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Proanthocyanidins from the bark of *Metasequoia glyptostroboides* ameliorate allergic contact dermatitis through directly inhibiting T cells activation and Th1/Th17 responses

Fengyang Chen [a], Xiaodi Ye [a], Yadong Yang [b], Tianli Teng [a], Xiaoyu Li [a], Shifang Xu [a], Yiping Ye [a,*]

[a] *Institute of Materia Medica, Zhejiang Academy of Medical Sciences, Hangzhou 310013, China*
[b] *Institute of Bioengineering, Zhejiang Academy of Medical Sciences, Hangzhou 310013, China*

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ABSTRACT

Objective: The leaves and bark of *Metasequoia glyptostroboides* are used as anti-microbic, analgesic and anti-inflammatory drug for dermatic diseases in Chinese folk medicine. However, the pharmacological effects and material basis responsible for the therapeutic use of this herb have not yet been well studied. The objectives of this study were to evaluate the anti-inflammatory effects of the proanthocyanidin fraction from the bark of *M. glyptostroboides* (MGEB) and to elucidate its immunological mechanisms.
Materials and methods: The anti-inflammatory activity of MGEB was evaluated using 2,4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD) in mice. Its potential mechanisms were further investigated by determining its effects on Con A-induced T cell activation and Th1/Th17 responses *in vitro*.
Results: Both intraperitoneal injection and oral administration of MGEB significantly reduced the ear swelling in DNFB-induced ACD mice. MGEB inhibited Con A-induced proliferation and the expression levels of cell surface molecules CD69 and CD25 of T cells *in vitro*. MGEB also significantly decreased the production of Th1/Th17 specific cytokines (IL-2, IFN-γ and IL-17) and down-regulated their mRNA expression levels in activated T-cells.
Conclusions: MGEB could ameliorate ACD, at least in part, through directly inhibiting T cells activation and Th1/Th17 responses.

Introduction

Allergic contact dermatitis (ACD), which is also known as contact hypersensitivity skin disease, is a T cell-mediated delayed-type hypersensitivity (DTH) response (Kim et al. 2013). ACD occurs in two phases: sensitization and elicitation phase. In the sensitization phase, the first contact of the skin with a hapten leads to binding of the hapten to an endogenous protein in the skin where they form immunogenic hapten–carrier complexes. The hapten–carrier complex is taken up by Langerhans cells and dermal dendritic cells, and transported from the epidermis to the draining lymph node. Here, they present the haptenated peptides to naive T cells which are subsequently activated. The newly activated T cells proliferate and migrate out of the lymph node and into circulation. In the elicitation phase, re-exposure of the skin to the hapten activates the specific T cells in the dermis and triggers the inflammatory process responsible for the cutaneous lesions (Scott et al. 2002). Classical ACD are generally thought to depend on T-helper (Th) 1 cells response that release pro-inflammatory cytokines, such as IFN-γ, and promote the killing of haptenized cells, resulting in the development of the inflammatory rash (Kaplan et al. 2012). Recently, Th17 cells response has also been described to be crucial in the development of ACD. Several studies with cells from human eczema lesions delivered experimental evidence for the presence of Th17 in ACD and isolated Th17 cells lines released significant amounts of IL-17 in response to chemical haptens. IL-17 deficient mice with reduced contact hypersensitivity reactions could be restored after transplantation of wild type $CD4^+$ T cells (Peiser 2013).

The leaves and bark of *Metasequoia glyptostroboides* Hu et Cheng belonging to Taxodiaceae family are traditionally used as anti-microbic, analgesic and anti-inflammatory drug for dermatic diseases in Chinese folk medicine. In our previous study, the proanthocyanidin enriched fraction from the bark of *Metasequoia glyptostroboides* (MGEB) was found to possess significant antioxidant activity and protective effect against CCl_4 induced acute liver injury (Chen et al. 2014). To elucidate whether proanthocyanidin is responsible for the anti-inflammatory effects of the bark of *M. glyptostroboides*, in this study, we evaluated the anti-inflammatory activities of MGEB

* Corresponding author. Tel.: +86 571 8821 5624; fax: +86 571 8821 5624
E-mail address: yeyiping2005@163.com (Y. Ye).

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on 2,4-dinitrofluorobenzene (DNFB)-induced mouse model of ACD, then further investigated its potential mechanisms by determining its inhibition on T cell activation and Th1/Th17 responses *in vitro*.

Materials and methods

MGEB preparation

The barks of *M. glyptostroboides* were collected in the Hangzhou Xiaoshan, Zhejiang Province, People's Republic of China, in May 2010, and were identified according to the Flora of China by one of the authors (Dr. Xiaoyu Li). A voucher specimen (No. 20100505) has been deposited at the Laboratory of Nature Drug, Institute of Materia Medica, Zhejiang Academy of Medical Sciences, China. MGEB was prepared as previously described (Chen et al. 2014). In detail, the pulverized bark of *M. glyptostroboides* (1.0 kg) was extracted three times with 60% ethanol under reflux for 2 h. The extract was filtered through Whatman filter paper. The filtrate was concentrated in a rotary evaporator under reduced pressure, and then lyophilized to obtain 60% ethanol extract (101 g). The extract (50 g) was then subjected to D101 resin column chromatography, and eluted subsequently with water, 30% and 50% ethanol. Finally, the eluates were concentrated and lyophilized to yield water (MGEA, 7 g), 30% (MGEB, 17 g) and 50% ethanol (MGEC, 2 g) fractions.

The total proanthocyanidin content of MGEB was determined by the *n*-butanol/HCl assay. The sample was dissolved in methanol and diluted appropriately. One millilitre of sample solution was added to 6 ml of a 95% solution of *n*-Butanol/HCl (95:5, v/v) in stoppered test tubes followed by 0.25 ml of a solution of $NH_4Fe(SO_4)_2$•$12H_2O$ in 2 M HCl. The tubes were incubated for 40 min at 95 °C. After cooled in the dark, the absorbance (*A*) was measured at 550 nm. Proanthocyanidin standard was used as a reference. The total proanthocyanidin contents of MGEB were 627.5 mg PE/g.

Ten proanthocyanidins including catechin, epicatechin, gallocatechin, epigallocatechin, catechin (4$\alpha\rightarrow$8) catechin, gallocatechin (4$\alpha\rightarrow$8) gallocatechin, gallocatechin (4$\alpha\rightarrow$8) epigallocatechin, gallocatechin (4$\alpha\rightarrow$8) catechin, catechin (4$\alpha\rightarrow$8) gallocatechin, gallocatechin (4$\alpha\rightarrow$8) epicatechin have been identified from MGEB.

Reagents

Concanavalin A (Con A), dexamethasone, 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); DNFB was purchased from BD Biosciences Pharmingen (CA, USA); Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Corp. (Hangzhou, Zhejiang, China). Hydrogen peroxide (H_2O_2) detection kit was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangshu, China). FITC-anti-CD3, PE-anti-CD4, PE-anti-CD8, PE-anti-CD69 and PE-anti-CD25 were purchased from eBioscience Inc. (San Diego, CA, USA); mouse cytokine (IL-2, IFN-γ and IL-17) detecting ELISA kits were from Wuhan Boster Biological Technology., Ltd. (Wuhan, Hubei, China); Trizol was purchased from Invitrogen (Carlsbad, CA, USA); PCR primers and other PCR reagents were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Experimental animals

Female BALB/c and ICR mice (6 weeks old) were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China) and acclimatized for 7 days before use. All the procedures were in strict accordance with the P.R. China legislation on the use and care of laboratory animals, and with the guidelines established by the Institute for Experimental Animals, and were approved by the Committee for Animal Experiments.

Induction and assessment of ACD

ICR mice were divided into four groups, each consisting of ten mice. Animals were initially sensitized with 50 μl of 1% DNFB dissolved in acetone/olive oil 1:1 on the shaved abdominal skin of recipients. Beginning on the day of immunization, mice were treated with MGEB at the doses of 25 mg/kg (i.g. or i.p.) or dexamethasone (Dex) at a dose of 3 mg/kg (i.g.) for 5 days once daily. The control groups received the same volume of saline. After 5 days, the ACD reaction was elicited by smearing 10 μl of 1% DNFB on both sides of the left ear. Twenty-four hours later, the ACD response to DNFB was evaluated by measuring the weight difference of right and left ear with an anal. Balance (Chen et al. 2012).

Splenocytes proliferation assay

Splenocytes prepared from BALB/c mice as previously described (Chen et al. 2012) were seeded into 4 wells of a 96-well flat-bottom plate at 5×10^6 cell/ml in 100 μl complete medium, thereafter Con A (3 μg/ml) or medium with MGEB were added giving a final volume of 200 μl. For investigating if MGEB's action was due to H_2O_2 production in the medium, the H_2O_2 scavenger catalase (1.25 μg/ml) was added in some cultures at 96-well plates. Dex (1 ng/ml) was used as the positive reference control. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. Cells were cultured for 48 h, and splenocytes proliferation was detected by MTT assay. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

H_2O_2 generation assay

In the presence or absence of catalase (1.25 μg/ml), MGEB and DMEM cell culture media were mixed and incubated at 37 °C in a humid atmosphere with 5% CO_2 for 24 h, H_2O_2 levels in culture medium were determined by H_2O_2 detection kit according to the manufacturer's instructions.

Cytotoxicity analysis

Splenocytes were seeded into 4 wells of a 96-well flat-bottom plate at 5×10^6 cell/ml in 100 μl complete medium, thereafter MGEB with or without catalase (1.25 μg/ml) were added giving a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. Cells were cultured for 48 h, and cell viability was detected by MTT assay.

Analysis of $CD3^+$ and $CD4^+$ T cells proliferation

Splenocytes were seeded into 24-well flat-bottom plate at 5×10^6 cell/ml in 1 ml complete medium, thereafter Con A (3 μg/ml) or medium with MGEB were added giving a final volume of 2 ml. The plates were incubated for 48 h. The cells were collected and stained with FITC-anti-CD3 plus PE-anti-CD4 or FITC-anti-CD3 plus PE-anti-CD8. After washed with PBS, samples were immediately detected by a FACScan flow cytometer.

Analysis of CD69 and CD25 cell surface expressions

Splenocytes were seeded into 24-well flat-bottom plate at 5×10^6 cell/ml in 1 ml complete medium, thereafter Con A (3 μg/ml) or medium with MGEB were added giving a final volume of 2 ml.