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Targeted delivery of chitosan nanoparticles to Peyer's patch using M cell-homing peptide selected by phage display technique

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ABSTRACT

This study is aimed to develop an efficient oral vaccine carrier which specifically targets the follicle-associated epithelium region of Peyer's patch (PP). M cell-homing peptide was selected by the phage display technique and its targeting efficiency was validated using chitosan nanoparticles (CNs) conjugated with the discovered peptide. A phage clone encoding CKSTHPLSC (CKS9) peptide sequence was selected by analysis of comparative superiority in transcytosis efficacy across the M cell layer *in vitro* and *in vivo* among the candidates. CKS9 was chemically conjugated to water-soluble chitosan (WSC) and the CKS9-immobilized chitosan nanoparticles (CKS9-CNs) were prepared by ionic gelation of CKS9-WSC with tripolyphosphate, yielding spherical nanoparticles around 226.2 ± 41.9 nm. The targeting ability of CKS9-CNs to the M cell and to the PP regions of rat small intestine was investigated by *in vitro* transcytosis assay and closed ileal loop assay, respectively, and was visualized by fluorescence-microscopy analysis. CKS9-CNs were transported more effectively across the M cell model and accumulated more specifically into PP regions in comparison with CNs, indicating that CKS9 peptide prominently enhanced the targeting and transcytosis ability of CNs to PP regions. These results suggest that the CKS9-CNs could be used as a new carrier for oral vaccine delivery.

1. Introduction

The intestinal epithelium is a physical barrier composed of tightly joined epithelial cells which prevent enteric environmental antigens from penetrating host tissue [1] On the other hand, there also exists a specialized cell type which can collect enteric antigens actively. The M cells, specifically located on the follicle-associated epithelium (FAE) of the Peyer's patch (PP) follicle, have a unique ability to uptake and deliver antigens from the enteric environment into the PP follicles via transcytosis. The Peyer's patch is referred as gut associated lymphoid tissues (GALT) in the immunological point of view because the antigens delivered through M cells consequently facilitate to induce antigen-specific immune response by activation of antigen-presenting cells and lymphocytes located in the PP follicles [2–4]. Therefore, targeting the M cells has been considered as one of the critical strategies for oral vaccination because the M cells play an important role in the mucosal immune system as a main portal for antigen-sampling in intestinal tract to protect the body from the invasion of pathogens [1,5,6].

Numerous studies have been performed to improve efficiency of oral vaccine delivery [7]. Encapsulation of the vaccine in nanoparticle system has been widely studied for oral vaccination because it offers many advantages such as non-invasive and patient-friendly method without sterile injection by qualified persons [6,8] and antigen-loaded nanoparticles induced mucosal IgA and systemic IgG antibody responses with a complete immune response [9,10]. Also, the nanoparticles can allow controlled release of the antigen and increase the duration of the contact between antigen and immune cells. Chitosan has already been used in the form of nanoparticles and nanocapsules for the transmucosal delivery of peptide drug across different mucosal surface [11–14] due to its biocompatibility, biodegradability, low toxicity and cost, the ability to open intercellular tight junctions, and strong mucoadhesive properties [15,16]. It was also reported that chitosan

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nanoparticles might be taken up by M cells in the Peyer's patches [17,18]. However, the specific delivery of vaccine molecules to M cells is still a challenging problem because of lack of knowledge in the molecular and cellular mechanisms underlying physiology of M cells [6,19–21]. In this point of view, M cell-homing moieties, such as small peptide ligands, which target M cells have a great potential to be applied in development of an efficient oral vaccine delivery system because they could enhance specificity and accessibility of given antigen or vaccine molecules to the M cells which are the first gate for induction of mucosal immune system in intestinal tract.

Phage display technique, in which uses a set of genetically recombinant bacteriophages expressing randomized foreign peptide sequences on their surface, is a promising method to find interacting peptide ligands to certain targets without knowledge about their molecular properties [22]. *In vitro* and *in vivo* phage display applications have been successfully conducted by a large number of research groups to identify homing peptide ligands targeting various organs or tissues including the M cells [23,24]. We hypothesized that the identification of novel M cell-homing peptide by phage display screening, combined with chitosan nanoparticles, could enable novel design and preparation of efficient vaccine carriers, which could be delivered specifically to the GALT through the M cells.

In this study, we aimed to find a novel M cell-homing peptide ligand via a phage display screening combined with *in vitro* human M cell model [25] and to validate its Peyer's patch-targeting efficiency using the peptide-conjugated chitosan nanoparticles for development of an effective oral vaccine carrier. Here, we described *in vitro* screening procedures for the M cell-homing peptide identification and showed the specificity of the peptide ligand to *in vitro* M cell model and *in vivo* Peyer's patch. Also, we investigated whether covalent conjugation of M cell-homing peptide, CKSTHPLSC (CKS9), to the surface of chitosan nanoparticles is capable of enhancing the targeting ability to *in vitro* M cell and *in vivo* PP regions by *in vitro* transcytosis assays, closed ileal loop assays, and confocal laser scanning microscopy analyses. It can be expected that the chitosan nanoparticles immobilized with M cell-homing peptide would be an effective carrier for the oral vaccination due to its specific targeting and accumulating property to the GALT in Peyer's patch.

2. Materials and methods

2.1. Materials

The human colon carcinoma cell line, Caco-2 cell subclone 1 (CRL-2102) and the human Burkitt's lymphoma cell line, Raji B cell (CCL-86) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture media (DMEM and RPMI) were purchased from Hyclone (Logan, UT. USA). Fluorescein isothiocyanate (FITC)-conjugated latex beads, streptavidin-conjugated Alexa 488, and isothiocyanate-conjugated *Ulex Europaeus Agglutinin* I lectin (UEA-I lectin) were purchased from Molecular Probes (Eugene, OR, USA). Ph.D.-C7C™ Phage Display Peptide Library was purchased from New England BioLabs (Beverly, MD, USA). The synthetic peptides biotinylated on the carboxyl termini, CKS9 (CKSTHPLSC) and CSK9 (CSKSSDYQC), were chemically synthesized from Peptron (Daejeon, Korea). Sprague–Dawley (SD) rats were purchased from Samtako (Osan, Korea). Water-soluble chitosan (WSC, $M_W = 10$ kDa; degree of deacetylation = 91.8%) was kindly donated by Prof. Nah (Sunchon National University, Korea). Alexa Flour 488 carboxylic acid succinimidyl ester and ProLong® gold antifade reagent were purchased from Invitrogen (Carlsbad, CA, USA). Sodium tripolyphosphate (TPP), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carboimide hydrochloride (EDC), and other chemicals were purchased from Sigma–Aldrich Co. (St. Luois, MO, USA).

2.2. Preparation and validation of in vitro M cell model

Construction of *in vitro* human M cell model was achieved by co-cultivation of Caco-2 cell subclone 1 (CRL-2102) and Raji B cell (CCL-86) in Transwell system (Corning Costar, NY, USA) with a procedure previously described by Gullberg et al. [25]. Briefly, 5.0×10^5 of Caco-2 cells suspended in DMEM were seeded on the upper side of Transwell polycarbonate filter inserts (pore diameter of 5.0 μm), and cultivated for 14 days to form confluent monolayers. Then, 5.0×10^5 of Raji cells suspended in RPMI:DMEM (v:v, 1:2) were added to the lower chamber, and the coculture was maintained for 4 days. Fresh medium was given and transepithelial electrical resistance (TER) was monitored using epithelial tissue voltohmmeter (EVOM, World Precision Instruments, Inc., Florida, USA) in every 24 h during cell culture.

For validation of *in vitro* M cell model construction, molecule-transport property and morphological change of Caco-2/Raji coculture were compared with Caco-2 monolayer culture by latex bead-transport assay and transmission electron microscopy (TEM), respectively. In the latex bead-transport assay, 1.6×10^{10} of FITC-conjugated latex beads (200 nm diameter) were added in the upper side of Transwell inserts of each cell culture layer and incubated at 37 °C and 4 °C alternately with 90 min of intervals for 240 min. During the incubation, the media samples were collected from the lower chambers in every 30 min for 4 h and the FITC-beads signals that had transported through the each cell layer were monitored using FACSCalibur with CellQuest software (BD Bioscience, San Diego, CA, USA). All flow-cytometric data were analyzed with Flow Jo software (Tree Star, San Carlos, CA, USA). TEM images were obtained from the vertical microsection of each cell layer using transmission electron microscope (JEOL, Tokyo, Japan).

2.3. Phage display screening using in vitro M cell model

Ph.D.-C7C™ Phage Display Peptide Library, which displays cyclic 9-mer peptides of 7 random amino acids and 2 flanking cysteines on the N-terminus of the pIII protein of M13 bacteriophage, was applied to screen M cell-homing peptides. 1.0×10^{11} pfu (approximately 100 copies for each peptide-encoding recombinant phage) of the Ph.D.-C7C library or wild type M13 phage was added into the upper side of Transwell inserts of Caco-2/Raji coculture (*in vitro* M cell model) and incubated for 30 min at 37 °C. Then, the media samples were collected from the lower chambers and the phage population transported through the cell layer was quantified by phage-plaque assay. The rest of media samples containing phage were amplified by infection into *Escherichia coli* ER 2738 for the next round of biopanning. The second round of biopanning was conducted by addition of newly amplified phages (1.0×10^{11} pfu) into the new cell layer preparation and the procedure was repeated as described above. After the third round of biopanning, individual phage-plaques were randomly selected from the lower chamber-media samples and were amplified separately. The peptide-encoding nucleotide sequence extracted from each individual phage recombinant was determined using ABI 3700, an automatic fluorescent sequencing system (PerkinElmer and Applied Biosystem, USA), with − 96gIII primer included in the Ph.D.-C7C phage library kit, then translated to peptide sequence. The M cell-homing peptide candidate was selected from the third round-phage population by analysis of its comparative superiority in transcytosis efficacy to the *in vitro* M cell model.

2.4. Targeting property-validation of M cell-homing peptide

To validate targeting property of the M cell-homing peptide candidate to the Peyer's patch, the closed ileal loop assay was conducted and assessed by quantification of the peptide-encoding phage titers specifically associated with PP region and fluorescence-microscopy analysis as below. For the quantification method, overnight-starved SD rats (12-week-old, male; $n = 4$) were incised abdominally under deep anesthesia (ketamine hydrochloride, 80 mg/kg bw; xylazine, 10 mg/kg bw) and the mid-ileal region of small intestine was tied at both ends with cotton threads in 3 cm length containing one or two Peyer's patches (a closed ileal loop) [24]. Then, 1.0×10^{11} pfu of homogeneously amplified peptide-encoding phage or wild type M13 phage suspended in 300 μl of PBS was injected into the closed ileal loops. After 30 min of retention, the closed ileal loops were excised and separated by PP (Peyer's patch) and non-PP (intestinal epithelial tissues with normal villi). The tissue preparations were washed extensively with PBS to remove unbound or loosely bound phage particles, and then homogenized. The phages specifically bound to or internalized into the tissues were quantified from the homogenized tissue samples by phage-plaque assay. For the fluorescence-microscopy analysis, after SD rats were incised and closed ileal loops formed, 0.1 μg of biotinylated synthetic peptide ligands (CKS9 or CSK9) were injected into the closed ileal loop. After 30 min, the tissue samples were isolated, fixed by 4% paraformaldehyde, and frozen-sectioned. The PP and non-PP tissue sections were visualized by streptavidin-conjugated Alexa 488 for the biotinylated peptide ligands and by rhodamine-conjugated UEA-I lectin for the mucus layer under confocal laser scanning microscopy (Carl Zeiss, Germany).

2.5. Preparation of CKS9-conjugated chitosan

CKS9-immobilized water-soluble chitosan (CKS9-WSC) was prepared by conjugation of WSC with CKS9 using NHS/EDC coupling agents. We used protected CKS9 peptide with acetyl group at the N-terminal (Ac-CKS9) to prevent coupling among peptides. Briefly, Ac-CKS9 (88 mg) was dissolved in 1.5 ml distilled water and activated with NHS (50 mg) and EDC (83 mg) at room temperature for 2 h. The resultant solution was added into the WSC solution (WSC 79 mg was dissolved in 1.5 ml distilled water) and stirred at room temperature for 24 h. The reaction mixture was dialyzed for 3 days using Spectra/Por® membrane (MWCO = 2000) against distilled water and then freeze-dried. A synthetic scheme is shown in Fig. 1. The composition of CKS9-WSC was determined by ^{1}H NMR spectroscopy (Avance