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An orally administrated nucleotide-delivery vehicle targeting colonic macrophages for the treatment of inflammatory bowel disease

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ABSTRACT

Tumor necrosis factor-alpha (TNF-α) plays a central role in the pathogenesis of inflammatory bowel disease (IBD). Anti-TNF-α therapies have shown protective effects against colitis, but an efficient tool for target suppression of its secretion - ideally via oral administration - remains in urgent demand. In the colon tissue, TNF-α is mainly secreted by the colonic macrophages. Here, we report an orally-administrated microspheric vehicle that can target the colonic macrophages and suppress the local expression of TNF-α for IBD treatment. This vehicle is formed by cationic konjac glucomannan (cKGM), phytagel and an antisense oligonucleotide against TNF-α. It was given to dextran sodium sulfate (DSS) colitic mice via gastric perfusion. The unique swelling properties of cKGM enabled the spontaneous release of cKGM& antisense nucleotide (ASO) nano-complex from the phytagel scaffold into the colon lumen, where the ASO was transferred into colonic macrophages via receptor-mediated phagocytosis. The treatment significantly decreased the local level of TNF-α and alleviated the symptoms of colitis in the mice. In summary, our study demonstrates a convenient, orally-administrated drug delivery system that effectively targets colonic macrophages for suppression of TNF-α expression. It may represent a promising therapeutic approach in the treatment of IBD.

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1. Introduction

Inflammatory bowel disease (IBD) is a group of chronic disorders within the gastrointestinal tract, caused by dysregulated immune responses [1]. Notably, tumor necrosis factor α (TNF-α), a versatile inflammatory cytokine involved in many physiological processes, plays a central role in the pathogenesis of IBD. In the colon tissue, TNF-α is mainly produced by colonic macrophages [2]. During the colitic process, the high level of TNF-α promotes the production of other pro-inflammatory cytokines, increases the leukocytes migration via stimulating the expression of adhesion molecules by endothelial cells and induces the formation of granuloma [3].

Current therapeutic strategies, aimed at blocking TNF-α for the treatment of IBD, have shown great promises in cell models but achieved limited clinical success [4]. A notable example is infliximab, a chimeric monoclonal antibody targeting TNF-α, which has exhibited satisfactory performances in alleviating IBD in clinical trials [5]. However, in systemically, non-selectively blocking TNF-α, this drug also brought about obvious side effects, such as causing immunodeficiency-related infections and generating antibodies against the drugs [6,7]. Therefore, anti-TNF-α therapies have proven effective, but they need to be limited at the specific site of inflammation.

To address this unmet medical need, we aimed to develop an orally-administrated vehicle for targeted delivery of anti-TNF-α nucleotides to colonic macrophages. The microspheric vehicle comprises three components - i) cationic konjac glucomannan (cKGM), ii) phytagel, and iii) an antisense nucleotide (ASO) specifically against TNF-α. cKGM can provide three functions. First, it is cationized to conjugate the negatively charged ASO to form the cKGM&ASO nano-complex core. Second, it has high mannose moieties that can be recognized by mannose receptors (MR) that are abundant on the macrophages [8]. The interaction between MR

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and cKGM can mediate the phagocytosis of the nano-complex core into macrophages. Third, cKGM has uniquely strong water-absorption and swells almost indefinitely [9]. In contrast, phytagel swells poorly. By mixing cKGM and phytagel, we aimed to fabricate a microspheric system that could expectedly collapse in the colon lumen, because of the contrast swelling properties of the two materials. Consequently, the core cKGM&ASO complex can be released and internalized into macrophages in the local tissue. In the present study, we fabricated this microspheric system, tested its drug release and transfection efficiency, and evaluated its therapeutic performance in a dextran sodium sulfate (DSS) induced murine colitis model. Our design offers a new, orally-administrative approach for targeted gene delivery to colonic macrophages, which may open up new avenues for development of patient-friendly gene therapy strategies for the treatment of IBD.

2. Materials and methods

2.1. Synthesis of materials and reagents

KGM was obtained from Megazyme (Wicklow, Ireland). cKGM was prepared by incorporating ethylenediamine within the hydroxyl groups of KGM by an N, N'-carbonyldiimidazole (CDI) activation method with different ratios between KGM and CDI, as previously report [10]. The cationic degrees were determined by element analysis of nitrogen (CHN—O-Rapid, Hanau, Germany). Besides this, KGM and cKGM were characterized by fourier transform infrared spectroscopy (FT-IR, Nicolet, 170SX, Thermal Fisher Scientific, USA) and NMR spectrometer (Bruker AVANCE DRX-500, Bruker Corporation, USA) to obtain the structure information. Phytagel, lipopolysaccharide (LPS), mannanase and cellulase were purchased from Sigma (St. Louis, MO). Other chemical reagents were purchased from Sangon Biotech (Shanghai, China).

The phosphorothioate-modified antisense oligonucleotide against TNF-α, ISIS25302 was synthesized by Life Technologies (La Jolla, CA, USA). The sequence of ISIS25302 is: 5′-AACCCATCGGCTGGCACCAC-3'. The ASO sequences have been proven to be effective in our previous study [11]. As a scrambled control oligonucleotide (SCO), ISIS 18154 (5′-TCAAGCAGTGCCACCGATCC-3′) was used. Alexa 546-labeled ASO (Alexa 546-ASO) was synthesized for *in vitro* transfection and *in vivo* cell localization and tissue distribution.

2.2. Preparation and characterization of cKGM&ASO complexes and the gel retardation assay

cKGM&ASO complexes were prepared by mixing an aqueous solution of cKGM with ASO according to the indicated weight ratio. Briefly, different weights of cKGM were dissolved in 500 μl saline, mixed with 500 μl saline containing 1000 μg ASO and incubated for 60 min at room temperature to obtain different complexes of cKGM and ASO. The diameters and zeta potential of cKGM&ASO complexes were examined by a 90 Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY, USA). Different complexes were prepared in saline at different N/P ratios. Transmission electron microscope (TEM, JEM-200CX, JEOL, Tokyo, Japan) was applied to observe the morphology of the complexes.

To examine whether the binding ability of cKGM to ASO, cKGM with different cationization degree was complexed with ASO. After incubating at room temperature, the cKGM/ASO complex solution of 10 μl was added to 2 μl of loading buffer and applied to a 3% agarose gel in a Trisborate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.3) containing 0.1 mg/ml ethidium bromide. The electrophoretic evaluation of the complex was carried out in TBE solution at 100 V for 30 min. The gel was imaged with a UV transilluminator (Gel Doc, 2000, BioRad Laboratories, Hercules, CA, USA). To examine whether the enzyme from gut flora degraded the cKGM&ASO complex, 1 mg/ml nano-complex ($M_{cKGM}/M_{ASO} = 5$) was incubated in 100 ml 0.05 M sodium citrate-hydrochloric acid buffer solution (pH = 5.0) containing 6 U/ml mannanase and 5 U/ml cellulase for 24 h at 37 °C. Then, the digested cKGM&ASO nano-complex was also performed with gel retardation assay. Moreover, 1 mg/ml cKGM or KGM was also digested by mannanase and cellulase, the total reducing sugar degraded by enzymes at indicated time points was examined by dinitrosalicylic acid colorimetric (DNS) protocol [12].

2.3. Fabrication cKGM/phytagel microsphere (KPM) containing ASO

The cKGM/phytagel microspheres were prepared using water-in-oil (W/O) emulsion method as previously described with minor modification [13]. Briefly, different ratios of phytagel, cKGM and ASO were completely dissolved in deionized water after being heated at 80 °C for 15 min. The hot polysaccharide solution (4 ml) containing 8 mg ASO was quickly added to pre-heated 90 °C peanut oil (50 ml) under stirring at 600 rpm for 30 min. Then the oil mixture was finally transferred into 200 ml amount of 1% $CaCl_2$ solution for 5 min to enhance the gel strength. The microspheres containing ASO or Alexa 546-ASO were collected by centrifugation and further washed with acetone before microscopic observation. The dried microspheres were obtained by gradient alcohol dehydration. The morphology of microsphere was observed by scanning electron microscope (SEM, S—3400N II, Hitachi, Tokyo, Japan). To evaluate the ASO loading efficiency, 100 mg dried KPM containing Alexa 546-ASO was pulverized by homogenizer, added to 100 ml 0.05 M sodium citrate-hydrochloric acid buffer solution (pH = 5.0) containing 60 U/ml mannanase and 50 U/ml cellulase and was incubated at 37 °C until a clear solution was obtained. The concentration of ASO was quantified by examining the fluorescence intensity at 573 nm.

2.4. In vitro drug release and swelling studies

In order to simulate the alimentary tract conditions, drug release and swelling studies were preformed in an orbital shaker (Thermo Scientific, Waltham, Massachusetts, USA) at 37 °C and 60 rpm. The different compositions of cKGM and phytagel formulated microspheres containing Alexa 546-ASO (dry weight: 100 mg) was first tested in simulated gastric fluid (NaCl/HCl buffer, pH 1.2) for the first 2 h. Then, the dissolution medium was changed to simulated intestinal fluid (K_2HPO4/NaOH buffer, pH 7.2) for 4 h and finally replaced by simulated colonic fluid (K_2HPO4/NaOH buffer, pH 6.8) for 18 h. The shape of KPM at different stages was also observed by Nikon microscope (TE-2000U, Tokyo, Japan) and its diameter was determined via Nis-element basic research software (Nikon). The dissolution medium was collected at indicated time points to quantify the released ASO by examining the fluorescence intensity at 573 nm. TEM was performed to study the morphology and size of contents released from KPM. The swelling index of KPM was calculated according to the following formula:

$$\text{Swelling index}(\%) = (Wt - Wi)/Wi * 100\%$$

where Wi is the weight of initial dried KPM and Wt is the weight of KPM at indicated time points which was removed the free water from its surface.

2.5. Cell culture and transfection experiments

Mouse leukemia monocytic macrophage cell line, Raw 264.7 cells and mouse colon cell line, CT-26 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 containing 10% FBS. The cell cultures were incubated in room air with 5% CO2 at 37°C and 95% humidity. Raw 264.7 cells and CT-26 cells were cultured in 24-well plates for the transfection experiment. Before transfection, complete medium was removed, and cells were rinsed once with PBS. The naked ASO, cKGM&ASO complex or microsphere released contents (MRC) containing 1 μg of ASO or Alexa 546-ASO was diluted with 0.3 ml medium and was used to refill the well. After incubation at 37 °C for 6 h, the medium containing the complex was removed. The cells were rinsed twice with PBS and refilled with medium. Transfection of the lipofectamine&ASO complex was performed as controls according to the manufacturer's protocol. The transferred cells were examined by Nikon confocal microscope (C2+, Nikon) and analyzed using Nis-element advanced research software (Nikon). To quantify the transfection efficiency, transferred cells were collected and analyzed via flow cytometer (BD Biosciences, San Jose, CA, USA). To further investigate the suppression effect of MRC on TNF-α production, macrophages transfected with naked ASO, naked ASO with simulated gastric fluid pre-treatment for 2 h &lipofectamine, cKGM&ASO complex, lipofectamine&ASO complex, MRC or cKGM/mismatch ASO complex were further stimulated by 100 ng/ml LPS for another 6 h. The supernatant was collected to determine TNF-α concentration by ELISA kit (R&D systems, Minneapolis, MN, USA).

2.6. Cell viability assay

Cytotoxicity of cKGM was examined using the cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan). Raw 264.7 cells and CT-26 cells were seeded in a 96-well plate at a number of 5000 cells/well and cultured overnight. Then the cells were incubated in 100 μl serum-free medium containing the set amount of cKGM or cKGM&ASO complex. The concentration of cKGM applied in this experiment is 20, 50, 100, 200, 500 and 1000 mg/ml, respectively and 50 mg/ml PEI was used as a control of cKGM. cKGM&ASO complex ($M_{cKGM}/M_{ASO} = 5{:}1$) at the concentration of 20, 50, 100, 200, 500 and 1000 mg/ml (containing ASO of 4, 10, 20, 40, 100 and 200 mg/ml), respectively were also tested. PEI/ASO complex ($M_{PEI}/M_{ASO} = 10{:}1$) at the concentration of 50 mg/ml (containing ASO of 5 mg/ml) was used as the control. After 6 h treatment, the medium was removed and the cells were rinsed twice with PBS. The wells were refilled with complete medium and the cells were cultured for another 24 h. Next, 10 μl CCK-8 solution was added into the well and was further incubated for another 1 h at 37 °C. Absorbance at 450 nm was measured with a microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.7. Establishment of dextran sodium sulfate (DSS)-induced colitic model

Female C57/B6 mice of the same background were obtained from Laboratory Animal Center of Nanjing University (Nanjing, China). All animals received human care according to Chinese legal requirements. Acute DSS colitis was induced by addition of 5% (w/w) DSS (MW 36,000—50,000, MP Biomedicals, Santa Ana, CA, USA) in the drinking water. Calculate 5 ml DSS solution per mouse per day and DSS solutions were replaced every two days. Control mice received the same drinking

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