Evaluation of Mdh1 Protein as an Antigenic Candidate for a Vaccine against Candidiasis

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Candida albicans malate dehydrogenase (Mdh1p) has been screened by previous proteome studies as a candidate for a vaccine against candidiasis. In this study, recombinant Mdh1 protein with a His-tag was produced in Escherichia coli and evaluated as an immunogenic protein against candidiasis. Mdh1p was administrated to mice by two methods subcutaneous injection and intranasal administration before challenging them with a lethal dose of C. albicans. After vaccination of Mdh1p, antibody responses were observed. To evaluate the vaccination effect of Mdh1p, survival tests were performed after 35 d. Although all control mice died within 24 d or 25 d, 100% and 80% of mice survived with subcutaneous and intranasal administration, respectively. Therefore, our results indicate that, among C. albicans antigens examined thus far, Mdh1p is currently the most effective antigen for use as a vaccine for C. albicans.

Key words : Mdh1p / Candida albicans / Candidiasis / Vaccine.

Candida albicans is an opportunistic fungal pathogen, causing superficial and systemic infections among immunocompromised patients (Suido and Miyao, 2008; Brown et al., 2007). The high mortality rate (up to 50%) induced by C. albicans in hospitalized patients indicates that this microorganism is one of the most detrimental pathogens to humans (Pfaller and Diekema, 2007). Commonly, pharmacotherapy of candidiasis involves the administration of antifungal drugs, such as caspofungin, micafungin, and amphotericin B. However, approximately half of the treated patients have a poor outcome in spite of antifungal therapy (Morschhäuser, 2002; Zaoutis et al., 2005), and as a result, mutants of Candida with reduced susceptibility to these drugs have emerged (Rodloff et al., 2011). Considering these situations, prevention of this fungal infection through vaccination is thought to be an important complementary strategy to pharmacotherapy.

In general, most of the available vaccines are categorized into 3 groups: killed or attenuated pathogenic cells, toxins, and polysaccharide-carrier protein conjugates (Fernandez-Arenas et al. 2004). Additionally, new approaches to vaccine development involve screening specific proteins. For example, a proteome study on hyphal induction (an infectious phenotype) in C. albicans shows characteristic cell wall protein profiles (Heilmann et al., 2011). Furthermore, we have investigated the virulent protein of C. albicans under the condition containing serum in the medium, by a liquid chromatography/mass spectrometry (LC-MS/MS) system equipped with a long monolithic capillary column (Aoki et al., 2012). In that study, we found a potential antigen candidate, malate dehydrogenase.
protein (Mdh1p, EC1.1.1.37). This protein was also identified in a proteome study for screening C. albicans immunogenic proteins by a two-dimensional electrophoresis (2-DE)/MS system (Fernandez-Arenas et al. 2004). Here, we showed that delivery of recombinant Mdh1p, by intranasal (i.n.) administration or subcutaneous (s.c.) injection increased the titers of anti-Mdh1p antibodies and prolonged the lives of mice infected with lethal levels of C. albicans.

The E. coli strain DH5α [F- endA1 hsdR17 (rK-, mK+)
supE44 thi1 recA1 gyrA96 ΔlacU169 δF80 lacZΔ M15] (Hanahan, 1983) was used as a host for manipulation of recombinant DNA. The E. coli strain BL21 [F-ompT hsdSB (rB+, mB+) gal dcm (DE3)] was used to produce recombinant Mdh1p. Both E. coli strains were grown in LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, and 0.1% (w/v) glucose, pH 6.7] (Matsumura et al., 2011).

The plasmid pQE-mdh1, which was used to produce Mdh1p in E. coli, was constructed by first using PCR to amplify the Mdh1p coding sequence using the primers
5′-CATCACCATCAGGATCTTCCGCAGGACTTTGTGCT
ACTAGTACATTTCTCTCT-3′ and 5′-GCTCACTTAAT
TAAGCCTT0TTATGGGTTTGGAGCACAAGTCAACAC
CACC -3′ and genomic DNA of C. albicans strain SC5314 (American Type Culture Collection), which was purified as described previously (Aoki et al., 2011). The amplified fragment of the Mdh1p coding sequence was inserted into the pQE30 plasmid (QIAGEN, Hilden, Germany) that had been digested with BamHI and HindIII, and the resultant plasmid was named pQE-mdh1. pQE-mdh1 was introduced into the E. coli BL21 strain as described previously (Hanahan, 1983) for propagation. The nucleotide sequence of the constructed plasmid was confirmed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Mdh1p was produced in the E. coli strain BL21 (DE3) as a fusion protein with an N-terminal His6 tag. The cells were inoculated in 14 ml of LB medium, containing 100 μg/ml ampicillin, and grown in shake flasks overnight at 37°C. Fresh LB medium, containing 100 μg/ml ampicillin (120 ml), was inoculated with 6 ml of the overnight cultures, and the cells were grown at 37°C until OD600 = 0.6–1.0. Gene expression was then induced by the addition of isopropyl β-D-thiogalactoside (IPTG; Wako Pure Chemical, Osaka, Japan) to a final concentration of 1 mM (Inaoka et al., 1999). After 3–4 h of cultivation at 37°C, the cell cultures were harvested by centrifugation (4,000 × g, 20 min, 4°C). The cell pellets were subsequently resuspended in 10 ml of bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific, IL, USA) and shaken gently for 10 min at room temperature (25°C). Soluble proteins were separated from insoluble proteins by centrifugation at 27,000 × g for 20 min. The supernatant containing soluble proteins was purified by recovering the His6-Mdh1 fusion protein after passage through nickel-chelated agarose (Thermo Fisher Scientific) columns. The column was equilibrated with 10 ml of B-PER before application of the supernatant. After washing the column with a wash buffer of the B-PER reagent (35 mM Tris, 150 mM NaCl, 10 mM imidazole, 5% (v/v) glycerol, pH 7.2), the bound proteins were released with an elution buffer (50 mM Tris, 300 mM NaCl, 200 mM imidazole, 10% (v/v) glycerol, pH 6.8). Endotoxins were removed from the eluate by passage through Detoxi-Gel endotoxin-removing columns (Pierce, Rockford, IL, USA), resulting in endotoxin levels of <0.1 U/ml, as indicated using Limulus Amebocyte Lysate (LAL) PYROGENT single-test vials (Lonz, Walkersville, MD, USA) as described in the manufacturer’s protocol.

Female C57BL/6 mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were kept in a specific-pathogen free manner and allowed to drink and eat ad libitum.

Seven-week-old female C57BL/6 mice (5 per group) were immunized with 30 μg of the recombinant Mdh1p by i.n. delivery or s.c. injection, administered in 20-μl volumes containing 1 μg of cholera toxin (CT) (Sigma-Aldrich, St. Louis, Mo., USA) or 100-μl volumes containing Freund’s incomplete adjuvant (IFA) (DIFCO Laboratories, Detroit, MI, USA) i.n. and s.c., respectively. Seven female C57BL/6 mice of the same age, which received the adjuvant alone mixed with phosphate-buffered saline (PBS, pH 7.4), served as the control group. Mice were immunized at weeks 0, 2, and 4. Blood samples were collected at week 6 from the tail vein, for determination of the titer of serum IgG that binds Mdh1p. The significance of the difference between groups was determined by Student’s t-test.

For survival studies, mice were infected with 1.1 × 10⁵ cells of C. albicans, resuspended in 100 μl PBS, by tail-vein injection 2 wk after the last immunization. Mice were observed daily for 5 wk after being infected. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee, and animal experiments were conducted according to the institutional ethical guidelines for animal experiments. The outcomes of the challenge with C. albicans were determined using the Kaplan-Meier method. Significant differences between groups were tested using the log-rank method.

Indirect enzyme-linked immunosorbent assay (ELISA) was conducted for antibody analysis of the antiseras collected at week 6 (i.n. or s.c.). Briefly, 96-well microtiter plates (Nalge Nunc International,
Rochester, NY, USA) were coated with 50 μl/well of E. coli-expressed recombinant Mdh1p (0.01 μg/μl). The plates were blocked with 1% bovine serum albumin (BSA) dissolved in PBS with 0.05% Tween-20. Serially diluted antisera and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1/4000, Promega, Madison, WI, USA), followed by its substrate, were added to wells. After 20-min incubation at room temperature, the reaction was stopped by addition of 1 M sulfuric acid, and the absorbance was measured at 450 nm (OD450) using a microplate reader (Bio-Rad Laboratories Inc., Redmond, WA, USA). The serum IgG antibody titer was defined as the serum dilution that gave an OD450 value equal to 0.1.

The use of PCR to amplify the Mdh1p-coding sequence, using genomic DNA isolated from C. albicans as a template, yielded a fragment of the expected size, which was cloned into the pQE30 vector that was designed to produce His-tagged recombinant proteins in E. coli. After confirmation that the nucleotide sequence of pQE-mdh1 was correct by comparison with the Candida genome database (http://www.candidagenome.org/), the plasmid was introduced into E. coli BL21, and the recombinant protein was produced in the cytosol and then recovered and purified by passage of the extract through a Ni-chelated column. The recombinant protein was further purified by passage through an endotoxin-removing column. The concentration of endotoxins in the eluate from the Ni-chelated column ranged from 0.06 to 0.125 endotoxin units (EU) per milliliter. Analysis of Mdh1p by SDS-PAGE and Western blotting indicated that the anti-His antibody recognized a purified protein (data not shown).

We used both s.c. injection and i.n administration to evaluate which delivery of recombinant Mdh1p could induce immunity effectively. The extent to which vaccination with Mdh1p could offer protection against candidiasis was determined by testing the survival after administration of a lethal dose of C. albicans. Administration of Mdh1p with the adjuvant (IFA) by s.c. administration enhanced titers of IgG bound to Mdh1p (Fig. 1a). At 35 d after challenge with a lethal dose of C. albicans, 100% of the mice vaccinated with Mdh1p were alive, while none of the control animals administered the adjuvant only and infected with the same dose of C. albicans survived longer than 24 d after infection (Fig. 1b). To date, the effects against systemic candidiasis of a vaccine based on several proteins derived from C. albicans have been examined in other studies in the same manner as in our evaluation. For example, GPI-anchored hyphal cell wall protein (Hyr1p) (Luo et al., 2010), fructose-bisphosphate aldolase (Fba), enolase 1 (Eno1p), glyceraldehyde-3-phosphate dehydrogenase (Gap1p), and phosphoglycerate kinase (Pgk1p) (Xin and Cutler, 2011) have been evaluated. However, none of those proteins, when administered after infection with C. albicans, resulted in more than 80% survival at 35 d. Therefore and in contrast to other previously tested proteins, Mdh1p is a promising potential vaccine candidate against candidiasis.

As was observed for s.c. injection, i.n administration of Mdh1p with the adjuvant (CT) increased titers of the Mdh1p-specific antibody (Fig. 2a). The average value of titers of the Mdh1p-specific antibody was 3.7 × 10³, while the value in the case of s.c. injection was 1.0 × 10⁷ (Fig. 1a). In addition, the survival test was performed, and approximately 80% as many animals
survived up to 35 d after infection with *C. albicans* after i.n. administration of the vaccine (Fig. 2b), although 100% of the animals survived up to same periods in the case of s.c. injection (Fig. 1b). Thus, s.c. injection with IFA was the more effective method of Mdh1p delivery as a vaccine against candidiasis than i.n. administration with CT.

In conclusion, our findings revealed for the first time that both s.c. and i.n. administration of Mdh1p appeared to induce protective immunity against *C. albicans*. We have narrowed our focus to a candidate antigen, Mdh1p, screened by two different proteome studies. This approach was thought to lead to finding a high vaccination effect. Convenient tools for further analysis of antigenic protein candidates generated by large-scale screening proteome studies are needed. For example, molecular display technology for microorganism cells such as the “arming yeast” (Shibasaki et al., 2009, 2010; Tamaru et al., 2006) would be a convenient tool to evaluate the efficacy of their antigenic properties against candidiasis.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES


