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Identification of a peptide sequence that improves transport of macromolecules across the intestinal mucosal barrier targeting goblet cells

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Abstract

In this study, we demonstrated that the CSKSSDYQC-peptide ligand which was identified from a random phage-peptide library through an *in vivo* phage display technique with rats could prominently improve the transport efficiency of macromolecules, such as large filamentous phage particles (M13 bacteriophage), across the intestinal mucosal barrier. Synthetic CSKSSDYQC-peptide ligands significantly inhibited the binding of phage P1 encoding CSKSSDYQC-peptide ligands to the intestinal mucosal tissue and immunohistochemical analysis showed that the CSKSSDYQC-peptide ligands could be transported across the intestinal mucosal barrier via goblet cells as their specific gateway. Thus, we inferred that CSKSSDYQC-peptide ligand might have a specific receptor on the goblet cells and transported from intestinal lumen to systemic circulation by transcytosis mechanism. These results suggest that CSKSSDYQC-ligand could be a promising tool for development of an efficient oral delivery system for macromolecular therapeutics in the carrier-drug conjugate strategy.

Keywords: Phage display; Intestinal mucosa; Receptor-mediated endocytosis; Transcytosis; Oral drug delivery system; Goblet cell

1. Introduction

Oral administration is one of the most convenient and acceptable ways for internal use of therapeutic compounds. However, conventional administration of macromolecular therapeutics should generally rely on parenteral injection because their absorption in gastrointestinal tract is mostly obstructed by enzymatic degradation and physical barrier function of intestinal epithelia (Fasano, 1998; Daugherty and Mrsny, 1999).

Transcytosis, a branch of receptor-mediated endocytosis, is a process by which receptor-bound cargo, even large protein molecules, internalized at one plasma membrane domain of a polarized cell is transported to the opposite side of plasma membrane via vesicular intermediates (Okamoto, 1998). Thus, transcytosis has been considered as a physiological means for transporting macromolecular therapeutics across endothelial and epithelial cells (Ivanenkov and Menon, 2000; Russell-Jones, 2001). From this point of view, the 'carrier-drug conjugate' strategy was established using well-known transcytotic ligands such as immunoglobulin G (IgG), lactoferrin, transferrin, or folic acid, *etc*., as carriers for macromolecular therapeutics (Swaan, 1998). For an instance, the transferring–insulin conjugates which were administered orally into diabetes-induced mice showed 28% decrease of initial blood glucose level (Wang et al., 1997).

In this study, we performed a modified phage display method to find novel peptide ligands which could induce effective transcytosis on the intestinal epithelia. Duerr et al. (2004) hypothesized that the introduction of a random phage-peptide library into the intestine would facilitate the identification of sequences that could induce transcytosis on intestinal epithelia and reported several peptide sequences identified from rat

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spleen by *in vivo* phage display. Higgins et al. (2004) identified novel peptide ligands targeting M cells on Peyer's patches through the intra-intestinal administration of random phage-peptide library into rats. As a similar approach with previous studies, we introduced random phage-peptide library into rats by oral administration and identified a group of peptide ligands from the four representative reticular organs including liver, lung, spleen, and kidney, which were known to have high trapping-capacity for M13 bacteriophage in systemic circulation (Molenaar et al., 2002). Among the peptide ligands selected by the *in vivo* phage display, CSKSSDYQC-peptide ligand greatly improved the transport efficiency of M13 bacteriophage across the intestinal mucosal barrier. The peptide ligands selected by the approach in this study have a potential to be applied in development of efficient delivery system for macromolecular therapeutics via gastrointestinal route.

2. Materials and methods

2.1. In vivo phage display

For selection of phage recombinants that traverse intestinal mucosal barrier, *in vivo* phage display experiments were conducted. Ph.D.-C7C phage display library (New England BioLabs, Beverly, MD, USA) was used through out this study, which displays cyclic 9 mer-peptide (random 7 amino acids with 2 flanking cysteines at both ends of the peptide) at the N-terminus of the pIII protein of M13 bacteriophage. For the initiation of *in vivo* selection (the first round of biopanning), 1.2×10^{12} pfu (approximately 1000 copies for each peptide-coding phage recombinant) of Ph.D.-C7C library in 500 μl PBS was orally administered into overnight-starved, 12-week-old male Sprague–Dawley (SD) rats ($n = 4$) (Samtako, Osan, Korea) using a flexible oral zonde (2.0 mm × 110 mm). After 1 h-retention, rats were sacrificed by abdominal incision under deep anesthesia (ketamine hydrochloride, 80 mg/kg bw; xylazine, 10 mg/kg bw) and perfused via left ventricle with 120 ml of heparin-added DMEM (GIBCO, USA) to remove whole blood from the organs. Four representative organs (liver, lung, spleen, and kidney) were extracted and minced on the petri dish separately and washed three times with 30 ml of ice-cooled PBS. Each drained organ sample was weighed and homogenized in 2 ml of Tissue-Suspension Buffer [TSB: DMEM, 1% (w/v) BSA (Sigma, USA), 10% (v/v) protease inhibitor cocktail (Sigma, USA)]. The phage trapped in each organ was eluted by vigorous voltexing with 2 ml of 0.1 M glycine (pH 2.0). After centrifugation at 14,000 × *g* for 8 min, supernatants were neutralized with 60 μl of 2 M Tris base. Eluted phage from each organ tissue was quantified by plaque assay, then was mixed together with same volume and amplified by infection into *Escherichia coli* ER 2738 for the next round of biopanning. The second round of biopanning was conducted by oral administration of newly amplified phage (1.2×10^{12} pfu) into rats ($n = 4$) and the procedure was repeated as described above. After the third round of biopanning, individual phage plaques were randomly selected from each organ tissue eluate and were amplified separately to identify peptide sequences from their genomes. Overall procedure of *in vivo* phage display in this study was depicted in Fig. 1. All applicable national laws and Seoul National University policies regarding the care and use of laboratory animals were observed.

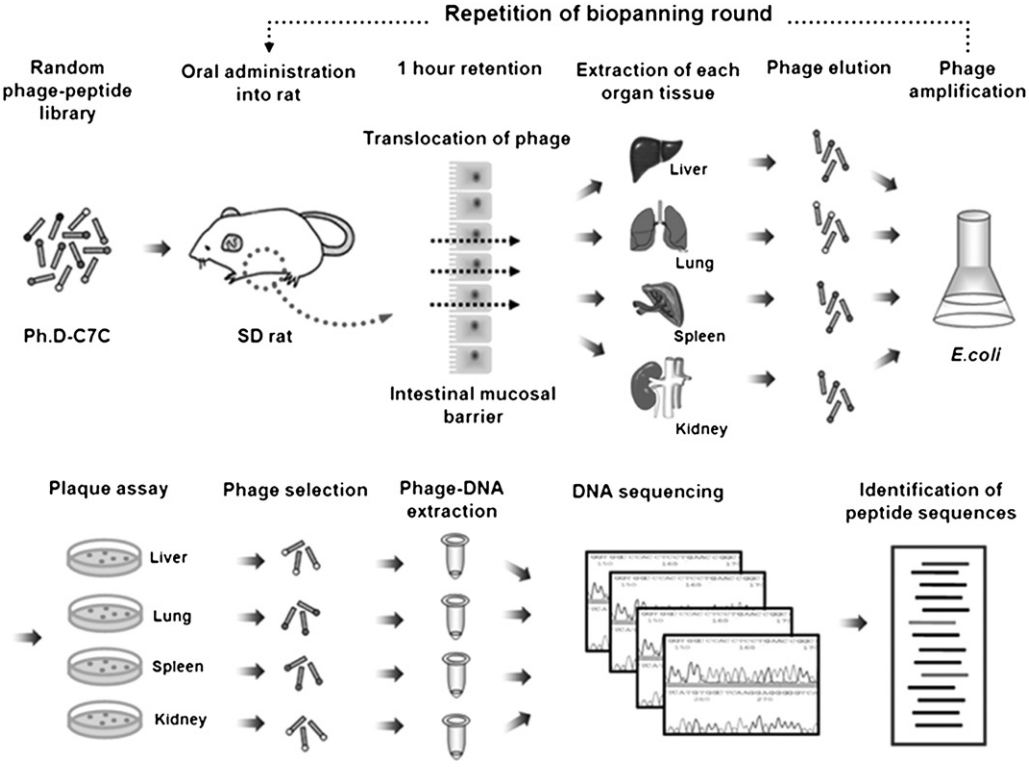

Fig. 1. A schematic diagram for *in vivo* phage display procedure by oral administration of random phage-peptide library into rats.

2.2. Sequencing of peptide-encoding DNA from selected phage recombinants

The peptide-encoding nucleotide sequences extracted from the third round individual phage recombinants were determined using ABI 3700, an automatic fluorescent sequencing system (PerkinElmer and Applied Biosystem, USA), with −96gIII primer included in Ph.D.-C7C phage display library (New England BioLabs, USA), then were translated to peptide sequences.

2.3. Competitive phage-binding analysis to small intestinal mucosal tissue

Rat small intestinal mucosal tissue (SIMT) was prepared as below. An overnight-starved adult SD rat (12 weeks old, male) was sacrificed, perfused, and small intestine (from duodenum to ileum) was extracted. SI was incised in longitudinal direction, dregs inside were washed out with PBS, and unfolded on the glass plate. SIMT was scraped out using sterile slide glasses, washed 3 times with 10 ml of DMEM, and resuspended with 20 ml of 1% BSA-added DMEM. The SIMT suspension was dispensed with 500 μl of aliquots, centrifuged at 14,000 × *g* for 2 min, drained, and weighed. All procedure above was conducted at 4 °C.

In vitro competitive analysis was performed to ascertain the binding property of selected phage encoding consensus peptide ligands or native M13 phage to SIMT with chemically synthesized peptide ligands (PEPTRON Inc., Daejoen, Korea). SIMT pellets were incubated with selected phage recombinants or native M13 phage (1.0×10^{10} pfu in 500 μl of 1% BSA-added DMEM, respectively) in presence (0.02 μg or 0.2 μg; >96% purity) or absence of the synthetic peptide ligands for 30 min at 4 °C. After the binding step, SIMT pellets were washed 5 times with 1 ml of DMEM containing 1% BSA and 0.1% Tween 20, drained, and resuspended with 500 μl of 1% BSA-added DMEM, then phage which bound to SIMT was eluted and neutralized by 0.1 M glycine (pH 2.0) and 2 M Tris base, respectively. The phage recovered from SIMT was quantified by plaque assay. All experiments were triplicated.

2.4. Validation of phage-transport efficiency across the intestinal mucosal barrier

Overnight-starved adult SD rats (12 weeks old, male; $n = 3$) were orally administered with 2.0×10^{11} pfu of consensus peptide ligand-encoding phage or native M13 phage which were suspended in 500 μl of PBS, respectively. After 1 h-retention, the rats were sacrificed, perfused, and phage titers were quantified from liver, lung, spleen, and kidney tissues. On the other hand, a modified procedure was performed to minimize the degradation of phage in gastrointestinal tract and in systemic circulation. Instead of oral administration, ligand-encoding phage or native M13 phage were injected into the distal side of ileum after abdominal incision of rats ($n = 3$) under anesthesia. After 30 min, liver and spleen were extracted and homogenized without blood perfusion. Samples were put on the ice until ready for use preventing cellular uptake or enzymatic degradation of phage. Trapped phage in liver and spleen, which transported from the intestinal lumen to each organ across the intestinal mucosal barrier, was eluted and quantified.

2.5. Immunohistochemistry

After 30 min from oral administration of ligand-encoding phage or native M13 phage (2.0×10^{11} pfu/of 500 μl of PBS, respectively), the rats (SD, 12 weeks old, male; $n = 3$) were sacrificed and perfused, then ileum region in small intestine was removed and fixed by 4% paraformaldehyde (PFA) for 2 h and immersed in 30% sucrose at 4 °C overnight. Samples were frozen quickly in liquid nitrogen-cooled OCT-compound (Optimal Cutting Temperature-compound, 'Tissue-Tek', Miles Laboratories Inc., USA). The small intestinal tissue-sections were prepared by Cryotome HM 505E (Microm, Germany) in 14 μm thickness and put onto the gelatin-coated slide glasses. The tissue-sections were blocked by 3% goat serum for 1 h, washed with PBS, and incubated with 50 ng/L of anti-M13 monoclonal antibody (Amersham Biosciences, USA) at 4 °C overnight. The samples were washed with PBS several times and treated by a fluorescein-5-isothiocyanate (FITC)-conjugated goat anti-mouse (Alexa 488, Molecular Probes, USA) secondary antibody or horseradish peroxidase (HRP)-linked anti-mouse antibody with DAB (diaminobenzidine) as a substrate (ABC kit, Santacruz, USA). Mucus droplets of goblet cells and mucus layer of intestinal epithelia were labeled by rhodamine-conjugated UEA-I lectin (Ulex Europaeus Agglutinin I lectin, Sigma, USA) and nuclei were stained by Hoechst dye (Sigma, USA). The tissue-sections were visualized by fluorescence microscope (Axiovert 40 CFL, Zeiss, Germany).

2.6. Statistical analysis

Results were expressed as the mean and S.D. (standard deviation). Significance testing (unpaired 2-tailed *t*-test) was performed using the PC analysis package, PRISM 3.0 software (GraphPad software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Selection of peptide ligands that facilitate molecule-transport across the intestinal mucosal barrier by in vivo phage display

At first, we determined 1 h for the optimal time point to recover translocated phage from inside organs after oral administration of random phage-peptide library into rats because the titers of translocated phage from library into liver showed its peak at 1 h after oral administration, then rapidly declined along the course of time (data not shown).

Three rounds of consecutive phage display biopanning were conducted to select peptide-ligands that could improve transport efficiency of macromolecules across the intestinal mucosal barrier (Fig. 2). After the third round of biopanning, total 850 peptide sequences were determined from randomly selected individual phage recombinants which were rescued

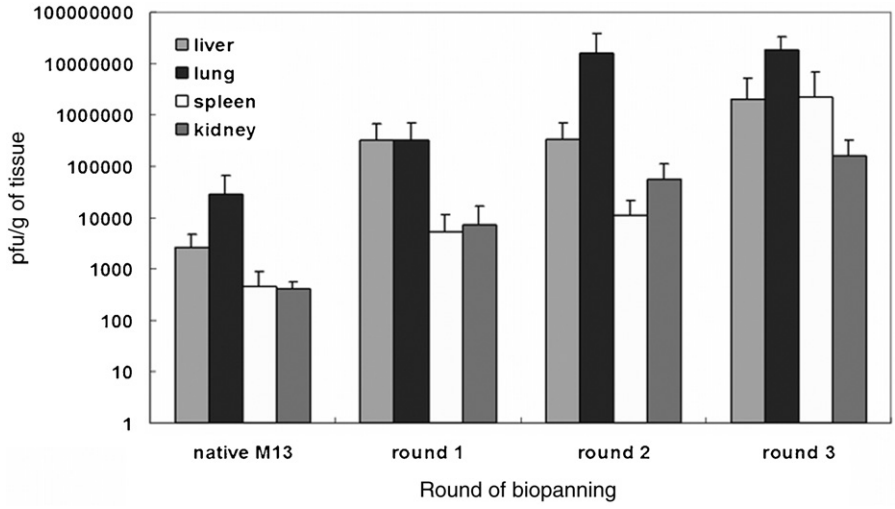

Fig. 2. Progressive enrichment of phage biodistribution in rat organ tissues (liver, lung, spleen, and kidney) along the rounds of biopanning.

from four representative organs, liver (from which 220 peptide sequences were identified), lung (218), spleen (204), and kidney (208). A multiple sequence-alignment analysis using 'Clustal X (version 1.81)' program (Thompson et al., 1997) revealed that the peptide sequences could be classified in various groups on the basis of their sequence similarity. Each group of peptide sequences shared consensus tri- or tetra-amino acid core motifs and some of identical peptide sequences appeared repeatedly in the alignment (data not shown). Such consensus amino acid motifs appearing in different sequence contexts or particular peptide sequences appearing with high frequencies were commonly observed in phage display experiments and considered as an important evidence of the phage display selection with the progressive enrichment in phage titers along the succeeding rounds of biopanning (Fig. 2) (Ivanenkov and Menon, 2000; Arap et al., 2002; Lee et al., 2002; Higgins et al., 2004; Arap, 2005). Thus, we inferred that the peptide sequences with high appearing frequency which identified by *in vivo* phage display in this study would have a potential property to traverse intestinal mucosal barrier more efficiently compared with unselected random peptide sequences.

3.2. Competitive binding analysis for CSKSSDYQC-peptide ligand to small intestinal mucosal tissue

In order to validate whether the consensus peptide ligands which selected from *in vivo* phage display would be related with high affinity to the small intestinal mucosal tissue, the affinity of peptide ligand-encoding phage to SIMT was compared with that of native M13 phage having no peptide insert. Among the selected phage recombinants, 'CSKSSDYQC' sequence-encoding phage (named as phage P1) showed a significantly higher affinity to SIMT compared with the native M13 phage (Fig. 3(a)). CSKSSDYQC was the peptide ligand which appeared 9 times (thrice in liver; twice in lung, spleen, and kidney) out of total 850 sequences selected by *in vivo* phage display.

For the next, *in vitro* competitive binding analysis for P1 or native M13 phage to SIMT was conducted with a chemically synthesized CSKSSDYQC-peptide ligand. If binding affinity of

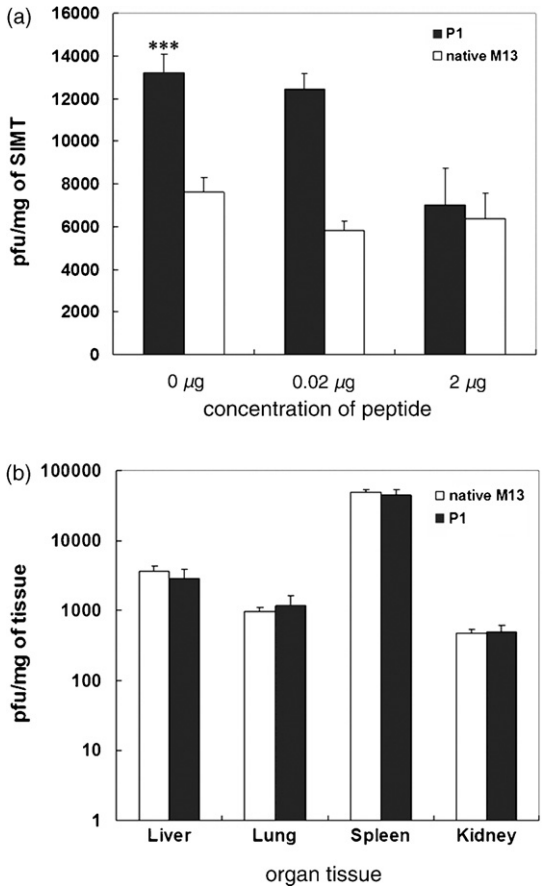

Fig. 3. *In vitro* phage-binding assays to rat organ tissues. (a) Competitive binding analysis of P1 and native M13 phage to small intestinal mucosal tissue in the presence or absence of synthetic CSKSSDYQC-peptide ligand. ***Tested group showed a statistical significance versus 2 μg of peptide by unpaired 2-tailed *t*-test ($P < 0.0001$). (b) Phage-binding assay to four representative organ tissues. No significance was observed between P1 and native M13 phage in binding affinity to each organ tissue.

the CSKSSDYQC-peptide ligand to intestinal epithelia were due to mediation by a specific membrane receptor, corresponding to the increase of synthetic CSKSSDYQC-ligand concentration, the accessibility of P1 to the receptor would be reduced by competition between P1 and synthetic ligand. Actually, the titers of P1 which bound to SIMT significantly decreased as concentration of synthetic CSKSSDYQC-ligand increased, however native M13 phage showed no response to increment of synthetic ligand concentration (Fig. 3(a)). It could be an indirect evidence that CSKSSDYQC-peptide ligand might have a specific receptor on the intestinal epithelia (Ivanenkov and Menon, 2000; Arap et al., 2002; Lee et al., 2002; Higgins et al., 2004).

We also investigated the binding affinity of P1 to four representative organ tissues (liver, lung, spleen, and kidney). Rat organ tissues were extracted after perfusion, homogenized, and drained, then P1 or native M13 phage (1.0×10^{10} pfu in 500 μl of 1% BSA-added DMEM, respectively) were added to the each organ tissue pellet, and recovered in a similar manner with the SIMT-binding assays ($n = 3$). In contrast to the SIMT-binding assays, P1 showed no significance in its binding capacity to all the organ tissues tested compared with native M13 phage (Fig. 3(b)), and it was consistent with the previous observation that P1 showed no specific organ-preference in its appearing frequency (P1 appeared three times in liver; twice in lung, spleen, and kidney).

Synthetically, the CSKSSDYQC-peptide ligand could be transported from intestinal lumen to systemic circulation via specific intestinal mucosal binding, and could access major organs without any particular organ-bias. This feature of the peptide ligand provides a potential advantage for development of efficient oral drug delivery system as a useful drug career.

3.3. *In vivo* validation of transport efficiency for phage P1 across the intestinal mucosal barrier

We investigated biodistribution of P1 after its oral administration into rats to validate the transport efficiency of the CSKSSDYQC-peptide ligand across the intestinal mucosal barrier. As expected, P1 showed significantly higher titers in all the organs tested compared with native M13 phage (Fig. 4(a)).

Molenaar et al. (2002), in their *in vivo* phage biodistribution report, demonstrated that the systemically injected M13 into mice rapidly lost its bioactivity with the lapse of time by cellular uptake and lysosomal degradation in liver, lung, and spleen, and we also monitored that the titers of translocated phage into rat organs after their oral administration rapidly declined along the course of time (data not shown). Thus, In spite of similar experimental approach, relatively lower scale of the translocated phage titers in study of Duerr et al. (2004) compared with this study was speculated due to the different time point of phage recovery between two studies (2 h after oral administration of phage in study of Duerr et al.; 1h after administration in this study). Accordingly, in order to prevent underestimation of the biodistribution result of P1, we adopted a modified method to economize the time from administration to rescue of the phage (see Section 2). Using the modified method, P1 showed approximately 1000-fold higher

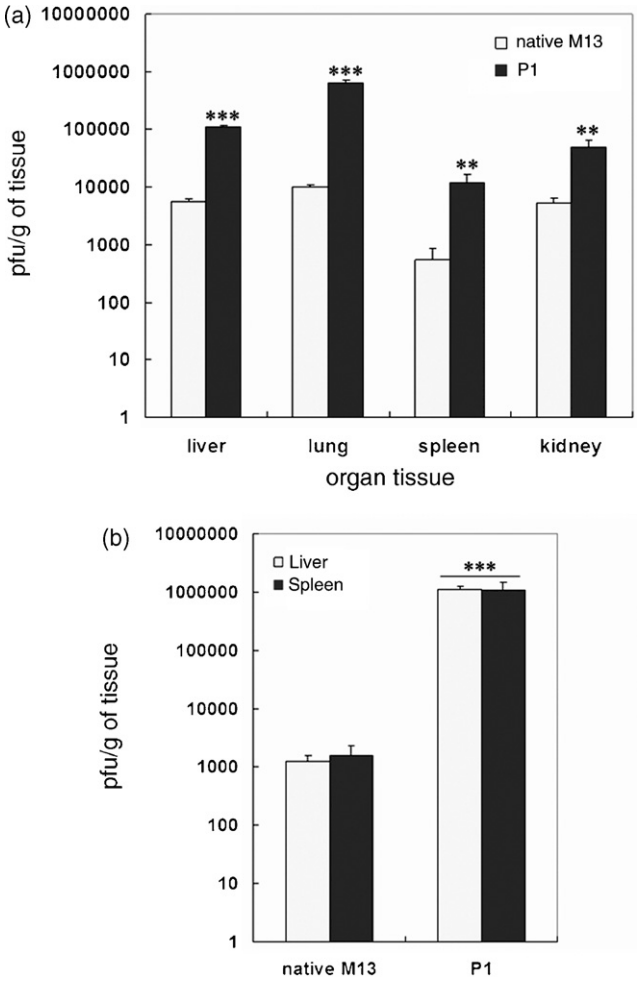

Fig. 4. Biodistribution of P1 and native M13 phage in rats. (a) Biodistribution of P1 and native M13 phage in four representative organs after 1 h post-oral administration of each phage into rats. ***, **Each tested group showed a statistical significance versus native M13 phage in each organ by unpaired 2-tailed *t*-test (***$P < 0.005$; **$P < 0.01$). (b) *In vivo* biodistribution of P1 and native M13 phage in liver and spleen after 30 min post-injection of each phage into ileum of rats. ***Each tested group showed a statistical significance versus native M13 phage titers in liver and spleen by unpaired 2-tailed *t*-test ($P < 0.001$).

titers compared with the native M13 phage in both liver and spleen (Fig. 4(b)). Because the only difference between P1 and native M13 phage is presence or absence of the peptide ligands (CSKSSDYQC) on their pIII coat proteins, the superiority of P1 to native M13 phage in transport efficiency across the intestinal mucosal barrier is obviously occurred by the peptide ligand.

3.4. Immunohistochemical analysis

Although intestinal epithelia have an exquisitely controlled barrier function to molecules in intestinal lumen, they also have a well-known nonspecific molecule-transporting gateway such like M cells, which associate with regulation of the mucosal immune response in gastrointestinal tract as an antigen-collecting portal (Neutra, 1998; Nagler-Anderson, 2001). Thus, detection of certain amounts of native M13 bacteriophage par-

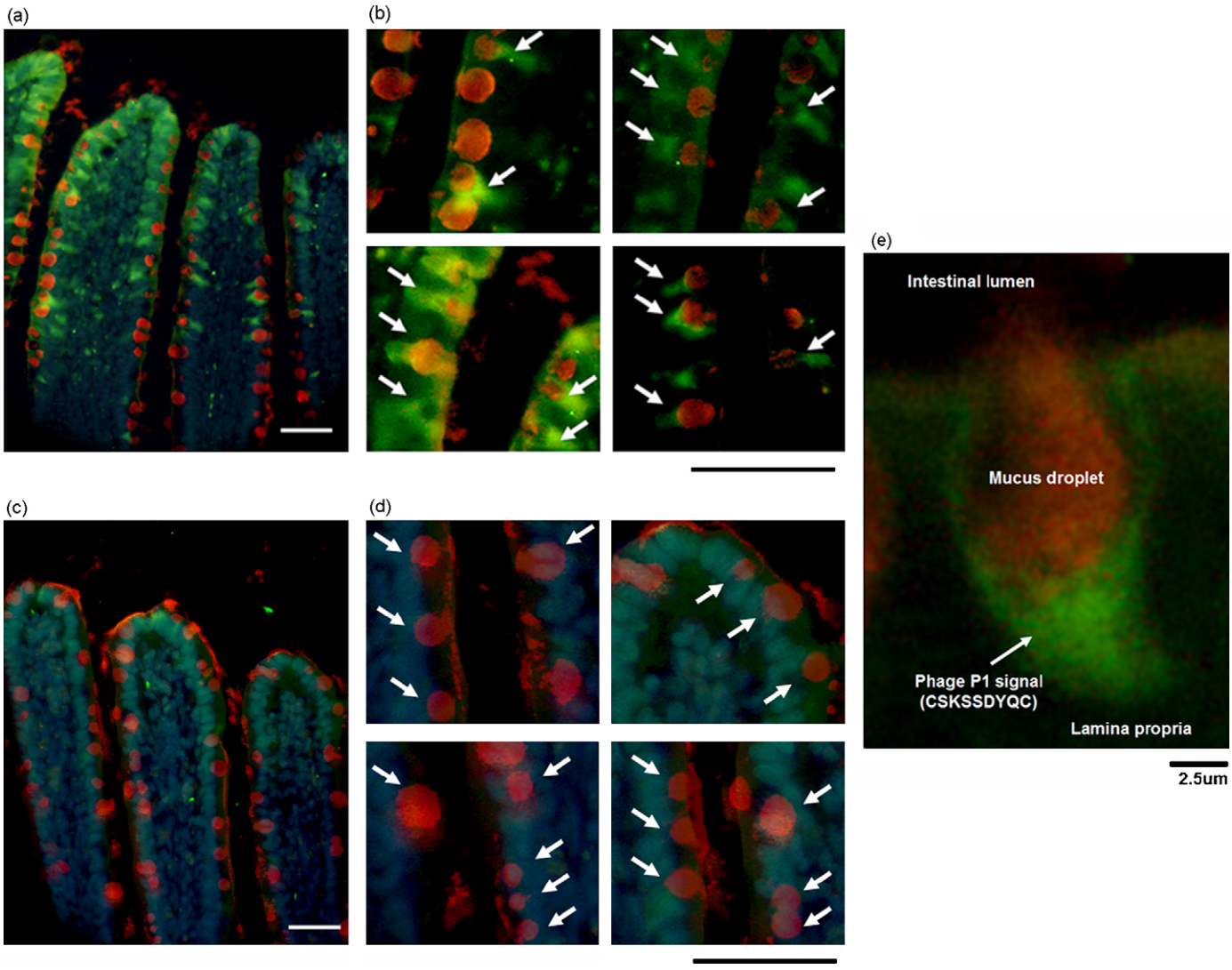

Fig. 5. Localization of P1 compared with native M13 phage using fluorescence microscopy. (a) and (b) P1. White arrows in (b) indicated positive phage signals (green) associated with goblet cells (red). (c) and (d) Native M13 phage. White arrows in (d) indicated goblet cells (red) without any positive phage signal. (e) Green fluorescence signal of P1 was detected on the both sides of the mucus droplet (red) on the location of goblet cell. Scale bars in (a)–(d): 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ticles from organs after their oral administration in our study could be explained by existence of such nonspecific routes in intestinal epithelia (Figs. 2 and 4). However, because the P1 encoding CSKSSDYQC-peptide ligands showed a significant superiority on transport efficiency across the intestinal mucosal barrier compared with native M13 phage (Fig. 4), we inferred that the CSKSSDYQC-peptide ligands would have another specific transporting route on intestinal epithelia. In immunohistochemical assays, P1 revealed intensive green signals along the intestinal lining on villi toward lamina propria, where primarily consists with enterocytes sealed by tight junction with each other (Fig. 5(a)). Interestingly, although the enterocytes are the most prevalent cells in intestinal epithelia and receptor-mediated endocytosis or transcytosis are mainly occurred from this absorptive cell type (Okamoto, 1998; Russell-Jones, 2001), most of strong positive P1 signals (green) were associated with location of the goblet cells (red) instead of enterocytes (Fig. 5(b) and (e)). Goblet cell is a secretory cell type in intestinal epithelia for mucin which is a large glycoprotein to protect the intestinal epithelia forming viscous mucus layer (Specian and Oliver, 1991). In contrast, native M13 phage showed only weak signal in background level and no positive signal associated with goblet cells (Fig. 5(c) and (d)). Similar result was also observed in assay using HRP-linked anti-M13 antibody with DAB. The positive signals (brown dots) were only detected with P1 on the villi corresponding to location of goblet cells (Fig. 6(a)–(f)).

Consequently, the CSKSSDYQC-peptide ligand could be available as a leading peptide for the carrier-drug conjugate strategy with macromolecular therapeutics to facilitate efficient transport of the drugs across the intestinal mucosal barrier. We expect that this approach could expand the availability of therapeutic macromolecules, such as protein drugs, as substituting for their parenteral injection in the future.

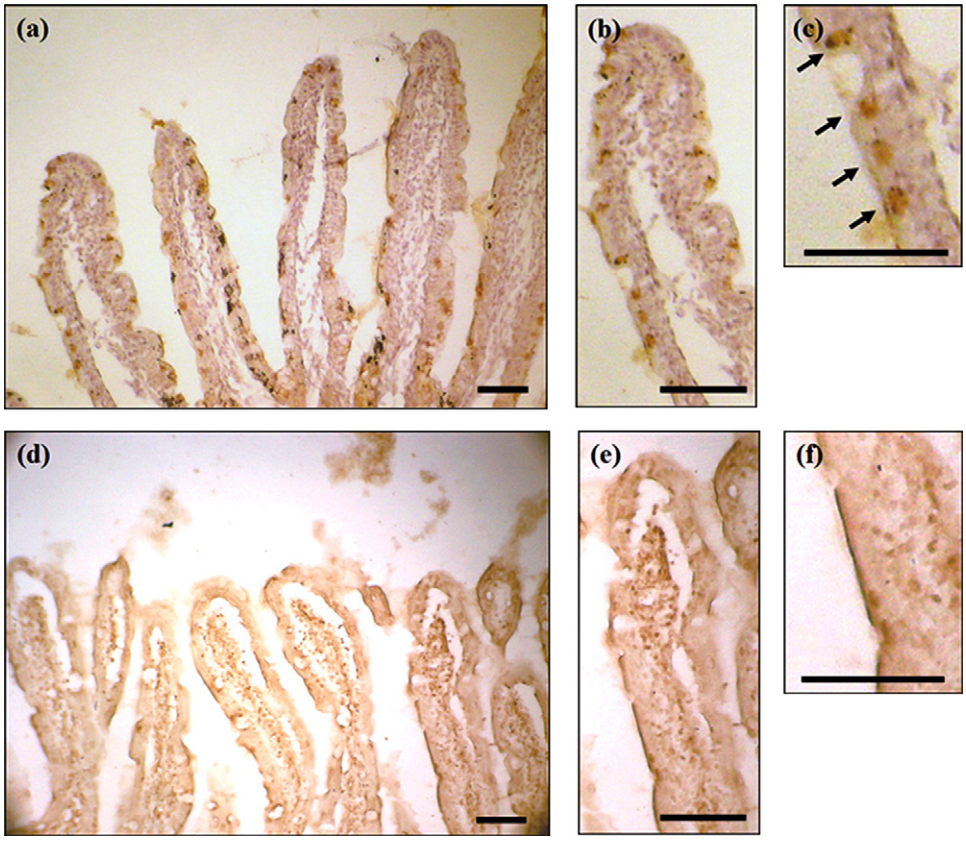

Fig. 6. Localization of P1 compared with native M13 phage using HRP-DAB reaction. (a)–(c) Positive signals of P1 were shown at the position corresponding with goblet cells along the intestinal lining (brown dots). Black arrows in (c) indicated the positive phage signals. (d)–(f) Any distinctive phage signal was not shown with native M13 phage along the intestinal epithelia. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Acknowledgements

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