Transport of Lipophilic Drug Molecules in a New Mucus-Secreting Cell Culture Model Based on HT29-MTX Cells

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Purpose. A new mucus-secreting *in vitro* drug absorption model based on monolayers of goblet-cell like sub-clones of the human colon carcinoma cell line HT29 obtained by methotrexate (MTX) treatment was investigated.

Methods. Twelve sub-clones were isolated and characterized by light microscopy (LM), transelectron microscopy (TEM), confocal laser scanning microscopy (CLSM), transepithelial electrical resistance (TEER) and the transport of a paracellular marker FITC-Dextran (Mw 4400) (FD-4).

Results. Significant differences of microscopical appearance, TEER-values and permeability of FD-4 between the sub-clones were evident. However, two of them, namely MTX-D1 and MTX-E12, formed tight confluent monolayers with a thick mucus-layer on the apical surface. They were used to compare the apparent permeability coefficient (P_{app}) of a series of lipophilic drugs, which should be affected by the mucus-layer, namely barbiturates (barbituric acid, barbital, phenobarbital, methylphenobarbital and heptabarbital) and testosterone, as a reference, to mucus-free Caco-2 cells. The permeability of drugs with a partition coefficient (log P) > 1 was decreased in the mucus-producing cell lines. Testosterone, the most lipophilic compound, showed a decrease of up to 43%.

Conclusions. We demonstrated that the mucus layer is a significant barrier to drug absorption for lipophilic drugs. In conclusion, our model may serve as a suitable in-vitro cell culture model to study the influence of the mucus layer on drug diffusion.

KEY WORDS: mucus layer; HT29-MTX; drug absorption; barbiturates; Caco-2; cell culture.

INTRODUCTION

Transport and absorption of lipophilic substances from the small intestine can be affected by the unstirred water layer (UWL) and the mucus layer. A number of studies have investigated the UWL (1,2), however, the significance of the mucus layer as a barrier to drug absorption is still incompletely understood. While some authors argue that the mucus layer is not relevant to drug absorption (3), Nimmerfall and Rosenthaler (4) have found a correlation between the absorption of a series of ergot alkaloids and their diffusion coefficient in isolated rat-mucus. Moreover, Wikman-Larhed *et al.* (5) found a significant decrease in the diffusion coefficient of testosterone, a high lipophilic compound, in pig intestinal mucin (PIM) as compared to purified pig gastric mucin (PPGM). Until now only a few studies have been carried out characterizing the influence of lipophilicity on drug diffusion in mucus (5,6).

Caco-2 cells are frequently used as an "in-vitro cell culture model" of the intestinal epithelium to characterize drug transport and metabolism. However, this model has some drawbacks compared to the *in vivo* situation, such as the lack of a nervous control, systemic blood flow, and motility of the intestine (7). Furthermore, it is based only on one cell type of the intestinal epithelium, namely enterocytes. Thus, mucus-secreting goblet cells, the second major cell type in the intestinal epithelium is absent (8). Another disadvantage is that the Caco-2 cell monolayer is too impermeable for hydrophilic, paracellularly transported compounds (8), since its paracellular permeability resembles colonic tissue more closely than small intestinal tissue.

Since the early 1980s, a number of methods have been developed to differentiate the adenocarcinoma cell line HT29 into mature intestinal cells under appropriate culturing conditions. Several clones have been established, some of them forming monolayers of enterocytes (9,10), whereas others differentiate into mature goblet cells (10,11). Of the goblet cell clones, only the HT29-H clone, obtained from HT29 by selected growth in a glucose-free, galactose-supplemented medium, has been widely used to investigate the influence of mucus on the drug absorption (6,12,13). Unfortunately, this clone was phenotypically unstable and currently grows in multilayers where only the outer layer forms mucus-producing goblet cells (13).

Nevertheless, interest in a mucus-layer containing cell culture model remains high, since this would permit a detailed study of the barrier properties of the intact human intestinal mucus layer. Moreover, in contrast to animal experiments, cell culture models provide an inexpensive and relatively simple tool to characterize transport properties of a large number of drug candidates under standardized conditions. This issue receives increasing attention in the context of high through put screening, since advances in combinatorial chemistry have revolutionized the field of drug discovery producing an increasing amount of compounds which need to be tested for their affinity to specific receptors and for their biopharmaceutical properties.

The aim of the present study was the development of a new mucus-producing cell culture model which can be used to study the permeability of drugs in mucus containing cell monolayers as well as elucidate the role of the mucus layer as a barrier for drug and particle uptake. We used HT29 cells that were differentiated into mature goblet cells under the influence of methotrexate (MTX) according to Lesuffleur *et al.* (11). Mucus-secreting HT29-MTX sub-clones were isolated from this cell clone and characterized with regard to tightness of the tight junctions, development of confluent monolayers and production of a mucus layer. Based on these results, we selected two sub-clones and investigated the effect

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ABBREVIATIONS: FD-4, fluoresceinisothiocyanate-dextrane Mw 4400; MTX, methothrexate; P_{app}, apparent permeability coefficient; PCR, polymerase chain reaction; PBS, phosphate buffered saline; TEER, transepithelial electrical resistance; log P, Partition coefficient (octanol/water); Mw, molecular weight.

of the mucus layer on the diffusion of a series of model compounds with different partition coefficients (log P) as a marker for their lipophilicity. Results obtained with the HT29-MTX sub-clones were compared to the data obtained in mucus-free Caco-2 cells.

MATERIALS AND METHODS

Materials

All barbiturates were obtained from Merck (Darmstadt, Germany). FITC-dextran (Mw 4400) and glutaraldehyde were from Sigma (Deisenhofen, Germany). Tissue culture reagents were from Gibco (Eggstein, Germany) and tissue culture articles from Nunc (Wiesbaden, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) in analytical quality.

Cell Culture

Caco-2 cells (passage: 47-50) were obtained from DKFZ (Deutsches Krebsforschungszentrum Heidelberg, Germany) and HT29 cells from ATCC (American Tissue Culture Collection, Rockville, MD USA). HT29-MTX cells (passage: 32-34) were a generous gift of Dr. A. Zweibaum, Paris. Both cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids and 1% L-glutamine at 10% CO_2, 95% r.h. and 37°C. Cells were passaged as described elsewhere (14).

For characterization and transport studies, cells were seeded at a density of $6*10^4$ cells/cm^2 on uncoated-polycarbonate Transwell filter inserts (Costar, 0.4 μm pore size, area: 4.71 cm^2) and cultivated over 21 days. Medium was changed every second day.

Subcloning of HT29-MTX Cells

For limiting dilution cloning of HT29-MTX cells, conditioned medium was used as described below. Medium obtained after two days incubation with non-confluent Caco-2 cell monolayers growing in plastic cell culture flasks was filtered (0.22 μm pore size, Millipore) to yield conditioned medium. HT29-MTX cells of passage number 18 were harvested from the culture flask and suspended in conditioned medium at a concentration of 100 cells/mL. 10 μL of the cell suspension and 100 μL of conditioned medium was added to each well of a collagen coated 96 well plate. A microscopic examination was carried out in order to discard all wells containing more than one cell. The cells were allowed to proliferate in the 96 well plate for approximately one month and subsequently transferred to a collagen coated 24 well plate. The conditioned medium was replaced every 3–6 days depending on the number of cells in the wells. When the cells reached ca. 50% confluency in the 24 well plate, they were transferred to 25 cm^2 plastic cell culture flasks and fed with a 50:50 mixture of conditioned and non-conditioned medium. This procedure resulted in 12 sub-clones of the original HT29-MTX cell line.

Selection of Appropriate Sub-Clones

To reduce the number of sub-clones six of 12 isolated sub-clones were selected showing a reduced proliferation rate, grown on filter inserts for 21 days and screened for their ability (i) to form confluent monolayers, (ii) to develop tight junctions and (iii) to secrete a continuos mucus layer.

Light Microscopy

Filters were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at 25°C. Afterwards, they were stained with Alcian Blue (Fluka, Deisenhofen, Germany), (1% in distilled water, adjusted to pH 2.5), counterstained with hematoxylin, embedded in OCT-Compound 4583 (Sakura, USA), frozen at –20°C, cut into cross sections of 20 μm using a cryo-microtome (Frigocut Mod. 2700, Reichert-Jung, Germany), mounted on a slide, embedded in PBS/glycerol (2:1) and examined using an inverted Nikon microscope (TMS) equipped with a phase contrast.

The thickness of the mucus-layer was measured in vertical cross-sections of filter inserts, stained with toluidine blue (Fluka, Germany) 0.25% in PBS for 1 min and observed under the light microscope as according to the method of Wikman *et al.* (12).

Transmission Electron Microscopy

Cells grown on permeable filter inserts were rinsed with PBS and fixed in 1.5% glutaraldehyde in phosphate buffer for 2h at 4°C. Specimens were rinsed with phosphate buffer, treated with 1% osmium tetroxide, dehydrated through a graded series of ethanol and embedded in Epon resin. Thin sections were cut and stained with uranyl acetate and lead citrate. The specimens were studied under a transmission electron microscope (EM 310, Philipps, Germany) operated at 60 kV.

Confocal Laser Scanning Microscopy

F-actin was stained using FITC-labeled phalloidin (Sigma, Germany) previously described by Anderberg *et al.* (15). Briefly, 10 μL of the stock solution (200 U/mL phalloidin in methanol) was evaporated and redissolved in 400 μL PBS, pH 7.4. Monolayers, grown on Transwell filter inserts for 21 days, were fixed in 3% paraformaldehyde in PBS for 30 min at room temperature, rinsed three times with PBS and treated with 1% Triton X-100 (Gibco) on ice for 5 min. After repeated washings and air-drying, the monolayer was stained with FITC-phalloidin under light-exclusion for 20 min. The sample was rinsed twice and examined under a confocal laser scanning microscope (Zeiss CLSM 501, Jena, Germany) equipped with a Zeiss Neofluor 40*/1.3 objective.

RNA Isolation and RT-PCR for MUC Gene Expression

Total RNA was extracted from cells incubated in 8.8 cm^2 polystyrene dishes for 21 days using a commercial RNA isolation kit (RNA Clean, AGS GmbH, Heidelberg, Germany) according to the manufacturer's protocol. The RNA integrity was confirmed by agarose-native gel (1 μg RNA, 100 V, 60 min). 1.0 μg total RNA was transcribed to cDNA by MMLV Reverse Transcriptase (Promega, Heidelberg, Germany), 500 μmol dNTP's, 3.5 μM Oligo(dT)-10, 1.0 μL 10x reaction

buffer, 0.5 U ribonuclease inhibitor and sterile water in a final volume of 25 μL for 60 min at 37°C. 1μL of cDNA was used per PCR reaction in a mixture of 5 μL buffer, 200 μM of each of the four dNTPs, 10 pM of each primer (actin + MUC1 or MUC2), 0.05 units of Taq polymerase Dynazyme II (Biometra, Göttingen, Germany). Mixtures were incubated in a thermal cycler (T Gradient, Biometra, Göttingen, Germany) for 30 cycles under the following conditions: 94°C 30 s, 60°C 30 s, 72°C 45 s. The sequence of primer pairs for MUC1 and 2 was taken from literature (16), actin primers were ATTTGGCACCACACTTTCTAC (sense strand) and TCACGCACGATTTCCCTCTCA (antisense strand) yielding a 380bp product.

After PCR reaction, an aliquot of 10 μL of the PCR product was separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide at 100 V for 45 min. To determine the relative ratios of the intensity of the MUC-bands to that of actin in the different cell lines, the gel was photographed, digitized and analyzed with Un-Scan-It software (version 4.1, Silk scientific cooperation, USA). Each experiment was carried out in triplicate.

Measurement of Transepithelial Electrical Resistance (TEER)

The integrity of the monolayer was checked on the 21th day after seeding as well as in the beginning and at the end of each transport experiment by measuring TEER using a volt-ohmmeter (EVOM, World Precision Instruments, Berlin) equipped with Endohm electrodes. The total TEER (cell monolayer + insert) was subtracted from background TEER (insert) to yield the monolayer resistance and multiplied with the area of the insert. Each experiment was done in triplicate.

Transport Studies

Transport studies were performed 21 days post seeding. Filter inserts were rinsed with PBS pH 7.2 supplemented with Ca^{2+}, Mg^{2+} and 15 mM glucose and allowed to equilibrate at 37°C for 15 min in the incubator. Barbiturates and testosterone were dissolved in PBS to a final concentration of 1 mM. To increase solubility, 0.2–0.4% di-methyl-sulfoxide (DMSO) was added, which did not affect cell viability (data not shown). Furthermore, the test solution contained 250 μg/mL FITC-dextran (Mw 4400) (FD-4) as a paracellular marker. Experiments were started by replacing the apical (1.5 mL) buffer with the test solution.

To diminish the unstirred water layer (UWL), transport experiments were carried out under agitation (70 Hz) in a shaking water bath tempered to 37°C (GFL, model 1083, Hannover, Germany) similar to Yee *et al.* (17). Every 20 min up to 120 min a 1.0 mL sample was collected from the basolateral chamber and replaced with fresh buffer. Each experiment was performed in triplicate.

Sample Analysis

Samples were analysed by reverse-phase HPLC consisting of a pump (model L-6200A), an automatic sampler (model As-200A), a column thermostat (model T-6300), a fluorescence detector (model F-1050) and an UV-light detector (model L-4000) (all from Hitachi, Merck, Darmstadt, Germany). FD-4 analysis was performed using a RP 18, 3 μm, 30 × 4 mm, column with a mobile phase of 10% acetonitrile and 90% KH_2PO_4 (2.0 mM, pH 7.2) and a flow of 1 mL/min. Barbiturates except for barbituric acid were separated on a 200 Supersphere LiChroCart cartridge 100 RP-18, 25 × 4 mm (Merck, Darmstadt) at a flow rate of 1mL/min. Mobile phase for barbiturates consisted of 0.1% trifluoro-acetic acid (TFA) in water and acetonitrile; 75:25 for barbital, 65:35 for phenobarbital and 55:45 for methyl-phenobarbital and heptabarbital. The acid mobile phase consisted of 55% 2.5 mM KH_2PO_4 adjusted to pH 5.0 and 45% acetonitrile, whereas for testosterone, it consisted of 65% methanol and 35% water. Samples were quantitated using a fluorescence- (FD-4: Ex: 490nm Em: 515nm) or UV-detector (barbituric acid (251 nm), other barbiturates (215 nm) and testosterone (242 nm)).

Calculations and Statistics

The apparent permeability coefficient (P_{app}) was calculated from concentration-time profiles using the following equation:

$$P_{app}\ (cm/s) = dC/dt * 1/A * V/C_o\ [cm/s]$$

where dC/dt (μg/s) represents the flux across the monolayer, A (cm^2) the surface area of the monolayer, V (cm^3) the volume of the receiver chamber and C_o (μg) the initial concentration in the donor compartment.

Results were depicted as mean value ± standard deviation of three experiments. Significance between the mean values was calculated using ANOVA one way analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, USA). Probability values $p < 0.05$ were considered as significant.

RESULTS

Selection of Mucus-Producing Sub-Clones of the Human Colon Carcinoma Cell Line HT29

According to Lesuffleur *et al.* (11), an important factor for the differentiation of the HT29 cell line into mucus producing sub-clones is their diminished proliferation rate. A selection of the sub-clones based on their proliferation rate was carried out, eliminating six of the twelve sub-clones. The remaining sub-clones were seeded on filter inserts and characterised with respect to their development of tight junctions, an important criterion for their differentiation. Three independent methods were used, namely (i) the measurement of the transepithelial electrical resistance (TEER), (ii) the determination of the permeability coefficient of FD-4, a paracellular marker and (iii) the staining of actin with FITC-labeled phalloidin.

Two of the six clones, namely MTX-D1 and MTX-E12, showed a higher TEER than the HT29 cell line and a decreased permeability coefficient of the paracellular marker FD-4 (Fig. 1). Moreover, a continuous shiny mucus layer on the apical surface could be observed microscopically. These two sub-clones of the HT29 cell line were selected for a more detailed characterization.

Characterization of Mucus-Secreting Sub-Clones MTX-D1 and MTX-E12

Light Microscopy

The HT29-MTX sub-clones, cultivated on 6 well plates for 21 days, appeared spindle-like and smaller in size than the

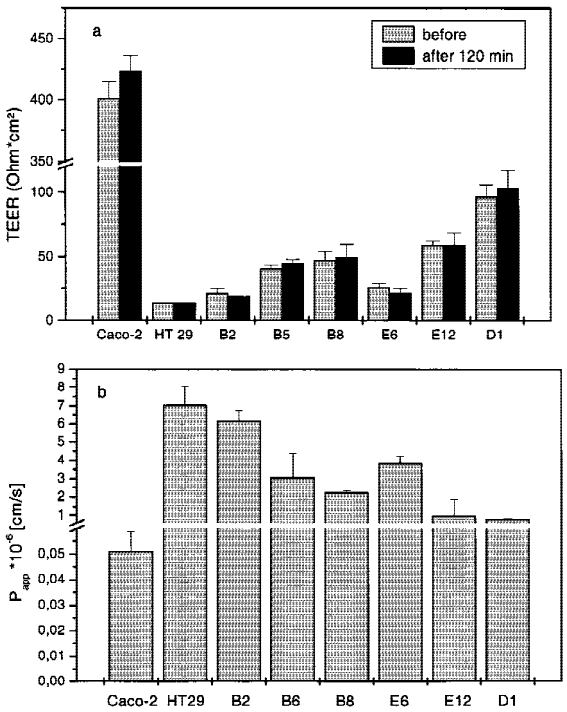

Fig. 1. Comparison of the development of tight junction between highly differentiated, enterocyte-like Caco-2 cells, undifferentiated HT29 cells (without tight junctions) and isolated, goblet -cell forming MTX-sub-clones (a) Transepithelial electrical resistance (TEER) values before and after transport of FITC-Dextran (Mw 4400) (FD-4) (n = 3). (b) Permeability of FD-4, a hydrophilic, paracellularly transported marker compound (n = 3).

Caco-2 cells, however, they were larger and less spherical than the HT29 cells. They were covered with a macroscopically visible, shiny mucus layer that spread across the entire apical surface (data not shown), demonstrated with and without Alcian Blue staining.

Studies of cryo-sections revealed that both sub-clones formed 20–25 μm thick monolayers. Staining with Alcian blue for acidic mucus components indicated that the cell layers consisted of a large number (70–80%) of mature goblet cells with mucin granules in the apical part of the cytoplasm (Fig. 2). No Alcian blue staining could be detected in Caco-2 and HT29. The thickness of the mucus layer was found to vary along the section in the range 142 ± 51 μm for MTX-E12 and 53 ± 52 μm for MTX-D1. Thus, the mucus layer thickness in MTX-E12 cells exceeded that in MTX D1 cells by a factor of ca. 3.

Transmission Electron Microscopy (TEM)

Both sub-clones formed layers of differentiated epithelial cells with tight junctions and some microvilli. Most of the cells resembled goblet cells and contained clusters of large mucin granules located in the apical region of the cells (Fig. 3). The MTX-E12 showed a much higher concentration of mucin granules, which filled at least half of the cell, compared to the MTX-D1 sub-clone. The monolayer was interspersed with a few premature cells (<10%) not showing microvillis and mucin granules.

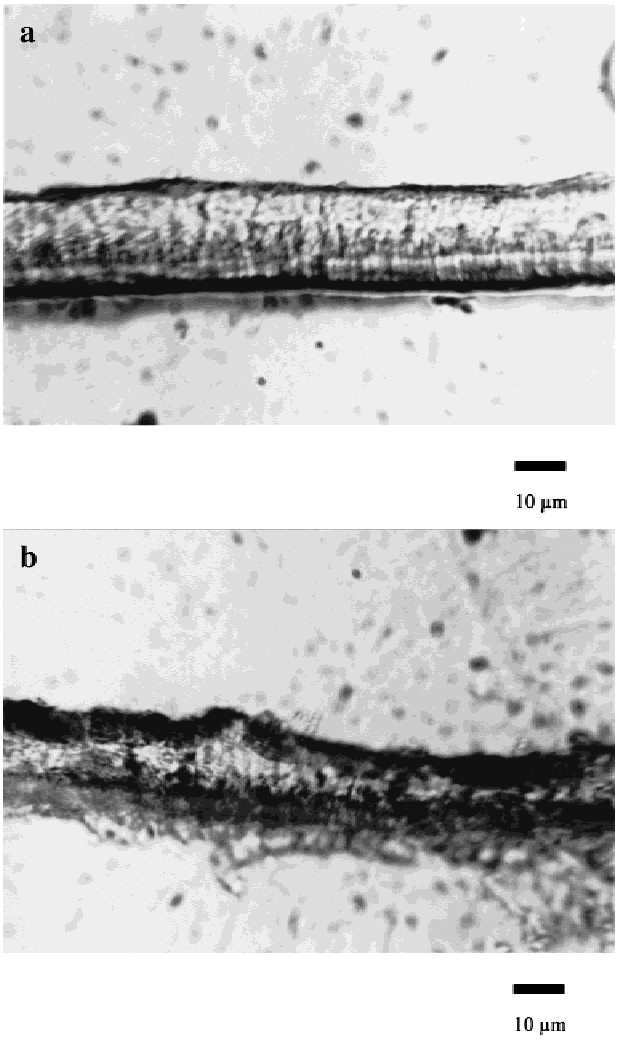

Fig. 2. Light micrographs of cryo-sections stained with Alcian Blue for acidic mucus components. Dark stained mucin granules are visible in the apical part of the monolayer. The bar indicates 10 μm. (a) MTX D1, (b) MTX E12.

Expression of Mucin Genes (MUC1 and MUC2)

To analyze the mucin production in the HT29-MTX sub-clones, the expression of two apomucin genes was studied in a semi-quantitative way by RT-PCR. While MUC1 can be found on the apical surface of a large number of epithelial tissues including the gastrointestinal tract (18), MUC2 mucin is primarily present in the small intestine (19). For semi-quantitative analysis of the PCR-products, the housekeeping gene actin was used as an internal standard, since it is widely used as a reporter gene to estimate the amount and integrity of RNA as well as for comparing gene expression among different tissues (20). Amplification performed on reverse-transcribed RNA yielded the expected size of 293 bp for MUC1 (Fig. 4a, lane 1-2) a second transcript, corresponding to the second MUC1 allele and 348 bp for MUC2 (Fig. 4b, lane 1-3) and 380 bp for actin (Fig. 4a and b, lane 1-4). Both sub-clones, MTX-D1 and MTX-E12, yielded a high expression of the two apomucin m-RNA, however, the expression of both genes was stronger in MTX-E12 than in MTX-D1. This correlates with the thicker mucus layer found in this sub-

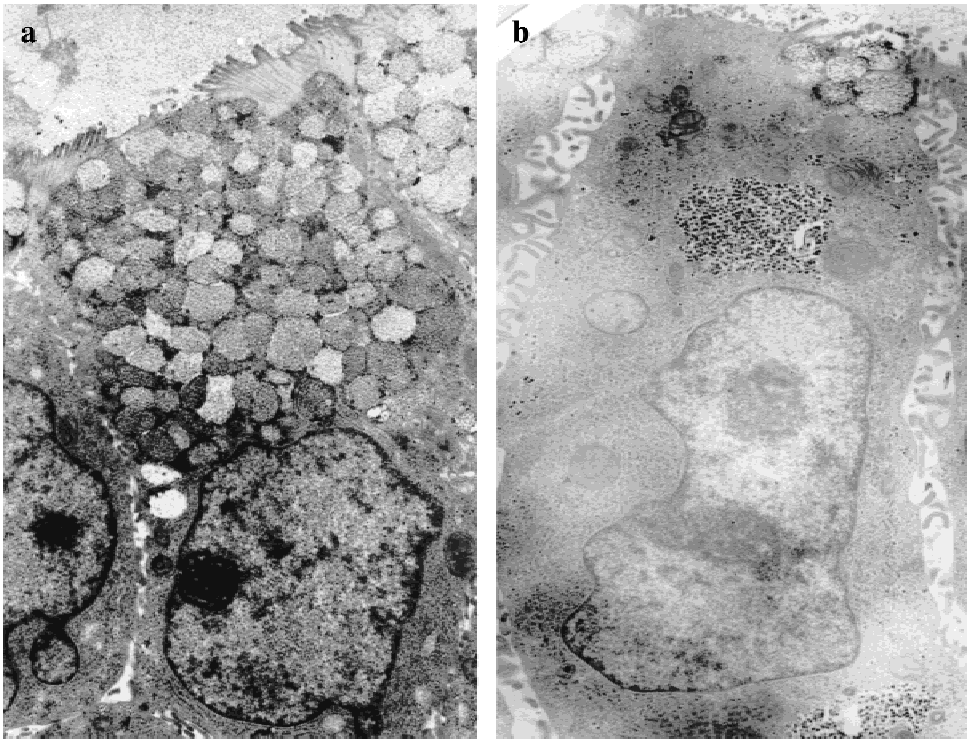

Fig. 3. Transmission electron micrographs of post-confluent cultures (21 days) of MTX sub-clones showing mature goblet cells with apical clustered mucin granules as well as tight junctions and sparse microvilli. In the MTX D1 sub-clone (b) only a small amount of mucin vesicles whereas in the MTX E12 (a) more than half of the cell is filled with vesicles. The bar indicates 1μm.

clone. MUC1 expression in mucus-free HT29 and Caco-2 was absent. The MUC1/actin ratio was 57% higher in MTX-E12 than in MTX-D1.

MUC2 expression varied between the different cell lines: HT29 and MTX-E12 showed a strong expression while MTX-D1 exhibited only a weak band. MUC2/actin ratio was 60% higher in MTX-E12 than in MTX-D1. No difference in the MUC2/actin ratios was found between MTX-D1 and HT29. No MUC2 expression was found in Caco-2.

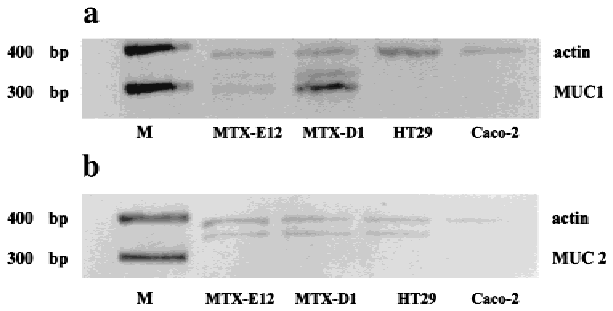

Fig. 4. mRNA expression of MUC1 and MUC2 by RT-PCR in Caco-2, HT29, MTX-D1 and MTX-E12 as described in Materials and Methods. Actin (380 bp) was used as an internal standard to confirm integrity and the amount of cDNA subjected to PCR reaction. PCR-products were investigated on a 1.5% agarose gel stained with ethidium bromide at 100V for 45 min. (a) MUC1(293 bp): lane 1 marker, lane 2-5 MUC1 and actin in the investigated cell lines. (b) MUC2 (348 bp): lane 1 marker, lane 2-5 MUC2 and actin in the investigated cell lines.

Monolayer Integrity and Development of Tight Junctions

As shown in Fig. 1, an inverse relationship between TEER and P_{app} for the paracellular marker FD-4, a widely used paracellular marker compound in Caco-2 cells (21), was observed. Thus, an increase in TEER was associated with a decrease of P_{app} of FD-4. TEER values were however not as high as those obtained in Caco-2 cell monolayer (658 ± 14 $\Omega*cm^2$), but they were significantly increased compared to the undifferentiated HT29 cell line (15 ± 0 $\Omega*cm^2$). Moreover, MTX-D1 (279 ± 32 $\Omega*cm^2$) developed tighter monolayers than MTX-E12 clones with 169 ± 22 $\Omega*cm^2$.

The permeability of FD-4 in the sub-clones was significantly increased as compared to Caco-2 cells (19 fold in MTX-D1 and 15 fold in MTX-E12), however, it was significantly diminished as compared to the HT29 cell line (7 fold in MTX-D1 and 9 fold in MTX-E12). Again MTX-E12 monolayers were approximately 50% leakier than MTX-D1 layers. Both parameters for the integrity of the monolayers remained constant over 120 min.

The presence of tight junctions was demonstrated using FITC-labeled phalloidin. Phalloidin attaches to actin on and within the cells. It stains actin fibers close to the cellular junctions, of the so called terminal web, another indicator for well differentiated epithelial cells, and of the cyto-sceleton. MTX-E12 and MTX-D1 showed an intensive staining of actin fibers close to or associated with the cellular junctions as shown in Fig. 5. Moreover, in the MTX-D1 sub-clone a tight network of the terminal web on the apical side was visible. In the MTX-E12 clone and to a lesser extent also in the MTX-D1 sub-

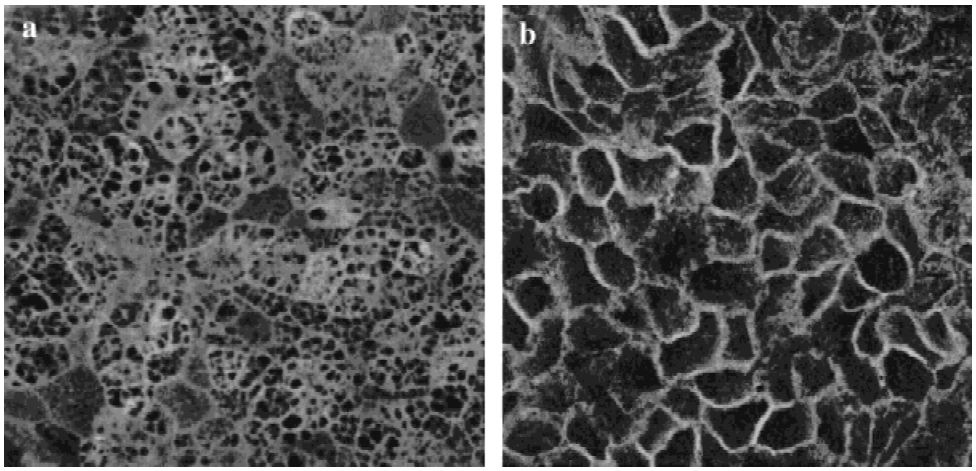

Fig. 5. Confocal laser scanning micrographs of MTX-sub-clones stained with FITC-phalloidin for actin fibres. MTX-E12 and MTX-D1 showed an intensive staining of actin fibers close to the cellular junctions. Moreover, in the MTX-D1 clone the tight network of the terminal web on the apical side was observed (a), whereas in the MTX-E12 clone intracellular, cystic structures were visible (b).

clone intracellular, cystic structures could be observed. All features remained constant over at least 7 passages (data not shown).

Permeability Experiments Using Barbiturates as Lipophilic Model Compounds with Different Partition Coefficients

The absorption of a series of barbiturates and testosterone with different log P as a marker for their lipophilicity was investigated. Barbiturates were selected because a broad range of partition coefficients is commercially available. Moreover, preliminary results indicate that barbiturates are transported primarily by passive diffusion (data not shown). Testosterone was chosen as a reference for comparison with earlier studies (6,12,22). All substances and their physical parameters are summarized in Table I.

To diminish the unstirred water layer (UWL), monolayers were stirred in thermostated water bath. P_{app} for each compound was determined in the mucus covered sub-clones and in the mucus-free Caco-2 cells. We found that P_{app} of barbituric acid and FD-4, molecules with negative log P values, were higher in the HT29-MTX sub-clones than in Caco-2 cells demonstrating paracellular transport. All other compounds exhibiting log P between + 0.5 and +3.3, showed a lower P_{app} in HT29-MTX sub-clones than in Caco-2 cells (Fig. 6), demonstrating an effect of the mucus layer on transport properties. With increasing lipophilicity of the drug, P_{app} reaches a plateau in Caco-2 cells and HT29-MTX sub-clones. However, the permeability in MTX-D1 was higher than in MTX-E12. P_{app} of testosterone, the most lipophilic drug (log P 3.32), was reduced as much as 43% in MTX-E12 and 29% in MTX-D1 as compared to Caco-2.

Table I. Physicochemical Parameters of the Drugs Used in the Transport Study [MW, pKa, log P (Partition Coefficient Between Octanol and Water)]

	Mw	pKa[a]	log P[a]
Barbituric acid	128.1	4.0	–1.47
Barbital	184.2	7.8	+0.65
Phenobarbital	232.2	7.3	+1.47
Methylphenobarbital	246	7.8	+1.84
Heptabarbital	250.3	7.4	+2.03
Testosterone	288.4	—	+3.32

[a] Martindale (31).

DISCUSSION

To investigate transport properties of lipophilic candidates and also the fate of particulate carriers in the human small intestine, a cell culture model mimicking more closely physiological conditions would be desirable. Therefore, twelve mucus-secreting sub-clones of a MTX-differentiated HT29 cell line were isolated by limiting dilution cloning. It was found that these sub-clones differed from each other with respect to the development of tight junctions, as demonstrated by the permeability of a paracellular marker FD-4 and the measurement of TEER. In accordance with previous studies (24), the HT29-MTX cell line consists of a variety of different sub-clones and cannot be considered as a reproducible model to investigate the function of the mucus barrier in the small intestine. We found that two of the isolated MTX-sub-clones (i) formed monolayers, (ii) developed tight junctions, (iii) consisted primarily of mature goblet cells (iv) secreted a continuous mucus layer on the apical surface of the cell and (v) was stable over at least 7 passages.

The thickness of the mucus layer was found to be 142 ± 51 μm for MTX-E12 and 53 ± 52 μm for MTX-D1, which is, especially for the MTX-E12 clone, close to the in-vivo situation, since in humans thickness of the mucosal layer is approximately 100–150 μm (25) and in rat 145 ± 22 μm (26). However, variations in the thickness of the mucus-layer of the sub-clones were large and comparable to the *in vivo* situation.

The main components of gastrointestinal mucus are the glycoproteins, composed of glyco-peptide sub-units (27). The protein core of a subunit is called apomucin. Presently 9 different core proteins are known (19). In our cell culture model, the expression of mucin was monitored by investigating the expression of two apomucins, MUC1 and MUC2, using RT-PCR. According to Lesuffleur *et al.* (28), a correlation between the amount of mRNA and the encoding MUC protein

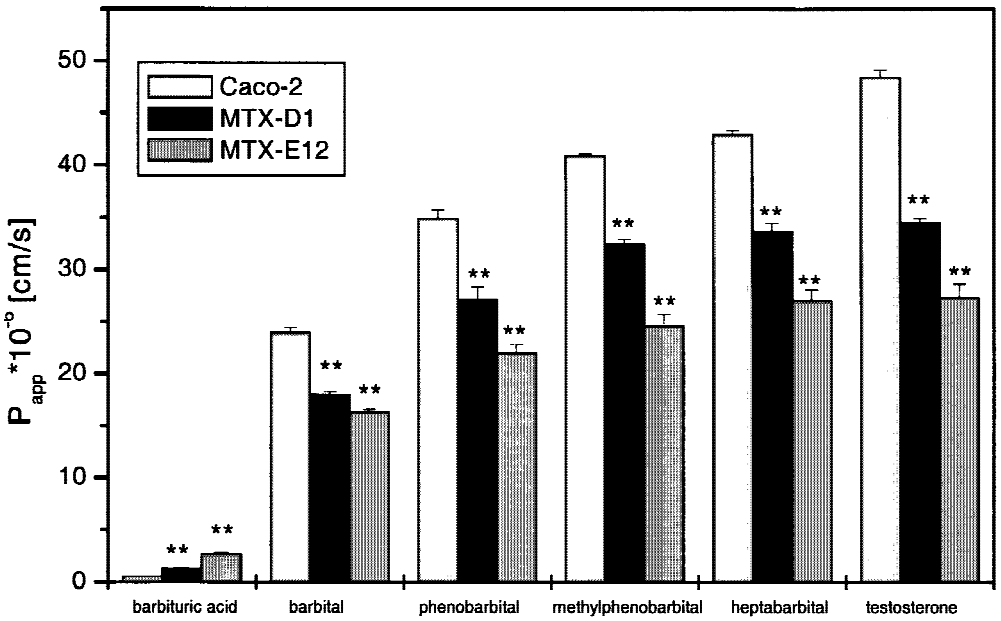

Fig. 6. Apparent permeability coefficient (P_{app}) of different barbiturates and testosterone in Caco-2, MTX-D1 and MTX-E12 cells during 120 min. The compounds are ordered from left to right according to increasing log P (octanol/water). The mean values of Papp between Caco-2 and the clones as well as between the clones were statistically significant (ANOVA). The bars indicate mean ± S.D. (n = 3).

can be found. Since the levels for MUC1 and MUC2 were much higher in the HT29-MTX sub-clones than in HT29 or Caco-2 cells without a mucus layer, RT-PCR of apoproteins seemed to be a suitable method to quantitate the mucin production. Especially, MUC1, which was absent in HT29 and Caco-2 cell monolayers, was shown to be a suitable marker. Moreover, semi-quantitative analysis revealed a 60% increase of MUC1 and MUC 2 expression in MTX-E12 in comparison to MTX-D1, which was combined with the observation of a thicker mucus layer in this sub-clone as analyzed by light microscopy. In contrast to van Klinken *et al.* (29) neither the expression of MUC1 nor MUC2 in the Caco-2 cell line was found.

The development of tight junctions is essential in differentiated cell layers of the small intestine, since they restrict the paracellular permeation of small hydrophilic molecules and allow the differentiation into polarized cells. Three methods were used to verify the development of tight junctions: measurement of TEER, transport of FD-4 and the staining of actin with FITC-phalloidin. As described for other goblet cell layers (6,12), it was found that the permeability of tight junctions is higher in the HT29-MTX sub-clones than in the Caco-2 monolayers. This might be due to a different structure of tight junctions between goblet cells compared to that between enterocytes, being more variable and more leaky, as found by Madara *et al.* (23) in the rat small intestine.

FITC-phalloidin staining for actin revealed the presence of actin fibers close to the cellular junctions, indicating the development of tight junctions. Moreover, especially in the MTX-E12, a large number of intracellular, apical cystic structures was visible. These might be connected with the production of mucin, since it is known that in the apical part of goblet cells a small layer of cytoplasm that contains actin filaments separates the membranes of mucin granules from the apical plasma membrane (30).

Based on these results, both sub-clones, MTX-E12 and MTX-D1 seemed to be attractive candidates in further, more detailed investigations. While the MTX-D1 clone formed tighter monolayers with less mucus, the MTX-E12 clone was more leaky and secreted a thicker mucus layer. However, it seems that the MTX-E12 subclone is the more promising model with regard to investigations of the mucus as a barrier to drug and particle absorption.

We found that the permeability of lipophilic drugs was dependent on the presence of a mucus layer. Moderately lipophilic drugs, such as barbital (log P +0.56) showed reduced permeability coefficients in goblet cell monolayers. Moreover, permeability decreased as a function of the amount of mucus, since P_{app} values in the MTX-E12 with a thicker mucus layer were lower than in MTX-D1.

However, the profile of the correlation of log P against P_{app} was similar in all investigated cell lines. These data suggest that the main barrier to drug transport of lipophilic substances remains the epithelial cell layer, while the mucus layer acts as an additional, yet more permeable diffusion barrier. Similar conclusions were reached by Specian *et al.* (30) who postulated that the mucus layer is a diffusion barrier for nutrients and small molecules, where the absorption of molecules is directly proportional to their diffusion rate. It will be of interest to study more closely the dependence of transport properties as a function of molecular weight and partition coefficient in these cell culture models.

Substances with a log P <1, showed a different ranking for P_{app} in the cell lines, which was mainly dependent on the development of tight junctions, revealing that the epithelial barrier plays an important role in drug absorption.

In summary, the MTX-sub-clones were shown to be a suitable model for the investigation of the drug absorption through the mucus layer since they form tight junctions, confluent monolayers and secret a continuos mucus layer.

Moreover, the transport of a series of lipophilic drugs with different log P values, indicates that the mucus layer is a significant barrier to drug absorption depending upon the lipophilicity of the drug.

Finally, this model might be more suitable for the investigation of mucus interactions with drugs or particles than the HT29-H, due to its growth as a monolayer and the reliability of mucus production. However, according to Wikman-Larhed *et al.* (31), its application in a co-culture with enterocyte-like Caco-2 cells needs further investigations.

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