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Expression of haptoglobin in *Trichosporon asahii* induced skin infection and its immunoregulatory role in mice

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To investigate the immunoregulatory role of haptoglobin (Hp) in the *Trichosporon asahii* induced skin infection. A total of 80 BALB/c mice were assigned into immunosuppression (IS) group and non-immunosuppression (non-IS) group. Mice in both groups were subcutaneously inoculated with 3.2×10^7 cfu/ml *T. asahii* suspension. 0.5, 1, 3 and 5 d after inoculation, mice was sacrificed and skin lesion was collected. mRNA expression of Hp gene was detected by RT-PCR. Microarray was employed to investigate immune related gene expression profile. In both groups, mRNA expression of Hp was detected as 0.5, 1, 3 and 5 d after *T. asahii* infection, but the Hp expression in IS group was significantly higher than that in non-IS group ($P<0.01$). One day after *T. asahii* infection, Hp expression was significantly higher than that of 0.5, 3 and 5 d after infection ($P<0.01$). A total of 11 immune related differentially expressed genes were identified in the microarray assay and the gene encoding Hp protein was up-regulated. Hp involves in the *T. asahii* infection of skin.

Key words: *Trichosporon asahii*, haptoglobin, skin, infection.

INTRODUCTION

Trichosporon species is an emerging pathogen in immunocompromised hosts, and *Trichosporon asahii* is the species most frequently involved in fatal disseminated trichosporonosis. In 2000, we reported the first case of disseminated trichosporonosis in China (Yang et al., 2001). In particular, disseminated trichosporonosis is becoming increasingly common in patients with underlying hematological malignancies, extensive burns, solid tumors, the wide application of cytotoxic drugs in chemotherapy, corticosteroid, and organ transplantation, accounting for approximately 10% of all confirmed cases of disseminated fungal infections (Thibeault et al., 2008; Shang et al., 2010; Heslop et al., 2011; Kumar et al., 2011; Abdala et al., 2005). Despite the use of antifungal drugs to treat trichosporonosis, infection is often persistent and is associated with high mortality (Di Bonaventura et al., 2006). With the wide use of azole and amphotericin B, the antifungal drug resistance has become a serious problem and brings new challenge to antifungal therapy (Sun et al., 2012). Neutrophil, macrophage and T lymphocyte mediated cell immune plays an important role in defense against invasion of pathogens. Among them, mononuclear macrophage is considered to be the main defense of the body against infection. Immune regulation therapy in recent years has become hotspot for the treatment of deep fungal infection (NIE et al., 2012).

Haptoglobin (Hp) is an active acute phase protein, and can bind to hemoglobin, exert anti-infection effect and promote angiogenesis. In addition, Hp also plays an important role in the immunoregulation (Langlois and

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DelaJlghe, 1996). Studies have found that Hp can inhibit the maturation of immune function of langerhans cells (LC) and thus; it may be involved in the immunoregulation via affecting the functions of LCs (Xie et al., 2000; Li et al., 2005). In the present study, the role of Hp in the *T. asahii* skin infection was investigated aiming to explore the immunoregulatory effect of Hp in focal infection.

MATERIALS AND METHODS

T. asahii (AS2.2174) was isolated in our department and stored at -80°C. Eighty male BALB/c mice weighing 20 ~ 28 g were purchased from the Experimental Animal Center of the Academy of Military Medical Sciences. Trizol, total RNA extraction kit, Access RT-PCR System kit (Promega), DEPC (Sigma), primers for Hp and β-actin (TaKaRa), Microarray hybridization kit and Probe labeling kit (Shanghai United Gene Biotech, Co., Ltd) were used in the present study. The primers for Hp were as follows (D'Anniento et al., 1997): forward: 5′-ACCTTAAAC GAC GAG AAG CAATGG-3′, reverse: 5′-AGC CAG ACA CGT AGC CCA CAC G-3′ (482 bp); the primers for β-actin were as follows: forward: 5'-GTGGGCCGCTCTAGGCACCAA-3' and reverse: 5'-CTCTTTGATGTCACGCACGATTTC-3' (540 bp).

Preparation of immunosuppresant and *T. asahii* suspension

All mice were randomly assigned into immunosuppression (IS) group and non-IS group. Mice in the IS group were intraperitoneally injected with cyclophosphamide (200 mg/kg) for 3 consecutive days followed by *T. asahii* inoculation. *T. asahii* was cultured in Sabouraud dextrose agar without addition of cyclohexamide medium at 37°C for 2 weeks. Steriel normal saline was used to flush the medium and the flushing fluid was filtered through sterile gauzes. The *T. asahii* concentration was adjusted to 3.2×10^7 cfu/ml (Yang et al., 2008).

Inoculation and sample collection

The skin on the back was prepared in each mouse and an area of hair of about 2.0 cm in diameter was removed followed by subcutaneous inoculation with 0.2 ml of *T. asahii* suspension. At 0.5, 1, 3 and 5 d after inoculation, mice were sacrificed (n = 10 at each time point) and the skin at the inoculation site was collected and rapidly stored in nuclease free tube which was pre-cooled with liquid nitrogen and then in liquid nitrogen (Wang et al., 2008).

Extraction of total RNA

Samples were ground in liquid nitrogen and then transferred into centrifuge tube. Total RNA was extracted with Trizol (invitrogen company) according to one step RNA extraction protocol and then stored at -80°C.

RT-PCR

PCR was conducted according to the manufacturer's instructions (Access RT-PCR System). The reaction mixture (50 µl) included 1 µl of dNTP, 1 µl of each primer, 2 µl of 25 mM $MgSO_4$, 1 µl of AMV reverse transcriptase, 1 µl of Tf1 polymerase, 1 µl of RNA template, 10 µl of 5 × PCR buffer and 32 µl of nuclease free ddH_2O. The reaction conditions were as follows: reverse transcription at 48°C for 45 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 68°C for 2 min and final extension at 68°C for 7 min. RT-PCR products were subjected to 1% agarose gel electrophoresis and images were captured with BioRad gel imaging system followed by analysis with Quantity One software. β-actin served as an internal reference. The optical density of Hp was normalized by that of β-actin. That is, the product of area and density of β-actin band was divided by that of Hp, which served as the relative mRNA expression of Hp.

Microarray assay

Pre-hybridization

The microarray and hybridization reagent I and II (6 µl for each) were independently heated in water bath (95°C) for 2 min. Then, the hybridization reagents were added to the microarray followed by addition of coverslip and incubation at 42°C for 5 h.

Probe labeling

In brief, 25 µl of double distilled water, 3 µl of primers for reverse transcription and 100 µg of total RNA were added to a 1.5 ml EP tube followed by incubation at 70°C for 10 min and then on ice. Then, 10 µl of reverse transcription buffer, 10 µl of DDT, 3 µl of dNTP, 2 µl of reverse transcriptase (addition in dark) and 3 µl of Cy5-dCTP (IS group)/Cy3-dCTP (non-IS group) were added to the tube followed by incubation at 42°C for 2 h. Then, addition of 4 µl of labeling reagent I was performed followed by incubation at 65°C for 10 min. Subsequently, 4 µl of labeling reagent II was added followed by vacuum pumping in dark until the volume of reaction mixture was 50 µl. DNA purification column was employed to purify DNA and 8 µl of labeling reagent was added followed by vacuum pumping.

Hydridization and washing

The labeled probe, hybridization reagent and microarray were heated in water bath (95°C) for 2 min and then mixed followed by hybridization at 42°C for 15 ~ 20 h. Then, the microarray was washed and dried for scanning.

Quality control: The microarray contained 4069 genes which contain 87 house-keeping genes. Blank spotting solution (3 spots) and plant genes (8 spots) severed as negative controls. The ratio of mean signal density to the density of background was not lower than 3 in both groups. Fluorescence scanning was performed with Scan array 4000 scanner. The fluorescence intensity of Cy3 and Cy5 was detected with GenePix Pro 3.0 image processing software. Following homogenization of raw data, statistical analysis was carried out.

Statistical analysis

SPSS version 11.0 for windows was employed for statistical analysis. Comparison of mRNA expression of Hp between two groups was done with two sample t test. For mRNA expression at different time points in both groups, comparisons were done with Least Significant Difference (LSD) test. A value of $P<0.05$ was considered statistically significant.

RESULTS

PCR amplification of total RNA

The products from RT-PCR were subjected to gel electrophoresis and results showed two bands with 28 S and 16 S. The density of band with 28 S was about 2 times higher

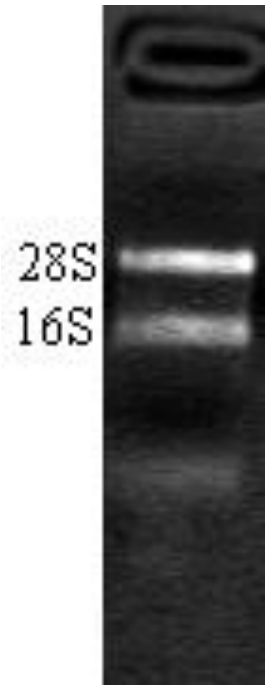

Figure 1. Formamide denaturing gradient gel electrophoresis of total DNA. Two bands with 28 S and 16 S, respectively. The density of 28 S band was 2 times higher than that of 16 S band.

than that with 16 S (Figure 1). The ratio of A260 to A280 (A260/A280) was 1.8 ~ 2.0, which suggested the integrity and purity of RNA were acceptable and met the requirement for further experiment.

mRNA expression of Hp and its relation with infection

After amplification with PCR, a band at 482 bp was noted in both IS group and non-IS group (Figure 2). Quantity-One software was used to detect the optical density of target bands and the relative mRNA expression of Hp was calculated. Statistical analysis showed marked difference in mRNA expression of Hp between two IS group and non-IS group (t = 8.97, P<0.01) and mRNA expression of Hp in IS group was markedly higher than that in non-IS group. In IS group, mRNA expression of Hp at 1 d after infection was significantly different from that at 0.5, 3, and 5 d after infection (P<0.05), but difference was not found at 0.5, 3 and 5 d after infection (P>0.05). Moreover, mRNA expression of Hp was comparable at different time points in non-IS group (P>0.05) (Table 1).

Microarray assay

Among 80 samples in two groups, a total of 11 immune related genes were identified to have differential expression. When compared with non-IS group, Hp gene was down-regulated in IS group, and the complement C2 gene, TNF-induced protein gene, interferon-induced protein gene, CD53 gene, CD22 gene, CD209a gene, CD79A binding protein 1b gene, Lymphocyte antigen 6 complex gene and prostaglandin E receptor 4 gene presented down-regulation (Figure 3).

DISCUSSION

Trichospporon is a genus of pathogenic yeast that has been identified from the mucosal surfaces of the human respiratory and gastrointestinal tracts and in stool and sputum, as well as on central venous catheters and hair (Yang et al., 2012). Over the past decades, it has emerged as a life-threatening pathogen and has been the second common cause of yeast infections among disseminated infection in humans following *Candida* (Girmenia et al., 2005). Disseminated Trichosporon infection is an uncommon but life-threatening fungal infection usually observed in immunocompromised hosts, especially in patients with hematological malignancies. The outcome of disseminated trichosporonosis is usually poor, with a mortality rate of approximately 80% (Kudo et al., 2011). This suggests that immune function plays an important role in defense against fungal infection. There is a close relationship between deep fungal infection and the immune defense and immune status. In recent years, immune regulation therapy has become a hot problem in treatment of deep fungal infection. However, the reasons that immune system of the host can not promptly clear the pathogens following *T. asahii* infection are still unclear. Haptoglobin (Hp) is an acid glycoprotein in serum α2 globulin family. As an acute phase protein, Hp involves in the anti-infection, repair of injured tissues and maintenance of homeostasis. Under pathological conditions such as infection, trauma, inflammation, tumor, myocardial infarction, etc, the serum Hp level significantly increases. Hp is mainly produced in the liver, but D'Anniento et al (1997) found that mRNA expression of Hp in the skin of healthy mice, and mRNA expression of Hp increased after LPS stimulation.

Xie et al. (2000) identified Hp in the plasma of LCs in the epidermis which could regulate the functional transformation of LCs under culture. In the present study, RT-PCR and microarray assay were employed to detect mRNA expression of Hp and Hp gene, respectively, in the skin after *T. asahii* skin infection. Results showed that mRNA expression of Hp in both mice with and without immunosuppression, but Hp expression in IS mice was markedly higher than that in non-IS mice. Microarray assay revealed 11 immune related genes with differential expression. Moreover, Hp gene was also up-regulated.

These indicated that Hp involved in the immune response of host after *T. asahii* skin infection. The *T. asahii* induced focal inflammation may cause production of Hp in the skin which exerts anti-inflammatory effect to

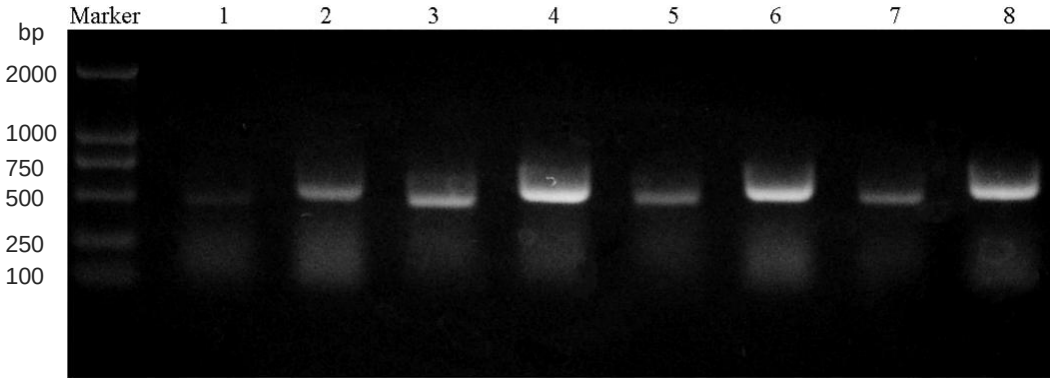

Figure 2. mRNA expression of Hp in IS and non-IS group. 1, 3, 5, 7: mRNA expression of Hp at 12, 24, 72 and 120 h after infection in non-IS group. 2, 4, 6, 8: mRNA expression of Hp at 12, 24, 72 and 120 h after infection in IS group.

Table 1. mRNA expression of Hp in IS mice and non-IS mice following *T. asahi* infection.

Group/Time point	n	Relative expression
IS group	40	0.383±0.025
0.5d	10	0.275±0.043
1d	10	0.537±0.054
3d	10	0.394±0.041
5d	10	0.327±0.046
Non-IS group	40	0.278±0.019
05.d	10	0.253±0.034
1d	10	0.304±0.036
3d	10	0.283±0.041
5d	10	0.272±0.043

In IS group, mRNA expression of Hp at 1 d after infection was significantly different from that at 0.5, 3, and 5 d after infection ($P<0.05$), but difference was not found at 0.5, 3 and 5 d after infection ($P>0.05$). Moreover, mRNA expression of Hp was comparable at different time points in non-IS group ($P>0.05$).

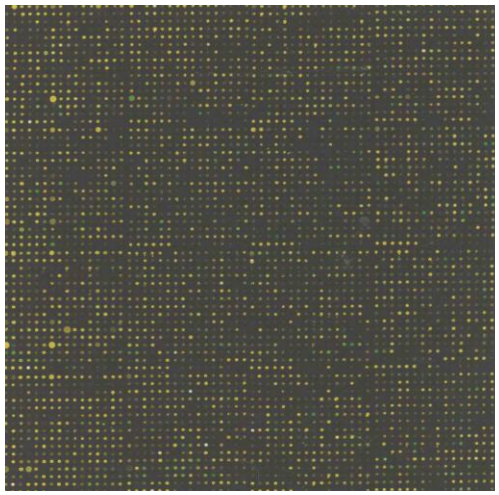

Figure 3. Scanning of microarray after hybridization. Red: up-regulation; green: down-regulation.

a certain extent aiming to prevent the over-inflammation induced injury. The anti-inflammatory effect of Hp is determined by the expression level and the duration of high Hp expression (Wang et al., 2001). Our results also showed that mRNA expression in IS mice at 1 d after infection was markedly higher than that at 0.5, 3 and 5 d after infection, and the Hp expression had a decreasing tendency over time. In the IS mice, the mRNA expression of Hp was increased and the Hp gene also up-regulated in the skin, which may attenuate the response of host to inflammtion to a certain extent. This condition provides chance for the survival, proliferation and dissemination of fungus.

In addition, there is evidence showing that Hp has multiple immunoregulatory effects; a fraction of which are conferred via binging to CD11B. Arredouani et al. (2001) investigated the effect of Hp on the production of cytokines by Th cells. Their *in vitro* experiment revealed that Hp could inhibit production of Th2 cytokines (IL-4, IL-

5, IL-10 and IL-13) in a dose dependent manner, but had no influence on Th1 cytokines (IL-2 and IFN-gamma) (Arredouani et al., 2003). Thus, they proposed that Hp possesses the ability to regulate Th1/Th2 balance in the immune system. In the IS mice, mRNA expression of Hp and Hp gene were up-regulated, which may inhibit production of Th2 cytokines attenuating the humoral immunity against *T. asahii*. On one hand, this may prevent excessive inflammatory reaction; on the other hand, this provides chance for the survival, infection and dissemination of *T. asahii*.

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