INDUCTION BY *EPIDERMOPHYTON FLOCCOSUM* OF HUMAN FIBROBLAST MATRIX METALLOPROTEINASE-9 SECRETION *IN VITRO*

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Abstract. Skin infection from pathogenic dermatophyte, *Epidermophyton floccosum,* can cause serious health complications, especially in immuno-compromised patients. Proteolytic enzymes secreted from *E. floccosum* are required for host tissue degradation, facilitating fungal invasion. However, little is known regarding host matrix metalloproteinase (MMP) expression during *E. floccosum* infection. In this study human foreskin fibroblast (HFF) cell line was used to determine MMP-9 protease activity by gelatin zymography and amount by ELISA. *E. floccosum* induced HFF secretion of MMP-9 in a time dependent manner, but HFF cell viability decreased. Treatment with an MMP inhibitor (SB-3CT) caused reduction in *E. floccosum*-induced secreted MMP-9 and improvement in HFF cell viability. These findings indicate a possible control measure for protecting skin from *E. floccosum* infection.

Keywords: *Epidermophyton floccosum*, antifungal activity, fungal infection, human foreskin fibroblast, matrix-metalloproteinase-9, MMP inhibitor (SB-3CT)

INTRODUCTION

Fungal infections, particularly from cutaneous mycoses (dermatophytes) can result in chronic and wide damage to skin, hair and nail (Gupta *et al*, 2004). The pathological conditions, often termed as ringworm or tinea, can cause high morbidity (Borgers *et al*, 2005). Recent studies have found that 25% of the world's population are affected by dermatophytes, found in almost 50% of patients > 25 years of age (Alió *et al*, 2005; Havlickova *et al*, 2008). Dermatophytes can be classified into three genera, namely, *Trichophyton, Microsporum,* and *Epidermophyton,* based on the formation and morphology of their conidia (structure of asexual reproduction). In addition, the species can be classified as zoophilic, geophilic, or anthropophilic, depending on their primary habitat or hosts (animal or humans) (White *et al,* 2008).

Epidermophyton floccosum is widely distributed in the tropics and subtropics, particularly in Thailand, and causes tinea cruris, tinea pedis, tinea corporis, and onychomycosis (Weitzman and Summerbell, 1995; Ungpakorn, 2005). A major event during dermatophyte infection is the secretion of endo- and exo-proteases to digest keratin tissue into short peptides and amino acids (Monod, 2008). Although

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infections by dermatophytes are usually restricted to the superficial epidermis, these fungi can be invasive and cause highly severe infections in immune-deficient patients, leading to the development of dermatophytic granulomas (Rodwell *et al*, 2008).

During tissue inflammation, matrix metalloproteinase-9 (a member of the gelatinase family) is secreted in response to the up-regulation of pro-inflammatory cytokines and is involved in tissue remodeling during the repair process (Ries *et al*, 2009). MMP-9 is expressed from macrophage in response to mycobacterial infection (Quiding-Järbrink *et al*, 2001), but little is known concerning MMP expression during fungal (dermatophyte) infection.

In general, dermatophyte infection can be cured with a wide variety of antifungal drugs. However, drug resistance and/or side effects of long-term use, such as liver damage, disturbed estrogen levels, and allergies (Guo *et al*, 2012), eventually lead to treatment failure and enhance the incidence of mycoses (Peres *et al*, 2010).

Thus, this study investigated secretion of gelatinolytic proteases from *E. floccosum*-infected human skin fibroblasts and the effects of the MMP-inhibitor SB-3CT.

MATERIALS AND METHODS

Fungal strains and growth conditions

Epidermophyton floccosum and *Candida albicans* were kindly provided by the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Thailand. Fungi were cultured on Sabouraud dextrose agar (SDA) (Sigma-Aldrich, St Louis, MO) at 30°C for 15 days, and then stored at 4°C until used. A small piece of SDA with sporulating colonies was inoculated into 500 ml of Sabouraud dextrose broth (SDB) (Sigma-Aldrich) and incubated at 25°C with shaking for 7-15 days to induce conidia formation. Both *E. floccosum* and *C. albicans* were diluted in the SDB broth to a concentration of 10^6 CFU/ml. Then each isolate was diluted with RPMI 1640 medium supplemented with 10 g/l glucose (Sigma® Chemical, St Louis, MO) to 1.0×10^5 CFU/ml for subsequent analysis.

Fungal growth kinetics analysis

Fungal growth in the liquid culture was examined using a microplate assay. The spore suspension was adjusted to 1.0×10^5/ml, centrifuged at 3,000*g* for 15 minutes at room temperature and the conidia pellet was suspended in 5 ml of SDB and incubated at 28°C in a well of a microplate. Absorbance at 620 nm was automatically recorded every 3 hours (Multiscan FC microplate-reader; Thermo Scientific, Waltham, MA) to generate a dermatophyte growth curve. In some experiments, *E. floccosum* was incubated with 200 nM of MMP inhibitor SB-3CT (Chemicon International, Temecula, CA).

Infection of human foreskin fibroblast (HFF) cells with fungi

A HFF cell line, obtained from American Type Culture Collection (ATCC CRL-2429™) was maintained in Dulbecco's modified minimum essential medium (DMEM; Gibco, Grand Island, NY), supplemented with 10% heat-inactivated (56ºC for 30 minutes) fetal bovine serum (FBS; Hyclone, Logan, UT), 1% L-glutamate, and 1% antibiotics (200 U/ml penicillin and 100 µg/ml streptomycin; Gibco) at 37°C under an atmosphere of 5% CO_2. Cells were detached using 0.25% trypsin/EDTA (PAA Laboratories GmbH, Pasching, Austria). A monolayer of 10^5 cells/ml was co-cultured with 10^5/ml of *E. floccosum* or *C. albicans* for 12 hours, washed twice

to remove excess fungi, and cultured as described above in DMEM without FBS. Cells and culture supernatants were harvested at various times. In some experiments, cells were incubated with 200 nM of SB-3CT. Following removal of culture medium, trypsinized cells were suspended in 10 ml of DMEM without FBS. The numbers of cells were counted using a hemocytometer. Viable cells were assessed using trypan blue (0.04%) dye exclusion method (Freshney, 2000).

Gelatinolytic protease assay

Gelatinolytic protease activities of *E. floccosum* and *C. albicans* were determined by culturing each organism for 24-48 hours on SDA in the presence of gelatin and Coomassie brilliant blue R-250 dye. Gelatinolytic protease activity was determined also by culturing 10^5cells/ml in SDB and culture supernatant was determined at various times for protease activity by SDS-PAGE gelatin zymography (Luplertlop and Missé, 2008). In brief, 500 µl aliquot of fungal culture medium was lyophilized, dissolved in loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 0.04% bromophenol blue dye) without prior heating and electrophoresed in 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatin for 45 minutes. Then the gel was stained with 0.25%-0.3% Coomassie brilliant blue R-250 dye and fixed in 30% (v/v) methanol/10% (v/v) acetic acid solution. Areas of protease activity are seen as clear bands (where protease has digested the substrate) against a dark blue background. Gels were scanned and the density of each band was semi-quantified with the aid of a computer-assisted image analysis program (1D Image Analysis Software, Kodak Digital Science v.3.0, Eastman Kodak, Rochester, NY). Cell culture supernatants, collected from HFF cells after being co-cultured with *E. floccosum* or *C. albicans* or mock infection, also were determined for gelatinase by gelatin zymography as described above. In addition, MMP-9 activity in human foreskin fibroblast (HFF) cell culture medium was determined using R&D Quantikine® ELISA kit (Cat. No. DMP900 for human MMP-9), according to the manufacturer's instructions. Absorbance was measured at

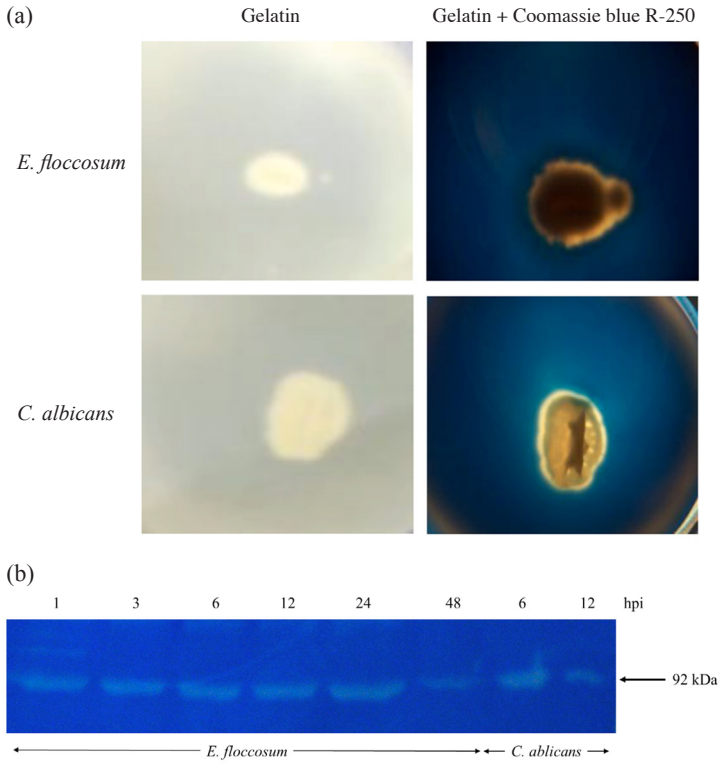

Fig 1–Secretion of gelatinolytic protease from *E. floccosum* and *C. albicans* in culture. (a) *E. floccosum* and *C. albicans* were grown for 12 hours at 30°C on SDA containing gelatin and gelatin stained with Coomassie brilliant blue R-250 dye. (b) Gelatin zymography of culture medium of *E. floccosum* and *C. albicans* grown in SDB. hpi, hour(s) post-incubation.

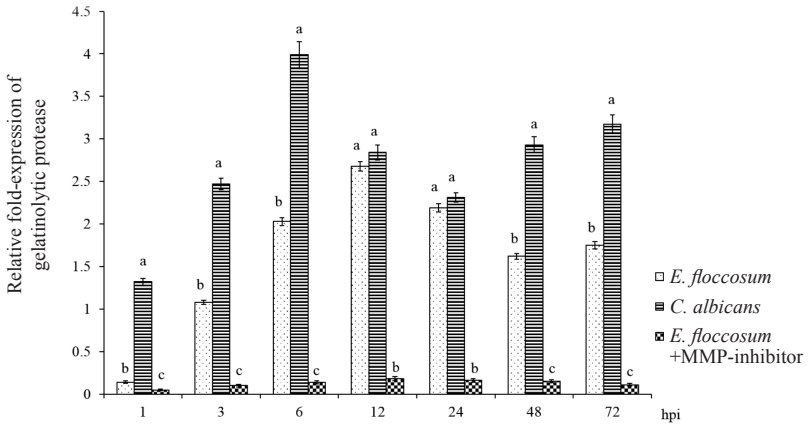

Fig 2–Secreted gelatinolytic protease activity of *E. floccosum*, *C. albicans* and *E. floccosum* treated with 200 nM of MMP inhibitor SB-3CT. Fungi (10^5/ml) were grown in SDB. Gelatinolytic protease activity in culture medium was determined by gelatin zymography. Data are presented as mean ± SD of an experiment conducted in triplicate. Different letters indicate that values are significantly different at $p < 0.05$ relative to mock. hpi, hour(s) post-incubation.

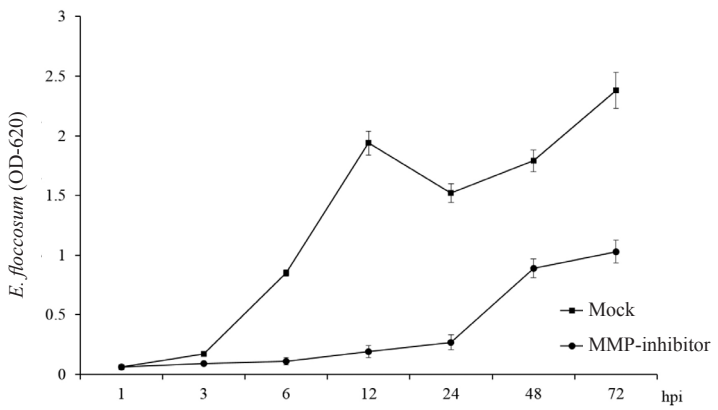

Fig 3–Growth of *E. floccosum* and *E. floccosum* treated with 200 nM of MMP inhibitor SB-3CT. *E. floccosum* conidia (10^5/ml) were grown in SDB. Data are presented as mean ± SD of an experiment conducted in triplicate. hpi, hour(s) post-incubation.

570 nm in a microplate reader (Sunrise™ TECAN, Männedorf, Switzerland).

Statistical analysis

Each independent experiment was carried out in triplicate and the results expressed as mean ± standard deviation (SD). Data were analyzed using SPSS version 16 software program (SPSS, Chicago, IL). The results of each experiment were compared using one-way analysis of variance (ANOVA). One-tailed Mann-Whitney *U* test was used for non-parametric data. Differences are considered statistically significant at a *p*-value < 0.05.

RESULTS

After incubating both *E. floccosum* and *C. albicans* on SDA containing gelatin and Coomassie brilliant blue R-250 dye, a clear zone against a dark blue background was apparent (Fig 1a), indicating that both *E. floccosum* and *C. albicans* can secrete gelatinolytic protease(s). Gelatin zymography revealed that *E. floccosum* and *C. albicans* produced gelatinolytic protease of 92 kDa (Fig 1b), which was similar in molecular size to MMP-9 secreted from HFF cells (Fig 5). The secretion of gelatinolytic protease by both fungi was highest at 6 hours post-incubation and declined thereafter (Fig 1b) as quantified using an image analyzer (data not shown). Gelatinolytic protease secretion of *E. floccosum*

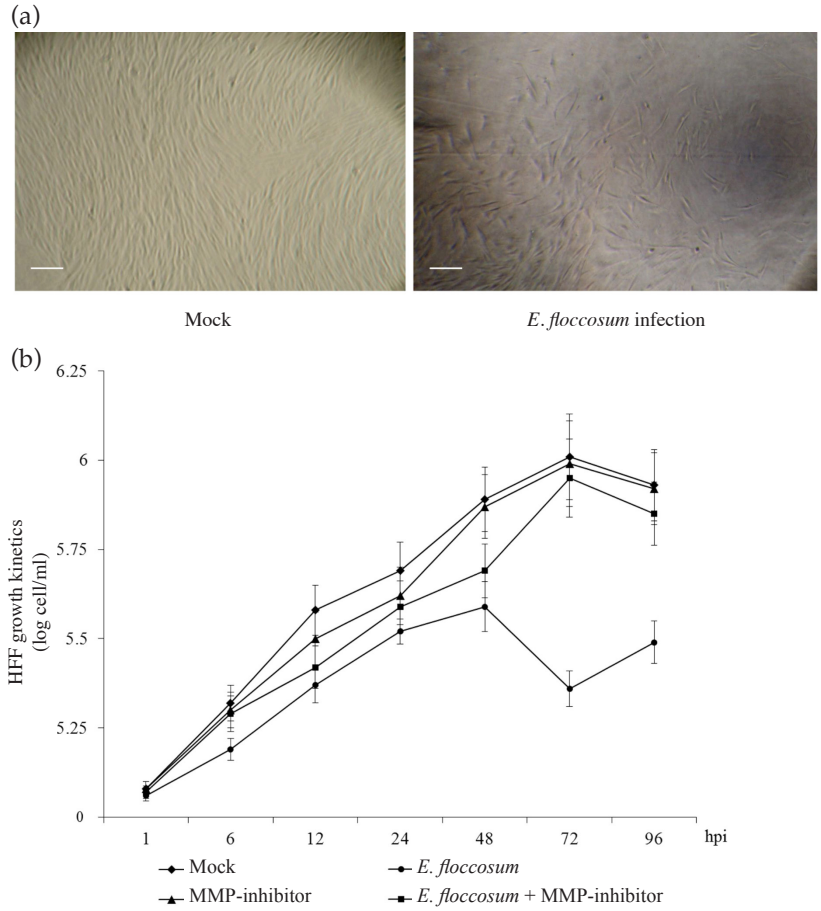

Fig 4–Effects of *E. floccosum* infection on HFF cells. (a) Light microscopy of mock (control) and HFF cells after 12 hours co-cultured with *E. floccosum* conidia (10^5 /ml). White bar, 10 μm. (b) Growth of mock control HFF cells, cells treated with 200 nM of MMP-9 inhibitor SB-3CT, cells co-cultured with *E. floccosum* conidia (10^5 /ml), and cells co-cultured with *E. floccosum* conidia (10^5 /ml) and treated with 200 nM of SB-3CT. Cell concentration was determined by staining the cells with 0.4% (w/v) of tryphan blue solution and counting with hemocytometer. Data are presented as mean ± SD of an experiment conducted in triplicate.

was lower than that of *C. albicans* (Figs 1b and 2). Activity of *E. floccosum* secreted gelatinase was completely inhibited by 200 nM of MMP inhibitor SB-3CT (Fig 2).

Growth kinetics of *E. floccosum* increased in a time-dependent manner (Fig 3). However, after treating *E. floccosum* with 200 nM of SB-3CT growth decreased significantly (Fig 3).

Growth of HFF cells increased with time and plateaued after 72 hours (Fig 4b) and was not affected by 200 nM of SB-3CT. Infection with *E. floccosum* produced a reduction in HFF cell growth, due in part to cell detachment from the culture plate surface (Fig 4a), even at 6 hours post-infection, with a drastic fall after 48 hours post-infection (Fig 4b). However, normal growth of *E. floccosum*-infected HFF cells was restored by 200 nM of SB-3CT.

Employing ELISA, MMP-9 secretion of HFF cells was discernable after 1 hour of growth, reaching a maximum at 12 hours and then declined, but was still higher at 96 hours than at 1 hour after culturing (Fig 5). The presence of 200 nM of SB-3CT resulted in a significant decrease in MMP-9 secretion at all time points. Infection with 10^5 cells/ml of *E. floccosum* stimulated HFF MMP-9 secretion above control levels, and this was inhibited by 200 nM of SB-3CT to levels significantly higher or comparable to those of SB-3CT-treated non-infected HFF cells (Fig 5). Infection with comparable amounts of *C. albicans* stimulated MMP-9 secretion at 6

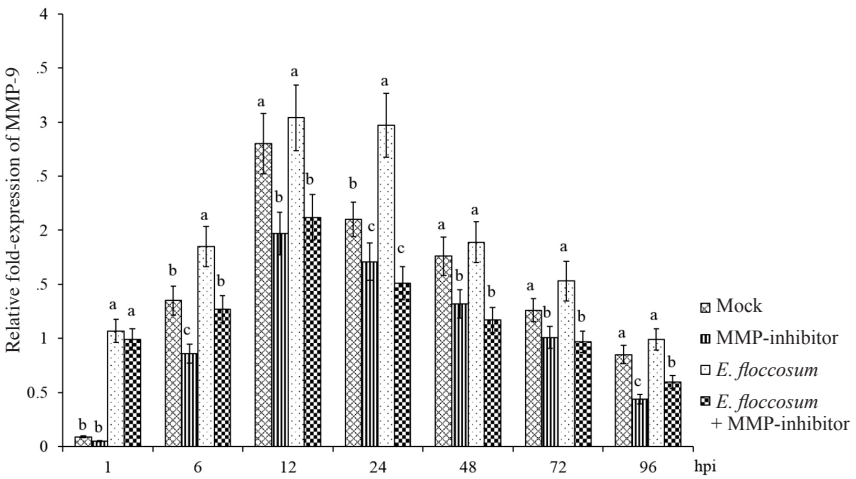

Fig 5–Secreted MMP-9 amount of mock control HFF cells, cells treated with 200 nM of MMP-9 inhibitor SB-3CT, cells co-cultured with *E. floccosum* conidia (10^5 /ml), and cells co-cultured with *E. floccosum* conidia (10^5 /ml) and treated with 200 nM of SB-3CT. MMP-9 amount in culture medium was determined by ELISA. Data are presented as mean ± SD of an experiment conducted in triplicate. Different letters indicate values are significantly different at $p < 0.05$ relative to mock control HFF cells.

hours post-inoculation to the same level as that seen with *E. floccosum* co-culture (data not shown).

DISCUSSION

E. floccosum can produce many different kinds of proteases, such as elastase, urease and sulphatase (Hopsu-Havu and Tunnela, 1977). It is possible that the gelatinolytic protease secreted from *E. floccosum* (and *C. albicans*) is MMP-9 as its molecular size was similar to that of HFF and was inhibited by the MMP-inhibitor SB-3CT. However, the properties of this secreted gelatinase enzyme need further investigation.

Candida spp, especially *C. albicans*, produces proteases and the latter has been used as a model organism to study the efficiency of drugs on adherence of *Candida* spp to various cell types (Naglik *et al*, 2003; Nobile *et al*, 2006; Tamai and Kiyoura, 2014). Protease expression of *C. albicans* is highest during 6-12 hours of growth and is reduced thereafter to undetectable level in time dependent manner (Pärnänen *et al*, 2011). Thus, *C. albicans* was employed as a positive control in this study.

It is of interest to note that reduction of *E. floccosum* gelatinase activity by a MMP inhibitor affected growth. Impairment of dermatophyte activity, either by mutation of protease genes (Grumbt *et al*, 2013) or by using protease inhibitor (Meevootisom and Niederpruem, 1979), limits growth.

In this study, the limitation to *E. floccosum* growth and inhibition of secreted gelatinase activity by SB-3CT protected HFF cells from fungal inflicted damage. Dermatophyte co-cultured skin cells produce a variety of inflammatory cytokines (*viz*, TNF-α, interleukins, and many growth factors) (Wagner and Sohnle, 1995), and failure to defend against such infection can lead to cell death (Calderon, 1989). In addition, it has been suggested that production of MMP-9 is a direct response to pro-inflammatory stimuli (as dermatophyte infections induce the secretion of pro-inflammatory mediators), which can be used as a marker for acute inflammation (Warner *et al*, 2004).

In summary, this study demonstrates that inhibition of *E. floccosum* gelatinase activity and cell growth by the MMP inhibitor SB-3CT provided protection against damage to *E. floccosum*-infected HFF cells, and, although SB-3CT reduced the levels of *E. floccosum*-induced MMP-9 secretion from HFF cells, MMP-9 levels did not fall below those secreted by uninfected HFF cells. Future research will focus on the role of SB-3CT in inhibiting and preventing dermatophytic skin infections.

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