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An inhibitor peptide of toll-like receptor 2 shows therapeutic potential for allergic conjunctivitis

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ABSTRACT

Allergic conjunctivitis (AC) is an inflammatory disease of the conjunctiva, which is characterized by antigen challenge and toll-like receptor 2 (TLR2) activation. Here, a designed small peptide ZY12 was found to contain therapeutic potential in staphylococcal enterotoxin B (SEB)-induced AC model. ZY12 treatment showed the remission of clinical signs, plasma total IgE levels, number of mast cells and the proportion of degranulated mast cell in AC mice. Levels of Th2 cytokines (IL-4, IL-5, IL-13) in the lymph nodes or spleen were significantly decreased while those of Th1 cytokine (IFN-γ) were increased in ZY12 treated group, suggesting a protective role of ZY12 in AC by mediating the balance of Th1/Th2 cytokines. Importantly, ZY12 significantly inhibited TLR2 expression in conjunctival tissue. Combined its therapeutic effects with TLR2 inhibitory function, ZY12 might be an ideal candidate for the development of new therapeutic agent for allergic disease.

1. Introduction

Allergic conjunctivitis (AC) is a common form of ocular allergy, as it affects nearly one-third of the world population [1]. Due to the prevalence of AC, it is a heavy burden on society and a great challenge to the healthcare resources [2]. AC affects the ocular surface, including the eyelid, conjunctiva and/or cornea, leading to the signs and symptoms that most commonly accompany with eye redness and itching and affecting visual function [3]. These clinical symptoms, if not treated properly, impair patients' quality of life.

As an inflammation condition of the conjunctiva tissue, AC is triggered by IgE-mediated and/or non-IgE-mediated allergic responses to allergens interacting with TLR2 [4,5]. Furthermore, AC is mediated by Th2-type cytokines and the role of *Staphylococcus aureus* colonization in the pathophysiology of chronic allergic conjunctivitis has also been suggested [6,7]. Patients with atopic dermatitis frequently experience colonization with *S. aureus* that has been identified as a trigger factor [8–10]. The products of *S. aureus*, such as staphylococcal enterotoxin (SE), toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxin, are correlated with allergic diseases. Bunikowski et al. reported that 80% patients with AC had SEA- or SEB-specific IgE antibodies in the plasma [11]. Staphylococcal enterotoxin B (SEB) reportedly increases eosinophilic cell number, which is the hallmark of allergic disease [12]. Activation of TLR2 signal by *S. aureus* induced Th2-type immune responses and accelerated experimental allergic conjunctivitis [6], suggesting that antagonism of TLR2 signal is a target to treat AC. More than 20 small antimicrobial peptides (AMP) have been designed in our previous work [13]. Among them, only ZY12 was found to exert inhibitory effect on TLR2 expression. In this study, the small peptide ZY12 was found to exert therapeutic potential for allergic conjunctivitis by inhibiting TLR2 expression.

2. Materials and methods

2.1. Peptide design and synthesis

ZY12 (VKRWKWKWRKWKKWV-NH2) was synthesized by solid phase synthesis on an Applied Biosystems model 433A peptide synthesizer by GL Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by

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reversed phase high performance liquid chromatography (RP-HPLC) and mass spectrometry to confirm the purity >98%.

2.2. Assays of hemolysis and cytotoxicity

Hemolysis assay was performed using human red blood cells inliquid medium according to our previous reports [14,15]. Briefly, serial dilutions of ZY12 were incubated with human red cells at 37 °C. After incubation for 30 min, the cells were centrifuged and the supernatant was removed. The released hemoglobin was then measured at 540 nm using spectrophotometer. Maximum hemolysis was determined by substituting the sample with 1% Triton X-100, which completely lyses the cells instantaneously. Hemolysis of ZY12 was calculated as the percentage of that of Triton X-100.

Human HEK293T cell and mouse RAW264.7 cell (cultured in Dulbecco's modified Eagle's medium, DMEM, Gibco) were used to evaluate the cytotoxicity of ZY12 in vitro. Two hundred microliter of cell suspension (1×10^5 cells/ml) was seeded in a 96-well plate (Nunc, Wiesbaden, Germany) and then cultured for 12 h. Series dilutions of ZY12 were added to the wells and the cells were then cultured for 24 h. After treatment by testing sample, 20 μl of 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml) was added to each well. The MTT solution was then removed after a 4-h incubation and 200 μl of dimethyl sulfoxide (DMSO) was added to solubilize the MTT-formazan crystals in living cells. The absorbance was recorded at 570 nm by using the microplate spectrophotometer system (Epoch, BioTek, USA). The experiments were performed in triplicate.

2.3. Allergic conjunctivitis model and treatment protocols

Eight-week-old female Balb/c mice were purchased from the Laboratory Animal Center of Kunming Medical University. The animals were maintained in specific pathogen-free conditions on a 12-h light-dark cycle with controlled temperature (25 °C) and humidity (55%).

Mice were immunized by intraperitoneal injection of 1 μg of ovalbumin (OVA, Sigma) and 1.5% aluminium hydrocide adjuvant diluted in 200 μl of sterile saline twice a week according to the reported model with modifications [16,17]. Mice were then challenged with direct instillation of 250 ng of SEB (Sigma) in 2.5 μl of sterile saline or vehicle (sterile saline) three times daily from post-immunization days 15 to 18. The mice were divided into 6 groups ($n = 8$): (1) normal group (untreated); (2) vehicle group (SEB challenge); (3) positive group (eye drops with 10 μl of 0.1% olopatadine (Sigma) after SEB challenge); (4)–(6) ZY12-treated groups (eye drops with 2.5, 5 and 10 mg/kg ZY12 in 10 μl of sterile saline after SEB challenge, respectively). All treatments were with direct instillation and were started 24 h after the last SEB challenge. Treatment was performed twice a day (morning and night) from days 19 to 25. Twenty four hours after the last treatment was administered, mice were anaesthetized to obtain whole blood from the eyeball using a 1.5-ml tapered plastic centrifuge tube for analysis of total serum IgE and anti-OVA IgE levels. The animals were then euthanized and the eyes, cervical lymph nodes and spleen were harvested. All procedures were carried out in accordance with the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences (Permit Number: 33-2397).

2.4. Clinical score in AC

To assess the therapeutic efficacy of ZY12, mice were examined for the occurrence and severity of conjunctivitis before the mice were euthanized. Physical and slit lamp biomicroscopic ocular examinations were performed. Three clinical signs were observed, conjunctival hyperemia, conjunctival edema and tearing. According to the methods reported by Magone et al. [18], each symptom was scored on a scale of 0–3 (0 = absence, 1 = mild, 2 = moderate, and 3 = severe symptoms). The sum of the individual scores range from 0 to 9, and the data were expressed as the mean ± *SD* for each group.

2.5. Determination of total serum IgE and anti-OVA IgE levels

Clotted blood was centrifuged at 3000 g for 10 min to obtain the serum. Total IgE and anti-OVA IgE in the serum in different experimental groups were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits (Dakewei, Beijing, China) according to the manufacturer's instructions. Each determination was made in triplicate and the data were reported as mean ± *SD*.

2.6. Histopathological assessment

The eyeballs together with the conjunctiva tissue from each group were resected and fixed in 10% buffered formalin. Tissues were dehydrated, fixed, embedded in paraffin wax, sliced into 6 μm-thick section, and then the mast cells was stained with aldehyde fuchsin (Maixin. Bio, China). For histopathological assessment, six random sections in each slice were examined and the number of mast cells and degranulated mast cells was counted under a light microscope with a magnification of 400. The percentage of degranulated mast cells was calculated according to the following formula: (the number of degranulated mast cells / the number of mast cells) × 100%.

2.7. Analysis of Th1/Th2 cytokines in cervical lymph nodes and spleen

To demonstrate the effect of ZY12 on allergic responses in the AC model, the mice in each group were soaked for 3 to 5 min in 75% ethanol after sacrifice by cervical dislocation, and then the cervical lymph nodes and spleen were removed and washed three times using sterile saline. The cervical lymph nodes and spleen were pulverized using a rubber syringe plunger and the cell suspension was then obtained by pushing through a 200-mesh sterile trap valve. Subsequently, the cell suspension was centrifuged at 3000 rpm for 20 min at room temperature to discard supernatant. Resuspension was performed by applying 100 μl of sterile saline to the precipitated cells. After washing the cells again by centrifugation, the cells were ultimately re-suspended with RPMI-1640 medium (containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin, Gibco Life Technologies). The cells (5×10^6 cells/well in 1 ml) were then seeded in a 24-well plate and cultured at 37 °C in a humidified 5%-CO_2 atmosphere for 72 h. The supernatants were then collected and the cytokine levels in the supernatants were determined by using ELISA kits (Dekewei, Beijing, China) for IL-4, IL-10, IL-17and IFN-γ.

2.8. THP-1 cells culture and stimulation

Human monocytic THP-1 cells (1 × 106 cells/ml, Shanghai Caoyan Biotechnology Co. Ltd., China) were cultured in 6-well plates containing serum-free medium (RPMI 1640 medium, Gibco Life Technologies). For stimulation, THP-1 cells were pre-challenged with SEB (2 μg/ml) for 2 h, and then incubated with ZY12 (0–200 μg/ml) for 36 h to assess TLR2 expression.

2.9. Western blot analysis

Conjunctival tissue pellets from mice were treated for 10 min on ice with 50 μl of RIPA lysis buffer (1% Triton X-100 (*v*/*v*), 0.5% deoxycholic acid (*w*/*v*), 10 mM EDTA in PBS) containing protease inhibitor cocktail. After brief sonication, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatants were stored at −80 °C. Protein concentration was determined by Bradford assay. To detect TLR2 and mitogen-activated protein kinases (MAPKs), 40 μg of protein extracts were firstly separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene