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Original article

Evaluation of recombinant MGL_1304 produced by *Pichia pastoris* for clinical application to sweat allergy

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M. globosa, Malassezia globosa; *E. coli*, *Escherichia coli*; *P. pastoris*, *Pichia pastoris*; AD, atopic dermatitis; ChU, cholinergic urticaria; HRT, histamine release test; TF, Trigger factor

ABSTRACT

Background: We previously identified MGL_1304 secreted by *Malassezia globosa* as a sweat antigen for patients with atopic dermatitis (AD) and cholinergic urticaria (ChU). However, purifying native MGL_1304 from human sweat or culture supernatant of *M. globosa* (sup-MGL_1304) is costly and time-consuming. Moreover, recombinant MGL_1304 expressed by using *Escherichia coli* (TF-rMGL_1304) needs a large chaperon protein and lacks the original glycosylation of yeasts. Thus, we generated a recombinant MGL_1304 by *Pichia pastoris* (P-rMGL_1304) and investigated its characteristic features.
Methods: Recombinant MGL_1304 proteins expressed by *E. coli* and *P. pastoris* were generated. Properties of these recombinants and native antigens were compared by western blot analysis, histamine release tests (HRT) of patients with AD and ChU, and β-hexosaminidase release tests with RBL-48 cells. P-rMGL_1304-specific IgE in sera of patients with AD were measured by sandwich ELISA.
Results: Western blot analysis revealed that IgE of patients with AD bound to all MGL_1304 recombinants and native antigens. The histamine releasing ability of P-rMGL_1304 was 100 times higher than that of TF-rMGL_1304, and was comparable to that of sup-MGL_1304. Degranulation rates of RBL-48 cells, sensitized with sera of patients with AD in response to the stimulation of P-rMGL_1304, were comparable to those of sup-MGL_1304, whereas those of TF-rMGL_1304 were relatively weak. The levels of P-rMGL_1304-specific IgE in sera of patients with AD were correlated with their disease severities.
Conclusions: P-rMGL_1304 has an antigenicity comparable to the native antigen, and is more useful than TF-rMGL_1304, especially in HRT and degranulation assay of RBL-48 cells.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with pruritus and characteristic distribution and morphology of skin lesions.[1,2] Sweat is one of the exacerbation factors of AD,[3,4] and reported as an antigen for type I allergy in 77% patients with AD.[5] Cholinergic urticaria (ChU) is a subtype of urticaria where small reddish wheals develop in response to sweating or the increase of body core temperature. We previously reported that the purified sweat antigen induces degranulation of basophils of patients with AD and those with ChU.[6] Type I antigenicity of the purified sweat antigen was endorsed by the sensitization of a human mast cell line with IgE purified from sera of patients with AD and neutralization of the histamine releasing activity of the purified sweat by sera of the patients.[5] We finally have identified MGL_1304 secreted by *Malassezia globosa* as a sweat antigen for patients with AD and ChU.[7,8] We also reported that levels of MGL_1304-specific IgE in sera of patients with AD were significantly higher than those of healthy subjects and correlated with the severity of AD by ELISAs using purified MGL_1304 and recombinant MGL_1304 expressed by *Escherichia coli* (TF-rMGL_1304).[8] In Japan, a histamine release test (HRT) against sweat antigen, which mainly contains MGL_1304, has been commercially available since 2010 (Allerport® HRT, MANUFACTURER: Shionogi & Co., Ltd., Osaka, Japan, DISTRIBUTOR: Kyowa Medex Co., Ltd., Tokyo, Japan). However, purification of MGL_1304 from human sweat or culture supernatant of *M. globosa* is both time-consuming and costly. Alternatively, TF-rMGL_1304 can be generated at low cost. However, it needs a large chaperon protein to be solubilized and lacks the original glycosylation of yeasts. Thus, another recombinant protein, that is

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similar to the native protein in both glycosylation and conformational structure, is desirable for clinical applications. In this study, we generated recombinant MGL_1304 using a yeast cell line, *Pichia pastoris*, and studied its features and usefulness for clinical applications to patients with sweat allergy.

Methods

Subjects

One-hundred thirty five subjects were included in this study. Nineteen patients with AD and 11 patients with ChU (15 men and 15 women; mean age ± SD: 31.7 ± 16.4 years) were investigated by HRT, and 34 patients with AD (13 men and 21 women; mean age ± SD: 26.2 ± 13.0 years; mean serum IgE ± SD, 4236.8 ± 5472.6 IU/ml) were studied by β-hexosaminidase release of RBL-48 cells. Fifty seven patients with AD (32 men and 25 women; mean age ± SD: 29.8 ± 11.7 years) and 18 healthy individuals (9 men and 9 women; mean age ± SD: 26.6 ± 7.2 years) were subjected to the measurement of the P-rMGL_1304-specific IgE in sera by the sandwich ELISA. Blood samples from patients with AD and ChU were obtained following written informed consent, and the studies were approved by the Ethics Committee of Hiroshima University Institute of Biomedical & Health Sciences.

Generation of recombinant MGL_1304 protein

A recombinant MGL_1304 using *P. pastoris* (P-rMGL_1304) was generated with EasySelect™ *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, USA). The cDNA of MGL_1304 were subcloned into pPICZα-A *Pichia* expression vector and the plasmid DNA were prepared using One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen). *P. pastoris* (G-115 strain) were transformed using the *Pichia* EasyComp™ procedure. After confirming that the phenotype of the transformants was Mut^+, recombinant *Pichia* strains were cultured for 4 or 5 days. For the secreted recombinant protein (P-rMGL_1304), the culture supernatant was purified by batch method using nickel resin (ProBond™, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instruction. For the intracellular P-rMGL_1304, cell pellets was lysed by the disposable homogenizer (Treff pellet mixers®, Treff AG, Degersheim, Switzerland), and then purified with the same procedure as the secreted recombinant protein. A recombinant human mucin-like 1 (P-rMUCL1), which has not been shown histamine release ability to peripheral blood basophils of patients with AD, was generated as a negative control with the same procedures as P-rMGL_1304. Trigger factor (TF)-fused rMGL_1304 protein (TF-rMGL_1304) was prepared as described previously.[7]

Preparation of native antigen (sup-MGL_1304)

MGL_1304 in the culture supernatant of *M. globosa* (sup-MGL_1304) was isolated as described previously.[7]

Determination of protein concentration

The concentrations of P-rMGL_1304, P-rMUCL1, sup-MGL_1304, and TF-rMGL_1304 were measured by bicinchoninic acid (BCA) protein assay. The immunological amount of MGL_1304 contained in P-rMGL_1304 was determined by the sandwich ELISA as described previously,[8] with a slight modification. Briefly, 10 μg/ml of the mouse monoclonal IgG antibody against purified sweat antigen (Smith-2) was used as capture antibody, and 1:40 diluted the AD standard serum was used as detection antibody. The concentration of sup-MGL_1304 measured by BCA protein assay was used as standard.

Western blot analysis

Samples were loaded into an SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane as reported elsewhere.[7] The membranes were incubated with anti-Penta-His antibody (Qiagen, Hilden, Germany), 1:100 diluted sera of patients with AD or healthy individuals, or 1 μg/ml of Smith-2 at 4 °C overnight. The membrane-bound primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. All images were adjusted by using "auto levels" in Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Histamine release test with peripheral blood basophils of patients with AD or ChU

HRTs with peripheral blood basophils were performed as described previously.[9] Cells were stimulated with 1 μg/ml of goat anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX, USA) and various concentrations of TF-rMGL_1304, P-rMGL_1304, sup-MGL_1304, or P-rMUCL1.

Measurement of β-hexosaminidase release of RBL-48 cells

RBL-48 cells, a rat basophilic leukemia cell line expressing the alpha chain of human high-affinity IgE receptor on the cell surface, were provided by Dr John Hakimi (F. Hoffmann-La Roche, Nutley, NJ, USA).[10] RBL-48 cells (0.3×10^6/ml) were sensitized with 30 times-diluted sera of patients with AD at 37 °C overnight. After the incubation, culture medium was removed and cells were washed for three times with PIPES-buffered saline (25 mM PIPES: pH 7.2, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA) and incubated with the same buffer at 37 °C for 10 min. The cells were then stimulated with 1 μg/ml of TF-rMGL_1304 and various concentrations of P-rMGL_1304 or sup-MGL_1304 for 15 min. The exocytosis of cells was quantified by measuring the hexosaminidase activity of the supernatants as previously described.[11]

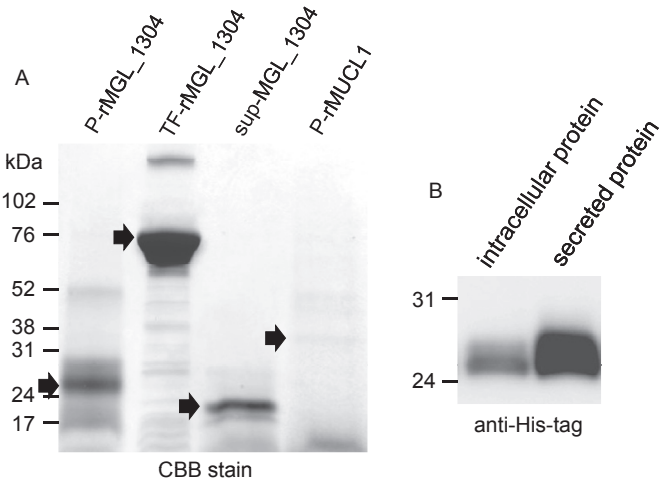

Fig. 1. (A) P-rMGL_1304, TF-rMGL_1304, supMGL_1304 and P-rMUCL1 were electrophoresed and stained with CBB. (B) The secreted and intracellular forms of P-rMGL_1304 were electrophoresed and blotted with anti-His-tag antibody. A representative data of three independent experiments was shown for each panel.