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Effect of chitosan on epithelial permeability and structure

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Abstract

Numerous studies have shown that chitosan, a mucoadhesive polymer, is a potential enhancer for transmucosal drug delivery. To further understand the mechanisms involved in chitosan action on the mucosal barrier, the activity of chitosan on the function and structure of monolayers of intestinal epithelial cells was investigated. In Caco-2 cells, chitosan caused a reversible, time and dose-dependent decrease in transepithelial electrical resistance. The effect of chitosan on tight junctions was confirmed by an increased permeability coefficient for mannitol transport when cells were treated with 0.1–0.5% w/v chitosan solution for 60 min compared to control cells. Involvement of tight junctions was visualized by confocal scanning microscopy using occludin and ZO-1, tight junctional proteins. Following an incubation with 0.01 or 0.1% w/v chitosan, labeling of both proteins varied in localization and decreased in fluorescent intensity at the cell periphery. In addition, a focal condensation of actin was observed preferentially at areas of cell-to-cell contacts. However, after 24-h recovery, the cell structure resembled untreated control cells. Simultaneous addition of cycloheximide, a protein synthesis inhibitor, prevented full recovery. This implied that protein synthesis was required for the cells to return to baseline levels. Chitosan treatment appeared to slightly perturb the plasma membrane as assessed by an increased release of lactate dehydrogenase. However, addition of 0.5% chitosan for 60 min did not affect cell viability as shown by Trypan blue dye exclusion. These data suggest that chitosan increases cell permeability by affecting paracellular and intracellular pathways of epithelial cells, in a reversible manner.

Keywords: Caco-2; Transport; Tight junction; Bioadhesive; Enhancer

1. Introduction

Chitosan, a high molecular weight cationic polysaccharide, has been reported to enhance the absorption of various compounds across the mucosal barrier. Illum et al. (1994) demonstrated the

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ability of glutamate chitosan to enhance the transport of insulin across the nasal mucosa of sheep and rat. The efficacy of chitosan as a nasal absorption enhancer was confirmed using salmon calcitonin in a rat model (Tengamnuay and Mitra, 1997). Chitosan hydrochloride has also been used to improve the bioavailibility of buserelin in rats, as described by Lueβen et al. (1996). The mechanism of action of chitosan was suggested to be a combination of mucoadhesion and an effect on the gating properties of tight junctions (TJ) (Artursson et al., 1994). It has also been shown that chitosan does not enhance drug absorption by reducing the metabolic activity of both intestinal proteases trypsin and carboxypeptidase B (Lueβen et al., 1997).

The objective of this study was to further elucidate mechanisms in a model of intestinal epithelium. Caco-2 cells represent a well-characterized in vitro transport model system for the small intestinal cellular barrier (Hidalgo et al., 1989). Therefore, we examined the effects of chitosan treatment at various times and concentrations on Caco-2 permeability and morphology by studying more complex aspects of the system not yet addressed in the limited data published on this subject (Borchard et al., 1996; Schipper et al., 1996; Lueβen et al., 1997). Namely, the requirement for protein synthesis was analyzed during the recovery process using cycloheximide as an inhibitor and plasma membrane perturbation was assessed by a lactate dehydrogenase (LDH) activity assay. In addition, an extensive study was performed by confocal scanning and electron microscopy to elucidate the effects of chitosan on cell morphology. In confocal scanning microscopy the label of three cell markers was followed: ZO-1, a TJ-associated protein (Stevenson et al., 1986), occludin, a transmembrane protein of the TJ (Furuse et al., 1993) and bodipy phalloidin to stain actin.

2. Materials and methods

2.1. Probes

Rabbit anti-occludin and rabbit anti-ZO-1 were obtained from Zymed Laboratories, San Francisco, CA. Cy-5 conjugated goat anti-rabbit IgG and FITC conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA. Texas Red®-X Phalloidin was obtained from Molecular Probes, Eugene, OR.

2.2. Chitosan solutions

Chitosan hydrochloride (Seacure CL110, degree of acetylation 20%) was obtained from Pronova, Portsmouth, NH and in this paper is referred to as chitosan. A 1.0% w/v stock solution of chitosan salt was prepared in water for injection. Chitosan solutions were prepared by combining chitosan stock solution, 2X Dulbecco's modified Eagle Medium (DMEM) and water. 2X DMEM was prepared from powdered medium (Gibco BRL, Grand Island, NY). This particular chitosan was soluble up to pH 6.5, therefore, all experimental solutions were prepared at pH 6.0–6.5. In addition, all solutions were iso-osmolar.

2.3. Cell culture

Caco-2 cells (originating from a human colorectal carcinoma) were obtained from the American Type Culture Collection, Rockville, MD. For electrical measurements, cells were seeded at a density of 2×10^5 cells/cm^2 on polyethylene teraphthalate filters (Falcon, Franklin Lakes, NJ) and cultured for 7–21 days. For fixed-cell confocal studies, Caco-2 cells were plated at a density of 700 cells/mm^2 on HTC Super Cured, 8-well slides (Cel-Line Associates, Inc., Newfield, NJ) for 3–4 days. Caco-2 cells were grown at 37°C/5% CO_2 in culture medium (CM; all ingredients were obtained from Gibco BRL) consisting of DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin-streptomycin and 1% Glutamax-1. Cells from passages 32–46 were used.

Low temperature and low pH have been reported to increase the monolayer permeability (Fischbarg and Whittembury, 1978; González-Mariscal et al., 1984, respectively). On that account, all experiments were performed at 37°C. All monolayers including control were preincu-

bated in pH 6.5 DMEM on the apical side 1 h prior to the experiments. The basolateral side of Caco-2 cells grown on filters was in contact with pH 7.4 DMEM.

2.4. Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TER) was measured using the Endohm™ tissue resistance measurement chamber coupled to an EVOM™ epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). All experiments were carried out in a 37°C/5% CO_2 incubator. An insert was placed in an Endohm™ chamber where it remained for the duration of the experiment and acted as an environmental control. The preincubation of Caco-2 monolayers with pH 6.5 DMEM alone resulted in a slight increase in TER. The resulting TER value was considered as the baseline in both control and treated monolayers. The medium was then changed to the appropriate chitosan solution apically. The experimental values of TER were expressed as a percentage of the baseline values. In all experiments, the control values remained within 5% of the baseline values. To dissociate the variations of resistance due to environmental conditions from the effects of chitosan, at each time point the differences of resistance in control cells were subtracted from those observed in treated cells for each set of experiments. The data were, therefore, expressed in net percent TER from baseline.

2.5. Dose/time/recovery studies

For dose/time studies, cells were incubated with various chitosan solutions and TER was measured every 15 min for 1 h. For time/recovery studies, cells were incubated with 0.00125 to 0.01% chitosan solutions. To address recovery following the 1 h time point, cells were washed twice with 0.1 M phosphate buffered saline, pH 7.4 (PBS) then incubated with CM at pH 7.4 and TER was measured at various time points up until 24 h.

2.6. Transport studies

2.6.1. Following chitosan treatment:

In 21-day-old Caco-2 cultures, the apical medium was changed to 0.01, 0.1 or 0.5% chitosan solutions containing 10 mM unlabeled mannitol and 0.33 μCi/ml ^{14}C-mannitol (New England Nuclear, Boston, MA).

2.6.2. Following recovery from chitosan treatment:

Twenty-one-day-old Caco-2 cultures were incubated with 0.005%, 0.1 or 0.5% chitosan solutions for 30 min. Cells were then washed twice with 0.1 M phosphate buffered saline, pH 7.4 (PBS) and incubated with CM at pH 7.4 for 24 h. The apical medium was changed to DMEM containing 10 mM unlabeled mannitol and 0.33 μCi/ml ^{14}C-mannitol.

All samples were taken from the basolateral sides of the membrane after 60 min and counted on a liquid scintillation counter (WALLAC, Inc., Gaithersburg, MD). Permeability coefficients (P) were calculated using Eq. (1):

$$P = \frac{dQ}{dt}\frac{1}{AC_0} \quad (1)$$

where dQ/dt is the flux across the monolayer, A is the surface area of the cell monolayer and C_0 is the initial concentration on the apical side of the membrane.

2.7. Protein synthesis inhibition

Time/recovery studies were performed as above on cells treated with 0.01 or 0.5% chitosan solution on the apical side. CM at pH 7.4 was used to wash the inserts twice and incubate the cells with or without 2 μM cycloheximide (Sigma, St. Louis, MO) during recovery. For these experiments, an additional control was used to monitor the effects of cycloheximide alone which consisted of three samples treated in the same manner as the experimental cultures recovering in 2 μM cycloheximide.

2.8. Lactate dehydrogenase release assay

Cells were treated with 0.005, 0.01 or 0.5% chitosan placed on the apical side of the mem-

brane for 30 min. To ensure that chitosan had the same effect as previously noted, TER was measured pre- and post-incubation. At the end of the 30-min incubation period, the monolayer was either used for LDH assay or rinsed and incubated in CM for recovery. Immediately following chitosan treatment or after recovery, the apical and basolateral media from individual wells were pooled and extracellular LDH activity was measured. All cell monolayers were treated with 0.1% Triton X-100 (Sigma) in PBS for 5 min, scraped from the permeable support membrane and centrifuged at $2000 \times g$ for 5 min at 4°C to evaluate the intracellular LDH activity. All samples were processed according to manufacturer's instructions (kit no. 340-LD, Sigma Diagnostics).

2.9. Trypan blue dye exclusion

Cells were treated with or without 0.005 or 0.5% chitosan as described in time/recovery studies. At each time point, cells were trypsinized and diluted in 0.4% Trypan blue dye solution (Sigma). Viable and non-viable cells were counted separately in a Neubauer chamber.

2.10. Immunocytochemistry

Cells were washed briefly with pH 6.5 DMEM and incubated with various chitosan concentrations for 60 min. After chitosan treatment, cells were processed either immediately or allowed to recover for 24 h in CM. All further experiments were performed at room temperature unless specified. Cells were washed and incubated with a fixative solution comprised of 3% formaldehyde (Polysciences Inc., Warrington, PA) in PBS for 30 min. Cells were washed three times with PBS and permeabilized with 0.2% Triton X-100 for 10 min. The wash was repeated and cells blocked with 5% each of normal goat serum (Jackson ImmunoResearch Laboratories, Inc.) and bovine serum albumin (Fraction V, Sigma) in PBS for 30 min. For dose/recovery studies, cells were treated with anti-occludin at 1:100 dilution overnight at 4°C or with anti-ZO-1 at 1:100 dilution for 60 min. Monolayers were washed with 0.5% Tween 20 (Sigma) in PBS and blocked as above. Cells were incubated in Cy-5 conjugated goat anti-rabbit IgG at 1:100 dilution and Texas Red®-X Phalloidin at 1:50 dilution for 60 min. Following three rinses with 0.5% Tween 20 in PBS, cells were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

2.11. Confocal analysis

The monolayers were viewed using a Leica TCS-4D confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with a 75 mW argon/krypton laser (Omnichrome, Chino, CA) and associated filters for simultaneous 488, 568 and 647 nm excitation. Serial sections of up to 0.5 μm thickness were observed through the monolayer and stacked together as a Z-section. The sections which contained label for ZO-1 and occludin were compiled to obtain a 3-D image of the entire junctional complex. For actin, basolateral sections were accumulated to form a 3-D image of the stress fibers while apical sections were stacked to acquire the actin ring. Micrographs were formatted in Adobe Photoshop.

2.12. Transmission electron microscopy

Time/recovery studies were performed as above on Caco-2 cells treated with 0.1% chitosan solution on the apical side for 30 min. Immediately after treatment or following 24-h recovery, cells were washed twice in PBS and fixed with 1% glutaraldehyde (Sigma)-2% paraformaldehyde (Polysciences) in PBS, pH 7.4 for 1 h at RT. Cells were thoroughly washed with PBS and processed for routine thin-section microscopy by Thomas A. Ardito, Boyer Center for Molecular Medicine, Yale University, New Haven, CT. In summary, samples were washed three times in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Fort Washington, PA), post-fixed in 1% osmium tetroxide buffered with 0.2 M *s*-collidine (Electron Microscopy Sciences) for 1 h at 4°C. After three rinses in 0.1 M *s*-collidine, cells were dehydrated in a series of graded ethanol to 100% ethanol, passed through two changes of propylene oxide before embedding in Epox-812 resin (Ernest Fullam, Latham, NY). Ultrathin sections were

performed on a Reichert Ultracut-E ultramicrotome (Reichert, Wien, Austria) and stained with aqueous uranyl acetate and lead citrate (Electron Microscopy Sciences). The sections were viewed on a Zeiss EM-910 electron microscope (Zeiss, Thornwood, NY).

2.13. Statistical analysis

All data are presented as mean ± S.E.M. Each graph is a composite of at least three experiments. Statistical evaluation of the effects of chitosan treatment on Caco-2 TER and LDH release was performed using a two way ANOVA with 95% confidence intervals and a least significant difference multiple comparison test. One way ANOVA with 95% confidence intervals was employed in the permeability studies.

3. Results

3.1. Dose/time studies

Chitosan caused an early and dose-dependent decrease in TER when placed apically (Fig. 1a). Even at the lower chitosan concentrations, there was a reduction in TER after only 15 min. At the highest concentration used in this experiment (0.01% chitosan), TER declined by 50% in 15 min and 75% after 1 h. However, additional studies showed no significant difference in the resistance values obtained between 0.1 and 0.5%, suggesting a threshold effect of chitosan above 0.1% (data not shown).

3.2. Recovery studies

To test the reversibility of chitosan modulation of cellular permeability, the recovery of the initial monolayer electrical resistance was monitored following cell incubation in CM without chitosan. In Caco-2 monolayers, the 1-h presence of apical chitosan resulted in a dose-dependent decrease of TER, which progressively returned to the baseline level throughout the remaining 24 h (Fig. 1b).

3.3. Transport studies

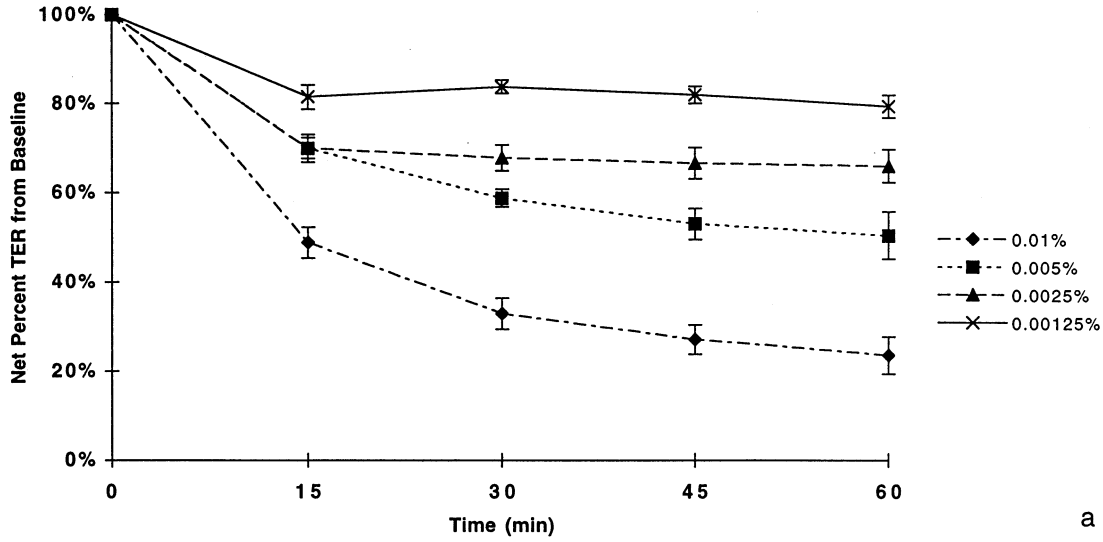
For a better understanding of chitosan alteration of epithelial barriers, we measured its effects on the permeability coefficient of mannitol, a marker of the paracellular transport. In control Caco-2 cultures, the coefficient was determined to be 0.29×10^{-6} cm/s (Table 1). Addition of 0.01–0.1% chitosan in the apical compartment resulted in a concentration-related rise in this permeability coefficient to achieve 4.38×10^{-6} cm/s at 0.1% chitosan. No significant difference in coefficient values was noticed between 0.1 and 0.5% chitosan. Following 24 h recovery, the permeability coefficient of mannitol was similar to that of control cells at the chitosan concentrations tested.

3.4. Protein synthesis inhibition

Since 30- and 60-min chitosan treatment induced a similar decrease in TER (see Fig. 1a), in the two following experiments, monolayers were treated for 30 min with chitosan. The extended time required for TER recovery observed in Caco-2 cells after chitosan incubation, prompted us to test whether protein synthesis was involved. Following chitosan removal, Caco-2 cells were incubated in the absence or presence of 2 μM cycloheximide, a protein synthesis inhibitor, during the 24 h recovery (Fig. 2). There was no significant difference in the recovery of Caco-2 cells treated with 0.01% chitosan with or without cycloheximide. However, protein synthesis was required after treatment with 0.5% chitosan.

3.5. Lactate dehydrogenase release assay-Trypan blue dye exclusion

The activity of LDH, a cytosolic enzyme released when cells are injured, was evaluated in the intra- and extracellular milieu. As reported in Table 2, Caco-2 cells exposed for 30 min to all chitosan concentrations had a slight increase in extracellular LDH activity. However, following 0.005 or 0.01% chitosan incubation, LDH activity at the 8 and 24 h recovery time points was similar to control. Despite a higher enzyme release observed in both recovery periods after 0.5% chi-



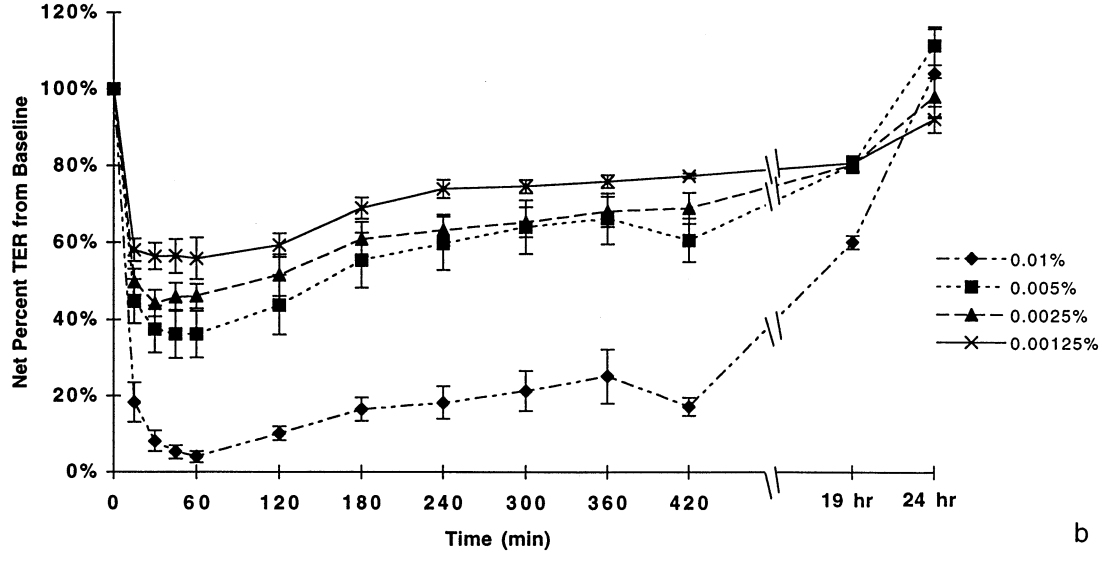


Fig. 1. Time course (a) and reversal (b) of chitosan effects on transepithelial electrical resistance in Caco-2 cells.TER baselines were 643 ± 8 and $1060 \pm 27\ \Omega \cdot \mathrm{cm}^2$ for time/dose study and recovery, respectively. $n \geq 10$.

tosan treatment, these data were markedly lower than those obtained subsequent to cell lysis (mean of 2824 ± 100 IU/l in both control and treated cells). In addition, plasma membrane integrity was also analyzed by performing Trypan blue dye exclusion which revealed 98% cell viability before and after 1-h exposure to both 0.005 and 0.5% chitosan concentrations.

Table 1
Mannitol transport

Sample ($n \geq 8$)	% Transport	P (10^{-6} cm/s)
Control	0.33 ± 0.04	0.29 ± 0.04
0.01%	1.40 ± 0.18	1.24 ± 0.16
0.10%	4.95 ± 0.56	4.38 ± 0.50[a]
0.50%	6.11 ± 0.90	5.42 ± 0.80[a]
Rec. 0.005%	0.35 ± 0.04	0.31 ± 0.03
Rec. 0.1%	0.30 ± 0.04	0.27 ± 0.03
Rec. 0.5%	0.30 ± 0.04	0.27 ± 0.03

[a] Significantly different from control and 0.01%.

3.6. Confocal microscopy

Confocal scanning microscopy was utilized to determine changes in structural proteins. Occludin, a transmembrane protein of TJ, and the TJ-associated protein, ZO-1, were found localized to a sharp, continuous band around the cell periphery in untreated cells (Fig. 3a,b). Filaments of actin, a cytoskeletal protein, were visualized throughout the cytosol as expected in control cells (Fig. 3c). After incubation with 0.1% chitosan, the pattern of occludin thickened at the cell periphery with a decreased fluorescent intensity in some areas (Fig. 3d). ZO-1, decreased in intensity and occasionally exhibited a diffuse staining within the cytoplasm (arrows, Fig. 3e). As shown in Fig. 3f, treatment with 0.1% chitosan also altered the fluorescent pattern of actin. There was a slight shortening of filaments and actin aggregates appeared specifically at cell-cell boundaries (arrows, Fig. 3f). However, the actin ring was not modified after treatment (data not shown). Even though 0.01% chitosan treatment caused a substantial decrease in TER (see Fig. 1), there were only slight differences in the actin pattern where the filaments seemed shorter (Fig. 3i) while occludin

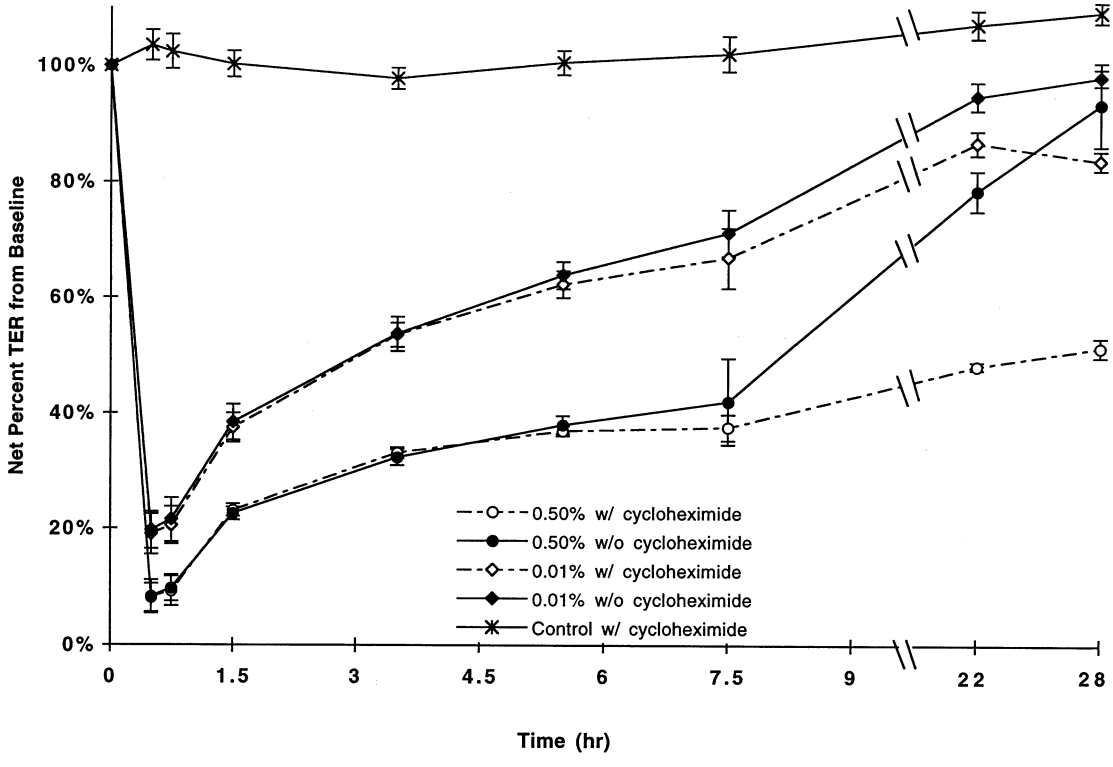


Fig. 2. TER recovery in the presence or absence of protein synthesis inhibitor, cycloheximide. TER baseline was $613 \pm 14\ \Omega \cdot \text{cm}^2$. $n = 9$.

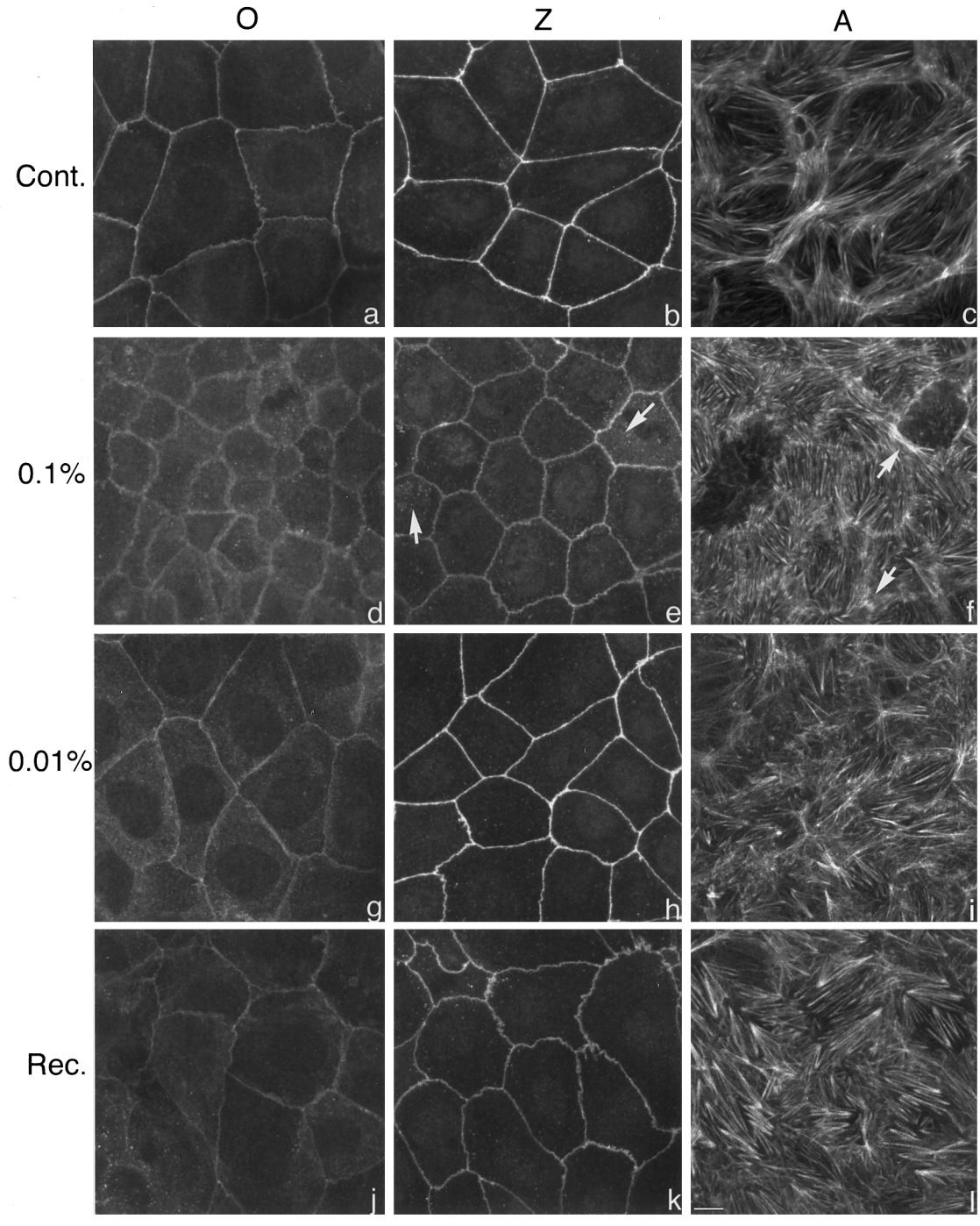


Fig. 3. Structural effects of chitosan on Caco-2 cells. Occludin (a,d,g,j), ZO-1 (b,e,h,k) and actin (c,f,i,l) were stained in control cells (1st row), cells treated with 0.1% (2nd row) or 0.01% chitosan (3rd row) or after 24 h recovery following a 0.1% chitosan incubation (4th row). Bar = 10 μm.

Table 2
LDH activity

Time	Sample ($n = 9$)	Extracellular (IU/l)
30 min	Control	9.49 ± 1.31
	0.005%	17.63 ± 1.99[a]
	0.01%	19.80 ± 2.64[a]
	0.50%	26.51 ± 3.42[b]
8-h Recovery	Control	24.60 ± 2.64
	0.005%	32.01 ± 1.25
	0.01%	36.17 ± 1.34
	0.50%	99.38 ± 7.93[c]
24-h Recovery	Control	32.88 ± 1.71
	0.005%	47.92 ± 4.97
	0.01%	58.10 ± 9.37
	0.50%	152.82 ± 16.80[c]

[a] Significantly different from the control.
[b] Significantly different from the control and 0.005%.
[c] Significantly different from the control, 0.005% and 0.01%.

and ZO-1 mimicked control cells (Fig. 3g–h). These data suggested that there were subtle changes below the level of detection by light microscopy. Following a 0.1% treatment and a 24-h recovery period (Fig. 3j–l), the cells displayed occludin, ZO-1 and actin patterns which resembled those observed in control cultures (Fig. 3a–c).

3.7. Transmission electron microscopy

Transmission electron micrographs of cells exposed to 0.1% chitosan for 30 min resulted in the appearance of large intracellular vacuoles (V) and swollen endoplasmic reticulum cisternae (arrowheads, Fig. 4b). However, the cells displayed a continuous apical membrane, normal microvilli, intact TJ (arrow) and organelles as observed in control cells (Fig. 4b vs. 4a). In addition, as confirmed by confocal microscopy, approximately 50% of the cells remained unaffected by 0.1% chitosan treatment. After 24 h recovery of 0.1% chitosan treatment (Fig. 4c), the cells had a morphology comparable to that observed in control cells.

4. Discussion

One of the limiting factors for the peroral delivery of peptides and proteins remains the poor permeability of these molecules across the intestinal epithelium. In order to enhance drug transport across the intestinal barrier many excipients have been considered. Chitosan has been shown to increase the absorption of diverse compounds in various in vivo models (Illum et al., 1994; Lueβen et al., 1996; Calvo et al., 1997; Odoriba et al., 1997; Tengamnuay and Mitra, 1997). To further understand the mechanisms involved in chitosan action on mucosal barrier, the activity of chitosan on the function and structure of monolayers of intestinal epithelial cells was investigated.

Our studies confirmed that chitosan increases cell permeability by affecting TJ. Indeed, the decrease in TER was associated with an increase in paracellular flux of the membrane-impermeant tracer, mannitol. In addition, as shown by confocal microscopy, staining for occludin and ZO-1 revealed a decrease in fluorescent intensity as well as a ZO-1 cytoplasmic localization in chitosan-treated monolayers versus controls. Furthermore, chitosan induced redistribution of F-actin. Specifically, discrete plaques of actin were identified and stress fibers were attenuated in the cells from chitosan-treated monolayers. Because actin has been shown to be important in regulating paracellular flow across cultured intestinal epithelia (Meza et al., 1980; Madara et al., 1986), the above effects of chitosan on epithelial barrier function might be due to a partial alteration of the cytoskeleton. Even though the mechanisms by which chitosan induces changes in cellular actin are still unknown, preliminary results demonstrated no difference in intensity between G- and F-actin (data not shown) suggesting that the assembly of monomeric G-actin to polymeric F-actin could not explain the results observed. The appearance of actin plaques at cell-cell boundaries could be associated with changes in focal adhesion. Further experiments are needed to investigate this hypothesis. The increased paracellular permeability was not accompanied by apparent changes in the junctional morphology as seen by

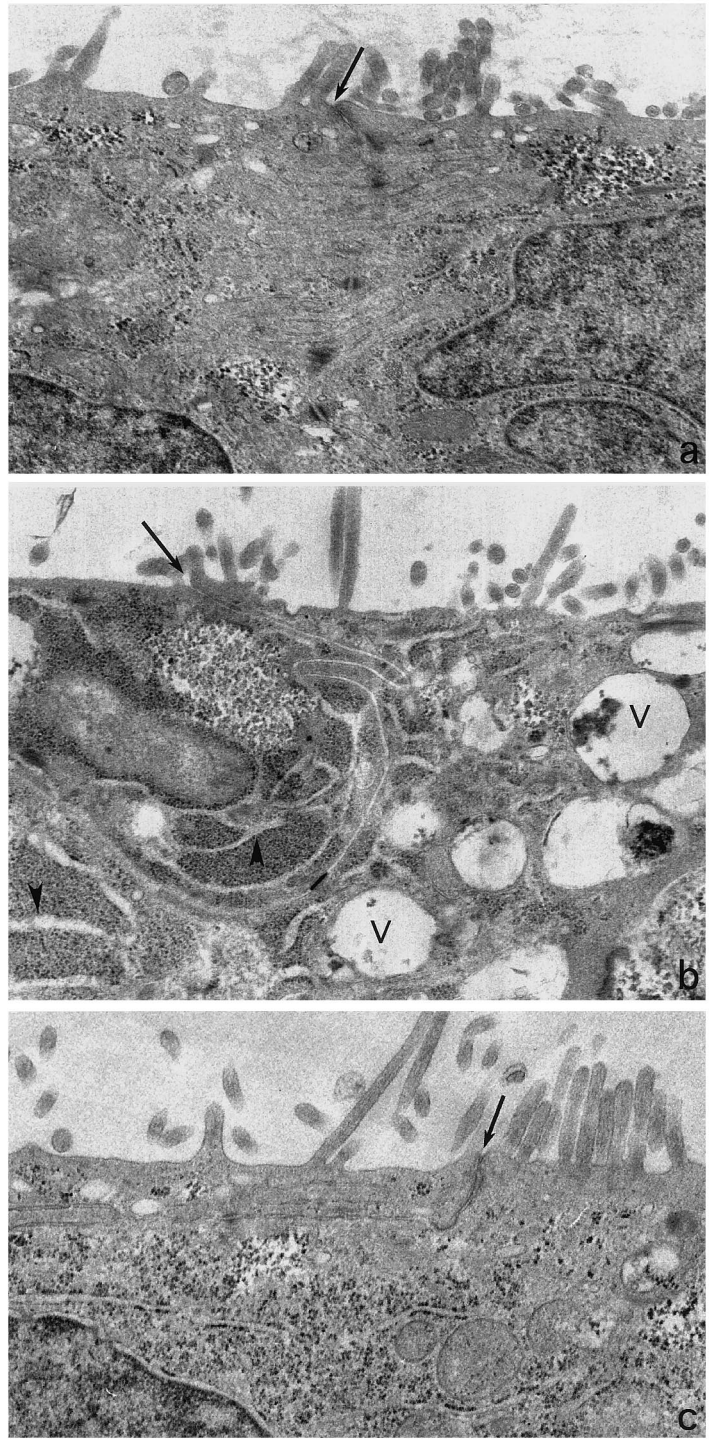


Fig. 4. Transmission electron micrographs of Caco-2 cells before (a), or after 30 min of 0.1% chitosan treatment (b) and 24 h recovery following 0.1% chitosan treatment (c). × 30 000.

electron microscopy. This observation, previously reported, reinforces the existence of additional factors in tight junction modulation such as, the number and the length of strands in the junctions, the existence of channels, the biochemical state of the junctional components and the regulation of the junctional complex by the cytoskeleton or secondary messenger systems (González-Mariscal, 1991).

The slight perturbation of the plasma membrane, as indicated by the rise in extracellular LDH release, implicates an increased intracellular uptake suggesting an action of chitosan on the intracellular pathway as well. These results correlate with those obtained by Schipper et al. (1996) where chitosan, with a degree of acetylation below 35%, elicited dose-dependent effects on dehydrogenase activity. The action of chitosan on the membrane integrity was confirmed by live cell studies performed using propidium iodide (PI), a fluorescent cell impermeant nuclear probe. Preliminary experiments demonstrated that within 40 min of 0.5% chitosan treatment, all cells displayed nuclear label, indicating a slight alteration of the epithelial membrane by chitosan (Koch et al., 1998). Despite this modification of membrane integrity, the need for de novo protein synthesis to obtain a complete recovery was observed solely following 0.5% chitosan treatment. As shown in Figs. 1–3, as well as Tables 1 and 2, the action of chitosan is dose-dependent. Therefore, it would stand to reason that a higher concentration of chitosan would require a more substantial mechanism to restore membrane integrity. No cell extrusion was observed by light, confocal scanning nor electron microscopy and cell death was not responsible for the increased permeability, as demonstrated by Trypan blue dye exclusion. The reversible effects of chitosan on permeability and structure of Caco-2 cells indicated that chitosan had a transient effect on the cellular barrier. All these data suggest that chitosan could be used as a permeability enhancer without causing membrane wounds such as the effect described after treatment with SDS (Anderberg and Artursson, 1993).

Electron micrographs were reviewed to determine whether there were toxic effects to the cells. No implication of damage was observed as shown by the normal appearing organelles and TJ. Two modifications of the intracellular milieu were the enlargement of the endoplasmic reticulum (a possible indication of increased cell metabolism) and the appearance of large vacuoles (a possible sign of enhanced endocytic activities). After extensive observation of monolayers by confocal and electron microscopy, it appears that chitosan incubation modified partial fields of monolayers, suggesting that only a certain population of cells was affected by the treatment. Additional experiments are currently being performed to address these issues.

In conclusion, this study demonstrates that chitosan increases cell permeability by affecting paracellular and intracellular pathways. Chitosan causes relatively mild and reversible effects on epithelial morphology which makes it a promising absorption enhancing compound for mucosal delivery of drugs. Moreover, the safety of ultrapure chitosan salts used in biomedical and pharmaceutical applications has been previously demonstrated (Dornish et al., 1997). Additional experiments are being pursued to further investigate chitosan mechanism of action which will facilitate its application in dosage form development.

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