Usefulness of a Novel Caco-2 Cell Perfusion System. I. *In Vitro* Prediction of the Absorption Potential of Passively Diffused Compounds

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ABSTRACT: A simple, reliable, and user friendly system was established to cultivate Caco-2 cell monolayer for epithelial transport studies. After an initial growth period of 1 week in a CO_2 incubator, Caco-2 cells were cultivated in an automated continuous perfusion system (Minucells and Minutissue, Germany). Medium was constantly renewed at the apical and basal side of the monolayers, which resulted in a continuous supply of nutrients as well as in a continuous removal of metabolite wastes. The monolayers obtained with the new perfusion culture system were evaluated to estimate the passive transport properties of a series of model compounds. The results produced were compared to those of monolayers obtained with the standard 21-day system. The integrity of cell monolayers was checked by measuring transepithelial electrical resistance (TEER) and by the transport of the paracellular leakage marker sodium fluorescein. The results of confocal microscopy as well as TEER measurements indicated the formation of a monolayer on various support filters. The growth and differentiation of Caco-2 cells were highly dependent upon the individual support filters and extracellular matrix proteins used for Caco-2 attachment. The permeability coefficients of several model compounds across Caco-2 cells obtained with the perfusion system were approximately two-fold higher than those obtained using the traditional 21-day Snapwell-based cultures. A good correlation was found between the transport of passively diffused drugs across Caco-2 monolayers differentiated in the perfusion system and the transport according to the standard method. The rank ordering of high permeable model compounds tested through Caco-2 monolayers, differentiated in a perfusion system, was similar to the standard 21-day culture method. J Pharm Sci 93:2507–2521, 2004

Keywords: cell culture; perfusion system; Caco-2 cell monolayer; polycarbonate; polyethylene terephtalate; support filter; Minucells (Minusheets); Snapwells; apparent permeability coefficient; transepithelial electrical resistance; HPLC

INTRODUCTION

Optimization of the bioavailability of drugs administered by the oral route is one of the important aims for the development of pharmaceuticals. Early intestinal absorption screening of drug candidates is currently implemented during

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drug discovery programs. The potential for intestinal drug absorption is estimated using a variety of methods including computational approaches, artificial membranes, isolated tissues, organ perfusion, and cultured cells.

The Caco-2 cell line, originally established from a well differentiated human colon adenocarcinoma, is presently the most popular *in vitro* cell line for predicting oral absorption of drug candidates in early drug discovery programs.[1–7] Unfortunately, the culturing of fully differentiated Caco-2 monolayers is time- and labor-intensive and relatively expensive, mainly due to the need for regular replacement of the culture media. The preparation of a fully differentiated confluent Caco-2 monolayer for conventional drug transport experiments across Caco-2 monolayer needs at least 21 days of cell culturing and requires an intensive cell feeding protocol.[8]

The preparation time of a fully functional Caco-2 monolayer can be shortened to 3 days using a modified system: Biocoat Intestinal Epithelium Differentiation Environment (BIEDE). By modifying both the coating material and growth medium, Caco-2 monolayers with acceptable barrier properties were obtained.[9,10]

The time required for Caco-2 cell differentiation has also stimulated the evaluation of alternative epithelial cell lines. MDCK and recently, 2/4/A1 cells are becoming popular alternatives for drug transport studies. MDCK, the Madin–Darby canine kidney cell line differentiates into columnar epithelial cells with the formation of tight junctions within 3 days[11] while the foetal rat intestinal cell line, 2/4/A1, immortalized with a temperature-sensitive mutant, differentiates within a period of 2–10 days.[12]

Under *in vivo* conditions, most tissues are continuously supplied with nutrients by capillaries. This physiological system is not sufficiently mimicked by the conventional culture dishes.[13] Due to this fact, stationary cultures have their limitation in successfully developing highly differentiated structures. The metabolic conditions within the culture medium are unstable and long-term cultures are at risk of bacterial or fungal contamination during manual medium replacement paradigms.[14] The degree of cellular differentiation in cultured epithelia depends not only on the right extracellular matrix, but also on a continuous supply of nutrients and growth factors, the elimination of harmful metabolic products and the prevention of an accumulation of synthesized paracrine factors.[15]

In an effort to obtain a simple, reliable, and less labor-intensive approach which mimics, as closely as possible, the *in vivo* situation, an advanced perfusion system technology[16] was explored for cultivating Caco-2 cells.

The Minuth perfusion culture system is a closed culture system, providing continuous perfusion with fresh culture medium. Nutrition is therefore optimized while metabolic products are removed from the cellular environment without fluid recycling, mimicking the physiological conditions.

The Minucell ring method[17] offers a versatile tool for selection of suitable support filters for cell growth. Support filters were coated with extracellular matrix proteins for optimal cell attachment and development of the Caco-2 cells. Caco-2 cells seeded on support filters placed in Minucell rings were grown for a short period of 1 week in a CO_2 incubator for monolayer formation and placed in a perfusion culture container.

The experimental procedure designed herein involves simply perfusing the luminal and basal sides of Caco-2 cells continuously with the fresh culture medium. It reduces workload, resources and manpower for obtaining full-differentiated Caco-2 monolayers. It also allows an easy cultivation of a large number of monolayers in parallel and can provide similar information for the ranking of high permeable compounds, as does the conventional Caco-2 cell culture.

MATERIALS AND METHODS

Chemicals

Atenolol, acycloguanosine, 4-acetamidophenol, griseofulvin, furosemide, ranitidine, caffeine, carbamazepine, metoprolol, and theophylline were purchased from Sigma (Bornem, Belgium). Sodium fluorescein was provided by Across (Geel, Belgium). All supplements and cell culture media were purchased from Invitrogen, Inc., Merelbeke, Belgium.

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection [ATCC (www.atcc.org)], and were used between passage 38 and 50. The cultures were mycoplasma free (mycoplasma detection kit; Roche Gmbh, Mannheim, Germany). The cells were routinely maintained in plastic culture flasks 175 cm^2 (Becton Dickinson Biosciences, Erembodegem, Belgium). The cells

were subcultivated before reaching confluence. Caco-2 cells were harvested with 0.25% trypsin and 0.2% EDTA (5–15 min) at 37°C and seeded in new flasks. The culture medium, Dulbecco's Modified Eagle Medium (DMEM), was supplemented with 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 100 U/mL penicilline/streptomycine and 10% foetal bovine serum (FBS).

For the experiments using the perfusion system, Caco-2 cells were grown for an initial period of 1 week in a CO_2 incubator (5% CO_2) at 37°C prior to mounting in the perfusion system. The cell monolayers were used for transport studies at day 21–28 post seeding.

For transport experiments in the conventional system, Caco-2 cells were seeded in Snapwell™ polycarbonate inserts (24 mm insert diameter, 0.4 μm pore size) (Corning Costar, Cambridge, UK) at a seeding density of 1.4×10^5 cells/mL. The culture medium was exchanged every 2 days.

Matrix Proteins

Poly-L-lysine hydrobromide 150000–300000 MW (PLL) and collagen IV were supplied by Sigma and matrigel was obtained from Becton Dickinson (BD Biosciences, Erembodegem, Belgium).

The following coating concentrations of matrix proteins were used: 2.5, 5, 10, and 20 μg/cm^2. The proteins were diluted in sterile water from a stock solution of 3 mg/mL or 0.3 mg/mL (0.1% acetic acid) for PLL and collagen IV, respectively.

For matrigel, the stock solution was diluted to the desired concentration in DMEM medium without serum. Two hundred and fifty microliters of the coating solutions were dispensed per insert and incubated at 37°C for 2 h. The coated support filters were thereafter washed with medium without serum.

Perfusion Culture System

The 0.45 μm pore size support filters of polycarbonate or a mixture of cellulose acetate and nitrate were obtained from Millipore (Brussels, Belgium). Polyethylene terephtalate was purchased from Cyclopore, Whatman (Louvain-La-Neuve, Belgium). The support filters were fixed into Minucell rings (Minucells and Minutissue Gmbh, Bad Abbach, Germany) and autoclaved. The sterile support filters were placed in Minucell rings (Fig. 1A) and then into 24-well plates (BD Biosciences) for initial Caco-2 cell culturing. Caco-2 cells were seeded at 5.26×10^5cells/cm^2 (7×10^5 cells/mL/insert). The effective growth surface of the support filter fixed in Minucell rings is 0.5 cm^2. Following an overnight incubation, the support filters were transferred into 6-well plates containing 6 mL of complete DMEM medium (10% FBS). This medium was replaced every other day. After a period of 1 week in a CO_2 incubator, Caco-2 cells grown on support filters were placed in Minucell rings and were transferred into the perfusion culture chambers (Fig. 1B) at 37°C. CO_2 independent medium supplemented with 5% FBS, 100 U/mL penicillin and streptomycin, 2 mM L-glutamine, and 1% non-essential amino acids was used as the perfusion medium.

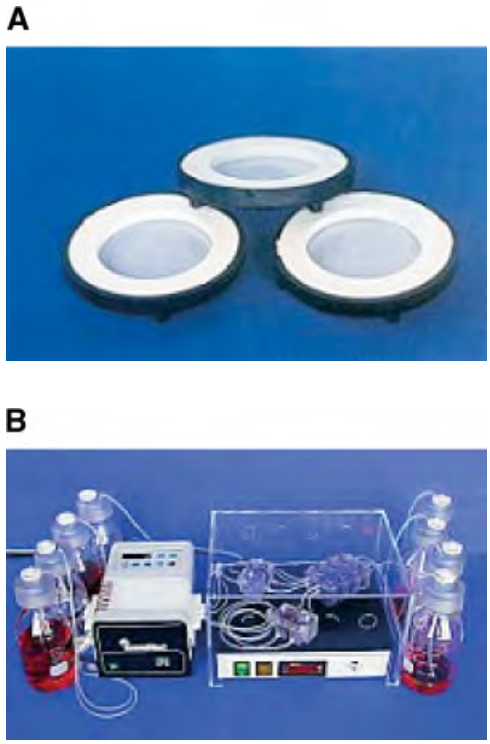

Figure 1. The perfusion system (Minucells and Minutissue) (A) the Minucell setup rings used to fix the support filters. The Minucells (Minusheets) can hold individually selected support filters of a diameter of 13 mm. (B) After an initial growth period of 1 week in a CO_2 incubator, Caco-2 cells seeded on support filters are placed in Minucell rings and mounted in a perfusion culture container maintained at 37°C. A container can hold 6 culture chambers of 24-inserts. At the left side, the fresh medium is continuously delivered via a peristaltic pump allowing a constant perfusion of 1 mL/h at the basal and apical side of the cells. Medium with metabolic products is removed at the upper right side.

A peristaltic pump guaranteed constant supply of 1 mL fresh medium/h. Fresh medium was taken

from a cooled reservoir and spent medium was pumped into a waste bottle. A heating plate at a constant temperature of 37°C maintained the temperature inside the perfusion culture system. The entire system operated on a laboratory table, at room temperature.

Assessment of the Growth Rate

The support filters were removed from Minucells, transferred in an empty 24-well plate and incubated with 250 μL of Trypsin-EDTA for 30 min at 37°C to detach Caco-2 cells. The cell suspensions were harvested, diluted in DMEM medium, stained with Trypan Blue, and counted using a hematocytometer.

Confocal Microscopy

The Minucell rings containing Caco-2 cells were removed from the incubator and put in an empty 24-well plate. The cells were incubated with 2×500 μL of 1% gluteraldehyde for 10 min at room temperature, in the dark. Then they were washed twice with 1 mL of Ca^{2+} and Mg^{2+} free PBS (Gibco, Inc.) and stained with 500 μL of propidium iodide (0.4 μg/mL in PBS) (Sigma). The cells were incubated for 5 min at room temperature in the dark and washed two times with milliQ water (Millipore). The support filters were removed from the inserts and put onto a microscopic glass slide with a drop of mounting medium, and covered. The mounting media is a solution of polyvinyl alcohol (MW = 10000) in saline, buffered to pH 7.2 and mixed with glycerol. The slides were analyzed on an inverted Laser Scanning Confocal Microscope (Carl Zeiss, Jena, Germany).

Measurement of Membrane Integrity

Measurement of transepithelial electrical resistance (TEER) was used to determine the integrity of the cell monolayers. The resistance of the cell monolayers grown on coated or uncoated support filters was measured using an Evom resistance volt-ohm meter (World Precision Instruments, Berlin, Germany). The background resistance values of the support filters without cells were subtracted from the resistance of support filters containing cells. Only the cell monolayers having TEER values above 100 Ωcm^2 were used in these experiments.

Transport Experiments

Prior to the transport of model compounds, the Minucell rings were placed in custom-made transport chambers. The transport experiments were set up using Caco-2 monolayers, which were either (i) differentiated in a perfusion system or (ii) grown in the Snapwells for a period of 21 days. The apical compartment of the cell monolayers was washed with Krebs buffer and allowed to equilibrate for 30 min at 37°C. For the apical to basolateral transport, the buffer was removed from the cells and replaced with 500 μL of the test compound (200 μM) on the apical side while for the basolateral to apical direction, the basolateral compartment was replaced with 4 mL of the test compound (200 μM). The monolayers were incubated for 120 min at 37°C, on a vibrax shaker at 80 rpm. At 0 and 120 min, the resistance was measured. The transport experiments were performed under "sink" conditions, when the concentration of the drug in the receiver side (4 mL) was <10% of the dosing concentration at all time points (30, 60, 90, and 120 min). The amount of drug accumulated in the basal compartment at 30, 60, 90, and 120 min was determined by LC/UV or LC/MS. The transport experiments were performed at least in triplicate.

Analytical Methods

The model compounds were analyzed using HPLC (Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector). Atenolol, 4-acetamidophenol, and ranitidine were also analyzed using a ThermoFinnigan LCQ DecaXP iontrap mass spectrometer equipped with an electrospray (ESI) ionization source. The LC eluent was introduced into the mass spectrometer, going through the LCQ's divert valve. Samples were analyzed for their content using the mass spectrometer and the data were processed using the Xcalibur software (version 1.3). The MS detection limits of atenolol, 4-acetamidophenol, and ranitidine were 25, 62.5, and 6.25 ng/mL, respectively.

Furosemide, acyclovir, and metoprolol were quantitatively determined with reversed-phase HPLC using an isocratic elution. The limits of detection for the analytical methods were 10 ng/mL for furosemide and acyclovir and 4 ng/mL for metoprolol. The columns used included an Xterra™ RP18 (4.6×100 mm) (Waters Corporation) with a mean particle size of 3.5 μm and a SymmetryShield™ Sentry RP8 (3.9×20 mm) guard column (Waters Corporation) with a mean particle size of 5 μm. The mobile phase consisted of an aqueous phase of a 0.5% (v/v) phosphoric acid

solution and acetonitrile (55:45, 95:5, and 83:17, respectively). The injection volume was 25 μL, the flow rates were 1.20, 0.70, and 1.20 mL/min, and the UV detection wavelengths 275, 255, and 224 nm, respectively.

Carbamazepine and griseofulvin were also analyzed with reversed-phase HPLC using an isocratic elution. The limits of detection for the analytical methods were 290 ng/mL for carbamazepine and 470 ng/mL for griseofulvin. The column used was Xterra™ MS C18 (3.0 × 50 mm) (Waters Corporation) with a mean particle size of 5.0 μm and a SymmetryShield™ Sentry RP8 (3.9 × 20 mm) guard column with a mean particle size of 5 μm. The mobile phase consisted of an aqueous 0.5% (v/v) phosphoric acid solution and acetonitrile (75:25). The injection volume was 25 μL, the flow rate 2.00 mL/min, and the detection wavelengths 285 and 295 nm, respectively.

Theophylline was analyzed with reversed-phase HPLC using a gradient elution. The limit of detection for the method was 160 ng/mL. The column was a Xterra™ MS C18 (3.0 × 50 mm) (Waters Corporation) with a mean particle size of 5.0 μm and a SymmetryShield™ Sentry RP8 (3.9 × 20 mm) guard column with a mean particle size of 5 μm. The mobile phases consisted of A (1% (v/v) phosphoric acid in water) and B (100% acetonitrile). The gradient profile was 0–2.5 min, 5–50% B; 2.5–5 min, 5% B. The injection volume was 25 μL, the flow rate 1.50 mL/min, and the detection wavelength 271 nm.

Caffeine was analyzed with reversed-phase HPLC using an isocratic elution. The column was a Hypersil ODS RP18 (4.0 × 100 mm) (Agilent Technologies) column with a mean particle size of 3.0 μm and a SymmetryShield™ Sentry RP8 (3.9 × 20 mm) guard column with a mean particle size of 5 μm. The mobile phase consisted of an aqueous 26 mM ammonium acetate solution and acetonitrile (88:12). The injection volume was 25 μL, the flow rate 1.20 mL/min, and the wavelength 274 nm. The method allowed the detection of at least 150 ng/mL of caffeine.

All the analytical methods were validated (J&J internal communication).

Transport of Sodium Fluorescein

The paracellular transport was assessed using sodium fluorescein. After an initial period of incubation with the test compound, the cell monolayers were rinsed and incubated with sodium fluorescein for an additional time of 60 min at 37°C, on a vibrax shaker at 80 rpm. The diffusion of sodium fluorescein into the basal compartment was determined by measuring the fluorescence intensity (excitation 485 nm, emission 535 nm).

Data Analysis

The transport rate (dQ/dt) was calculated by plotting the amount of drug transported to the receiver side versus time (30, 60, 90, and 120 min) and then determining the slope of this plot. The apparent permeability coefficients (P_{app}, cm/s) were calculated from the following equation: $P_{app} = \frac{dQ/dt}{C_o \cdot A}$ where dQ/dt is the permeability rate (μmol/s), C_o is the initial drug concentration on the apical side of cell monolayers (200 μM), and A is the surface area of the membrane filter in cm^2.[18]

Statistical Analysis

Statistical analysis were done using Anova test (Statgraphics Plus, version 3.3). The multiple range tests was applied to determine which means were significantly different from others. The method currently used to discriminate among means was the Tukey's honestly significant difference (HSD) procedure.

RESULTS

Development of Caco-2 Cell Monolayers Grown Onto Minucell Rings Under Static Conditions

Growth Rate

Caco-2 cells were pre-cultivated on polycarbonate (0.45 μm pore size) support filters fixed in Minucell rings. The support filters were coated with extracellular matrix proteins and the cells were grown for 1 week in a CO_2 incubator in the standard Caco-2 medium as described in the "Materials and Methods" section. The ability of Caco-2 cells to attach and grow on polycarbonate support filters was analyzed. The results in Figure 2 illustrate the attachment and growth rate of Caco-2 cells seeded on polycarbonate (Fig. 2A) or polyethylene terephtalate (Fig. 2B) mounted on Minusheets and coated or not with extracellular matrix proteins. Counting of detached cells showed various results depending on the type of support filters and of the coating reagents:

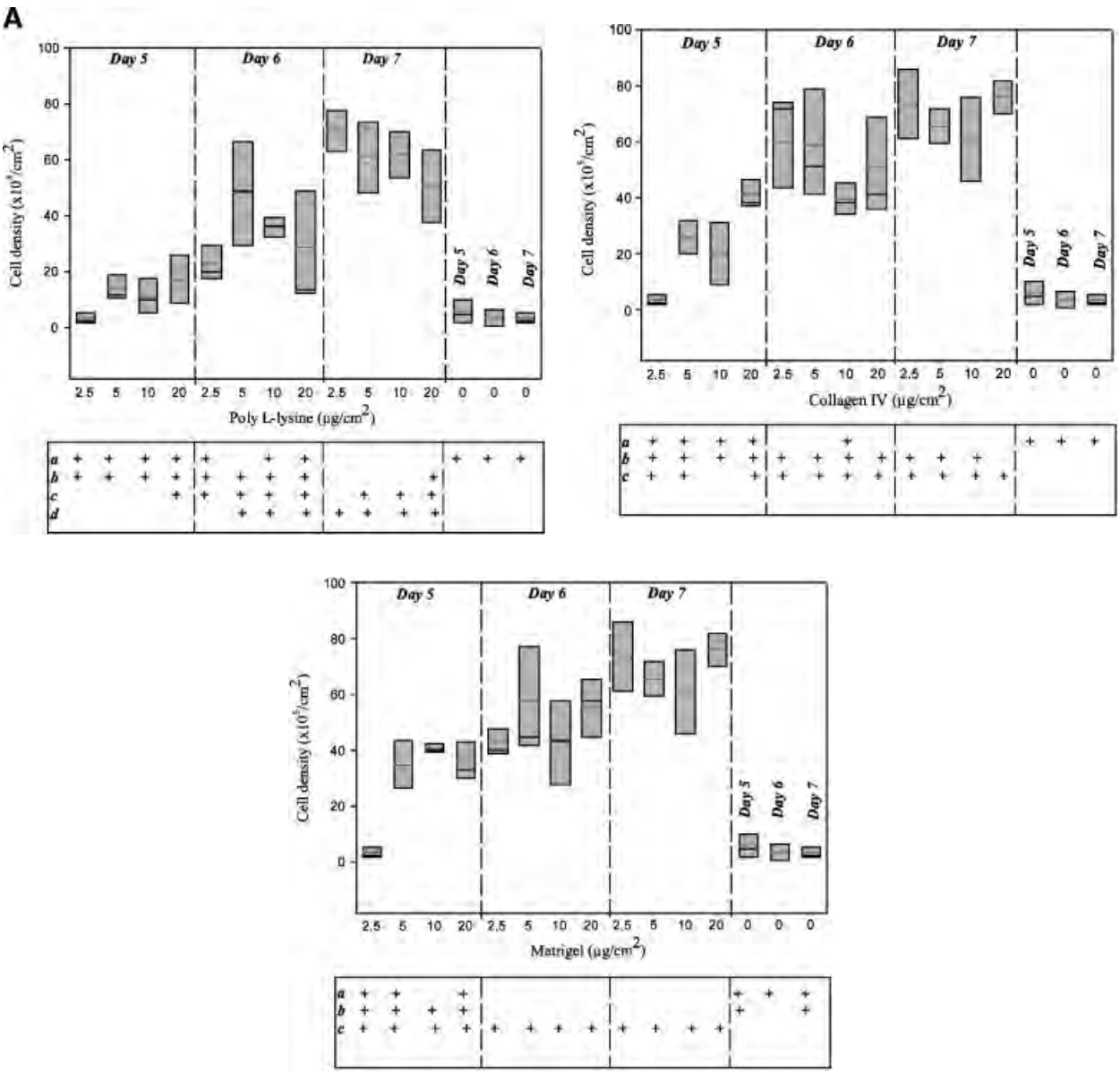

Figure 2. Growth of Caco-2 cells on polycarbonate support filters (A) or polyethylene terephtalate (B) placed in Minucell rings. The support filters were coated with poly-L-lysine, collagen IV, and matrigel. The cells were incubated for 5, 6, 7 days in a CO_2 incubator and the number of attached cells was counted as described in "Materials and Methods." Each bar represents the mean and standard deviation of three individual experiments. At the bottom of each graph, homogenous groups are identified using the letters a, b, c, or d. There are no statistically significant differences within homogenous groups.

(i) At day 5, the growth of Caco-2 cells seeded on polycarbonate support filters coated with 2.5, 5, or 10 µg of poly L-lysine, showed statistically significant differences compared to day 7 ($p < 0.05$).

(ii) Caco-2 cells seeded on polycarbonate support filters coated with 10 µg of collagen IV revealed a statistically significant difference at day 5 compared to the growth of the cells seeded on inserts coated with 20 µg of collagen IV, at day 7.

(iii) There were no significant differences regarding the growth of Caco-2 cells seeded on polycarbonate support filters coated with increasing amounts of matrigel at day 5 compared to day 6 and 7.

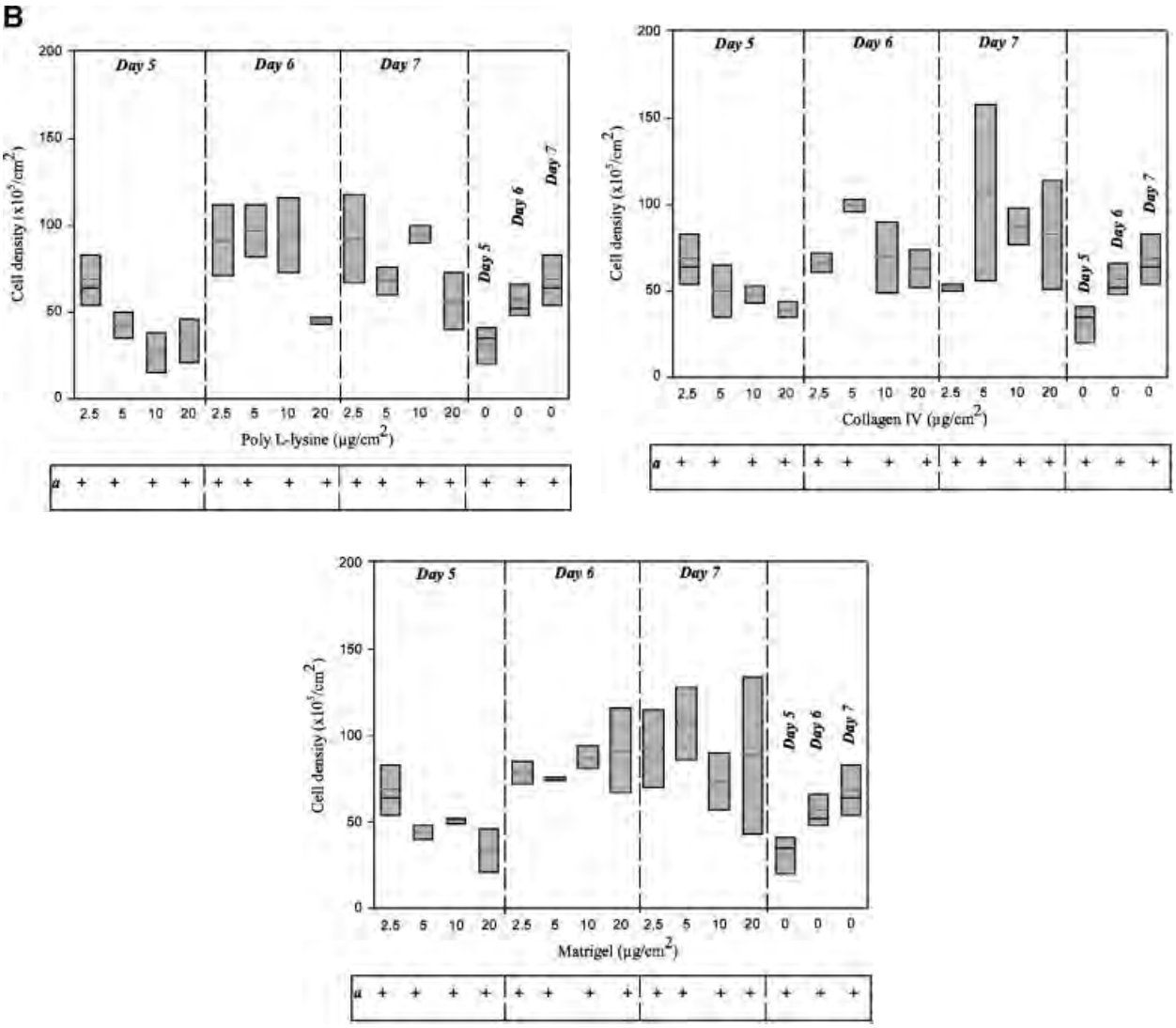

Figure 2. (*Continued*)

(iv) Strikingly, unlike Snapwells^{TM}, Caco-2 cells do not grow on uncoated polycarbonate support filters fixed in Minucell rings. The overall growth of the cells was statistically significantly different on non-coated polycarbonate support filters mounted on Minusheets compared to the cells grown on similar material coated with increasing amounts of poly L-lysine, collagen IV or matrigel at any time during the experiments ($p < 0.05$) (Fig. 2A).

(v) The cell density on polyethylene terephtalate support filters determined at day 5, 6, and 7 indicates however that Caco-2 cells reached a monolayer density 5–7 days after seeding on uncoated as well as polyethylene terephtalate support filters coated with poly L-lysine, collagen IV, or matrigel proteins (Fig. 2B). These results indicate that regarding the growth of Caco-2 cells seeded on polyethylene terephtalate support filters, there were no statistically significant differences at day 5 compared to day 6 and 7.

Laser Scanning Confocal Microscopy

Caco-2 cells were cultivated on non-coated or coated polycarbonate filters for a period of 1 week in a CO_2 incubator. The cells were morphologically analyzed using a scanning confocal microscope. Substantial phenotypic difference could be observed between Caco-2 cells grown on non-coated polycarbonate support filters and those

grown on support filters coated with poly-L-lysine, collagen IV, or matrigel. As shown in Figure 3A, Caco-2 cells seeded on non-coated polycarbonate support filters placed in Minucell rings, did not form a monolayer. However, propidium iodide staining of Caco-2 cells showed that the precoating of polycarbonate support filter with poly-L-lysine, collagen IV, or matrigel (Fig. 3) facilitated the monolayer formation.

The structure of the cell monolayers was highly dependent on the support filters used for Caco-2 cell attachment. Caco-2 cells cultivated on non-coated polyethylene terephtalate support filters formed a confluent monolayer (Fig. 4). The cell monolayers were more uniform on support filters coated with poly-L-lysine, collagen IV, or matrigel (Fig. 4).

Differentiation of Caco-2 Cells in a Perfusion System

TEER

The development of Caco-2 cell monolayers in the perfusion system was assessed by the measurement of TEER for cells grown on polycarbonate support filters coated with 2.5 μg/cm^2 collagen IV (21 days in perfusion culture) and for cells grown according to the traditional method.

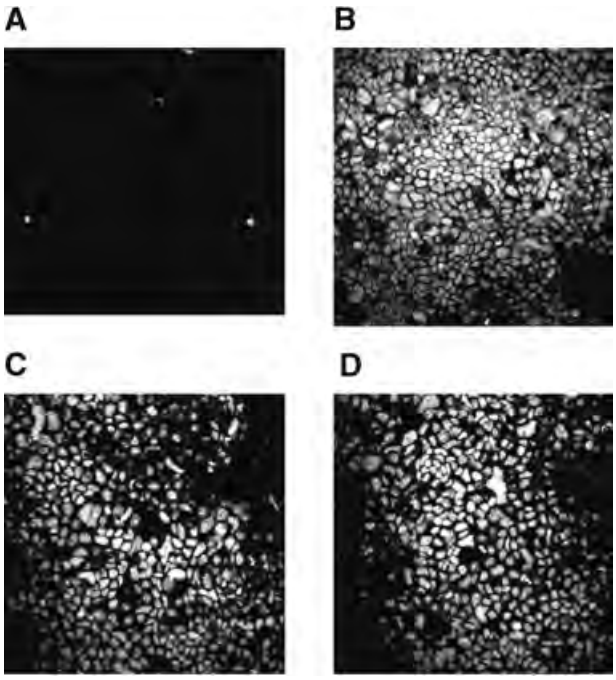

Figure 3. Confocal microscopy images of Caco-2 cells grown on polycarbonate support filters placed in Minucell rings not coated (A) or coated with 10 μg/cm^2 of poly-L-lysine (B), collagen IV (C), or 2.5 μg/cm^2 of matrigel (D) matrix proteins. At day 7 following preculture in a CO_2 incubator, cells were stained by propidium iodide as described in "Materials and Methods" and analyzed on a confocal scanning microscope (magnification 40×).

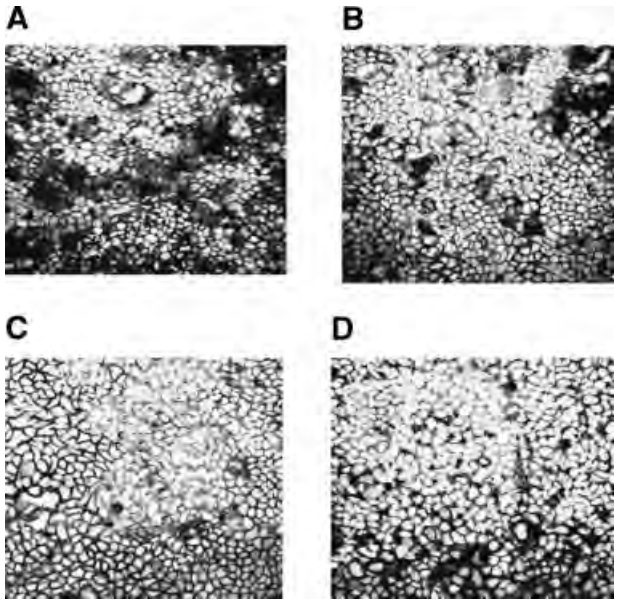

Figure 4. Confocal microscopy images of Caco-2 cells grown on non coated (A) or polyethylene terephtalate support filters coated with 10 μg/cm^2 of poly-L-lysine (B), collagen IV (C), or 2.5 μg/cm^2 of matrigel (D) proteins. At day 7 following pre-culture in a CO_2 incubator, the cells were stained by propidium iodide as described in "Materials and Methods" and analyzed on a confocal scanning microscope (magnification 40×).

Following the perfusion culture, the TEER value of Caco-2 cells grown on polycarbonate support filters was $224 \pm 84\ \Omega\ cm^2$. This value is lower ($p < 0.05$) than the value of Caco-2 cells grown in traditional Snapwells^{TM} ($856 \pm 78\ \Omega.cm^2$) under static conditions (Fig. 5).

The lower TEER value of Caco-2 cells grown in perfusion system suggests that the perfusion system provides a relatively leaky system resembling the functional colon.[19]

Transport of Theophylline

The influence of drug diffusion properties through the blank support filters placed in Minucell rings, was important to consider prior to the passive diffusion transport studies of model compounds. Figure 6 indicates that the transport across polycarbonate support filters fixed in Minucell rings (0.45 μm pore size), in absence of cells, showed no restriction for the diffusion of theophylline while polyethylene terephtalate support filter, 0.45 μm

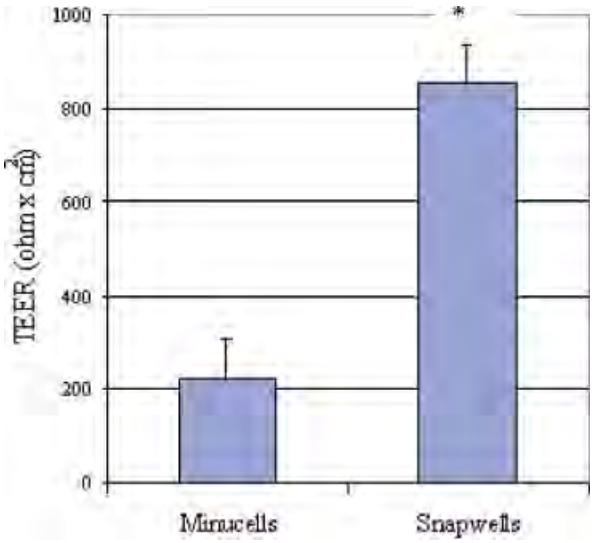

Figure 5. Transepithelial electrical resistance (TEER) of Caco-2 cells seeded on polycarbonate support filters placed in Minucell rings or on polycarbonate Snapwells. All the inserts were coated with 2.5 μg/cm^2 of collagen IV. The cells were cultivated according to the standard procedures or in the perfusion system as detailed in "Materials and Methods." Prior to drug transport experiments, the resistance of the cell monolayers was controlled. Each bar represents the mean and standard deviation of 15 Minucell and 6 individual Snapwell inserts. * means $p < 0.05$.

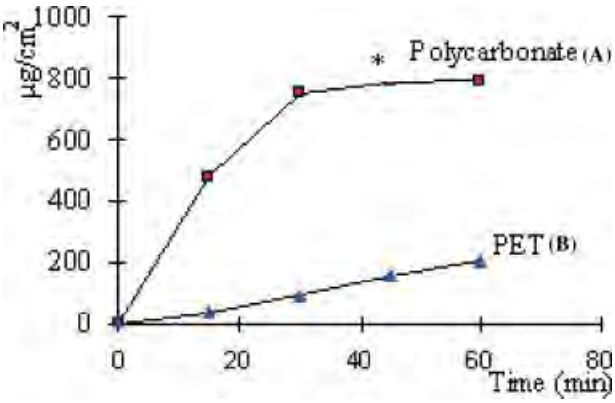

Figure 6. Schematic representation of the rate of theophylline transported through polycarbonate (A) or polyethylene terephtalate (B) support filters placed in Minucell rings free of cells. The experimental calculation is made with cumulative fraction of the drug transported. The results are plotted as μg/cm^2 in function of time and show statistically significant difference between polycarbonate and polyethylene terephtalate ($p < 0.05$).

pore size, constituted a limiting factor for drug transport. Therefore, the hydrophilic polycarbonate support filters were used to analyze the passive diffusion transport because these support filters do not restrict the diffusion of most drugs.[20]

Theophylline is used as a marker molecule to assess passive transcellular transport. The transport from apical (A) to basolateral (B) direction of Caco-2 cell monolayers allows ranking of compounds while the basolateral to apical transport in combination with the apical to basolateral transport, allows the identification of whether the compound is a potential substrate of intestinal efflux mediated by proteins such as P-glycoprotein (Pgp). The result of theophylline transport from A to B as indicated in Figure 7 emphasized the ranking of theophylline in the high permeability class of drugs. The similar apparent permeability coefficients obtained for the transport of theophylline from A to B and B to A, respectively 31.4×10^{-6} and 31.9×10^{-6} cm/s, suggested that no effect of efflux carrier on transepithelial transport of theophylline was present.

Transport of Passively Diffused Model Compounds

The passive diffusion of model compounds was assessed from the apical to the basolateral side of the cell monolayers. The apparent permeability coefficients obtained with the perfusion system were compared to those obtained in the traditional

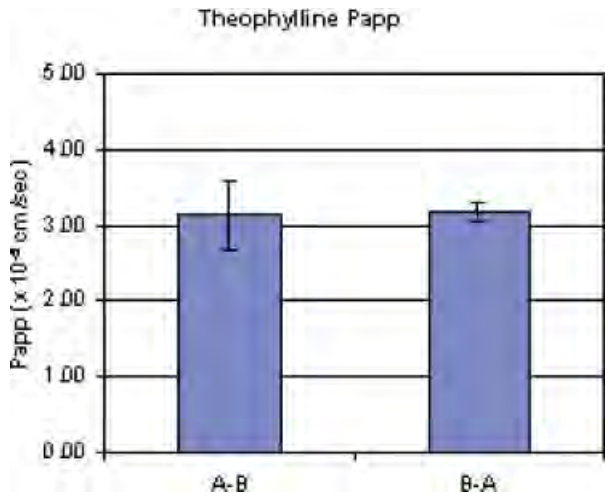

Figure 7. Apparent permeability coefficients of theophylline. The transport of theophylline (200 μM) was performed from apical to basolateral (A–B) or from basolateral to apical (B–A) of the cell monolayers seeded on polycarbonate support filters, placed in Minucell rings and grown for 21 days in the perfusion system. Each bar represents the mean and standard deviation of four individual inserts.

system. The results showed that the P_{app} of highly permeable drugs ranged from 30.8 to 85.4×10^{-6} cm/s in the perfusion system (Fig. 8). The P_{app} of caffeine, carbamazepine, griseofulvine, and metoprolol were approximately two times higher compared to their transport across Caco-2 cells differentiated in the standard system ($p < 0.05$), while theophylline and acetamidophenol showed a similar P_{app} in the two systems.

The transport of low permeable model compounds, acyclovir, atenolol, ranitidine, and furosemide was also assessed using Caco-2 monolayers differentiated in the perfusion system and cells from the traditional 21-day system. Higher P_{app} values were obtained for these model compounds using the perfusion system than using the standard Snapwell inserts (Table 1). These results indicate an increase of apparent permeability coefficients of several compounds assessed using Caco-2 cells differentiated in the perfusion system, indicative of an enhancement of permeability in the perfusion system probably related to the increase of paracellular junctional permeability.

Effect of Extracellular Protein Coating Concentration on the Apparent Permeability Coefficient

Caco-2 cells were cultivated on polycarbonate or polyethylene terephtalate support filters. To determine whether the coating concentration could influence the permeability of drugs, the support filters were precoated by increasing concentrations of extracellular matrix proteins; 2.5, 5, 10, and 20 μg/cm^2 of poly-L-lysine, collagen IV, or matrigel. The cells were grown in the perfusion system for 1 month. The transport of theophylline from the apical to the basal side of the cell monolayers is presented in Figure 9. While the apparent permeability coefficients of theophylline obtained from the cells seeded on polycarbonate support filters and grown in the perfusion system were statistically different compared to the values obtained with polyethylene terephtalate support, there was however no influence regarding the amounts of poly-L-lysine, collagen IV, or matrigel used for coating the Minucell devices. It is also interesting to note that the P_{app} of theophylline

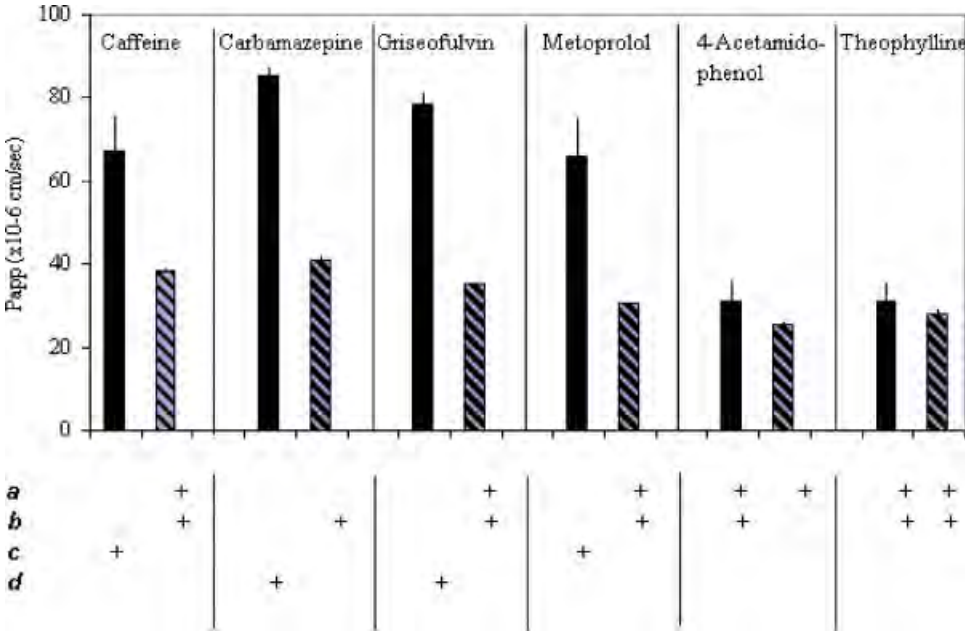

Figure 8. Transport of passively diffused model compounds through Caco-2 cells. The cells were cultivated on polycarbonate support filters, placed in Minucell rings and maintained 21 days in the perfusion system (back bars) or on Snapwells™ (hatched bars). The transport of the model compounds (200 μM) was performed from the apical to the basolateral direction of the cell monolayers. Each bar represents the mean and standard deviation of four individual inserts for the perfusion system and three inserts for the Snapwells™. At the bottom of the graph, homogenous groups are identified using the letters a, b, c, and d. There are no statistically significant differences between data belonging to a homogenous group.

Table 1. Apparent Permeability Coefficient (P_{app}: cm/s) Values of Model Compounds Across Caco-2 Cells Grown on Polycarbonate Support Filters Placed in Minucell Rings ($n = 6$) or Snapwells ($n = 3$)

		$P_{app} \times 10^{-6}$ (cm/s)			
		Minucell Rings		Snapwells	
Compound	Permeability	Mean	SD	Mean	SD
Acetoamidophenol	High	30.9	5.5	25.5	0.9
Caffeine	High	67.3	8.6	38.5	0.6
Carbamazepine	High	85.4	2.2	40.8	1.2
Griseofulvin	High	78.3	3	35.4	0.2
Metoprolol	High	65.8	9.4	30.8	0.0
Theophylline	High	31.4	4.7	28.2	1.5
Acyclovir	Low	1.7	85	<1	N.D.
Atenolol	Low	1.98	14	0.1	0.0
Furosemide	Low	1.94	62	<0.02	N.D.
Ranitidine	Low	6.78	1.48	0.003	0.0

The filters were coated with 2.5 μg/cm^2 of collagen IV. P_{app} values were calculated as described in "Materials and Methods."
N.D., not determined.

obtained from the cells seeded on non-coated polyethylene terephtalate support filter was similar to their counterpart coated with extracellular matrix proteins (Fig. 9).

Transport of Sodium Fluorescein

TEER values are indicative for the transjunctional flux of ions that are much smaller than the tight junction dimensions. Therefore, TEER values alone are not sufficient to assess the integrity of monolayers when considering drug transport. An alternative and more relevant methods to assess monolayer integrity is to study the transport of hydrophylic marker molecules such as mannitol, sodium fluorescein, lucifer yellow, PEG, and dextran.[21,22] Sodium fluorescein (MW 376) was chosen to assess the paracellular permeability

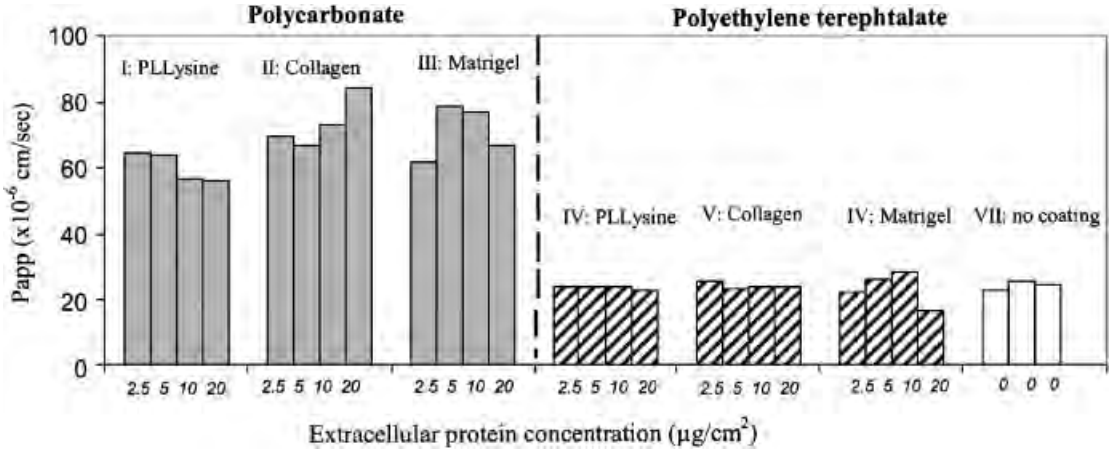

Figure 9. Effect of coating concentration on the apparent permeability coefficient of theophylline. The polycarbonate or polyethylene terephtalate support filters placed in Minucell rings were coated by increasing concentration of poly-L-lysine, collagen IV, or matrigel. Caco-2 cells were seeded and grown for 1 month in the perfusion system. The transport of theophylline (2.5 mM) from apical to basal direction was performed. Data are representative of individual experiments. The groups I versus III and II versus III belong to homogenous groups a and b, respectively while IV, V, VI, and VII belong to the homogenous group c. There were no statistically significant differences within a homogenous group.

and the tightness of the monolayers. The use of sodium fluorescein is advantageous because after the drug transport experiments, the cells can be rinsed and reused. The sodium fluorescein allows one to monitor the effect of drugs on the integrity of the cell monolayers.

As shown in Figure 10, sodium fluorescein transport across Caco-2 cells differentiated in a perfusion system was significantly different ($0.7 \pm 0.68\%$) compared to the transport across Caco-2 cells cultivated according to the standard procedure ($0.08 \pm 0.05\%$) ($p = 0.014$).

DISCUSSION

In this study, a perfusion system was implemented to generate functional and highly differentiated Caco-2 cells. The system uses special cell and tissue carriers (Minucells and Minutissue) to attach different support filters, allowing the selection of the optimal conditions for Caco-2 cell attachment and growth. In the perfusion system, the medium is constantly renewed at the apical and basal side of the cell monolayers, thus resulting in a continuous removal of metabolite wastes.

Optimal support and cell adhesion is an important prerequisite for differentiation. The quality of the cell monolayers was highly dependent upon the support filters to which Caco-2 cells were attached as well as the extracellular matrix proteins.

Caco-2 cells did not grow on uncoated polycarbonate support filters placed in Minucell rings while they did grow on uncoated polycarbonate Snapwells™. The Snapwell devices are modified Transwell™ culture inserts with 12 mm cell culture treated membranes housed in a detachable ring. The devices are gamma-irradiated for optimal cellular adhesion, allowing convenient polarized cell culture on both polycarbonate and polyester support filters.

The optimal attachment of Caco-2 cells required coating of polycarbonate support filters placed in Minucell rings with poly-L-lysine, collagen IV, or matrigel.

Poly-L-lysine as well as collagen IV and matrigel (a basement membrane matrix containing laminin, collagen IV, and entactin) promoted cell attachment and differentiation. The effect of those extracellular matrix proteins indicates that Caco-2 cells find an optimal anchorage when polycarbonate support filters placed in Minucell rings were coated.

Unlike polycarbonate, Caco-2 cells grown on non-coated polyethylene terephtalate support filters placed in Minucell rings were confluent as determined by confocal microscopy. In contrast, Caco-2 cells did not grow either on non-coated, or

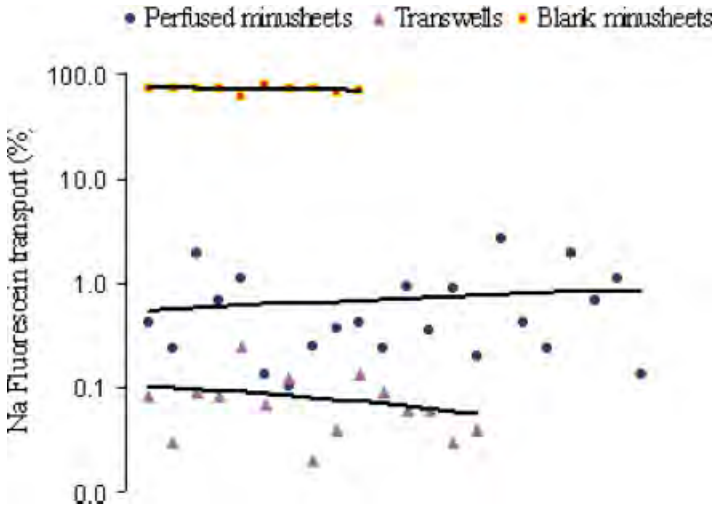

Figure 10. Transport of sodium fluorescein through Caco-2 cells. Polycarbonate support filters placed in Minucell rings or Snapwells™ were coated with 2.5 μg/cm^2 of collagen IV. The cells were allowed to grow and were maintained 21 days in the perfusion culture or in the CO_2 incubator. The blank polycarbonate support filters did not contain cells. Following the transport of model compounds for 2 h, the apical compartment containing the drug substance was replaced by sodium fluorescein. The cells were incubated for an additional hour at 37°C. Fluorescence intensity was determined.

on coated nitrocellulose acetate support filters placed in Minucell rings (data not shown).

The degree of cellular differentiation in cultured epithelia depends not only on the appropriate extracellular matrix proteins, but also on a continuous supply of nutrients and growth factors, the elimination of metabolic products and the prevention of an accumulation of synthesized paracrine factors.[23] It is clear that optimal differentiation can only be obtained, if the culture environment is adapted, as close to the natural situation as possible. Furthermore, the *in vitro* differentiation of cells and tissues depends not only on a single morphogenic substance but also on several environmental factors.[24] The *in vitro* culture of immortalized cell lines on tissue culture dishes often does not reflect the microenvironment of the cells *in vivo*. In the stagnant environment of conventional culture dishes, Caco-2 cells undergo enterocytic differentiation, which makes them attractive as a potential model system of polarized small intestinal epithelium.[25] However, due to the loss of morphological, physiological, and biochemical features by dedifferentiation following extensive long-term cultures, the limited shelf life of Caco-2 cells differentiated in the conventional inserts led to the implementation of culture protocols which speed up the Caco-2 cell differentiation process, usually less than 1 week.[9,10,26] Nevertheless the limited published information about the performance of Caco-2 monolayers cultivated under the accelerated protocols suggests that the cells are not fully differentiated and therefore have to be used at a certain time point.[27]

One of the advantages of the perfusion system developed for Caco-2 cell culture is that it is semiautomated. It offers a user-friendly, simple and easy way for long-term culturing (up to 2 months) with lower manipulation and risk of contamination. The system mimics the *in vivo* situation with the delivery of growth factors via a permanent perfusion of the culture medium on the basal and apical side of the cells.[28]

Another advantage is that the culture containers are closed, so a CO_2 incubator is not needed.[14] Cultures in a CO_2 incubator are usually buffered using a high amount of $NaHCO_3$ and a 5% CO_2 atmosphere to maintain a constant pH of 7.4.[13] The use of such a medium in perfusion culture in the absence of a CO_2 incubator decreased the pH from a physiological range to acidic values due to the low content of CO_2 in room air (0.3%). For that reason, the medium used was independent of CO_2 supply. It contains a buffering system that maintains pH at 7.0–7.6, thus preserving the viability of Caco-2 cells as judged microscopically (data not shown). The differentiation of Caco-2 cells is accompanied by the coordinate down-regulation of genes involved in cell cycle progression and DNA synthesis, which reflected the concomitant reduction in cell proliferation.[29] The perfusion system used herein yielded high specimen viability and retention of cellular structure.

Some limitations for the use of Caco-2 cells in predicting intestinal drug absorption, have been mentioned. Compounds of intermediate or low permeability have a lower permeability in the Caco-2 model than *in vivo*[30] and the paracellular route in Caco-2 cells is tighter than in the small intestine. Because new drugs discovered by combinatorial chemistry and high throughput screening generally are larger and have more groups that form hydrogen bonds than historical drugs, the paracellular route contributes significantly to their transepithelial transport. Caco-2 cells as cultivated in the standard Snapwells demonstrated tight junctions, which restrict the permeability of poorly absorbed drugs. Ranitidine, atenolol, furosemide, and chlorothiazide, which are adequately absorbed in humans (greater than 50% of dose),[8] showed poor permeability in the standard 21-day Caco-2 cell monolayers. The lower paracellular permeability of Caco-2 cell monolayers cultivated in the conventional inserts has been attributed to the colonic origin of the cell line. Some studies suggest that the Caco-2 cell line has significant permeability via the paracellular route, comparable to the tips of the villi of the small intestinal mucosa, and structural differences between the cell cultures and the intestine *in vivo* are the cause of the lower apparent paracellular permeability in the Caco-2 model.[30,31] While the average pore radius of the tight junctions in the human intestine is around 8–13 Å, the corresponding radius in Caco-2 cells is about 5 Å.[27]

The Minuth perfusion system as implemented here for Caco-2 cell culture showed functional differences in the transport of poorly permeable drugs when compared to the Snapwell system. The increased apparent permeability coefficients of those compounds might suggest that the continuous perfusion of fresh culture medium with an optimal nutrition and a removal of metabolites from the cellular environment impacted the Caco-2 cell differentiation, which might mimic *in vivo* conditions. The dynamic flow in the perfusion system has no effect on the barrier functionality of Caco-2 cells as the cells seeded on Minucell devices

and cultivated in a CO_2 incubator also displayed low TEER values (see article II). The barrier function of the tight junctions contributes significantly to the total barrier function of the intestinal epithelium and it is generally accepted that the variations in tight junction permeability explain differences in barrier function.[32]

Acyclovir, atenolol, furosemide, and ranitidine were transported through Caco-2 cells grown in the perfusion system. These poorly permeable drugs were detectable in Caco-2 cells from the perfusion system by LC/UV, avoiding the use of LC/MS/MS.

The apparent permeability of highly permeable drugs, metoprolol, carbamazepine, and griseofulvin was increased across Caco-2 cells grown in the perfusion system compared with the Snapwell inserts. The passive diffusion transport of those model compounds showed a good correlation ($r^2 = 0.9592$) between the perfusion system and the static system; the ranking of permeability for the high permeable drugs was similar in the two systems. Although, the rank-order of permeability of model compounds was maintained in the perfusion system and higher P_{app} values were obtained for caffeine, carbamazepine, griseofulvine and metoprolol probably due to the increased paracellular permeability, theophylline and acetomidophenol showed similar P_{app} values in the two systems. Other transport mechanisms might be involved. The active transport of theophylline for instance is a matter of debate. In our experiments, no efflux mechanism was observed in the transport of theophylline as similar P_{app} were obtained from apical to basolateral versus basolateral to apical direction. A few animal studies suggest, however, that theophylline may be partially absorbed by an active transport mechanism. Carbohydrates have been found to increase the absorption rate of phenytoin, another acidic drug with a pKa similar to that of theophylline. This emphasizes the complexity of factors regulating the absorption of drugs and their interactions with dietary components.[33] Theophylline may also increase intracellular cyclic AMP to stimulate H^+ and Cl^- secretion. Cyclic AMP, outside the serosal membrane, stimulates active transport of Na^+.[34]

In conclusion, the experimental procedure designed here is straightforward and reduces the workload, resources and manpower for obtaining full-differentiated Caco-2 monolayers. It also allows an easy long-term cultivation of a large number of monolayers and provides similar information for ranking passively diffused compounds compared to the conventional 21-day culture method. This system is arguably a better reflector of the *in vivo* situation based on morphologic and functional features.

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