JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 909–915
© 2006 The Authors
Received January 5, 2006
Accepted March 20, 2006
DOI 10.1211/jpp.58.7.0005
ISSN 0022-3573

Transepithelial transport of morphine and mannitol in Caco-2 cells: the influence of chitosans of different molecular weights and degrees of acetylation

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Funding: This work was supported by grants from the Norwegian Research Council (139610/300,160579/V50, 164032/V50, 147218/320).

Conflicts of interest: OD, TN, GO, KT, DA and FS have no conflicts of interest.

Abstract

The object of this study was to compare the effect of chitosans of different number-average molecular weights (MWs) and degrees of acetylation (F_A) on transepithelial transport of morphine in Caco-2 cells. Caco-2 monolayers on polycarbonate (PC) membranes (0.5 cm^2) were incubated with morphine (10 μM) or mannitol (55 μM) for 180 min. Samples for analysis of morphine (LCMSMS) and mannitol (liquid scintillation) were drawn at 45, 90, 120 and 180 min. Transepithelial electrical resistance (TEER) and transmission electron microscopy were used to monitor cell integrity. In controls, morphine transport was half that of mannitol. Chitosans affected the transport of morphine and mannitol similarly. For chitosans with similar F_A (0.32–0.43) and varying MWs (7–200 kD), transport was increased at MWs of 29 kD or more. Among chitosans of similar MWs (180–300 kD) and varying F_A (0.01–0.61), those with the highest F_A (0.61) had the least effect, while chitosans with F_A/MW 0.01/250 and 0.17/300 promoted the greatest transport. An F_A/MW of 0.32/200 and 0.43/170 induced a high and stable transport rate. Chitosans may enhance transepithelial transport of morphine by the same mechanism as for mannitol. Chitosans with F_A of 0.3–0.4 and MW of approx. 200 kD seem favourable in this respect.

Introduction

Morphine is a first-line opioid for the treatment of cancer pain. More hydrophilic than other opioids, such as fentanyl and methadone, its uptake and onset of action is thus slower. The treatment of some pain conditions, such as breakthrough pain characterized by rapid onset and short duration, ideally requires a rapid onset of analgesia, which can sometimes be achieved by nasal administration, although morphine is not well absorbed in man (Illum et al 2002). The problem has been overcome by using absorption enhancers in a nasal morphine formulation; chitosans are promising in this respect (Artursson et al 1994; Illum 1998; Dodane et al 1999; Smith et al 2004).

Chitosans are a family of cationic polysaccharides containing amino groups with a pK_a of 6.5, and acetylated amino groups in varying proportions (Varum & Smidsrod 2005). They are able to attach to negatively charged surfaces, such as cells, DNA and certain proteins. Their properties are crucially affected by chemical composition, compositional distribution within and among the polymer chains, and molecular weight and molecular weight distribution (Varum & Smidsrod 2005). Chitosans have mucoadhesive properties, probably as a result of ionic interactions between their positively charged amino groups and negatively charged components (sialic acid) in mucus or on cell surfaces (Schipper et al 1996). They enhance the absorption of hydrophilic drugs across the nasal epithelium, probably via a combination of mucoadhesion and the opening of tight junctions in the epithelium (Artursson et al 1994; Illum et al 1994). A glutamate salt with a molecular weight (MW) of about 250 kD and a degree of acetylation (F_A) of more than 0.2 has been used for nasal delivery (Illum 2003). Improved methods for their production and characterization (MW and F_A) allow analysis of the significance of these factors for the biological properties of chitosans.

Caco-2, a cell line based on human colorectal carcinoma cells, is a common intestinal epithelium model for the study of transepithelial drug transport. Caco-2 cells undergo spontaneous differentiation when seeded onto permeable supporting membrane and the resulting homogeneous epithelial layer displays epithelial characteristics.

Mannitol is a metabolically inert and hydrophilic molecule. It is absorbed through the alternative aqueous paracellular pathway (i.e. through tight junctions) and is not significantly distributed into membrane cells (Artursson et al 1994), and can thus be used as a control for paracellular transport (Schipper et al 1997). Thus, the aim of this study was to investigate the influence of chitosan MW and F_A on the ability to facilitate transepithelial transport of morphine and mannitol.

Materials and Methods

Materials

The CACO-2 cells (cat. no. HTB-37; passage 20) were supplied by ATCC (VA). The chitosans of varying F_A were prepared by homogeneous deacetylation and subsequently converted to chitosan hydrochloride salts. Chitosans of varying MW were prepared by nitrous acid depolymerization and subsequent reduction (Anthonsen et al 1993) (Table 1). The number-average molecular weights of the chitosans (MW of 170 kD and higher, Table 1) were found by determination of the intrinsic viscosity, which was converted to number-average molecular weight as previously described (Anthonsen et al 1993). The number-average molecular weights of the chitosans (MW of 66 kD and lower, Table 1) were determined by SEC-MALLS as previously described (Fredheim & Christensen 2003). Morphine (cat. no. M-35-HC-10) was obtained from LIPOMED AG (Arlesheim, Switzerland). Fetal calf serum (FCS) (cat. no. PET 10106169), penicillin/streptomycin (cat. no. PL15140122), Dulbecco's modified Eagle's medium (DMEM) (cat. no. 21969-035), non-essential amino acids (NEAA) (cat. no. 11140-035) and Hank's balanced salt solution (HBSS) (cat. no. 24020-091) were obtained from Gibco BRL Life Technology (NY, USA). Trypsin/EDTA (cat. no. L11-004) was from PAA Laboratories GmbH (Linz, Austria). Insulin (cat. no. I-5500), L-glutamine (cat. no. G-3126), *N*-(2-hydroxyethyl)-piperazine-*N*-(2-ethanesulfonic acid (HEPES) (cat. no. H-4034), 2*N*-morpholino-ethanesulfonic acid (MES) (cat. no. M-8250), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) (cat. no. M-2128), NADH (cat. no. N-8129), EDTA di-sodium salt (Triplex III), Trizma base (cat. no. T-1503) and dimethyl sulfoxide (DMSO) (cat. no. D-8418) were from Sigma (MO). ^{14}C Mannitol (cat. no. ARC 127 A) was from American Radio Labeled Chemicals Inc. (MO). Scint Hei 3 (cat. no. 1007400) scintillation liquid was from Zinsser Analytic (Frankfurt, Germany).

Cell culture flasks, 24-well plates, cryotubes and the cell supporting membranes polycarbonate (PC) Nunc N-24137052, (ϕ10 mm, 0.4 μm pore size) were obtained from NUNC Brand Products (Denmark). Due to delivery failure from Nunc PC membranes, cell supporting membranes polyethylene terephthalate (PET) 353495 and 353095 (ϕ 6.2 mm, 0.4 μm pore size) from BD Biosciences (MA, USA) were also used in one set of experiments. Experiments to compare these supporting membranes were performed. The pH-electrode used was from Mettler Toledo 6030-M3/180/1M/BNC and the pH meter PHM 210 was from Radiometer, Copenhagen.

Table 1 Denomination and characteristics of chitosans

Chitosan	F_A[a]	MW (kD)
0.01/250	0.01	250
0.17/300	0.17	300
0.32/200	0.32	200
0.43/200	0.43	170
0.61/180	0.61	180
0.43/7	0.43	7
0.39/12	0.39	12
0.35/29	0.35	29
0.35/66	0.35	66

F_A, degree of acetylation; MW, number-average molecular weight.

Cell cultivation passage 20–45

Cells from ATCC (passage 20) were cultivated according to protocol: 5% CO_2 in DMEM containing 4.5 g L^{-1} glucose, 3.7 g L^{-1} sodium bicarbonate and 1 mM Na-pyruvate supplemented with 20% FCS, 1% NEAA, L-glutamine (2 mM), insulin (10 mg mL^{-1}) and 1% penicillin/streptomycin. Only cells from passages 35–45 were used.

Incubation of Caco-2 cells in polycarbonate well inserts

Cells were seeded (1.75×10^5 cells/insert) into the well inserts (0.4 μm PC membranes) in 24-well plates. Further cultivation in the same medium as above allowed the cells to spontaneously differentiate and polarize into the epithelial monolayer within 21 days. Cultivation medium was changed every 48 h. Cells were used for experiments after 21–23 days on membranes.

Preparation of chitosans/drug solutions

Chitosans (250 μg mL^{-1}) were dissolved in HBSS buffered with MES (20 mM, pH 5.7). The stock solutions were stored at −20°C. After appropriate dilution and the addition of the transport substrate morphine 10 μM or mannitol 55 μM, all solutions were filtered (0.2 μm, sterile). The specific activity of the purchased mannitol solution was 55 mCi mmol^{-1}. Non-radiolabelled mannitol was added to obtain a final specific activity of 13.6 mCi mmol^{-1}. The dissolution of chitosans in HBSS/MES was assessed by a ninhydrin test in both the filtered and the non-filtered solutions (Prochazkova et al 1999). No significant loss of

chitosans at the concentration of 250 μg mL^{-1} was observed (data not shown), indicating full dissolution of all chitosans at this pH.

Transport studies and sampling procedures

The transport study was performed on PC cell supporting membranes (Nunc) with the nine different chitosans listed in Table 1. Chitosans at two different concentrations (50 and 250 μg mL^{-1}) were tested. All experiments were done with four parallels on 24-well plates. In each plate four controls, containing all ingredients but no chitosans, were included. The cell epithelial layers were conditioned (by replacing the DMEM with HBSS/MES and HBSS/HEPES at the apical and basolateral side, respectively) and left for 15 min in at 37°C and 5% CO_2. Transepithelial electrical resistance (TEER) was measured (see below). The transport experiments were initiated by replacing the HBSS/MES solutions with 500 μL of the chitosans/substrate solution on the apical side. Volumes of 25 μL from both the apical and basolateral compartments were withdrawn at 45, 90, 120 and 180 min. Between sampling, the cells were kept in an incubator at 37°C and 5% CO_2 environment with very mild shaking on a Multi-tube Vortexer (Baxter Scientific Products).

pH measurements

The solubility of chitosans depends on the pH and at lower F_A values a pH value < 6 is required (Varum et al 1994). Thus, apical HBSS solution with chitosans was buffered to pH 5.7 with MES and the basolateral HBSS solutions were buffered with HEPES to pH 7.4. The pH was also measured at the start and the end of the experiment for chitosans 1–5 to identify any damage to wells.

TEER (Ω cm^{-2}) measurements

TEER was measured to assess the integrity of the cell monolayer on the membrane support just before and immediately after the experiments. TEER was measured by using the Millicell electrode (Millipore Corporations, MA, USA) designed for this purpose. Net resistance was obtained by subtracting the resistance obtained in the solutions over the membrane without cells. Only data from epithelial layers with $\Omega > 300$ were used for data analysis.

Quantification of morphine

Morphine was quantified by high-performance liquid chromatography ion spray tandem mass spectrometry (LC-MS/MS, Sciex API-300). The limit of quantification (LOQ) was 2.8 nmol L^{-1}. The calibration range was 3.5–175 nmol L^{-1}. The correlation coefficients (r^2) were > 0.99. The average coefficients of variation (CV) found by inter-run quality controls (QCs, n = 12) at 4 levels distributed over the calibration range were < 9.7%. Samples (25 μL) from the basolateral compartment were mixed with 125 μL mobile phase (MF, 5% acetonitrile with 0.1% formic acid). The HPLC column was a Zorbax SB-C18 (7.5 cm, 5 μm, 4.6 mm i.d.). The injection volume was 80 μL and the RT was 1.7 min. The ion spray source was operated with a spray voltage of 4.8 kV, an orifice voltage of 54 V and nebulizer gas of 1.04 L min^{-1}. The ring voltage was 380 V. The mass spectrometer was operated in MS-MS mode (multiple reaction monitoring, MRM) and the product ion was m/z 286.2. The fragmentation occurred at collision energies of −27 eV. Nitrogen was used as collision gas.

^{14}C mannitol quantification

Samples were counted in a liquid scintillation analyser (Tricarb 2300TR; Packard Instruments Laborel, Oslo, Norway). Twenty-five microlitres of the basolateral solution was mixed with 2 mL of the scintillation liquid and counted for 5 min.

Apparent permeability coefficient (P_{app}) of mannitol and morphine

Calculations of transepithelial transport rate is given as P_{app} (cm s^{-1}), the apparent permeability coefficient, described by Artursson et al (1994) and is calculated using equation 1:

$$P_{app} = (dQ/dt)/(A \times C_0) \quad (1)$$

where dQ/dt is the permeability rate (mol min^{-1}), C_0 (mol mL^{-1}) is the initial concentration at the apical side (donor chamber) and A (cm^2) is the surface area of the cell support membrane. P_{app} was independently calculated at each time point separately.

Light microscopy and transmission electron microscopy (TEM)

The differentiated cell layer on PET membranes (BDH) where fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. It was then exposed for 1 h to 2% osmium tetraoxide in 0.1 M phosphate buffer, dehydrated and embedded in resin. Semi-thin sections (1000 nm) stained in toluidine blue were examined by a light microscope. Ultra-thin sections (70 nm) were cut and collected on 75 mesh formvar-coated copper grids, contrasted for 25 min with 4% uranyl acetate and for 5 min with 1% lead citrate, before examination with a JEOL 100CX transmission electron microscope (2000×).

Statistics

The P_{app} value at 180 min was the primary outcome measure. One-way analysis of variance was used to determine differences from control incubations. Bonferroni

correction was used for post-hoc multiple comparisons; *t*-test was used for group comparisons.

Results

Figure 1 shows the data for mannitol, a general marker for transepithelial transport. The transepithelial transport rate of mannitol (P_{app} in cm s^{-1}), at chitosan concentrations of $50\,\mu g\,mL^{-1}$ (Figure 1A), indicated that within a relatively narrow MW range (170–300 kD), chitosans increased the transport rate (within-group difference $P = 0.033$). The difference is seen more clearly in Figure 1B, which compares the same chitosans at $250\,\mu g\,mL^{-1}$. The P_{app} values for chitosans with 0.01/250, 0.17/300 and 0.32/200 (F_A/MW) differed from control ($P < 0.05$) at 180 min. A 3-fold increase in P_{app} value with time was observed for chitosans 0.01/250 and 0.17/300, while the P_{app} values were stable for the others. When testing chitosans ($250\,\mu g\,mL^{-1}$) at MWs in the range 7–200 kD (Figure 1C), and a narrow range of F_A (0.32–0.43), chitosan 0.43/7 did not differ statistically from control at 180 min; however, those of MW 29 kD or more gave greater values ($P < 0.05$).

The corresponding data for the transepithelial transport of morphine is shown in Figure 2. Chitosans had similar effects on the transepithelial transport of morphine to those of mannitol. However, the P_{app} values of morphine controls ($1.07 \times 10^{-6}\,cm\,s^{-1}$) were less than those of mannitol ($2.63 \times 10^{-6}\,cm\,s^{-1}$, $P < 0.001$, *t*-test). Transepithelial morphine transport was more sensitive to chitosans than mannitol, as the P_{app} values for chitosans 0.01/250, 0.17/300 and 0.32/200 differed from control ($P < 0.05$) at 180 min at $50\,\mu g\,mL^{-1}$ (Figure 2A), in contrast to the lack of findings for mannitol (Figure 1A). This was supported by the observation (Figure 2B) that by increasing the chitosan concentration to $250\,\mu g\,mL^{-1}$, all chitosans except 0.61/180 differed statistically from control ($P < 0.05$). Finally (Figure 2C), in experiments with chitosans at $250\,\mu g\,mL^{-1}$ (MW 7–200 kD, F_A 0.32–0.43), that with MW of 29 kD or more differed statistically from control ($P < 0.05$).

TEER measurements (data not shown) conducted before and after incubation with morphine displayed a reduction similar to the pattern of morphine transport rate with respect to the effects of chitosans ($P = 0.022$ and 0.002 for 250 and $50\,\mu g\,mL^{-1}$, respectively). Post-hoc analysis was negative for the $250\,\mu g\,mL^{-1}$ experiments. For the $50\,\mu g\,mL^{-1}$ experiments chitosan 0.01/250 differed from both control ($P = 0.020$), and chitosans 0.32/200 and 0.61/180 ($P = 0.027$ and 0.001, respectively).

In separate experiments, P_{app} values for mannitol and morphine were almost identical with BD (PET) and Nunc PC membranes. Experiments (with BD PET membranes due to NUNCs delivery failure) similar to those shown in Figures 1 and 2C at chitosans concentrations of $50\,\mu g\,mL^{-1}$ showed no significant effects (data not shown). Since morphine was analysed by a specific

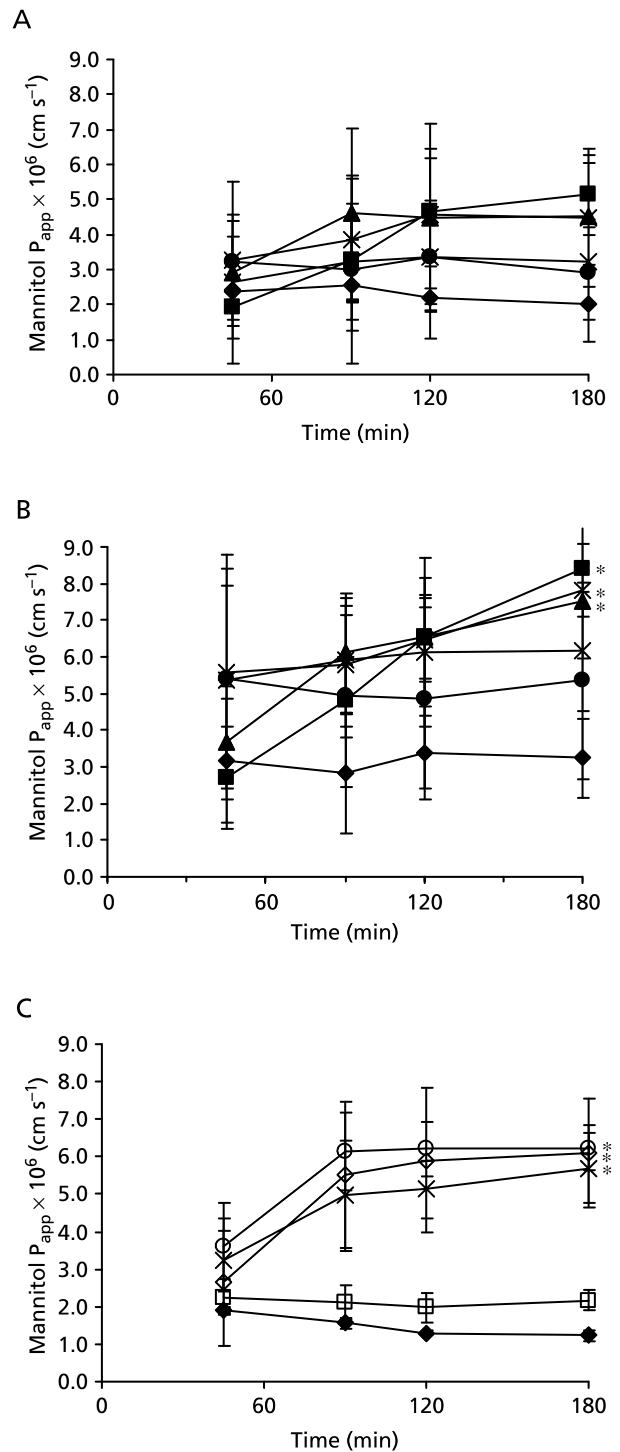

Figure 1 Effect of chitosans on transepithelial transport rate of mannitol across Caco-2 cell monolayer. A. Effect of chitosans $50\,\mu g\,mL^{-1}$. Control (◆) and chitosans F_A 0.01/MW 250 kD (■), F_A0.17/MW 300 kD (▲), F_A0.32/MW 200 kD (×), F_A0.43/MW 170 kD (∗), F_A0.61/MW 180 kD (●). Data are means ± s.d., n = 12. B. Effect of chitosans $250\,\mu g\,mL^{-1}$. See Figure 1A for symbols. Data are means ± s.d., n = 12. *$P < 0.05$ vs control (analysis of variance with Bonferroni correction). C. Effect of chitosans $250\,\mu g\,mL^{-1}$. Control (◆) and chitosans F_A0.43/MW 7 kD (□), F_A0.35/MW 29 kD (○), F_A0.35/MW 66 kD (◇), F_A0.32/MW 200 kD (×). Data are means ± s.d., n = 4. *$P < 0.05$ vs control.

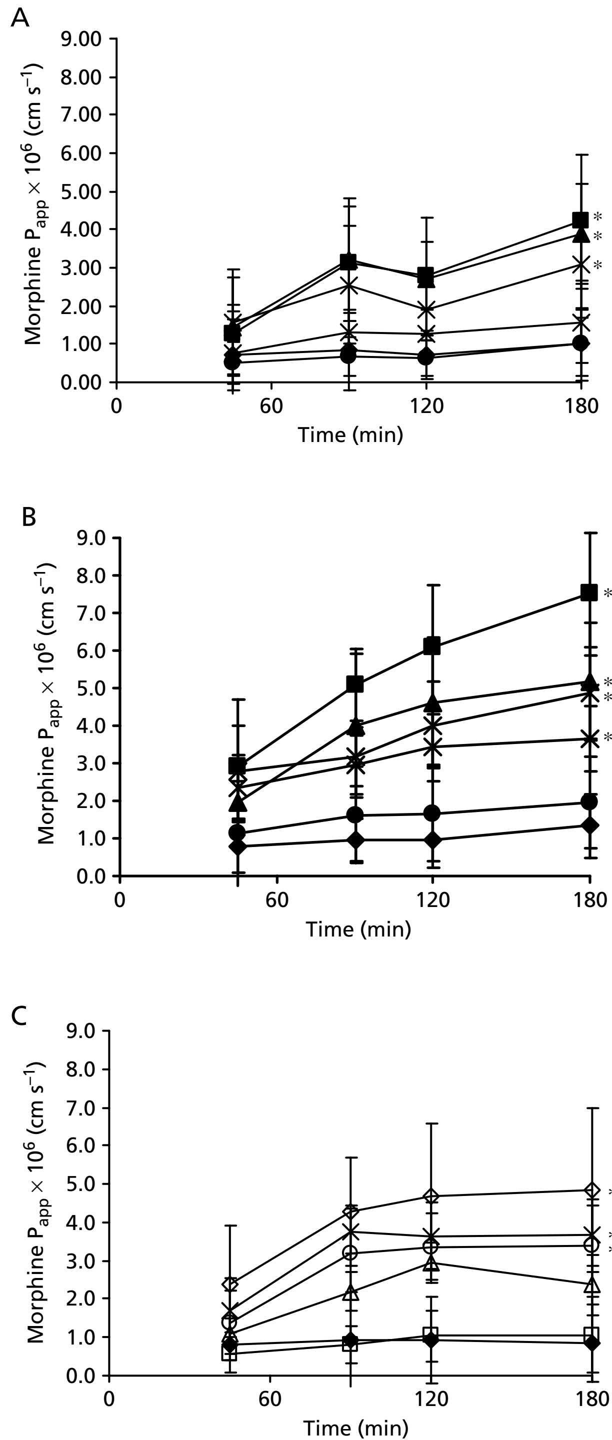

Figure 2 Effect of chitosans on transepithelial transport rate of morphine across Caco-2 cell monolayer. A. Effect of chitosan 50 μg mL^{-1}. Ctrl (◆) and chitosans F_A0.01/MW 250 kD (■), F_A0.17/MW 300 kD (▲), F_A0.32/MW 200 kD (×), F_A0.43/MW 170 kD (∗), F_A0.61/MW 180 kD (●). Data are means ± s.d., n = 12. *P < 0.05 vs control. B. Effect of chitosan 250 μg mL^{-1}. See Figure 2A for symbols. Data are means ± s.d., n = 12. *P < 0.05 vs control. C. Effect of chitosan 250 μg mL^{-1}. Ctrl (◆) and chitosans F_A0.43/MW 7 kD (□), F_A0.39/MW 12 kD (△), F_A0.35/MW 29 kD (○), F_A0.35/MW 66 kD (◇), F_A0.32/MW 200 kD (×). Data are means ± s.d., n = 8. *P < 0.05 vs control.

LC-MS/MS method, it was possible to document that the morphine metabolites M-3-G and M-6-G were not present in the basolateral compartment in detectable amounts.

Histological examination revealed a continuous layer of cuboidal cells (Figure 3). Microvilli were found on the apical cell surface (Figure 3) and each cell was typically in close contact with the opposed lateral domains of neighboring cells, often forming extensive interdigitating folds (Figure 4). The cells were further anchored to each other by numerous desmosomes (maculae adherentes).

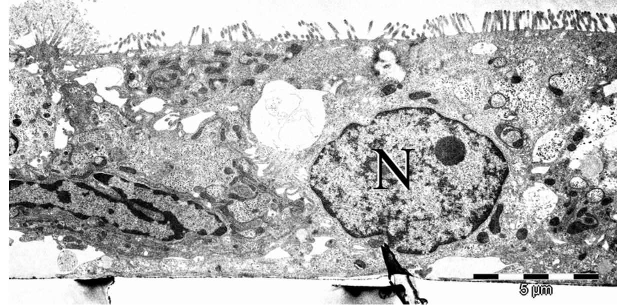

Figure 3 Typical TEM low power micrograph of Caco-2 monolayer epithelium. Microvilli are evident on the apical surface of the cells. N, nucleus.

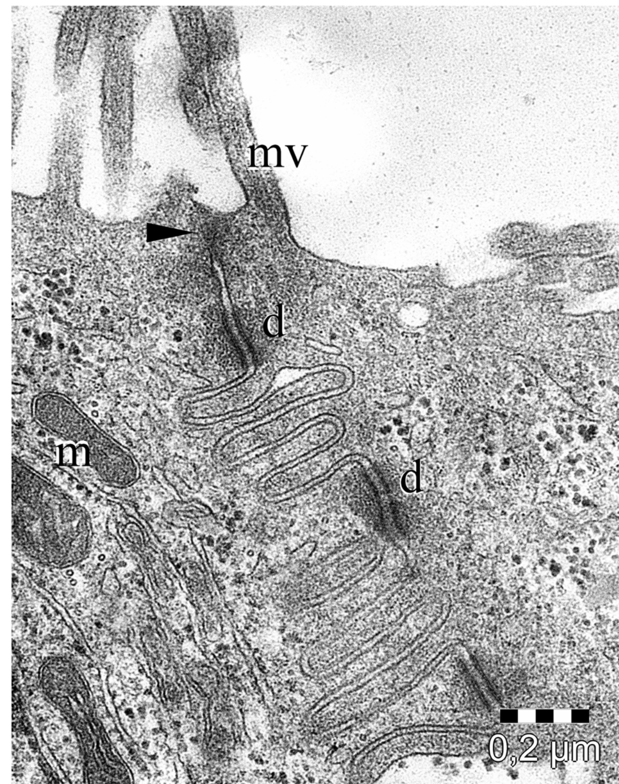

Figure 4 High power TEM micrograph of a typical apicolateral site, showing two cells in close contact, anchored by extensive interdigitating folds and numerous desmosomes (d). Most apically, a tight junction (arrowhead) is seen. mv, microvillus, m, mitochondrion.

Apicolaterally, tight junctions (zonulae occludentes) were easily demonstrated. The integrity of the monolayer was further verified with TEER measurements recorded at the start of each experiment. Less than 10% of the wells were excluded from analysis due to low electrical resistance. Similar TEER was observed when Caco-2 cells differentiated on supporting membranes from both BD (PET) and Corning (PC), indicating that the experimental model was robust. Also the minor changes in mean pH from 5.70 to 5.90 (n = 96, s.d. 0.05) on the apical side and from pH 7.40 to 7.37 (n = 48, s.d. 0.05) on the basolateral side after 180 min incubation with chitosans verify the integrity of the wells.

Discussion

To the best of our knowledge, this is the first published study of the interaction of chitosans over Caco-2 epithelial monolayers on the transport of morphine. It shows that their effect on transepithelial transport of morphine depends on the chitosan concentration, degree of acetylation, molecular weight and also incubation time. Mannitol was used as a positive control. Morphine concentrations were measured directly in the buffer with a method also capable of detecting metabolites of morphine. The integrity and differentiation of the monolayer was documented with transmission electronic microscopy. The light and electron microscopic findings suggest a well-differentiated, polarized epithelial monolayer, with advanced junctional complexes. This was also supported by TEER recordings both before and after incubations. Also, the transepithelial mannitol transport rate is a hallmark for the functionality of the Caco-2 model epithelial monolayer (Schipper et al 1996; Darwin et al 1998; Phillips et al 2002). The rate for the controls was within the previously reported range ($\leq 4 \times 10^{-6}\,cm\,s^{-1}$) (Schipper et al 1996; Darwin et al 1998; Phillips et al 2002), indicating that the experiments had been carried out under adequate conditions.

The concentrations of chitosans, as well as the variation in F_A and MW used in this study, are within previously published ranges (Schipper et al 1996; Holme et al 2000; Thanou et al 2000). Our results confirm earlier findings (Schipper et al 1996) that chitosans stimulate the transepithelial transport rate of mannitol and also that their characteristics have varying effects on mannitol transport. The morphine concentrations used are also in the same range as reported by Wandel et al (2002). However, these authors did not report P_{app} values and it is therefore not possible to compare their data with ours. Moreover, since an analysis of the metabolites of morphine was included in this study, it can be concluded that the Caco-2 cells do not significantly metabolize morphine.

Chitosans affect transepithelial transport of morphine in the same way they affect mannitol transport. It was least stimulated by chitosans with the highest F_A (0.61), especially for morphine. Those with a low F_A (and consequently a high degree of protonation) and a MW of 200–300 kD clearly showed the highest transport rate at 180 min, although the effect was modest at 45 min. An increase in transepithelial transport rate with time may be explained by an increased facilitation of physiological transport, but this only occurred with chitosans with an F_A much below 0.32, which were previously shown to be more toxic than those with an F_A close to 0.35 (Schipper et al 1996). Although no direct experimental support was provided in our study, this observation is possibly a subtle sign of incipient deleterious effects on cell function (Schipper et al 1996).

Schipper et al (1996) showed that the impact of MW on mannitol transport at 20–60 min was greatest for chitosans with F_A 0.35. At this F_A, MW 12 was equal to control; at 170 kD the rate was increased about 10 times. Our study supports this finding, as chitosans in the same F_A range (0.32–0.43) displayed no effect unless their molecular weight was 29 kD or more. Schipper et al (1996) concluded that chitosans with intermediate F_A (about 0.35) and a high molecular weight (about 200 kD) showed good enhancement of transepithelial transport of mannitol combined with low cytotoxicity. Our data confirm this for mannitol, and can be extended to morphine transport, as chitosans with F_A/MW values of 0.32/200 were the only ones with $F_A > 0.30$ that showed any effect at the lowest concentrations ($50\,\mu g\,mL^{-1}$). They also performed well in the other experiments. Chitosans used clinically to enhance nasal absorption of morphine (Illum et al 2002; Pavis et al 2002) usually have a MW of about 250 kD and $F_A > 0.2$.

Chitosans specifically affect the tight junctions and thus stimulate paracellular transport of mannitol (Artursson et al 1994; Dodane et al 1996; Illum 1998). This may also be true for morphine, since the effects on mannitol and morphine are similar in this study. Illum et al (2002) showed that chitosans increase the nasal bioavailability of morphine from 10 to 60%. It seems therefore likely that increased paracellular transport is the major mechanism for increased transepithelial transport of morphine.

However, there are also differences in the effect of chitosans on the transepithelial transport of morphine and mannitol, in that morphine exhibits a lower control rate, and seems more sensitive to the effects of chitosans. This may be caused by their different structures and physiochemical characteristics. Another explanation might be the possible metabolization of morphine by Caco-cells; however, no metabolites of morphine were found in this study. A third explanation may be that morphine is a substrate for P-glycoprotein, a membrane structure that counteracts the transcellular transport of morphine. This mechanism exists in man (Kharasch et al 2003) and also operates in Caco-2 cells (Crowe 2002; Wandel et al 2002). Whether chitosans have any effect on the activity of P-glycoprotein remains unresolved.

In conclusion, chitosans stimulate transepithelial transport of mannitol and morphine in a similar manner, suggesting similar mechanisms of action. The reasons for the observed differences between the effects of chitosan

remain unclear; they depend on molecular weight, degree of acetylation and, in some instances, the duration of incubation. Chitosans with an acetylation of 0.3–0.4 and molecular weight about 200 kD seem to have favourable characteristics for enhancement of transepithelial transport of morphine.

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