Microbial Pathogenesis 113 (2017) 365–371

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Polysaccharides from Citrus grandis L. Osbeck suppress inflammation and relieve chronic pharyngitis

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ARTICLE INFO

Keywords:
Polysaccharides
Citrus grandis L. Osbeck
Chronic pharyngitis
Anti-inflammatory
IKKs

ABSTRACT

Chronic pharyngitis, a common inflammation of the pharyngeal mucosa, is often caused by bacteria, viruses, alcohol abuse, overuse of the voice and cigarettes. This study aimed to explore the effects of polysaccharides of Citrus grandis L. Osbeck (PCG) in relieving chronic pharyngitis and illustrate the underlying mechanisms. Polysaccharides were obtained from PCG by column chromatographic extraction. Six clinical symptom scores, such as the severity of itchy throat, hoarseness, pain, odynophagia, cough and otalgia were evaluated in chronic pharyngitis patients after the oral intake of PCG. The effects of polysaccharides on chronic pharyngitis were investigated in ammonia-stimulated rabbits through pathology analysis. The levels of inflammatory markers in the peripheral blood T cells were analyzed by ELISA. The total and phosphorylated levels of ERK1/2, JNK and p38 were assessed by Western blot. Protein amount of IKKα and p65, IKKα/β activity and p65 activity were evaluated by Western blot and luciferase assay. The clinical studies presented that PCG significantly relieved the six symptoms of chronic pharyngitis patients. Pathology analysis of chronic pharyngitis animals showed that the PCG treatment groups showed a significant decrease in the number of pathologic cells and the reduction of pathologic cells was dose-dependent ($p < 0.01$). ELISA analysis showed that PCG significantly inhibited the αCD3-induced increase of IFN-γ, IL-2 and IL-4 expression in a dose-dependent manner. Moreover, Western blot and luciferase assay suggested that the phosphorylation of IKKα/β in peripheral blood T cells was inhibited by the administration of PCG. These results indicate that polysaccharides exhibit anti-inflammatory effects by inhibiting the phosphorylation of IKKs, subsequently suppressing the NF-κB pathway activation and decreasing the expression of inflammatory mediators.

1. Introduction

Chronic pharyngitis, one of the most common causes for visit to otorhinolaryngology physicians, can be caused by bacteria, viruses, alcohol abuse, overuse of the voice and cigarettes [1,2]. Chronic pharyngitis, a common inflammation of the pharyngeal mucosa, may last for at least one year and adversely affects the quality of the lives of patients. The clinical manifestations of chronic pharyngitis contain itchy throat, hoarseness, pain, odynophagia, cough and otalgia [3]. According to the epidemiological data, at least 20% of the adult population suffers from chronic pharyngitis in the world [4]. Among various therapeutic strategies for chronic pharyngitis, anti-inflammatory medications are commonly taken to reduce throat swelling, ameliorate pathophysiological processes and improve patient comfort [1].

Naturally occurring polysaccharides, high molecular-weight compounds consisting of long chains of monosaccharide units, have been reported to possess potential anti-inflammatory effects. For example, momordica charantia polysaccharides suppress inflammation during myocardial infarction by inhibiting the NF-κB pathway [5]. Moreover, the anti-inflammatory activities of polysaccharides from plants, such as smilax glabra, ulva lactuca and astragalus, may be used by inhibiting the release of inflammatory mediators [6–8].

Citrus grandis L. Osbeck belongs to the family Rutaceae and is native to Southeast Asia and China. Citrus grandis L. Osbeck, which is originally recorded for the Chinese medicinal use in the Tang Dynasty (659 A.D.), is widely used in traditional Chinese medicine and health foods [9]. With polysaccharide component, Citrus grandis L. Osbeck

Abbreviations: PCG, polysaccharides of Citrus grandis L. Osbeck

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https://doi.org/10.1016/j.micpath.2017.11.018
Received 17 October 2017; Received in revised form 10 November 2017; Accepted 10 November 2017
Available online 13 November 2017

possesses anti-inflammatory activity, removes phlegm, promotes digestion and has been widely used for the treatment of cough [10]. However, few reports are found regarding the anti-inflammatory activity of the polysaccharides from Citrus grandis L. Osbeck (PCG).

The aim of present study was to examine the effects of PCG on chronic pharyngitis and explore the underlying mechanisms. Therefore, we extracted polysaccharides from Citrus grandis L. Osbeck by column chromatography in this report. Six clinical symptom scores, such as the severity of itchy throat, hoarseness, pain, odynophagia, cough and otalgia, were evaluated in chronic pharyngitis patients after the oral intake of PCG. The clinical studies showed that PCG significantly relieved the symptoms of chronic pharyngitis patients. In order to reconfirm the role of PCG on chronic pharyngitis and investigate the possible mechanisms, the ammonia induced rabbit model was established to mimic the clinical pathology of chronic pharyngitis. Pathology analysis showed that there were lots of inflammatory cells in the pharyngeal tissue following ammonia stimulation and the number of lymphocytes increased dramatically. Compared with the vehicle group, the PCG treatment groups showed a significant decrease in the number of pathologic cells and the reduction of pathologic cells was dose-dependent. The results of ELISA, Western blot and luciferase assay showed that the administration of PCG strikingly down-regulated the expression of inflammatory markers (IFN-γ, IL-2 and IL-4) and inhibited the IKKα/β activity.

2. Methods & materials

2.1. Extraction of polysaccharides from citrus grandis L. Osbeck

Dried Citrus grandis L. Osbeck was purchased from Huazhou Native Pomelao Products Company (Huazhou, China). Column chromatographic extraction with gradient elution followed by automatic separation of polysaccharides was established as previously described [10]. 0.5 g dried Citrus grandis L. Osbeck was added into 5 ml extraction solvent (ethanol: pure water = 6:4) and incubated in a 30 °C water bath for 1 h with shaking once every 10 min by hands, and then centrifuged for 10 min at 5000 g. The above solution was used for determination of target substances. Extraction of polysaccharides was conducted in glass chromatographic columns. Citrus grandis L. Osbeck (5 g unless for scale up experiments) was put into a column (H/D = 10:1) by the wet column preparation method, with a minimum volume (MV) of solvent. MV, which was used as a basic volume unit in extraction, indicates the minimum volume of solvent for Citrus grandis L. Osbeck to be completely absorbed (a MV = mL solvent/g material) without excess solvent. The polysaccharides reached dynamic equilibrium in solution after 2 h. The same solvent was used to rinse the column at a flow rate of 1 MV/h, and the elutes were subsequently collected in fractions. The extraction mixture was adjusted to 80% ethanol and kept at 4 °C overnight. The polysaccharides were harvested by centrifuged for 20 min at 5000 g and washed twice by 80% ethanol.

2.2. Plasmid construction

The rabbit IL-2 promoter was amplified from rabbit genomic DNA and inserted into the luciferase reporter plasmid. The rabbit IKKβ DNA and p65 DNA were amplified from rabbit genomic DNA and inserted into the pSG5-basic plasmid, respectively.

2.3. Transfection and luciferase reporter assay

Transfection and luciferase reporter assay were performed as previously described [11], with some minor adjustment. The HEK293 cells were plated at 2×10^5 cells per well in 24-well plates. Cells were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Briefly, cells were transfected with either a pSG5 empty control plasmid or IKKβ expression vector or p65 expression vector and with an IL-2 promoter luciferase reporter plasmid. In this study, 1 μg pRL-TK promoter Renilla luciferase reporter plasmid was added to each transfection for normalization of transfection efficiency. At 34 h post-transfection, cells were incubated with different concentrations of PCG (0, 10, 20 μg/ml) for another 2 h. The HEK293 cells were harvested and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activities were normalized by dividing the measured firefly luciferase activity by the measured renilla luciferase activity. All transfections were performed in at least 3 independent experiments.

2.4. Clinical studies

Clinical studies were performed as previously described, using a double-blinded method [4]. 14 chronic pharyngitis outpatients, who met all the initial selected criteria, were enrolled in this study. All patients should attend the baseline visit (day 0) and day 7 and 14 after orally taking 10 mg PCG, 3 times/day for each patient. The physical examination was carried out by direct observation and by using a fiberscope to evaluate the state of mucosa. Each symptom was linked with an ordinal scale of intensity at specified time points: 0, absent; 1, low intensity; 2, intense; 3, very intense. Clinical symptoms, such as itchy throat, hoarseness, pain, odynophagia, cough and otalgia had been assessed. The clinical study protocol was carried out in accordance with World Medical Association Declaration of Helsinki, 2004 and approved by the Ethics Committee of Yantai Yuhuangding Hospital. Written content was acquired from the participants.

2.5. The ammonia-induced rabbit chronic pharyngitis model

The rabbit models with chronic pharyngitis were established as previously described [1]. New Zealand white rabbits weighting 2.5 ± 0.3 kg were purchased from the Experimental Animal Center of Shandong Luye Pharmaceutical Co., Ltd. Rabbits were sprayed with 2.5% ammonia water into the pharynx mucosal 2 times daily (600 μl total), for a period of 15 days. On the eighth day, 0.5 ml turpentine oil was injected into the rabbit pharynx mucosa. The animals were randomly assigned into 4 groups: Sham, CP + Veh, CP + PCG (1 mg/kg) and CP + PCG (2 mg/kg) (n = 5 per group). Each treatment group received treatment of vehicle or PCG (1 or 2 mg/kg) sprayed into the pharynx mucosa 4 times a day for a period of 14 days, respectively. Rabbits were anesthetized with urethane (1 g/kg) at 24 h after the last administration and the pharyngeal tissue was removed. All the tissues were fixed in 4% formalin, sliced into 4 μm-thick sections, and stained with hematoxylin and eosin. All cases were examined under a light microscope blindly by three independent researchers (Olympus Co., Ltd., Beijing, China).

This study was performed in accordance with the standard guidelines for the Care and Use of Laboratory Animals from Yantai Yuhuangding Hospital and was approved by the Ethics Committee of Yantai Yuhuangding Hospital. All the rabbits were housed in cages at 22–24 °C and 20% humidity with a 12 h light/dark cycle and food and tap water were provided ad libitum throughout the study.

2.6. Measurement of the levels of cytokines in rabbit peripheral blood T cells

2×10^5 CD4 T lymphocytes in the peripheral blood were sorted by flow cytometry. The $CD4^+$ T lymphocytes were exposed to αCD3 (1 μg/ml) and different PCG (0 μg/mL, 10 μg/mL, 20 μg/mL) as indication. After 24 h of incubation, the T cell supernatant was collected. The levels of IL-2, IL-4, IL-17 and IFN-γ were measured by ELISA kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instruction. Finally, the absorbance at 570 nm (A570) was measured with a multifunction microplate reader, and the cytokine concentrations were calculated from the standard curve.