Effect of Chitosan on Epithelial Cell Tight Junctions

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Purpose. Chitosan has been proposed as a novel excipient for transepithelial drug-delivery systems. Chitosan is thought to disrupt intercellular tight junctions, thus increasing the permeability of an epithelium. The effect of chitosan on tight junction complex was investigated at the molecular level.

Methods. Changes in barrier properties of Caco-2 cell monolayers, including transepithelial electrical resistance and permeability to horseradish peroxidase (HRP), were assessed in response to chitosan treatment. Changes in subcellular localization of the tight junction proteins zona occludens 1 (ZO-1) and occludin by immunofluorescence and Western blotting of cellular fractions were also assessed.

Results. Chitosan was found to cause a dose-dependent reduction in transepithelial electrical resistance of Caco-2 monolayers of up to 83%. A corresponding increase in horseradish peroxidase permeability of up to 18 times greater than the control was also observed across the monolayer. Immunofluorescent localization of ZO-1 revealed loss of membrane-associated ZO-1 from discrete areas. Analysis of cellular fractions revealed a dose-dependent loss of ZO-1 and occludin from the cytosolic and membrane fractions into the cytoskeletal fraction. These changes did not occur because of chitosan-mediated ATP depletion.

Conclusions. Chitosan-mediated tight junction disruption is caused by a translocation of tight junction proteins from the membrane to the cytoskeleton.

KEY WORDS: chitosan glutamate; tight junctions; TEER; ZO-1; occludin.

INTRODUCTION

Chitosan is the deacetylated product of the naturally occurring polysaccharide, chitin, which is commercially derived from crustacean shells (1). Partial deacetylation of chitin results in the production of chitosan, which is a linear polymer that consists of *N*-acetyl glucosamine and glucosamine units. Chitosan induces little cellular toxicity (2,3) and is naturally biodegradable because it is metabolized by lysozyme both *in vitro* and *in vivo* (4). As a result of its well-characterized biocompatibility, chitosan has been investigated for use in a number of pharmacologic applications (for review, see Ref. 5), including its use as a novel excipient for drug-delivery systems. The development of new transepithelial drug delivery routes across epithelia may increase the availability of many drugs. Drugs applied in this manner avoid hepatic first-pass metabolism and enzymatic degradation in the gastrointestinal system (6).

However, epithelial membranes provide a significant barrier to the free diffusion of substances across them. The presence of tight junctions between neighboring epithelial cells prevents the free diffusion of hydrophilic molecules across the epithelium by the paracellular route. The ability of chitosan to increase the permeability of model drug compounds across monolayers of cultured epithelial (Caco-2) cells has been investigated in numerous studies (7–10). Caco-2 cells, when grown to confluence and allowed to polarize, form intercellular tight junctions and therefore prevent the passage of substances across them. The prevention of passage of small electrolytes also leads to the generation of transepithelial electrical resistance (TEER) (11). Application of chitosan to Caco-2 monolayers results in a well-characterized decrease in TEER and a simultaneous increase in the permeability of model drug compounds. It is assumed that these chitosan-mediated events are caused by a disruption of tight junctions; however, little direct evidence exists to substantiate this assumption.

Tight junctions are located at the boundary between apical and basolateral domains in epithelial cells and appear as a continuous apical belt around the cell periphery. On a molecular level, tight junctions consist of a number of complexed membrane, (occludin, claudin, and junctional adhesion molecule) and cytoplasmic proteins (e.g., zona occludens 1 [ZO-1], ZO-2, ZO-3) and numerous other associated proteins (reviewed in Refs. 12 and 13). In intact tight junctions, these proteins are strongly associated with the plasma membrane. However, under conditions precluding tight junction formation, such as low extracellular calcium concentration or after ATP depletion, these proteins appear to be relocated from the membrane into other cellular compartments (14,15). The aim of the current study was to investigate on a molecular level the effect of chitosan on tight junction integrity in Caco-2 cells.

MATERIALS AND METHODS

Materials

Chitosan glutamate (Protasan UP G113, degree of deacetylaton = 85%, $\overline{Mw}$ = 128,000 Da, specific viscosity 12 mPas) was obtained from FMC BioPolymer AS (Oslo, Norway). Rabbit anti-ZO-1 was obtained from Zymed (San Francisco, CA, USA) and goat anti-occludin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti Rabbit-HRP and FITC and anti goat-HRP secondary antibodies were obtained from Sigma (Poole, Dorset, UK). All other standard reagents and chemicals were obtained from Sigma (Poole, Dorset, UK) unless specified otherwise.

Cell Culture

Caco-2 cells were obtained from European collection of cell cultures and used for experimental purposes between passages 45–55. Cells were grown at 37°C in 5% CO_2 in Modified Eagle's Medium (MEM) supplemented with 10% fetal calf serum, 0.5 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown to postconfluence before experimentation. For cell treatment, chitosan was dissolved in H_2O and diluted (1:1) in 2× MEM to obtain final concentrations of 0.05–0.5%. An acidified control sample was prepared (pH 6.3.) to match

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the pH of the highest (0.5%) chitosan sample to ensure that the changes following application of chitosan were not due to a change in pH.

TEER and Permeability Assays

1×10^5 Caco-2 cells per cm^2 were seeded on polycarbonate inserts with 0.4-μm pores (Costar, Cambridge, MA, USA) and medium was replenished every other day (16). Resistance across the insert membrane was measured using the Millicell ERS system (Millipore, Bedford, MA, USA). When resistance readings were between 1000–1500 ohms/cm^2 (typically between days 10–14) test solutions along with 100 μg/ml horseradish peroxidase (HRP) were applied to the apical chamber and TEER measured at 5, 15, 30, and 60 min. Data were expressed as a percentage of the initial (baseline) values. The presence of HRP in the basolateral medium after 1 h of treatment was determined by means of a HRP-sensitive colorimetric assay (11). The amount of HRP in the basolateral medium was determined by comparison with a HRP standard curve and data were expressed as a percentage of the total applied HRP. The use of culture medium as a solvent did not affect the colorimetric assay. Samples were incubated with substrate solution (50 mM sodium phosphate, 542 μM *o*-dianisidine dihydrochloride, 0.003%v/v hydrogen peroxide) at 37°C for 20 min. Fifty microliters of stop solution (0.1% sodium azide in phosphate-buffered saline [PBS]) was added and absorbance read at 450 nm. To monitor recovery, test solutions were removed from the cells after treatment and replaced with culture medium. TEER was monitored over the next 48 h. After 24 h of recovery, HRP was again applied to the apical surface of the cells and its presence in the basolateral medium assessed after 1 h. Data were analyzed using unpaired, two-tailed *t* tests.

Assessment of Cell Viability

After the 1-h treatment, Caco-2 monolayers were assessed for cell viability. Cells were removed from insert surface by trypsinization. Trypsin activity was quenched by scraping of the cells into MEM supplemented with 10% fetal calf serum to give rise to a single cell suspension. This was diluted 1:1 with Trypan blue dye solution and cells were viewed under an inverted microscope using a hemocytometer. Viable cells were detected by their exclusion of Trypan blue.

Immunofluorescence

Caco-2 cells were grown to confluence and treated as described above. Cells were fixed in 10% formalin, and permeabilized in 0.5% Triton X-100/PBS. Nonspecific binding was blocked in blocking solution (5% powdered nonfat milk in PBS) for 1 h. ZO-1 antibody at a concentration of 1:1000 was applied in blocking solution for 1 h followed by anti-rabbit-HRP at 1:1000. For actin staining, after the permeabilization step cells were incubated with FITC-conjugated phalloidin diluted 1:100 in PBS for 1 h at room temperature. Cells were viewed using a Leica TCS NT spectral confocal imaging system coupled to a Leica DM IRBE inverted microscope. Lamp intensity and exposure length were kept constant during capture of images to provide accurate information regarding intensity of staining. Images shown are representative of three independent experiments.

Preparation of Cell Fractions

Cells were grown to confluence and treated with chitosan. Cell fractions were prepared as described by Tsukamoto and Nigam (15). Monolayers were incubated in 0.5 ml of lysis buffer A (20 mM Tris, pH 7.5, 0.25 M sucrose plus Roche "Complete mini" inhibitors) for 30 min on ice. The lysates were centrifuged at 80,000 rpm (200,000g) in a Beckman Optima TL-Ultracentrifuge at 4°C for 10 min. The supernatant (the cytosolic fraction) was removed. The remaining cell pellet was washed 3× by resuspension in lysis buffer A and centrifugation as above. The pellet was then re-suspended in 200 μl of lysis buffer B (lysis buffer A containing 0.5% Triton X-100) and incubated on ice for 30 min. The suspension was centrifuged as described above and the supernatant (the membrane fraction) was removed. The remaining pellet was washed three times in lysis buffer B. Proteins were extracted from the remaining pellet by incubation in 100 μl of lysis buffer C (lysis buffer B containing 0.2% sodium dodecyl sulfate) for 1 h on ice followed by centrifugation. The supernatant (the triton-insoluble fraction) was removed.

Western Blotting

Equal amounts of protein (approx. 20 μg) were separated by 6% (for ZO-1) or 10% (for occludin) sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transfer of proteins onto nitrocellulose membrane, blots were blocked in 5% nonfat powdered milk in TBS–0.05% Tween. Primary antibodies (ZO-1 at 1:3000 overnight at 4°C, occludin 1:1000 for 1 h at room temperature) were applied followed by appropriate secondary antibodies (anti-rabbit-HRP at 1:10,000 and anti-goat-HRP at 1:5000 respectively, for 1 h at room temperature). Bands were visualized using an enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Inc.) and exposure in a Fujifilm LAS1000 Intelligent Dark Box and captured digitally. Images shown are representative of three independent experiments.

ATP Assay

Assessment for intracellular ATP was performed using a commercially available kit (Enliten™, Promega, Southampton, UK). Treated Caco-2 monolayers were lysed in 0.1% TCA and neutralized by dilution 1:25 in 100 mM Tris-acetate. Luciferin/Luciferase test solutions (supplied in kit) were added to lysates automatically in a Mediators-PHL Luminometer. Samples were read after a 10 s lag. ATP concentration was expressed in arbitrary luminescence units.

RESULTS

Chitosan Caused a Dose-Dependent Decrease in Tight Junction Integrity

Treatment with control or acidified control media did not significantly affect TEER. However, treatment with 0.05, 0.1, and 0.5% solutions of chitosan resulted in reductions in TEER of 12, 54, and 83%, respectively, after 60 min (Fig. 1). Changes in TEER after treatment with 0.1 and 0.5% chitosan were highly significant compared with control ($p = 4.5 \times 10^{-4}$, 4.6×10^{-9}, respectively). A dose-dependent increase in permeability of the monolayer was also observed after treatment.

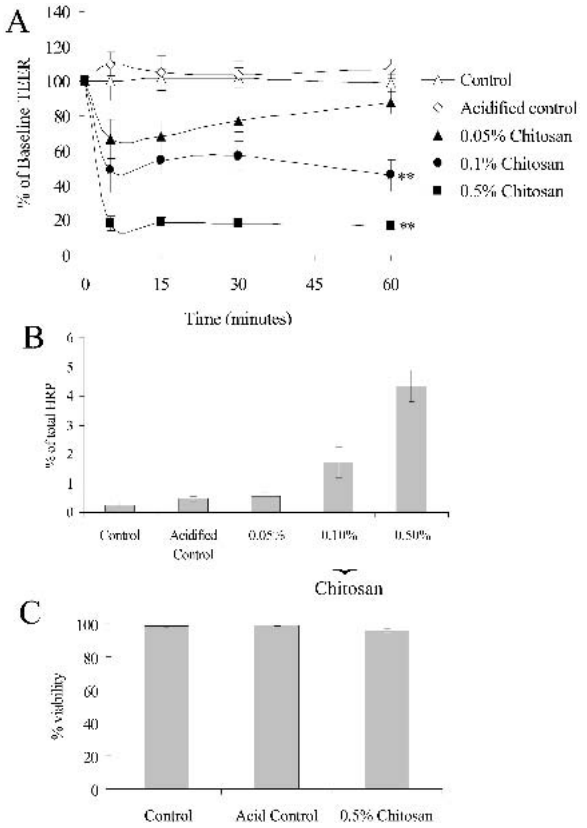

Fig. 1. Dose-dependent effect of chitosan on Caco-2 monolayer barrier properties. Caco-2 monolayers were treated with control, acid control, and chitosan at 0.05%, 0.1%, and 0.5%, and the change in resistance was measured over 1 h (A). The passage of HRP across the monolayers (B) and changes in viability (C) over 1 h were also assessed. n ≥ 6 ± SEM *p > 0.05, **p > 0.01.

Only 0.25 and 0.47% of the total applied HRP permeabilized across control and acidified control treated monolayers, respectively, over 1 h. However, incubation with 0.05, 0.1, and 0.5% solutions of chitosan resulted in passage of 0.58, 1.33, and 4.5% of total applied HRP, respectively. The passage of HRP in response to 0.1 and 0.5% solutions of chitosan were significantly greater than the control (p values of 0.018 and 1.96×10^{-5}, respectively). The viability of treated cells was not significantly different from control (Fig. 1C), indicating that loss of cell viability was not responsible for this dose-dependent response.

Recovery of Chitosan-Exposed Caco-2 Monolayers

The TEER of control monolayers did not change significantly from baseline TEER over 48 h. 0.1% chitosan-treated monolayers fully recovered their barrier properties by 24 h as assessed by TEER and 0.5% chitosan-treated monolayers recovered over 80% of their baseline resistance values within 48 h (Fig. 2A). A corresponding assessment of the permeability of the monolayer to HRP was conducted after 24 h. The permeability of the 0.5%-treated monolayers to HRP had fully recovered to control values after 24 h of recovery as indicated by the reduced passage of approximately 0.36% of total applied HRP (compared to 4.5% immediately after 1 h of treatment with chitosan; Fig. 2B). This was not significantly different from the control (p = 0.438).

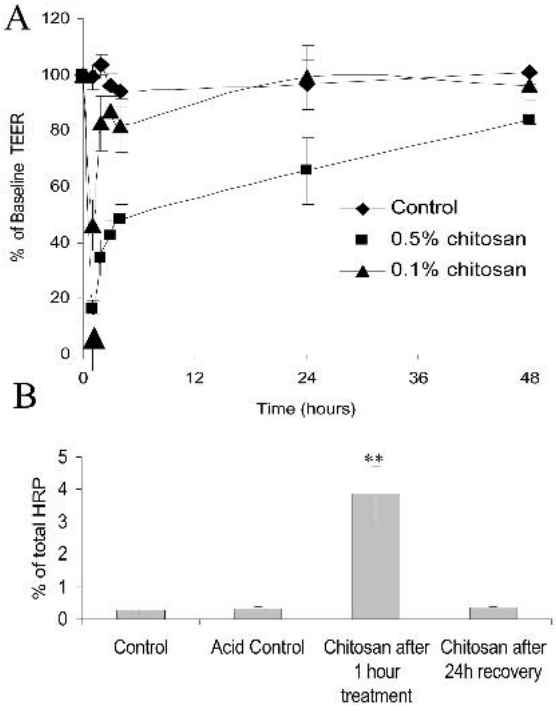

Fig. 2. Recovery of Caco-2 monolayer barrier properties after removal of chitosan. After 1 h of treatment with control or chitosan at 0.5% and 0.1%, the medium was changed and chitosan washed off (arrow) and recovery of monolayer TEER was monitored over time (A). Permeability to HRP was assessed after treatment of 1 h with control, acid control, and 0.5% chitosan and 24 h after recovery from treatment with 0.5% chitosan (B). n ≥ 6 ± SEM **p > 0.01.

Changes in Caco-2 Cell Actin Architecture Mediated by Chitosan

Treatment with 0.5% chitosan had no effect on actin ring integrity compared to control and acidified control samples (Fig. 3). The actin ring located at the apical side of the cell monolayers was continuous around the periphery of individual cells. Some areas of actin ring staining were weaker than others but these were consistently distributed between cells regardless of treatment. After treatment with 0.5% chitosan, there was a slight increase in perinuclear actin localization (arrowheads). The arrangement and localization of the basolateral microfilament was also investigated. In control cells, these actin filaments were laterally arranged and relatively long. Some truncation of these filaments was observed in both acidified control treated cells and cells treated with 0.5% chitosan although no changes in distribution were observed.

Changes in the Immunofluorescent Staining Pattern of ZO-1 After Treatment with Chitosan

Caco-2 monolayers were treated with control, acid control or 0.5% chitosan for 1 h and immunofluorescently stained for ZO-1. ZO-1 localization in control and acidified control cells was consistent with the plasma membrane and delineated points of cell-cell contact around the periphery of the cells at the apical surface (Fig. 4A and B). After treatment with 0.5% chitosan, some loss of continuity of staining was

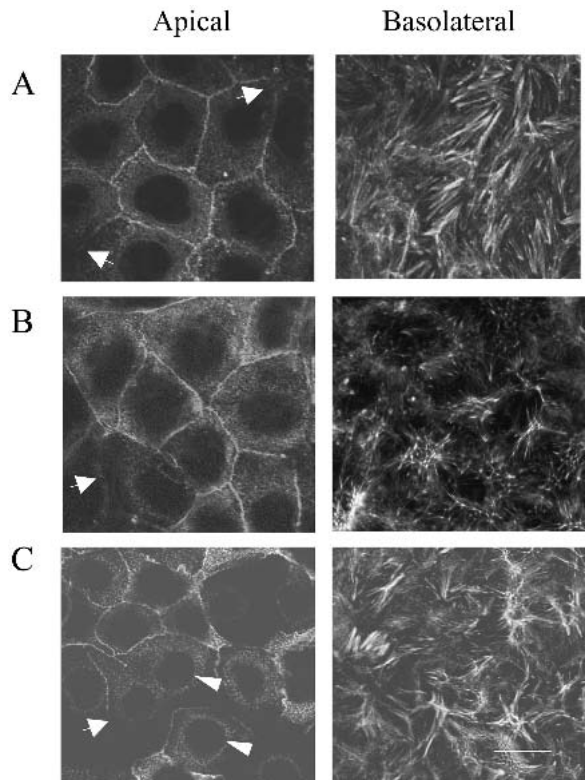

Fig. 3. Changes in actin architecture mediated by chitosan. Control (A), acid control (B), or 0.5% chitosan (C) was applied to Caco-2 monolayers for 1 h and cellular actin stained with FITC-phalloidin. Apical actin ring (left) and basolateral (right) actin filaments are shown separately. Arrowheads represent areas of increased perinuclear staining. Bar = 10 μm.

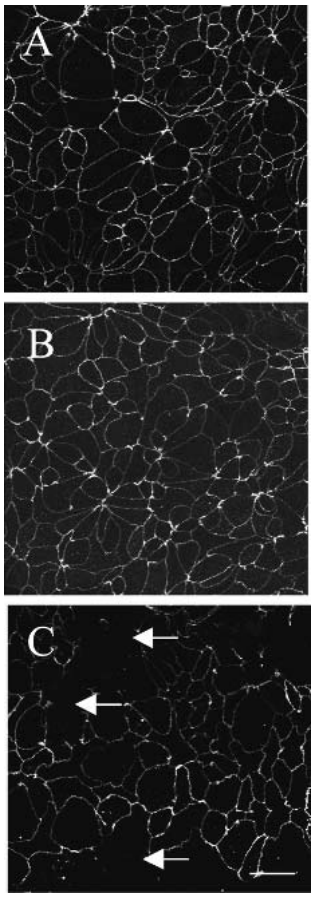

Fig. 4. Changes in the subcellular distribution of ZO-1. ZO-1 subcellular distribution in control cells (A) and acid control cells (B) is continuous and delineates the points of cell–cell contact. After treatment with 0.5% chitosan (C) ZO-1 staining is less intense and some punctate peripheral staining can be observed (arrows). Bar indicates 10 μm.

observed (arrows) possibly, indicating loss of tight junction function in these localized areas (Fig. 4C).

Redistribution of ZO-1 and Occludin After Treatment with Chitosan Glutamate

Caco-2 monolayers treated with control medium or chitosan at 0.05, 0.1, or 0.5% were fractionated into cytosolic, membrane, and triton-insoluble fractions and western blotted for ZO-1 (Fig. 5) or occludin (Fig. 6). Both ZO-1 and occludin appeared predominantly in the membrane fraction of control cells with a small amount present in the cytosolic and triton-insoluble fractions. A dose-dependent loss of ZO-1 and occludin from both the cytosolic and membrane fractions was observed after treatment with chitosan. A concurrent dose-dependent increase in intensity of the bands present in the triton-insoluble fraction was observed, possibly indicating an increased association with the cytoskeleton. After treatment with 0.05, 0.1, or 0.5% chitosan the amount of ZO-1 present in the cytosolic fraction had decreased to 83 ± 19%, 51 ± 8%, and 44 ± 10% of control values, respectively. A decrease of ZO-1 present in the membrane fraction was also observed with 77 ± 3%, 64 ± 6%, and 52 ± 8%, respectively, compared with the control. However, an increase in ZO-1 associated with the triton-insoluble fraction was observed. After treatment with 0.05, 0.1, and 0.5% chitosan, an increase to 111 ± 1%, 136 ± 14%, and 144 ± 14% of control values was observed.

A similar pattern of redistribution was observed with occludin. After treatment with 0.05, 0.1, and 0.5% chitosan, the amount of occludin associated with the cytosolic fraction decreased to 74 ± 12%, 66 ± 11%, and 38 ± 12%, respectively, of control values. A decrease in membrane-associated occludin was also observed with 81 ± 8%, 64 ± 8%, and 64 ± 3% of control values, respectively. An increase of occludin associated with the triton-insoluble fraction after treatment with 0.05, 0.5, or 0.5% chitosan was observed with 154 ± 16%, 221 ± 16%, and 215 ± 21% of control values, respectively.

Chitosan-Mediated Changes in ATP Levels

The pattern of ZO-1 distribution in chitosan-treated cells was reminiscent of the pattern of events associated with the ATP depletion model of tight junction disruption model (15). To investigate the possibility that chitosan may exert its effects on Caco-2 tight junctions via a depletion of intracellular ATP, the ATP content of chitosan-treated cells was investigated Intracellular levels of ATP in control and chitosan treated Caco-2 cells was assessed. Control and acid control cells contained similar amounts of ATP (Fig. 7). In addition, no significant change in ATP levels was observed in Caco-2 cells treated with 0.05, 0.1 and 0.5% chitosan ($p = 0.178$, 0.725, and 0.256, respectively, compared with control).

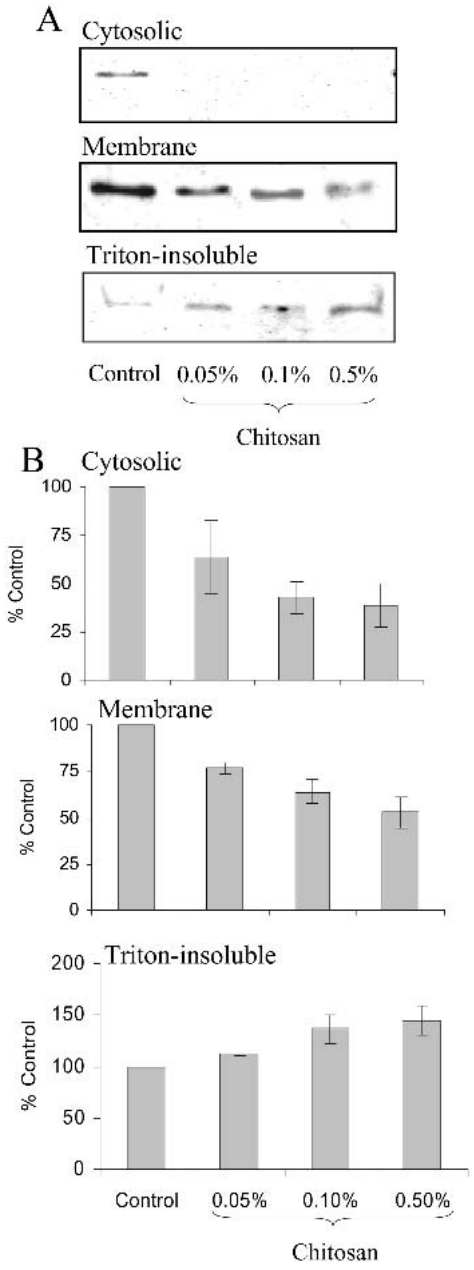

Fig. 5. Redistribution of ZO-1 after treatment with chitosan. Caco-2 monolayers were treated with control medium or chitosan for 1 h and fractionated into cytosolic, membrane and cytoskeleton fractions and ZO-1 detected by western blotting (A). Blots were quantitated by densitometry and expressed as percentage of the control. n = 3 ± SEM (B).

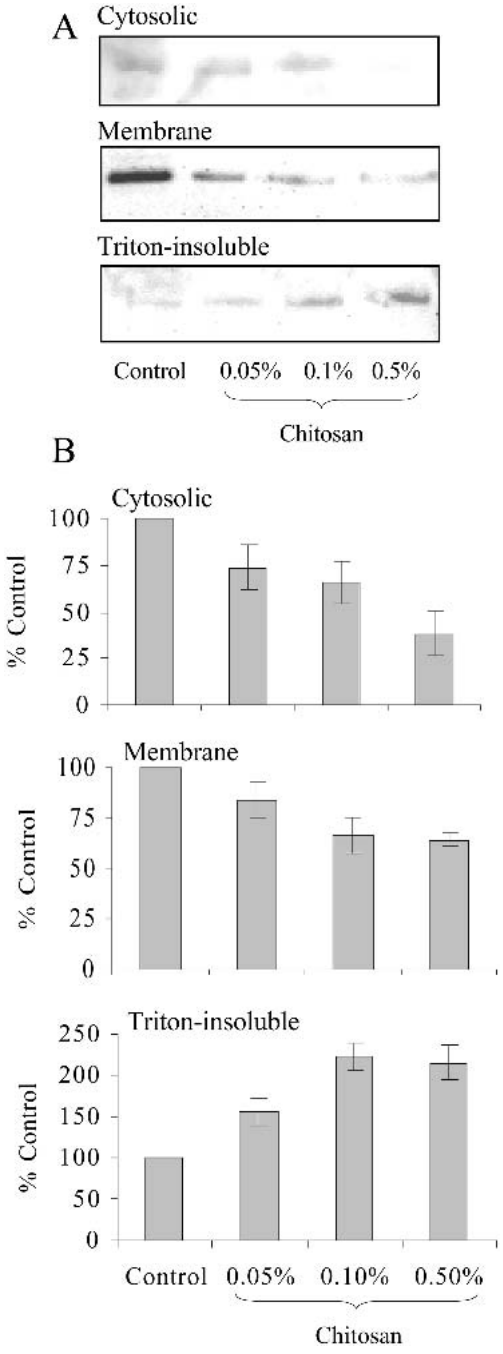

Fig. 6. Redistribution of occludin after treatment with chitosan. Caco-2 monolayers were treated with control medium or chitosan for 1 h and fractionated into cytosolic, membrane, and cytoskeleton fractions and occludin detected by western blotting (A). Blots were quantitated by densitometry and expressed as percentage of the control. n = 3 ± SEM (B).

DISCUSSION

Previous studies have not investigated in detail the tight junction events that are thought to accompany chitosan-mediated changes in barrier properties. Data presented here show clear evidence that chitosan causes tight junction disruption at the molecular level.

The effect of chitosan on Caco-2 monolayer tight junction integrity, as assessed by changes in TEER and permeability of HRP, was found to be dose-dependent up to a concentration of 0.5% (Fig. 1). This was not caused by the acidity of the chitosan samples (pH 6.3) as treatment with the "acidified control" solution also at pH 6.3 had no significant effect either on TEER or the permeability of HRP. The extent of the TEER response to concentrations of chitosan of up to 0.5% was consistent with previously published observations (9,17). However, Ranaldi *et al.* (10) observed similar reductions in TEER (approx. 80%) with concentrations of chitosan as low as 0.01%. This may be explained in part by the lower molecular weight chitosan used by Ranaldi *et al.* (approx.

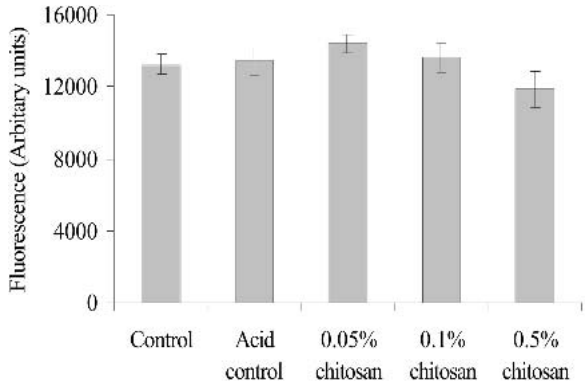

Fig. 7. Chitosan mediated changes in ATP levels. Caco-2 monolayers were treated and ATP content was assessed. n = 6, ± SEM.

25,000 Da) compared with molecular weight of 128,000 Da in the current study. In a comparison of chitosans with different molecular weights Holme *et al.* (9) demonstrated that molecular weight did affect the extent of the chitosan-mediated decrease in TEER. This variation may be explained by the greater solubility of lower molecular weight chitosan, which may facilitate closer interaction with the surface of the epithelial cells. Other factors that appear to affect the extent of the response to chitosan include degree of deacetylation. The general consensus regarding this variable appears to be that a degree of deacetylation of higher than 80% provides the greatest effect on cells in culture and is consistent among the majority of articles in this field.

Lack of recovery of barrier properties after treatment of an *in vivo* epithelium could lead to problems such as increased risk of infection of the area. Therefore the ability of Caco-2 cells to recover from treatment with chitosan was investigated. The permeability of HRP across chitosan-treated monolayers had reduced to control values after 24 h of recovery (Fig. 2B). However complete recovery of TEER (i.e., return to 100% baseline TEER) was not observed during the 48-h observation period. This is consistent with previous studies (17) in which chitosan-treated monolayers reached a maximum of 80% of their initial baseline value after full recovery. This discrepancy may be the result of the greater ease of passage of smaller electrolytes through newly forming tight junctions compared to the passage of the relatively large protein, (40 kDa) HRP. Recovery may have been impeded by the action of a chitosan residue because of its inability to completely remove all chitosan from the surface of the cell, and that this may have been exacerbated by the use of highly deacetylated chitosan samples that display greater mucoadhesion.

Reorganization of the actin ring (18,19) results in loss of tight junction integrity. Chitosan was found not to affect the actin ring of Caco-2 cells (Fig. 3). This is consistent with a study by Dodane *et al.* (8) but inconsistent with studies by Ranaldi *et al.* (10) and Artursson *et al.* (20), who demonstrated a decrease in fluorescence intensity of actin staining in Caco-2 cell actin ring after treatment with chitosan chloride and glutamate respectively. Dodane *et al.* (8) also reported shortening of the basolateral actin filaments and appearance of actin aggregates after treatment with chitosan. This is consistent with the data shown in Fig. 3 where some shortening of the basolateral filaments was also observed. The lack of reorganization of the actin ring suggests that chitosan does not affect tight junctions via a change in actin architecture. The relevance of the changes in basolateral filaments with regard to tight junction integrity remains to be established.

The effect of chitosan on tight junction proteins was examined to attempt to clarify the mode of action. The loss of ZO-1 immunofluorescence from conserved membrane regions in chitosan-treated Caco-2 cell monolayers indicates a loss of tight junction integrity and therefore barrier properties in these areas and was consistent with data presented by Ranaldi *et al.* (10). Further evidence for a change in subcellular location of the tight junction protein ZO-1 was provided by the Western blotting of Caco-2 cell fractions. The ZO-1 content of the cytosolic and membrane fraction decreased with treatment with chitosan in a dose-dependent manner (Fig. 5). Concurrently, the ZO-1 content of the triton-insoluble (cytoskeleton) fraction increased in a dose-dependent manner, indicating that ZO-1 translocates from the membrane to the cytoskeleton in response to treatment with chitosan. This trend was true also of occludin (Fig. 6).

This shift of ZO-1 and occludin into the cytoskeleton fraction is also a phenomenon observed in Caco-2 cells following ATP depletion (15,21). This was explained in these studies by increased association of ZO-1 with fodrin in a location immediately beneath the plasma membrane. If this is true in the present study, the close proximity of the fodrin-ZO-1 complex to the plasma membrane may explain why minimal changes in ZO-1 immunofluorescence were observed. This lead to speculation that chitosan may cause a depletion of intracellular ATP. A previous study by Lekka *et al.* (22) reported a cell-specific, chitosan-mediated decrease in intercellular ATP. However a decrease in intracellular ATP was not observed in Caco-2 cells in response to chitosan (Fig. 7).

In conclusion, the mode of action of chitosan with regard to its ability to disrupt epithelial cell tight junctions has been shown to involve the translocation of the proteins ZO-1 and occludin from the plasma membrane where they are available to form tight junctions, into the triton-insoluble fraction.

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