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Research paper

Increase of polypeptide transport by lectin conjugation across rabbit Peyer's patches

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Abstract

The transport of bovine serum albumin (BSA) across Peyer's patches (PP) was increased by conjugate synthesis with lectin concanavalin A (Con A). The transport of the intact form was increased by the conjugation and significantly reduced by colchicine. The increase is thought to be due to the endocytosis activity of PP in response to lectin Con A. The transport of the conjugate across the jejunal epithelium (JE) was also increased, and it was concluded that the increase was due to the increase in stability against enzymatic degradation. These results suggest that conjugate synthesis with lectin is an effective method to improve the poor absorption of polypeptides and protein drugs in the intestine. © 1997 Elsevier Science B.V.

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1. Introduction

Intestinal Peyer's patches (PP) have attracted special interest as absorption sites for high molecular peptide drugs which are hydrolyzable and poorly permeable in the gastrointestinal tract and in the epithelial membrane. M cells in PP have the following characteristics [1–3]: (1) short microvilli which make the macromolecule readily accessible to the membrane surface; (2) endocytosis of extracellular macromolecules through binding to specific receptors or by adsorption to the membrane surface; and (3) a paucity of lysosomal hydrolyzing enzymes which enables macromolecules to permeate virtually intact. These characteristics explain the effectiveness of PP as intestinal absorption sites for polypeptides or protein drugs after oral dosing. Horseradish peroxidase [4] and glycoprotein (RU41740) [5] have been reported to be more effectively absorbed in the intact form in piglet PP than in the jejunal epithelium (JE) and in rabbit PP than in the duodenum, respectively.

In our previous study [6], the transport of fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) and FITC–concanavalin A (Con A) was compared in the rabbit PP and JE. The transport amount of total (intact and degradation forms) of FITC–Con A was greater than that of FITC–BSA in the PP. The transport of total FITC–Con A across the PP was superior to that across the JE, but that of FITC–BSA was not different in the PP and JE. The stability against enzymatic degradation of FITC–Con A was superior to that of FITC–BSA both in the PP and JE, especially in the PP.

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In the present study, to increase polypeptide transport in the PP using affinity of Con A for the PP, the conjugate of FITC–BSA as a model polypeptide with Con A was synthesized, and the transport across the PP and the stability were compared with those of FITC–BSA.

2. Materials and methods

2.1. Chemicals

FITC–BSA (apparent molecular weight (M_w) 67 000), Con A (apparent M_w 102 000) and colchicine were purchased from Sigma (St. Louis, MO, USA). FITC–BSA and Con A were fractionated with Sephadex G-75 as described in our previous paper [6] and used after lyophilization. Sephadex G-25, G-75 and G-200SF were obtained from Pharmacia (Uppsala, Sweden); TSK-GEL G3000 SWxL from Tosoh (Tokyo, Japan); immobilized D-mannitol gel from Pierce (Rockford, IL, USA); Centriprep-30 (filter for ultrafiltration) from Amicon (Beverly, MA, USA). Other reagents were of an analytical grade or better.

2.2. Membrane permeation experiment

The JE and proximal PP were removed from male New Zealand white rabbits (2–3 kg) which were fasted for about 24 h. The isolation of the tissues, the set-up using Ussing-type chambers and the permeation experiments were performed as previously described [6,7]. After 1-ml samples were taken from the serosal side in the chamber at 20-min intervals, the serosal volume was kept constant by supplying 1 ml of Ringer's solution. The concentrations used in the experiments were 0.1 and 0.01% for FITC–BSA, and 0.01% for FITC–BSA–Con A conjugate, in accordance with the detection limit of the serosal appearance amount. To inhibit the permeation, 0.1 mM colchicine was added to the mucosal solution.

2.3. Degradation of FITC–BSA–Con A conjugate

After the completion of the permeation experiment (100 min), the serosal samples were lyophilized and redissolved in a small amount of 0.1 M phosphate buffer solution (pH 6.8) before application to gel filtration. For the gel filtration, Sephadex G-200SF was used under the following conditions: column length, 2.5 × 90 cm; mobile phase, 20 mM phosphate buffer (pH 8.0); flow rate, 0.15 ml/min.

2.4. Assay of intact and degraded FITC–BSA–Con A conjugate

To determine the total amounts of intact and degraded FITC–BSA–Con A conjugate and the intact amount of the conjugate, the concentrations of the serosal samples and the intact fraction obtained by the above gel filtration were determined fluorometrically at 490 nm for excitation and 520 nm for emission.

2.5. Synthesis of FITC–BSA–Con A conjugate

2.5.1. Addition of maleimide group to FITC–BSA

After 23.5 mg of FITC–BSA was dissolved in 2.1 ml of 0.1 M phosphate buffer solution (pH 7.0), 1.4 mg of sulfosuccinimidyl-4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate (sulfo-SMCC) was added to the solution and the mixed solution was incubated at 30°C for 30 min. The free SMCC was removed by gel filtration with Sephadex G-25 (prepacked PD-10 column, Pharmacia LKB) and 0.1 M phosphate buffer solution (pH 6.0) as a mobile phase.

2.5.2. Addition of thiol group to Con A

After 35.0 mg of Con A was dissolved in 2.5 ml of 0.1 M phosphate buffer solution (pH 6.5), 10 ml of *N*,*N*-dimethylformamide solution containing 0.35 mg of *S*-acetyl mercaptosuccinic anhydride was added to the solution and the mixed solution was allowed to stand for 30 min at room temperature. To the mixed solution, 0.1 ml of 0.1 M EDTA aqueous solution, 0.5 ml of 0.1 M Tris–HCl buffer solution (pH 7.0) and 0.5 ml of 1.0 M hydroxylamine–HCl solution (pH 7.0) were added, and the final solution was left standing for 4 min at room temperature. The final solution was fractionated by gel filtration (Sephadex G-25 prepacked PD-10 column; 0.1 M phosphate buffer solution (pH 6.0) and 5 mM EDTA solution as mobile phase), and the fraction containing Con A–SH was concentrated by Centriprep-30.

2.5.3. Conjugation of FITC–BSA–maleimide and Con A–SH

The concentrated phosphate buffer solutions of FITC–BSA–maleimide (23.5 mg) and Con A–SH (35.0 mg) were mixed and allowed to stand for 20 h at 4°C, followed by the addition of 70 ml of 0.1 M *N*-ethylmaleimide to the mixed solution to stop the conjugation reaction. The final solution was fractionated by immobilized D-mannnose gel affinity chromatography. The final solution was applied and fixed to an immobilized D-mannitol gel and allowed to stand for fixation for 1 h. Thereafter, excess FITC–BSA–maleimide was eluted with 0.1 M phosphate buffer solution (pH 7.0) and the gel was allowed to stand for 1 h. The fraction containing Con A was eluted using 0.1

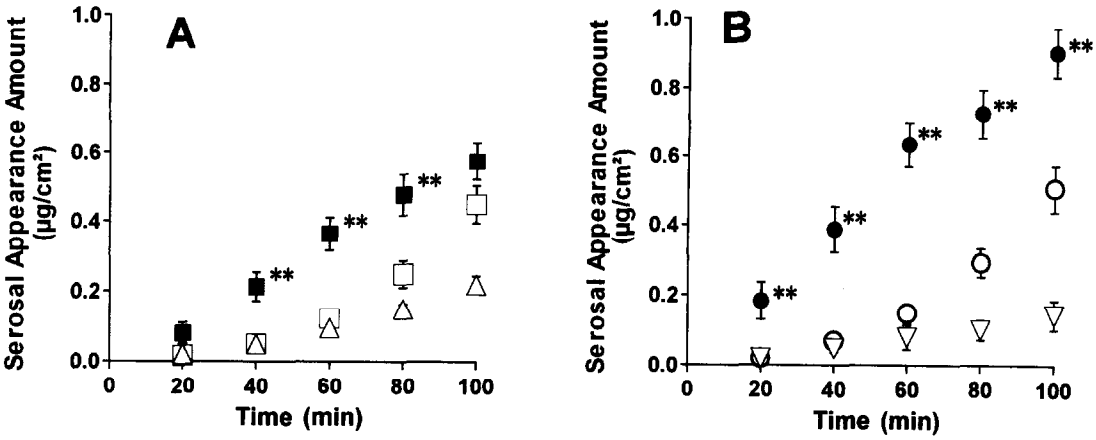

Fig. 1. Time course of the transport of FITC–BSA and FITC–BSA–Con A conjugate across rabbit jejunal epithelium (JE) (A) and Peyer's patches (PP) (B), as determined by an in vitro Ussing-type chamber method. (□) 0.1% FITC–BSA; (△) 0.01% FITC–BSA; (■) 0.01% FITC–BSA–Con A conjugate; (○) 0.1% FITC–BSA; (▽) 0.01% FITC–BSA; (●) 0.01% FITC–BSA–Con A conjugate. Data represent the transport amounts of total (intact + degradation) forms of FITC–BSA and FITC–BSA–Con A conjugate and the mean ± S.E. of more than six experiments.

** $P < 0.01$ vs. FITC–BSA.

M phosphate buffer solution (pH 7.5) including excess D-glucose (0.5 mol/l). Finally, the fraction was concentrated with Centriprep-30 and desalted. As a result of high-performance liquid chromatography (HPLC) analysis, the peak of the conjugate (molecular weight, about 170 000) was obtained at a retention time of 17.6 min. The conditions of the HPLC assay were as follows: column, TSK-GEL G3000SWxL; mobile phase, 0.1 M phosphate buffer solution (pH 6.8); flow rate, 0.5 ml/min; detector, fluorophotometer (Ex 490 nm, Em 520 nm). Finally, the concentrated solution was lyophilized to obtain the FITC–BSA–Con A conjugate.

3. Results and discussion

The maleimide method used for the conjugation reaction of FITC–BSA and Con A gave the conjugate of the equal molar ratio of BSA and Con A, avoiding the irregular binding of these peptides which occurs in the general cross-linking method with glutaraldehyde. The time courses of the amount of total (intact and degradation) forms of 0.01 and 0.1% FITC–BSA and 0.01% FITC–BSA–conjugate transported across JE and PP are shown in Fig. 1. Since the average molecular weight of BSA–Con A conjugate was about 2.6 times greater than that of BSA, the permeation experiment at the molar concentration equal to 0.01% conjugate should be performed at the FITC–BSA concentration of about 0.004%. However, mucosal to serosal transport of FITC–BSA was detected in both the JE and PP at the initial mucosal concentration of more than 0.01%. The transport rates, in decreasing order, were 0.01% FITC–BSA–Con A conjugate in PP > 0.01% FITC–BSA–Con A conjugate in JE > 0.1% FITC–BSA in JE and PP > 0.01% FITC–BSA in JE and PP. The amount of the conjugate transported over 100 min was about 1.5 times greater in the PP than in the JE. Since the transport amount of FITC–BSA was approximately equal in the JE and PP regardless of the initial concentration in the mucosal side, the conjugate of FITC–BSA with Con A was shown to increase the permeability of FITC–BSA in the PP. Thus, conjugation with Con A can be used for improving polypeptide absorption in the PP. The conjugate increased the transport of FITC–BSA in the JE as well as in the PP. The separation of the intact and degradation forms of the serosal appearance amount was performed only in the case of 0.1% FITC–BSA, since the appearance amount at 0.01% FITC–BSA was so small. The increase in the stability of the peptide by conjugate synthesis was considered to be one of the increasing factors in the JE, i.e. the intact percent to the total transport amount in the serosal side was increased to 2-fold by the conjugation in both the PP and JE (Table 1).

The inhibitory effect of colchicine, an endocytosis inhibitor, on the intact percent to the total transport amount of the conjugate in the JE was present to a slight extent but not significantly so (Table 1). In contrast, in the PP, the intact percent was reduced to one-third of that in the absence of colchicine. This marked decrease indicates the significance of the contribution of endocytosis in the PP to the transport of the conjugate.

In conclusion, it was shown that the conjugate synthesis of the polypeptide with Con A is an effective method of improving poor intestinal absorption of the

Table 1
Inhibitory effects of colchicine on intact percentage of FITC–BSA and FITC–BSA–Con A conjugate appearing at the serosal side across rabbit jejunal epithelium (JE) and Peyer's patches (PP) over 100 min[a]

	JE	PP
FITC–BSA[b]		
Control	8.37 ± 1.37	17.2 ± 1.8
+Colchicine	6.20 ± 1.30	5.47 ± 1.21*
FITC–BSA–Con A conjugate		
Control	18.1 ± 5.6	34.6 ± 5.4
+Colchicine	10.7 ± 4.0	11.4 ± 1.9*

[a] Each value represents the mean ± S.E. of more than eight rabbits.
[b] Data for FITC–BSA are quoted from Ref. [6].
* $P < 0.01$ vs. the control without colchicine.

polypeptides. The main factor in this improvement is due to potent adsorptive endocytosis activity in response to lectin [8] in the PP, but the increase in the stability against enzymatic degradation cannot be ruled out as a factor. The stability factor was also observed for the increase in jejunal absorption of the conjugate. The mechanism of improving the resistance of BSA to enzymatic degradation by conjugation is unclear. However, the fact that Con A was more resistant to enzymatic degradation than was BSA, especially in the PP, may be related to the improvement of the stability of the BSA–Con A conjugate [6]. The practical use of the conjugate with lectin remains to be investigated further, but the above information could be useful for identifying the characteristics of the PP as an effective absorption site.

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