Chitosans as Absorption Enhancers for Poorly Absorbable Drugs 2: Mechanism of Absorption Enhancement

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Purpose. It has recently been shown that the absorption enhancing and toxic effects of chitosans are dependent on their chemical composition. In this study, the mechanisms underlying these effects were investigated at the cellular level.

Methods. The effects on epithelial cells of chitosans with different chemical composition, absorption enhancing properties and toxicities were studied in Caco-2 monolayers. Chitosan C(1:31) has a low degree of acetylation (DA) (1%) and a low m.w. (31 kD), and displays dose-dependent absorption enhancement and cytotoxicity; chitosan C(35:170) has a higher DA (35%) and a higher m.w. (170 kD), is less dose-dependent in absorption enhancement, and is not cytotoxic. A third non-toxic chitosan C(49:22) with a high DA (49%), a low m.w. (22 kD), and no influence on epithelial permeability was used as control.

Results. C(1:31) and C(35:170) bound tightly to the epithelium. Cellular uptake of the chitosans was not observed. Both chitosans increased apical but not basolateral cell membrane permeability and induced a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1. This resulted in increased paracellular permeability of hydrophilic marker molecules of different molecular weights. Addition of negatively charged heparin inhibited the cellular and the absorption enhancing effects of the chitosans, indicating that these effects are mediated via their positive charges. The onset of the effects of C(35:170) on apical membrane permeability and tight junction structure was much faster than that of C(1:31). C(49:22) did not influence any of the properties of the Caco-2 cell monolayers studied.

Conclusions. The binding and absorption enhancing effects of chitosans on epithelial cells are mediated through their positive charges. The interaction of chitosans with the cell membrane results in a structural reorganisation of tight junction-associated proteins which is followed by enhanced transport through the paracellular pathway.

KEY WORDS: chitosan; Caco-2; absorption enhancer; drug transport; drug absorption; tight junction; epithelial permeability.

INTRODUCTION

Chitosans are linear polysaccharides which can be prepared by alkaline N-deacetylation of chitin, an abundant biopolymer in, for example, crab and shrimp shells. Chitosans consist of linear 1–4 linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-β-D-glucopyranose (GlcN) units. It has been shown that water-soluble chitosans have a random distribution of GlcNAc units (1). Chitosan is a polycation at acidic pH values, with an intrinsic pKa value independent of degree of acetylation (DA; i.e. the molar content of GlcNAc) of 6.5 (2). They have been employed in many studies as a pharmaceutical excipient for the dissolution and sustained release of drugs (3). Chitosans have mucoadhesive properties which are probably mediated through ionic interactions with negative charges in mucus or on cell surfaces (4,5). They have potential as enhancers of mucosal drug absorption (6,7). The DA and the molecular weight of chitosans determine their absorption enhancing and cytotoxic properties (8). Chitosans with a low DA (1 to 15%) promote drug absorption at both low and high molecular weights but also show clear dose-dependent toxicity. Chitosans with DAs of 35 to 49%, on the other hand, only increase the absorption of drugs with high molecular weights. These chitosans display low toxicity (8). Thus, absorption enhancement properties and toxicity may be controlled by selecting a chitosan with the optimal chemical composition (DA) and molecular weight.

Only preliminary information regarding the binding and cellular effects of chitosans has been published. Artursson et al. showed that a chitosan with a DA of 11% and a molecular weight of 162 kD (Seacure +210 chitosan, (9)) induced the redistribution of cytoskeletal F-actin at a concentration that enhanced mannitol transport in Caco-2 cell monolayers, and speculated that this effect may result in changes in paracellular permeability (10). In contrast, recent results from Borchard et al. showed that a similar chitosan-glutamate increased the apical membrane permeability but not the transport of fluorescent marker molecules through Caco-2 monolayers (11). Thus, further studies investigating the mechanism of this potentially important new series of absorption enhancers are required.

In this study, the mechanisms underlying absorption enhancement and toxicity were investigated at the cellular level. Three chitosans with different absorption enhancing properties and toxicities were studied in Caco-2 cell monolayers. This cell culture model has been used previously to obtain mechanistic insight into the effects of pharmaceutical additives and absorption enhancers on the intestinal epithelium (12).

MATERIALS AND METHODS

Materials

The chitosans were prepared by heterogeneous N-deacetylation. The acid-soluble fractions were isolated as previously described (13) and converted to the chloride salts, and the intrinsic viscosities were determined. The number average molecular weights were determined from viscosity measurements. The chitosans used in this study are differentiated by using the abbreviation C followed by two numbers between brackets. The first number is the degree of acetylation (%) and the second is the mean molecular weight in kDa. The chitosans studied were C(1:31), C(35:170) and C(49:22).

^{14}C-mannitol (54 mCi/mmole) was obtained from New England Nuclear, Boston, MA. Lissamine™-rhodamineB sulfo-

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nyl chloride, bisbenzimide (H33258), rhodamine phalloidin and propidium iodide (PI) were obtained from Molecular Probes, Eugene, OR. Streptavidine Texas red, and biotinylated sheep anti-rabbit IgG were purchased from Amersham, Buckinghamshire, UK. Rabbit anti-ZO-1 polyclonal antibodies were obtained from Zymed, San Francisco, CA. Paraformaldehyde was obtained from Merck, Darmstadt, Germany. Sodium heparin was purchased from Sigma, St. Louis, MO. Fluorescein isothiocyanate dextran 4,000 (FD-4) and 20,000 (FD-20) with average molecular weights of 4,300 and 19,000, respectively, were purchased from Sigma. The fluorescein isothiocyanate (FITC) content was 0.006 mole FITC/mole glucose for FD-4 and 0.01 mole FITC/mole glucose for FD-20.

Cells

The Caco-2 cell line was obtained from American Type Culture Collection, Rockville, MD. The cells were cultured on polyethylene terephtalate filters (Falcon cell culture inserts, mean pore diameter: 0.045 μm; Becton Dickinson, Oxford, UK) for actin staining and on polycarbonate filters (Transwell or Snapwell cell culture inserts, Costar, Cambridge, UK) for all other experiments as described earlier (14). Cells of passage number 93-105 were used throughout, unless otherwise stated. The cells were used for experiments 21–35 days after seeding.

Preparation of Chitosan Solutions

Chitosans were dissolved in Hanks balanced salt solution (HBSS) buffered with 25 mM 2N-morpholino-ethanesulfonic acid (MES) to pH 5.5 at concentrations of 0.5 and 1.0 mg/ml by vigorous shaking for 24 to 48 hours. Stock solutions were stored at −20°C. Fresh chitosan solutions were prepared for each experiment by thawing a portion of the stock solution and diluting to the appropriate concentrations with HBSS pH 5.5. Control solutions consisted of HBSS pH 5.5 without addition of chitosan.

Confocal Laser Scanning Microscopy (CLSM)

Chitosans on Intestinal Epithelium

Chitosans were labelled with lissamine™ rhodamine according to the manufacturer's instructions. The degree of substitution for the conjugation reaction was 0.04 moles dye/mole C(1:31), 0.18 moles dye/mole C(35:170), and 0.0024 moles dye/mole C(49:22). The absorption enhancing activity of the conjugated chitosans was similar to that of unconjugated chitosans as demonstrated by mannitol permeability experiments. Mannitol permeability after 60 min exposure to approx 50 μg/ml chitosan increased by a factor 5 to 10 for C(1:31) and C(35:170), and was not different from exposure to 50 μg/ml unlabelled chitosan. The effect on permeability of the labelled chitosans was completely inhibited by addition of heparin. Moreover the lissamine label itself had no effect on mannitol permeability. Caco-2 cell monolayers grown on 6.5 mm Transwell filters were incubated on the apical side with chitosan-lissamine rhodamine at a concentration of approximately 50 μg/ml in HBSS pH 5.5 for 60 min at 37°C. The basolateral solutions were HBSS buffered with 25 mM Hepes to pH 7.4. Following incubation, the monolayers were washed twice with PBS and fixed with 3% w/v paraformaldehyde. The monolayers and the supporting filter were cut from the filter inserts and were directly mounted under a cover glass. The fluorescence-labelled chitosans were localised using confocal laser scanning microscopy (CLSM) (Molecular Dynamics Multiprobe 2001 confocal unit equipped with an argon/krypton laser, Sunnyvale, CA). The 568 nm excitation line was selected and emitted light at wavelengths longer than 590 nm was passed through. Confocal images were obtained with a 60 × 1.4 Nikon Oil Plan Apo lens. All images were means of 3 scans without image enhancement and were obtained using similar laser intensity, filter block, lens, black level and scan speed. Gain and aperture opening were kept constant. Experiments were performed in duplicate, at least.

Transport of Hydrophilic Marker Molecules

Caco-2 cell monolayers of passage number 84 grown on 12 mm Transwell cell culture inserts were used. The monolayers were used 24 to 28 days after seeding, and had an electrical resistance of >300 ohms.cm^2. Monolayers were incubated with 10 mg/ml FD-4 or FD-20 in HBSS pH 5.5 containing 50 μg/ml chitosan on the apical side for 60 minutes at 37°C. The chitosans were omitted in the control experiments. Following incubation, apical and basolateral solutions were aspirated off, and the monolayers were washed with PBS. Monolayers and supporting filters were cut from the cell culture inserts. The monolayers were mounted on glass slides without additional tissue processing, and were prepared for CLSM evaluation as described elsewhere (15). A Biorad MRC-600 confocal unit equipped with an argon-ion laser (excitation lines 488 and 518 nm) (Biorad, Richmond, CA) mounted on a Zeiss IM35 inverted microscope (Carl Zeiss, Oberkochen, Germany) was used. The FITC-label was detected with a Biorad blue high sensitivity filter block, which selects the 488 nm laser line for illumination and allows emitted light with wavelengths longer than 515 nm to pass. Confocal images were obtained with a Zeiss Plan-Neofluar 63 × 1.25 oil lens. All images were means of 10 scans. The images were not enhanced after acquisition and were obtained using similar laser intensity, filter block, lens, black level and scan speed. In addition, gain and aperture opening were kept constant. Autofluorescence of the Caco-2 cell monolayers was very low at the settings used for imaging. Images were taken within 20 min of termination of chitosan exposure. The cells remained viable during this period as indicated by PI exclusion (15). All experiments were performed in duplicate.

Fluorescence Microscopy

The Caco-2 cell monolayers were exposed to 50 μg/ml chitosan on the apical side as described above. For reversibility experiments, sodium heparin was added to HBSS pH 5.5 at a concentration of 52 μg/ml (10 U/ml). Heparin solutions were added to the apical side of Caco-2 cell monolayers either simultaneously with the chitosans or after the monolayers had been exposed to the chitosans for 20 min and then washed.

Cells with increased membrane permeability were stained with the fluorescent probe PI. Cytoskeletal F-actin was stained with rhodamine phalloidin. Staining was performed according to the manufacturer's specifications, as described elsewhere (14). The tight-junction protein zonula occludens I (ZO-I) was identified using polyclonal antibodies (14). A Zeiss fluores-

cence microscope (Axioskop, Zeiss, OberKochen, Germany) was used to examine all preparations. Photographs were taken with a microscope camera (MC100, Zeiss, OberKochen, Germany). PI-stained cells were counted as the relative proportion of the total number of cells ($5.4 \pm 0.7 \times 10^5$ cells/cm^2) using the NIH Image 1.41, a public domain image analysis program for Macintosh.

Electrical Measurements

Caco-2 cells grown on 12 mm Snapwell inserts were placed in horizontal type diffusion chambers (Costar, Cambridge, MA) and maintained at 37°C on a heating block. The basolateral solution consisted of 1 ml HBSS pH 7.4; the apical solution was 2 ml HBSS pH 5.5. Measurement of electrical parameters was performed as described earlier (16). Shortly, the chambers were connected with KCl salt bridges to voltage sensitive Ag/AgCl electrodes and to platina electrodes for application of current. Five current pulses between −30 and 30 μA of 235 msec each were generated every minute. Transepithelial resistance (TER) was determined by a linear least square fit of U = PD + TERxI, where PD is the spontaneous potential difference across the epithelium. The short circuit current (Isc) was calculated from Isc = PD/TER. After measuring the electrical parameters of the cell layers for 15 min, 100 μl chitosan solution in HBSS pH 5.5 were added resulting in an apical chitosan concentration of 50 μg/ml. For controls 100 μl HBSS pH 5.5 without chitosan were added after 15 min.

Transport Studies

All transport experiments were performed using prewarmed HBSS at 37°C. Solutions used on the basolateral side of the monolayers were buffered with 25 mM Hepes to pH 7.4. The apical solutions were buffered with 25 mM MES to pH 5.5. Prior to the experiments the cells were washed with HBSS. The experiments were initiated by replacing the apical blank HBSS pH 5.5 solution with a chitosan solution containing a transport marker (^{14}C-mannitol or FITC-dextrans). Transport studies were performed over 60 min in air at 37°C and 95% relative humidity. ^{14}C-mannitol was analysed by liquid scintillation counting (Tricarb, 1900CA, Packard Instrument, Downers Grove, IL). FD-4 and FD-20 concentrations were determined using fluorescence spectroscopy at excitation and emission wavelengths of 488 and 512 nm, respectively. The apparent permeability (Papp) was calculated using the following equation: Papp (cm/sec) = $dQ/dt \times 1/AxC_0$, in which dQ/dt (mmole/sec) is the rate of appearance of the transport marker on the basolateral side, C_0 (mmole/ml) is the initial marker concentration on the apical side, and A (cm^2) is the surface area of the monolayer. Since the effects of chitosans on permeability were time dependent, a "mean" Papp was calculated for the first 60 min period of transport experiments for comparison of data. For reversibility experiments, sodium heparin was added to ^{14}C-mannitol solutions in HBSS pH 5.5 at a concentration of 52 μg/ml (10 U/ml) as described above for fluorescence microscopy experiments.

RESULTS

Binding of Chitosans to Caco-2 Cell Epithelium

In order to study the binding of chitosan to Caco-2 cell monolayers, chitosans labelled with lissamine-rhodamine were prepared which can be localised by confocal laser scanning microscopy (CLSM). Following a 60 min incubation, the chitosans labelled with lissamine-rhodamine [C(1:31) and C(35:170)] were found in close contact with the apical membrane of Caco-2 cell monolayers. Small aggregates of the fluorescent chitosans were observed over the entire monolayer. The chitosans were not detected intracellularly, and only low fluorescence was seen in the intercellular space (Fig. 1). C(49:22), which was demonstrated to be an ineffective absorption enhancer in an earlier study (8), did not bind to the monolayer and was completely washed away in the rinsing step before preparation for microscopy (not shown). Similarly, the unconjugated lissamine-rhodamine control (of similar fluorescent strength as the labelled chitosans) was not detected after 60 min incubation and subsequent washing (Fig. 1).

Effects of Chitosans on Membrane Permeability

Exposure of Caco-2 cells to 50 μg/ml C(1:31) and C(35:170) increased the permeability of the apical cell membrane as shown by staining experiments using the membrane-impermeable DNA stain, PI (Table 1). The effects of C(35:170) were stronger than those of C(1:31). After a 20 min exposure period, the effects on apical membrane permeability were not reversed by replacement of chitosan solutions with regular HBSS, but were reduced significantly by replacement with HBSS containing 52 μg/ml heparin. The membrane permeabilities of C(1:31) and C(35:170) were approximately 30 and 70% of those obtained without heparin. Addition of C(1:31) and C(35:170) to the apical incubation solutions did not affect the permeability of the basolateral membrane (Table 1). The number of stained cells following incubation with 50 μg/ml C(49:22) was less than 0.5% and similar to that seen with control treatment (Table 1).

Effects of Chitosans on the Distribution of F-Actin and ZO-1

Effects of chitosans on cytoskeletal F-actin were demonstrated by staining with rhodamine phalloidin. As described previously, control cells displayed the typical continuous perijunctional F-actin rings required for maintenance of integrity (10). Exposure to 50 μg/ml C(1:31) and C(35:170) for 60 min resulted in disbandment of the actin. Discontinuities in actin staining seemed to be more frequent for C(1:31) than for C(35:170). However, effects on F-actin appeared as early as 20 min after the start of the experiment for C(35:170), whereas C(1:31) did not appear to cause any alterations in the actin distribution at this time point. Cytoskeletal actin appeared normal following incubation with chitosan solutions containing 52 μg/ml heparin (data not shown).

In control cells, the tight junction protein ZO-1 was discretely and continuously distributed around the apical cell borders. Monolayers treated with C(1:31) and C(35:170) developed areas where the ZO-1 staining was completely absent, while in other areas the intensity of staining was reduced compared to control (Fig. 1). Exposure of Caco-2 monolayers to C(49:22) did not cause changes in F-actin and ZO-1 distribution compared to control treatment (not shown).

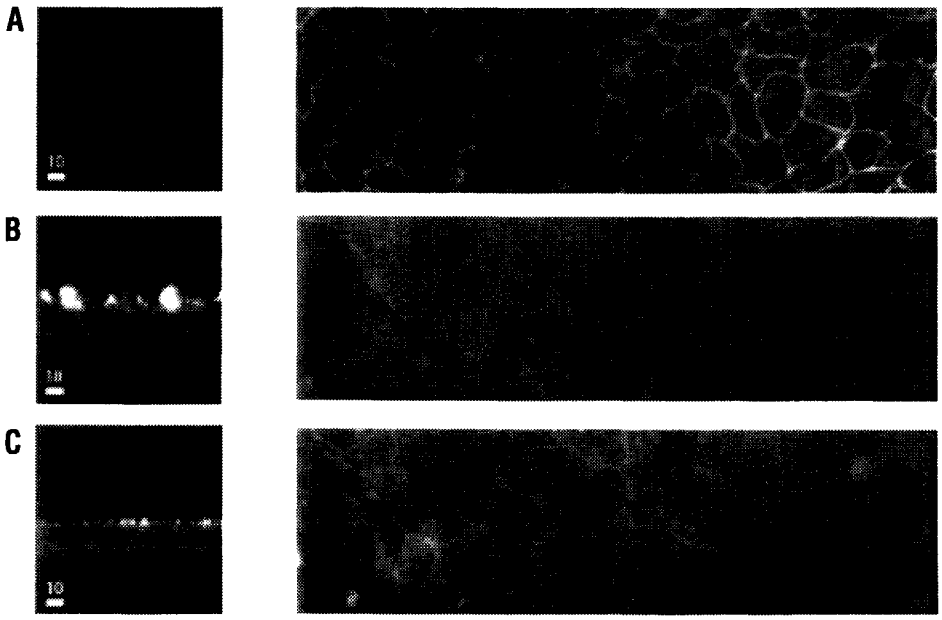

Fig. 1. Binding of chitosans to Caco-2 cell monolayers and the effect on the tight junction protein ZO-1. Caco-2 cells were exposed for 60 min to (A) control; (B) 50 μg/ml C(1:31); and (C) 50 μg/ml C(35:170). Left panel: confocal micrographs of Caco-2 cell monolayers exposed for 60 min to chitosans labelled with lissamine-rhodamine. The control was exposed to lissamine-rhodamine alone at a similar fluorescent strength as the labelled chitosan solutions. The graphs represent optical vertical cross sections with a step size in the z-direction of 0.29 μm. Top is apical, bottom is basolateral. The scale bar indicates 10 μm. Fluorescence is mainly restricted to the apical side of the monolayer. Small amounts of fluorescence can be observed in the paracellular space, no intracellular fluorescence was detected. Right panel: Caco-2 monolayers stained for ZO-1. The ZO-1 protein in control cells was found discretely and continuously distributed along the apical borders of the cells. Exposure to C(1:31) and C(35:170) resulted in loss of the typical ZO-1 staining pattern. Effects on ZO-1 staining were similar for the two chitosans. The magnification of the pictures was 660x.

Effects of Chitosans on the Electrical Properties of Caco-2 Cell Monolayers

The electrical parameters of Caco-2 cell monolayers following exposure to chitosans are shown in Fig 2. Under the experimental conditions, the initial transepithelial electrical resistance (TER) was >300 ohm·cm^2. The resistance was higher than reported previously for our Caco-2 cell monolayers (16). This was simply due to the different buffer composition on the apical side of the cells. Resistances were comparable with earlier data (200 to 300 ohm·cm^2) with the HBSS pH 7.4 buffer. Addition of chitosan (50 μg/ml) to the apical bathing solution of the Caco-2 cell monolayers resulted in an immediate decrease in the electrical resistance of the epithelium. The resistance continued to decrease over the 60 min of the experiments. The 35% acetylated chitosan C(35:170) showed a more pronounced effect during the first ten minutes, while C(1:31) had a stronger effect in the latter part of the experiment (Fig. 2). Chitosan exposure resulted in a strong increase in inward short circuit current (Isc) and potential difference (PD). The maximum effect was reached at 5 and 20 min for C(35:170) and C(1:31), respectively. Isc was increased from 4.5 ± 1.5 to 10 ± 2 μA/cm^2 for C(35:170) and to 8.0 ± 1.8 μA/cm^2 for C(1:31). PD increased from −3.5 ± 2.0 to −5.5 ± 2.5 mV and from −2.7 ± 0.2 to −4.5 ± 2.0 mV for C(35:170) and C(1:31), respectively. After reaching the maximum values, Isc and PD returned slowly to control values.

Transport of Hydrophilic Marker Molecules

As reported earlier (8), C(1:31) and C(35:170) increased the permeability of Caco-2 cell monolayers to ^{14}C-mannitol. In agreement with previous data, C(35:170) had the stronger effect on mannitol transport during the first 60 min. Replacement of chitosan solutions with HBSS buffer after a 20 min pretreatment period did not reduce the effects on mannitol transport as compared to continuous exposure (Table 2). Addition of negatively charged heparin, on the other hand, completely inhibited this effect on epithelial permeability. Addition of heparin after a 20 min pretreatment period with chitosans also inhibited ^{14}C-mannitol transport. The fraction of mannitol absorbed in the latter case, however, was not reduced completely to control values (Table 2).

Transport of the hydrophilic macromolecules FD-4 and FD-20 across Caco-2 cell monolayers was very low. Although the molecular weight of FD-20 is about 4.5 times higher than the molecular weight of FD-4, the baseline permeability for the two markers was the same, with a Papp of approximately 0.1 × 10^{-7} cm/sec (Table 3). As observed for mannitol, chitosans increased the epithelial permeability of FD-4 and FD-20 in a time dependent manner. A 60 min exposure to 50 μg/ml C(1:31) or C(35:170) to the apical compartment resulted in 4.2 and 3.0 fold increases in FD-4 transport, respectively. The permeability of the monolayers to FD-20 increased less on addition of the chitosans. After 60 min

Table 1. Effects of Chitosans at a Concentration of 50 μg/ml on Apical and Basolateral Membrane Permeability

Chitosan	Exposure time (min)	Recovery	PI staining side	PI stained cells
None[a]	20	none	apical	0.0 ± 0.0
C(1:31)[a]	20	none	apical	0.2 ± 0.2
C(35:170)[a]	20	none	apical	2.9 ± 1.5*
C(49:22)[a]	20	none	apical	0.1 ± 0.2
None[b]	60	none	apical	0.2 ± 0.3
C(1:31)[b]	60	none	apical	4.6 ± 2.1*,**
C(35:170)[b]	60	none	apical	8.7 ± 1.6*,**
C(49:22)[b]	60	none	apical	0.1 ± 0.0
None[c]	20	60 min in HBSS	apical	0.3 ± 0.3
C(1:31)[c]	20	60 min in HBSS	apical	5.3 ± 2.6*,**
C(35:170)[c]	20	60 min in HBSS	apical	8.6 ± 3.1*,**
C(49:22)[c]	20	60 min in HBSS	apical	0.1 ± 0.1
None[d]	20	60 min in HBSS with 52 μg/ml heparin	apical	0.4 ± 0.5
C(1:31)[d]	20	60 min in HBSS with 52 μg/ml heparin	apical	1.7 ± 0.7***
C(35:170)[d]	20	60 min in HBSS with 52 μg/ml heparin	apical	5.9 ± 2.2*,***
C(49:22)[d]	20	60 min in HBSS with 52 μg/ml heparin	apical	0.4 ± 0.7
None[e]	60	none	basolateral	0.0 ± 0.0
C(1:31)[e]	60	none	basolateral	0.2 ± 0.2†
C(35:170)[e]	60	none	basolateral	0.0 ± 0.0[e]
C(49:22)[e]	60	none	basolateral	n.d.

Note: The Caco-2 cell monolayers were stained with PI added at the apical side after:
[a] exposure for 20 min, or
[b] 60 min to chitosans in HBSS pH 5.5, or
[c] exposure for 20 min and subsequent replacement of apical chitosan solution with HBSS pH 5.5 without heparin, or
[d] with 52 μg/ml (10 U/ml) heparin.
[e] Monolayers were stained with PI added to the basolateral side after exposure to apically added chitosan for 60 min. Three areas of 0.45 mm^2 were calculated on each of 2-3 different filters, giving n = 6–9. The percentage of stained cells is given as the mean ± s.d.
*Significant different from control, or
**Significantly different from treatment[a], or
***Significantly different from treatment[c] ($P < 0.05$, one factor Anova).
† Significantly different from treatment[b] ($P < 0.05$, Mann-Whitney).

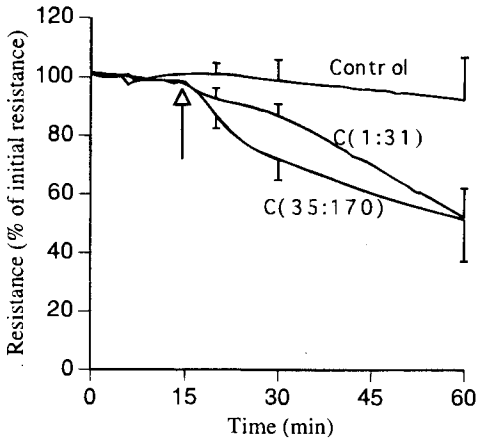

Fig. 2. Effects of 50 μg/ml chitosans on the transepithelial electrical resistance of Caco-2 cell monolayers. Chitosans were added apically after 15 min equilibration in HBSS solutions (arrow). The data are given as the means of 4 experiments. Error bars indicate standard deviations at 3 representative time points.

Table 2. Effect of Chitosans at a Concentration of 50 μg/ml on Mannitol Transport Across Caco-2 Cell Monolayers

	Papp × 10^7 (cm/sec)[a]			
Chitosan	Treatment B[b]	Treatment C[c]	Treatment D[d]	Treatment E[e]
None	0.9 ± 0.3	1.1 ± 0.06	1.5 ± 0.2	1.1 ± 0.06
C(1:31)	2.8 ± 0.03[f]	1.5 ± 0.08	9.0 ± 4.7[f]	3.1 ± 1.1
C(35:170)	10.1 ± 2.2[f]	0.8 ± 0.2	22.9 ± 4.4[f]	10.1 ± 1.5[f]

Note: The Papp values are given as the mean ± s.d. of 4 experiments.
[a] Mean Papp values are calculated for 60 min during continuous exposure to chitosans in treatment B and C, and during the recovery period after exposure to chitosans for 20 min in treatment D and E.
[b] Continuous exposure to chitosans without heparin.
[c] Continuous exposure to chitosans with 52 μg/ml heparin.
[d] Pretreatment with chitosans for 20 min. recovery for 60 min in HBSS.
[e] Pretreatment with chitosans for 20 min, recovery for 60 min in HBSS with 52 μg/ml heparin.
[f] Significantly different from control ($P < 0.05$), one factor Anova.

Table 3. Transport of FD-4 and FD-20 Across Caco-2 Cell Monolayers

Chitosan	FD-4 Papp $\times 10^7$ (cm/sec)[a]	FD-20 Papp $\times 10^7$ (cm/sec)[a]
none	0.11 ± 0.003	0.11 ± 0.01
C(1:31)	0.46 ± 0.06[b]	0.14 ± 0.02[b]
C(35:170)	0.33 ± 0.06[b]	0.19 ± 0.006[b]

Note: Chitosans were used at a concentration of 50 μg/ml.
[a] The mean Papp values for FD-4 and FD-20 were calculated over a 60-min period. The Papp values are given as the means ± s.d. of 4 experiments.
[b] Significantly different from control ($P < 0.05$, one factor Anova.)

exposure, Papp values were only 1.3 and 1.7 times larger than control values for 50 μg/ml C(1:31) and C(35:170), respectively (Table 3).

Transport Route of Hydrophilic Marker Molecules

Transport of FD-4 and FD-20 across the Caco-2 cell epithelium was demonstrated using CLSM. This technique has proven very useful in the elucidation of transport pathways through mucosal tissue (11,15,17). CLSM pictures were taken after incubation of the monolayers with FD-4 or FD-20 with or without C(1:31) or C(35:170). Typical examples of CLSM images are shown in Fig. 3. In control monolayers, FD-4 permeated Caco-2 cell monolayers via the paracellular route. Intracellular uptake of fluorescence was not generally observed. FD-20 was detected intercellularly and to a small extent also intracellularly. Addition of the chitosans to the FD solutions resulted in increased intracellular uptake of fluorescence. Most of the FD-4 and FD-20, however, remained restricted to the intercellular space. Some cells (5–10%) showed a very strong uptake of the fluorescence-labelled dextrans (Fig. 3).

DISCUSSION

In summary, this investigation revealed that two chitosans with different chemical composition and molecular weights, which induce absorption enhancement with different kinetics and display different toxicities (8), have very similar mechanisms of action at the cellular level. Thus, both chitosans bound to the epithelial cell membrane through a charge-dependent mechanism, resulting in F-actin depolymerization and disbandment of the tight junction protein ZO-1. The binding of the chitosans to the membrane resulted in enhanced permeability of the apical membrane and tight junctions. Since no increase in the basolateral membrane permeability was observed, we conclude that the enhancement of absorption occurred through the paracellular pathway, a finding corroborated by the demonstration of paracellular transport of marker molecules in the confocal microscope and by earlier studies using the transmission electron microscope (8).

Due to ion condensation, one would expect that the difference in the effective charge densities for chitosans are higher between C(1:31) and C(35:170) than between C(35:170) and the non-binding C(49:22) (18). Therefore, the present results may be explained by more specific binding of chitosans to the cell surface (receptors) where consecutively GlcN-units are involved. In addition, a large increase in lysozyme degradation rates of partially N-acetylated chitosans with increasing Da has been found (19) meaning that lysozyme degradation rates of C(49:22) would be about 4 times higher than C(35:170). This may explain why C(49:22) does not bind to the cell surface.

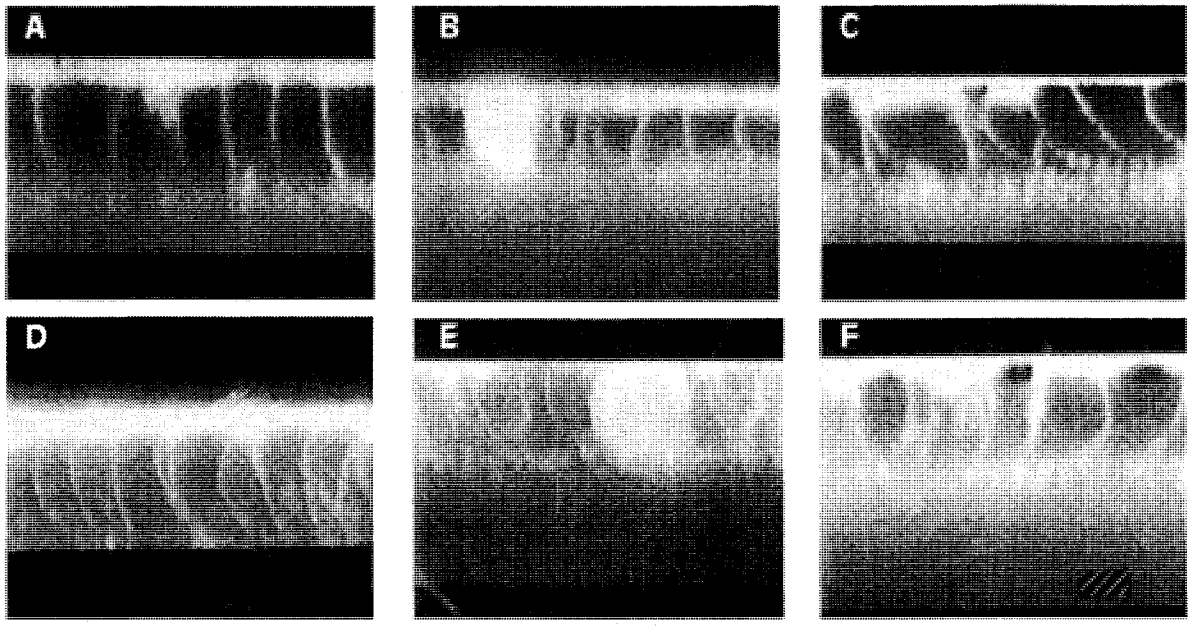

Fig. 3. Confocal micrographs of Caco-2 cell monolayers after 60 min incubation with (A) 10 mg/ml FD-4; (B) 10 mg/ml FD-4 and 50 μg/ml C(1:31); (C) 10 mg/ml FD-4 and 50 μg/ml C(35:170); (D) 10 mg/ml FD-20; (E) 10 mg/ml FD-20 and 50 μg/ml C(1:31); and (F) 10 mg/ml FD-20 and 50 μg/ml C(35:170). The pictures represent optical vertical cross sections with a step size in the z-direction of 0.24 μm. Top is apical, bottom is basolateral. The scale bar indicates 20 μm. See text for description of the pictures.

Two clear differences between the two chitosans with absorption enhancement activity were found. Firstly, the previously observed difference in onset of action between the two chitosans was demonstrated more clearly using electrophysiological measurements. Secondly, the cell surface binding of the chitosan with the more rapid onset of action [C(35:170)] resulted in increased apical membrane permeability in a larger percentage of cells than was apparent in C(1:31). The addition of the counter-ion heparin reversed this effect by no more than about 30%. In contrast, the increase in apical membrane permeability induced by the chitosan with a slower onset of action [C(1:31)] was reversed by almost 70% after addition of heparin. We tentatively conclude that although the positive charge is important for the cell surface binding and chitosan effects, the higher proportion of N-acetylated monomers in C(35:170) results in modified binding to the cell surface, possibly due to lower positive charge density, or to steric effects caused by the larger proportion of acetyl moieties. This chitosan has different intracellular signalling, and a more rapid onset of action and lower toxicity as a result.

The effects of C(1:31) and C(35:170) on the proteins of Caco-2 cell monolayers associated with tight junctions were similar to the effects reported for other polycations, such as poly-l-lysine and poly-l-arginine, on epithelial monolayers of MDCK cells (20). In addition, changes in the electrical properties of the monolayers, such as an immediate decrease in TER and increases in Isc and PD, were similar. These effects thus appear to be shared by several polycations. In the same study, the number but not the type of cationic moieties presented to the epithelium was found to be the determining factor for the extent of effect (20). Reduced paracellular resistance was only found with poly-L-lysine after increases in the permeability of the apical epithelial cell membrane. This suggests that changes in paracellular barrier properties occur as a cellular action mediated by polycations rather than as a consequence of direct action on the junctional complex (20).

Addition of heparin to the incubation solutions inhibited the effects of the chitosans on apical cell membrane permeability and tight-junction integrity. In addition, the enhancement of ^{14}C-mannitol absorption was abolished. We conclude that heparin neutralises the positively charged chitosans and thus inhibits their actions. Removal of chitosans from the incubation medium after a pretreatment period of 20 min did not reduce their effects. Addition of heparin after pretreatment with chitosans, on the other hand, reduced the effects on epithelial permeability and tight-junction structure to a large extent. Apparently, the chitosans bind to the cell epithelium during preincubation. After removal of the chitosans from the incubation medium, they do not readily dissociate from the cell membrane but continue to exert their effects. Addition of heparin to the incubation medium reverses chitosan binding, and consequently reverses chitosan actions on epithelial permeability. Binding of the chitosans to the Caco-2 cells thus precedes absorption enhancement, and is mediated by the positive charges on the polymer. In a study by Hammes and Singh, binding of polycations to cultured monolayers of glomerular epithelial cells could also be reversed by negatively charged heparin. Removal of the polycation after initial binding failed, however, to protect the monolayers from paracellular leakage of macromolecules (21). Leakage could be inhibited in this study by incubation at 4°C, despite equal cation binding at 4 and 37°C. Thus, these data also suggested the role of active intracellular processes in the impairment of tight-junction regulated epithelial permeability (21).

In conclusion, the results present for the first time a general mechanism of action for a new group of absorption enhancers, the chitosans. Only small differences in mechanism were observed for two chemically different chitosans with different absorption enhancing properties and toxicities. An explanation for these differences may be found after more extensive investigation of the intracellular responses to the chitosans, as has recently been undertaken for other series of absorption enhancers (22,23).

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