolished immunomodulatory effects of the drugs. Antifungal agents affect the immune reaction caused by A. fumigatus conidia in stimulated monocytes at clinically relevant drug concentrations. Because drugs with different mechanisms of action produced this effect, this suggests that it is the result of factors mediated by the cells. The impact of these immunomodulatory effects needs assessment.

**Keywords** antifungals, immunomodulation, TNF-α, GM-CSF
Materials and methods

Reagents

Amphotericin B (AmB, Sigma, St. Louis, USA) and miconazole (MF, Fujisawa Pharmaceutical Company, Osaka, Japan) were diluted in RPMI-1640 medium. Voriconazole (VCZ, Choongwae Pharmaceutical Corporation, Seoul, Korea) was dissolved in isopropyl alcohol before dilution. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Calbiochem (Darmstadt, Germany) and dissolved in distilled water. All reagents used in cell treatment experiments were certified LPS-free, or <100 pg/mg. Mouse anti-human TNF-α as a capture antibody and biotinylated goat anti-human TNF-α as a detection antibody (R&D Systems, Minneapolis, MN) were used to detect production of TNF-α and 1% BSA in PBS was employed as a blocking solution. Streptavidin-horseradish peroxidase (HRP) was purchased from R&D systems; sulfuric acid (2N) was used to stop the action of HRP on biotin.

Culture of THP-1 cell line

The THP-1 cell line (KCLB 40202) was purchased from Korea Cell Lines Bank, Seoul, Korea. THP-1 cells were maintained in RPMI-1640 with 10% (vol/vol) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in an atmosphere containing 5% CO₂.

Aspergillus fumigatus

A. fumigatus (isolate IFO 30870, originally recovered from soil) was incubated on Sabouraud’s dextrose agar plates at 37°C to form conidia. The conidia were collected in 0.05% Tween 80 in saline and filtered through sterile gauze. The conidia were washed, diluted in saline, and counted.

Cell viability assay

Cell viability was determined by the Cell Proliferation Reagent WST-1 and assay kit (Roche, Mannheim, Germany) [16] that measures the metabolic activity of cells. The cells (10⁵/well) in 100 μL culture medium were treated with antifungal drugs for 0, 24, 48, or 72 h at 37°C and 5% CO₂. Ten μl of WST-1 was added, and the cells incubated for 4 h at 37°C and 5% CO₂. Absorbance was measured at 440 nm. The results were scored as ‘viability’, a summation of effects on cellular viability, proliferation, metabolic activity in viable cells, apoptosis and death in different cells in the population.

RT-PCR

THP-1 cells were first treated with drugs for 24 h and then conidia (Effector (E):Target (T)=1:1) were added for 8 h at 37°C, 5% CO₂. Total RNA was isolated from cells (Qiagen GmbH, Hilden, Germany) and the concentration of total RNA determined spectrophotometrically. mRNAs were captured by an mRNA capture commercial kit (Roche, Mannheim, Germany). mRNA expression of TNF-α from 2 μg of total RNA was determined by a one step RT-PCR kit (Takara, Shiga, Japan). GAPDH was used as a control for the PCR studies. A negative control (distilled water substituted for cell extract) was included in each run. RT-PCR assays were performed using 30 cycles, in which each cycle consisted of the following steps: cDNA synthesis at 42°C for 30 min, denaturation at 94°C for 20 sec, annealing at 60°C for 10 sec, and extension at 72°C for 30 sec. Five μl of RT-PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Primer sequences were as follows; (1) sense, 5′-CTTGTTCCTCAGCCTCTTCT-3′ and (2) anti-sense, 5′-ACTCGGCAAGTCGAGATA-3′.

Enzyme linked immunosorbent assay (ELISA)

THP-1 cells (10⁶ cells/ml) were treated with antifungal drugs for 24 h at 37°C, 5% CO₂. After washing three times with PBS, conidia (E:T=10:1) were added for 8 h at 37°C, 5% CO₂. Supernatants were then stored at −80°C and cytokine production was determined by using a commercial ELISA kit (R&D Systems) according to the manufacturer’s protocols. Briefly, 96-well plates were coated with 100 μl of capture antibody overnight and reagent diluent added for blocking. Then 100 μl of samples were added to wells for 2 h at room temperature, and detection antibody added to the wells. After washing, streptavidin-HRP was added, the wells incubated for 20 min, and 100 μl of 3,3′,5,5′-tetramethylbenzidine was included. After an additional 20 min, the reaction was stopped by adding the stop solution and results per well were measured at 450 nm using an ELISA reader.

Preparation of cell extracts

THP-1 cells (8×10⁶) were treated with antifungal drugs for 24 h at 37°C, 5% CO₂. After washing three times with PBS, conidia (E:T=10:1) were added for 2 h at 37°C, 5% CO₂. Cell extracts were prepared as previously described [17]. Cells were washed twice with ice-cold PBS, and pelleted by centrifugation at 1,500 g for 10 min. The pellet was resuspended in 160 μl of buffer A (10 mM HEPES (N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid) hemisodium salt), pH 7.9, 10 mM KCl, 0.1 mM EDTA (ethylenediamine tetraacetic acid), 0.1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′′-tetraacetic acid, 1 mM DTT (dithiothreitol), 0.5 mM PMSF (phenylmethylsulfonyl fluoride). The cells were allowed to swell on ice for 15 min, after which 40 μl of a 2.5% solution of thiol and protease inhibitors were added.
4-nonylphenolpolyethyleneglycol (Nonidet P-40, Roche Diagnostics Corporation, Indianapolis, IN) was added and the tube vigorously vortex-mixed for 10 sec. The homogenate was centrifuged at 12,000 g for 5 min, with the supernatant saved as the cytoplasmic extract. The nuclear pellet was resuspended in 40 μl of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) and the tube vigorously rocked at 4°C for 20 min on a shaking platform. The nuclear extract was centrifuged at 12,000 g for 5 min at 4°C and aliquots of the supernatant were frozen at −80°C. The protein concentration was determined by the Micro BCA™ protein assay reagent kit (Pierce, Rockford, IL).

Western blot analysis

After quantification of protein, protein separation was performed by electrophoresis on a 10% SDS-polyacrylamide gel. The samples were then transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were washed three times in TBST (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) and then blocked for 1 h in TBST containing 5% skim milk. After washing the membranes with TBST, the membranes were incubated with 1:500 dilution of rabbit polyclonal anti-IκB-α antibody (cytoplasmic extracts) or rabbit polyclonal anti-NFκB p65 antibody (nuclear extracts) (Cell Signaling Technology Inc, Beverly, MA) in TBST overnight at 4°C. After washing the membranes in TBST, they were incubated in a 1:1,000 dilution of HRP-conjugated anti-rabbit immunoglobulin G (Zymed, Camarillo, CA) for 1.5 h at room temperature. Chemiluminescent detection was performed with SuperSignal West chemiluminescent substrates (Pierce).

Statistical analysis

Tukey’s method or the Scheffe test were used for pairwise comparison after the analysis of variance. A P value of <0.05 was judged statistically significant. The data reported were pooled from three independent (cellular extraction to Western blotting) experiments.

Results

Effects of antifungal drugs on THP-1 cell viability

To investigate the direct effects of antifungal drugs, the ‘viabilities’ (as defined in Methods) of THP-1 cells treated with antifungal drugs were evaluated by the WST-1 assay. All concentrations tested were in the desirable serum therapeutic range [18]. AmB did not affect THP-1 cells at the concentrations studied (Fig. 1, top). Although MF had no effect on cell viability at low concentrations, there was a decrease in viability at MF concentrations >0.1 μg/ml at 48 h (inhibition percentage, 17.2%; Fig. 1, middle). As shown in Fig. 1, bottom panel, cell viability decreased over
Effects of antifungal drugs on TNF-α production in THP-1 cells

To determine the effects of antifungal drugs on cytokine production, *A. fumigatus* conidia were added to wells containing THP-1 cells with or without antifungal drug treatment. Pretreatment with antifungal drugs significantly reduced TNF-α as assessed by both mRNA and protein production (Fig. 2). The TNF-α protein level of cells pretreated with AmB or MF was inhibited 16.2% and 19.0%, respectively.

VCZ pretreatment inhibited TNF-α protein level 32.6% (Fig. 2B). Three additional experiments were performed to assess the effects of the antifungal drugs, at the concentrations studied, on TNF production in the absence of conidia. We noted that TNF production in treated cells was not significantly different than that found with untreated cells (data not shown). Inhibition of TNF-α mRNA expression correlated with protein production (Fig. 2A). Treatment with GM-CSF with antifungal drugs abolished the decreased production of TNF-α induced by conidial stimulation seen with antifungal agents alone (Fig. 2B).

Effects of antifungal drugs on NFκB activation/translocation and IκBα degradation in THP-1 cells

Recent evidence suggests that exposure to *A. fumigatus* conidia results in activation of macrophages and TNF-α secretion through the Toll-like receptor (TLR) signaling pathway which involves the activation of NFκB [8–11]. Therefore, to determine whether the antifungal drugs’ immunomodulatory effects might be associated with the NFκB signaling pathway, Western blot analysis was performed to assess the activation/translocation of NFκB by degradation of IκBα. As shown in Fig. 3, NFκB activity of conidia-stimulated THP-1 cells was enhanced, but was clearly reduced by antifungal drug pretreatment, at least in the cases of MF and VCZ. Treatment of conidia-stimulated cells with GM-CSF alone did not appear to affect the levels of NFκB compared to conidia alone. GM-CSF treatment of conidia-stimulated cells, combined with antifungal drugs, appeared to reverse the effect of the NFκB-suppressing drugs. Changes in IκBα levels were not prominent.

Discussion

We found that representatives of all three classes of antifungal agents reduced the important proinflammatory...
cytokine, TNF-α, production, in THP-1 cells stimulated by A. fumigatus conidia. Apparently with some drugs this effect was brought about through inhibiting NFκB activation. These findings are concordant with a previous report of the immunosuppressive effects of AmB, itraconazole, and MF in RAW264.7 cells exposed to A. fumigatus conidia [13]. Simitsopoulou et al. reported VCZ treatment enhanced TNF-α production in THP-1 cells stimulated with A. fumigatus hyphae, suggesting another mechanism for VCZ eradication of A. fumigatus [8].

Conidia (the infectious form of the fungus) and germlings (the initial step in the formation of invasive hyphae) are known to have quite different cellular structures and induce different immune responses compared to hyphae (the ultimate form of disease invasion) [15]. Activation of the mammalian cell receptor for β-glucan would presumably lead to increased cytokine production via such triggering transcription factors as NFκB and activator protein-1 [19,20]. Echinocandins act by inhibiting the synthesis of 1,3-β-D-glucan, one of the common macromolecules comprising the fungal cell wall. Treatment of A. fumigatus conidia with caspofungin results in less TNF-α and CXCL2 secretion by macrophages, attributed to decreased β-glucan exposure [9]. As conidia germinate, less 1,3-β-D-glucan is present at the surface, a virulence mechanism that results in a lessened host inflammatory response. Echinocandin treatment of hyphae reduces fungal 1,3-β-D-glucan levels but, paradoxically, increases the 1,3-β-D-glucan at the cell surface. The result is an increased host response.

VCZ and other azoles exert their antifungal effects by inhibiting synthesis of the key fungal membrane component, ergosterol, and amphotericin B and polyenes act at preformed ergosterol sites that results in leaky membranes [18]. Our finding that three antifungals with quite different mechanisms of action produce the same immunological effects suggests the hypothesis that these effects may be a result of the drugs’ antifungal effects, mediated by the monocyctic cells during the 8 h fungal-cell contact period. Enough of the drugs may be taken up by the monocytic cells during the prior drug-cell treatment period such that the drugs may each (although by different antifungal mechanisms) then influence conidial metabolism or nascent morphogenesis. This in turn would affect the result of the subsequent fungal-cell interaction, causing a diminished cellular proinflammatory response to the conidia. Since the stage and metabolic activity of the conidial forms markedly affect the immune response [15,21], we demonstrated that antifungal agents can have a profound, and probably under-appreciated, effect on the host response to infection. The antifungal drug effect on host cells could be relevant to patients receiving the drugs for either prophylaxis or therapeutic purposes.

The antifungal agents themselves can also directly affect the immune system [11]. AmB can promote inflammatory cytokines and this could explain the frequent and sometimes severe systemic toxicity of this antifungal. The effects differ, depending on dose and cells targeted [22,23]. Kinoshita et al. have reported that MF inhibits the THP-1 cell increase in TNF production in response to LPS [24].

Fungal species may differ in the immune reactions that they evoke. For example, Candida conceals β-glucan beneath a mannoprotein layer during the growth of yeast cell and pseudohyphae. Therefore, the effect on the immune response by the same antifungal could differ; comparing Candida with Aspergillus [17,25]. Recognition of fungi by TLRs and activation of NFκB are the essential steps in the activation of immune system, as is the case in bacterial infection [26–28]. TLR-2 and TLR-4 are known to be pattern recognition receptors for opportunistic fungal pathogens such as Candida and Aspergillus [26–28]. The TLR occupied differs for Aspergillus and Candida, and for conidia and hyphae of A. fumigatus. These differences could lead to different profiles of cytokines produced.

GM-CSF reversed the immunosuppressive effect of antifungal drugs in the present study. We previously reported similar results with GM-CSF in dexamethasone-treated macrophages with an effect of GM-CSF on the activation of NFκB [14,29]. The opposing effect of GM-CSF on the drugs suppressing NFκB is the presumed mechanism of action in the present study. The immunomodulatory effects of antifungal agents and their interaction with GM-CSF on the response to the hyphal stage of Aspergillus will be our next issue. The effects on freshly isolated effector cells and other types of host effector cells (e.g., neutrophils) will also be of interest. The results of immunomodulating effects of antimicrobials, including antifungal agents, is still not clear. The enhancement of proinflammatory cytokines could be beneficial in eradicating invading pathogens. However, a strong immune reaction could have deleterious effects on the host [22,23].

In conclusion, agents from three different classes of antifungal agents, i.e., AmB, VCZ, and MF, reduced TNF-α mRNA expression and production in THP-1 cells challenged with A. fumigatus conidia. The clinical implications of this immunosuppressive effect of antifungal agents needs further study.

Acknowledgment

Funded in part by the Foundation for Research in Infectious Diseases.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
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This paper was first published online on Early Online on 12 February 2010.