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Effect of Chitosan on the Permeability of Monolayers of Intestinal Epithelial Cells (Caco-2)

Per Artursson,[1] Tuulikki Lindmark,[1] Stanley S. Davis,[2,3] and Lisbeth Illum[2,3]

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INTRODUCTION

Chitosan is a cationic polysaccharide chemically derived by the deacetylation of chitin; a natural material found for example in crab and shrimp shells. Chitosan has been used for a range of applications such as a food ingredient, as a water purification aid and as a pharmaceutical excipient. The compound has been shown to be mucoadhesive at physiological pH a property that is probably mediated by an ionic interaction between the positively charged amino groups in chitosan and the negatively charged sialic acid residues in mucus (1). Recently, it has been reported by Illum (2,3) that chitosan enhanced considerably the absorption of peptides such as insulin and calcitonin across the nasal epithelium. The mechanism of action was suggested to be a combination of mucoadhesion and an effect on the gating properties of the tight junctions. The latter proposal was based on reports in the literature that cationic molecules such as protamine can exert an effect on the integrity of the tight junctions in the brain endothelial barrier (4).

The purpose of the present study was to investigate the mechanism of the enhancement of the transport of peptides across the nasal mucosal membrane. It is extremely difficult to grow confluent layers of nasal epithelial cells in culture for drug transport studies (5). Consequently, a human intestinal cell line, Caco-2, was selected as a model epithelial cell layer. This cell line readily forms confluent monolayers of polarized epithelial cells (6). Caco-2 monolayers have previously been used as a model to study the effects of various pharmaceutical additives and absorption enhancers on the intestinal epithelium (7). More recently, it has also been used to study the mechanisms of nasal absorption enhancers (8). Carbon-14 labelled mannitol was selected as the model drug due to its low baseline membrane permeability (7).

MATERIALS AND METHODS

Materials

^{14}C-mannitol (MW 182; specific radioactivity 271 mCi/g) was obtained from New England Nuclear, Boston, U.S.A. through du Pont, Scandinavia AB, Kista, Sweden. Rhodamine-phalloidin were purchased from Molecular Probes Inc., Eugene, OR, USA. Chitosan glutamate (Seacure 210 +, batch number 902-572-05) with a viscosity at 2% of 550 cps, was obtained from Pronova Ltd., Hampshire, UK.

Cells

Caco-2 cells originating from a human colorectal carcinoma (9) were obtained from American Type Culture Collection, Rockville U.S.A. The cells were cultivated on polycarbonate filters (Transwell cell culture inserts, Costar, Cambridge, MA, USA, mean pore diameter 0,45 μm) for absorption experiments, and on polyethyleneterephtalate filters (Cyclopore cell culture inserts; Falcon, Lincon Park, NJ, USA; mean pore diameter of 0,45 μm) for fluorescence microscopy, as described previously (10,11). Cells of passage 90-100 were used.

Absorption studies

The absorption of ^{14}C-mannitol across Caco-2 cell monolayers was studied as described previously (7). Solutions containing the radioactive marker with or without added chitosan were added to the apical side of the monolayer and samples were taken at regular time intervals from the basolateral side. Chitosan was dissolved in phosphate buffered saline adjusted to pH 4.0, 4.9 or 6.0 with NaOH. The solutions were iso-osmolar. The effects of chitosan (0.10, 0.25 and 0.50 % w/v final concentrations) on the absorption rate of ^{14}C-mannitol (0.20 μM) were studied for 60 minutes. The radioactive contents of the samples were analyzed in a liquid scintillator (Tri-carb 1900 CA, Packard Instrument, Downers Grove, IL).

The apparent permeability coefficient (P_{app}) was determined according to the following equation:

$$P_{app} = dQ/dt \ 1/AC_o$$

where dQ/dt is the permeability rate (steady state flux mol/s), C_o is the initial concentration in the donor chamber (mol/ml) and A is the surface area of the monolayer (cm^2).

Fluorescence microscopy

F-actin was stained with rhodamine-labelled phalloidin according to the manufacturers specification. 10 μl of a stock solution (200 U/ml rhodamine phalloidin in methanol) was evaporated under nitrogen gas. The residue was redissolved in 400 μl (PBS). The monolayers were rinsed three times with PBS, fixed for 10 min in 4% formaldehyde in PBS on ice, rinsed with PBS three times and extracted over 5 min with 1% Triton X 100 on ice. The cell monolayers were rinsed with PBS twice, air dried and stained with rhodamine phalloidin for 20 min in the dark. The monolayers were then rinsed with PBS three times and finally mounted on glass slides in a 1:1 solution PBS and glycerol.

All preparations were examined with a fluorescence microscope (Zeiss Axioskop, Oberkochen, Germany) fitted with a x 40 objective for oil immersion. The photographs

[1] Department of Pharmaceutics, Biomedical Centre, Uppsala University, Box 580, 751 23 Uppsala, Sweden.

[2] Department of Pharmaceutical Sciences, Nottingham University, Nottingham NG7 2RD, UK.

[3] DanBioSyst UK Ltd., Albert Einstein Centre, Highfield Science Park, Nottingham NG7 2TN, UK.

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were taken with a microscope camera (MC 100, Zeiss, Oberkochen, Germany) and Kodak T-Max 400 film.

Statistics

All figures are expressed as means ± SD. The results were analyzed by one way ANOVA.

RESULTS AND DISCUSSION

The permeability of the Caco-2 cell monolayers was investigated using 14C-mannitol as a marker. Mannitol is a metabolically inert and very hydrophilic molecule that has been used previously to follow changes in the epithelial integrity of the intestinal mucosa (7). Mannitol does not distribute into the cell membranes to a large extent but is absorbed through the alternative aqueous paracellular pathway (i.e through the tight junctions). As a result the baseline permeability of mannitol in Caco-2 monolayers is very low and therefore, small changes in permeability can be detected, making this probe ideal in studies of absorption enhancement. The transepithelial electrical resistance of the monolayers in the present study was approximately 200 ohms . cm^{-2}, a value close to the 100-200 $ohms.cm^{-2}$ recently reported for sheep nasal mucosa by Reardon et al. (12). However, the mannitol permeability was approximately three fold lower in the Caco-2 monolayers as compared to the sheep nasal mucosa, a difference well within the variances observed between different epithelial preparations.

In general, chitosan increased the absorption of mannitol in a concentration ($p<0.003$) and pH ($p<0.05$ for 0.10% and 0.25%; $p>0.05$ for 0.5%) dependent way (Table 1 and Figure 1). An increase in the chitosan concentrations resulted in an increase in the P_{app} with a plateau level reached between 0.25% and 0.50%. P_{app} was highest for the lowest pH applied (pH = 4) and the concentration dependency was not as pronounced as observed for previously investigated absorption enhancers (7,13). In previous studies, small increases in the concentration of various anionic surface active agents used as absorption enhancers such as sodium dodecyl sulphate and sodium taurodihydrofusidate resulted in more than a 100-fold increase in absorption rates. In contrast, in the present study, exposure of the epithelial model to 0,25 % and 0.50 % chitosan gave a maximum of a seven fold increase in the absorption rate of mannitol as compared to control as indicated by the P_{app}. Moreover, when the chitosan concentration was increased from 0.25% to 0.50%, the increase in P_{app} was not significant at any of the different pH-values studied. This shallow dependence on concentration of the effect of chitosan is in agreement with previous results performed in the in vivo rat and sheep models, where chitosan was shown to provide an apparent absorption plateau level at concentrations of about 0.2% and 0.5%, respectively (3).

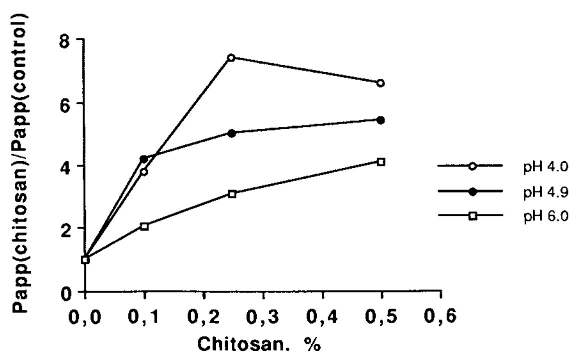

Fig. 1 Ratios between apparent permeability coefficients obtained from chitosan treated and untreated Caco-2 monolayers as function of chitosan concentration. Data are from 3-4 different experiments. (The standard deviations are given in Table 1)

Table 1. Effect of chitosan in phosphate buffered saline on the permeability of mannitol across a Caco-2 cell monolayer[1]

pH[3]	Chitosan conc	Papp[4] cm/s × 107	SD[5] cm/s × 107
7.2	Control[2] HBSS	0.49	0.04
4.0	Control buffer	0.66	0.06
4.0	0.10%	2.52*	0.29
4.0	0.25%	4.86*	1.33
4.0	0.50%	4.33*	0.55
4.9	Control buffer	0.69	0.07
4.9	0.10%	2.89*	0.77
4.9	0.25%	3.46*	1.63
4.9	0.50%	3.73*	1.15
6.0	Control buffer	0.8	0.06
6.0	0.10%	1.71	0.46
6.0	0.25%	2.47*	0.49
6.0	0.50%	3.23*	1.14

[1] Apical buffer: NaCL 0.9% + PBS (4:1); Basolateral buffer:HBSS
[2] Apical = Basolateral = HBSS
[3] Chitosan was dissolved at pH 4.0 and the pH was set with NaOH
[4] The absorption of mannitol was followed for 60 min. Mean values from 3–4 filters
[5] Standard deviation
* Significantly different from control at 95%; one factor ANOVA

In the present work the transport of mannitol across the membrane increased with decreasing pH as seen by the P_{app} values of 1.7, 2.9 and 4.9 for 0.25% chitosan concentrations at pH 6.0, 4.9 and 4.0 values, respectively. This phenomenon is most likely due to the pH-dependent change in molecular configuration in conjunction with the degree of ionisation of the chitosan molecule. Chitosan has an apparent pKa of about 5.6. At the higher pH the chitosan molecule exists in a more coiled configuration but as the pH decreases and the molecule becomes more ionised the molecule uncoiles and assumes a more elongated shape (14). Hence, at the lower pH values the chitosan has a higher charge density and will have a better possibility for intimate contact with the epithelial membrane. A low pH value of 4.0 does not in itself lead to changes in membrane permeability (13).

Pharmacological agents that interact with cytoskeletal F-actin have often been shown to induce a simultaneous increase in paracellular permeability (16). This is in agreement with the hypothesis that F-actin is directly or indirectly associated with the proteins in the tight junctions (17). We have shown recently that absorption enhancers that induce structural separation of the tight junctions in Caco-2 cells induce parallel changes in F-actin distribution (7,18).

The exposure of the apical cell surface to a 0.50 % chi-

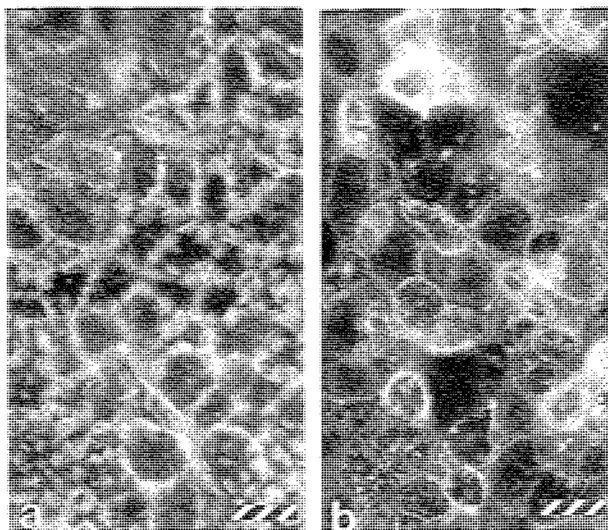

Fig. 2 En face view of Caco-2 monolayers stained with rhodamine-phalloidin for visualization of filamentous actin. a. control cells with typical apical perijunctional rings and microvilli. b. After exposure to chitosan (0.5%, pH=4, 60 min), cells with disbanded perijunctional rings appeared. In some cells, the staining of the microvilli was also weaker, suggesting that chitosan caused a redistribution of F-actin in the microvilli. The bars indicate 20 μm.

tosan solution for 60 min also induced clear changes in the F-actin distribution (Figure 2). In general, the staining of F-actin was weaker after chitosan exposure and areas with markedly diminished or disbanded actin staining could be observed. The changes in F-actin distribution were comparable to those reported previously, suggesting that chitosan affected the permeability of the tight junctions (7,18)

The results obtained suggest that chitosan has a different mechanism of action of enhancing the transport of drugs across epithelial membranes to that displayed by "classical" materials in the form of surfactants and bile salts, such as sodium dodecyl sulfate and sodium taurodihydrofusidate. The interaction between the apical membrane of the epithelial cell and chitosan appear to be specific and saturable, as opposed to the non-specific and non-saturable effects seen for surfactants and bile salts. Because of their positive charge, cationic macromolecules such as protamine, polylysine and chitosan can interact with the anionic components (sialic acid) of the glycoproteins on the surface of the epithelial cells. Recent studies of brain capillaries have shown that cationic macromolecules such as protamine interact with the endothelial cells and neutralise the luminal anionic charge thereby producing a differential opening of the blood-brain barrier to make it more permeable to large molecules (4). In a similar study an intravenous infusion of protamine produced large increases in lymph flow and in lymph protein clearance. This enhanced transcapillary protein flux was explained to be primarily due to the neutralisation of fixed anionic sites on the capillary wall (19). Further, it has been suggested that cationic macromolecules are able to displace cations from electronegative sites (such as tight junctions) on a membrane which require coordination with cations for dimensional stability (20). In addition it has been shown that the interior of the tight junction channels (pores) are highly hydrated and contain fixed negative charges. Thus, a relatively modest alteration in the relative concentration of specific species of ions within the volume of the pore could result in substantial alterations in tight junction resistance leading to loosening or opening of the pore (21). Further studies will be conducted to see if chitosan acts in such a manner on cultured epithelia.

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