Permeability and modulation of the intestinal epithelial barrier in vitro

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Erwin Duizer
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Cover: Confocal laser scanning microscopy image of filter-grown IEC-18 cells labeled with a polyclonal anti-ZO-1 antibody and visualized with a TRITC-conjugated streptavidin-biotin system.

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STELLINGEN

behorende bij het proefschrift
"Permeability and modulation of the intestinal epithelial barrier in vitro"
van Erwin Duizer,
te verdedigen op woensdag 16 juni 1999, om 16.00 uur te Wageningen.

1 Voor het modeleren van de paracellulaire permeabiliteit van de dunne darm voor hydrofiele macromoleculen voldoet het twee-compartimenten systeem beter als het wordt uitgerust met dunne darm cellen (IEC-18) dan met, van oorsprong, dikke darm cellen (Caco-2) *(dit proefschrift).*

2 Veranderingen in de transepitheliale elektrische weerstand (TER) zijn geen goede kwantitatieve indicatie voor veranderingen in de transepitheliale permeabiliteit voor macromoleculen *(dit proefschrift).*

3 Het voorspellen van de orale bio-beschikbaarheid van een stof, ongeacht de absorptie route, aan de hand van één enkel *in vitro* model is nu nog niet mogelijk *(dit proefschrift).*

4 Cadmium heeft enkele eigenschappen gemeen met de ideale "drug absorption enhancer" *(dit proefschrift).*

5 Het gebruik van "permeation enhancers" kan gemakkelijk leiden tot een verhoging van het aantal mensen met voedselallergie.
(Pena and Crusius. Food allergy, coeliac disease and chronic inflammatory bowel disease in man. Vet. Q. 1998;20 Suppl 3:S49-52.
Ahmed and Fuchs. Gastrointestinal allergy to food: a review. J. Diarrhoeal Dis. Res. 1997;15:211-23).

6 Dat verminderde gluthation peroxidase activiteit door Selenium-deficiëntie kan leiden tot enterovirale myocarditis, is eerder toe te schrijven aan een verhoging van de virulentie van de enterovirussen dan aan een verminderd functioneren van het immuunsysteem.
(Beck *et al.* Increased virulence of a human enterovirus (Coxsackievirus B3) in selenium-deficient mice. J. Inf. Dis. 1994;170:351-357.
Beck *et al.* Glutathione peroxidase protects mice from viral-induced myocaritis. FASEB J. 1998;12:1143-1149).

7 In een celkweek laboratorium is er een positieve correlatie tussen de reproduceerbaarheid van de experimenten en het alcohol gebruik.
(Ojeh *et al.* Evaluation of the effects of disinfectants on rotavirus RNA and infectivity by the polymerase chain reaction and cell-culture methods. Mol. Cell Probes 1995;9:341-346).

8 De angst dat chimpansees een reservoir zullen vormen voor levend poliovirus en daarmee de eradicatie van polio in gevaar brengen is ongegrond, het is waarschijnlijker dat de chimpansees eerder zijn ge-eradiceerd dan het poliovirus.
(Dowdle and Birmingham. The biological principles of poliovirus eradication. J. Infect. Dis. 1997;175:S286-S292).

9 Een turner staat het liefst niet met beide benen op de grond.

10 Voor zowel cabaret programma's als wetenschappelijke artikelen geldt "gebruik altijd de teksten van een ander.....tenzij die van jezelf beter zijn" en "schrijven is schrappen".
(Herman Finkers en *dit proefschrift*).

In dankbare herinnering,
voor mijn vader

A good place to begin is at the beginning,
because otherwise things can get confuzzled.
Winnie-the-Pooh

Contents

Section I: Introduction and Methodology

Section II: Permeability of the Intestinal Epithelial Barrier *In Vitro*

Section I

Introduction and Methodology

Chapter 1

General Introduction

Manuscript based on the chapters 1, 2 and 8 in preparation; to be submitted to ATLA.

In a challenging effort to gain a better insight in oral bioavailability of food components, toxicants, and drugs, we studied the absorption and barrier characteristics of two intestinal cell lines, Caco-2 and IEC-18, cultured in a two-compartment system. We wondered whether the rat small intestinal cell line IEC-18 could provide a better model than the Caco-2 cell line, in obtaining *in vitro* permeation rates to predict oral bioavailability *in vivo*, for non-nutrient hydrophilic molecules.

Secondly, in order to gain insight in the mechanisms by which the integrity of the epithelial barrier may be compromised, the filter-grown cells are exposed to a drug absorption enhancer (palmitoyl carnitine) and to a possible food contaminant (cadmium). In these experiments the relation between increased permeability for hydrophilic macromolecules, an altered paracellular barrier structure, and the viability of the epithelial cells was assessed.

§ 1.1 Introduction

The bioavailability of all ingested compounds is to a great extend determined by the ability of these compounds to pass the intestinal epithelium. A high bioavailability is guaranteed for most nutrients and electrolytes since they are actively absorbed by the epithelium. The same epithelium, however, presents an almost impermeable barrier to non-nutrient (potentially harmful) hydrophilic (macro-) molecules, viruses and bacteria, rendering their entrance into the systemic circulation very low. To perform its function as a selective absorber (or a selective barrier) on the interface between the intestinal lumen and the internal host milieu, the intestinal epithelium has evolved into a highly specialized structure. The single cell layer forming the intestinal epithelium consists of several cell types, each with a specific morphology and function. For example, the intestinal epithelial absorptive cells, the enterocytes, are polarized to allow vectorial transport, they posses microvilli to enlarge the absorptive area, and the cells are sealed together by a junctional complex to maintain a barrier in order to avoid absorption of potentially harmful compounds and to prevent back-flow of the actively absorbed nutrients.

The complexity of the gastrointestinal tract, and the complications caused by pre-and post-absorptive kinetics when studying the absorption and barrier functions of the intestinal epithelium *in vivo*, were great stimuli to develop *in vitro* models for the intestinal epithelium. One of the established *in vitro* models consists of epithelial cells grown on a permeable support in a two-compartment system. The development, characterization and application of this model was recently boosted by the need for a high-throughput screen to select drugs with oral bioavailability, and by the recognition that the filter-grown intestinal epithelial Caco-2 cells allowed the determination of *in vitro* permeation rates, that were predictive for *in vivo* oral bioavailability, for a wide variety of compounds (Artursson and Karlsson, 1991).

However, for hydrophilic molecules, which were not transported across the epithelium by transporters or carriers, the permeation rates obtained with these colonic carcinoma derived Caco-2 cells, were very low and did not allow for an accurate determination of the expected bioavailability. The absorption of these hydrophilic compounds must occur by the paracellular pathway, i.e. the route along the cells. However, the rate limiting barriers in the paracellular pathway, the tight junctions, are much more restrictive (colon-like) in the Caco-2 cell layers than in the small intestinal epithelium *in vivo*. Since permeation will occur at sites posing the lowest resistance, permeation of hydrophilic compounds will occur in the small intestine. At the moment there is no established model available which mimics the permeability of the small intestinal epithelium for non-nutrient hydrophilic molecules. The IEC-18 cells might pose such a model because these cells are derived from the small intestine and they were found to be able to grow on filters. We, therefore, chose to study the paracellular permeability of the small intestinal cell line IEC-18 and compare this to the permeability of the colonic carcinoma cell line Caco-2.

The permeability of the intestinal epithelium is not a fixed measure, but it is subjected to

various modulations. The absorptive and barrier functions are regulated physiologicaly, for example, by serosal peptide hormones (Hochman and Artursson, 1994) or luminal D-glucose (Madara and Pappenheimer, 1987), but it can also be hampered in patho-physiological conditions (Hollander, 1988; Schulzke *et al.*, 1998). It has also been recognized that nutritional and xenobiotic compounds can modulate the epithelial integrity by causing lytic mucosal damage, or by opening the paracellular pathway (Hochman and Artursson, 1994). The opening of the paracellular pathway depends on changes in the structure and function of tight junctions, and it is now understood that a great variety of cellular signaling pathways is involved in controlling the barrier function of the tight junctions (see § 1.6). Especially the interest of pharmaceutical companies in making drugs with a low intestinal absorption rate, available as oral dosing, has triggered the development of an *in vitro* system to screen drug absorption enhancers for effects on the intestinal barrier function, i.e. to establish the relation between efficacy and (mucosal) toxicity. However, perturbation of the intestinal epithelial barrier function is not a feature of drug absorption enhancers alone, but it can also be caused by food additives or food contaminants. In the studies described in this thesis the filter-grown Caco-2 and IEC-18 cells have been evaluated as *in vitro* models to determine the effects of drug absorption enhancers and food components on the intestinal epithelial barrier. Furthermore, a method allowing the assessment of effects which occurred concomitantly with a reduced barrier function, when the epithelial-like cell sheets were exposed to barrier disruptors (e.g. drug absorption enhancers or food contaminants), would help to gain insight in the mechanism of barrier disruption, i.e. to determine whether permeation enhancement was due to increased paracellular or transcellular permeation.

§ 1.2 Transepithelial permeation routes in the intestine

The anatomy of the gastrointestinal tract with the presence of structures that greatly increase the total surface area of the intestinal epithelium (i.e. plicae circularis, mucosal crypts and villi, and the microvilli at the apex of the absorptive cells), indicates that the intestinal tract is structured to perform its absorptive function. For most nutrients, the villus tips of the small intestine suffice to absorb most of the ingested load. The epithelium at the villus tips consists of a single cell layer of highly differentiated columnar absorptive enterocytes and several (5-10%) mucus secreting goblet cells (Madara and Trier, 1994; Kato and Owen, 1994). Absorption across this cell layer can occur by two different routes: one through the cells, the transcellular pathway and one along the cells, the paracellular pathway. In the intestinal epithelium the transcellular pathway occupies more than 99.9% of the total surface area, leaving less than 0.1% of the total surface area for the paracellular pathway.

Lipophilic molecules ($0 < \log D < 4$, where D is the octanol/water partition coefficient) can be absorbed by energy-independent (passive) diffusion (route A in Figure 1.1) due to their

solubility in the lipid bilayers (Wils *et al.*, 1994b and references therein). Hydrophilic (log D <0) molecules can not readily pass the lipid bilayers but their transcellular absorption can be facilitated by substrate specific active transporters (route E in Figure 1.1), carriers (i.e. energy-independent carrier proteins, route C in Figure 1.1) or by passive membrane passage through channel proteins (i.e. ion channels) in the apical and/or basolateral membrane. The absorption of nutrients such as mono-saccharides, amino acids and di/tripeptides, and of electrolytes such as sodium, chloride and potassium is mostly energy-dependent (active) transport, and is performed by substrate specific and saturable transporters (reviewed in Alberts *et al.*, 1989; and Sadee *et al.*, 1995). It is important to note that passive and facilitated diffusion is driven by a concentration gradient and that it can take place in both directions, while active transport is energy-dependent, vectorized and can take place against a concentration gradient. The pathways and mechanisms by which the various compounds may cross the intestinal epithelium are shown in Figure 1.1.

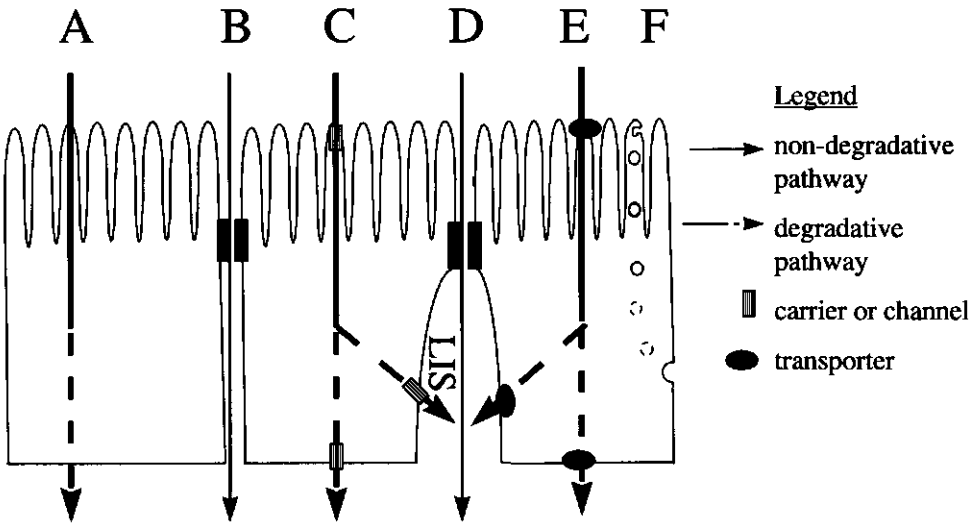

Figure 1.1: Transepithelial permeation routes in the intestine:A; passive transcellular diffusion, B; passive paracellular diffusion, C; facilitated transcellular diffusion, D; solvent drag, E; active transcellular transport, F; transcytosis, LIS; Lateral Intercellular Space.

The combination of a large surface area and the activity of apical and basolateral carriers and transporters brings about an absorptive capacity for small molecules which generally exceeds the intake. Whereas the epithelial permeability for lipophilic and small hydrophilic molecules (nutrients) is generally high, the epithelial permeability for macromolecules is low and the nutritional significance of the absorption of intact peptides (>3 amino acids), proteins or other macromolecules (> 400 Da) is considered to be negligible (i.e. < 1% bioavailability; Lennernas, 1998; He *et al.*, 1998). However, even low transepithelial passage of these molecules can be of

great importance due to the possibly high intrinsic biological, immunological, toxicological or therapeutical activity of those molecules. Furthermore, many data are based on the low bioavailabilities found in animal or human volunteer experiments. These data may underestimate the actual transepithelial passage of the compounds due to, for example, the inappropriate extrapolation of incomplete urinary recovery profiles (Nasrallah and Iber, 1969) or instability of the compounds in the gastrointestinal tract or in the body.

The transepithelial permeation of macromolecules can occur by the transcellular or paracellular route. The transcellular route for macromolecules comprises the passive trans-membrane route for lipid-soluble compounds and (active) receptor mediated transcytosis (route F in Figure 1.1) for proteins and viruses (Okamoto, 1998). Transcellular passage of bio-active macromolecules is, however, greatly diminished by cytoplasmatic or lysosomal digestive activities in the enterocytes (Gardner, 1994). Increased transepithelial permeation of macromolecules is found to occur in neonates and in the M cells in the small intestine of adults. In neonates of many mammal species, considerable amounts of intact orally ingested proteins can be absorbed due to the incompleteness of the intestinal closure at the time of birth. The higher permeability to macromolecules of the intestinal epithelia of neonates most likely has a transcellular (transcytotic) and a paracellular component, and is important for acquiring passive and active immunity (Henning *et al.*, 1994). In the adults' small intestine, the M cells overlay the Peyers patches and seem to be specialized in transporting luminal antigens over the epithelium to the underlying immunocompetent cells. They differ from the absorptive villus enterocytes not primarily by a higher transcytotic capacity, but by a lower degradative activity (Madara and Trier, 1994).

Transepithelial passage of macromolecules by the paracellular route (Route B in Figure 1.1) would get round the intracellular degradative processes. The significance of the paracellular pathway to the absorptive capacity of the intestinal epithelium is still uncertain. This pathway, through an "aqueous pore", is available to hydrophilic molecules. Transepithelial passage by this extracellular route depends on passive diffusion, but might be enhanced by the induction of solvent drag. The tight junctional permeability is size- and charge- (cation-)selective, but the influence of the size of the molecules is dominant over that of the charge in the intestinal epithelia (Pauletti *et al,*. 1997). The permeability and size selectivity of the paracellular route varies along both, the longitudinal and the crypt-villus, axis of the gastrointestinal tract. The diameter of the junctional pore decreases from an estimated 30-40Å in the most leaky part of the small intestine, the jejunal crypts, to 3-6 Å in the human colon (Chadwick *et al.*, 1977; Powell, 1987; Cerejido, 1992; Ma *et al.*, 1995; Thillainayagam, 1998). In the small intestine the paracellular permeability decreases from crypt to villus (Madara and Trier, 1994; Schulzke *et al.*, 1998). Marcial *et al.* (1984) calculated that in the ileum, 73% of paracellular conductance was attributable to the crypts. The contribution of the highly permeable crypts to the total intestinal paracellular absorption is, however, questionable due to the low availability of the crypt surface area and the secretory nature of the crypts (Bjarnason *et al.*, 1995; Madara and Trier, 1994; Barrett, 1997). Even though larger

pores are suspected to be present within the gastrointestinal epithelium as a whole, paracellular passage *in vivo* is generally found to be restricted to molecules < 11 Å (Madara, 1989; Hochman and Artursson, 1994; Fasano, 1998).

Although paracellular passage is essentially passive, the combined activity of digestive enzymes, apical and basolateral carriers and transporters may cause an additional absorptive paracellular process called "solvent drag" (route D in Figure 1.1). Solvent drag is the presumed result of a high osmotic load of electrolytes and nutrients in the lateral intercellular space (LIS), caused by the activity of Na-coupled nutrient transporters. The high osmotic load in the LIS leads to an osmotic gradient between the lumen and the LIS and to morphological alterations in the junctional complex, which in turn allow a flow of luminal fluid through the tight junctions into the hyper-osmotic LIS. It has been suggested (Pappenheimer and Reiss, 1987; Atisook and Madara, 1991; Pappenheimer, 1993) that this route contributes considerably to the absorption of nutrients (up to 60 to 80% for monosaccharides and amino acids) in the small intestine. However, the significance of the by solvent drag increased intestinal permeability is questioned by others (Fine *et al.*, 1993; Uhing, 1998).

§ 1.3 The transcellular barriers of the intestinal epithelium

Being the first contact site of all orally ingested compounds, the epithelium of the gastrointestinal tract has an important absorptive function for nutrients but it also needs to present a barrier to absorption of potentially harmful compounds. It is this barrier that also limits the bioavailability of many orally administered drugs. The barrier related features of small intestinal villus epithelial enterocytes are shown in Figure 1.2 and will be discussed in more detail in this paragraph. In the barrier presented by the intestinal epithelium, as in the epithelial absorptive function, it is possible to discriminate a transcellular and a paracellular component. Other, non-enterocytal, barriers such as the luminal degradation, the aqueous boundary layer, the mucus layer (Karlsson, 1995) and the immunological barriers (e.g. IgA secretion) are beyond the scope of this thesis.

Some of the barrier features mentioned could also be presented in the absorptive function. Brush border enzymes (C in Figure 1.2) such as peptidases and oligo- and disaccharidases, for intact absorbtion. example, enhance the absorption of di/tripeptides, amino acids and monosaccharide, but they do so by breaking down proteins and carbohydrates. Furthermore, the cell membranes are essentially permeable for lipophilic compounds but present an impermeable barrier for hydrophilic compounds.Not only in the brush-borders, but also in the cytoplasm and lysosomes, the cells contain all kinds of digestive enzymes which can degrade macromolecular compounds to prevent intact absorbtion.

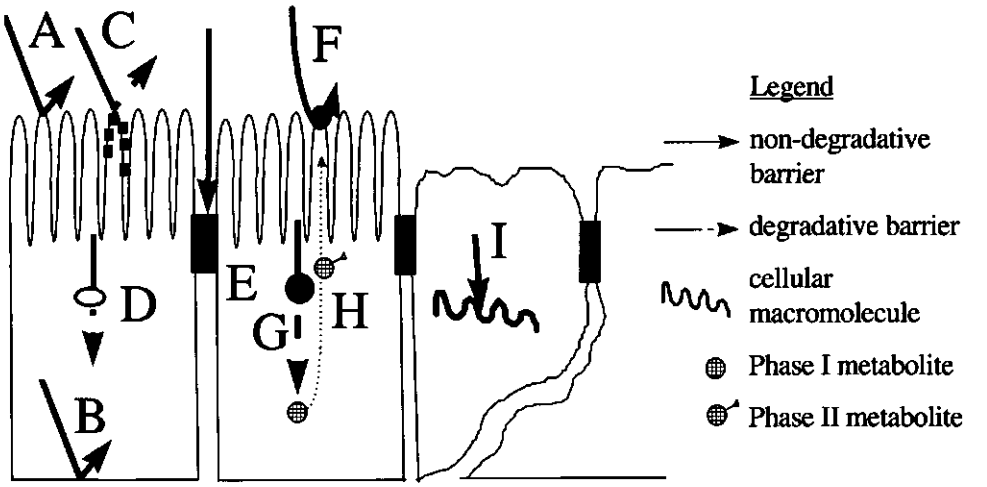

Figure 1.2: Epithelial permeation barriers of the intestine: Epithelial permeation barriers of the intestine: A; apical cell membrane, B; basolateral cell membrane, C; degradative brush border enzymes, D; intracellular degradative enzymes, E; tight junctions, F; apical efflux systems, G; phase I metabolizing enzymes, H; phase II metabolizing enzymes, I; intracellular sequestration of toxic compounds and extrusion of dead cells.

Besides the general structural barriers (membranes) and the digestive enzymes, mature enterocytes also posses several features more specifically involved in limiting oral bioavailability of potentially harmful compounds. This barrier activity depends on cellular metabolizing enzymes (G and H in Figure 1.2) such as glutathione S-transferases and cytochrome P450, and on efflux proteins in the apical membrane (F in Figure 1.2). Recently there has been much attention for the expression and functions of efflux systems, and several were found to be expressed in the intestine. Examples are, the permeability-glycoprotein (P-gp) which was found to be expressed in the apical cell membrane of intestinal epithelial cells (Terao *et al.*, 1996), the multidrug resistance associated protein 1 (MRP1) and MRP2/cMOAT (canicular multispecific organic anion transporter, Makhey *et al.*, 1998). The activity of the metabolizing enzymes might even be orchestrated with the activity of the efflux systems in the intestinal apical cell membrane. For example, the significant overlap in substrate specificities and the co-localization of CYP3A4 and P-gp in the villus tip enterocytes, suggest a combined action in limiting oral bioavailability of their substrates (Watkins, 1997; Wacher *et al.*, 1998). A similar combined action is suggested for glutathione S-transferases and MRP in MCF7 cells (Morrow *et al.*, 1998), and this enzymatic

barrier function might also prevail in the intestine.

An additional, intestinal epithelium specific, transcellular barrier function results from the high turnover rate of the intestinal cells. Potentially toxic compounds, which have entered the cells but cannot be detoxified or effluxed to a sufficient extent, can be sequestered in the cells by specific proteins, or bound covalently to cellular macromolecules (I in Figure 1.2). In such cases the bound toxic compounds can be drained into the intestinal lumen by the extrusion of the dead cells.

§ 1.4 The paracellular barrier of the intestinal epithelium

The intercellular transepithelial permeation route is called the paracellular pathway. This pathway has long been thought to be completely sealed for permeation of all compounds by the junctional complex. Only in the nineteen-sixties came the recognition that there were tight and leaky epithelia and that the existence of an aqueous extracellular permeation route was likely in the leaky epithelia such as that of the intestinal mucosa. By now, it has become clear that paracellular solute permeation does occur (see §1.2), that it is regulated physiologically (Bentzel *et al.*, 1992; Madara, 1998), and that it can be modulated by lumenal contents (see §1.6; Ballard *et al.*, 1995). The paracellular pathway can be seen as a two-component system (Figure 1.3): one of the components is the junctional complex and the other is the lateral intercellular space (LIS or ICS, Inter-cellular space). In epithelial cells the junctional complex consists of tight junctions, adherens (intermediate) junctions and desmosomes (Figure 1.3). Desmosomes (macula adherens) provide the epithelial cell layer with tensile strength by anchoring the intermediate filaments of the cytoskeleton, and attaching neighboring cells to each other. The cadherin based adherens junctions form a continuous belt (zonula adherens) around the apex of the epithelial cell. The adherens junctions link the cytoskeleton to the cell membrane and provide structural integrity to the epithelia. Junctional localization of E-cadherin (uvomorulin) is believed to be an early trigger in the cascade which leads to epithelial cell differentiation, polarization and assembly of tight junctions (Gumbiner, 1992; Balda, 1992; Nybom, 1996). The most apical (luminal) structure in the junctional complex is the tight junction. Just as adherens junctions, tight junctions form a continuous belt (zonula occludens) which encircles the cells. At the site of the tight junctions the lateral cell membranes of adjacent cells are in very close apposition and electron microscopy revealed the presence of extracellular material at these sites (Madara, 1992). Therefore, it was suggested that the tight junctions are the sealing elements between the epithelial cells and the rate limiting barrier in the paracellular pathway. By restricting paracellular diffusion along the cells ("gate" function) and by restricting free diffusion of membrane components from the apical to the basolateral membrane ("fence" function), these structures are essential for the barrier and for the absorptive functions of the intestinal epithelium (Balda and Matter, 1998).

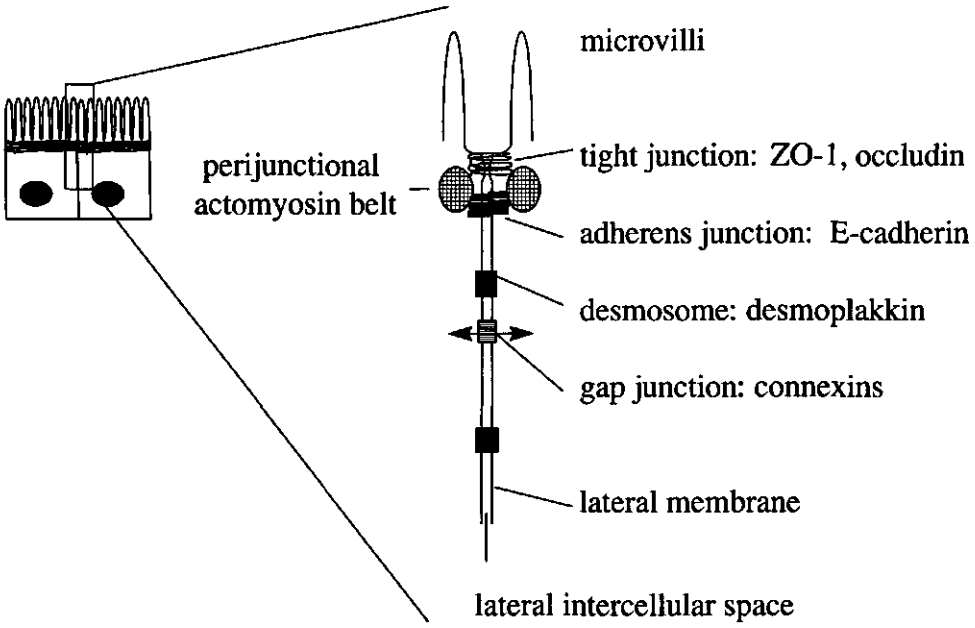

Figure 1.3: The seperate junctions and some of their building proteins in the junctional complex of epithelial cells.

§ 1.5 Tight Junctions: The gate-keepers of the paracellular barrier

Due to the leading role of the tight junctions in the maintenance of the epithelial barrier, a separate paragraph will be dedicated to this intriguing cell-structure. Study of the molecular composition of tight junctions and regulation of the paracellular permeability barrier is now proceeding at a rapid pace. This paragraph will deal only with those aspects of tight junction composition, and permeability regulation with direct relation to the thesis. For detailed information and the state of the art on this subject refer to: Stevenson and Keon, 1998; Madara, 1998; Mitic and Anderson, 1998; Cereijido *et al.*, 1998; Furuse *et al.*, 1998 and references therein.

At low magnification, the tight junctions appear as kissing lateral membranes of adjacent cells, and at higher magnification (approximately 50.000x) the membranes appear to fuse. In freeze fracture replicas the tight junctions can be seen as strands in the cytoplasmic leaflet of the plasma-membrane (P-face) and correlating grooves in the exoplasmic leaflet (E-face; Madara, 1992). Ultrastructural studies showed that the P-face strands formed a cell-circumscribing network with varying features which correlated to paracellular barrier function of the epithelium. The morphological factors which are now considered to contribute to the permeability of this junctional pathway are (Figure 1.4): the junctional density (junctional length per cm^2, i.e. relative surface area of the paracellular pathway; A and B in Figure 1.4), the number of junctional strands

(or fibrils) per lateral membrane (C in Figure 1.4), the junctional complexity (density of anastomoses between strands, i.e. number of crosses per µm junctional strand), depth of the junctional area in the lateral membrane (in µm; E in Figure 1.4), and the width and length of the lateral intercellular space. A popular model for junctional permeability assumes pores (in the junctional strands) with various, but fixed, sizes and a regulated "open probability" (Reuss, 1992; Gonzalez-Mariscal, 1992). Along the small intestinal crypt-villus axis it has been shown that the junctional density decreases from 91 to 34 m/cm^2 in rats, due to the increase in cell size in the villi (Collettt *et al.*, 1997). However, the junctional strand number increases from 4.7 to 5.5 strands (in human jejunum; Schulzke *et al.*, 1998), the junctional complexity increases, and junctional pores size and number decrease, altogether resulting in a more tight paracellular barrier in the differentiated villus epithelium (Madara and Trier, 1994; Bjarnason *et al.*, 1995, Schulzke *et al.*, 1998).

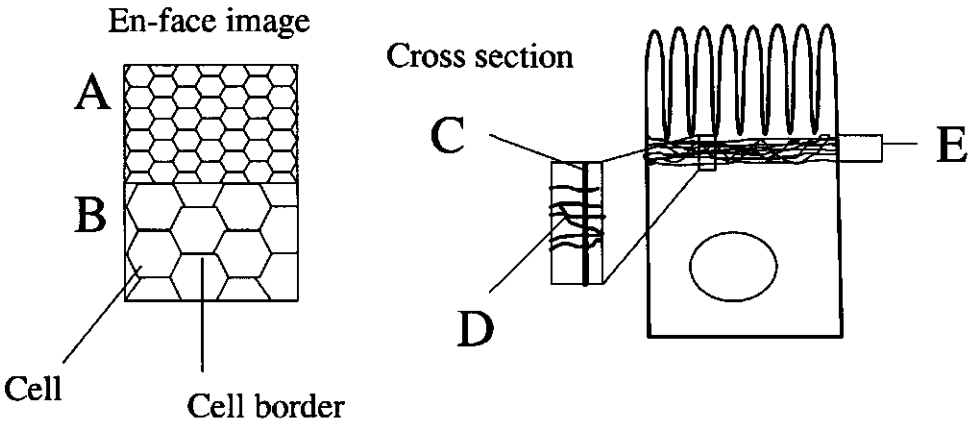

Figure 1.4: Morphological factors determining the permeability of the tight junctions. A; a small apical surface area of the cells results in a relative high junctional density, B; a large apical surface area of the cells results in a relative low junctional density, C; junctional strand number in the lateral membrane (n=6), D; anastosome, E; junctional depth.

Biochemical studies into the constitution of the tight junctions have revealed that the strands (fibrils) are proteinaceous in nature (Anderson and Stevenson, 1992), but the lipid composition of the proximate bilayers is also of importance. By now, using immunocytochemical and biochemical techniques, 12 proteins have been localized to the tight junctional region in epithelial cells. The best-studied is ZO-1, a 210-225 KDa protein which localizes in the cytoplasmic plaque of tight junctions. Two isoforms of ZO-1, ZO-1α+ and ZO-1α-, are described and these isoforms differ in the absence or presence of the α domain and the expression in different epithelia and endothelia. In intestinal epithelia the ZO-1 α+ form is expressed which is

indicative for a structurally less dynamic tight junction (Balda and Anderson, 1993). In epithelia ZO-1 forms a complex with ZO-2 and ZO-3 (formerly known as p130/130k), and all three are phosphoproteins and members of the Membrane-Associated GUanylate Kinase (MAGUK) protein family. Members of this protein family are all involved in (the regulation of) protein-protein interactions and/or signal transduction, and they are all localized at the cytoplasmic surface of the plasma membrane. ZO-1 is now known as (one of) the bridging protein between occludin and cytoskeletal actin (Fanning *et al.*, 1998). Occludin was the first known tight junctional transmembrane protein, it contains 4 transmembrane domains, 2 extracellular loops and a C-terminus which interacts at the intercellular side with the ZO-1/ZO-2/ZO-3 complex (Mitic and Anderson, 1998; Stevenson and Keon, 1998). But very recently 2 novel integral membrane proteins were discovered and designated claudin-1 and claudin-2 (Furuse *et al.*, 1998a). These small (22 kD) proteins also contain 4 transmembrane domains but do not show sequence similarity with occludin. It is now suggested that the claudin-1 and claudin-2 proteins are responsible for tight junction strand formation and that occludin is an accesorry protein (Furuse *et al.*, 1998b). Other tight junction associated proteins are cingulin, 7H6 (only in polarized epithelia), Rab3B, Symplekin (only in polarized epithelia), AF-6 and 19B1. Another cellular component which is associated with the tight junctional complex is actin. Actin is not an exclusively junctional protein, but a major component of the cytoskeleton. Actin prevails in two states, globular actin (G-actin) and its cellular polymer, filamentous actin (F-actin). In differentiated epithelial cells the F-actin at the apical cell side is organized in the terminal web and a circumferential ring of actin and myosin II, named the perijunctional actin-myosin ring. This ring is an important factor in regulation of paracellular permeability (Madara, 1992; Mitic and Anderson, 1998). Actually, the relationship between the tight junctions and the cytoskeleton was among the first discovered interactions involved in the regulation of tight junction function. It is now clear that junctional permeability can be regulated physiologically and that it can be increased by nutritional, pharmaceutical or toxicological compounds (see § 1.6). Furthermore, a decreased intestinal paracellular barrier function is found to play a role in the etiology and pathogenesis of Celiac disease (gluten sensitive enteropathy or non-tropical sprue, Schulzke *et al.*, 1998) and inflammatory bowel diseases (e.g. Crohn's disease and ulcerative colitis, Hollander, 1988; Wild *et al.*, 1997).

§ 1.6 Modulation of the intestinal epithelial barrier: The relationship between increased permeability and toxicity

Oral dosing is the most convenient and preferred way to administer drugs. However, many hydrophilic drugs (e.g. peptides and proteins, peptidomimics) have a very low bioavailability and therefore, several ways to improve bioavailability have been explored. One way to increase oral

bioavailability of drugs is to enhance the transepithelial passage (permeation) of the intact drug (reviewed by Hoogdalem *et al.*, 1989; Swenson and Curatolo, 1992; and Fix, 1996). Other mechanisms and methods to improve oral bioavailability are beyond the scope of this thesis and will not be discussed here. In the remaining part of this paragraph the terms "permeability" or "permeation enhancement", will be used instead of "absorption enhancement", this to refer more precisely to the mechanism of absorption enhancement (thus excluding other mechanisms as for example inhibition of efflux systems present in the apical cell membrane, inhibition of degradative enzymes in the cytoplasm, or the use of prodrugs).

In order to accomplish a higher transepithelial permeation of a drug, the barrier function of the epithelium must be reduced. Irrespective of the mechanism by which the barrier is disrupted, the effect is clearly a toxic one since one of the main functions, the barrier function, of the epithelium is compromised. In order to minimize adverse systemic effects, it was stated that a permeation enhancer should act rapidly and site specifically (preferably in the small intestine), it should not be cytotoxic (or at least allow a rapid recovery of epithelial integrity), and the permeation enhancement should be size specific, allowing the drugs to pass the epithelium but still abolishing the permeation of viruses and bacteria (Fix, 1996; Fasano, 1998). Since the barrier consists of a transcellular and a paracellular component, the permeation enhancers can be divided in two groups. The first group acts on the transcellular pathway, and decreases the barrier presented by the cell membranes, whereas the second group of permeation enhancers can act on the paracellular pathway by decreasing the barrier presented by the tight junctions, or by increasing the fluid flow through the paracellular pathway (solvent drag, e.g. oral rehydratation solutions).

The transcellular permeation enhancers are in general surfactant-like compounds such as surfactants (e.g. SDS, Triton X-100), bile salts, phospholipids, medium chain glycerides, fatty acids and their derivatives (Swenson and Curatolo, 1992; LeCluyse and Sutton, 1997). Since the very mechanism of surfactant-like absorption enhancers is the disruption of the transcellular barrier by membrane solubilization and cell lysis, efficacy of these compounds is likely to be directly related to epithelial damage (Swenson and Curatolo, 1992; Uchyama *et al.*, 1996; Lindmark *et al.*, 1997). Moreover, absorption enhancement obtained by lytic activity can not be specific for the (size of the) drug of interest, and thus the intestinal barrier to entry of toxins, viruses and bacteria is also compromised. It was therefore suggested (Hochman and Artursson, 1994; LeCluyse and Sutton, 1997) that the preferable route to be enhanced was the paracellular route. This preference was stimulated by the observation that paracellular permeability could be increased by nutritional components such as glucose (Madara and Pappenheimer, 1987) and by the recognition that intestinal tight junctions could be loosened in a non-cytolytic and size selective manner by compounds such as sodium caprate or tumor necrosis factor (Lindmark *et al.*, 1997; Mullin *et al.*, 1997).

Until recently the only compounds known to selectively enhance paracellular permeation were Ca-chelators (EGTA, EDTA), but, by now, many common cellular effectors, including intra-

and extracellular Ca^{2+}, tyrosine- and serine/threonine phosphokinases and phosphatases, protein kinase C and cAMP, are found to be involved in the physiological regulation of tight junction permeability (reviewed in: Lutz and Siahaan, 1997; Mitic and Anderson, 1998; Madara, 1998). Additionally, all these cellular regulatory pathways of tight junctions can be activated or inhibited by nutritional or xenobiotic compounds (reviewed in: Hochman and Artursson, 1994; Ballard *et al.*, 1995; Anderson and Itallie, 1995; LeCluyse and Sutton, 1997). Some interesting examples are the paracellular permeability-increasing effects of natural toxins such as okadaic acid (diarrhetic shellfish poisoning, Tripuraneni *et al.*, 1997), presumably by increasing junctional protein phosphorylation, or the zonula occludens toxin (ZOT) of vibrio cholerae (Fasano *et al.* 1997). ZOT interacts with a specific surface receptor in the small intestine, but not in the colon, and induces a PKC dependent polymerization of actin filaments in the junctional region. Due to the specific non-cytotoxic effects of ZOT, this toxin is studied as absorption enhancer for insulin (Fasano, 1998). A similar mechanism involving PKC activation and actin polymerization was suggested for capsianoside, a component of sweet peppers (Hashimoto *et al.*, 1997).

Even though many compounds are found to decrease the intestinal epithelial barrier function, and much research effort has been put in elucidating their mode of action, often the exact mechanism of barrier disruption is unknown. The introduction of appropriate *in vitro* models, most of all the Caco-2 cell culture model, has made it possible to study the relation between permeation increasing en (cyto-) toxic effects in more detail (Anderberg *et al.*, 1992; Schasteen *et al.*, 1992; Lindmark *et al.*, 1997; Quan *et al.*, 1998). Without doubt these studies have contributed greatly to the knowledge on the transepithelial permeation enhancing mechanisms of drug absorption enhancers. However, it has still proven difficult to differentiate between paracellular and transcellular permeation enhancement (LeCluyse and Sutton, 1997).

§ 1.7 Intestinal epithelial cell lines as model for the intestinal epithelium: Paracellular permeation prognosis beyond Caco-2.

To date, several *in vitro* methods have been developed to study the intestinal absorptive and barrier functions. Some models such as everted sacs, intestinal rings and intestinal mucosa preparations in Ussing chambers, are discussed by Stewart *et al.* (1995) and Grass (1997), but due to difficulties in obtaining viable human tissues, most studies are now being performed with cells cultured on permeable supports. This so-called two-compartment cell culture system (see § 2.1) is by now a widely used model to study permeability and metabolism of drugs and nutrients, to study mechanisms of drug absorption enhancement, or to study effects of toxins on barrier or absorptive functions (reviewed by Quaroni and Hochman, 1996). Especially, the human carcinoma derived Caco-2 cells are, due to their enterocyte-like differentiation, among the most widely used cells, as model for the intestinal epithelium. Transepithelial transport rates for various compounds,

obtained in the two-compartment system with Caco-2 cells, are successfully correlated to human absorption data (Artursson and Karlsson, 1991; Artursson *et al.*, 1996). This suggested that during the enterocyte-like differentiation, the Caco-2 cells obtain many absorptive functions which are also operative in the intestinal epithelium *in vivo*. However, the cell line is derived from a colonic adenocarcinoma and some features may be fetal colonic, instead of enterocytal (Pinto *et al.*, 1983; Quaroni and Hochman, 1996). Moreover, the barrier presented by the junctional complex which limits the passive diffusion of hydrophilic macromolecules across the cells is un-physiologically tight, or at best, resembling colonic epithelium (Hidalgo *et al.*, 1989; Tanaka *et al.*, 1995; Artursson *et al.*, 1996). However, paracellular permeation of hydrophilic molecules occurs predominantly in the small intestine, due to the fact that ingested compounds first reach the small intestine, prior to the colon, and the epithelium of the small intestine has a higher paracellular permeability. Therefore, permeability rates obtained with the Caco-2 model could lead to an underestimation of intestinal absorption of hydrophilic drugs and toxins. Thus, there is a clear need for an *in vitro* model with a small intestinal paracellular permeability. For that purpose, we chose to study the absorption and barrier characteristics of the small intestinal cell line IEC-18. It was found that the TER of the ileal IEC-18 cells more closely resembles the TER of the small intestine (Ma *et al.*, 1992; Tanaka *et al.*, 1995), and that the transport of various paracellular markers, such as the hydrophilic molecules inulin and mannitol and the macromolecule albumin, was limited by filter-grown IEC-18 cells, albeit to a lesser extent than by Caco-2 cells. This suggests a more permeable paracellular pathway in filter-grown IEC-18 cells than in the Caco-2 cells. To establish whether the IEC-18 cells could be used to model small intestinal permeability, the permeability and the barrier functions of the IEC-18 cells were further determined and evaluated in this thesis. Furthermore, it was found that IEC-18 cells could be induced to acquire (differentiated) villus enterocyte characteristics by matrix components (Benya *et al.*, 1993) or a small intestinal extract (Sintani *et al.*, 1989). Therefore, it was also investigated whether the filter-grown IEC-18 cells could be used as a model to study the development of the intestinal epithelial barrier along the crypt-villus axis.

§ 1.8 Objectives and Approach

The two experimental sections in this thesis deal with the *in vitro* model based on Caco-2 and IEC-18 cells in a two-compartment system. In Section II the permeability of the filter-grown cells is determined. In Section III the cellular effects co-occurring with the modulation of the barrier function of the filter-grown cells are assessed.

The aims of this thesis were

- To investigate and evaluate the filter-grown small intestinal IEC-18 cells as model for small intestinal permeability, with special emphasis on the transepithelial passage of hydrophilic (macro-) molecules whose transport is dependent on paracellular permeability.

- To study the effects of intestinal epithelial cell differentiation on paracellular barrier development, i.e. to develop a model to study paracellular permeability along the crypt-villus axis.

- To investigate and evaluate filter-grown Caco-2 and IEC-18 cells as models for the assessment of effects of ingested compounds on the small intestinal epithelial barrier.

and,

- To assess the mechanism of barrier perturbation in a cell culture system with emphasis on the relation between permeation enhancement (barrier function), the disruption of the tight junctional morphology (paracellular barrier structure), and cytotoxicity.

In chapter 3 and 4 the cell layer morphology, brush border enzyme activities, active and passive absorption properties, and the barrier functions of both cell lines were determined and correlated to the differentiation status of the cells. In chapter 5, the effects of an epithelial cell differentiation inducer (dexamethasone) on the paracellular barrier of the crypt-like IEC-18 cells are assessed, and correlated to changes in the F-actin organization, ZO-1 localization and cell (layer) morphology. In chapter 6 and 7 the barrier function of the filter-grown Caco-2 and IEC-18 cells is compromised by treatment with the drug permeation enhancer palmitoyl carnitine (PCC) or the possible food contaminant cadmium. Subsequently, the relation between permeation enhancement, transepithelial electrical resistance, tight junction structure and cell viability is studied.

Chapter 2

Methodology

Application of cell culture systems in studies of intestinal permeability and permeability modulation

§ 2.1 The two-compartment cell culture system

The two-compartment cell culture system consists of a permeable cell culture (filter)-insert which is placed in a well of a normal cell culture plate. The cells are seeded on the filter-inserts and cultured to cover the whole surface area. When cells are grown to confluence, the total cell number is fixed and the cell layer forms a barrier between the two compartments. In the case of polarized intestinal epithelial cells, the two compartments are designated the apical or luminal compartment and the basolateral or serosal compartment.

Several advantages of this system include (Karlsson, 1995): 1/ small amounts of the compound suffice to perform a transport or effect experiment, 2/ experiments are relatively rapid and reproducible compared to other absorption models, 3/ real intestinal epithelial permeation rates can be determined (i.e. no gastrointestinal degradation, hepatic first-pass metabolism or complicating whole-body kinetics do not obscure the results), 4/ several parameters can be determined almost simultaneously for each filter-insert.

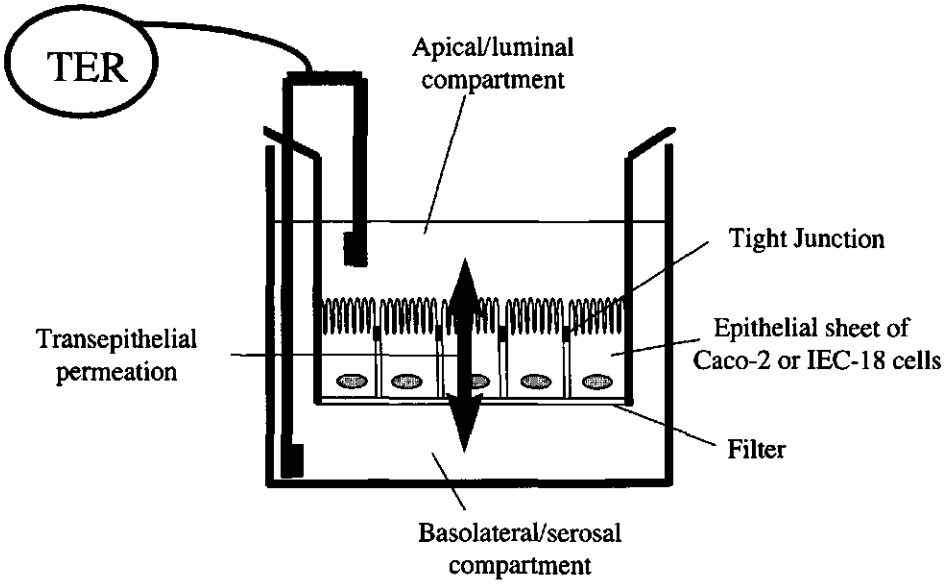

Figure 2.1: The configuration of the two-compartment cell culture system.

The configuration of the two-compartment cell culture system allows determination of:

- transport of several classes of compounds in two directions, thus both, absorption and secretion can be studied.
- effects on the cell layer after exposure to the apical (luminal), basolateral (serosal) or both cell sides
- cell layer integrity within seconds on the basis of the transepithelial electrical resistance (TER)
- cell viability (cytotoxicity) with several assays (for example: LDH leakage, NR uptake and PI uptake) on basis of a fixed number of cells
- cell morphology by light-, and/or electron microscopy
- cellular localization of proteins using immunocytochemistry and fluorescence microscopy.

Within this thesis combinations of measurements with respect to absorptive permeability, integrity and morphology of layers of Caco-2 and IEC-18 cells in the two-compartment system are explored.

Without doubt the use of intestinal epithelial cells in the two-compartment system has contributed to the knowledge on the differentiation and functions of the intestinal epithelium. However, some factors need to be considered in the interpretation of the measured transport

rates. One factor is the difference in the absorptive surface area between the flat cell culture model in which the absorptive surface area = anatomical surface area, and the *in vitro* models, using intestinal tissue preparations, where the absorptive surface area << anatomical surface area due to the crypt-villus configuration (Artursson *et al.*, 1996). This can be of particular interest to the absorption of compounds with a low permeability, such as hydrophilic macromolecules. As a result of their low permeation rate across the epithelium of the villi, these compounds might be able to penetrate into the direction of the mid-villus or crypt. By doing so they not only increase the available absorptive surface area, but they possibly also reach regions with a higher intrinsic permeability (Figure 2.2).

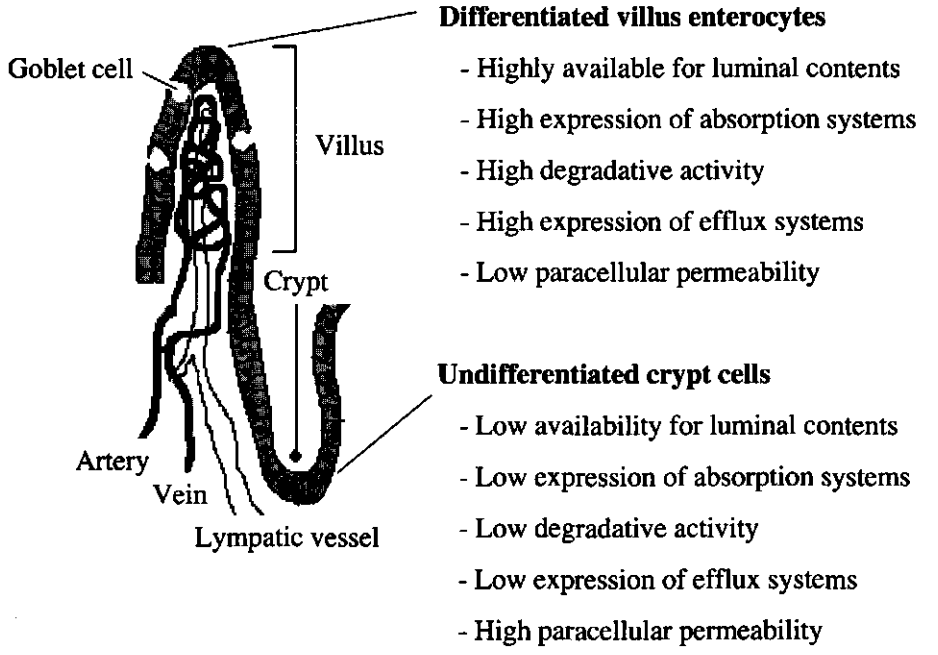

Figure 2.2: Structures and functions along the small intestinal crypt-villus axis.

Another factor which needs to be considerer in the use of the two compartment system, is that after seeding, the cells are cultured for a fixed period of time and it can thus be assumed that most cells obtain a similar degree of differentiation, with other words, each filter might represent a model for only one region along the crypt-villus axis. This information can in principle be used to obtain knowledge on the permeability of the different regions (e.g. crypt, mid-villus, villus tips). Within this thesis we would like to develope a model with which the

relation between the differentiation of the enterocytes and the development of their permeability and barrier functions can be studied.

§ 2.2 The intestinal epithelial cell lines Caco-2 and IEC-18

The intestinal cell line most intensively studied and used for its applicability as intestinal transport model is the Caco-2. This human colon carcinoma cell line made it to the forefront of intestinal research due to its autonomous differentiation (Pinto *et al.*, 1983). When seeded on a filter and given time to grow to confluence and to differentiate (2-3 weeks), these cells display many structural and functional characteristics of differentiated small intestinal enterocytes. The Caco-2 cells form polarized columnar cells with a well developed brush border and junctional complex at the cells' apex (Pinto *et al.*, 1983). The brush borders were found to exhibit digestive enzymes (e.g. sucrase isomaltase, lactase, dipeptidyldase IV and alkaline phosphatase) and many intestinal carrier systems (e.g. carriers for glucose, amino acids and di/tripeptides, nucleosides, bile acids and vitamin B12)(Quaroni and Hochman, 1996; Hidalgo and Li, 1996). Furthermore, Caco-2 cells express efflux systems (e.g. P-glycoprotein), Phase I metabolic enzymes (cytochrome P450s) and Phase II conjugating enzymes (sulfotransferase, UDP-glucuronyltransferase, glutathione S-transferase) and thus represent an intestinal-like biochemical barrier (Gan and Thacker, 1997). Some disadvantages of the Caco-2 cells are that the cell line is morphologically and functionally very heterogeneous (Vachon and Beaulieu, 1992) and that functional and structural features are variable with passage number (Briske-Anderson *et al.*, 1997; Yu *et al.*, 1997).

The rat ileal crypt-derived non-transformed cell line IEC-18, was originally isolated by Quaroni and co-workers and used to investigate cytotoxic effects of chemical carcinogens (Quaroni and Isselbacher, 1981). They found that IEC-18 cells were able to metabolize dimethylbenz[a]anthracen and benzo[a]pyrene and that the cells were very sensitive for the cytotoxic effects of these compounds. Since then, the IEC-18 cells were found to express P-450 reductase, microsomal and cytosolic epoxide hydrolases, glutathione S-transferase and UDP-glucuronosyl transferase (Glatt and Gemperlei, 1987). The cells were also used as model for the small intestinal epithelium to study mechanisms of carrageenan or reactive oxygen species induced injury in the intestine (Ling *et al.*, 1988; Ma *et al.*, 1991). In the early nineties Ma *et al.* (1992) started using filter-grown IEC-18 cells as a model to study small intestinal epithelial permeability. It was found that filter-grown IEC-18 cells expressed the junctional proteins ZO-1 and desmoplakin, that they developed a TER comparable to that of the small intestine (approximately 20 to 45 $\Omega*cm^2$) and that the cells formed a permeation barrier for hydrophilic molecules.

§ 2.3 Methods to assess the functionality of the Intestinal epithelium *in vitro*

§ 2.3.1 Measurement and meaning of the Transepithelial electrical resistance

The TER is generally used to determine the integrity of the epithelial barrier. Since the resistance of biomembranes, i.e. the apical and basolateral cell membranes, are usually very high (>1000 $\Omega.cm^2$), in fully functional confluent cell layers the TER reflects the paracellular barrier towards electrolytes (Reuss, 1992; Madara, 1998). The TER is therefore a very sensitive measure to detect changes in the paracellular permeability. However, when the cells are exposed to membranolytic compounds such as surfactants, fatty acids and bile salts, the resistance of the biomembranes are decreased. Due to the fact that the area of the transcellular pathway is more than 1000 times larger than the area of the paracellular pathway, a decreased resistance of the membranes (transcellular pathway) may result in a decreased TER. Furthermore, extensive opening or activation of ion channels in the cell membranes can decrease the TER substantially (Madara, 1998).

The transepithelial electrical resistance (TER) was measured using the Millicell-ERS epithelial voltohmmeter with Ag/AgCl electrodes (Millipore Co., Bedford, USA) in iso-osmotic (approximately 300 mOsm) media. The TER of the cell layers was calculated according to the following equation:

$TER = (R_{total}-R_{blank}) \times A\ (\Omega.cm^2)$,

R_{total} = the resistance measured,

R_{blank} = resistance of control filters without cells (approximately 140 $\Omega.cm^2$)

A = surface area of the filter (1 cm^2).

The TER can be expressed as absolute value ($\Omega.cm^2$) to compare different regions of the intestine or different cell lines, but within an experiment the TER is preferably expressed as % of control (i.e. control is untreated filter or the same filter prior to exposure). The Caco-2 monolayers obtain a TER between 300 and 600 $\Omega.cm^2$ after prolonged culture, which is un-physiologically high for the intestine (Artursson *et al.*, 1996). The IEC-18 cells develop a TER of approximately 50 $\Omega.\ cm^2$ which is in good agreement with the TER of the small intestine (Ma *et al.*, 1992).

§ 2.3.2 Assessment of transepithelial permeation rates

The transepithelial passage of all kinds of compounds in both directions is easily studied in the two-compartment system. A detectable tracer is added to the donor

compartment (in case of absorption: at the apical/luminal side) and its appearance in the acceptor compartment (basolateral/serosal) can be followed in time. The transport experiments were carried out at 37°C in Transwell inserts with 0.5 ml medium with tracers apically and 1.8 ml medium basolaterally. With these volumes there is no hydrostatic pressure since the media levels apically and basolaterally are even. A reduction of the unstirred water layer and homogeneous mixing of the probes was achieved by rotating the two-compartment transport system at 30 rpm on a rotating platform device. For the studies in this thesis the Polyethyleneglycols PEG-900 (±900 D) and PEG-4000 (± 4000 D) and the fluoresceine-isothiocyanate-labeled Dextrans FD4 (4400 D), FD20 (19400 D), FD70 (71200 D), and FD500 (487 kD) were used as model compounds for hydrophilic macromolecules (i.e. proteins; Noach, 1994). As models for smaller hydrophillic compounds, mannitol (182 D) and fluoresceine (332 D) were used. These compounds were found to be stable in the systems used, and transepithelial passage of these compounds occurred mainly paracellularly under physiological conditions (Schasteen *et al.*, 1992; Bjarnason *et al.*, 1995; Noach, 1995).

Permeation rates frequently used in the literature are the flux representing the nett passage of compound per unit of time ($mol.s^{-1}$), the clearance ($flux/C_0$ in $\mu l.s^{-1}$) and the apparent permeability coefficient (P_{app} in $cm.s^{-1}$) representing the steady-state flux under sink conditions (i.e. before 10% of the total amount of tracer has appeared in the receptor compartment), normalized for the start concentration of the tracer, and for the area. Thus the P_{app} is the flux divided by A and C_0.
In formula:

$P_{app} = P/(A*C_0)$ $(cm.s^{-1})$

P = permeability rate or flux $(mol.s^{-1})$

C_0= Initial apical concentration of test substance (M)

A = area of filter (cm^2)

In this thesis the P_{app} will be used to express the permeability rates since it was already shown in 1991 (Artursson and Karlsson, 1991) that for a broad range of chemical compounds the P_{app} found in a Caco-2 system correlated well with human absorption data.

§ 2.3.3 Qualitative Immunofluorescence localization of junctional proteins

Indirect immunolabeling was performed to establish the organization and localization of junctional proteins (ZO-1, occludin and E-cadherin) and filamentous actin (F-actin). The tight junctional proteins ZO-1 and occludin were immunolabeled to verify the presence of tight junctions at sites of cell-cell contact. Many reports describe the structural expression and changes therein of these proteins using immunolabeling and western blotting (reviewed by

Mitic and Anderson, 1998). For both proteins it has been shown that disappearance, reduced junctional expression or altered phosphorylation status was concomitant with reduced paracellular barrier functionality (Balda and Matter, 1998; Anderson and Itallie, 1995). However, it was also shown that junctional ZO-1 expression not always correlated to barrier function (Itoh *et al.*, 1993) and that a discontinuous occludin ring could suffice to form electrically tight MDCK monolayers (Balda *et al.*, 1996). Nonetheless, we hypothesize that if non-physiological permeation enhancement is achieved mainly by paracellular permeation enhancement, this should be accompanied by structural changes in the tight junctions and these structural changes can be visualized by altered immunolocalization of ZO-1 and/or occludin.

For immunocytochemistry the cells were fixed in 1% formaldehyde in HBSS for 5 min and permeabilized in 0.2% Triton X-100 in HBSS for 30 min. Subsequently the first antibodies (Polyclonal rabbit anti-ZO-1, polyclonal rabbit anti-Occludin, polyclonal Goat anti human E-Cadherin) were diluted in 0.1% Triton X-100 in HBSS and incubated for 1-3 h at room temperature or at 4^0C overnight. After rinsing, the secondary antibodies (anti-rabbit IgG conjugated with biotin, anti-goat IgG conjugated with biotin) were diluted in HBSS and incubated at RT for 1 h. In the final step the biotin-groups were stained with streptavidin conjugated with TRITC or FITC. F-actin was stained with phalloidin conjugated with TRITC or FITC in a one step procedure. All immunofluorescence label protocols could be combined with the staining of nuclei using Hoechst 33258.

§ 2.3.4 Quantification of junction-related ZO-1 using confocal scanning laser microscopy and image processing

In addition to the qualitative descriptions of patterns, and changes therein, of labeled proteins, we developed a method which allowed for quantification of changes in the junction related ZO-1 pattern in CLSM images of filter-grown epithelial cells. Earlier reports mentioned the use of CLSM to determine the transport route of fluorescent compounds (Hurni *et al.*, 1993) or for the semi-quantitative evaluation of cellular ZO-1 and F-actin content (Nybom and Magnusson, 1996).

Confocal fluorescence microscopy was performed with the Bio-Rad MRC600, using the PlanApoChromat 40x objective with variable diaphragm (Zeiss, Oberkochen, Germany). All images were recorded at standardized acquisition conditions; per cell line and per experiment settings for pinhole, gain and blacklevel were optimized and applied throughout that experiment. Images are single plains (a depth of 7 um covers >90% of the total intensity) recorded using the Kalman filter mode at n=5 with Bio-Rad software (COMOS version 7.0a). After transfer to an image processing workstation, the original grey value images (Figure

2.3A) were processed by a custom written program, based on the image processing toolbox SCIL-Image (version 1.3, Free University of Amsterdam, Netherlands). The program was developed to discriminate between junction-related immunolabeled ZO-1 and non-junction-related immunolabeled ZO-1 and background and consists of three sequential steps. In the first step, two methods are applied to differentiate between immunolabeled ZO-1 and background using a threshold value of 1.4. The two resulting binary images are combined to a usually honeycomb-like skeleton. This intermediate skeleton represents most of the junction-related ZO-1 pattern, sometimes with small discontinuities and some not junction-related appendixes. In the second part of the program, the skeleton can be improved, first by closing the small discontinuities of the borderlines using a maximum cost procedure (adding less than 0.5% to the total pixel count) and secondly, by interactive and automatic removal of spurious line fragments. Finally, from the binary image (Figure 2.3B) of the optimized skeleton the total number of pixels is determined and used as measure for junctional density. The final binary image is subsequently superimposed on the original grey value image to determine the average pixel intensity over the actual cell borderlines throughout the monolayer. This measure is an indirect quantification of the amount of junctional protein (comparable to the number of junctional strands) in the lateral membranes.

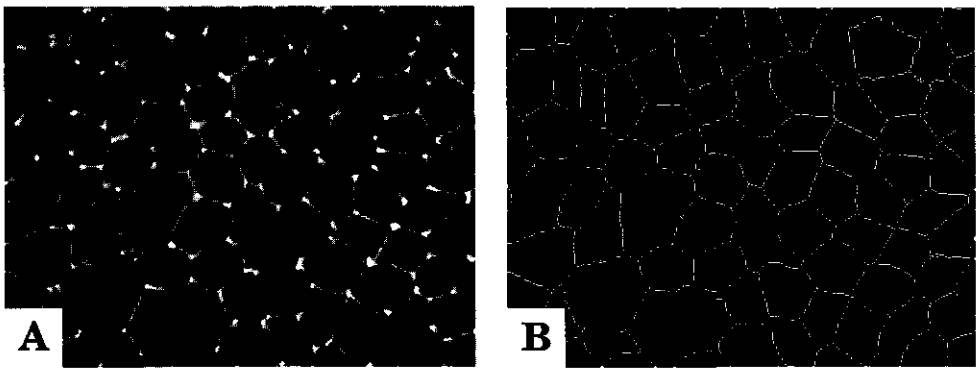

Figure 2.3: The immunocytochemical localization of ZO-1 in a Caco-2 cell monolayer in an original CLSM image (A) and in a binary image after processing by a cutsom written program which selected the honeycomb-like tight junctional pattern (B).

§ 2.3.5 Assessment of adverse effects on the intestinal epithelium *in vitro*

There are many assays available to detect cytotoxic effects of chemicals on cells in culture (LDH leakage, NR or PI uptake, MTT assay etc.) and all these assays can be performed on filter-grown cells too. Additionally, in the two-compartment system the transepithelial permeation of tracer molecules and the transepithelial electrical resistance can

be used to detect adverse effects on the absorptive or barrier function of the epithelial cell layers. Furthermore, changes in expression or immunolocalization of structural and functional proteins can be interpreted as adverse effects on the cells or cell layers.

Whereas in most studies these assays are used to assess cytotoxicity, or cell viability, in general, in this thesis the LDH leakage, the NR and PI uptake assays are considered with respect to the actual effect they are pointing at. The LDH leakage assay is based on the release of cytosolic LDH into extracellular medium and thus indicates damage to the cell membrane. Damage can be expressed as units, or percentage, of LDH released from the cells into the medium, or remaining in the cell. This assay has been shown to be extremely sensitive since many LDH units are present in the cell layers and the activity is determined with great sensitivety. Since LDH leakage is such a direct measure of increased (apical) membrane permeability (or cell lysis), and the surface area of the cell membranes is at least 1000 times larger that the paracellular surface area, we assume a correlation between LDH leakage and disruption of the transcellular barrier.

Another membrane damage assay used is the propidium iodide (PI) uptake assay. Functional biomembranes do not allow cellular uptake of propidium iodide, however, when the permeability of these membranes is increased, this dye can enter the cells. Staining can be determined by counting the stained nuclei under a fluorescence microscope, cytotoxicity is then expressed as number (%) of nuclei stained (Hochman *et al.*, 1994). It is also possible to quantify total fluorescence of a PI exposed cell layer and to express cytotoxicity as increase in fluorescence (chapter 6). An advantage of this method is that it can be used together with the immunofluorescent localization of junctional proteins, allowing determination of functional and structural effects on the transcellular and the paracellular barrier.

The neutral red uptake assay is based on the passive diffusion through the cell membranes, and subsequent intracellular sequestration, of this weakly cationic dye in lysosomes (Babich and Bohrenfreund, 1990). The uptake is dependent on the functionality of the membrane on the side of NR exposure while the sequestration is dependent on the functionality of the lysosomes. Both, uptake and sequestration are dependent on cell number. In the two-compartment cell system the cells are cultured to confluence thus a fixed number of cells is present at the start of the experiment. Reduced NR uptake readings may indicate a reduction in cell number (severe lysis), or more subtle effects as reduced lysosomal functionality. NR uptake data are usually presented as % uptake relative to NR uptake by control cell layers.

Section II

Permeability of the Intestinal Epithelial Barrier *In Vitro*

Chapter 3

Comparison of permeability characteristics of the human colonic Caco-2 and rat small intestinal IEC-18 cell lines

Erwin Duizer, Andre H. Penninks, Wilma H. Stenhuis, and John P.Groten.
Journal of Controlled Release, 1997;49:39-49.

Abstract

Several human colonic carcinoma cell lines are used to study the intestinal transport of compounds *in vitro*. However, the major site of absorption of drugs and nutrients is the small intestine, which differs greatly from the colon in paracellular permeability. Several hydrophilic compounds, of which intestinal uptake *in vivo* was found, are not transported across filter-grown colonic cells *in vitro*. The aim of this study was to compare transport properties of the low resistance rat small intestinal cell line IEC-18 to those of the high resistance human colonic cell line Caco-2.

After prolonged culture of Caco-2 and IEC-18 on Transwell polycarbonate filters enzyme activity determinations revealed the presence of sucrase-isomaltase and mature enterocyte alkaline phosphatase in Caco-2 and fetal alkaline phosphatase in IEC-18. Both cell lines formed a confluent layer as confirmed by transepithelial electrical resistance measurements (TER_{Caco-2}= 350±14 Ω/cm^2, TER_{IEC-18}= 55±4 Ω/cm^2) and fluorescence microscopy on immunolabeled F-actin. The tight junctional protein ZO-1 is organized into cell circumscribing strands in both cell lines.

Transport rates of lipophilic compounds transported transcellularly were almost similar in both cell lines but transport rates of hydrophilic compounds transported paracellularly were clearly higher in IEC-18 cells. IEC-18 cells also allowed for a better discrimination on the basis of molecular size between several compounds which are transported paracellularly.

Introduction

The intestine forms the main port of entrance for many nutrients and drugs. Several *ex vivo* and *in vitro* models have been developed and utilized to study intestinal permeability. One frequently used *in vitro* model is the two-compartment transport system which is based on filter-grown intestinal cell lines. The popularity of this system is mainly due to the ability of some human colonic tumor cell lines to undergo differentiation into polarized epithelial cells in culture. Well known cell lines being used in the two-compartment cell culture model are Caco-2 (Hidalgo *et al.*, 1989; Hilgers *et al.*, 1990; Artursson and Karlsson, 1991), T84 (Dharmsathaphorn and Madara, 1990; Madara *et al.*, 1987) and HT-29 (Karlsson *et al.*, 1993; Wils *et al.*, 1994a).

For drug absorption studies the Caco-2 cell line is most frequently used. This cell line is derived from a human colon adenocarcinoma and differentiates spontaneously into polarized columnar enterocyte like cells after reaching confluence (Pinto *et al.*, 1983; Wilson *et al.*, 1990). The differentiated Caco-2 cells grown on permeable filters, present a mixed set of small intestinal and colonic characteristics. With respect to the passive and active transcellular transport of electrolytes, sugars, amino acids and lipophilic drugs, the Caco-2 monolayer displays several features of the small intestinal epithelial barrier (Hillgren *et al.*, 1995). Also, a good correlation between *in vivo* human absorption data and transport in Caco-2 cells was found for several compounds (Artursson and Karlsson, 1991). However, many of the newly synthesized drugs or nutrients are neither transported by active transcellular processes nor are they absorbed by passive diffusion through the cells due to their size and/or hydrophilicity (Hillgren *et al.*, 1995; Artursson and Karlsson, 1991). The only remaining and most likely port of entrance of these compounds is the paracellular pathway, i.e. along the cells, through the tight junctions and the intercellular spaces. Especially with respect to this paracellular pathway the Caco-2 monolayer displays the tightness of colonic epithelium. The transepithelial electrical resistance (TER) of the colonic epithelium, a direct measure of monolayer resistance to passive electrolyte and ion permeation, is much higher than that of small intestinal epithelium. This means the Caco-2 monolayer excludes the passage of several hydrophilic molecules, which are probably transported to some extend by the leaky small intestinal epithelium *in vivo* (Artursson and Karlsson, 1991; Blomqvist *et al.*, 1993; Bijlsma *et al.*, 1995; Ma *et al.*, 1995). Therefore the suitability of the colonic Caco-2 cells as a model to study paracellular permeability of drugs that are absorbed incompletely, is probably limited (Artursson and Karlsson, 1991; Nellans, 1991). This indicates there is a clear need for additional cell culture models resembling the paracellular transport properties of the small intestinal epithelium more closely.

IEC-18, a small intestinal crypt-derived cell line, was originally isolated by Quaroni and co-workers and used to study cytotoxic effects of chemical carcinogens (Quaroni and

Isselbacher, 1981), mechanisms of carrageenan or radical injury (Ling *et al.*, 1988; Ma *et al.*, 1991) and oxidant-dependent bactericidal activity (Deitch *et al.*, 1995). Just recently Ma and coworkers (1992) started using filter-grown IEC-18 cells as a model to study small intestinal epithelial permeability. The TER of these IEC-18 cells more closely resembles the TER of the small intestine, which could indicate a comparable leakiness/tightness of the paracellular pathways. Transport of various paracellular markers, such as the hydrophilic molecules inulin and mannitol and the macromolecule albumin, was limited by filter-grown IEC-18 cells, albeit to a lesser extend than by Caco-2 cells. This suggests a more permeable paracellular pathway in IEC-18 than in the colonic Caco-2 cells. Whether the differences in TER and paracellular permeability between IEC-18 and Caco-2 cells are related to the structure of the junctional complexes is unknown.

In order to confirm the suitability of the IEC-18 cell line as an *in vitro* model for small intestinal transport of lipophilic and hydrophilic compounds we compared transport properties of this cell line to those of the Caco-2 cell line. This characterization is not restricted to paracellular and transcellular transport alone, but is also based on the differentiation stage and the development of the junctional complexes of both cell lines. For that purpose brush border enzyme activities, transepithelial electrical resistance and morphological features of both cell lines are compared during prolonged culture under similar test conditions.

Materials and methods

Cell culture

The Caco-2 cell line originating from a human colorectal carcinoma, and IEC-18 cells originating from rat ileum epithelium were obtained from the American Type Culture Collection. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Maintenance medium was DMEM with high glucose (4.5 g/l) and 25 mM Hepes, supplemented with 1% (v/v) MEM nonessential amino acids, 6 mM L-glutamine, 50 µg/ml gentamycin (all from Gibco, Paisley, Scotland), and 10% (v/v) foetal calf serum (FCS, Integro, Zaandam, Netherlands) for Caco-2, and 5% (v/v) FCS and 0.1 U/ml insulin (Sigma, Beers, Belgium) for IEC-18 cells.

Cells grown in 75 cm^2 flasks (Costar, Badhoevedorp, Netherlands) were passaged weekly at a split ratio of 1:10 using 0.05% trypsin in PBS with 0.022% EDTA. For transport experiments approximately 2 $*10^5$ cells/cm^2 (IEC-18) or 1 $*10^5$ cells/cm^2 (Caco-2) were seeded on Transwell polycarbonate cell culture inserts with a mean pore size of 0.4 µm (Costar, Badhoevendorp, Netherlands). Cells were cultivated for 18-29 days. Caco-2 cells were used at passages 85-97, IEC-18 at passages 18-30.

Transepithelial electrical resistance (TER)

The transepithelial electrical resistance of filter grown cells was measured using the Millicell-ERS epithelial voltohmmeter (Millipore Co., Bedford, USA) at different time intervals (n=6). The TER of the cell monolayers was calculated according to the following equation: TER = $(R_{total}-R_{blank})$ X A ($\Omega.cm^2$), R_{total} is the resistance measured, R_{blank} is resistance of control filters without cells (approximately 140 $\Omega.cm^2$), A = surface area of filter (1 cm^2).

Cell number

Cell counting of filter grown cells was performed using fluorescence microscopy after staining the cell nuclei with Hoechst 33258 (2.5 µg/ml). For each time point at least 8 area's (>300 cells) were counted on two different filters. Nucleus staining was combined with a F-actin label (phalloidin-FITC from Sigma, Beers, Belgium) to check for monolayer confluency. Cell numbers of plastic grown cells (25 or 75 cm^2 flasks, Costar, Badhoevedorp, Netherlands) were determined after trypsinization using a heamatocytometer.

Light microscopy

Filter grown cells were fixed in 4% formaldehyde in PBS. Cells were dehydrated using an ascending series of ethanol with the final step in 100%. Filters with cells were embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany) with 1% polyethyleneglycol-4000. Cross sections (thickness is 2-4 µm) of filter grown cells were stained with trypan blue.

Immunocytochemistry

For immunocytochemistry the filters with cells were fixed in 1% formaldehyde in HBSS and permeabilized in 0.2% triton X100 in HBSS. F-actin was stained with phalloidin conjugated with TRITC or FITC (Sigma, St.Louis, USA). Polyclonal rabbit anti-ZO-1, anti-rabbit IgG-biotin and streptavidin-FITC (Zymed, San Francisco, USA) were used to visualize ZO-1 in both cell lines. Monoclonal antibody to desmoplakin I and II was obtained from Boehringer Mannheim Biochimica (Mannheim, Germany) and rhodamine-conjugated rabbit-anti-mouse IgG from Dako (Glostrup, Denmark).

Confocal Scanning Laser Microscopy

Confocal fluorescence microscopy was performed with the Biorad MRC600, using Plan-neofluar objective 63/1.25 and PlanApoChromat 40/1.0 (Zeiss, Germany). Images were recorded with Biorad software and processed using the image processing toolbox SCIL-Image (VU Amsterdam, Netherlands).

Enzyme activity

Activities of alkaline phosphatase were determined according to Tietz (1983), on the BM/Hitachi 911 using p-nitrophenylphosphate as a substrate. Cell homogenates were diluted to 30 mU ALP/mL and incubated with levamisol (10 min, 0-10 mM) at room temperature to discriminate between the differentiated enterocyte isotype of ALP and the fetal or

liver/kidney/bone isotype (Hoffman *et al.*, 1994). A modification of the method of Dahlqvist (1984) was used to determine sucrase-isomaltase activity. In short, the cell homogenate was incubated 30 min at 37°C with 100 mg/ml sucrose as substrate, the reaction was stopped by boiling for two minutes. The glucose formed was measured with the BM/Hitachi 911 using the hexokinase/G6P-DH assay (Boehringer, Mannheim, Germany). Total protein contents were determined according to Lowry with bovine serum albumin as standard.

Transepithelial transport

^{14}C-polyethyleneglycol-4000, ^{14}C-salicylic acid, ^{14}C-acetyl salicylic acid, ^{14}C-testosterone, ^{3}H-mannitol, and ^{3}H-propranolol (NEN products, Boston, USA), were mixed with unlabelled compounds to yield final concentrations of 1 to 100 µM with specific activities of 30 Ci/mol to 300 Ci/mol. Specific activities of the probes shown in fig 5 were: 270 Ci/mol for ^{14}C-polyethyleneglycol-4000 and ^{3}H-mannitol, and 180 Ci/mol for ^{14}C-testosterone. ^{3}H- and ^{14}C-labelled probes were analyzed using a Wallac 1409 Liquid Scintillation Counter. Fluorescein and the fluorescein isothiocyanate dextrans (FD) with average mol weights of 4,400 (FD-4), 19,400 (FD-20), 71,200 (FD-70) and 487,000 (FD-500) Da were from Sigma, St.Louis, USA. Fluorescein and the FD samples were analyzed using HPLC and fluorescence detection (seize exclusion columns 500G and sec30XL in series in this order, a mobile phase of 25 mM NaH_2PO_4 buffer pH 9, flow rate 0.5 ml/min, and detected with excitation at 495 nm and emmission at 519 nm with a Jasco FP920 Fluorescence detector). All dextrans showed one peak in the HPLC profiles. Retention times of the transport solution and the basolateral samples were the same in all cases.

Transport experiments were carried out at 37°C in Transwell inserts with 0.5 ml medium apically and 1.8 ml medium without test compound basolaterally. The medium used in transport experiments was DMEM with non-essential amino acids, gentamycin, and 0.1% BSA. A slight reduction of the aqueous boundary layer and homogeneously mixing of the probes was achieved by rotating the two-compartment transport system at 30 rpm on a rotating platform device. Apparent Permeability coefficients (P_{apps}) were determined on basis of appearance of the probe in the basolateral receiver compartment before 10% of the probe was transported (i.e. under sink conditions) according to the following equation (Artursson and Karlsson, 1991): $P_{app} = P/A*C_0$ (cm/s), P = permeability rate (mol/s), C_0= Initial apical concentration of test substance (mol/ml), A = area of filter (cm^2). The effect of metabolism on P_{apps} was neglected for compounds of which cellular uptake was known to be very low (mannitol, fluorescein, FD4, PEG-4000, FD20, FD70 en FD500). The P_{apps} of salycilic acid, acetyl salycilic acid, testosterone and propranolol were determined under conditions where effects of drug metabolism were minimized i.e. under sink conditions and at different concentrations.

Results

Cell growth and development of transepithelial electrical resistance

The cell number and transepithelial electrical resistance were determined of both cell lines grown on Transwell permeable cell culture inserts at several timepoints after seeding.

Data of a typical experiment are presented in Figure 3.1. Cells were seeded at 1 to $1.5 \cdot 10^5$ cells/cm^2. The cell number of Caco-2 cells increased up to day 15 and remained relatively constant from day 15 to day 29 at approximately $8 \cdot 10^5$ cells/cm^2. The TER of the Caco-2 monolayer showed a clear increase during the whole culture period of 29 days. Even from day 15 onwards, when cell numbers were constant, the TER increased steadily. IEC-18 cells proliferate quickly up to a density of $5 \cdot 10^5$ cells/cm^2 on day 4, after which a slow gradual increase to $\pm 7 \cdot 10^5$ cells/cm^2 on day 29 was observed. However, the TER of filter grown IEC-18 cells reached a maximum value of approximately 50 $\Omega \cdot$cm^2 within 4 days after seeding and stayed relatively constant up to day 29.

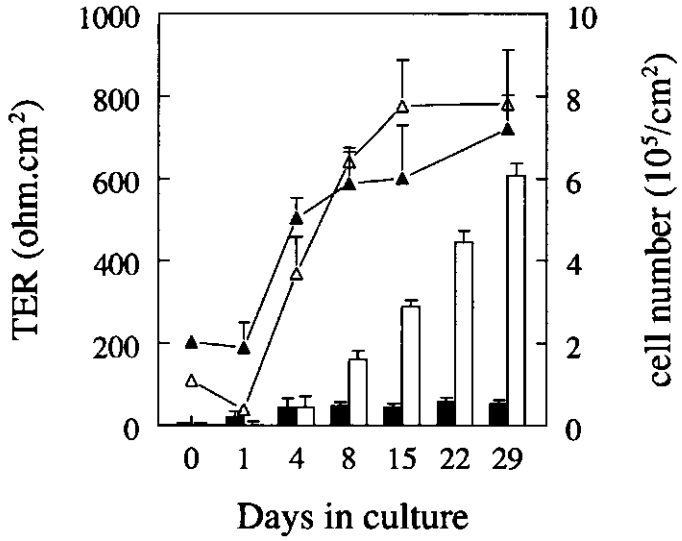

Figure 3.1: Time-dependent growth pattern and Transepithelial electrical resistance (TER) development of Caco-2 and IEC-18 cells grown on 12mm-Transwell polycarbonate filters. Cell numbers (Caco-2 -▵- and IEC-18 -▲-) are expressed as means±SD for 10 areas (5 areas/filter). The TER (Caco-2 open columns and IEC-18 black columns) is shown as means±SD for 6 filters..

In Figure 3.2 the total protein contents and activities of alkaline phosphatase and sucrase-isomaltase of whole-cell homogenates are presented. Both cell lines showed a gradual increase in alkaline phosphatase activity in time. Lack of inhibition with levamisole revealed that the alkaline phosphatase of Caco-2 cells was the intestinal isoenzyme (data not shown).The alkaline phosphatase activity of IEC-18 was dose-dependently inhibited by levamisole, indicating that this was not the mature enterocyte intestinal alkaline phosphatase. Sucrase-Isomaltase activity showed a gradual increase in time in Caco-2 cells whereas no

sucrase-isomaltase activity could be detected in the IEC-18 cells so far (detection limit of method used is 1 mU/cm^2).

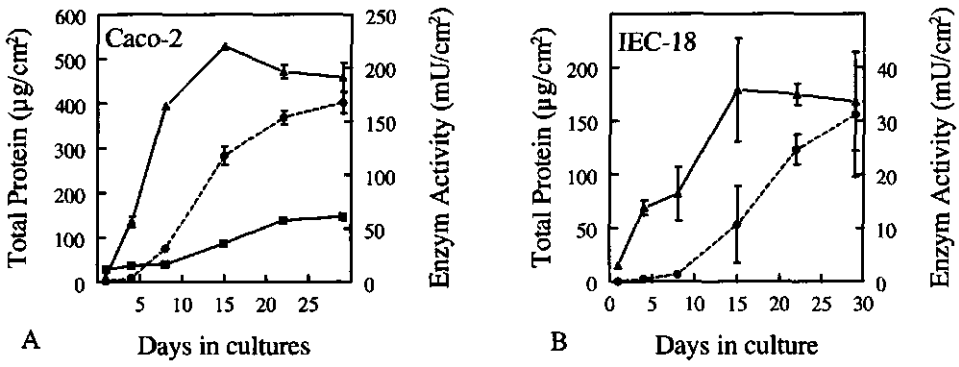

Figure 3.2: *Total protein contents (-▲-) and enzyme activities (sucrase-isomaltase (-■-), alkaline phosphatase (-●-)) of Caco-2 (A) and IEC-18 (B) cells grown on 12mm-Transwells for 29 days. Data are expressed as means±SD (n=3).*

Light microscopy and confocal scanning laser microscopy

Plastic imbedded filters with 22 days old Caco-2 cells (Figure 3.3) showed a well polarized monolayer of ±58 µm high cells with well developed brush borders. The filter grown IEC-18 cells showed a less organized pattern and seemed to be pseudo-stratified or even double layered. The single cell height was approximately 14 µm but the height of the whole cell layer came close to 25 µm. Confocal images of immunofluorescent labelled cells as shown in Figure 3.4, indicate that the tight junctional protein ZO-1 was present at all stages of differentiation in both cell lines. However, the organization of ZO-1 is changing in time. The images of Caco-2 cells 1 day after seeding show strand-like tight junctions at locations of cell-cell contacts. At sites lacking cell-cell contacts the ZO-1 protein is localized in a vesicle-like manner in the proximity of the lateral cell membrane (Figure 3.4A). The typical pattern of cell circumscribing tight junctional strands was complete from day 8 onwards (Figure 3.4B and 3.4C), which coincides with the observed starting increase in TER. The overall intensity of ZO-1 label does not seem to increase from day 8 to day 22, while the TER almost triplicates. At all stages of growth the Caco-2 cells presented a peripheral actin belt (data not shown) which is characteristic for epithelial cells. In contrast with ZO-1 there is also an actin belt present in cells lacking cell-cell contacts. During differentiation there is a decrease in cell-stretching fibrils of F-actin and an increase of sharpness and intensity of the peripheral belt. No remarkable changes were seen in the F-actin morphology from day 14 onwards.

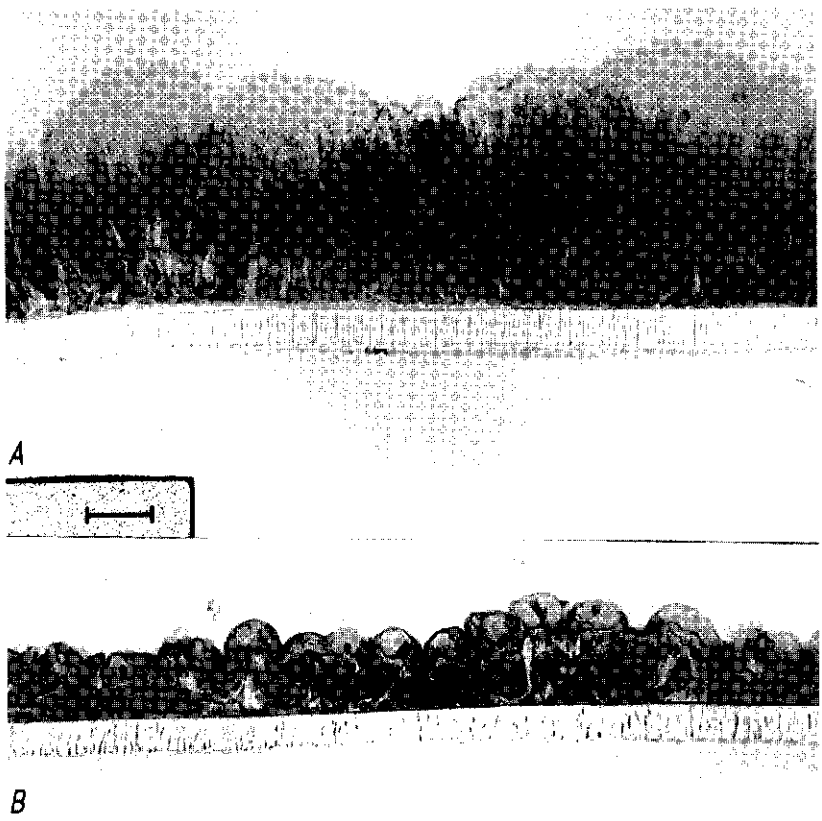

Figure 3.3: Light micrographs of cross sections through 22 days old filter grown Caco-2 (A) or IEC-18 cells (B). Magnification 200X, bar is 25 µm.

The tight junction development in IEC-18 cells seems to start with small ZO-1 strand-fragments at day 1 (Figure 3.4D) at sites of cell-cell contact but without any detectable ZO-1 at sites lacking these contacts. Tight junctions appear to be formed by connecting the small fragments to a cell circumscribing network at day 8 (Figure 3.4E), followed by an increase in total ZO-1 protein in the junctional strands as indicated by a more intense fluorescence signal at day 22 (Figure 3.4F). However, in IEC-18 cells the changes in ZO-1 organization do not coincide with a change in TER, which remains relatively constant from day 4 onwards. One day after seeding there is a belt-like organization of F-actin at the periphery of many cells. During growth the total amount of F-actin in IEC-18 seems to increase but this does not lead to a clear actin belt. The presence of desmosomes in the lateral cell membrane was also confirmed in this study at day 15 and 22 by the patchy staining with desmoplakin antibodies in both cell lines (data not shown).

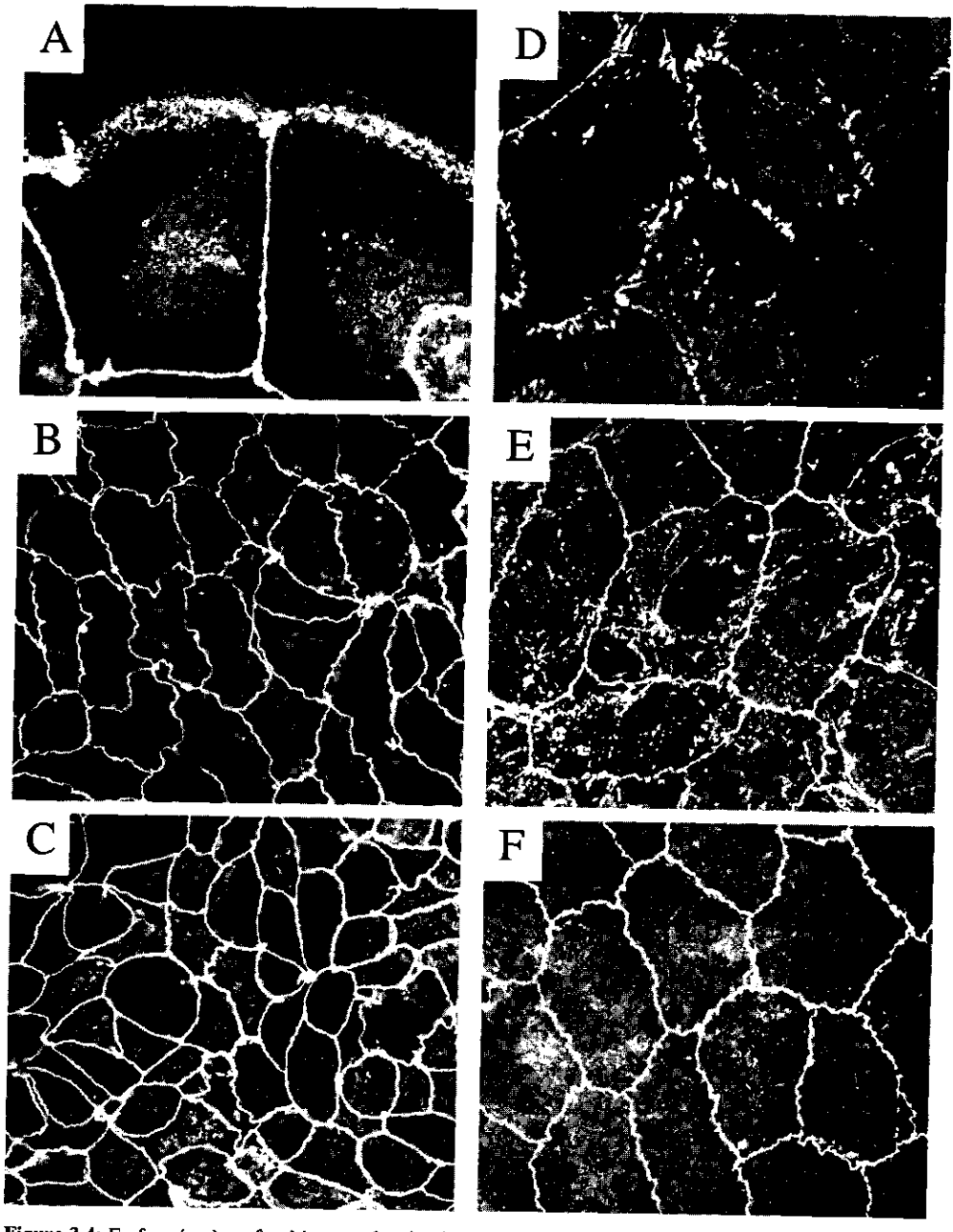

Figure 3.4: En face (x-y) confocal images showing immunolocalization of ZO-1 in filter grown Caco-2 (A-C) and IEC-18 (D-F) cells. Cells were seeded on day 0 and fixed and immunostained for ZO-1 on day 1 (A,D), 8 (B,E) and 22 (C,F). Magnification 200X.

Transepithelial transport

Transport of various compounds across filter grown cells was studied in apical to basolateral direction (Table 3.1). For all compounds tested at concentrations ranging from 1 to 100 µM, the apparent permeability coefficients were not found to be concentration dependent, and no cytotoxicity was indicated as measured by LDH leakage. The transport pathway for the hydrophilic compounds fluorescein, mannitol, PEG-4000 and the FDs is thought to be the paracellular route. Testosterone and the drugs salicylic acid, acetyl salicylic acid and propranolol are mainly transported by a (passive) transcellular pathway, although there might be a variable contribution of the paracellular pathway. In Caco-2 monolayers there are clear differences between P_{apps} for hydrophilic ($P_{app} < 7.6 \cdot 10^{-7}$ cm/s) and lipophilic compounds ($6 \cdot 10^{-6}$ cm/s$< P_{app} < 4 \cdot 10^{-5}$cm/s). The P_{apps} found for lipophilic compounds across IEC-18 cells are in the same order of magnitude as those found for the Caco-2 cells. In contrast, the P_{apps} for the hydrophilic compounds are at least 4 times higher in IEC-18 cells than in Caco-2 cells. The time course of transport of the hydrophilic markers mannitol and PEG-4000 and the lipophylic marker testosterone is shown in Figure 3.5.

Table 3.1: Permeability coefficients (P_{apps}) of various test compounds as calculated from transport studies across filter grown IEC-18 (TER ± 55 Ω.cm^2) and Caco-2 (TER ± 350 Ω.cm^2) cells.

compound	Mw(D)	Mr(Å)	P_{app} (cm.s^{-1} x 10^{-6})[a] IEC-18	Caco-2
Mannitol	182	3.6	8.3	0.76
Fluorescein	332	5.5	4.1	0.21
FD4	4,400	14	1.77	0.03
PEG-4000	4,000	?	2.3	0.58
FD20	19,400	31	0.12	< 0.01
FD70	71,200	60	0.05	< 0.01
FD500	487,000	135	< 0.01	< 0.01
Salicyilic Acid	138	5.7	9.6	6.0
Acetyl salicylic acid	180	6.9	14.1	6.0
Propranolol	259	9.0	36.6	30.6
Testosterone	288	8.0	24.9	18.0

Mw (D) Molecular weight in Daltons

Mr (Å) Molecular radius of the molecules in Å. Stokes radius was used for mannitol, fluorescein, FD4, FD20, FD70 and FD500, vanderWaals radius was used for for salycilic acid, acetyl salycilic acid, propranolol and testosterone

[a] Values are means of at least three experiments performed in triplicate, standard deviations were less than 10%.

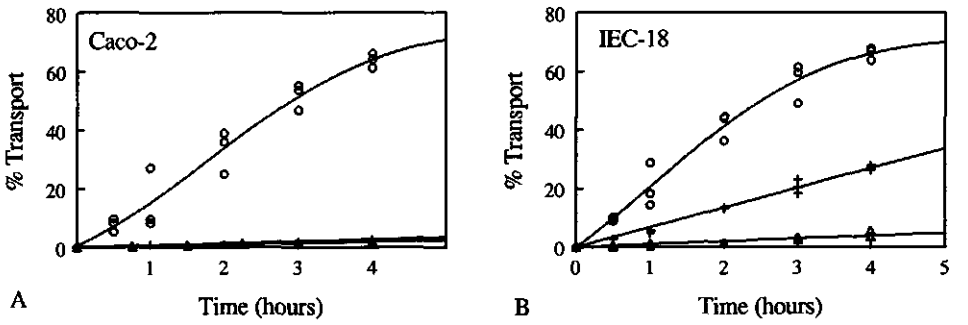

Figure 3.5: Apical to basal transport of Mannitol (-+-), PEG-4000 (-△-) and testosterone (-o-) across filter grown Caco-2 (a) and IEC-18 cells (b) in time. For each sampling time individual datapoints are shown. Lines represent calculated best fits for n=3 per time point.

Figure 3.6 shows a plot presenting the relation between the molecular radius (Mr) and the P_{apps} for the hydrophilic markers. PEG-4000 is not depicted in the figure because it is impossible to calculate the exact molecular radius. There is a gradual decrease in P_{app} with increasing Mr for both cell lines. However, the curve for IEC-18 cells is shifted towards higher P_{app} values and the slope of the curve for IEC-18 cells is less steep than that for Caco-2 cells. This indicates that the number and size of the junctional pores is higher in IEC-18 than in Caco-2 cell layers.

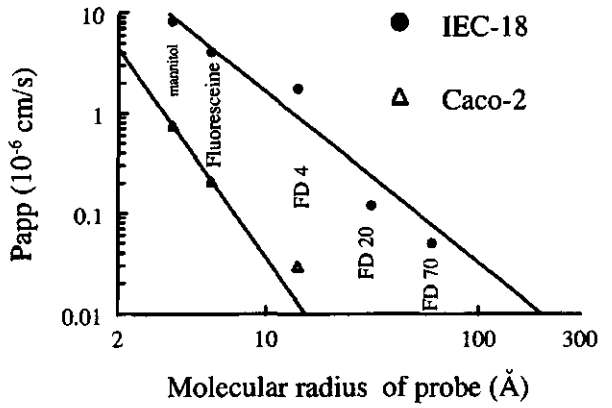

Figure 3.6: Relation between molecular radius (Mr) and apparent permeability coefficient (P_{app}, see Table 3.1) for hydrophilic probes across filter grown Caco-2 and IEC-18 cells.

Discussion

The aim of the present study was to compare filter grown IEC-18 and Caco-2 cells, with emphasis on differentiation, development of the tight junctional complex and transport properties. Histomorphological examination of cross sections of filter grown cells revealed that the Caco-2 monolayer consisted of well polarized, columnar epithelial cells with microvilli on the apical side. On the other hand, IEC-18 cultures seemed to be pseudo-layered or consisted of multilayers of less differentiated epithelial cells. Previous studies using electron microscopy have shown that these IEC-18 cells had a patchy appearance of microvilli (Quaroni and Isselbacher, 1981; Groten *et al.*, 1992).

Determinations of enzyme activities indicated that both cell lines express alkaline phosphatase, albeit the fetal isotype in IEC-18 and the mature enterocyte isotype in Caco-2. Levels of sucrase-isomaltase activities were found in Caco-2 cells in the same order of magnitude as in the literature (Pinto *et al.*, 1983; Chantret *et al.*, 1994), but we were unable to detect sucrase-isomaltase activity in IEC-18 cells. This is supported by Quaroni *et al.* (1979) who described that also with immunocytotochemical methods no sucrase-isomaltase complexes could be detected in IEC cells.

The differentiation stage of the two cells was not only reflected by the activity and isotype of the brush border enzymes, but also by the TER development, ZO-1 expression and actin-belt formation. The F-actin in Caco-2 cells is organized into a peripheral actin-belt at the apical cell side, a characteristic for mature epithelial cells. In IEC-18 cells F-actin is not organized into an actin belt but expressed as cell crossing fibres. Both cell lines do form a cell circumscribing network of ZO-1 strands. The intensity of the ZO-1 signal in IEC-18 seems to increase in time without a concomitant increase in TER while no clear change in ZO-1 pattern or intensity was seen in Caco-2 during a time-period in which the TER almost triplicates. If the intensity of the fluorescence signal of ZO-1 might be considered a good measure for the quantity of junctional proteins, these findings do not support the structure-function hypothesis for tight junctions which states that the TER is directly related to the number of tight junctional strands (Claude, 1987). Our results indicate that neither the pattern nor the intensity of the immunofluorecence signal of this tight junctional protein can be used as indicators of relative junctional tightness when comparing different cell lines. This was also shown by Stevenson *et al.* (1988) who found that the ZO-1 contents of two MDCK strains was identical while the TER of these strains differed remarkably.

In the present study it was shown that the differences in TER between IEC-18 and Caco-2 clearly correspond to differences in paracellular permeability. In IEC-18 the paracellular permeability is not only higher than in Caco-2 cells, but it even offers the possibility of a more clear discrimination between hydrophilic compounds which are poorly absorbed *in vivo*. For example, in Caco-2 monolayers the P_{app} for PEG-4000 (not absorbed in

humans) differed only slightly from that for mannitol (more than 60% absorption in humans; Nasralla and Iber, 1969), whereas in IEC-18 cells the P_{app} for mannitol is much higher than for PEG-4000. The relation between the molecular radius of the hydrophilic markers and the P_{apps}, would thus allow for a more accurate prediction of transport rate on basis of molecular dimensions in IEC-18 than in Caco-2.

The paracellular permeability in IEC-18 cells may be more comparable to that of the small intestine than the permeability in the Caco-2 cells. The IEC-18 cells appear to have a tight junctional network resembling that of the ileum, the area of the gastro-intestinal tract where permeation of hydrophilic molecules is most likely. The TER of Transwell grown IEC-18 cells (±50 $\Omega.cm^2$) is slightly lower than the TER for the rat ileum (88 $\Omega.cm^2$) as reported by Powell (1981). The low TER might be the result of the crypt origin and the low differentiation stage of the IEC-18 cells. Within the ileum the TER in the crypts is lower than at the villus tips (Heresbach *et al.*, 1994), therefore one might assume that the paracellular pathway through the ileal crypt epithelium is a favourable route for hydrophillic macromolecules. However the accessibility of the crypt epithelium is generally very low thus making the physiological importance of this route questionable. Nevertheless, based on our present knowledge, absorption of macromolecular hydrophilic drugs through the small intestinal crypts can not be excluded.

The transport of lipophilic compounds is quite similar in both cell lines. Considering that passive diffusion of these compounds will occur transcellularly, no relation between transport and TER was expected. The slightly lower transport rates of the lipophilic compounds in Caco-2 cells might be due to a thicker aqueous boundary layer, which is known to be a barrier for lipophilic compounds (Karlsson and Artursson, 1991). We did not try to reduce the aqueous boundary layer by stronger agitation since the layer is part of the system, both *in vivo* and *in vitro*. It was also shown that a rather slow agitation rate of approximately 30 rpm was sufficient to allow for diffussion of highly lipophylic PCB congeners through the aqueos boundary layer (Dulfer *et al.*, 1996).

Not much is known so far about active transport properties of IEC-18 cells but considering the low state of differentiation one might assume that these cells lack the expression of many of the active transport systems known to be present in human intestinal epithelium and in Caco-2 cells (Griffith *et al.*, 1994; Hillgren *et al.*, 1995). Among our future interests with implications to the paracellular pathway are the relevance and possible mechanisms of IEC-18 and Caco-2 cells to induce solvent drag (Pappenheimer and Reiss, 1987).

In summary, although the Caco-2 system has proven to be a powerful tool in absorption studies concerning transcellular transport processes (Artursson and Karlsson, 1991; Wilson *et al.*, 1990; Stewart *et al.*, 1995), its use as model in predicting uptake of poorly absorbed compounds which are transported paracellularly is probably limited. This is due to

the poor discrimination in P_{apps} between various compounds with clearly different values of absorption rates in *in vivo* experiments (Artursson and Karlsson, 1991; Griffith *et al.*, 1994). In contrast, IEC-18 cells seem to be more useful as a model to obtain information on transport rates of paracellularly transported compounds. Discrimination between several hydrophilic macromolecules on basis of transport rate is possible using filter-grown IEC-18 cells whereas this was not possible using Caco-2 cells.

Chapter 4

Carrier-mediated transport in rat small intestinal IEC-18 cells: Comparison with the human colon carcinoma Caco-2 cell line.

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Submitted.

Abstract

Previous studies have shown that the rat small intestinal cell line IEC-18 provides a size-selective barrier for paracellular permeation of hydrophilic macromolecules. In order to determine the utility of IEC-18 cells as a model to study intestinal carrier mediated transport, we have now examined the transport of GlySar (H^+-coupled di/tripeptide carrier), *O*-methyl-D-glucose (glucose carrier), vincristine and rhodamine 123 (P-glycoprotein), and calcein and DNPSG (MRP/cMOAT). The transepithelial permeation of these compounds in IEC-18 cells was compared with the permeation across the human colon carcinoma Caco-2 cells. In IEC-18 cells, the permeation of GlySar and methylglucose was as fast as that of mannitol, which permeates passively via the paracellular route. The transepithelial passage of mannitol was much lower than that of GlySar and methylglucose in Caco-2 cells. In contrast to Caco-2 cells, no H^+-coupled transport of GlySar could be measured in IEC-18 cells.

P-glycoprotein mediated transport was characterized in Caco-2 cells by an enhanced transport of vincristine and rhodamine 123 in the basolateral to apical direction and by inhibition of this transport by verapamil. In IEC-18 cells, permeability of vincristine and rhodamine 123 was similar in both directions and verapamil had no effect on the transepithelial permeation of these compounds. IEC-18 and Caco-2 cells showed transport of the organic anions calcein and DNPSG, which could be inhibited by probenecid.

In conclusion, no carrier mediated transport of GlySar, methylglucose, vincristine and rhodamine 123 could be determined in IEC-18 cells in contrast to Caco-2 cells. However, both IEC-18 and Caco-2 cells showed MRP/cMOAT mediated efflux system(s) in the apical and basolateral membrane.

Introduction

The small intestinal epithelium forms a permeability barrier influencing oral drug absorption. The predominant absorption route for compounds is the transcellular route. This includes compounds that are sufficiently lipophilic to pass the cell membranes by passive diffusion and hydrophilic compounds including many nutrients such as glucose, amino-acids and dipeptides, which are transported by carrier-mediated processes. Another possible way for hydrophilic compounds to pass the intestinal epithelium is the passive paracellular route along the epithelial cells. In addition to selective transport systems for the absorption of nutrients, the intestinal epithelium presents a first line of defense against the absorption of xenobiotics. This defense consists of the detoxification of the xenobiotics by metabolizing enzymes and/or extrusion of the (metabolized) xenobiotics from the mucosa cells into the intestinal lumen by transport proteins such as P-glycoprotein and MRP/cMOAT (Hunter *et al.*, 1997). The substrate specificity of P-glycoprotein is very broad but a general feature is that the compounds are hydrophobic (cations). Like P-glycoprotein, the multidrug resistance-associated protein (MRP) is a membrane glycoprotein which transports various hydrophobic anti-cancer agents out of tumor cells (Versantvoort *et al.*, 1992; Zaman *et al.*, 1993). In contrast to P-glycoprotein, MRP/cMOAT is able to transport not only hydrophobic compounds but also compounds conjugated with glutathione, other organic anions, and bile salts such as taurocholate (Jedlitschky *et al.*, 1994; Müller et al, 1994; Versantvoort *et al.*, 1995; Büchler *et al.*, 1996; and Paulusma *et al.*, 1996). RNA analysis showed that MRP/cMOAT is expressed in small intestine but the precise localization in the small intestine is not known at this moment (Paulusma *et al.*, 1996; Kool *et al.*, 1997).

To anticipate the intestinal absorption of compounds and to investigate their mechanism of transport, monolayers of intestinal cell lines grown on permeable filter supports are increasingly used (Hillgren *et al.*, 1995). One of the most commonly used cell lines is the differentiated human colon adenocarcinoma Caco-2. In several studies the permeability of compounds in Caco-2 monolayers correlated with the human oral absorption *in vivo*. Caco-2 cells are an useful model for studying the functionality of carrier mediated transport in the small intestine, although these carriers are often expressed to a lesser extent in Caco-2 cells than in normal enterocytes (Hillgren *et al.*, 1995; Artursson *et al.*, 1996; Hunter *et al.*, 1997). However, due to the tightness of the Caco-2 monolayer, the paracellular barrier presented by Caco-2 cells is much higher than that of the small intestinal epithelium. For that reason we have used the rat small intestinal crypt cell line IEC-18, to study the transport of poorly absorbed hydrophilic compounds. The transepithelial electrical resistance of filter grown IEC-18 cells resembles the resistance for rat ileum and these cells seem to be a useful model to obtain information on the small intestinal permeability barrier for paracellularly transported hydrophilic macromolecules (Ma *et al.*, 1992; Duizer *et al.*, 1997). The IEC-18 cells are less

well differentiated than the Caco-2 cells, but not much is known about the expression of carrier mediated transport systems in this cell line.

In the present study, we have investigated the utility of IEC-18 cells as a model to study intestinal carrier mediated transport. Thereto, the transport of GlySar (H^+-coupled di/tripeptide carrier), methylglucose (glucose carriers), vincristine and rhodamine 123 (P-glycoprotein), and calcein and DNPSG (MRP/cMOAT) is characterized in IEC-18 cells. The transport of these compounds is compared with the transport in Caco-2 cells.

Methods

Chemicals

Radiolabeled chemicals used were obtained from the following suppliers ^{3}H-vincristine sulphate (Amersham, 0.3 GBq/mmol), ^{3}H-O-methyl-D-glucose (Amersham, 185 GBq/mmol), ^{3}H-mannitol (ICN, 1 GBq/mmol), ^{14}C-Gly-Sar (Zeneca Cambridge Research Biochemicals, 2.1 GBq/mmol). Calcein acetoxymethyl ester was obtained from Molecular Probes. Rhodamine 123, verapamil hydrochloride, probenecid and chlorodinitrobenzene were from Sigma, phloridzin dihydrate was from Aldrich.

Cells

The human colon carcinoma cell line Caco-2 and the rat epithelial cell line IEC-18 were obtained from the American Type Culture Collection. Caco-2 cells were used at passages 26-38, IEC-18 at passages 25-33. Cells were maintained at 37°C in an atmosphere of 5% CO_2 in Hepes buffered DMEM (4.5 g/l glucose), supplemented with 1% (v/v) MEM nonessential amino acids, 6 mM L-glutamine, 50 µg/ml gentamycin (all from Gibco), and 10% (v/v) FCS (Integro) for Caco-2, and 5%(v/v) FCS and 0.1 U/ml insulin (Sigma) for IEC-18 cells. For transport experiments, cells (1-2 x 10^5 cells/insert) were seeded on Transwell polycarbonate cell culture inserts (12 well format) with a mean pore size of 0.4 µm (Costar). Cells were then cultured for 17-24 days.

Drug transport and cellular accumulation

Transepithelial drug transport in IEC-18 and Caco-2 cells was measured as described before (Duizer *et al.*, 1997). Transport of 10 µM compound (unlabeled compound plus tracers of radiolabel) was determined in Transwell inserts with apical 0.5 ml transport medium (Hepes buffered DMEM supplemented with 1% (v/v) MEM nonessential amino acids, 6 mM L-glutamine, 50 µg/ml gentamycin (all from Gibco), and 0.1% bovine serum albumin (Sigma) and basolateral 1.8 ml transport medium on a rotating platform (30 rpm) at 37°C. In case of transport studies using a medium pH of 6.0, DMEM was replaced by Hanks Balanced Salt Solution (Gibco). Apparent permeability coefficients (P_{app} values) were determined on basis of the appearance of the probe in the receiver compartment (Duizer *et al.*, 1997). ^{3}H- and ^{14}C-labeled compounds were analyzed by using a Wallac 1409 liquid scintillation counter. Transport of rhodamine 123 was measured by fluorescence detection using Cytofluor 2300 (Millipore) with excitation filter at 485 nm and emission filter at 530 nm.

For cellular accumulation studies, the medium was removed at the end of the incubation period, and the monolayers were washed rapidly with 1 and 2 ml of ice-cold PBS

in the apical and basolateral compartment, respectively. The filters with cells were detached from the inserts, and immerged in scintillation liquid.

Calcein efflux

Calcein transport was studied after loading the cell layer by incubation with 2 µM calcein acetoxymethyl ester (calcein-AM) (apical and basolateral exposure) for 30 min at 37°C. Cells exposed to calcein-AM become fluorescent following cleavage of the calcein-AM by cellular esterases to produce the fluorescent derivative calcein. In order to maximally load the cells with calcein, 2 mM probenecid and 20 µM verapamil were added to the calcein-AM solution in order to inhibit the transport of calcein and calcein-AM, respectively, during the loading period (Versantvoort *et al.*, 1995). Subsequently, the apical and basolateral calcein-AM solutions were removed, the filters were rinsed with ice-cold PBS and the inserts were transferred to new wells containing fresh pre-warmed medium with or without 20 µM verapamil or 2 mM probenecid. Efflux of calcein from the cells into the medium was measured by sampling from the apical and basolateral compartments at indicated time points. Samples were analyzed by fluorescence detection using a Cytofluor 2300 (Millipore) with excitation filter at 485 nm and emission filter at 530 nm.

Cellular accumulation and efflux of DNPSG

Transport of DNPSG out of the cells into the medium was studied after the monolayers were incubated with 25 µM CDNB in Hanks at 37°C in a CO_2 incubator. At the indicated time points, 400 µl samples were taken from both the apical and basolateral compartment and were subsequently precipitated with 100 µl 20% (w/v) TCA. The remaining medium was then removed, and the cells were washed with ice-cold PBS. DNPSG (and other CDNB metabolites) were extracted from the cells by addition of 400 µl 5% (w/v) TCA in Hanks. DNPSG was analyzed by HPLC (Pharmacia LKB HPLC system) on a Zorbax ODS column (4.6*250 mm, Alltech Applied Science Group, Emmen, The Netherlands), eluted at a flow rate of 1 ml/min with 0.1% (v/v) trifluoro acetic acid (solvent A) and 0.1% (v/v) trifluoro acetic acid in methanol (solvent B) isocratically at 30% B for 5 min, followed by a linear gradient of 30-90% B in 22.5 min (retention time 14 min for DNPSG and 21 min for CDNB). Quantification of DNPSG was performed by peak area integration at 340 nm, using concentration/absorbance curves of a DNPSG standard. To prevent hydrolysis of DNPSG by γ-glutamyl transferase, 2 mM acividin was added to incubation medium (Oude Elferink *et al.*, 1993).

Results

H^+-coupled GlySar and O-methyl-D-glucose transport

Transport characteristics of the small hydrophilic compounds GlySar (MW 146), *O*-methylglucose (MW 194) and mannitol (MW 182) were compared in IEC-18 and Caco-2 cells. These compounds can not permeate the cell membrane easily because of their hydrophilicity and are transported exclusively via the paracellular route unless a carrier system is present to facilitate transcellular permeability. Time course of the drug transport was

examined when the drug was added to either the apical or basolateral side of the monolayers. Figure 4.1A shows that in IEC-18 cells the transepithelial transport of GlySar, methylglucose and mannitol was similar. We also found that transport of all three compounds was as fast in the apical to basolateral direction as the other way around. In Caco-2 cells, transport of methylglucose was faster than that of mannitol and GlySar (Figure 4.1B). Furthermore, phloridzin (100 µM) inhibited the transport of methylglucose to 77± 8 % of control ($P<0.01$ Students paired t-test (n=3)) in Caco-2 cells, without affecting the methylglucose transport in IEC-18 cells (105±13%). In Caco-2 cells, no difference was found in transport of methylglucose and mannitol in apical to basolateral or basolateral to apical direction, whereas GlySar transport was significantly faster from the apical to basolateral direction than that from the basolateral to apical side (Table 4.1).

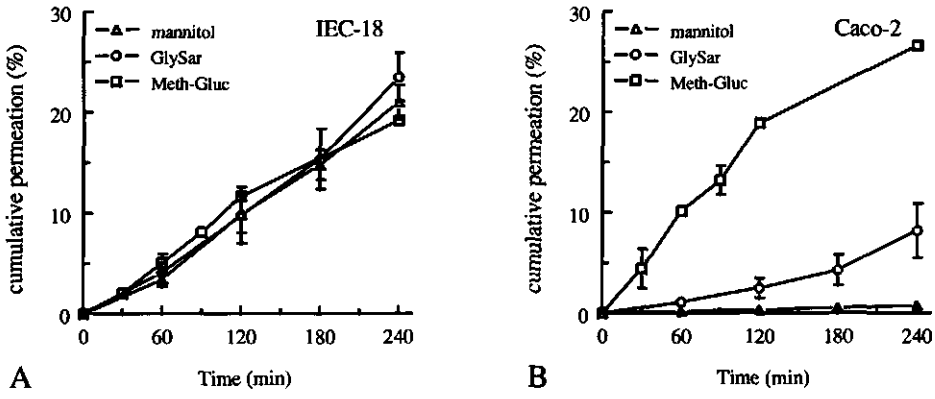

Figure 4.1: Transepithelial permeability of GlySar, methylglucose and mannitol in IEC-18 and Caco-2 cells. Apical to basal transport of 10 µM GlySar, methylglucose (Meth-Gluc) and mannitol was determined from 0 to 240 min in IEC-18 (A) and Caco-2 cells (B). Each point is the mean ±sd of at least two experiments each performed in triplicate.

It has been shown that an H^+ gradient is the driving force for the transport of dipeptides in the intestine and Caco-2 cells (Thwaites *et al.*, 1993; Matsumoto *et al.*, 1994). The effect of H^+ gradient on the transepithelial transport and cellular accumulation of GlySar is summarized in Table 4.1. The transport of GlySar in either direction was 2-fold increased in Caco-2 cells by introduction of a H^+ gradient (pH 6 and pH 7.4 in donor and receiver compartment, respectively). Moreover, the transport of GlySar was decreased when transport was measured against a H^+ gradient ($P_{basolateral\text{-}to\text{-}apical}$ was 2.8 vs 2.1 $x10^{-6}$ cm/s in case pH7.4-to-pH7.4 vs pH7.4-to-pH6.0, respectively). The accumulation of GlySar after 4 hours of incubation was 2.5 to 3-fold lower when GlySar was administered at the basolateral

compartment and could be increased only 1.2- to 1.4-fold, by introduction of a H^+ gradient. Acidifying the donor incubation medium to a pH 6 had no effect on the permeability and accumulation of GlySar in IEC-18 cells. Furthermore, the accumulation of GlySar in IEC-18 cells (5-6 pmol / filter) is much lower compared to the accumulation in Caco-2 cells (224-278 pmol / filter). These results suggest that GlySar transport in Caco-2 cells is mediated by the H^+-coupled di/tripeptide carrier, while it is not in IEC-18 cells.

Table 4.1. pH-dependent GlySar transport and accumulation in IEC-18 and Caco-2 cells.
Monolayers were incubated for 4 hours with 10 μM GlySar added to the apical or basolateral compartment with pH 6 or pH 7.4. The appearance of GlySar in the receiver compartment (pH7.4) was measured in time and the cellular accumulation was determined at the end of the experiment. Data are mean ± sd, number of independent experiments in parentheses, in each experiment at least two monolayers were used.

	IEC-18		Caco-2	
apical to basolateral	Pab (10^{-6} cm/s)	accumulation (pmol/filter)	Pab (10^{-6} cm/s)	accumulation (pmol/filter)
pH7.4->pH7.4	12.1 ± 2.7 (4)	6.0 ± 0.7 (4)	4.6 ± 1.1 (4)	224 ± 33 (4)
pH6.0->pH7.4	11.3 ± 2.5 (3)	5.1 ± 1.9 (3)	8.7 ± 2.0 (4)*	278 ± 32 (4)*
basolateral to apical	Pba (10^{-6} cm/s)	accumulation (pmol/filter)	Pba (10^{-6} cm/s)	accumulation (pmol/filter)
pH7.4->pH7.4	11.9 ± 2.7 (4)	7.2 ± 2.5 (4)	2.8 ± 0.8 (4)#	78 ± 15 (4)#
pH6.0->pH7.4	12.5 ± 3.6 (2)	8.9 ± 1.5 (2)	5.5 ± 1.0 (2)*	109 ± 5 (2)

* Transepithelial permeability and cellular accumulation along the H^+ gradient are significantly different compared to pH 7.4 in donor compartment; $P<0.05$, Student's *t*-test.
# Transport and cellular accumulation of GlySar is significantly different when applied at the basolateral side of the cell layer than when applied at the apical side; $P<0.01$, Student's paired *t*-test.

P-glycoprotein mediated transport

Transport of the P-glycoprotein substrates vincristine and rhodamine 123 were compared in IEC-18 and Caco-2 cells. P-glycoprotein is expressed in Caco-2 cells at the apical cell membrane, which is consistent with the *in vivo* functionality of P-glycoprotein mediating the transport of compounds into the intestinal lumen (Hunter *et al.*, 1993, Meyers *et al.*, 1991). The present study shows that transport of vincristine and rhodamine 123 in Caco-2 cells was much faster in the basolateral to apical direction than vice versa, whereas it was similar in both directions in IEC-18 cells (Figure 4.2). Verapamil, an inhibitor of P-glycoprotein mediated transport, decreased the difference in bidirectional transport of

rhodamine 123 and vincristine in Caco-2 cells whilst having no effect in IEC-18 cells (Figure 4.2).

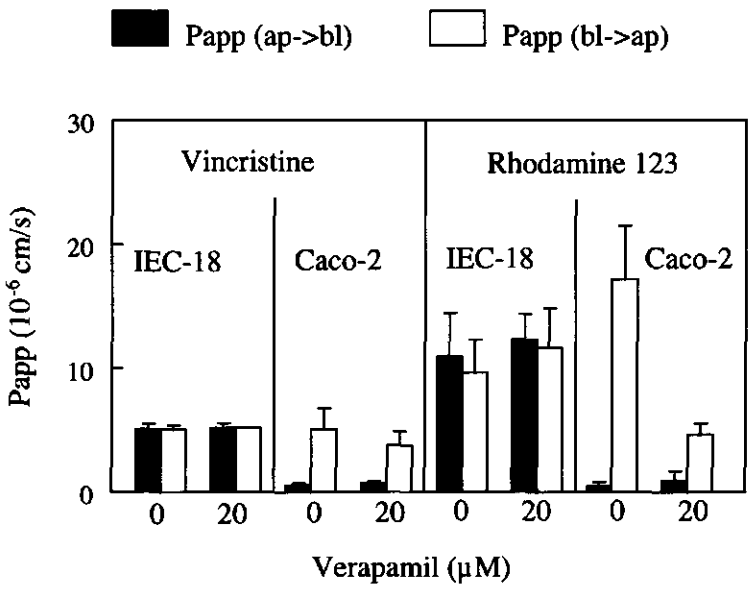

Figure 4.2: Bidirectional apparent permeability coefficients of vincristine and rhodamine 123 across IEC-18 and Caco-2 cells. The cells were incubated for 4 h in the presence or absence of 20 μM verapamil with the substrate added to the apical or basolateral compartment. Data are mean ± sd, n=2 to 6 experiments, with 3 filters per experiment.

Caco-2 cells undergo differentiation and polarization during culture of the cells on permeable filter supports (Hillgren *et al.*, 1995; Hunter *et al.* 1997; Duizer *et al.*, 1997). In order to determine whether the expression of P-glycoprotein was related to the differentiation and polarization status of Caco-2 monolayers, the transport of rhodamine 123 was determined at 6, 13 and 20 days after seeding. The ratio Pba/Pab increased from 10.8 at day 6 to 29.2 at day 20 after seeding of the cells, indicating that the P-glycoprotein expression was indeed correlated to differentiation.

MRP/cMOAT mediated transport

The organic anion calcein and the glutathione *S*-conjugate DNPSG were used as substrates to study the function of MRP/cMOAT in the two intestinal cell lines. Figure 4.3 shows the calcein efflux out of the IEC-18 and Caco-2 cells in presence or absence of

probenecid, an inhibitor of organic anion transport. Calcein efflux out of IEC-18 cells was as fast to the apical as to the basolateral compartment. Incubation with probenecid resulted in a reduced efflux to either side (Figure 4.3A). In Figure 4.3B is shown that the efflux of calcein from Caco-2 cells was approximately 4-fold higher to the apical than to the basolateral compartment and that the apical efflux was more susceptible for inhibition with probenecid. Verapamil (20 µM) had no effect on the efflux of calcein in either cell line (data not shown). In contrast to the increased P-glycoprotein mediated rhodamine 123 transport, the efflux of calcein was not affected by the differentiation and polarization status of Caco-2 monolayers. At day 6 and day 20, the apical over basolateral efflux of calcein was 3.8.

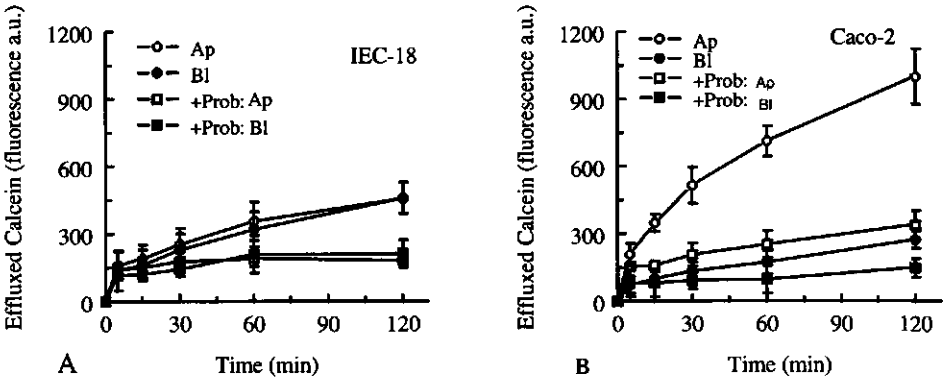

Figure 4.3: Calcein efflux from IEC-18 and Caco-2 cells. IEC-18 (A) and Caco-2 (B) cells were loaded for 30 min with 2 µM calcein-AM in presence of 20 µM verapamil and 2 mM probenecid. Release of calcein to the apical (Ap) and basal (Bl) compartment was measured in absence or presence (+Prob) of 2 mM probenecid after transfer of the filter-grown cells into prewarmed calcein-free medium. Each point is mean ± sd of three independent experiments.

Because of its hydrophilicity, DNPSG can not pass the cell membranes easily by passive diffusion and, therefore, transmembrane transport systems are essential for the elimination of cellular DNPSG. Cells were exposed apically to 25 µM CDNB. In the cells CDNB is rapidly conjugated to glutathione by GST and the transport of the glutathione *S*-conjugate, DNPSG, was followed in time. DNPSG was preferentially effluxed to the apical compartment in both cell lines (Figures 4.4A and 4.4B). Probenecid blocked the efflux of DNPSG to both directions, and thus retaining DNPSG in the cells. Incubation of Caco-2 cells with 2 mM probenecid resulted in a large decrease (>60%) in DNPSG efflux to the basolateral compartment, whilst having only a small effect (5-25% inhibition) on the transport to the

apical side. The DNPSG transport to the apical (and basolateral) compartment could be inhibited more efficiently by increasing the concentration of probenecid (Figure 4.4B).

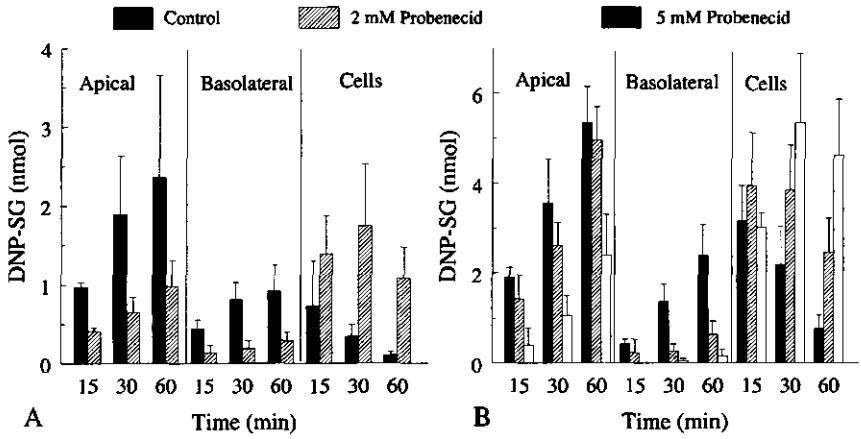

Figure 4.4: DNPSG transport and cellular accumulation in IEC-18 and Caco-2 cells. IEC-18 (A) and Caco-2 (B) cells were apically exposed to 25 µM CDNB. Cellular accumulation and transport of DNPSG to the apical and basal compartment was measured during 60 min in the absence or presence of probenecid. Data are mean ± sd of at least three experiments.

In Table 4.2 is shown that the preferential efflux of DNPSG to the apical compartment was dependent on the side of exposure of the cells to CDNB. When CDNB was applied at the basolateral side of the monolayers, DNPSG was effluxed to the same extent to both compartments in both cell lines. This was due to combination of a reduced DNPSG efflux to the apical compartment and an increased DNPSG efflux to the basolateral compartment compared to the DNPSG efflux when CDNB was applied at the apical side. The relative effects of probenecid on DNPSG transport, however, were independent of the side of CDNB exposure (Table 4.2).

Table 4.2: DNPSG efflux after apical or basolateral exposure of the cells to CDNB. Cells were incubated for 30 min with 25 μM CDNB applied at the apical or basolateral compartment in the absence or presence 2 mM probenecid. After 30 min, the DNPSG content was measured in the apical, basolateral and cellular compartment. Data are mean ± sd of 3-6 independent experiments.

	IEC-18		Caco-2	
CDNB exposure	apical	basolateral	apical	basolateral
ratio DNPSG efflux apical/basolateral	2.34 ± 0.67	1.16 ± 0.41*	2.55 ± 0.68	1.17 ± 0.38*
ratio probenecid/control				
apical	0.39 ± 0.15	0.40 ± 0.06	0.82 ± 0.07	0.77 ± 0.26
basolateral	0.23 ± 0.11	0.45 ± 0.14	0.20 ± 0.07	0.15 ± 0.02
cells	5.00 ± 1.50	4.32 ± 0.96	1.60 ± 0.12	1.51 ± 0.02

* Ratio of DNPSG efflux is significantly different when CDNB was applied at the apical resp. basolateral side of the monolayer; $P<0.05$, Student's *t*-test.

Discussion

Intestinal absorption of drugs and nutrients occurs predominantly in the small intestine. Previously, we have studied passive transcellular and paracellular transport characteristics in rat small intestinal IEC-18 cells (Duizer *et al.*, 1997). Grown on permeable filters, IEC-18 cells are less differentiated than the Caco-2 cells as was determined by histomorphology (not very well polarized) and brush border enzyme activities (no expression of sucrase-isomaltase or intestinal isotype of alkaline phosphatase (Duizer *et al.*, 1997). However, the IEC-18 cells, originating from rat ileum epithelium, appear to more closely resemble the permeability for paracellularly permeating compounds (Ma *et al.*, 1992; Duizer *et al.*, 1997). In this paper, we have investigated the usefulness of IEC-18 cells as a model to study carrier mediated transport processes.

First, the transepithelial permeability characteristics of the small hydrophilic compounds GlySar (substrate for H^+-coupled di/tripeptide carrier), methylglucose (glucose carriers) and mannitol (paracellular route) were examined in IEC-18 and Caco-2 cells. These three compounds, which differ in molecular weight only from 146 to 194, passed equally fast across the IEC-18 cells ($P_{app} \approx 12 \times 10^{-6}$ cm/s), and there was no difference between the transport rates from apical to basal and basal to apical direction. Moreover, an H^+ gradient did not affect GlySar permeation (Table 4.1) and phloridzin did not inhibit the transepithelial passage of methylglucose. This suggests that the paracellular route is the predominant route for the absorption of GlySar and methylglucose in IEC-18 cells. In contrast to IEC-18 cells,

the transepithelial passage of mannitol in Caco-2 cells was much lower than that of GlySar and methylglucose (P_{app} is 0.4, 4.6, and 20.2 x 10^{-6} cm/s, respectively). Indeed, Caco-2 cells are known to express carrier-mediated systems for the transport of GlySar and methylglucose (Thwaites *et al.*, 1993; Hillgren *et al.*, 1995). Note that although the transport of GlySar and methylglucose in Caco-2 cells is facilitated by carriers, the apparent permeability of GlySar and methylglucose was almost as high in IEC-18 cells as in Caco-2 cells (Figure 4.1 and Table 4.1). The high transport in IEC-18 cells might be explained by the fact that the transepithelial electrical resistance of the IEC-18 cells (small intestine) is much lower than that of the Caco-2 cells (large intestine), 50 versus 500 $\Omega.cm^2$, respectively (Duizer *et al.*, 1997). These "loose" tight junctions in the small intestine allow small hydrophilic compounds (MW<350) to be absorbed via the paracellular route (He *et al.*, 1998). Thus, the results from the transport experiments with GlySar, methylglucose and mannitol indicate that these compounds can be absorbed by the passive paracellular route (IEC-18 cells) and by the carrier-mediated transcellular route (Caco-2 cells).

The H^+-di/tripeptide co-transporter plays an essential role in the absorption of several important pharmaceuticals such as the cephalosporin ß-lactam antibiotics (reviewed by Dantzig, 1997). Studies with the ß-lactam antibiotics loracarbef, ceftibuten and cephadrine suggested that the transcellular absorption depends on at least two distinct oligopeptide transporters, an apical H^+-dependent co-transporter and a basolateral H^+-independent carrier, and that the basolateral carrier forms the rate-limiting step for transcellular transport in Caco-2 cells (Hu *et al.*, 1994; Matsumoto *et al.*, 1994). In these studies, however, the transport of the cephalosporins in basolateral-to-apical direction was measured against a H^+-gradient (basolateral pH 7.4 and apical pH 6.0), whereas the transport in apical-to-basolateral direction was determined along a H^+-gradient. Our data showed that when transport was determined along a H^+-gradient in both directions in Caco-2 cells, the transport in either direction was equally proton dependent (Table 4.1). These data are in agreement with the results reported by Thwaites *et al.* (1993), showing that the transport of GlySar in Caco-2 cells was mediated by expression of an H^+-coupled carrier at both apical and basolateral membranes. Cellular accumulation of GlySar in Caco-2 cells was higher when GlySar was applied at the apical compared to the basolateral side of the cells (Table 4.1). A similar side-specific cellular accumulation in Caco-2 cells has been measured for several cephalosporins (Inui *et al.*, 1992; Matsumoto *et al.*, 1994). The lower cellular accumulation and lower transepithelial permeability of GlySar when GlySar was applied at the basolateral side indicate indeed that the transport across the basolateral membrane forms the rate-limiting step for transcellular transport in Caco-2 cells.

Besides the function of the intestinal epithelium in the absorption of nutrients, the intestinal epithelium plays an important role in protecting the body from against the absorption of ingested xenobiotics. Transport proteins such as P-glycoprotein and

MRP/cMOAT may contribute to such line of defense by expelling xenobiotics into the intestinal lumen (Hunter and Hirst, 1997). In the intestine P-glycoprotein is localized in the secretory domain of the enterocytes (van der Valk *et al.*, 1990), and its expression is very low in the crypt but increases along the crypt-villus axis (Meyers *et al.*, 1991). Thus, the lack (or low) P-glycoprotein activity in IEC-18 cells correlates with the low differentiation status and the origin of these cells from crypt ileum epithelium (Quaroni *et al.*, 1981; Duizer *et al.*, 1997). Moreover, the increased P-glycoprotein activity during ongoing differentiation of the Caco-2 cells (from day 6 to day 20 after seeding) correlated with the *in vivo* expression of P-glycoprotein in the small intestine.

MRP1 expression, like P-glycoprotein expression, has been associated with the efflux of lipophilic agents out of tumor cells (Versantvoort *et al.*, 1992; Zaman *et al.*, 1993). Recently, a number of homologues of MRP1 (MRP2-MRP5) have been identified (Kool *et al.*, 1997). The precise localization and function of for example MRP1 and MRP2/cMOAT in the intestine is not known at this moment. MRP1 is expressed in most tissues (Zaman *et al.*, 1993; Flens *et al.*, 1996) and the substrate specificity of MRP1 is very broad. Not only hydrophobic compounds can be transported by this protein but also organic anions and compounds conjugated with glutathione (Jedlitscky *et al.*, 1994; Müller *et al.*, 1994; Versantvoort *et al.*, 1995). MRP2 (cq. cMOAT) is highly expressed in the canalicular membrane of hepatocytes and has been shown to be responsible for the transport of taurocholate out of the cells into the bile (Büchler *et al.*, 1996; and Paulusma *et al.*, 1996). In the small intestine only low RNA expression has been found so far (Kool *et al.*, 1997).

In this study we have characterized intestinal MRP/cMOAT-mediated transport by studying the transport of the model substrates calcein and DNPSG. Calcein efflux from IEC-18 cells was similar in both directions whereas calcein was preferentially effluxed into the apical compartment by the Caco-2 cells (Figure 4.3). A similar preferential, apical efflux of calcein was found in rat jejunum (Fujita *et al.*, 1997), suggesting that enterocytes express a MRP-like transport system at the apical membrane. However, the transport of DNPSG and the inhibition by probenecid (Figure 4.4 and Table 4.2) indicates that IEC-18 and Caco-2 cells express MRP-like transport systems at not only the apical but also the basolateral membrane. Since the DNPSG and calcein efflux from Caco-2 cells to the apical and basolateral compartment was not equally susceptible for inhibition by probenecid, it is suggested that different transport systems are expressed in the apical and basolateral membrane of the cells.

Transfection experiments with MRP1 and MRP2/cMOAT in polarized kidney tumor cells showed that MRP1 was localized at the basolateral membrane whereas MRP2/cMOAT was localized at the apical membrane (Evers *et al.*, 1996 and 1998). When the routings found by the transfection experiments are representative for the localization of MRP1 and cMOAT in the intestine, these findings suggest that MRP1 is expressed at the basolateral membrane and MRP2/cMOAT at the apical membrane of Caco-2 cells. However, the identity and

localization of the MRP/cMOAT like transport systems in intestinal Caco-2 and IEC-18 cells will have to be determined by immunocytochemical techniques. Our results further indicate that MRP/cMOAT mediated transport in the intestine is not confined to the differentiated enterocytes, but that MRP/cMOAT transport systems are likely to be present along the whole crypt-villus axis.

In conclusion, the bidirectional permeation rates of GlySar, methylglucose, vincristine and rhodamine 123 in IEC-18 cells gave no indication for the expression of, respectively, the H^+-coupled di/tripeptide carrier, glucose carriers or P-glycoprotein in these small intestinal crypt cells. However, a probenecid-sensitive efflux of calcein and DNPSG to both cell sides did suggest the presence of MRP/cMOAT mediated efflux system(s) in the apical and basolateral membrane of the crypt and villus-like intestinal epithelial cells. For Caco-2 cells, the presence of H^+-coupled di/tripeptide carrier, glucose carriers and P-glycoprotein were confirmed, and our data suggested a differential expression of MRP homologues over the apical and basolateral membranes in these differentiated intestinal epithelial cells.

Chapter 5

Dexamethasone inhibits cell proliferation and enhances the paracellular barrier function of rat ileal IEC-18 cells.

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Submitted

Abstract

The paracellular pathway in the small intestine has been recognized to contribute significantly to the oral bio-availability of hydrophilic (macro-)molecules. The paracellular barrier was found to increase with the ongoing differentiation along the crypt-villus axis. Our aim was to develop a cell culture model allowing the study of small intestinal paracellular permeability and to correlate cell proliferation and differentiation to paracellular barrier development.

IEC-18 cells were seeded on polycarbonate filter inserts and treated with 0.1-10 μM dexamethasone for 4-19 days. Measures of cell differentiation (morphology and brush border enzyme activities) and proliferation (cell number) were correlated to paracellular barrier development. Dexamethasone induced growth arrest in filter-grown IEC-18 cells. This was concomitant with a reduction of non-intestinal alkaline phosphatase expression, an increased organization of F-actin and the tight junctional protein ZO-1, and a decrease in the paracellular permeability of electrolytes and macromolecules. Furthermore, TPA and EGTA completely abolished the dexamethasone induced increase in paracellular barrier function without causing cytotoxic effects.

Dexamethasone reduced cell proliferation, induced an epithelial-like cell morphology and increased the paracellular barrier function of filter-grown IEC-18 cells. The dexamethasone-induced changes suggest a maturation of the junctional complex in these ileal crypt cells.

Introduction

The epithelium in intestinal crypts is one of the most rapidly proliferating tissues. As cells move away from the lower part of the crypts towards the villi, they stop proliferating and start to differentiate into several cell types of which the enterocyte is the most abundant. This differentiation process is regulated by matrix factors, cell-cell interactions, growth factors and hormones (Koldovsky, 1994; Quaroni and Hochman, 1996) and it is characterized by an irreversible loss of the proliferative potential, by an increase in the expression and polarization of many enzymes and transporter systems, and by the development of the epithelial barrier function (Henning *et al.*, 1994). The epithelial barrier is composed of a transcellular and a paracellular component. The cell membranes and cellular contents form the transcellular component, while the apical junctional complex and the lateral intercellular space form the paracellular component (Reuss, 1992). Along the small intestinal crypt-villus axis the increase in paracellular barrier function is concomitant with an increase in cell-circumscribing tight junctional strand number at the apical cell side (Madara and Trier, 1994).

One of the most widely used *in vitro* models to study enterocytic differentiation is the Caco-2 cell line (Pinto *et al.*, 1983; Peterson and Mooseker, 1993). But, even though use of these cells have greatly advanced insight in intestinal brush-border development and intestinal transport and permeability properties (Hidalgo *et al.*, 1989; Artursson *et al.*, 1996), this cell line is not a very valuable tool for studying the (development of the) small intestinal paracellular barrier function. One important reason is that the tight junctional barrier formed by this colonic carcinoma cell line restricts the passage of hydrophilic macromolecules known to pass to some extent the small intestinal epithelium *in vivo*. The rat ileum derived cell line IEC-18 was found to be a more appropriate model for small intestinal permeability of hydrophilic macromolecules (Ma *et al.*, 1992; Duizer *et al.*, 1997). These epithelial cells have a normal rat karyotype and express intestinal epithelial specific cell surface antigens (Quaroni and Isselbacher, 1981) and junctional proteins such as ZO-1 and desmoplakin (Ma *et al.*, 1992; Duizer *et al.*, 1997). Furthermore, it was found that IEC-18 cells could be induced to acquire villus enterocytic characteristics by extracellular matrix components (Benya *et al.*, 1993) or a small intestinal extract (Shintani *et al.*, 1989). On the other hand, IEC-18 cells cultured on un-coated filters under standard conditions grew in multilayers and showed a low expression of brush-border enzymes and a low organization of F-actin (Duizer *et al.*, 1997).

Additionally, while small intestinal paracellular permeability can be regulated *in vivo* to increase the uptake of nutrients (Madara and Pappenheimer, 1987; Madara, 1987; Ballard *et al.*, 1995) or to improve bioavailability of orally administered drugs (Hochman and Artursson, 1994; Lutz *et al.*, 1997), specific modulation of paracellular permeability in IEC-18 cell layers was not readily achieved (Duizer *et al.*, 1998; Duizer *et al.*, 1999). In mature enterocytes, the regulation of paracellular permeability is, at least partly, dependent on the peri-junctional actin belt. This

peripheral ring of actin microfilaments is part of the cytoskeleton and it is connected to junctional membrane components in polarized epithelial cells (Madara and Trier, 1987; Mitic and Anderson, 1989). The very low transepithelial electrical resistance (TER) and the lack of a recognizable perijunctional actin belt in filter-grown IEC-18 cells, suggests that the paracellular barrier has not yet developed to the full extent in these undifferentiated cells. In order to be able to study modulation of small intestinal paracellular permeability in more detail, a further maturation of the paracellular barrier of these cells is required.

The synthetic glucocorticoid dexamethasone was found to cause an increase in TER and a concomitant decrease in mannitol and inulin permeability in the mouse mammary epithelial 31EG4 cells. These dexamethasone-induced effects could not be correlated to changes in F-actin, E-cadherin or ZO-1 production or location using indirect immunofluorescence (Zettl *et al.*, 1992). However, under comparable conditions, a 2.3 fold increase in levels of ZO-1 protein was found using quantitative Western blotting (Singer *et al.*, 1994). Thus the mechanism by which the paracellular barrier function was increased in these mammary epithelial 31EG4 cells has not been resolved, but it was suggested that glucocorticoids provide a signal for rearrangement of regulatory or structural junctional components (Zettl *et al.*, 1992). Moreover, dexamethasone is reported to have maturing effects on brush-border enzyme expression in the developing mammalian gastrointestinal tract *in vivo* (Beaulieu and Calvert, 1985; Koldovsky, 1994), and on differentiating human fetal intestinal crypt cells *in vitro* (Quaroni and Beaulieu, 1997).

The aim of the present study was therefore to differentiate filter-grown IEC-18 cells to obtain a better model for the small intestinal paracellular barrier. Using dexamethasone as a culture medium supplement, we want to prevent growth into multilayers and to induce a more mature paracellular barrier in this undifferentiated crypt cell line. The functionality of the barrier formed in the presence or absence of dexamethasone was determined by measuring the permeability of the cell layers for electrolytes (TER) and hydrophilic macromolecules. Immunofluorescence microscopy was used to assess the localization of the tight junctional protein ZO-1 and the organization of filamentous actin (F-actin). Additionally we studied the effects of the paracellular barrier perturbants EGTA (Cassidy and Tidball, 1967; Gorodeski *et al.*, 1997) and TPA (Ojakian, 1981; Anderson and Itallie, 1995) on the permeability of hydrophilic macromolecules, to determine whether tight junction-specific effects could be evoked in dexamethasone treated IEC-18 cells.

Materials and Methods

Cell Culture

IEC-18 cells originating from rat ileum epithelium were obtained from the American Type Culture Collection. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and >90%

relative humidity. Standard medium was DMEM with high glucose (4.5 g/L) and 25 mM Hepes, supplemented with 1% (v/v) MEM nonessential amino acids, 6 mM L-glutamine, 50 μg/ml gentamycin (all from Gibco, Paisley, Scotland) and 5% (v/v) heat-inactivated fetal calf serum (batch: 5-41101 Integro b.v., Zaandam, Netherlands), and 0.1 U/mL insulin (Sigma, Beers, Belgium). Cells grown in 75 cm^2 flasks were passaged weekly at a split ratio of 1:10 using 0.05% trypsin in PBS with 0.022% EDTA. Cells were used at passages 18-28. For experiments the cells were resuspended in culture medium at a concentration of 3 x 10^5 cells/ml. The IEC-18 cells were subcultured onto Transwell cell culture inserts (growth area of 1 cm^2 per filter, 0.5 ml cell suspension apical and 2 ml medium basolateral) and allowed to settle for 12-16 h under standard culture conditions. Subsequently, the medium was replaced by standard culture medium without (basolateral) or with (apical) dexamethasone (0.1-10 μM) every 2 or 3 days. The mouse fibroblast L929 cells and human colonic carcinoma Caco-2 cell line were cultured as described previously for Caco-2 (Duizer *et al.*, 1997).

Light microscopy

Filter-grown cells were fixed in 4% phosphate-buffered formaldehyde. The cells were dehydrated in an ascending series of 70 to 100% ethanol and subsequently embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrein, Germany). The 2-4 μm thick cross-section were stained with toluidine blue.

Enzyme Activity

Cell homogenates were obtained by sonicating filter-grown cells in ice-cold PBS. Activities of alkaline phosphatase were determined spectrophotometrically on the BM/Hitachi 911 using p-nitrophenylphosphate as a substrate. Cell homogenates were diluted to 20 mU ALP/mL and incubated with levamisol (0-10 mM, 10 min, room temperature) to discriminate between the (levamisol resistant) adult intestinal isotype of ALP and the (levamisol sensitive) fetal or liver/kidney/bone isotype (Hoffman *et al.*, 1994). LDH was measured at the BM/Hitachi 911 using pyruvate as substrate. A modification of the method of Dahlqvist (1984) was used to determine sucrase-isomaltase activity. In short, the cell homogenate was incubated 30 min at 37°C with 100 mg/ml sucrose as substrate. The reaction was stopped by boiling for two minutes and the glucose formed was measured with the BM/Hitachi 911 using the hexokinase/G6P-DH assay (Boehringer, Mannheim, Germany). Total protein contents were determined according to Lowry with bovine serum albumin as standard.

Transepithelial Paracellular Transport and Transepithelial Electrical Resistance

The radioactive tracers ^{14}C-polyethyleneglycol-4000, ^{3}H-polyethyleneglycol-900 (NEN products), ^{3}H-mannitol (ICN), were mixed with unlabeled compounds to yield final concentrations of 10 μM with specific activities of 10-30 Ci/mol. ^{3}H- and ^{14}C-labeled probes were analyzed using a Wallac 1409 Liquid Scintillation Counter. Fluorescein and the fluorescein isothiocyanate dextrans (FDs) with average molecular weights of 4,400 (FD-4), 19,400 (FD-20), 71,200 (FD-70) and 487,000 (FD-500) Da were from Sigma (St.Louis, USA). Fluorescein and the FD samples were analyzed using fluorescence detection on a Cytofluor 2300 (Millipore,

Bredford, MA) with excitation at 485 nm and emission at 530 nm. The percentage of free fluorescein isothiocyanate (FITC) in the FDs was determined on a HPLC system, using a 500G size exclusion column as stationary phase. The mobile phase consisted of a linear acetonitril gradient (2.5-25% in 20 min) in a 0.01 mM phosphate buffer. Fluorescence was detected by excitation at 519 nm and emission at 495 nm on a Jasco FP-929 Intelligent Fluorescence detector. Corrections were made for the free FITC impurity in the FDs.

The transport experiments were carried out in Transwell inserts on a rotating platform device (30 rpm, 37 C, and >90% relative humidity) with 0.5 ml DMEM-BSA (standard medium without insulin and FCS but with 0.1% bovine serum albumin) with marker apically and 1.8 ml DM-BSA without marker basolaterally. Initial transport rates, expressed as apparent permeability coefficients, were determined on basis of appearance of the tracer in the basolateral compartment. The transepithelial electrical resistance of filter grown cells was measured using the Millicell-ERS epithelial voltohmmeter (Millipore Co., Bedford, USA) in standard culture medium at room temperature.

Immunocytochemistry

For immunocytochemistry the cells were fixed for 5 min in 1% formaldehyde in HBSS and permeabilized for 20 min in 0.2% Triton X-100 in HBSS. F-actin was stained with FITC- or TRITC-conjugated phalloidin (Sigma, St.Louis, USA). Polyclonal rabbit anti-ZO-1 and polyclonal anti-Occludin, anti-rabbit IgG-biotin and streptavidin-FITC (Zymed, San Francisco, USA) were used to visualize ZO-1 and occludin, respectively. E-Cadherin expression was analyzed with a polyclonal Goat anti-E-cadherin (RDI, Flanders, USA) stained with biotin-conjugated rabbit anti-goat IgG and streptavidin-FITC. Cross-reactivity with rat was confirmed for all primary antibodies using cryostat cross-sections of rat ileum. The nuclei of filter grown cells were stained with Hoechst 33258 to perform cell counting. The filters were analyzed with a standard fluorescence microscope from Zeiss equipped with a PlanApoChromat 40/1.0 (Zeiss, Oberkochen, Germany) objective and a COHU monochrome CCD camera. The nuclei were counted manually from the display screen. Confocal laser fluorescence microscopy was performed with the Bio-Rad MRC600, using a Plan-Neofluar 63/1,25 objective (Zeiss, Oberkochen, Germany).

Exposure to EGTA and TPA

The filter-grown cells were treated with 5 mM EGTA in DMEM-BSA at both cell sides for 45 min. During the transport experiment 5 mM EGTA was maintained basolaterally only. The effects of 0.1-10 μM TPA were studied after 90 min preincubation with TPA in 0.5 ml DMEM-BSA at the apical cell side and 1.8 ml DMEM-BSA basolateral. After the preincubation the LDH leakage was measured and after the transport experiment a neutral red uptake assay was performed to measure cytotoxic effects of the treatment.

Results

Cell proliferation and enzyme activities

Cells seeded at a density of $1.5x10^5$ cell/cm^2 on polycarbonate filters and cultured in standard culture medium grew in several layers of cells (Figure 5.1A). When dexamethasone was added to the culture medium at a concentration of 0.1 to 10 μM, cell number did not increase and a monolayer of cells was formed (Figure 5.1B). Since we did not find a concentration dependency in the range from 0.1 to 10 μM dexamethasone, only 1 μM dexamethasone was used throughout all further experiments.

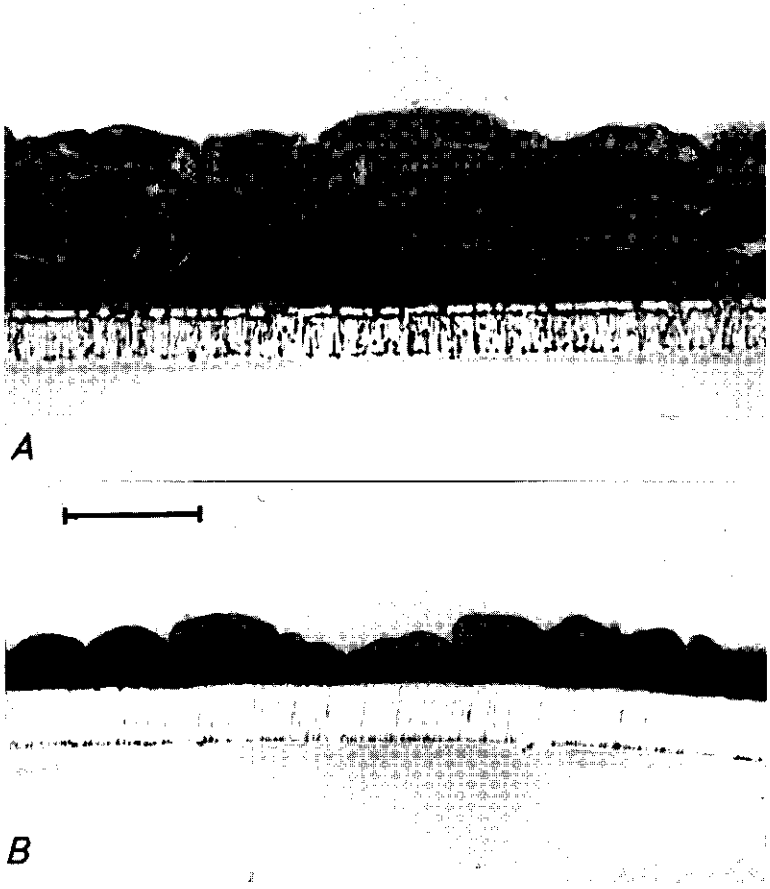

Figure 5.1: Light photomicrographs of cross-sections of filter-grown IEC-18 cells. Cells were cultured in the absence (A) or presence (B) of 1 µM dexamethasone for 19 days (bar = 25 µm).

The observation that dexamethasone treated cells did not grow into multi-layers was also reflected by the cell number per cm^2 which was approximately 2.5 times as high in control cell layers than in dexamethasone treated cells after 20 days of culture (Figure 5.2A). The activity of ALP was found to increase in control cell layers from day 5 to day 8 but no further increase was detected to day 20 (Figure 5.2B). The addition of dexamethasone led to a significant decrease in ALP activity, both in time and in comparison with control cell layers. The ALP activity in homogenates of control and dexamethasone treated cells was inhibited almost completely by 10 mM levamisol (data not shown). This indicates that the ALP iso-enzyme found in control IEC-18 and dexamethasone treated IEC-18 is not the adult intestinal iso-enzyme (Hoffman *et al.*, 1994). The activity of sucrase-isomaltase was below the detection limit of 1 mU/cm^2 in all samples of control and dexamethasone treated cells.

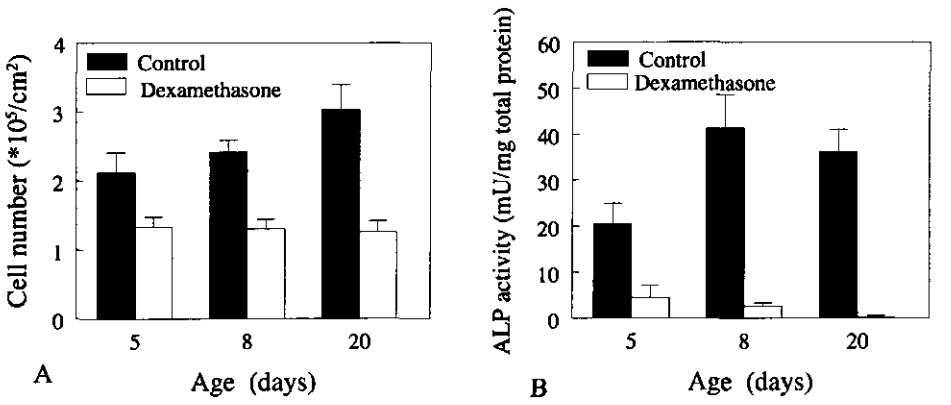

Figure 5.2: Cell number (A) and activities of ALP (B) of filter-grown IEC-18 cells. Cells were seeded at day 0 at a density of $1.5*10^5$ cells/cm^2 and cultured in the absence (■) or presence (□) of 1 μM dexamethasone. Data are presented as means±SD (n=6).

Paracellular Barrier

The TER in control cell layers was constant between day 5 and 20 at 48±7 $\Omega.cm^2$ (n=48). The apparent permeability coefficients (Papps) of a series of hydrophilic macromolecules showed a clear dependency on molecular weight (Figure 5.3). The TER and transport rates found at day 8 and day 20 did not significantly alter from those found at day 5 (data not shown). Culture in the presence of dexamethasone resulted in monolayers with a TER of 57±6 $\Omega.cm^2$ ($p<0.05$, students t-test, n=48). As in control cell layers, the Papps were dependent on molecular weight and were

independent of culture time. Dexamethasone decreased permeabilities for the whole series of compounds tested ($p<0.05$, students t-test).

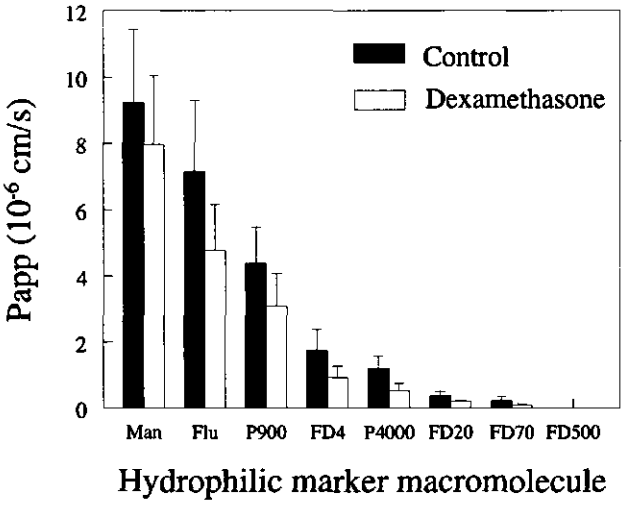

Figure 5.3: Apparent permeability coefficients (Papps) of hydrophilic macromolecules across filter-grown IEC-18 cells at 5 days of age. Cells were cultured in the absence (■) or presence (□) of 1 μM dexamethasone. Data are presented as means±SD (n=6).

Additional transport experiments to study size-selectivity of the paracellular barrier in the IEC-18 cells were performed with all markers using empty filter inserts and filter-grown fibroblastic cells (*L929*). For that purpose the *L929* cells were grown for 1 week to obtain a confluent cell layer. Even in the confluent state the TER was not significantly increased compared to empty filters and no junctional ZO-1 could be detected in these fibroblastic cells, indicating the absence of tight junctions. Figure 5.4 presents the Papps for mannitol divided by the Papps for the other hydrophilic macromolecules tested (the markers) in IEC-18 (control and dexamethasone treated), L929 and empty filters. In this way a permeability ratio is obtained which represents the size-selectivity of the paracellular barrier relative to mannitol. This parameter is in essence comparable to the lactulose-mannitol ratio which is used *in vivo* (Ma, 1997). The figure shows that the barrier function was almost independent of molecular weight of the marker

in the empty filter or filter-grown L929 system. In control IEC-18 cells, the barrier function increased with the molecular weight (and radius) of the marker. In the dexamethasone treated monolayers the barrier increased more pronounced with molecular weight (Figure 5.4). This indicates that IEC-18 cells developed a size-selective paracellular barrier and that the size-selectivity was increased by dexamethasone treatment.

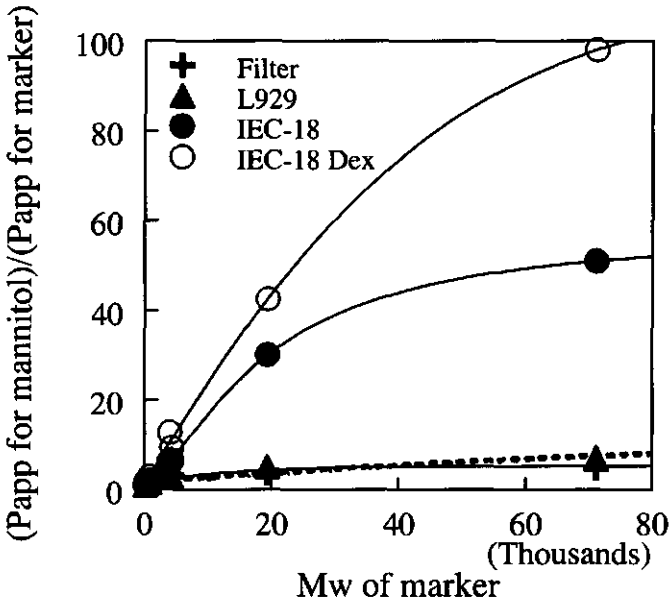

Figure 5.4: Relative barrier function of filter-grown cells. The Papps of hydrophilic macromolecules (see Figure 5.3) were divided by the Papps for mannitol. IEC-18 cells were cultured for 5 days in the absence (-●-) or presence (-○-) of 1 µM dexamethasone, L929 cells (-▲-) were cultured for 7 days, empty filters (-✚-)were maintained in culture medium under standard culture conditions for at least 1 day.

Localization of ZO-1 and F-actin

In control cell layers at day 5, the tight junction protein ZO-1 was distributed as small fragments with numerous interruptions at regions of cell-cell contacts (Figure 5.5A). After prolonged culture the number of fragments had increased and, using low magnification, appeared to have formed a cell-circumscribing belt at day 20. However, at higher magnification the "belt" had the appearance of a string of fragments (Figure 5.5B). F-actin could be found throughout the cells and displayed a low degree of organization at day 5 and day 20 (Figures 5C and 5D).

In dexamethasone treated IEC-18 cultures at day 5, ZO-1 was localized mainly at sites of

cell-cell contact (junctions) but with fragments of non-junction related ZO-1. The peripheral pattern of ZO-1 was thin, but belt-like and virtually cell-circumscribing (Figure 5.6A). During prolonged culture the staining of ZO-1 intensified but the pattern was not subject to substantial changes until day 20 (Figure 5.6B). In dexamethasone treated cells the F-actin was concentrated at the periphery and it appeared organized in a peripheral actin-belt. The degree of organization reached at day 5 (Figure 5.6C) did not seem to alter significantly until day 20 (Figure 5.6D).

We did not detect junctional E-cadherin or occludin at regions of cell-cell contact in control and dexamethasone treated IEC-18 cells. This indicated that the junctional expression of both proteins was very low or absent, even in the presence of dexamethasone.

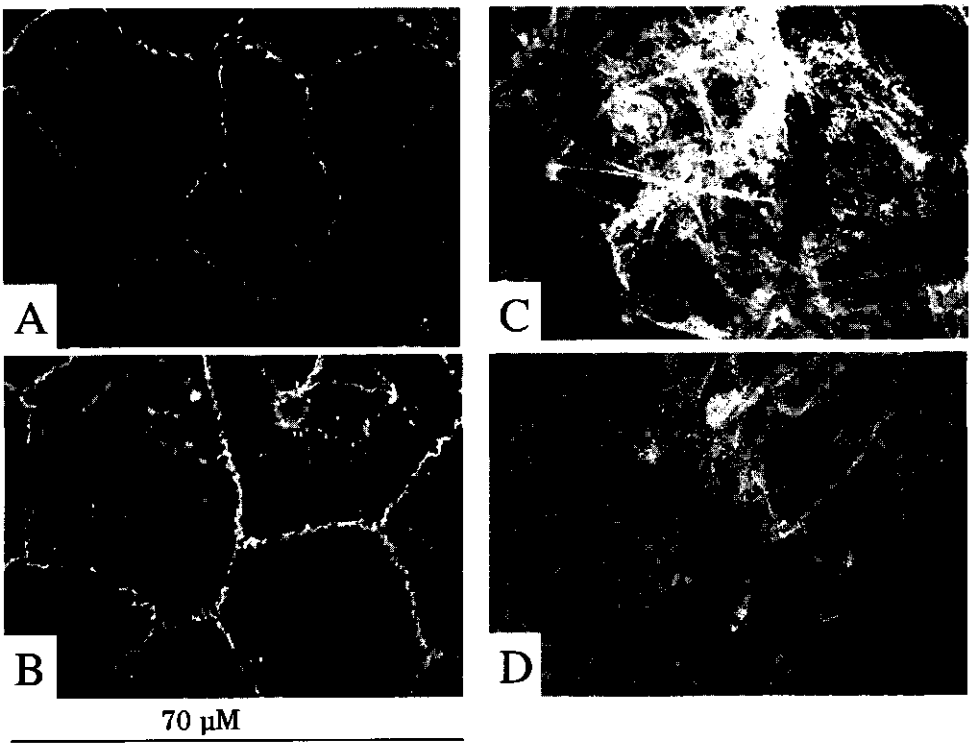

Figure 5.5. Immunocytochemical staining of ZO-1 (A and B) and filamentous actin (C and D) in filter-grown IEC-18 cells. Cells were cultured in control medium for 5 days (A and C) or 20 days (B and D) (original magnification 630X: bar =70 µm).

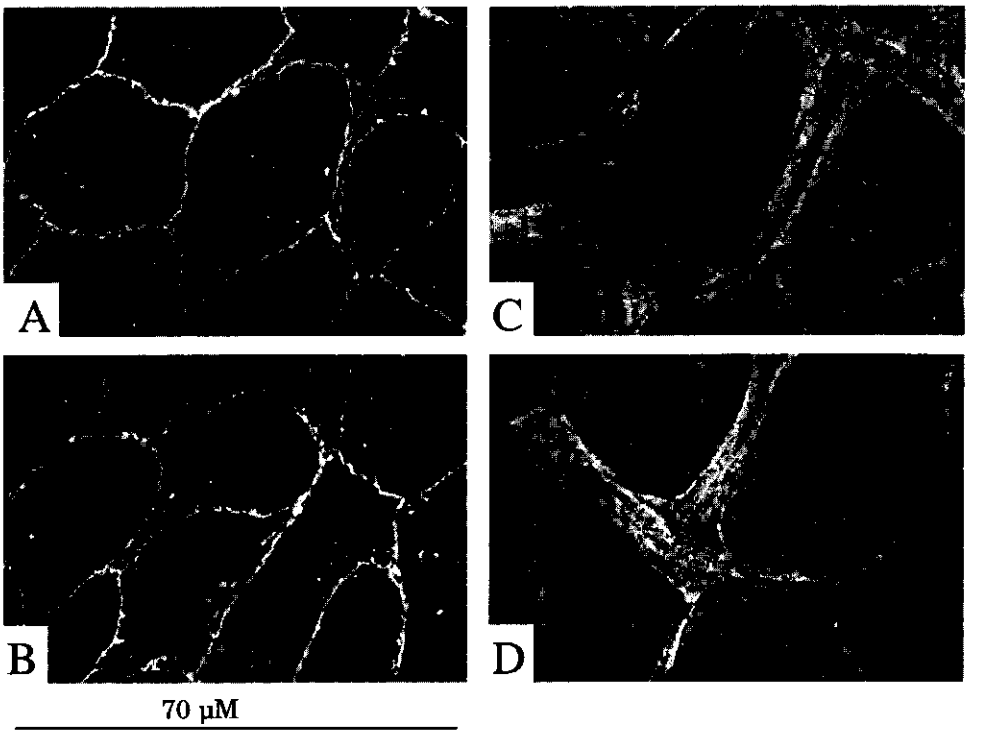

Figure 5.6: Immunocytochemical staining of ZO-1 (A and B) and filamentous actin (C and D) in filter-grown IEC-18 cells. Cells were cultured in the presence of 1 µM dexamethasone for 5 days (A and C) or 20 days (B and D) (original magnification 630X: bar =70 µm).

Modulation of the paracellular barrier by TPA and EGTA

In order to determine whether the barrier of dexamethasone treated monolayers was subject to junction-specific modulation by paracellular barrier perturbants, the effects of TPA and EGTA on transport rates of mannitol, PEG-4000 and FD-20 were assessed. Preincubation with TPA resulted in increased Papps for mannitol, PEG-4000 and FD-20 in control and dexamethasone treated filter-grown IEC-18 cells (Figure 5.7). The increased permeability was dose-dependent for controls but dose-independent for dexamethasone treated cells in the range from 0.1 to 10 μM TPA. In control and dexamethasone treated cells the transport enhancement factor of TPA (Papp of tracer in the presence of TPA divided by the Papp for the control) was more pronounced with increasing molecular weight of the marker. Nonetheless, the permeability was still dependent of the molecular weight of the markers, indicating preservation of some size-selectivity. In cells cultured in the presence of dexamethasone, the Papps for mannitol, PEG-4000

and FD-20 were increased by TPA to the level of control filter-grown IEC-18 cells. None of the TPA concentrations caused LDH leakage or a reduced neutral red uptake (data not shown) indicating that cell viability was maintained throughout the experiment.

Similar to TPA, EGTA exposure caused increased Papps for all three markers in control and dexamethasone treated IEC-18 cells, with increased transport enhancement factors for increased molecular weight of the markers. In control cell layers the Papps for PEG-4000 and FD-20 were almost equal after EGTA exposure, indicating that the size-selectivity for the larger molecules was diminished. In IEC-18 monolayers cultured in the presence of dexamethasone the effects of EGTA were similar to those of TPA, i.e. the Papps for mannitol, PEG-4000 and FD-20 were increased to the level of control IEC-18 cell layers. EGTA caused a slight reduction in neutral red uptake by control cell layers while this parameter was unaffected in the dexamethasone treated cells. No LDH leakage was detected as a result of EGTA exposure (data not shown).

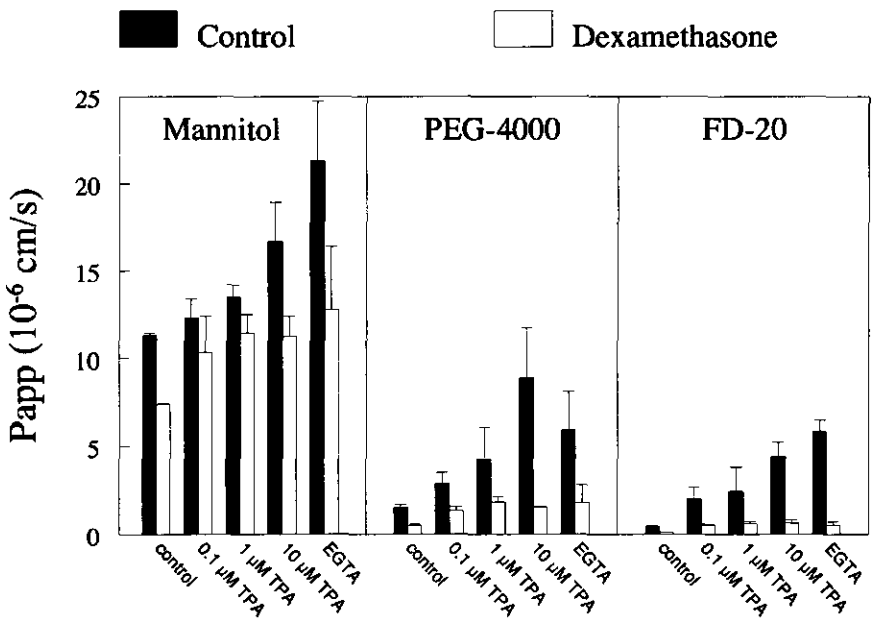

Figure 5.7: Apparent permeability coefficients of mannitol, PEG-4000 and FD-20 across filter-grown IEC-18 cells preincubated with several concentrations TPA or 5 mM EGTA. Cells were cultured in the absence (■) or presence (□) of 1 µM dexamethasone for 5 days. Data are presented as means±SD (n=6).

Discussion

Among the most striking effects of dexamethasone on rodent intestinal organ cultures (Beaulieu and Calvert, 1985; Beaulieu and Calvert, 1987; Albert *et al.*, 1994) and on immortalized human intestinal epithelial cells (Quaroni and Beaulieu, 1997) are the reduction of cell proliferation rate and induction, or increase in expression, of brush-border enzymes. IEC-18 cells seeded at high density in the presence of 5% FCS and insulin on uncoated polycarbonate permeable inserts grow in multilayers of cells. Addition of dexamethasone to the standard culture medium resulted in the formation of a monolayer and a complete stop of cell proliferation at a cell density of 1.4 $x10^5$ cells/cm^2. This cell density is close to the density originally reported by Quaroni (1981) for IEC-18 cells grown on plastic for several days. Thus, dexamethasone stimulated the formation of a confluent monolayer of filter-grown IEC-18 cells. The results of this study indicate that medium components can induce growth arrest in this primitive ileal crypt cell line. This growth arrest might be the result of a direct inhibition of DNA synthesis by dexamethasone as was reported by Beaulieu and Calvert (1987) to occur in fetal mouse intestinal crypt epithelium after dexamethasone exposure.

Earlier studies reported that the loss of proliferative activity of small intestinal crypt cells occurs concomitant with the onset of, or increase in, brush-border enzyme expression (Beaulieu and Calvert, 1985; Beaulieu and Calvert, 1987; Albert *et al.*, 1994; Quaroni and Beaulieu, 1997). In this study we found a reduced activity of non-intestinal alkaline phosphatase as a result of dexamethasone exposure. We consider the reduction in expression of this presumably fetal isoenzyme an intermediate step in the process of intestinal cell differentiation. The brush-border enzyme sucrase-isomaltase, which is absent in small intestinal crypt cells in rat *in vivo* (Chandrasena, 1992) and in IEC-18 cells (Quaroni and Isselbacher, 1981; Duizer *et al.*, 1997) could not be induced to detectable levels by dexamethasone. It was, however, shown that activity of this enzyme was induced in the IEC-18 cells by an acid extract of small intestine (Shintani *et al.*, 1989), indicating that this enzyme is inducible in these cells. Thus, even though one of the most striking reported effects of dexamethasone was the induction or increased expression of brush-border enzymes (Beaulieu and Calvert, 1985; Albert, 1994; Quaroni and Beaulieu, 1997), we did not find this to be a clear effect on IEC-18 cells.

Besides the effects on cell proliferation and brush-border enzyme expression, dexamethasone was found to modulate tight junction assembly and paracellular barrier development in cultured hepatoma cells (Porvaznic *et al.*, 1979) and mammary epithelial cells (Zettl *et al.*, 1992; Singer *et al.*, 1994). In the present study, the development of a barrier in the IEC-18 cells was followed during prolonged culture in the absence or presence of dexamethasone. The paracellular barrier for hydrophilic macromolecules formed by filter-grown IEC-18 cells was found to be size-selective, even though the tight junctional ZO-1 strands appeared discontinuous.

Moreover, the ZO-1 pattern did change during prolonged culture but this was not accompanied by a change in transport rates. This implies that even incomplete junctional strands are able to maintain a size-selective paracellular barrier, probably by bringing lateral membranes of neighboring cells in very close apposition (i.e. forming "kisses"). Further biochemical and ultrastructural studies will be necessary to verify this hypothesis. The paracellular barrier formed in IEC-18 cells after culture in the presence of dexamethasone is more tight and has a higher size-selectivity than untreated cell layers. Structural features accompanying the increased barrier functionality in IEC-18 cells are: decreased cell densities, increased continuity of the junctional ZO-1 strands and increased organization of F-actin. Thus in contrast to findings for the mouse mammary epithelial 31EG4 cells where no clear effects of dexamethasone treatment on ZO-1 and F-actin localization were found (Zettl *et al.*, 1992), we found structural changes in both proteins in this small intestinal cell line. Inhibition of cell proliferation, formation of a peri-junctional actin belt and increased tight junctional sealing as induced by dexamethasone in IEC-18 cells, are all natural events reported to occur in the absorptive cells in the small intestine along the crypt-villus axis (Madara and Trier, 1987; Quaroni and Hochman, 1996). From our observations we speculate that the junctional fragments as found in the en-face images of ZO-1 labeled control IEC-18 cell layers are isolated free-strands and fasciae occludentes, which are common in lateral membranes of crypt cells (Madara and Trier, 1987). These fasciae occludentes are then fused to form continuous tight junction strands under the influence of dexamethasone. Thus dexamethasone induced a more mature paracellular barrier by inducing a continuation of tight junctional strands and formation of a peri-junctional actin-belt.

IEC-18 cells cultured in the absence or presence of dexamethasone were exposed to TPA and EGTA in order to explore the possibilities to selectively regulate paracellular permeability. The exact mechanism by which the phorbol ester TPA increases the paracellular permeability of epitheliums is unknown, but PKC activation, junctional(-related) protein phosphorylation and/or contraction of the perijunctional actin-belt ultimately lead to a diminished paracellular barrier (Ojakian, 1981; Anderson and Itallie, 1995; Mullin *et al.*, 1997; Mullin *et al.*, 1998). In the present study, TPA indeed increased the permeability of control IEC-18 layers to levels which are comparable to those of the confluent fibroblastic L929 cells. The same TPA treatment increased the permeability of dexamethasone treated cells to the level of that of control IEC-18 cell layers. Similar findings were found after Ca^{2+} depletion by EGTA treatment. Ca^{2+} depletion has a direct effect on junctional elements (Gorodeski *et al.*, 1997) and causes microfilaments in the peri-junctional actin belt to contract. Both actions can lead to a separation of the tight junctional strands of neighboring cells and thus increased junctional permeability (Contreras *et al.*, 1992). Thus, the paracellular permeability of IEC-18 cells cultured in the presence of dexamethasone is increased by TPA and EGTA treatment, but an epithelial-like barrier (i.e. that of control IEC-18 cell layers) seemed to be maintained. These paracellular barrier modulation

studies with TPA and EGTA, and the increased organization of ZO-1 and F-actin suggest that the tight junctional barrier in dexamethasone treated cells is more mature than the barrier in control IEC-18 cells.

The development of a tighter paracellular barrier as a result of glucocorticoid exposure as we found for IEC-18 cells, was also suggested to occur in intestinal epithelium of rats during the suckling-weanling transition. During the suckling period, just after a rise in plasma levels of glucocorticoids, the small intestinal epithelium is subject to major changes (Henning *et al.*, 1994). Prior to weaning the epithelium is highly permeable and allows intact uptake of macromolecules such as insulin (6 kD) epidermal growth factor (6 kD) or IgGs (150 kD) from the mother's milk (Jarett and Hall, 1979; Henning *et al.*, 1994). However, with weaning starts the intake of non-sterile food and arises the need to make the epithelium less permeable for macromolecules such as bacterial toxins and antigens. It is particularly the barrier for these very large molecules which is mostly increased by dexamethasone exposure in IEC-18 cells, cells obtained from rats in the suckling-weaning transition. Additionally, a decrease in alkaline phosphatase activity was found in the ileum of rats at the time of weaning and in IEC-18 cells after dexamethasone exposure. Thus it might be that the changes induced by dexamethasone exposure in IEC-18 cells resemble the first steps along the crypt-villus axis in the development of the rodent gastrointestinal tract during the suckling-weaning transition phase. This is a phase of which knowledge on barrier function is very limited, in contrast to the vast amount of knowledge on the maturation of digestive and absorptive functions (Henning *et al.*, 1994).

In conclusion, dexamethasone inhibited cell proliferation and allowed long-term culture of confluent monolayers of IEC-18 cells. Moreover, dexamethasone induced formation of a circumferential continuous tight junctional network and an organized cytoskeleton with a perijunctional actin-belt. However, no clear induction of the brush-border enzymes sucrase-isomaltase or intestinal-ALP could be found. Although the physiological significance of the reported findings remains to be elucidated, we believe that the results obtained with the IEC-18 cell line are of considerable interest and will advance studies of the relationship between paracellular permeability and intestinal epithelial cell differentiation.

Section III

Modulation of the Intestinal Epithelial Barrier *In Vitro*

Chapter 6

Absorption Enhancement, Structural Changes in Tight Junctions and Cytotoxicity Caused by Palmitoyl Carnitine in Caco-2 and IEC-18 Cells.

Erwin Duizer, Cees van der Wulp, Carolien H. M. Versantvoort, and John P. Groten.
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Abstract

Palmitoyl carnitine (PCC) has been shown to be an effective enhancer of intestinal transport of hydrophilic molecules. The exact mechanism by which the epithelial barrier function is decreased is not clear. In an attempt to elucidate the mechanism of action of PCC, we studied the relationship between absorption enhancement, cell viability, and tight junction protein localization in the human colonic Caco-2 cell line and the rat small intestinal cell line IEC-18.

Filter grown cells were exposed to 0 - 1 mM PCC for 30 minutes and the efficacy of PCC treatment was determined by assessing the transepithelial electrical resistance (TER) and the apparent permeability (Papp) for mannitol and PEG-4000. Membrane lysis and cytotoxicity were assessed by measurements of lactate dehydrogenase (LDH) leakage and uptake of propidium iodide (PI) and neutral red (NR). The immunolocalization of the tight junctional protein ZO-1 was quantified using a CSLM and image processing software.

In both cell lines PCC caused a dose-dependent decrease in TER and a concomitant increase in the permeability for mannitol and PEG-4000. The transport enhancement was accompanied by an increase in apical membrane permeability and reduction in cell viability. At higher PCC concentrations (≥0.4 mM) the distribution of the tight junctional protein ZO-1 was changed and cells were unable to recover viability. PCC is effective as absorption enhancer for hydrophilic macromolecules, however, lytic effects on the cell membrane and reduced cell viability were concomitant with transport enhancement.

Introduction

Transport across the intestinal epithelium is often the rate limiting step in the total process of absorption for orally administered hydrophilic macromolecular compounds. Passage of these compounds is most likely to occur extracelluarly by passive diffusion through the paracellular pathway. However, tight junctions abolish passage of large molecules almost completely. Co-administration of an absorption enhancer is a potential way to increase bioavailability of these compounds and considerable effort has been directed towards identifying agents able to loosen tight junctions, thus increasing paracellular permeability.

Although exact mechanisms of action are not fully identified, in general two classes of absorption enhancers are distinguished: surfactants and calcium chelators (Hochman and Artursson, 1994). Surfactants (bile acids and salts, derivatives of fatty acids, Triton X-100 etc.) act by increasing the solubility of hydrophobic macromolecules in the aqueous boundary layer or by increasing the fluidity of the apical (and the basolateral) membrane. Calcium chelators (EGTA, EDTA) reduce the extra-cellular calcium concentration which leads to disruption of cell-cell contacts. In general, the surfactants increase transcellular permeability while the chelators increase paracellular permeability. Acylcarnitines seem to be an exception, since they are suggested to enhance paracellular permeability in a calcium independent way.

The absorption enhancing properties of these fatty acid derivatives of L-carnitine have been studied extensively (Fix *et al.*, 1986; LeCluyse *et al.*, 1993; Sutton *et al.*, 1993a+b). The C16 conjugate palmitoyl carnitine (PCC) was found to be very effective in increasing transepithelial transport of poorly absorbed drugs *in vivo* in rats and dogs (Fix *et al.* 1986; Sutton *et al.*, 1993b) and *in vitro* in intestinal segments (Sutton *et al.*, 1993a; LeCluyse *et al.*, 1993) and Caco-2 cells (Raeissi and Borchardt, 1993; Hochman *et al.*, 1994). Histological and ultra-structural examination of PCC exposed sections of rat jejunum and colon showed slight alterations in the microvillus border but an intact cytoplasmic integrity and un-compromised junctional complexes (Fix *et al.*, 1986; Sutton *et al.*, 1993). To elucidate the mechanism by which PCC exerts its action, *in vitro* studies were performed using Caco-2 cells (Raeissi and Borchardt 1993; Hochman *et al.*, 1994). These studies have shown an immediate drop in transepithelial electrical resistance (TER) and increase in mannitol transport after apical exposure to sub-millimolar concentrations of PCC. The drop in TER proved to be transient and returned to approximately control values in 10-12 hours. No visible damage to the epithelium or alterations in the filamentous actin were seen, however, the appearance of tight junctions in freeze fracture replicas seemed to be altered; i.e. junctional strands showed a beaded appearance, several discontinuities and diminished cross-bridging. These results might indicate that PCC is able to reversibly increase permeability without causing major morphological alterations in the intestinal epithelium. On the other hand, other studies have shown that PCC induces damage to erythrocytes (Cho and Proulx, 1971) and the vaginal

epithelium (Richardson *et al.*, 1992) at concentrations likely to be used to achieve absorption enhancing effects. In addition, LeCluyse *et al.*(1993) reported that after PCC treatment lucifer yellow transport could be enhanced without observed exfoliation of cells, however, an enhancement factor of 18 for lucifer yellow transport was accompanied by extensive cell exfoliation from rat colonic mucosa.

The exact mechanism of action by which PCC exerts its permeability enhancing action has still not been elucidated. Moreover, the sequence of events (i.e. transport enhancement, membrane damage and tight junction disruption) is unknown. Therefore, we studied the relationship between absorption enhancement, cell viability, and tight junction protein (ZO-1) distribution in the human colonic Caco-2 cell line (Hidalgo *et al.*, 1989; Baily *et al.*, 1996; Artursson *et al.*, 1996) and the rat small intestinal cell line IEC-18 (Ma *et al.*, 1992; Duizer *et al.*, 1997). These two intestinal epithelial cell lines differ greatly in state of differentiation, transepithelial electrical resistance and paracellular permeability. Both cells express ZO-1 and discriminate paracellular transport rates on basis of molecular radius (Duizer *et al.*, 1997). Filter grown cells were exposed to 0 - 1 mM PCC for 30 minutes, TER and transport of mannitol and PEG-4000 were used as parameters for epithelial permeability to hydrophilic (macro-)molecules. Lactate dehydrogenase (LDH) leakage into the apical medium, propidium iodide (PI) and neutral red (NR) uptake were used to detect reduced integrity of the apical cell membrane and cell viability. For Caco-2 cells, permeability enhancement, TER reduction and LDH leakage resulting from PCC exposure were compared to the effects of the surfactant Triton X-100 and the chelator EGTA. Additionally, the immunolocalization of the tight junctional protein ZO-1 was correlated with PCC exposure and a method was developed to quantify ZO-1 staining using confocal laser scanning microcopy (CSLM) images.

Methods

Cell culture

The Caco-2 cell line originating from a human colorectal carcinoma, and IEC-18 cells originating from rat ileum epithelium were obtained from the American Type Culture Collection. Cells were maintained at 37^{0}C in an atmosphere of 5% CO_2 and 90% relative humidity. Maintenance medium was DMEM with high glucose (4.5 g/l) and 25 mM HEPES, supplemented with 1% (v/v) MEM nonessential amino acids, 6 mM L-glutamine, 50 µg/ml gentamycin (all from Gibco, Paisley, Scotland), and 10% (v/v) foetal calf serum (FCS, Integro, Zaandam, Netherlands) for Caco-2, and 5% (v/v) FCS and 0.1 U/ml insulin (Sigma, Beers, Belgium) for IEC-18 cells.

Cells grown in 75 cm^2 flasks (Costar, Badhoevendorp, Netherlands) were passaged weekly at a split ratio of 1:10 (IEC-18) or 1:5 (Caco-2) using 0.05% trypsin in PBS with 0.022% EDTA. For transport experiments both cell types were seeded on Transwell polycarbonate cell culture inserts with a mean pore size of 0.4 µm (Costar, Badhoevendorp,

Netherlands) at 2-3 $*10^5$ cells/cm^2 and cultivated for 17-24 days. Caco-2 cells were used at passages 30-40, IEC-18 at passages 25-32.

TER measurements

Filter grown cells were adapted to room temperature to allow for more standardized transepithelial electrical resistance (TER) measurements using the Millicell-ERS epithelial voltohmmeter (Millipore Co., Bedford, USA). The TER was measured prior to and during the experiments to monitor cell layer confluence and the integrity of the tight junctions. The TER of the cell layers was calculated according to the following equation: TER = $(R_{total}-R_{blank})$ X A (Ω.cm^2), R_{total} is the resistance measured, R_{blank} is resistance of control filters without cells (approximately 140 Ω.cm^2), A = surface area of filter (1 cm^2).

Exposure to palmitoyl carnitine

Caco-2 cells with a TER > 600 Ω.cm^2 and IEC-18 cells with a TER > 40 Ω.cm^2 (both determined at room temperature) were transferred to new wells with 1.8 ml basal medium (HEPES buffered DMEM with non-essential amino acids, 6 mM L-glutamine, gentamycin, and 0.1% BSA). The apical medium was changed for 0.5 ml basal medium supplemented with 0-1 mM freshly dissolved palmitoyl-L carnitine hydrochloride (Aldrich, Milwaukee, USA). After 30 minutes preincubation at room temperature, the apical test medium was removed and used for LDH leakage measurements. Subsequently, the inserts with cells were used for assessment of transepithelial transport or immunocytochemistry.

Exposure to Triton X-100 and EGTA

The protocol for Triton X-100 exposure (0 - 0.05% v/v) was exactly the same as that for PCC incubation. The studies with EGTA (0 - 5 mM) were performed by preincubation with EGTA for 45 min at room temperature. During the preincubations EGTA was applied apically and basolaterally. After the preincubation the TER was measured and apical medium was collected for LDH measurements. Transport of mannitol and PEG-4000 was performed with EGTA basolateral.

Transepithelial transport

Transport experiments were carried out at 37°C in Transwell inserts with 0.5 ml basal medium with tracers apically and 1.8 ml basal medium basolaterally. A reduction of the unstirred water layer and homogeneous mixing of the probes was achieved by rotating the two-compartment transport system at 30 rpm on a rotating platform device in an incubator (Dulfer *et al.*, 1996). Apparent Permeability coefficients (Papps) were determined on basis of appearance of the probe in the basolateral receiver compartment before 10% of the probe was transported (i.e. under sink conditions) according to the following equation (Artursson and Karlsson, 1991): P_{app}= P/A*C_0 (cm/s), P = permeability rate (mol/s), C_0= Initial apical concentration of test substance (mol/ml), A = area of filter (cm^2). The probes ^{14}C-polyethylene glycol-4000 and ^{3}H-mannitol (Amersham, Little Chalfont, UK) were mixed with unlabeled

compounds to yield final concentrations of 2.5 µM with specific activities of 2.5 GBq/mmol for ^{14}C-polyethylene glycol-4000 and 1.5 GBq/mmol for ^{3}H-mannitol. ^{3}H and ^{14}C labeled probes were analyzed using a Wallac 1409 Liquid Scintillation Counter after addition of Ultima Gold Scintillation liquid (Packard, Groningen, Netherlands).

Immunocytochemistry

For immunocytochemistry the cells were fixed in 1% formaldehyde in HBSS and permeabilized in 0.2% Triton X-100 in HBSS. F-actin was stained with phalloidin conjugated with TRITC or FITC (Sigma, St.Louis, USA). Polyclonal rabbit anti-ZO-1 and polyclonal anti-Occludin, anti-rabbit IgG-biotin and streptavidin-FITC (Zymed, San Francisco, USA) were used to visualize ZO-1 and occludin respectively, in both cell lines.

Confocal scanning laser microscopy and image processing

Confocal fluorescence microscopy was performed with the Bio-Rad MRC600, using the PlanApoChromat 40x objective with variable diaphragm (Zeiss, Oberkochen, Germany). All images were recorded at standardized acquisition conditions; per cell line and per experiment settings for gain and blacklevel were optimized and applied throughout that experiment. Images are single plains (a depth of 7 µm covers >90% of the total intensity) recorded using the Kalman filter mode at n=5 with Bio-Rad software (COMOS version 7.0a). After transfer to an image processing workstation, the images were processed by a custom written program, based on the image processing toolbox SCIL-Image (version 1.3, Free University of Amsterdam, Netherlands). The program developed to select junction-related immunolabeled ZO-1, consists of three sequential steps. First, two methods are applied to differentiate between borderline and background using a threshold value of 1.4. Both resulting binary images are combined to a usually honeycomb-like skeleton. This intermediate skeleton represents most of the junction-related ZO-1 pattern with small discontinuities and some not junction-related appendixes. In the second part of the program, the skeleton can be improved, first by closing the small discontinuities of the borderlines using a maximum cost procedure (adding less than 0.5% to the total pixel count) and secondly, interactive and automatic removal of spurious line fragments. Finally, from the binary image of the optimized skeleton the total number of pixels is determined and the original grey value image was used to determine the average pixel intensity over the borderlines.

Cytotoxicity and membrane damage assays

LDH leakage into the apical medium resulting from PCC, Triton X-100 or EGTA exposure was determined with the Boehringer Mannheim kit for LDH at the BM/Hitachi 911. Total LDH content was determined after sonicating the inserts with cells in basal medium. In several experiments 5 µg/ml propidium iodide was added to the PCC exposure medium and uptake of this membrane impermeant fluorescent dye during PCC exposure was determined using images made with a standard fluorescence microscope from Zeiss equipped with a PlanApoChromat 40/1.0 (Zeiss, Oberkochen, Germany) objective and a COHU monochrome CCD camera. Quantification was performed with the Colourmorph program (Perceptive

Instruments, Halstead, UK.). Each image yielded 130-160 Caco-2 cells or 40-60 IEC-18 cells, 5 images were recorded per filter (n=6 per exposure group).

Neutral red uptake was determined directly after the transport experiments. For that purpose, cells were incubated with neutral red (50 µg/ml) in control medium for 30 minutes (37^0C, 5%CO_2, 95% relative humidity). Subsequently, the cell layer was rinsed once with HBSS and the neutral red was extracted with 1% (v/v) acetetic acid in a 1:1 water-ethanol mixture. The A_{540nm} was measured spectrophotometrically.

Statistics

Data on PCC (TER, LDH leakage, transport of mannitol and PEG-4000 and NR uptake) were obtained in 4 independent experiments with both cell types using 3 filters per exposure group. Data on EGTA and Triton X-100 reflect the means of 3 independent experiments. Data on immunolabeling of ZO-1 were obtained in 2 experiments using 2 filters per exposure group per experiment. Per filter 5 areas were recorded for junction-related ZO-1 specific fluorescence quantification. Presented data are pooled data of all experiments. Data were analyzed using a two-tailed Students (paired) t-test.

Results

Effects of PCC on transepithelial electrical resistance

In both cell lines the TER was decreased by PCC in a dose-dependent manner (Figure 6.1). The reduction of the TER caused by 30 min PCC exposure was statistically significant at concentrations as low as 0.1 mM in IEC-18 and 0.4 mM in Caco-2. Most of the TER reduction was already accomplished within 5 min after exposure (data not shown). At the higher PCC concentrations the decrease in TER was more pronounced in Caco-2 then in IEC-18. For instance, 1 mM PCC reduces the TER in Caco-2 to 29% (190 $\Omega.cm^2$) and in IEC-18 to 62% (40 $\Omega.cm^2$). Note that the absolute TER of the 1 mM exposed Caco-2 monolayer was still higher than the TER of control IEC-18 cell layers (64.5 ± 12 $\Omega.cm^2$).

Effects of PCC on transepithelial transport rates

Transport rates of mannitol and PEG-4000 were increased by PCC in a dose-dependent manner in both cell lines (Figure 6.1). The apparent permeabilities of both compounds were significantly increased at PCC concentrations ≥0.2 mM in IEC-18 and at ≥0.4 mM in Caco-2 cells. Although the IEC-18 cells were affected at lower concentrations of PCC, the maximum relative increase in transport obtained with 1 mM PCC was larger in Caco-2 cells. The fluxes of mannitol (except for the higher PCC concentrations) and PEG-4000 were constant throughout the 4 hours that transport was monitored, indicating that the effects on transport of 30 min PCC exposure did not change within 4 h after removal.

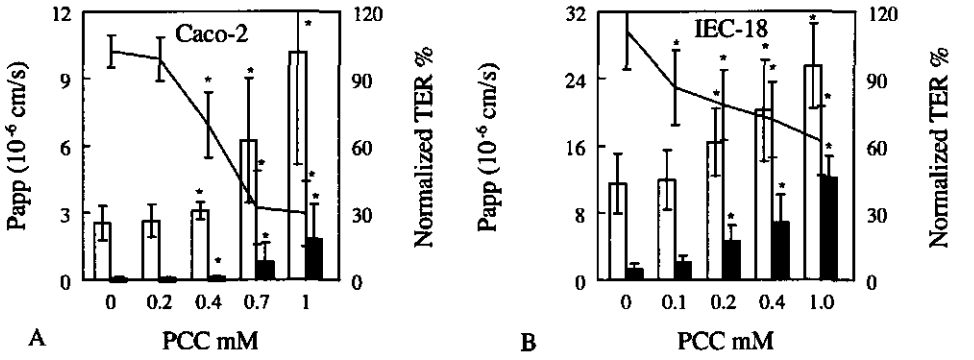

Figure 6.1: Effects of 30 min PCC exposure on apparant permeabilities of mannitol (□) and PEG-4000 (■) and TER (-) of Caco-2 (A) and IEC-18 (B) monolayers. TER was normalized with the TER prior to PCC exposure as 100%. Data are presented as mean±SD (n=4 experiments).
* Significantly different from control, P<0.05 according to two-tailed Student's paired t-test.

Effects of PCC on ZO-1 distribution

Immunocytochemical localization of ZO-1 in untreated layers of Caco-2 and IEC-18 cells showed a belt-like cell circumscribing pattern throughout the total area (Figure 6.2 A and 3 A). In Caco-2 cells only local and subtle modifications of the ZO-1 pattern were seen at lower PCC concentrations. Up to a concentration of 0.4 mM PCC no interruption of the cell circumscribing pattern were detected. At 0.7 mM small holes, the size of only few cells, in the monolayer were found and the pattern of junction-related ZO-1 was locally disturbed (Figure 6.2 B). Exposure to 1.0 mM PCC caused holes varying in size from a few up to many cells, but cells with a cell circumscribing ZO-1 pattern were still present (Figure 6.2 C).
In control and PCC-treated cell layers, the effect of PCC on immunolocalization of ZO-1 was quantified according to the method described in the methods section, the data are presented in Figure 6.4 (see also Figure 6.2 D, E, F and Figure 6.3 D, E, F). In Caco-2, the selected pixel number was slightly reduced in a dose-dependent manner at PCC concentrations ≥0.4 mM (Figure 6.4 A). The average pixel density of the selected cell borders did not change in Caco-2, indicating that the junction-related ZO-1 density of the cell circumscribing pattern did not change. Immunolabeled monolayers of Caco-2 cells exposed to 0.4-1 mM PCC and PI at the same time showed that whenever the cell circumscribing ZO-1 pattern was disrupted, the nuclei were always stained with PI.

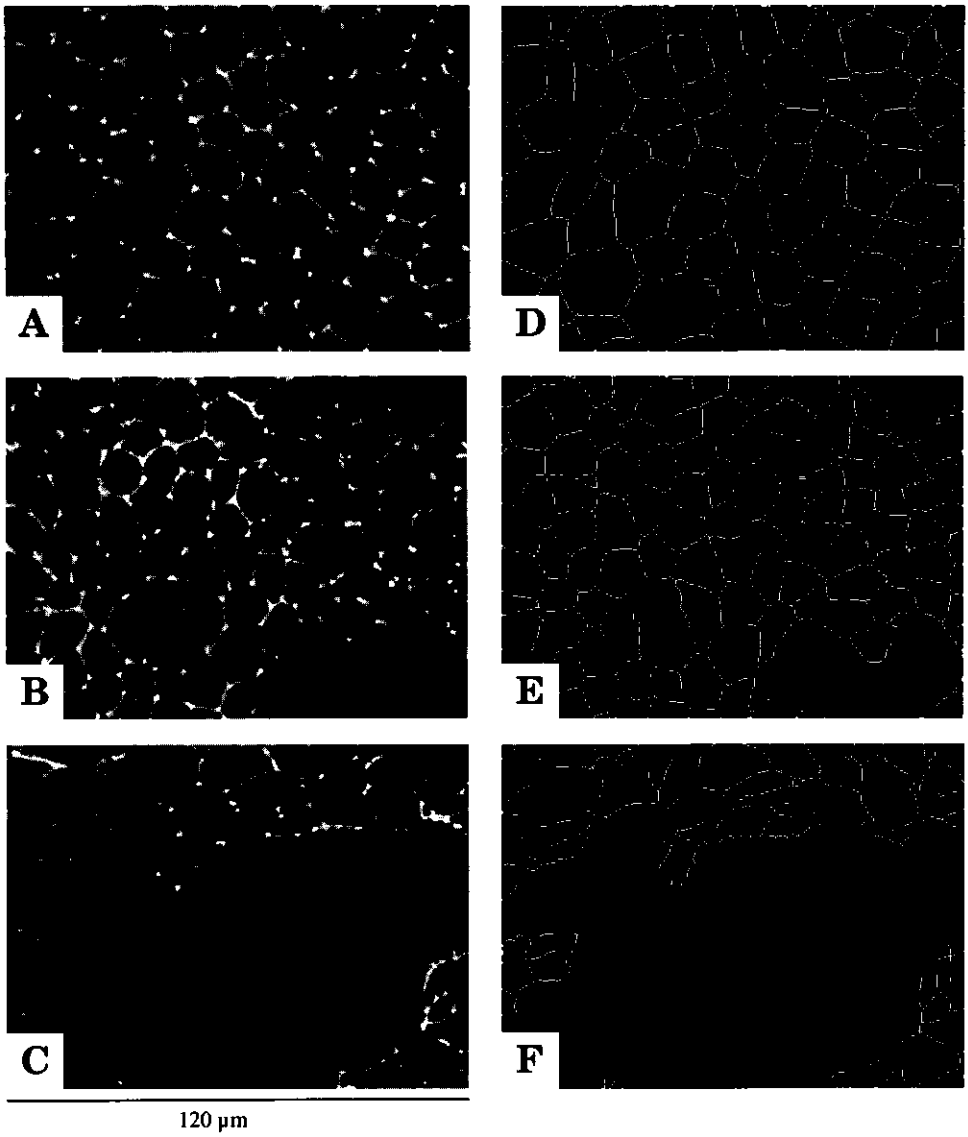

Figure 6.2: CSLM image showing immunolocalization of ZO-1 in control (A), 0.7 mM (B) and 1.0 mM (C) PCC treated Caco-2 monolayers. D, E and F are the binary images of A, B and C respectively after processing: the custom written program selected the honeycomb-like tight junctional pattern.

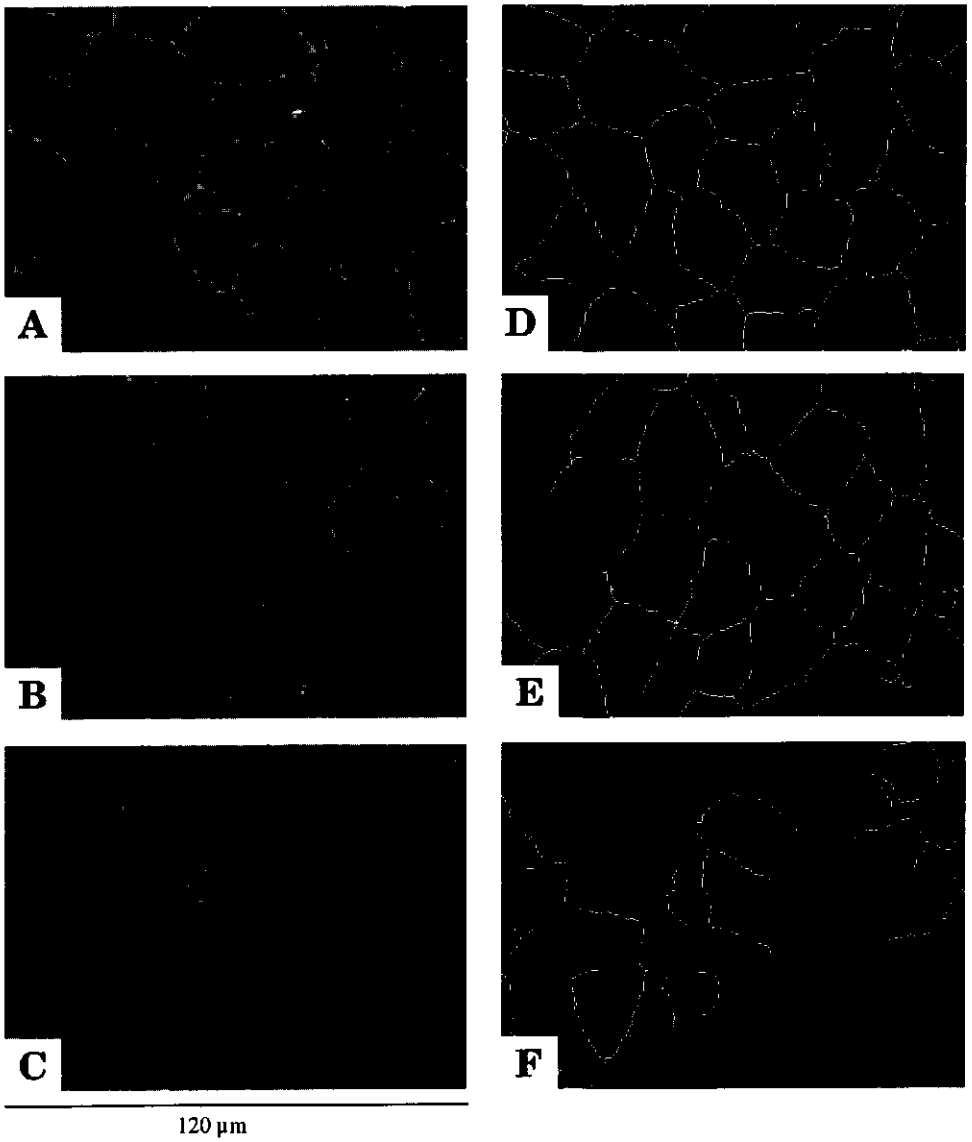

Figure 6.3: CSLM image showing immunolocalization of ZO-1 in control (A), 0.4 mM (B) and 1.0 mM (C) PCC treated IEC-18 cell layers. D, E and F are the binary images of A, B and C respectively after processing, the custom written program selected the honeycomb-like tight junctional pattern.

In IEC-18 cells the ZO-1 pattern was locally disturbed in cell layers exposed to PCC concentrations as low as 0.1 mM. The localized interruptions in the belt-like structure became more pronounced with increasing PCC concentrations (Figure 6.3 B and C) and resulted in a significant decrease in selected pixel number (Figure 6.4 B) at PCC concentrations ≥0.4 mM. At these concentrations the average pixel densities (Figure 6.4 B) were also reduced, which indicates that the immunolocalization of the ZO-1 protein was less stringently organized in the cell periphery. Co-incubations with PCC and PI, followed by immunolabeling of ZO-1, showed that PI staining correlated with a disrupted ZO-1 pattern.

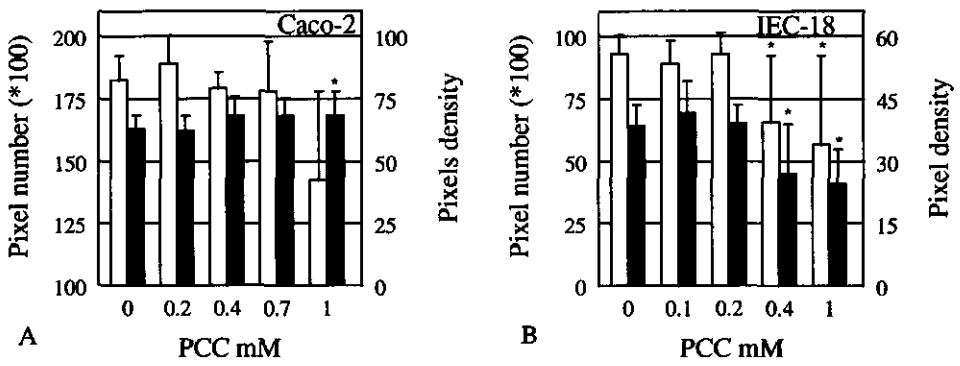

Figure 6.4: Effects of PCC exposure on immunolocalization of ZO-1 in Caco-2 (A) and IEC-18 (B) cells. A custom written program selected the junction-related ZO-1 pattern, shown are selected pixel number (□) and selected pixel density (■). Data are presented as means±SD (n= 10).
* *Significantly different from control, P<0.05 according to Student's t-test.*

Effects of PCC on apical membrane integrity and cell viability

Cytotoxic effects of PCC were determined by three different assays. LDH leakage and PI staining are based on detection of apical membrane damage and the neutral red assay is based on two processes: non-ionic passive diffusion through the apical membrane and intracellular accumulation in lysosomes (Babich and Bohrenfreund, 1990). Figure 6.5 shows that the release of the cytosolic enzyme lactate dehydrogenase into the apical medium increased in a dose-dependent manner after exposure to PCC. Leakage of this enzyme (MW 140 kDa) is significantly increased at doses of ≥0.2 mM PCC in Caco-2 and ≥ 0.1 mM in IEC-18 cells. Staining of cells with the DNA dye propidium iodide (MW 668 Da) occurred at higher PCC concentrations and was significantly increased at ≥0.4 mM PCC in both cell lines. Next to apical membrane damage, PCC caused a dose-dependent reduction of cell viability as assessed by the neutral red assay. Since we found increased apical membrane permeability

(LDH leakage and increased PI staining) after PCC exposure, we assume that lower A_{540} readings in the NR assay indicate a reduced functionality of the lysosomes (NR retention) and not a reduced uptake of NR.

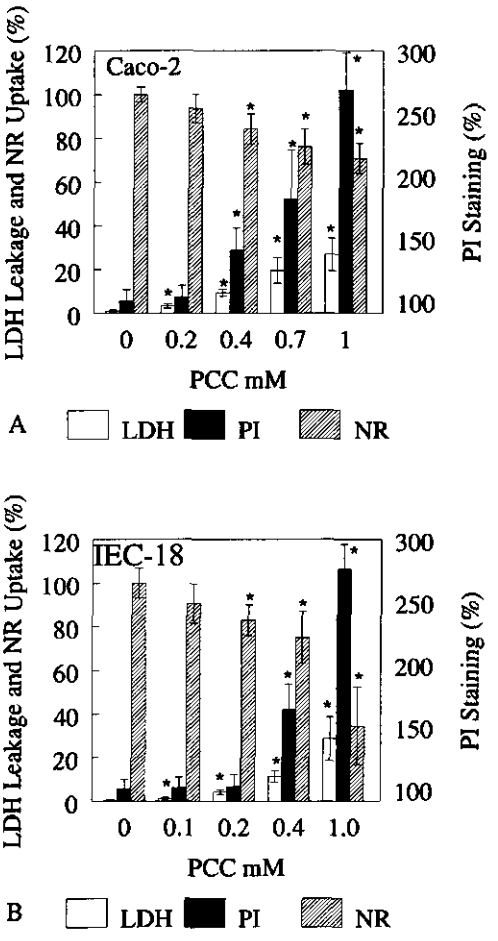

Figure 6.5: Effects of 30 min. PCC exposure on apical membrane integrity and cell viability of Caco-2 (A) and IEC-18 (B) cells. LDH leakage (n=4 experiments) is normalized with sonificated untreated cells as 100%. NR uptake (n=4 experiments) and PI staining (n=6 filters from 2 independent experiments) are presented with the control set to 100%. Data are presented as means±SD.

* Significantly different from control, $P<0.05$ according to Student's paired t-test.

Comparison of effects of PCC, Triton X-100 and EGTA on Caco-2 cells

In Figure 6.6 A the LDH leakage into the apical medium is presented as a function of the transport enhancing factor for PEG-4000. For PCC and Triton X-100 a similar relationship was found showing increased LDH leakage with increased transport enhancing factors. For EGTA the increase in transport enhancing factors was independent of LDH leakage. The relationship between the TER and the enhancing factor for PEG-4000 (Figure 6.6 B) is almost linear after exposure to Triton X-100, whereas after exposure to low concentrations of PCC or EGTA the limited decrease in TER is not accompanied by increased PEG-4000 permeability. Figure 6.6 C shows that when Caco-2 cells are exposed to PCC or Triton X-100, increased LDH leakage correlates with a decreased TER. Data on EGTA exposure show that it is possible to decrease the TER without causing membrane damage.

Recovery of TER and cell viability after PCC exposure

To investigate whether the effects of PCC on TER and cell viability were transient, both cell lines were allowed to recover for 22 h after 30 min exposure to 0, 0.4 and 1 mM PCC. In Caco-2 cells TER recovered completely within 6 h after exposure to 0.4 mM PCC (table 6.1) while recovery of TER after exposure to 1 mM PCC was only partial even after 22 h. Cell viability as determined with the NR assay was still not restored 22 h after exposure to 0.4 or 1 mM PCC (Table 6.1).

The TER of IEC-18 cells recovered slow, but completely within 22 h after exposure to 0.4 mM PCC, while no significant reconstitution of its barrier function after exposure to 1 mM was observed within 22 h (Table 6.2). Exposure to 0.4 or 1 mM PCC resulted in significantly reduced NR uptake in IEC-18, indicating an incomplete recovery of cell viability within 22 h.

Table 6. 1: Recovery of TER and cell viability of Caco-2 cells after 30 min palmitoyl carnitine hydrochloride (PCC) exposure.

PCC	Normalized TER %			Neutral Red Uptake
mM	30 min	6 h	22 h	22 h (%)
0	96.2±3.1	90.6±0.8	88.6±2.9	100±1.5
0.4	53±9.2*	94.8±1.5	88.8±4.6	89.8±7.5
1	42±6.8*	76.2±2.3*	82.7±0.7*	84.5±1.5*

Data are presented as mean ± SD (n=3). TER was normalized by using the TER value prior to PCC exposure as 100%. Timepoints are given with start of luminal exposure to PCC as t=0 min.
* Significantly different from control, $P<0.05$ according to Student's t-test.

Table 6.2: Recovery of TER and cell viability of IEC-18 cells after 30 min palmitoyl carnitine hydrochloride (PCC) exposure.

PCC	*Normalized TER* %			Neutral Red Uptake
mM	30 min	6 h	22 h	22 h (%)
0	101±5.7	107±7.8	115.3±14	100±2.7
0.4	48.6±9.6*	81.3±4.9*	102.3±15	79.2±5.1*
1	26.3±4.1*	46.4±15*	37.4±9.5*	39.4±5.8*

Data are presented as mean ± SD (n=3). TER was normalized by using the TER value prior to PCC exposure as 100%. Timepoints are given with start of luminal exposure to PCC as t=0 min.

* Significantly different from control, $P<0.05$ according to Student's t-test.

Discussion

The present study was intended to correlate absorption enhancing effects of PCC with effects on tight junction morphology and cytoxicity on the intestinal epithelium. For that purpose a small intestinal (IEC-18) and a colonic (Caco-2) cell line were apically exposed to PCC and TER and transport of mannitol and PEG-4000 were determined. Under similar conditions the immunolocalization of ZO-1, uptake of PI and NR, and release of LDH were assessed. It was found that PCC caused a decrease in TER and a concomitant increase in the permeability for mannitol and PEG-4000 in both cell lines. In IEC-18 cells, the PCC induced transport enhancement of the hydrophilic markers mannitol and PEG-4000 was slightly lower than in Caco-2. The finding that the absorption enhancement was smaller for small intestinal then for colonic cells is supported by studies of Sutton *et al.* (1993a) who used isolated ligaments of the rat and showed that ileal bioavailability of the drug cefoxitin was increased less than colonic bioavailability after co-administration with PCC. This indicates that the IEC-18 cell line could not only be a useful model for small intestinal paracellular transport (Duizer *et al.* 1997) but also for the study of transport enhancement across the small intestinal epithelium.

Both parameters, increased transport of mannitol and PEG-4000 and decreased TER, are generally considered to be indicators of increased paracellular permeability in experiments using cell lines. Nevertheless, it has been shown by Bernards and Kern (1996) that an increased transmeningeal flux of hydrophilic compounds caused by PCC exposure resulted from an increased transcellular flux. Furthermore, increased transport of PEG-4000 and decreased TER are seen as the result of affected membrane integrity, rather than a specific effect on paracellular permeability (Fagerholm *et al.*, 1996). Thus, even though hydrophilic

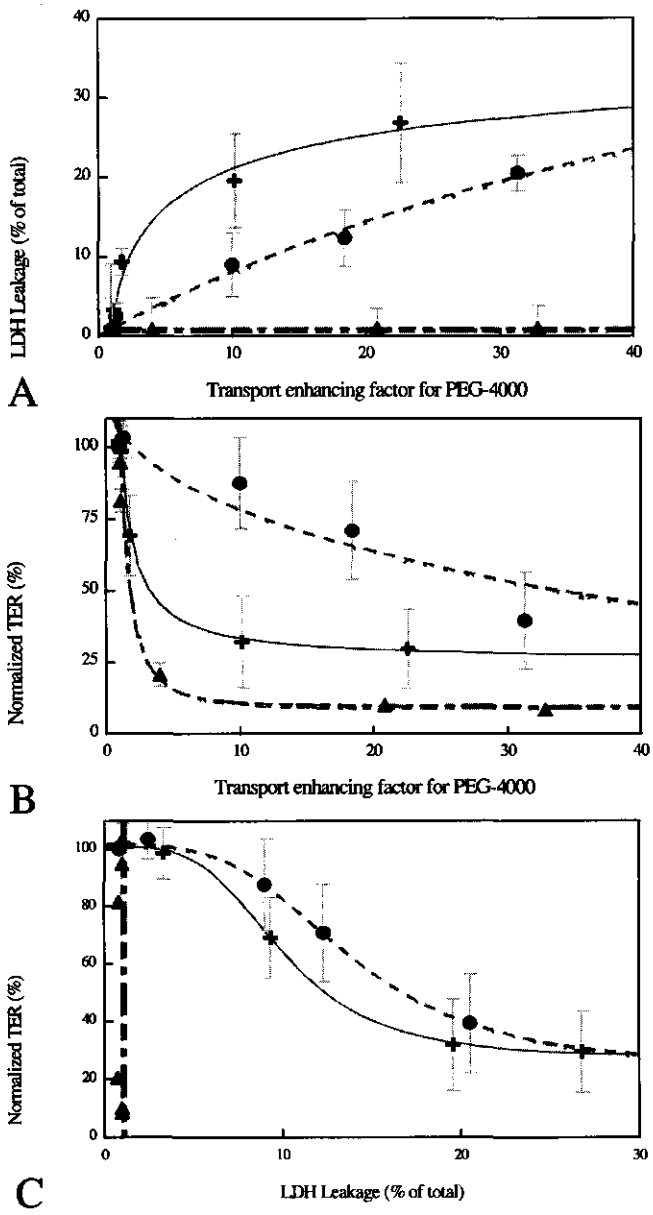

Figure 6.6: Effects of various concentrations of PCC (✚) , Triton-X-100 (●) or EGTA (▲) on Caco-2 cells. Expressed are the LDH leakage (A) and Normalized TER (B) as a function of the enhancing factor for PEG-4000, and the normalized TER as a function of LDH leakage (C). Data are presented as best fitted curves and mean ± SD (n=4 experiments for PCC, n= 3 experiments for Triton X-100 and EGTA).

macromolecular permeability and TER are, under physiological conditions, indicators of paracellular permeability, in case of increased membrane permeability these parameters can indicate increased transcellular permeability as well.

In an attempt to visualize alterations in the paracellular pathway as a result of PCC exposure, immunolocalization of ZO-1 was studied. It was hypothesized that if the permeation enhancing effect of PCC was mainly due to increasing the paracellular permeability, changes would be visible in the distribution of the tight junctional proteins. Indeed, PCC caused small irregularities in the ZO-1 pattern of Caco-2 and discontinuities in the ZO-1 pattern of IEC-18 cells. In order to quantify the changes in the ZO-1 pattern, we have developed a method with which we were able to determine pixel number and intensity as a measure for the immunolocalization of this tight junctional protein. The very local disruptions caused by 0.1-0.2 mM PCC in IEC-18 cells, could not be quantified by this method. However, as soon as the effects on ZO-1 pattern became more apparent (0.4 and 1 mM PCC) the pixel density and pixel number were reduced. Quantification of immunolocalization of junction-related ZO-1 seemed to correlate with transport of mannitol and PEG-4000 in IEC-18 cells after exposure to PCC. It appeared thus that the quantification of this tight junctional protein might be a valuable tool to assess changes in the tight junctional complex complementary to the determination of TER and apparent permeability of hydrophilic compounds.

In Caco-2 no reduction in pixel density as a result of PCC exposure was detected; either the ZO-1 pattern was located in the cell periphery and cell circumscribing or whole cells were dislodged or lysed. This shows the heterogeneity of the Caco-2 cell line, clearly some cells are more sensitive to PCC than others. But it probably also indicates that PCC does not selectively cause alterations in the intracellular ZO-1 distribution in Caco-2 cells. Since all cells with detectable defects in ZO-1 or occludin immunolocalization also had increased propidium iodide uptake, while not all cells with increased propidium iodide uptake showed an altered ZO-1 or occludin pattern, we conclude that increased permeability of the apical membrane is an earlier effect of PCC exposure than disruption of tight junction structure. In general, interactions of PCC with biological membranes are widely studied and are held responsible for most of the effects, such as blocking of inward rectifier K^+ channels in myocytes (Sato *et al.*, 1993), inhibition of insulin receptor tyrosine phosphorylation (McCallum and Epand, 1995) and protein Kinase C (Nakadate and Blumberg, 1987) but also for enhancement of drug absorption as shown clearly in this study and in the study of LeCluyse *et al.* (1991).

To assess the severity of the membrane damaging effects and cytotoxicity of PCC, three tests were performed. A clear indicator for apical membrane damage is leakage of cytosolic enzymes into the apical medium. This type of assay has been shown to be very sensitive in studies on the effects of surfactants on intestinal epithelial cells (Anderberg *et al.* 1992 and this study). In the present study we show that in both intestinal cell lines, leakage of

the 140 kDa enzyme LDH through the apical membrane started at lower PCC concentrations then transport enhancement of mannitol and PEG-4000 across the cell layer. Similar findings as with the LDH leakage assay were obtained with the PI uptake assay, although the latter was slightly less sensitive. In pilot studies we were unable to detect an increase in PI stained nuclei in Caco-2 cells after exposure to PCC concentrations up to 0.4 mM, similar as reported by Hochman *et al.* (1994). However, we detected an increased cytosolic PI staining prior to nucleus staining and therefore choose to determine the total PI fluorescence intensity in the final studies instead of performing a nuclei count. Using this method the PI uptake was shown to be increased at 0.4 mM PCC in Caco-2 and IEC-18 cells. In addition to LeCluyse *et al.* (1991) who found that perturbation of the lipid order of biological membranes correlated well with the absorption enhancing action of PCC, we report a good correlation between the absorption enhancing action of PCC and the membrane damage assays LDH leakage and PI uptake.

The neutral red assay showed that in both intestinal cell lines cytotoxicity of PCC is concomitant with increased transepithelial permeability of mannitol and PEG-4000. In Caco-2, cell damage as measured by LDH leakage and NR uptake was of the same order of magnitude, while in IEC-18 cells the reduction in NR uptake is much larger than would be anticipated from the LDH leakage. One possible explanation is that PCC has additional (toxic) effects on IEC-18 cells which occur during or after the immediate lysis of the membrane. For example, PCC has been proposed to deplete ATP (Hochman and Artursson, 1994) which might finally result in cell death and thus in lower NR uptake or retention in the cells.

In an attempt to establish the balance between selective paracellular transport enhancement and transcellular transport enhancement we exposed Caco-2 monolayers to PCC, Triton X-100 and EGTA and compared the TER, the transport enhancing factors for PEG-4000 and the LDH leakage (Figure 6.6). We found that increased PEG-4000 transport correlated with LDH leakage into the apical medium in cells exposed to PCC or Triton X-100, whereas no correlation was found for EGTA treatment. This similarity between the effect of PCC and Triton X-100 suggests transcellular transport enhancement. On the other hand, at lower concentrations of PCC and EGTA the decrease in TER did not correlate with an increased transport of PEG-4000, which suggests a selectivity of PCC and EGTA for increased electrolyte permeability (indicated by the TER) prior to macromolecule permeability. This generally indicates involvement of the paracellular pathway. However, it also shows that the initial reduction in TER is not necessarily indicative for paracellular transport enhancement of macomolecules. The parallelism in the correlation between LDH leakage and the TER after exposure to PCC and Triton X-100, show that the lytic effect of PCC contributes to the decrease in TER to almost the same extent as the lytic effect of Triton X-100.

In both cell lines the reversibility of the PCC effect on permeability and cell viability

was tested in additional experiments. In both cells the TER reduction caused by 0.4 mM PCC was transient (Hochman *et al.*, 1994; Raeissi and Borchardt, 1993; and this study). However, at this PCC concentration the cells were unable to recover viability to the full extent, as shown by decreased readings in the NR uptake assay. After exposure to 1 mM PCC, neither TER, nor cell viability could be recovered in IEC-18 or Caco-2.

The present study shows clear signs of cell damage at all effective (i.e. mannitol or PEG-4000 transport enhancing) concentrations of PCC. Although we show the cytotoxicity of PCC on intestinal epithelial cells, one should be careful with direct extrapolation of our results to the *in vivo* situation. It has been shown previously that the Caco-2 cells were much more sensitive and responsive to PCC than intestinal mucosa mounted in Ussing chambers (Sutton *et al.*, 1992; Hochman *et al.*, 1994) and intestinal tissues of rats and beagle dogs *in vivo* (Fix *et al.*, 1986). And even though PCC damages cells in the mucosa, the intestinal tract may be assumed to be able to deal with loss of cells to a certain extent due to the high rate of cell turn-over. Thus further studies will be needed to elucidate the balance between absorption enhancement and cytotoxicity in the intestinal mucosa *in vivo*.

In conclusion, PCC is effective as absorption enhancer for hydrophilic macromolecules, however, lytic effects on the apical cell membrane and reduced cell viability were concomitant with transport enhancement at all concentrations. Although the peripheral tight junctional network and the paracellular pathway are altered by PCC, our results indicate that this is not an initial effect but rather the result of interaction of PCC with the cell membrane, suggesting that enhancement of the transcellular route or cell lysis contributes considerably to the increased permeability.

Chapter 7

Effects of Cadmium Chloride ($CdCl_2$) on the Paracellular Barrier Function of Intestinal Epithelial Cell Lines

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Abstract

In the present study we characterized the functional and structural disruption of the paracellular barrier of intestinal epithelium *in vitro* in relation to cytotoxicity after apical Cd^{2+} exposure. For that purpose filter-grown Caco-2 and IEC-18 cells were apically exposed to 5 to 100 µM $CdCl_2$ for 4 or 14 hr. It was found that the effects of Cd^{2+} on the epithelial barrier were concentration- and time-dependent. The first detected effects of Cd^{2+} in Caco-2 cells after 4 hr exposure were a decrease in transepithelial electrical resistance, increased permeabilities of mannitol and PEG-4000 and changes in intercellular localization of ZO-1, occludin and e-cadherin. The effects were far more pronounced after prolonged exposure. The disruption of the paracellular barrier by 5-30 µM Cd^{2+} was detected without a significant loss of viability of the Caco-2 cells. In the IEC-18 cells, Cd^{2+} concentrations affecting the barrier (50 and 100 µM) also affected cell viability. In both cell lines the effects on the cell layers continued to develop after removal of extracellular Cd^{2+}. This correlated with the cellular retention of Cd^{2+}, which was high for the 12 hr following 4 hr accumulation. This study showed that the decreased epithelial barrier function of intestinal epithelial cells is accompanied by tight junction disruption. It is concluded that Cd^{2+} causes increased paracellular permeability by disruption of junctional function and structure. The initial junctional effects of Cd^{2+} suggest that Cd^{2+} increases its own bioavailability by causing disruption of the intestinal paracellular barrier.

Introduction

The intestinal epithelium is the main port of entrance for nutrients and at the same time the main barrier for absorption of other orally administered compounds such as food additives, drugs and toxicants. The epithelial cell itself with its membranes forms the barrier in the transcellular route, while the junctional complexes between the cells seal the paracellular route and regulate its permeability. The junctional complex consists of desmosomes, adherens junctions and tight junctions. The apical tight junction contributes mostly to the barrier/occluding function of this complex. Several studies into barrier functionality have shown reduced paracellular barrier function in various epithelia as a result of exposure to cadmium (Cd^{2+}). For instance, Janecki *et al.* (1992) found a reduced development of transepithelial electrical resistance (TER) in Sertoli cell monolayers when grown in the presence of cadmium. Also, the collapse of domes and reduction in TER was reported in several renal epithelial cell lines (Prozialeck and Niewenhuis, 1991; Niewenhuis *et al.*, 1997). Structural studies with renal epithelial cells revealed disrupted actin filaments, an increased lateral intracellular space and a decreased density of adherens junction-associated plaques (Mills and Ferm, 1989; Prozialeck and Niewenhuis, 1991; Hazen-Martin *et al.*, 1989). However, none of these studies showed a clear disruption of the structure of the tight junctions which could account for the significant reduction in the barrier function.

Damage of the intestinal epithelium was reported to occur after exposure to high concentrations of Cd^{2+} in mice (Valberg *et al.*, 1977). Moreover, epithelial accumulation and absorption into the serosal compartment are increased at higher concentrations *in vivo* (Goon and Klaasen, 1989; Groten *et al.*, 1994) and in Caco-2 cells (Rossi *et al.*, 1996; Pigman *et al.*, 1997). In a study of the toxicity of heavy metals, Rossi *et al.* (1996) described a reduction in TER and increase in transepithelial mannitol passage in the Caco-2 monolayers after exposure to Cd^{2+}. It is, however, not yet established whether the selective effects of Cd^{2+} on the paracellular barrier function, as found in several epithelia, can account for the increased absorption of Cd^{2+} in the intestinal epithelium.

In this paper, we would like to establish the relation between a reduced tissue functionality (decreased TER and increased Cd^{2+} and mannitol and PEG-4000 transport), the structural disruption of the junctional complex (immunolocalization of ZO-1, occludin and e-cadherin) and the cytotoxicity (LDH leakage and neutral red (NR) uptake) in intestinal epithelial cells after Cd^{2+} exposure. We use filter-grown intestinal epithelial cells as a model to determine the sequence of events in the modification of the junctional barrier function of the intestinal epithelium in vitro as a result of Cd^{2+} exposure. The human colon carcinoma cell line Caco-2 was used for its enterocytic differentiation (Hildalgo et al., 1989) and widespread use as a transport model in pharmacology and toxicology (reviewed by Artursson *et al.*, 1996). In addition to the villus-like Caco-2 cells, we used the rat small intestinal crypt cell line IEC-

18 because it was used earlier to study Cd^{2+} toxicity and accumulation (Groten *et al.*, 1992). Furthermore, the relation found between the molecular size of several hydrophilic molecules and the Papps, allowed for a more accurate prediction of transepithelial transport rates on the basis of molecular size in IEC-18 than in Caco-2 cells (Duizer *et al.*, 1997).

Materials and methods

Cell culture

The Caco-2 cell line originating from a human colorectal carcinoma, and IEC-18 cells originating from rat ileum epithelium were obtained from the American Type Culture Collection. Cells were maintained as described previously (Duizer *et al.*, 1997). For experiments both cell types were seeded on Transwell polycarbonate cell culture inserts with a mean pore size of 0.4 µm (Costar, Badhoevedorp, Netherlands) at 2-3 $*10^5$ cells/cm^2 and cultivated for 17-24 days. Caco-2 cells were used at passages 30-40, IEC-18 cells at passages 20-30.

TER measurements

Filter grown cells were adapted to room temperature to allow for more standardized transepithelial electrical resistance (TER) measurements using the Millicell-ERS epithelial voltohmmeter (Millipore Co., Bedford, USA). The TER was measured prior to and during the experiments to monitor cell layer confluency and the integrity of the tight junctions. The TER of the cell layers was calculated according to the following equation: TER = $(R_{total}-R_{blank})$ x A ($\Omega.cm^2$), R_{total} is the resistance measured, R_{blank} is resistance of control filters without cells (approximately 130 $\Omega.cm^2$), A = surface area of filter (1 cm^2).

Exposure to $CdCl_2$.

Two exposure scenarios were used to study the mechanism of action of Cd^{2+}. In both cases the basolateral medium was DMEM (Gibco, Paisley, Scotland) supplemented with non-essential amino acids, 6 mM L-glutamine, gentamycin and 0.1% BSA (basal medium). The apical medium was protein free basal medium with freshly dissolved $CdCl_2$ in concentrations ranging from 0-100 µM (test medium). In the first scenario, in which acute effects are studied, the inserts with cells were transferred to new wells with 1.8 ml basal medium in the basolateral compartment and cells were apically exposed to 0.5 ml test medium for 4 hr or 14 hr (37°C, 5%CO_2, 95% relative humidity). After the exposure time the TER was determined and the apical test medium was removed and used for LDH leakage measurements. Subsequently, the inserts with cells were used for assessment of transepithelial transport and neutral red (NR) uptake or immunocytochemistry. In the second exposure scenario the cells were apically exposed for 4 hr after which time the apical and basolateral medium were carefully removed and fresh basal medium was added to both sides. Cells were incubated overnight (12-14 h, 37°C, 5%CO_2, 95% relative humidity) and subsequently the same measurements were performed as for the 4 or 14 hr incubations. This was done to study acute and delayed effects of a 4 hr Cd^{2+} exposure period.

Transepithelial transport of mannitol and PEG-4000

Transport experiments were carried out as described previously (Duizer *et al.*, 1997). Apparent Permeability coefficients (Papps) of mannitol and PEG-4000 were determined on the basis of appearance of the probe in the basolateral receiver compartment before 10% of the probe was transported (Artursson and Karlsson, 1991).

Transepithelial transport and cellular accumulation of $^{109}Cd^{2+}$

The effect of Cd^{2+} exposure on transepithelial transport of $^{109}Cd^{2+}$ (DuPont, Boston, USA, kindly provided by F. Schulz, University of Utrecht, Netherlands) was followed for 24 h. The cellular sequestration of $^{109}Cd^{2+}$ was determined after 4 hr exposure to 5 and 30 μM Cd^{2+}. After the exposure time cells were washed with basal medium and PBS with 0.022% (w/v) EDTA to remove all extracellular Cd^{2+} (Prozialeck and Lamar, 1993). Subsequently, cells were incubated in basal medium and release of $^{109}Cd^{2+}$ was measured for 12 hr (37°C, 5%CO_2, 95% relative humidity). Prior to the experiments the $^{109}Cd^{2+}$ was mixed with unlabeled Cd^{2+} to yield specific activities of 5-30 mCi/mol for experiments with Caco-2 cells, and 1 mCi/mol for IEC-18 cells. All $^{109}Cd^{2+}$ samples were counted for 3 min in a 1272 Clinigamma counter from Wallac.

Immunocytochemistry

For immunocytochemistry the filters with cells were fixed in 1% formaldehyde in HBSS (5 min, RT) and permeabilized in 0.2% Triton X-100 in HBSS (20 min, RT). Polyclonal rabbit anti-ZO-1 and polyclonal rabbit anti-Occludin, anti-rabbit IgG-biotin and streptavidin-FITC or-TRITC (Zymed, San Francisco, USA) were used to visualize ZO-1 and occludin respectively. E-cadherin was labeled in Caco-2 cells with monoclonal anti-uvomorulin (Sigma, St.Louis, USA), biotin conjugated goat-anti-rat IgG and streptavidin-FITC (Zymed, San Francisco, USA).

Confocal scanning laser microscopy and image processing

Confocal fluorescence microscopy was performed with the Bio-Rad MRC600, using the PlanApoChromat 40x objective with variable diaphragm (Zeiss, Oberkochen, Germany). Images were recorded with Bio-Rad software and transferred to an image processing workstation. The images were processed by a custom written program, using the image processing toolbox SCIL-Image (Free University of Amsterdam, Netherlands) as a basis. The program developed to select junction-related immunolabeled ZO-1, consists of three sequential steps (Duizer *et al.*, 1998). In brief, a threshold value (1.4 for Caco-2 cells and 1.6 for IEC-18 cells) was applied to differentiate between junction-related ZO-1 pattern and background. This step usually results in a honeycomb-like skeleton with small discontinuities, representing most of the junction-related ZO-1 pattern. In the second step of the program, the skeleton can be improved by closing the small discontinuities of the borderlines and removal of spurious line fragments. Finally, from the binary image of the optimized skeleton representing only junction-related ZO-1, the total number of pixels is determined (as a measure of the junctional length) and the original grey value image was used to determine the average pixel intensity over the junctional pattern (as a measure of the junctional density).

Cytotoxicity and membrane damage assays

LDH leakage into the apical medium resulting from Cd exposure at the apical cell side was determined with the Boehringer Mannheim kit for LDH at the BM/Hitachi 911. Total LDH content was determined after sonicating the inserts with cells in basal medium. Neutraluptake was determined directly after the transport experiments. For that purpose, cells were incubated with NR (50 µg/ml) in basal medium for 30 minutes (37°C, 5%CO_2, 95% relative humidity). Subsequently, the cell layer was rinsed once with HBSS and the NR was extracted with 1% (v/v) acetic acid in a 1:1 water-ethanol mixture. The A_{540nm} was measured spectrophotometrically.

Statistics

Data on TER, LDH leakage, transport of mannitol and PEG-4000 and NR uptake were obtained in 2 independent experiments with both cell types using 3 filters per exposure group in each experiment. For the figures and statistical analysis data were calculated for n=6. Data on immunocytochemistry were obtained in 2 experiments using 2 filters per exposure group for each experiment, 5 areas per filter were recorded to quantify ZO-1 specific fluorescence. Presented data are mean ± SD of two pooled independent experiments, unless mentioned otherwise. Data were analyzed using two-tailed students t-test.

RESULTS

Effects of Cd^{2+} on Transepithelial Electrical Resistance and Paracellular Transport in Caco-2 cells

Apical exposure to 0-100 µM Cd^{2+} caused a small but dose-dependent decrease in TER and slightly increased Papps for mannitol and PEG-4000 directly after a 4 hr preincubation (Figure 7.1A). The decrease in TER and increased Papps for mannitol and PEG-4000 caused by a 4 hr preincubation with Cd^{2+}, and subsequently maintained in basal medium, were more severe when measured 14 hr later (Figure 7.1B). This indicates that the effects induced by a 4 hr incubation period are irreversible and initial effects of Cd^{2+} are decisive, even in the absence of a continued Cd^{2+} exposure. The 14 hr full-time exposure resulted in decreases in TER and increased transport rates of mannitol and PEG-4000 at Cd^{2+} concentrations of 10 µM and higher.

Effects of Cd^{2+} on Transepithelial Electrical Resistance and Paracellular Transport in IEC-18 cells

In IEC-18 cell layers apical exposure to 50-100 µM Cd^{2+} resulted in significantly increased permeability rates for mannitol and PEG-4000, when compared to 0 µM Cd^{2+}, in all three exposure scenarios (Figure 7.2 A, B and C). The decrease in TER after exposure to 50-

100 µM Cd^{2+} was statistically significant in the 14 h full-time exposure only. The 14 h full-time preincubation with 0, 5 and 10 µM Cd^{2+} resulted in increased Papps for mannitol and PEG-4000, whereas these concentrations had no effect after 4 h preincubation.

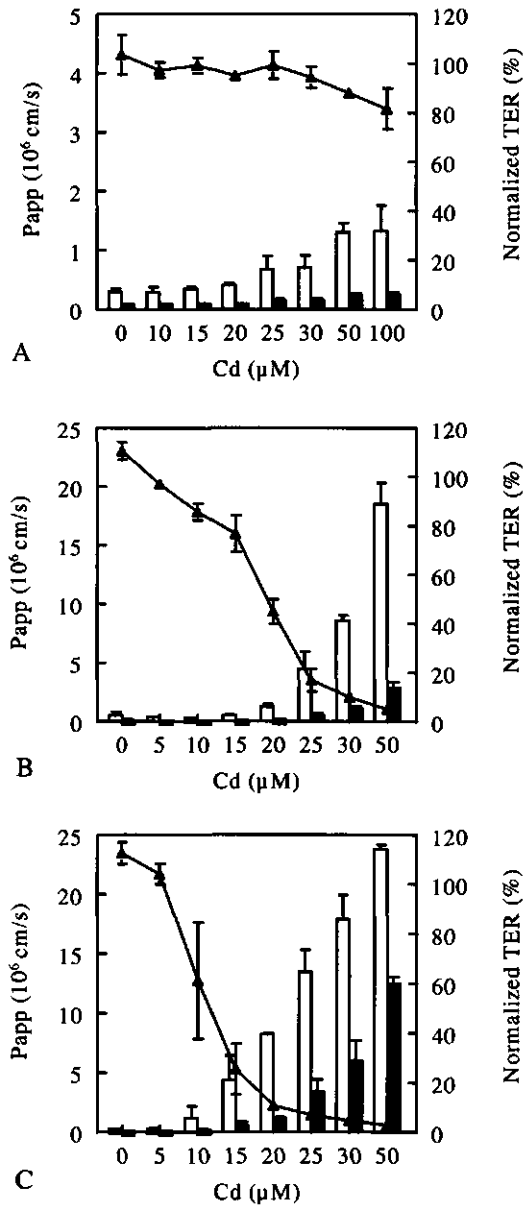

Figure 7.1: Effects of apical $CdCl_2$ exposure on apparent permeabilities of mannitol (□) and PEG-4000 (■) and TER (-▲-) of Caco-2 cells. Cells were exposed for 4 hr and used immediately (A) or after 14 hr recovery (B), or cells were exposed for 14 hr (C). TER was normalized with the TER prior to $CdCl_2$ exposure set to 100%. Data are presented as mean±SD (n=6).

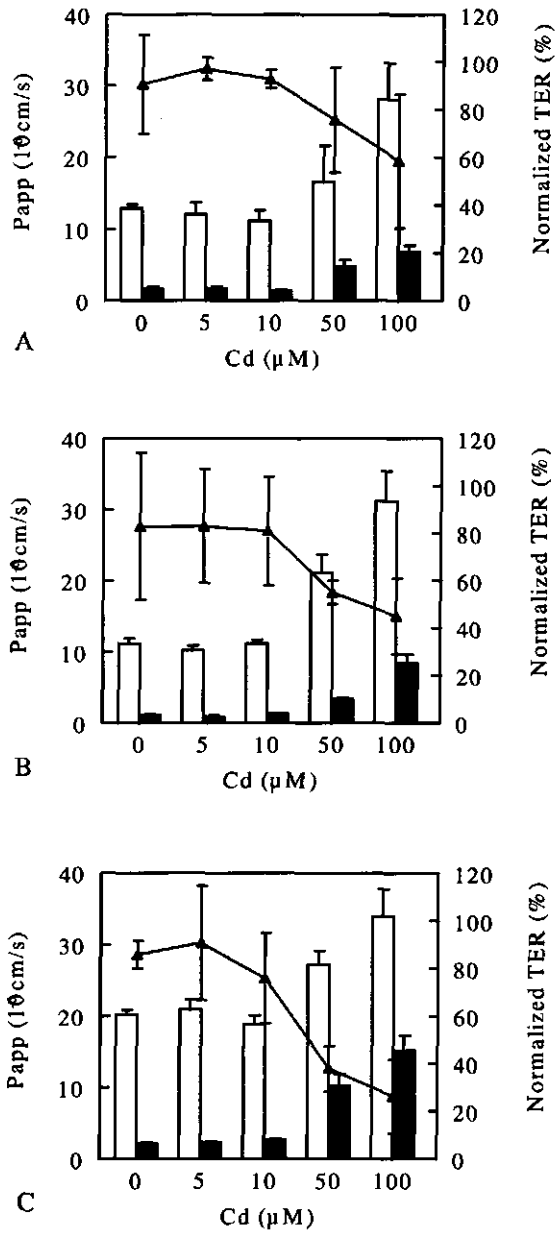

Figure 7.2: Effects of apical $CdCl_2$ exposure on apparent permeabilities of mannitol (□) and PEG-4000 (■) and TER (-▲-) of IEC-18 cells. Cells were exposed for 4 hr and used immediately (A) or after 14 hr recovery (B), or cells were exposed for 14 hr (C). TER was normalized with the TER prior to $CdCl_2$ exposure set to 100%. Data are presented as mean±SD (n=6).

Effect of Cd^{2+} on Transport of $^{109}Cd^{2+}$

Figure 3 shows the transepithelial transport of Cd^{2+} across Caco-2 and IEC-18 cells. The 5 µM Cd^{2+} was considered as control, no unlabeled Cd^{2+} was added and this concentration was found to be ineffective towards TER and permeability of mannitol and PEG-4000 in both cell lines (Figure 7.1 and 7.2). Compared to 5 µM Cd^{2+}, transepithelial transport of Cd^{2+} is increased at 15 and 30 µM Cd^{2+} in Caco-2 monolayers. In these cells the first signs of the effect of Cd^{2+} on its transport occurred between 6 and 9 hours after onset of exposure. The steady-state flux of Cd^{2+} across Caco-2 cells (determined between 12-18 h) was 5.1±0.7 pmol/h when the Cd^{2+} concentration was 5 µM. The flux was increased to 43±9 pmol/h and 327±89 pmol/h, after addition of 15 and 30 µM Cd^{2+}, respectively.

Transport of Cd^{2+} across IEC-18 cell layers was higher than across Caco-2 monolayers but was also dependent on the concentration. The lag-phase of approximately 6 hr as found for transport across Caco-2 cells was not found for Cd^{2+} transport across IEC-18 cells. Cd^{2+} fluxes were 89±13 pmol/h and 1003±146 pmol/h (determined between 0-9 h), after addition of 5 and 30 µM Cd^{2+}, respectively.

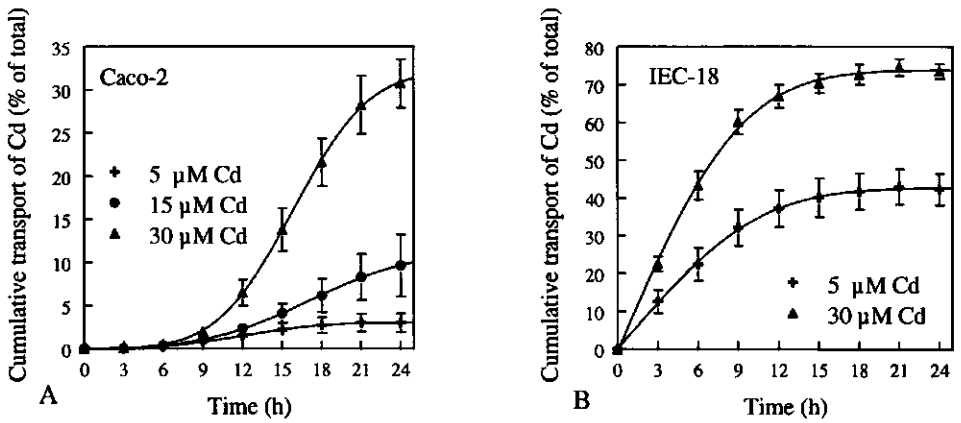

Figure 7.3: Cumulative apical to basolateral $^{109}Cd^{2+}$ transport profiles across Caco-2 (A) and IEC-18 cell layers (B). $^{109}Cd^{2+}$ was added apically at t=0 hr at a concentration of 5 µM (-✚-), 15 µM (-●-) or 30 µM (-▲-) and appearance in the basolateral compartment was measured. Data are presented as mean±SD (n=3).

Effects of Cd^{2+} on the Cellular Distribution of ZO-1 in Caco-2 cells

Immunocytochemical localization of ZO-1 in untreated layers of Caco-2 cells showed a continuous belt-like cell circumscribing pattern at all sites of cell-to-cell contact (Figure 7.4A). After 4 hr exposure to 30 µM Cd^{2+} the first signs of fragmentation of the ZO-1 pattern became visible (data not shown). Locally, densely stained spots of immunofluorescent ZO-1

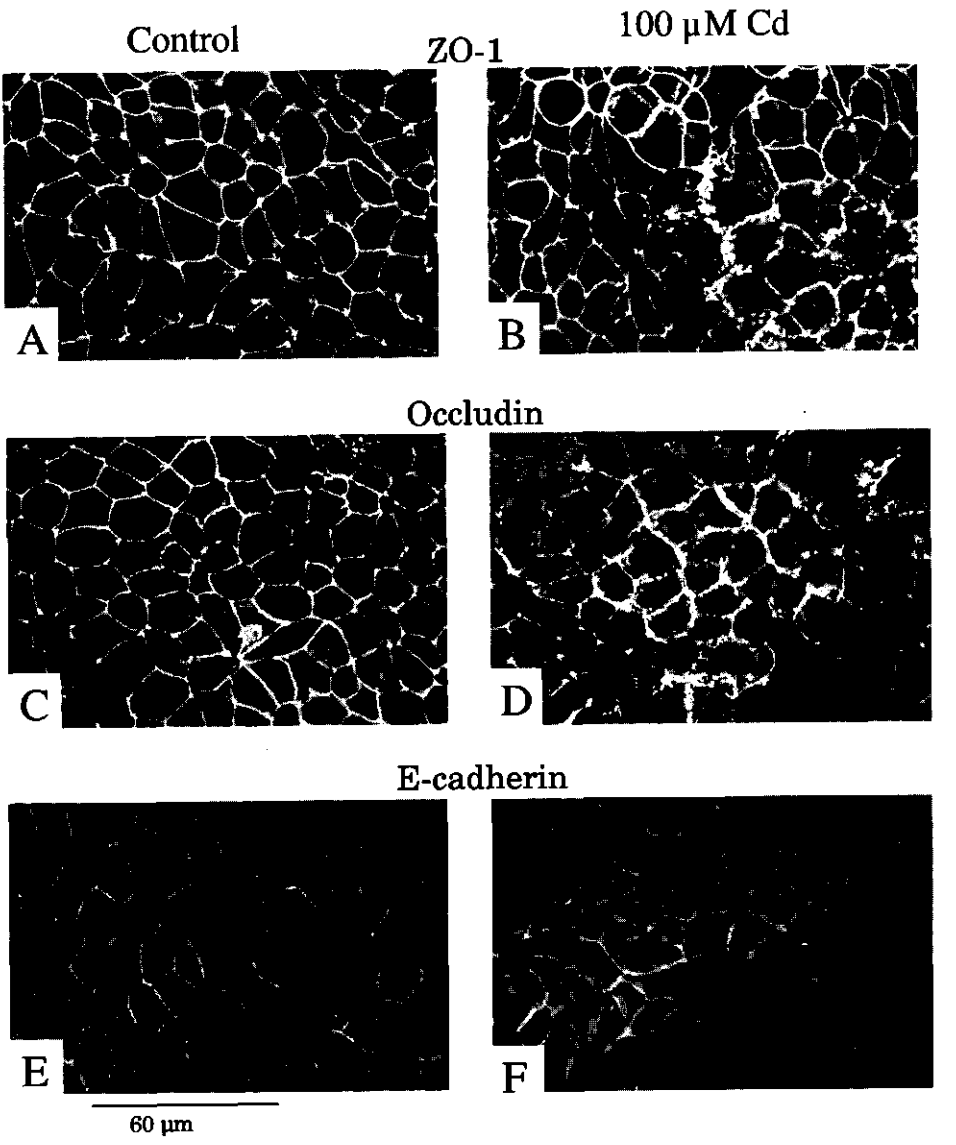

Figure 7.4: Immunofluorescent staining for junctional proteins in Cd^{2+} exposed Caco-2 cells. Cells were labeled for ZO-1 (A and B), Occludin (C and D) or E-cadherin (E and F) after apical exposure to 0 (A, C, E) or 100 µM (B, D, F) Cd^{2+} for 4 h.

appeared in the cell periphery, but the belt-like pattern was in general still continuous and cell surrounding. After 4 hr exposure to 100 μM Cd^{2+}, clusters of Caco-2 cells showed a clearly damaged ZO-1 pattern (Figure 7.4B). This fragmentation was more severe than after 30 μM exposure, and the cell surrounding pattern showed interruptions. Exposure to 15 μM Cd^{2+} or higher concentrations for 14 hr led to severe disruptions of the ZO-1 pattern (Figure 7.5A). In substantial parts of the monolayer the belt-like structure was no longer recognizable, but the ZO-1 protein could still be detected as intensely stained spots throughout the affected cells. The ZO-1 pattern of the Caco-2 monolayers exposed to 15 μM Cd^{2+} for 4 hr but maintained in culture for 14 hr in basal medium, showed a few localized spots of severe disturbances of the cell surrounding pattern in an overall normal appearing monolayer. The cells surrounding the affected spots appear to extend into the area to close the holes in the monolayer (Figure 7.5B).

The quantitative analysis of ZO-1 localization in Caco-2 cells (Table 7.1) showed that immediately after 4 hr exposure to 100 μM Cd^{2+} the junctional length was decreased. This effect became more apparent after 14 hr exposure, even at lower concentrations of Cd^{2+}. After a 4 hr exposure to 15 or 30 μM Cd^{2+} followed by a recovery period of 14 h, the net junctional length was not affected.

The junctional density was reduced by 4 hr exposure to 100 μM Cd^{2+} but remained unchanged after 14 hr exposure to 15 or 30 μM. The 4 hr exposure followed by 14 hr in culture resulted in a slight decrease in net junctional density at 15 μM Cd^{2+} but not at 30 μM.

Effects of Cd^{2+} on the Cellular Distribution of ZO-1 in IEC-18 cells

The high background of non-junctional ZO-1 in control cell layers of IEC-18 (Figure 7.6A) makes it difficult to characterize early changes in the junctional pattern. The Cd^{2+} dependent disruption of the ZO-1 pattern in IEC-18 cells appeared to evolve in a less organized manner than Caco-2 cells. The 14 hr exposure to 50 μM Cd^{2+} led to rounded cells with a vaguely stained nucleus and a complete lack of cell-surrounding junctional staining (Figure 7.6B). After 14 hr exposure to 10 μM Cd^{2+}, the junctional ZO-1 pattern has become invisible in several patches throughout the cell layer and a decreased junctional length as determined by the quantified immunolocalization of ZO-1 (Table 7.1) was observed.

Effects of Cd^{2+} on the Cellular Distribution of Occludin and E-cadherin in Caco-2 cells

Since the undifferentiated IEC-18 cells did not express detectable levels of occludin and E-cadherin, the effects of Cd^{2+} on the cellular distribution of these two proteins was studied only in Caco-2 cells. Immunocytochemical localization of occludin in untreated Caco-2 monolayers showed a continuous belt-like cell-circumscribing pattern at all sites of cell-to-cell contact (Figure 7.4B). Occludin appeared in punctuates near the sites of cell-cell contacts after

4 hr exposure to 100 µM Cd^{2+} (Figure 7.4D) and as punctuates throughout the cell after 14 hr exposure to 15 µM Cd^{2+} (not shown). The intercellular localization of occludin was indiscernible from that of ZO-1, in control and Cd^{2+} exposed monolayers after all exposure scenarios. All disturbances of the patterns of the junctional proteins tested (ZO-1, occludin and e-cadherin) started locally, i.e. in patches in an overall normally appearing monolayer. Only after exposure to 100 µM Cd^{2+} for 4 hr or >20 µM Cd^{2+} for 14 hr effects are more general throughout the monolayers.

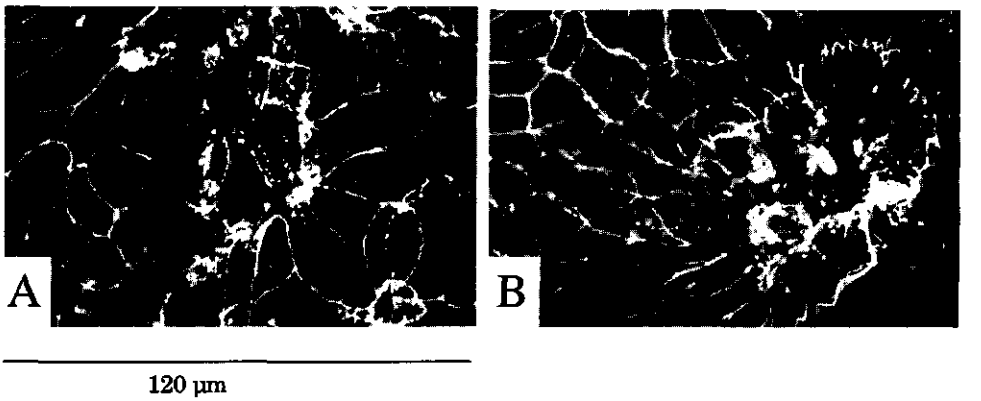

Figure 7.5: Immunofluorescent staining for ZO-1 in Cd^{2+} exposed Caco-2 cells. Cells exposed to 15 µM Cd for 14 h(A) or for 4 hr followed by 14 hr recovery (B).

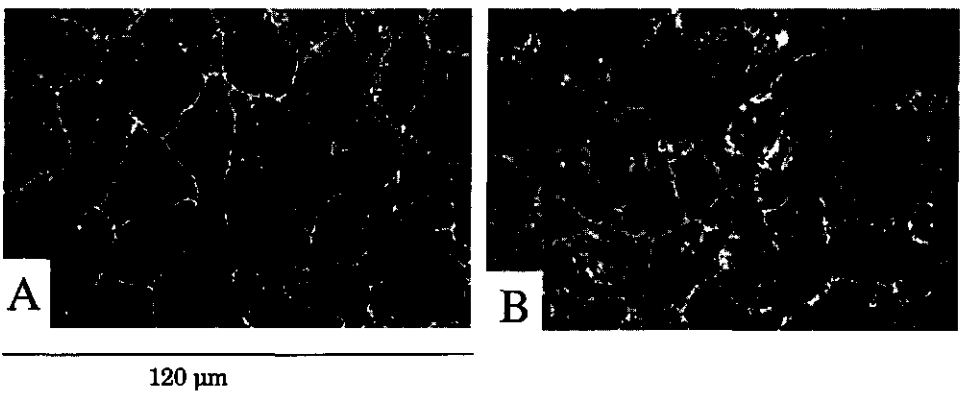

Figure 7.6: Immunofluorescent staining for ZO-1 in Cd^{2+} exposed IEC-18 cells. Control (A) or cells exposed to 50 µM Cd^{2+} for 14 hr (B).

In contrast to the punctuated pattern of ZO-1 and occludin, the junctional organization of e-cadherin as found in control cell layers at sides of cell-to-cell contacts (Figure 7.4E) faded after 4 hr exposure to 100 µM Cd^{2+}, leading to vaguely recognizable cell nuclei without peripheral staining (Figure 7.4F). Immunofluorescent double labeling of Caco-2 cells for ZO-1 and e-cadherin revealed that in these cases a punctuate ZO-1 pattern co-occurred with a vague or undetectable e-cadherin labeling. Thus the localization of e-cadherin was changed but in a different way than that of ZO-1 and occludin.

Table 7.1: Quantitative analysis of immunolocalization of ZO-1 in Caco-2 and IEC-18 cells.

Cells	Treatment Exposure time, concentration	junctional length[a] %	junctional density[b] %
Caco-2	Control	100 ± 6.04	100 ±11.8
	4 h, 30 µM	98.2 ± 5.3	92.9 ±8.5
	4 h, 100 µM	89.8 ± 9.4*	77.3± 14.8*
	4 h, 15 µM, 14 h[c]	108.1 ± 6.6	85.1 ±14*
	4 h, 30 µM, 14 h	97.3 ± 9.8	102.5 ±14
	14 h, 15 µM	71.5 ± 23.9*	113.6 ± 17.7
	14 h, 30 µM	42.3 ± 21.3*	103.7± 17.7
IEC-18	Control	100 ± 6.0	100 ± 11.5
	14 h, 10 µM	80.3 ± 16.7*	111.3 ± 14.6
	14 h, 50 µM	< 10	nd[d]

[a] Junctional length is based on the number of pixels belonging to the junctional pattern as selected by a custom written program after immunolabeling the ZO-1 protein
[b] Junctional density is based on the pixel intensity of the pixels forming the junctional pattern.
[c] Time after exposure to cadmium, cells were maintained in culture prior to fixation.
[d] not determined because of the limited junctional length
* Statisticaly significant with $p<0.05$.

Cell Viability after Cd^{2+} Exposure

Figure 7 A-C show that Cd^{2+} exposure did not cause LDH leakage in Caco-2 cells, thus in this cell line none of the exposure scenarios resulted in membrane damage. Cell viability, as determined by the NR assay performed after the transport experiments (i.e. 4 hr after end of exposure), was slightly, but significantly, reduced after 4 hr exposure to 50 and 100 µM Cd^{2+}. Cells exposed to Cd^{2+} for 4 hr and maintained in culture for another 14 hr showed similar viabilities as the 14 h full time exposure.

The IEC-18 cells exposed to 10 - 100 µM Cd^{2+} for 4 hr did not show a significant

increase in the LDH leakage but the NR uptake was reduced in a dose-dependent manner directly after the transport experiment. Cells exposed to 50-100 μM Cd^{2+} for 4 hr and maintained in culture for 14 hr did show a clear increase in LDH leakage and reduction in NR uptake. A similar tendency was observed after 14 hr Cd^{2+} exposure.

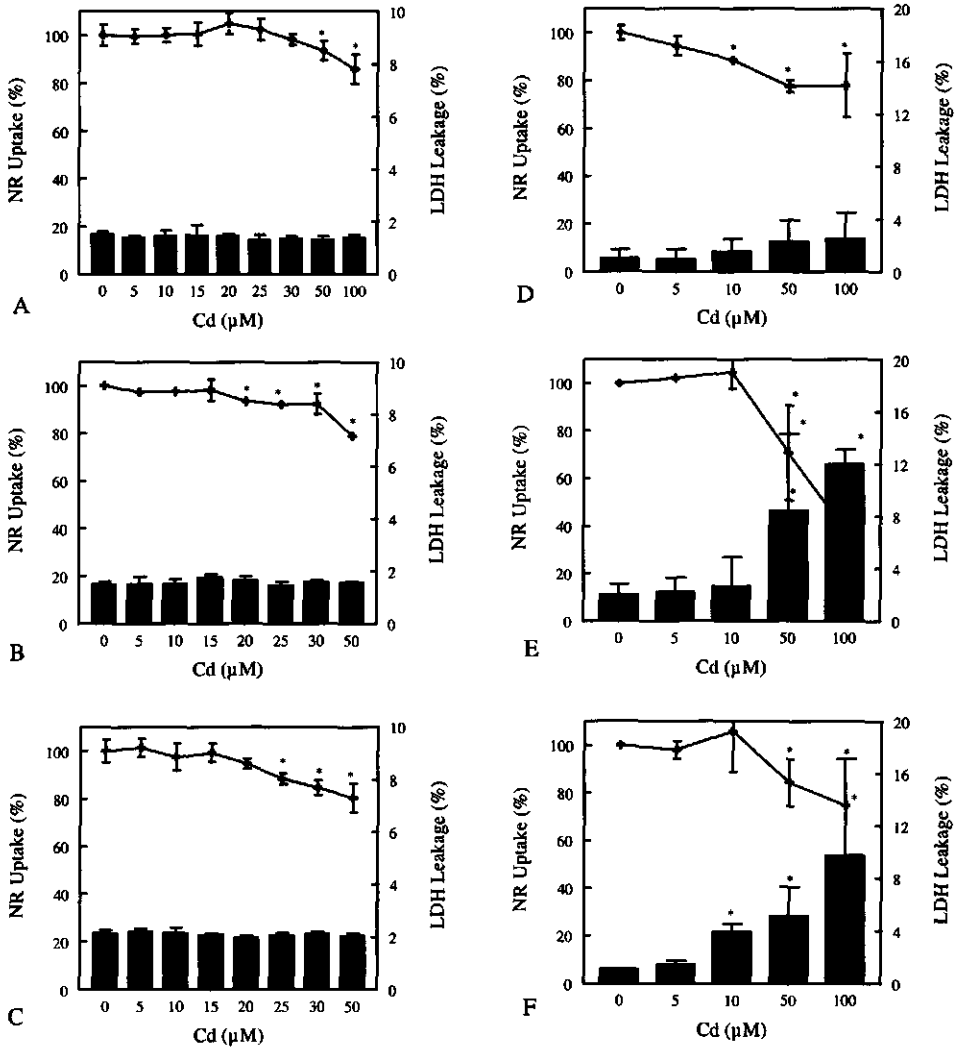

Figure 7.7: Effects of apical $CdCl_2$ exposure on LDH leakage (bars) and NR uptake (lines) of Caco-2 (A, B, C) and IEC-18 (D, E, F) cells. Cells were exposed for 4 hr and used immediately (A, D) or after 14 hr recovery (B, E), or cells were exposed for 14 hr (C, F). Data are presented as mean±SD (n=6). *, $p<0.05$ in two-tailed Students t-test.

Fate of $^{109}Cd^{2+}$ after 4 hr exposure

Since we found that even after removal of extracellular Cd^{2+}, the effects on the cell layers continued to develop, the cellular retention of $^{109}Cd^{2+}$ was followed for 12 h. After 4 hr incubation with Cd^{2+} a dose-dependent cellular accumulation of $^{109}Cd^{2+}$was found in Caco-2 cells (Figure 7.8). In IEC-18 cells the cellular Cd^{2+} accumulation was increased 1.6-fold when the Cd^{2+} concentration was increased 6-fold (from 5 to 30 μM). During the 12 hr recovery period in culture medium, 65-85% of the Cd^{2+} was retained in the cells. More than 70% of the released fraction was found in the apical medium in both cell lines at all concentrations (data not shown).

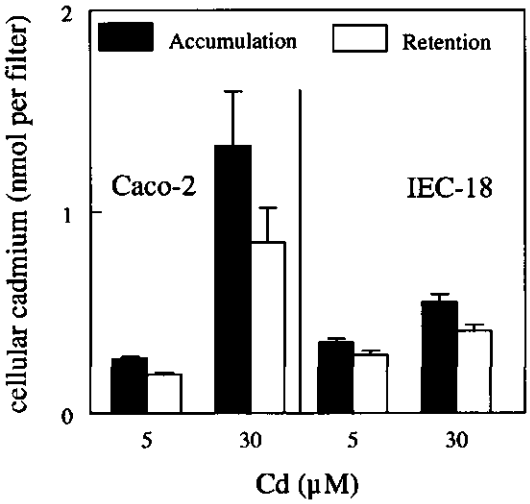

Figure 7.8: Cellular accumulation of $^{109}Cd^{2+}$ in cells exposed apically to 5 and 30 μM Cd^{2+} for 4 hr (■) and subsequent retention during maintenance in basal medium for 12 hr (□). Data are presented as mean±SD (n=3).

DISCUSSION

In the present study we characterized the effects of apical Cd^{2+} exposure on the barrier function of intestinal epithelium. For that purpose a small intestinal crypt cell line (IEC-18) and a colonic (Caco-2) cell line with enetrocytic-like differentiation, were apically exposed to $CdCl_2$ and the permeability of electrolytes (TER), Cd^{2+} and medium-sized and large hydrophilic molecules (mannitol and PEG-4000) were determined. Under similar conditions the immunolocalization of junctional proteins (ZO-1, occludin and e-cadherin), the release of LDH and uptake of NR were assessed.

It was found that the effects of Cd^{2+} on the epithelial barrier function were concentration- and time-dependent. Although the first effects of Cd^{2+} on Caco-2 cells (i.e. decrease in TER, increased permeabilities of mannitol and PEG-4000 and qualitative and quantitative changes in intercellular ZO-1, occludin and e-cadherin localization) were already apparent after a 4 hr incubation, the effects were far more pronounced after prolonged exposure. These changes were detected without a significant loss of cell viability of the Caco-2 cells. Whereas Cd^{2+} appeared to interfere particularly with the epithelial barrier function of Caco-2 cells, this specific effect was not found in the IEC-18 cells. In this cell line of poorly differentiated small intestinal epithelial cells, Cd^{2+} concentrations affecting the barrier function and ZO-1 expression also caused a reduced cell viability and membrane damage. Although IEC-18 cells do express ZO-1 in a junctional pattern, we were unable to detect a peripheral expression of occludin and e-cadherin. The low expression of junctional proteins and the relatively high permeability for hydrophilic macromolecules in IEC-18 cells indicate that the junctional complexes are less tightly organized, which might explain the lack of early specific effects of Cd^{2+} on the paracellular barrier function in IEC-18 cells. Furthermore, the relative early beginning of cytotoxic effects in IEC-18 cells, compared to Caco-2 cells, may be enhanced by the two-sided exposure due to the high permeability of Cd^{2+}. The higher sensitivity due to basolateral or two-sided exposure was already found for epithelial kidney cells (Prozialeck and Niewenhuis, 1991; Hazen-Martin *et al.*, 1993; Prozialeck and Lamar, 1997) and for Caco-2 cells (Rossi *et al.*, 1996).

In this study we found relatively high transepithelial passage of mannitol, PEG-4000 and Cd^{2+} across IEC-18 cells, compared to Caco-2 cells. Since cellular accumulation and retention were comparably low in both cell lines after exposure to 5 μM Cd^{2+}, we suggest that the extra Cd^{2+} transport across IEC-18 cells compared to Caco-2 cells, occurs by the paracellular pathway. It was found earlier that the paracellular permeability of hydrophilic macromolecules across IEC-18 cells correlated with the intestinal permeability *in vivo* (Duizer *et al.*, 1997). The transepithelial permeability of Cd^{2+}, however, seemed to be more closely resembled by the permeability across Caco-2 cells. This suggest that other mechanisms limit the absorption of Cd^{2+}. For example, Cd^{2+} can be bound by the well-organized brush border, present on small intestinal villus and Caco-2 cells, or to a variety of macromolecules in the luminal contents of the gastrointestinal tract. In both cases the Cd^{2+} availability in the more permeable crypts will be decreased and therefore absorption will be low. This also explains the overestimation of Cd^{2+} absorption on basis of Cd^{2+} permeability in the ileal crypt cell line IEC-18.

In order to be able to relate the decrease in barrier function to changes in tight junction patterns, we have developed a method which enabled us to quantify junctional length (pixel number) and density (pixel intensity) on the basis of the immunolocalization of the tight junctional protein ZO-1. The quantification of the tight junctional protein ZO-1 was used

previously in a study into the mechanism of the absorption enhancing effect of palmitoylcarnitine (Duizer *et al.*, 1998). It was found that the sequence of events after palmitoylcarnitine exposure was first membrane damage (increased LDH leakage), followed by reduced barrier function and decreased junctional ZO-1 expression. This led to the conclusion that transcellular transport enhancement or cell lysis contribute significantly to the increase in intestinal permeability. In the present study we found that in Caco-2 cells the barrier function was decreased concomitantly with decreased junctional ZO-1 expression but without LDH leakage after Cd^{2+} treatment. Thus, the increased permeability, observed at relatively low Cd^{2+} concentrations, is most likely attributable to increased paracellular permeability.

In our view several adverse effects can lead to reduced junctional length or reduced junctional density in confocal images. Obviously, effects as cell lysis, junctional protein breakdown or changes in the epitope would result in decreased pixel density and/or number. But even more subtle effects as a reduced connection to the cytoskeleton may result in decreased pixel density and/or number due to the permeabilization step with Triton-X100 in the labeling procedure. One reason for the reduction in pixel density of the fluorescent signal of tight junctional proteins after exposure to Cd^{2+} was already proposed by Hazen-Martin *et al.* (1993). They found that the sealing tight junctional strands of human proximal tubule cells were overall intact after Cd^{2+} exposure, however, the distance between the strands was increased, i.e. the junctional strands were distributed over a larger area of the lateral membrane. Such an increase in junctional width would lead to a decrease in intensity in a confocal image as observed in the present study.

The early effects of low concentrations of Cd^{2+} were detected as altered junctional patterns in localized patches of cells. At higher concentrations these effects became more general throughout the monolayer, whereas after prolonged incubation to the lower concentrations the effects remain more localized. Detection of the localized early effects can be considered as one of the major advantages of the integration of immunolabeling and fluorescence microscopy compared with electron microscopy or light microscopy using cross sections. Within a reasonable amount of time and effort the total area of monolayer of cells can be screened for irregularities. This may be the reason why we were able to detect morphological changes in the tight junctions whereas Rossi *et al.* (1996), and Niewenhuis *et al.* (1997) did not detect changes in zonula occludens by using electron microscopy. Prozialeck and Niewenhuis (1991) did detect a decrease in membrane associated e-cadherin in LLC-PK_1 cells by using immunolabeling and fluorescence microscopy. In Caco-2 cells the immunofluorescent pattern of e-cadherin is somewhat vague or disorganized in patches (clones) of cells in the control monolayers. It was, therefore, difficult to determine early effects on basis of changes in the e-cadherin pattern in the Caco-2 cells. However, all exposures which caused a decrease of junctional ZO-1 also led to a decrease in membrane

associated e-cadherin.

Despite the differences between the responses of IEC-18 and Caco-2 cells to Cd^{2+} exposure with respect to paracellular permeability and cytotoxicity, in both cell lines the effects following the "4 hr Cd^{2+} exposure + 14 hr culture" scenario, resemble the effects of a 14 hr incubation more than the effects directly after 4 hr incubation. This shows that the effects of Cd^{2+} develop rather slowly and that the cells are unable to immediately start recovery after removal of extracellular Cd^{2+}. The lack of recovery can be explained by the high cellular Cd^{2+} retention after termination of exposure. The fact that cellular Cd^{2+} retention in Caco-2 cells leads to selective effects on the barrier function might indicate that junction-related molecules are involved in Cd^{2+} retention. The inability of the known Cd^{2+}-binding compound gluthatione to reduce these effects in other epithelial cell lines (Prozialeck and Lamar, 1995) suggests that these junction-related molecules have a high affinity for Cd^{2+}. The binding of Cd^{2+} with a polypeptide analog of a Ca-binding side of e-cadherin was studied by Prozialeck *et al.* (1996), but the binding of Cd^{2+} seemed to have a low affinity (Kd ~ 20 μM). Furthermore, best known Cd^{2+} binding polypeptides have a high affinity due to their cysteine groups, making it reasonable to assume a cysteine-rich peptide to be an early target of Cd^{2+} toxicity. Although ZO-1 and occludin are as a whole poor in cysteine residues, ZO-1 and occludin are possible targets since ZO-1 has a cysteine cluster at the c-terminus region (Itoh *et al.*, 1993) and occludin has, among others, 2 cysteine-groups in one of the extracellular loops (Mitic and Anderson, 1998). The involvement of junctional proteins in Cd^{2+} binding may serve as protective mechanism in vivo. Conformational changes in these proteins as a result Cd^{2+}-binding could reduce cell adhesion and thereby facilitate exfoliation of Cd^{2+} containing cells into the lumen. More studies will be necessary to elucidate the relation between the cellular Cd^{2+} retention and the selective early junctional effects.

In this study we have shown that Cd^{2+} exposure to the apical side of intestinal epithelial cells in vitro compromises the epithelial barrier function. The decreased barrier functionality is characterized by a decrease in TER, an increased permeability for mannitol, PEG-4000 and Cd^{2+}, a disorganization of the honeycomb-like pattern of the tight junctional proteins ZO-1 and occludin, a loss of adherens junctional e-cadherin, and the lack of membrane damage. These changes in cell layer integrity and permeability are being considered as an initial effect and appeared to be a general phenomenon of acute Cd^{2+} dosing, as it was found for the vascular endothelium, Sertoli cell monolayers, several epithelial kidney cell lines and intestinal epithelial cells. To the best of our knowledge we are the first to show that the decreased epithelial barrier function is accompanied by tight junction disruption. We conclude that Cd^{2+} causes increased paracellular permeability and that adverse effects on the junctional complexes are early effects of Cd^{2+} on intestinal epithelium *in vitro*.

Section IV

Summarizing Discussion and Conclusion

Chapter 8

Summary and concluding remarks

The studies described in Section II of this thesis were aimed at determining the "pros and cons" of filter-grown small intestinal IEC-18 cells, relative to the colonic Caco-2 cells, as model for small intestinal permeability. In Section III both cell types were used to study the effects of barrier perturbants on the intestinal epithelium and a set of parameters was composed allowing the assessment of the most likely mechanism of permeation enhancement. In this chapter, the Section II and III are summarized, some overall conclusions are drawn and the implications of these conclusions are presented.

§ 8.1 Transepithelial permeability of the filter-grown Caco-2 and IEC-18 cells.

In **Section II** we characterized growth, morphology, differentiation, absorption and barrier functions of the cell lines Caco-2 and IEC-18. In the studies described in **Chapter 3** we found that Caco-2 cells formed a monolayer of polarized epithelial cells with brush borders with sucrase-isomaltase and intestinal alkaline phosphatase activity, an obvious peripheral F-actin belt and a cell circumscribing pattern of the tight junctional protein ZO-1. The IEC-18 cells grew into several layers of cells, did not express sucrase isomaltase activity and alkaline phosphatase activity was present as the fetal isoform. In these cells the F-actin was not organized into a peripheral actin belt, but the tight junctional protein ZO-1 was organized into virtually cell circumscribing strands. In addition to the morphological differences, both cell lines differed significantly in the TER they developed: the Caco-2 cells obtained a TER of 350±14 Ω*cm^2 while the TER of IEC-18 cells was 55±4 Ω*cm^2. This marked difference was also reflected by the permeability rates of the paracellular permeation of all hydrophilic compounds (mannitol, fluorescein, FD4, PEG-4000, FD20, FD70 and FD500), which were low in the Caco-2 monolayers, but higher and clearly size specific in IEC-18 cell layers. The permeability rates of the transcellular permeation of lipophilic compounds (salicylic acid, acetyl salicylic acid, propranolol and testosterone) were, on the other hand, high and comparable in both cell lines.

In **Chapter 4** we examined the activity of several intestinal transporters in the two cell lines. In order to determine H^+-coupled di/tripeptide carrier activity we have examined the pH-dependent bidirectional transport of glycylsarcosine (GlySar). Transport was enhanced by a proton-gradient, it was faster than the permeation of mannitol which is approximately the same size, and transport in the absorptive direction (from apical to basolateral) was higher than transport in the secretive direction in Caco-2, but not in IEC-18 cells. We concluded that the H^+-coupled di/tripeptide carrier was present in Caco-2 cells but not in IEC-18 cells. The activity of glucose carriers was studied by the bidirectional transport of *O*-methyl-D-glucose, this activity again, was found in Caco-2 but not in IEC-18 cells. Next to these two absorptive activities, we also studied the activity of two secretive systems (P-glycoprotein and MRP/cMOAT). The preferential transport in the secretive direction of vincristine and rhodamine 123, and the fact that this could be inhibited by verapamil, revealed P-glycoprotein (P-gp) activity in Caco-2 cells. These experiments gave no indication for the expression of P-gp in IEC-18 cells. However, both, IEC-18 and Caco-2, cells showed probenecid-sensitive transport of the organic anions calcein and DNPSG, across the apical and basolateral membranes, indicating the activity of MRP/cMOAT in both cell lines at both cell sides. The presence of an H^+-coupled di/tripeptide carrier, glucose carriers and P-glycoprotein in Caco-2 cells confirms the enterocytic differentiation of these cells. Likewise, the lack of these activities in the IEC-18 cells is in agreement with their, crypt-like, and thus low state of

differentiation. The expression of MRP/cMOAT mediated efflux activity in the human small intestine is still unknown. Perhaps the expression in the crypt (IEC-18) and villus-like (Caco-2) intestinal epithelial cells, as shown in **Chapter 4**, is indicative for the expression of MRP/cMOAT along the intestinal crypt-villus axis *in vitro*. However, more studies will be needed to assess the relation between intestinal epithelial cell differentiation and the exact intestinal expression of this efflux system.

In conclusion, experiments with the filter-grown Caco-2 cells yielded discriminative permeation rates for the transcellular permeation of lipophilic, and actively transported (nutritional) hydrophilic compounds on the one hand, and paracellular permeation of hydrophilic (non-nutritional or) macromolecular compounds on the other hand (Figure 8.1).

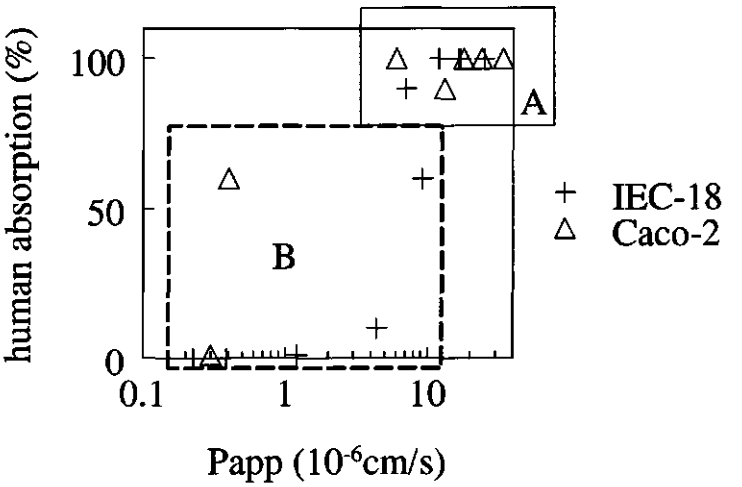

Figure 8.1: Human absorption data for several drugs and model compounds as a function of the apparent permeability coefficients obtained in the Caco-2 or the IEC-18 system.
A: Transcellularly permeating compounds; high P_{apps} and high absortion due to lipophylicity or active transport.
B: Paracellularly permeating compounds; low or moderate absorption and variable P_{apps}.

In general, this is in good agreement with human absorption data and Caco-2 monolayers are considered an appropriate model to study the intestinal permeation rates for many different classes of compounds (Artursson and Karlsson 1991; Artursson *et al.*, 1996; and chapter 3 and 4 of this thesis). In practice, it is suggested to divide human oral absorption levels in 3 groups (Yee, 1997), for example as follows:

a) less than 5 or 10% absorption correlates with Papps < 0.1 $*10^{-6}$ cm/s,

b) 5-70% absorption for 0.1$*10^{-6}$ cm/s < Papp < 1 $*10^{-6}$ cm/s, and

c) more than 70% absorption for Papp > 1 $*10^{-6}$ cm/s.

However, an important drawback of the Caco-2 system is the improper ratio between the different transepithelial permeation routes. As shown in **Chapter 3 and 4**, the paracellular permeability is relatively low in comparison with the passive transcellular permeability for lipophilic molecules. Additionally, the expression of the active absorptive systems (glucose, di/tripeptide transporters) is low compared to the small intestinal epithelium *in vivo* (Hidalgo and Li, 1996; Chong *et al.*, 1996), while the expression of the MDR1 gene product P-gp is relatively high, probably due to the fact that the cell line is colonic carcinoma derived (Gan and Thacker, 1997; Yee, 1997). This implies that for different pathways (i.e. classes of compounds) different cut-off values need to be assessed in the Caco-2 model. Furthermore, due to the heterogeneity of the Caco-2 cell line there will be considerable inter-laboratory variations in permeability rates and the suitable cut-off values (Gres *et al.*, 1998).

Whereas, the Caco-2 cells present a good model for a general screen on intestinal permeation for a wide variety of compounds, the IEC-18 cells did allow for a better discrimination between the hydrophilic compounds on the basis of their molecular weight (Figure 8.2). Consequently, experiments with the filter-grown IEC-18 cells yielded discriminative permeation rates for the paracellular permeation of hydrophilic compounds with differing bioavailabilities (Figure 8.3). Thus, data presented in this thesis suggest that the filter-grown IEC-18 cells could be valuable as model for paracellular permeability in the small intestine, but more data will be necessary to verify this hypothesis.

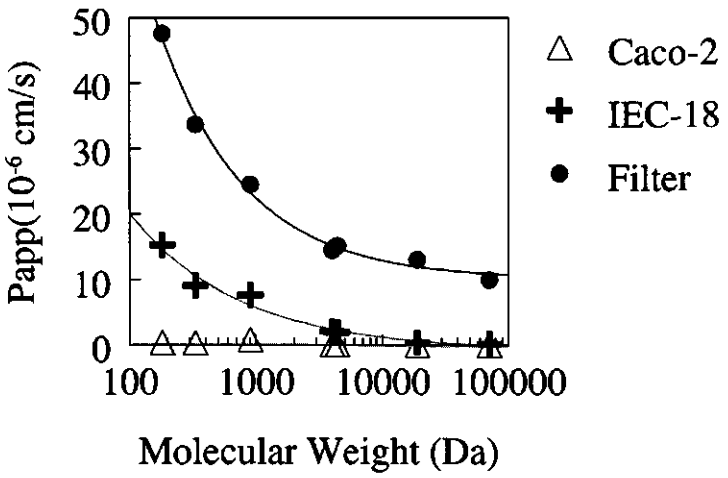

Figure 8.2: Apparant permeability coefficients of hydrophilic model compounds as a function of their molecular weight. Permeation was determined in the apical to basal direction across Caco-2 or IEC-18 cells, or across empty filters. The IEC-18 system forms a size-dependend barrier.

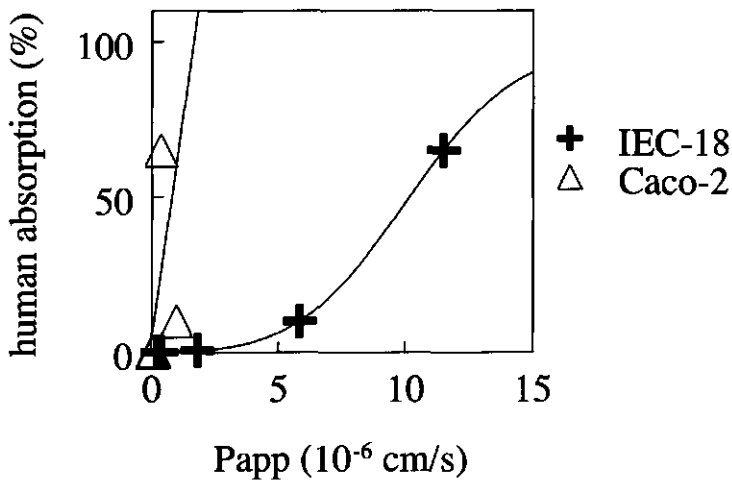

Figure 8.3: Human absorption data of hydrophilic model compounds as a function of their apparent permeability coefficients. The IEC-18 system allows for a more accurate prediction of human absorption rates than the Caco-2 system does.

The combined results concerning IEC-18 cells as presented in chapter 3 and 4 indicate that these cell are very primitive with respect to their differentiation status. Especially the fact that they did not form a monolayer but instead grew into several cell layers, indicates that the cells do have a high proliferative potential which is a characteristic of the stem cells, positioned low in the intestinal crypts. The availability of the lower parts of the intestinal crypts for luminal contents is low, and it might thus be argued that more detailed studies to transport processes with these undifferentiated crypt cells, are of little physiological importance. Therefore, several experiments were performed to try to differentiate these cells in order to establish a cell model with villus-epithelium characteristics. In **Chapter 5** we describe the differentiation of the ileal crypt cell line IEC-18 by exposure to dexamethasone, and correlated cell proliferation and differentiation to paracellular barrier development. Addition of dexamethasone to the standard culture medium resulted in the formation of a monolayer of morphologically epithelial-like cells. In these cells the F-actin was organized in a peripheral actin-belt, and the localization of the tight junctional protein ZO-1 was more strand-like than in non-dexamethasone exposed cell layers. The morphological maturation of the IEC-18 cells co-occurred with an increase in paracellular barrier function for electrolytes (increase in TER) and hydrophilic macromolecules. Inhibition of cell proliferation, formation of a peri-junctional F-actin belt and increased tight junctional sealing as induced by dexamethasone in IEC-18 cells, are all natural events reported to occur in the enterocytes in

the small intestine along the crypt-villus axis. It might thus be concluded that the IEC-18 cells can be induced to form a small intestinal villus-like phenotype when cultured in the presence of dexamethasone. These cells might therefore present an *in vitro* model to study the relation between intestinal epithelial cell differentiation and paracellular barrier development along the crypt-villus axis. We were, however, unable to detect sucrase isomaltase or intestinal alkaline phosphatase activity. The activity of fetal ALP, however, was reduced, which might be an early sign of the cells transition to a more differentiated status. Therefore, additional studies are required to determine the exact differentiation status of the dexamethasone treated IEC-18 cells and to assess the value of the more villus epithelial-like monolayers as *in vitro* model for the small intestinal epithelium. It should also be mentioned that the differentiation process evoked in the IEC-18 cells by dexamethasone, might be representative for the intestinal closure known to occur during the suckling-weaning transition in rodents. During that stage the permeability of the small intestinal epithelium for macromolecules is greatly reduced. This reduction in permeability is the result of a diminished transcytotic capacity of the epithelial cells, and of a tightening of the paracellular pathway, probably by a maturation of the junctional complexes. An *in vitro* model for the study of the changes in permeability during the intestinal closure would be of considerable interest since a vast amount of data is available on brush border enzyme activity development, but only limited information is available on the development of the barrier functions (Henning *et al.*, 1994).

§ 8.2 Modulation of the intestinal epithelial barrier: The relation between increased permeability and toxicity in cell culture systems.

In **Section III** we have studied the effects of several compounds on the epithelial barrier function of filter-grown intestinal epithelial cells, with emphasis on the structure and function of the paracellular barrier. These experiments are also used to explore combined determinations of epithelial permeability (TER and hydrophilic macromolecular permeation), membrane damage and cell viability (LDH leakage and PI or NR uptake) and tight junction structure (immunolocalization of tight junctional proteins) to determine the mechanism of permeation enhancement (i.e. to discriminate between paracellular and transcellular). Thereto, we determined the sequence of events during and after the exposure of filter-grown cells to PCC, Triton X-100, EGTA (**Chapter 6**), and cadmium (**Chapter 7**), by applying the following general protocol:

1/ Measurement of the TER prior to exposure
2/ Start of (apical) exposure
3/ End of exposure: Measurement of TER and LDH leakage
4/ Start of 4 h transport experiment or fixation for immunolocalization of junctional proteins (for PCC and cadmium only)
5/ End of transport experiment: Measurement of neutral red uptake or medium refreshment for the determination of recovery of cell viability (i.e. assessment of the reversibility of the cytotoxic effects).

The disruption of the epithelial barrier, measured as enhancement factor for PEG-4000 permeation, will be evaluated with the other parameters in order to determine the mechanism of barrier disruption. The TER was found to be a sensitive indicator for the detection of decreased cell layer integrity. In all cases that the permeation of the hydrophilic molecules was increased the TER was decreased (Figure 8.4). However, significant decreases in TER could be measured without concomitant increase in tracer permeation at high TER values, and significant increases in PEG-4000 permeation were detected without decrease in TER at low TER values. Thus, the TER, as such, is not a good quantitative predictor for macromolecular permeability.

The immunolocalization of the tight junctional protein ZO-1 was used to indicate structural changes in the tight junctional barrier. It was hypothesized that if the permeation enhancing effect was mainly due to increasing the paracellular permeability, changes would be visible in the distribution of the tight junctional proteins. An advantage of immunofluorescence microscopy is that it allows the screening of the whole monolayer within an acceptable amount of time, making this method more convenient for the screening of

localized (clonal) effects compared to electron- or light microscopy using cross-sections. An important restriction is that the changes in the tight junction structure might be so subtle that they can not be visualized by this method. Furthermore, the high background of non-junctional ZO-1 in control cell layers of IEC-18 makes it difficult to characterize early changes in the junctional pattern. Nonetheless, we were able to detect changes in ZO-1 localization after cadmium, and after PCC treatment in both cell lines. It was also found that all detectable changes in the ZO-1 pattern started as localized disruptions which became more omnipresent at higher concentrations of cadmium or PCC.

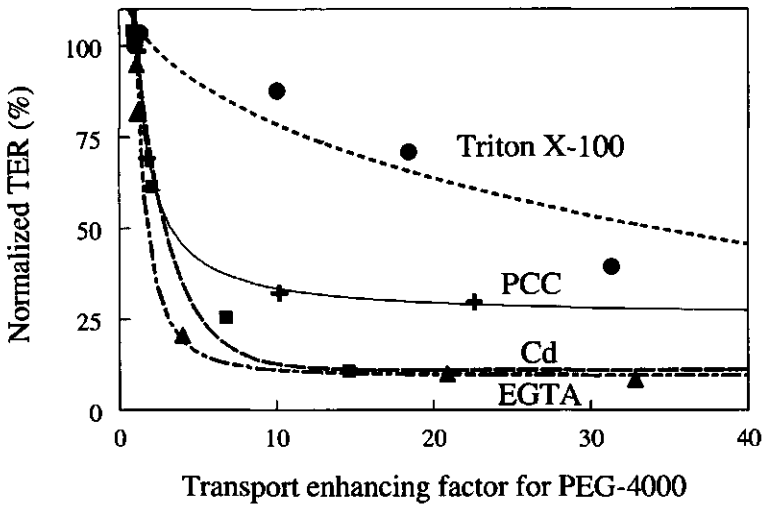

Figure 8.4: The TER as a function of the increased permeation of PEG-4000 across Caco-2 monolayers after exposure to PCC, Triton X-100, Cd or EGTA (Chapter 6 and 7).

In order to quantify the changes in the ZO-1 pattern, we have developed a method with which we were able to determine pixel number and intensity as a measure for the immunolocalization of this tight junctional protein. It was found that very local disruptions could not be quantified by this method. However, as soon as the effects on ZO-1 pattern became more generalized, changes in the pixel density and pixel number could be quantified. It appeared that in Caco-2 cells, PCC (**Chapter 6**) caused a dose-dependent decrease in junctional pixel number (junctional length) without having an effect on junctional pixel

density, while cadmium (**Chapter 7**) caused a dose-dependent decrease in both, pixel number and density after 4 h exposure. These findings could indicate that different mechanisms are involved in the barrier disruption by PCC or cadmium. Since both, pixel number and density, are affected by cadmium, it is likely that the effects of cadmium are more selective on the tight junctions. Since a functional reduction of barrier integrity (i.e. increased macromolecular permeation) could be detected prior to morphometric changes in the junctional network, it must be concluded that the quantification of ZO-1 immunolocalization was not a very sensitive method for detecting localized disruptions of the junctional pattern. Nevertheless, with the morphometric analysis using the custom written image processing program, generalized changes in pixel density could be determined while this is very hard to do with CLSM images only.

To assess membrane damage, the LDH leakage into the apical compartment, i.e. at the side of exposure, was determined. LDH leakage was found to be a sensitive indicator for membrane damage. Since LDH leakage is such a direct measure of increased (apical) membrane permeability (or cell lysis), and the surface area of the cell membranes is at least 1000 times larger that the paracellular surface area, we assume a correlation between LDH leakage and transcellular permeation enhancement. In **Chapter 6** we describe that after PCC exposure, increased LDH leakage preceded the permeation enhancement in both cell lines. Cadmium exposure (**Chapter 7**), on the other hand, induced no LDH leakage in Caco-2 cells at all, thus there was no relation between membrane damage and permeation enhancement found (Figure 8.5). In IEC-18 cells cadmium induced a dose-dependent increase in LDH leakage, however, after short term exposure the increase in transepithelial permeability was more pronounced, and occurred prior to, the small increases in LDH leakage. The PI uptake assay as measure for membrane damage proved very useful to detect cell lysis when used in combination with ZO-1 immunolocalization. The combination of those two stains showed that the decrease in junctional pixel number after PCC exposure was mainly due to the lysis of whole cells and not to a specific effect on the junctions.

In order to assess cell viability after the transport experiment, a quantitative neutral red uptake assay was performed. In Caco-2 cells it was found that after exposure to PCC (**Chapter 6**) or cadmium (**Chapter 7**) the NR uptake correlated well with the LDH leakage, although after exposure to high levels of cadmium the NR uptake was slightly reduced even when no LDH leakage was found. This might indicate that one of the cytotoxic effects of cadmium involves the functionality of the lysosomes (i.e. sequestration of NR). In IEC-18 cells, effects on NR uptake were in general more severe than effects on LDH leakage. Based on the fact that NR uptake was determined after 4 h recovery (4 h transport), whereas the LDH leakage was determined directly at the end of exposure, it can be concluded that no recovery of cell viability during the 4 h transport took place. When the cells were allowed to recover

from PCC exposure for 22 h, some recovery of TER and cell viability was found, indicating that the cell layers were able to partly restore the barrier integrity.

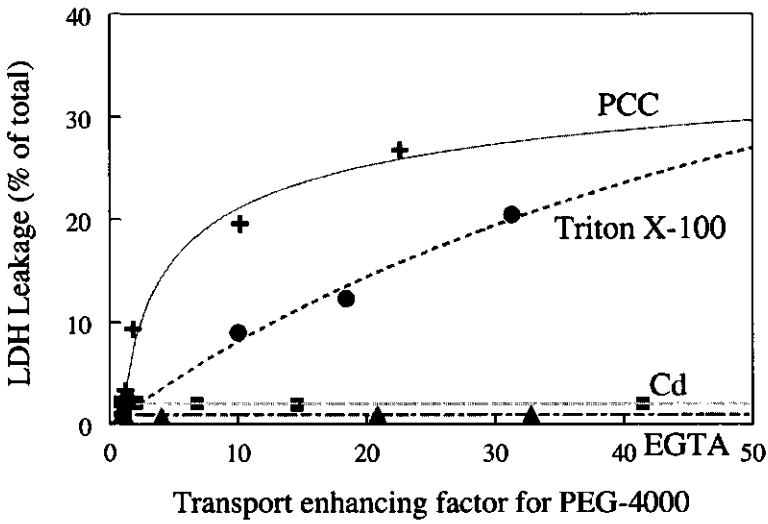

Figure 8.5: The LDH leakage (membrane damage) as a function of the increased permeation of PEG-4000 across Caco-2 monolayers after exposure to PCC, Triton X-100, Cd or EGTA (Chapter 6 en 7).

In summary, it was found that the sequence of events after palmitoyl carnitine exposure (**Chapter 6**) was first membrane damage (increased LDH leakage), followed by reduced barrier function (increased hydrophilic macromolecular permeability and decreased TER) and decreased junctional ZO-1 expression. After cadmium exposure (**Chapter 7**) the barrier function was decreased concomitantly with decreased junctional ZO-1 expression but without LDH leakage. Chapter 6 and 7 illustrate that a combination of several parameters was needed to elucidate the mechanism of permeation enhancement (i.e. increased permeation of tracers as mannitol and PEG-4000) and to assess epithelial (cell) damage. For example, the TER was a sensitive indicator for the detection of decreased cell layer integrity but not a good indicator to determine the mode of action of the enhancers. Figure 8.4 and 8.5 show that a decreased TER was indeed found when the permeability was increased without concomitant membrane damage (EGTA and Cd), but also when the permeability was enhanced with concomitant membrane damage (Triton X-100 and PCC). Thus, hydrophilic macromolecular permeability and TER are, under physiological conditions, indicators of paracellular permeability, however, in case of increased membrane permeability, these parameters can

indicate increased transcellular permeability as well. Furthermore, when the integrity of the tight junctional network, as assessed by the ZO-1 immunolocalization, was affected, macromolecular permeation was increased. However, macromolecular permeation could be increased without detection of effects on ZO-1 distribution. Thus, an disturbed junctional ZO-1 localization is an indicator for enhanced macromolecule permeabilty, but it is not a prerequisite.

In conclusion, the occurrence of increased hydrophilic macromolecular permeability concomitant with LDH leakage but without alterations in junctional ZO-1 expression as early effect, strongly suggests the enhancement of the transcellular pathway. On the other hand, increased hydrophilic macromolecular permeability and a decreased TER, concomitant with alterations in junctional ZO-1 expression but without LDH leakage strongly suggests the enhancement of the paracellular pathway.

With respect to studies on the modulation of intestinal epithelial barrier function, the Caco-2 proves to be a more convenient model than the IEC-18 cells. The most important reason is the high basal barrier function for hydrophilic molecules of the Caco-2 cells relative to the low barrier function of IEC-18 cells. In IEC-18 cells it proved difficult to measure TER accurately and the window for decreases in TER or increases in permeability rates were much more narrow than in Caco-2 cells. The IEC-18 cells were also found to be somewhat more sensitive to cell damage than Caco-2 cells, and both cell lines are probably more sensitive than the intestinal epithelium *in vivo*. The higher sensitivity may be due to the lack of a mucus layer in cultures of these two cell lines. Attempts to tackle this problem by the co-culture of enterocyte-like and mucus secreting Goblet-like cells, met with limited success (Wikman-Larhed and Artursson, 1995). Additionally, it must be noted that Caco-2 cells are adenoma carcinoma derived and some of the cell signaling pathways, involved in for example tight junction regulation, may not be operative in these cells. Other cell lines such as HT-29 or T84 or perhaps, differentiated IEC-18 cells, will prove to be more appropriate for studies to physiological barrier integrity regulation.

§ 8.3 Conclusions and Perspectives

From the work described in this thesis the following conclusions are drawn:

- *In vitro* permeation rates obtained with filter-grown IEC-18 cells allowed for a better discrimination between hydrophilic compounds, which differed in their molecular size, than the rates obtained in the Caco-2 system did. Consequently, filter-grown IEC-18 cells can be valuable as model to assess small intestinal paracellular permeability.

- IEC-18 cells can be induced to acquire a more villus-like phenotype when cultured in the presence of dexamethasone. After the transition from flat, stapling cells, to cuboidal cells forming a monolayer, the cells had achieved a higher organization of junction-related proteins (F-actin and ZO-1). Moreover, the monolayer of cells presented an increase in paracellular barrier function for electrolytes (increase in TER) and hydrophilic macromolecules. The IEC-18 cells might thus present an *in vitro* model to study the relation between intestinal epithelial cell differentiation and paracellular barrier development along he crypt-villus axis.

- The intestinal Caco-2 and IEC-18 cells in the two compartment system present a valuable model to screen for effects of ingested compounds on intestinal epithelial barrier integrity. Due to their high basal barrier function, the Caco-2 cells present a more convenient model to assess enhancement of epithelial permeability than the IEC-18 cells.

- Increased hydrophilic macromolecular permeability concomitant with LDH leakage but without alterations in junctional ZO-1 expression (as early effect), is indicative for transcellular permeation enhancement. Increased hydrophilic macromolecular permeability and a decreased TER, concomitant with alterations in junctional ZO-1 expression but without LDH leakage, is indicative for paracellular permeation enhancement.

With the evaluation of filter-grown IEC-18 cells as a model to study transepithelial permeability of hydrophilic macromolecules, we show that "paracellular permeation prognosis beyond Caco-2" is possible. The IEC-18 cells, cultured under standard conditions or in the presence of dexamethasone, might offer the possibility to close one of the gaps left open by the Caco-2 system. The Caco-2 cells present a good first screen to distinguish between paracellular or transcellular permeation and to determine permeation rates for the transcellular permeating compounds. Permeation experiments with IEC-18 cells should allow for the assessment of *in vitro* permeation rates which can be used to predict oral bioavailability, of

hydrophilic compounds which depend on paracellular permeation. These two intestinal cell lines together, offer the possibility to obtain permeation rates enabling bioavailability modeling for virtually all classes of compounds. Without doubt it would be much more convenient if permeability rates for all compounds could be assessed with one cell culture model. Perhaps the IEC-18 cells cultured in the presence of dexamethasone present the basis for that model.

Furthermore, the filter-grown intestinal epithelial cell lines can be used to determine adverse effects of, for example, drug absorption enhancers, food contaminants or nutraceuticals on the intestinal epithelium. Since this system offers the possibility to assess several parameters, such as TER, LDH leakage, and macromolecular permeation or junctional protein localization within one experiment, it allows for the determination of the most likely mechanism of barrier disruption. Additionally, the fact that it is now possible to elucidate the ratio between the efficacy and the toxicity may advance the search for a save drug permeation enhancer. Since the co-administration of absorption enhancers has met with little success, due to the surfactant-like lytic properties, most recently much attention has been paid to other, maybe more selective strategies, such as receptor-mediated transcytosis for macromolecules (Swaan, 1998) and the development of peptidomimetric drugs which can be substrates for the di/tripeptide transporters (Eddy *et al.*, 1995). Nevertheless, the search for drug absorption enhancers with a specific effect on the paracellular permeation is still continuing, and some promisfull compounds (e.g. zonula occludens toxin and chitosans) are still under investigation. The *in vitro* systems and research strategies presented in this thesis might help to elucidate the modes of action of these, and other compounds, in the future.

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Samenvatting

Doorlaatbaarheid en beïnvloeding van het darmepitheel *in vitro*

Inleiding

Niet alle stoffen die we via de mond binnen krijgen, worden in gelijke mate opgenomen. De hoeveelheid van een stof die wordt opgenomen wordt voor een belangrijk deel bepaald door de snelheid waarmee die stoffen de darmwand kunnen passeren en deze darmwand bestaat uit verschillende lagen weefsel, waarvan het epitheel de belangrijkste is. Het epitheel is slechts één cellaag dik en vormt de bekleding van de darm en dus de eigenlijke afscheiding tussen de inhoud van de darm (het darm lumen), en de systemische bloedsomloop (het inwendige lichaam). Het epitheel beschikt over allerlei transport-systemen die zorgen voor de actieve opname van voedingstoffen, zouten en water, maar het epitheel verhindert ook de opname van giftige stoffen en voorkomt het binnendringen van virussen en bacteriën. Het is dus duidelijk dat inzicht in de eigenschappen die de permeabiliteit van het darm epitheel bepalen, belangrijk is om in te kunnen schatten wat de actuele opname (de bio-beschikbaarheid) van een voedingsmiddel, een medicijn, een voedingsupplement of juist een giftige stof, zal kunnen zijn. In de darm en het lichaam van vrijwilligers en proefdieren spelen echter zoveel complexe processen door elkaar, dat het moeilijk is om op die manier, dus *in vivo*, aan voldoende nauwkeurige gegevens te komen. Daarbij worden tegenwoordig zoveel medicijnen en andere chemische producten ontwikkeld dat het ondoenlijk en on-ethisch is deze allemaal *in vivo* te testen op hun bio-beschikbaarheid.

Om deze redenen is een eenvoudig, goedkoop, nauwkeurig en reproduceerbaar model nodig die het mogelijk maakt om *in vitro* de permeabiliteit van het darm epitheel voor die stof te bepalen. In de experimenten beschreven in dit proefschrift wordt een *in vitro* systeem gebruikt waarbij darm epitheel cellen worden gekweekt op een filter dat in een kweekbakje hangt. Zodra de cellen tegen elkaar aangroeien, een confluente cellaag vormen, hechten ze aan elkaar en vormen ze een barrière waardoor ze het systeem verdelen in twee compartimenten. Als, zoals hier het geval is, gebruik wordt gemaakt van darm epitheel cellen wordt het bovenste compartiment, de apicale of luminale zijde genoemd en de het onderste compartiment de basolaterale of serosale zijde. Dit twee-compartimenten systeem maakt het mogelijk om de passage van allerlei stoffen door de epitheliale cellaag te bepalen. Deze trans-epitheliale passage kan plaatsvinden langs twee verschillende wegen, dwars door de cel heen, de transcellulaire route, of tussen de cellen door, de paracellulaire route.

In hoeverre de in het twee-compartimenten *in vitro* systeem vastgestelde

transepitheliale passage van een bepaalde stof een goede maat is voor de permeabiliteit van die stof in de menselijke darm *in vivo*, wordt dan ook in belangrijke mate bepaald door het cel type dat gebruikt wordt. Een inmiddels bekende en veel gebruikte cel is de Caco-2, deze epitheliale cel is oorspronkelijk afkomstig van een humane dikke darmkanker, maar in een kweekbakje en in het twee-compartimenten systeem groeien de cellen uit tot een cellaag die veel lijkt op het dunne darm epitheel. Aangezien het epitheel van de dunne darm verantwoordelijk is voor het belangrijkste deel van de opname van voedingsstoffen, lijkt het Caco-2 systeem een goed model voor de bestudering van de intestinale absorptie. Voor heel veel stoffen is de snelheid van passage door de Caco-2 cellaag bepaald en voor veel van deze stoffen is de passage snelheid een goede maat voor de bio-beschikbaarheid van die stof. Echter, de passage van de grotere wateroplosbare stoffen (hydrofiele macromoleculen zoals eiwitten en sommige medicijnen) is relatief te langzaam in het Caco-2 systeem vergeleken met *in vivo* waarden. Deze hydrofiele stoffen worden niet actief opgenomen en kunnen ook niet passief door de cel heen, dus voor opname zijn deze stoffen afhankelijk van de paracellulaire route. Dit is een route die niet door de cel(membraan) heen gaat maar langs de cellen, dus tussen de cellen door. De barrière in de paracellulaire route, die vooral bepaald word door de tight junctions, is te groot in de Caco-2 cel laag en daardoor is de paracellulaire permeabiliteit te laag. Dit komt doordat de structuur van deze tight junctions in de Caco-2 cellen meer lijkt op de structuur van de tight junctions in dikke darm cellen dan op die van de tight junctions van dunne darm cellen. Aangezien de bio-beschikbaarheid van de hydrofiele macromoleculen vooral bepaald wordt door de permeabiliteit van de dunne darm is voor deze stoffen de Caco-2 niet de meest geschikte cel. Daarom was een van de doelen van het hier beschreven onderzoek het opzetten en evalueren van een *in vitro* model voor de transepitheliale permeabiliteit van de dunne darm. Hierbij werd extra aandacht besteed aan de permeabiliteit van de hydrofiele macromoleculen. In het hier beschreven onderzoek is gebruik gemaakt van een epitheliale cellijn uit de dunne darm van een rat, de IEC-18 cellijn. In de **hoofdstukken 3 en 4** zijn verschillende, aan de permeabiliteit gerelateerde karakteristieken van Caco-2 en IEC-18 cellen in het twee-compartimenten systeem met elkaar vergeleken. In **hoofdstuk 3** ligt daarbij de nadruk op de ontwikkeling en groei van de cellen en de passieve diffusie van verschillende modelstoffen over de gekweekte epitheellagen. Tevens wordt met behulp van immunocytochemische technieken de intracellulaire lokalisatie van het structurele tight junction eiwit ZO-1 bestudeerd. In **hoofdstuk 4** worden enkele actieve transport systemen voor bestudeerd waarvan er twee van belang zijn voor de opname van voedingsstoffen (absorptie functie van de darm) en twee juist van belang voor de uitscheiding of beperking van de opname van mogelijk schadelijke stoffen (barriere functie van de darm).

Naast het verschil in permeabiliteit tussen de dikke en de dunne darm zijn er ook binnen de dunne darm langs de crypt-villus as weer locale verschillen in de epitheliale permeabiliteit. In de crypten worden de cellen gevormd door celdeling, de cellen verplaatsen

zich richting de villus en aan de top van de villus laten de cellen los en worden afgevoerd met de bolus (de darm inhoud). Tijdens de verplaatsing van de crypte naar de villus rijpen de cellen, ze krijgen steeds meer eigenschappen die van belang zijn om de uiteindelijke taak van de volwassen (gedifferentieerde) darmcellen, de opname van voedingsstoffen en het tegenhouden van schadelijke stoffen, uit te kunnen voeren. Door het rijpen (differentiëren) van de cellen langs de crypt-villus as, is ook de permeabiliteit van het epitheel in de crypten anders dan de permeabiliteit van het epitheel aan de villi (zie ook figuur 2.2). het is echter nog niet duidelijk hoe de relatie tussen de differentiatie van de darmcellen en de paracellulaire permeabiliteit van het epitheel is. Daarom zijn we vervolgens in **hoofdstuk 5** nagegaan in hoeverre de IEC-18 cellen kunnen worden aangezet zich te differentiëren tot volgroeide dunne darm cellen en hoe tijdens dat proces de permeabiliteit van de cellaag veranderd.

Zoals in de vorige alinea's is beschreven zijn er verschillen in de permeabiliteit van het darmepitheel tussen de verschillende regio's van de darm en langs de crypt-villus as in de dunne darm. De permeabiliteit kan echter ook beïnvloed worden door stoffen die we via de mond kunnen binnenkrijgen zoals sommige voedsel-componenten, gifstoffen of (additieven van) medicijnen. De meeste stoffen waarvan bekend is hoe ze de permeabiliteit van de darm verhogen blijken dat te doen vanwege de oppervlakte-spanning verlagende eigenschappen van die stoffen. Doordat ze geheel of gedeeltelijk de celmembranen oplossen, zorgen ze voor een verlaging van de transcellulaire barrière en dus voor een verhoging van de passieve transcellulaire diffusie van hydrofiele macromoleculen. Er zijn echter ook stoffen die juist de paracellulaire barrière, de tight junctions, verstoren en dus zorgen voor een verhoogde permeabiliteit van de paracellulaire route. Het is recentelijk ontdekt dat de tight junctions fysiologisch gereguleerd worden en dat het dus wellicht mogelijk is om de permeabiliteit van de darm te verhogen zonder de cellen zelf ernstig te beschadigen of op te lossen. Deze manier van de permeabiliteit verhogen wordt gezien als goede methode om de opname van medicijnen die normaal slecht worden opgenomen, te verhogen. Het blijkt echter tot op heden moeilijk om onderscheid te maken tussen de stoffen die de transcellulaire permeabiliteit verhogen en stoffen die de paracellulaire permeabiliteit verhogen.

Om meer inzicht te krijgen in mogelijke effecten van oraal ingenomen stoffen op het darmepitheel, wordt in de **hoofdstukken 6 en 7** nagegaan of het mogelijk is met behulp van het twee-compartimenten systeem met Caco-2 of IEC-18 cellen effecten van bijvoorbeeld transepitheliale passage-versnellers (permeatie verhogers) of voedselcontaminanten op het darmepitheel *in vitro* te bestuderen. Uit de gegevens van **hoofdstuk 6 en 7** willen we tevens bepalen welke parameters gemeten moeten worden om een uitspraak te kunnen doen of de verhoogde permeabiliteit vooral te danken is aan een verhoogde transcellulaire permeabiliteit of aan een verhoogde paracellulaire permeabiliteit.

Samenvatting van de hoofdstukken

Transepitheliale permeabiliteit van Caco-2 en IEC-18

Hoofdstuk 3
Vergelijking van de permeabiliteits-karakteristieken van Caco-2 (humane colon) en IEC-18 (rat dunne darm) cellen.

In de literatuur worden verschillende *in vitro* modellen beschreven om het transport over, en de doorlaatbaarheid van, het darmepitheel te bepalen. In een van die *in vitro* systemen, het twee-compartimenten celkweek-systeem, wordt veelvuldig gebruik gemaakt van cellen afkomstig van een colon carcinoom (Caco-2). Deze cellen lijken in veel opzichten op de absorptie-cellen (enterocyten) van de dunne darm, maar ze hebben ook verschillende eigenschappen die bij de dikke darm horen. Een belangrijk verschil tussen de dunne en de dikke darm is bijvoorbeeld de zeer geringe passieve diffusie van wateroplosbare stoffen door de paracellulaire route in de dikke darm en de daarvan afgeleide celkweek-systemen zoals de Caco-2 cellen in vergelijking met de paracellulaire diffusie in de dunne darm. Om in deze paracellulaire permeabiliteit meer inzicht te krijgen hebben we dikke (Caco-2) en dunne darm (IEC-18) cellen gekweekt in het twee-compartimenten systeem en de permeabiliteit van verschillende, water- en vetoplosbare, stoffen bestudeerd.

Tijdens langdurige kweek (2 tot 3 weken) op filters, groeien de Caco-2 cellen uit tot een monolaag van volwassen enterocyt-achtige cellen: de cellen zijn gepolariseerd en hebben een borstelzoom waarin de enzymen sucrase-isomaltase en het darm-specifieke alkalische fosfatase zijn aangetoond. Daarnaast is met immunofluorescentie aangetoond dat het F-actine (een eiwit van het cytoskelet dat betrokken is bij cel-cel contacten) is georganiseerd in een perifeer gelokaliseerde ring. Bovendien komt het tight junction eiwit ZO-1 op een cel-omgevende manier tot expressie en is het gelokaliseerd op plaatsen waar cellen onderling contact hebben. De IEC-18 cellen daarentegen, vormen tijdens langdurige kweek, meerdere cellagen op elkaar, hebben geen borstelzoom of sucrase-isomaltase activiteit maar ze vertonen wel, zij het niet darm-specifieke, alkalische fosfatase activiteit. Het F-actine in IEC-18 cellen vormt geen perifere ring maar het meeste ZO-1 is, net als in Caco-2, wel zichtbaar als een cel-omgevende ring. Naast deze structurele verschillen is er ook een duidelijk verschil in de transepitheliale elektrische weerstand (TER), de Caco-2 monolaag ontwikkeld een TER van ongeveer 350 $\Omega.cm^2$ terwijl de TER van IEC-18 cellen slechts ongeveer 55 $\Omega.cm^2$ is. Dat wil zeggen dat de permeabiliteit voor de elektrolyten van de IEC-18 cellaag overeenkomt met de permeabiliteit van de dunne darm, terwijl de permeabiliteit van de Caco-2 cellaag meer op de permeabiliteit van de dikke darm lijkt. De permeabiliteit van beide cellen voor hydrofiele macromoleculen (met molecuul-gewichten van 180 tot 500.000 Da) verschilt ook aanzienlijk.

De permeabiliteit is voor alle hydrofiele macromoleculen laag in het Caco-2 systeem, maar duidelijk hoger en afhankelijk van de grootte van het molecuul, in het IEC-18 systeem. De permeabiliteit van vetoplosbare stoffen is daarentegen hoog en vergelijkbaar in beide systemen. Het blijkt dat Caco-2 cellen, met betrekking tot opname snelheid, een goed onderscheid maken tussen vet en wateroplosbare stoffen, maar slecht discrimineren tussen de paracellulair passerende wateroplosbare stoffen onderling. De IEC-18 cellen discrimineren minder goed tussen water en vetoplosbare stoffen maar maken wel goed onderscheid tussen de wateroplosbare stoffen die verschillen in molecuul-grootte. Naar aanleiding van deze resultaten concluderen wij dat voor het modelleren van de opname van de vetoplosbare stoffen het Caco-2 systeem, geschikt is, maar dat voor het modelleren van de opname van wateroplosbare stoffen, die alleen via de paracellulaire route kunnen worden opgenomen, beter het IEC-18 systeem gebruikt kan worden.

Hoofdstuk 4

Vergelijking van transport activiteiten in Caco-2 (humane colon) en IEC-18 (rat dunne darm) cellen.

In hoofdstuk 3 is beschreven hoe de doorlaatbaarheid is van de Caco-2 en IEC-18 cellen voor stoffen die door passieve diffusie het darm epitheel passeren. Met de proeven beschreven in dit hoofdstuk wordt bestudeerd in hoeverre beide cellen beschikken over de enzymsystemen die in de darm zorgen voor de goede opname van voedingsstoffen, of er juist voor zorgen dat mogelijk schadelijke stoffen niet worden opgenomen. Als voorbeelden van de voedingsstoffen hebben we gekeken naar het transport van het dipeptide (verbinding van 2 aminozuren) Glycyl-Sarcosine (GlySar) en de suiker O-methyl-D-Glucose (Meth-Gluc). We hebben gevonden dat het enzym systeem dat zorgt voor het transport van GlySar, de proton-afhankelijke di/tripeptide carrier, wel aanwezig is in Caco-2 cellen maar niet in de IEC-18 cellen. Hetzelfde geldt voor de glucose carrier; er is wel actief transport van Meth-Gluc in het Caco-2 systeem maar niet in het IEC-18 systeem.

Als voorbeelden van de transportsystemen die betrokken zijn bij uitscheiding, of beperking van de opname vanuit de darm, van mogelijk schadelijke stoffen hebben we de activiteiten van de transport-eiwitten P-glycoproteïne en MRP2/cMOAT bestudeerd. Vincristine en rhodamine 123 zijn gebruikt als modelsubstraten waarvan bekend is dat de opname in de darm actief wordt beperkt door het P-glycoproteïne. Dit eiwit is ontdekt doordat het in sommige tumorweefsels in hoge mate voorkwam en er voor zorgde dat cytostatica (tumorgroei-remmende middelen) niet werkte doordat ze actief de tumorcel werden uitgepompt. P-glycoproteïne is ook aangetoond in de darm waar het de opname van mogelijk schadelijke stoffen kan verminderen. We vonden dit eiwit wel in Caco-2 cellen maar niet in

IEC-18 cellen. De activiteit van het andere eiwit met een mogelijk beschermende functie, het MRP2/cMOAT, is bestudeerd aan de hand van de efflux (het transport de cel uit) van de modelsubstraten calceïne en DNP-SG. Beide substraten werden in het apicale en in het basolaterale compartiment teruggevonden bij zowel Caco-2 als IEC-18 cellen. Tevens was de efflux van calceïne en DNP-SG te remmen met probenecid, een remmer van MRP2/cMOAT. We concluderen dan ook dat dit MRP2/cMOAT aanwezig is in de apicale en de basolaterale membraan in beide typen darmcellen.

Hoofdstuk 5

Dexamethason remt cel-proliferatie en verhoogd de paracellulaire barrière functie van de ratte-ileum cellijn IEC-18.

Nadat uit de resultaten van hoofdstuk 3 en 4 bleek dat de IEC-18 cellen slechts een lage differentiatie graad bereikten, is geprobeerd deze cellen aan te zetten tot differentiatie waardoor ze meer eigenschappen gaan vertonen die horen bij een darm-villus cel in plaats van bij een darm-crypte cel. Door toevoeging van 1 µM dexamethason aan het kweekmedium van de IEC-18 cellen groeiden de cellen in een mooie mono-laag over het filter terwijl de cellen over elkaar heen groeiden en stapelen, als er geen dexamethason is toegevoegd. Bovendien veranderde door dexamethason toevoeging de vorm van de cellen van vrij platte, naar kubus-achtige cellen en de activiteit van het foetale alkalische fosfatase werd onderdrukt. Tevens werd het in de cel aanwezige fibrillaire actine georganiseerd in een perifere actine ring en het tight junction eiwit ZO-1 werd zichtbaar als een echte cel-omgevende-ring in plaats van als een gefragmenteerde ketting zoals in de onbehandelde cellen. Naast al deze morfologische veranderingen, verminderde de permeabiliteit van de cellaag voor zowel de elektrolyten (lagere TER), als voor de grotere wateroplosbare moleculen. Tevens werd aangetoond dat de toegenomen barrière functie van de cellaag weer verlaagd kon worden, tot het niveau van de onbehandelde cellen, als de cellaag behandeld werd met TPA of EGTA. Omdat deze stoffen specifiek de permeabiliteit van de paracellulaire route verhogen door een effect op de tight junctions werd verondersteld dat toevoeging van dexamethason leidde tot een verhoogde functionaliteit van de tight junctions. Omdat ook in de darm waarschijnlijk de permeabiliteit van de paracellulaire route bij de villus lager is dan in de crypt, wordt gesuggereerd dat het kweken van deze IEC-18 cellen in aanwezigheid van dexamethason kan leiden tot een model waarmee de permeabiliteit van de paracellulaire route langs de crypt-villus as in de darm kan worden bestudeerd.

Effecten op de intestinale epitheliale barrière *in vitro*

In de experimenten die beschreven zijn in de hoofdstukken 6 en 7 zijn de op de filters gekweekte cellen blootgesteld aan een zogenaamde "drug absorption enhancer" (opname verhoger, hoofdstuk 6) en aan het zware metaal cadmium (Hoofdstuk 7), waarvan bekend is dat het effecten heeft op het junctional complex en de permeabiliteit van niercellagen *in vitro*. Een "drug absorption enhancer" is een stof die aan bijvoorbeeld medicijnen kan worden toegevoegd om er voor te zorgen dat de opname van het medicijn in de darm tijdelijk wordt verhoogd. Voor beide stoffen hebben we bestudeerd wat de effecten zijn op de gekweekte cellen die model staan voor het epitheel van de darm. Er werd daarbij vooral gelet op welke manier en in welke mate de barrière wordt aangetast. Hierbij werd gebruik gemaakt van functionele parameters (de TER en de permeabiliteit voor hydrofiele macromolculen), van morfologische parameters (kwalitatieve en kwantitatieve immunohistochemische localizatie van tight junction eiwitten en F-actine) en van cytotoxiciteits-testen (LDH lekkage, opname van neutral rood of propidium jodide).

Hoofdstuk 6

Verhoogde absorptie, structurele verandering in de tight junctions en cytotoxiciteit veroorzaakt door palmitoyl carnitine in Caco-2 en IEC-18 cellen.

Van palmitoyl carnitine (PCC) is bekend dat het de opname van wateroplosbare stoffen in de darm kan verhogen, dus dat het de barrière functie verlaagt. Het is echter niet bekend wat het werkingsmechanisme is waarmee de barrière functie word verminderd. Om meer inzicht te krijgen in dat werkingsmechanisme hebben we de cellen, Caco-2 en IEC-18, vanaf de darm-lumen kant blootgesteld aan PCC en bepaald wat de effecten zijn op de doorlaatbaarheid voor grote wateroplosbare moleculen, wat er vervolgens gebeurd met de viabiliteit ("gezondheid" of "levensvatbaarheid") van de cellen en wat er gebeurd met de expressie of de lokalizatie van het ZO-1 eiwit.

We hebben gevonden dat door blootstelling aan PCC de transepitheliale elektrische weerstand van de cellagen werd verlaagd en dat de doorlaatbaarheid voor grotere wateroplosbare moleculen werd verhoogd. Daarnaast bleek dat PCC de cel-membranen beschadigde. Bij hoge concentraties ($\geq$ 0.4 mM) was ook de verdeling van het ZO-1 eiwit in de cellen veranderd waardoor plaatselijk de tight junctions geen duidelijke cel-omcirkelende structuren meer waren. Voor een goede analyse van de lokalisatie van het ZO-1 eiwit hebben we een methode ontwikkeld waarmee we in staat waren om te bepalen hoeveel van het ZO-1 eiwit gerelateerd was aan het tight junction netwerk. Hiervoor werd gebruik gemaakt van met de confocale laser scanning microscoop gemaakte opnamen en een speciaal ontwikkeld

beeldverwerkings-programma. Hieruit bleek dat bij de hogere concentraties PCC de lengte van het tight junction gerelateerd ZO-1 netwerk inderdaad was verminderd.

We concluderen dat PCC inderdaad de doorlaatbaarheid van de darm cel lijnen voor wateroplosbare moleculen verhoogt. De verhoging is echter meer gecorreleerd aan de mate waarin de celmembranen zijn beschadigd dan aan veranderingen in de tight junctions. We veronderstellen dan ook dat de verhoogde doorlaatbaarheid wordt veroorzaakt door een verhoging van de transcellulaire doorlaatbaarheid en niet in de eerste plaats door een verhoging van de paracellulaire doorlaatbaarheid.

Hoofdstuk 7

Effecten van cadmium chloride op de paracellulaire barrière van intestinale epitheliale cel lijnen.

In de experimenten voor dit hoofdstuk hebben we in grote lijnen hetzelfde gedaan voor cadmium als in het vorige hoofdstuk is beschreven voor PCC. Van het zware metaal cadmium was bekend dat het in hoge concentraties het darm-epitheel van muizen beschadigde en dat het in niercellen *in vitro* de paracellulaire permeabiliteit verhoogde. Het was echter nog niet bekend of cadmium ook de structuur van de tight junction aantastte en of de effecten op de permeabiliteit vroege of late effecten waren. De cellen zijn blootgesteld aan cadmium en er is bepaald wat de effecten zijn op de TER, op de doorlaatbaarheid voor grote wateroplosbare moleculen, wat er gebeurd met de viabiliteit van de cellen en wat er gebeurd met het ZO-1 eiwit.

We hebben gevonden dat door blootstelling aan 5-10 uM cadmium de doorlaatbaarheid van de IEC-18 en Caco-2 cellagen voor zowel kleine geladen moleculen (bepaald met de TER) als voor grote wateroplosbare moleculen, werd verhoogd. Deze verhoging van de doorlaatbaarheid ging gepaard met zichtbare verandering in de verdeling van het ZO-1 eiwit maar niet met schade aan de cel membranen. De effecten van cadmium kwamen langzaam op gang (pas na ongeveer 4 uur) en ontwikkelde zich verder in de tijd ook als het extra-cellulaire cadmium werd verwijderd na 4 uur. Het bleek ook dat de cellen erg veel cadmium opnamen en dit niet meer uitscheidde.

We concludeerden dat cadmium de doorlaatbaarheid van de cellagen verhoogde door vooral de paracellulaire route te vergemakkelijken en niet de transcellulaire route. Het is ook waarschijnlijk dat cadmium op deze manier zijn eigen passage door de darmwand kan verhogen bij niet direct cytotoxische concentraties. Dat de echte opname van cadmium desalniettemin toch laag is komt waarschijnlijk voor een belangrijk deel doordat de cellen veel cadmium kunnen opnemen en vasthouden.

Conclusies en perspectieven

Uit dit onderzoek is gebleken dat het goed mogelijk is om het darmepitheel na te bootsen met gekweekte darm cel lijnen in het twee-compartimenten systeem. Het gebruik van Caco-2 cellen in dit systeem leidt tot een model dat geschikt is als eerste screening voor de bepaling van de opname route (dus of de transepitheliale passage transcellulair of paracellulair plaatsvind). Daarnaast voldoet het Caco-2 syteem goed voor de kwantitatieve bepaling van de te verwachte opname van de transcellulair passerende stoffen. Voor bepaling van de opname van de grotere wateroplosbare moleculen lijkt het IEC-18 systeem echter een beter model omdat met dit systeem een beter onderscheid kan worden maakt tussen de paracellulaire passage snelheden van moleculen die verschillen in molecuul-grootte. Door gebruik te maken van beide cel lijnen is het mogelijk om voor vrijwel alle klassen van stoffen de te verwachte opname te modelleren. Het zou zonder twijfel handiger zijn als dit met één cel-systeem zou kunnen, dit is tot nu toe nog niet goed mogelijk, wellicht bieden de met dexamethason behandelde IEC-18 cellen, de basis voor een dergelijk compleet model.

Daarnaast heeft het in dit proefschrift beschreven onderzoek aangetoond dat het twee-compartimenten systeem met Caco-2 en IEC-18 cellen ook gebruikt kan worden om de effecten van stoffen te bestuderen die de integriteit van het darmepitheel beinvloeden (bijvoorbeeld toevoegingen aan de voeding of aan medicijnen). Een belangrijk voordeel van deze *in vitro* systemen is dat verschillende metingen aan dezelfde darmcellaag bepaald kunnen worden, waardoor het mogelijk is om het meest waarschijnlijke mechanisme achter de verhoogde doorlaatbaarheid te bepalen. Daardoor is het nu bijvoorbeeld beter mogelijk om bij een verhoogde permeabiliteit te bepalen wat de verhouding is tussen de effectiviteit (voor hoeveel extra opname zorgt het product) en de toxiciteit (hoeveel schade aan het darm-epitheel veroorzaakt het product). Wellicht dat de in dit proefschrift beschreven systemen en strategieën kunnen bijdragen aan de zoektocht naar veilige toedienings-vormen van nieuw ontwikkelde medicijnen of voeding-supplementen, met een suboptimale (lage) opname in de darm.

Curriculum vitae

Erwin Duizer is geboren in Gorinchem op 11 februari 1969. In 1987 behaalde hij het VWO diploma aan de Rijksscholengemeenschap Wijdschild te Gorinchem en in de zomer van datzelfde jaar werd begonnen met de studie Biologie aan de Landbouwuniversiteit in Wageningen (LUW). Tijdens de doctoraalfase werd door hem onderzoek verricht bij de vakgroepen Toxicologie (onder leiding van Dr. Ir. C den Besten), Biochemie (Prof. Dr. Ir I.M.C.M. Rietjens) en Erfelijkheidsleer (Dr. Ir. P de Boer) van de LUW. De eerste stageperiode werd doorgebracht op het Department of Drug Metabolism and Kinetics van Organon (AKZO Pharma Division) in Oss (Dr. Ir. R. Vos). Hier op volgend werd een tweede stageperiode doorgebracht bij het Department of Zoology van de University of Tennessee (Knoxville, USA, Prof. Dr. M.A. Handel). De doctoraalfase van de oriëntatie Celbiologie werd afgerond in september 1993.

Na een korte periode als medewerker van de vakgroep Erfelijkheidsleer van de LUW trad Ir. E. Duizer in mei 1994 in dienst als assistent in opleiding bij de vakgroep Toxicologie (LUW). Het in dit proefschrift beschreven onderzoek werd uitgevoerd bij TNO Voeding, Toxicologie Divisie, te Zeist, onder begeleiding van Dr. Ir. J.P. Groten, Prof. Dr. P.J. van Bladeren en Prof. Dr. J.H. Koeman. Naast het verrichten van het promotieonderzoek, heeft hij de nodige modules van de postdoctorale opleiding toxicologie gevolgd om voor erkenning als toxicoloog in aanmerking te komen.

Sinds september 1998 is hij werkzaam als postdoctoraal onderzoeker bij het Laboratorium voor Infectieziektenonderzoek van het RIVM op het PAD-project "Development of advanced human intestinal cell culture models for multiplication of enteropathogenic viruses".

Curriculum vitae

Erwin Duizer was born on February 11, 1969 in Gorinchem, the Netherlands. After graduation from secondary school at the Rijksscholengemeenschap Wijdschild in Gorinchem, he began his study Biology at the Wageningen Agricultural University (WAU) in 1987. As part of this study he conducted research projects at the departments of Toxicology (supervisor: Dr. Ir. C. den Besten), Biochemistry (supervisor: Prof. Dr. Ir. I.M.C.M. Rietjens) and Genetics (supervisor Dr. Ir. P. de Boer) of the WAU. Subsequently, practical periods were fulfilled at the Department of Drug Metabolism and Kinetics of Organon, AKZO Pharma Division (Oss, the Netherlands, supervisor: Dr. Ir. R. Vos), and at the Department of Zoology of the University of Tennessee (Knoxville, USA, supervisor: Prof. Dr. M.A. Handel). He obtained his M.Sc. degree in Cell Biology in September 1993.

In may 1994 he started as a Ph.D. student at the Toxicology Group of the WAU on the research presented in this thesis under supervision of Dr. Ir. J.P. Groten, Prof. Dr. P.J. van Bladeren and Prof. Dr. J.H. Koeman. The research was conducted at the Toxicology Division of TNO Nutrition and Food Research Institute (Zeist, the Netherlands). During this period he successfully completed the postdoctoral education program in Toxicology.

From September 1998 he is appointed as a post-doc at the Research Laboratory for Infectious Diseases of the National Institute of Public Health and the Environment (Bilthoven, the Netherlands) on the project entitled "Development of advanced human intestinal cell culture models for multiplication of enteropathogenic viruses" which is financed by the Dutch Alternatives to Animal Experimentation Platform.

List of Publications

Besten, C. den, van Bladeren, P.J., Duizer, E., Vervoort, J., and Rietjens, I.M.C.M. Cytochrome P450 mediated oxidation of penta-fluoro-phenol to tetra-fluoro-benzoquinone as the primary reaction product. Chemical Research in Toxicology 1993;6: 674-680.

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Duizer, E., van der Wulp, C., Versantvoort, C.H.M., and Groten, J.P. Absorption enhancement, structural changes in tight junctions and cytotoxicity caused by palmitoyl carnitine in Caco-2 and IEC-18 cells. The Journal of Pharmacology and Experimental Therapeutics 1998;287:395-402.

Duizer, E., Gilde, A.J., Versantvoort, C.H.M., and Groten, J.P. Effects of cadmium chloride ($CdCl_2$) on the paracellular barrier function of intestinal epithelial cell lines. Toxicology and Applied Pharmacology 1999;155:117-126.

Duizer, E., Versantvoort, C.H.M., Gilde, A.J., and Groten, J.P. Dexamethasone inhibits cell proliferation and enhances the paracellular barrier function of rat ileal IEC-18 cells. Submitted.

Versantvoort, C.H.M., Duizer, E., Gilde, A.J., and Groten, J.P. Carrier-mediated transport systems in rat small intestinal IEC-18 cell line. Comparison with human colon carcinoma Caco-2 cell line. Submitted.

Duizer, E., and Groten, J.P. Application of the two-compartment cell culture system is studies of intestinal permeability and permeability modulation. Manuscript in preparation, to be submitted as review to ATLA.

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