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Usefulness of a novel Caco-2 cell perfusion system II. Characterization of monolayer properties and peptidase activity

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Received April 26, 2008, accepted May 30, 2008

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Pharmazie 64: 36–42 (2009) *doi: 10.1691/ph.2008.8592*

In this study, the enzymatic activity and the influence of support filters and extracellular matrix proteins on the differentiation of Caco-2 cells grown in a perfusion system (Minucells and Minutissue™) were examined and compared to traditional culturing approaches. Differences were observed regarding the differentiation of Caco-2 cells using the traditional approach and perfusion system such that the cell monolayers grown in a perfusion system showed a significant increase in dipeptidase activities (18.20 ± 0.43 nmol $\cdot$ min^1 $\cdot$ cm^{-2}) compared to the cells cultivated using the 21-day protocol (9.45 ± 0.50 nmol $\cdot$ min^{-1} $\cdot$ cm^{-2}). The peptidase activity of Caco-2 cells was strikingly inhibited when Matrigel extracellular protein was used for coating polycarbonate support filters. While the enzymatic activities of the cell monolayers differentiated in the perfusion system were up-regulated, the transepithelial electrical resistance values of the cell monolayers (171 ± 52 and 251 ± 62 $\Omega \cdot$ cm^2 for polycarbonate and polyester, respectively) decreased compared to the traditional Snapwell inserts (644 ± 119 $\Omega \cdot$ cm^2). The results suggested that the perfusion systems were useful permeability models which reduce workload, resources and manpower needed to obtain useful Caco-2 monolayers. In addition, the approach offers an efficient tool for long-term culturing of highly differentiated Caco-2 cell monolayers.

1. Introduction

Caco-2 cell lines are the most popular *in vitro* model to explore the mechanism of intestinal drug absorption and metabolism of drugs. The cell line spontaneously transforms into enterocyte-like structures and expresses a number of drug metabolising enzymes. These include alkaline phosphatase, disaccharide hydrolases, aminopeptidase A, aminopeptidase N, dipeptidylpeptidase IV as well as cytochrome P450 1A1 and 1A2 (Fogh et al. 1977; Hauri et al. 1985; Rousset 1986; Hidalgo and Ji 1996). The aminopeptidase and dipeptidyl peptidase families are two of the numerous enzymes of the intestinal brush border of the small intestine responsible for digesting amino acids and dipeptides located at the amino terminus of peptides and proteins. These enzymes contribute to the metabolism of peptides and proteins, and thus limit intestinal absorption of peptides. Both enzymes are present in their active form in Caco-2 cell culture model (Howell et al. 1993b; Ingels et al. 2002) and can be used as an indication for the differentiation of a cell monolayer.

Although Caco-2 cell lines are a useful model for studying intestinal epithelial drug transport, the traditional culturing of fully differentiated Caco-2 monolayers is time- and labor-intensive due to the need for frequent replacement of the supportive medium. The preparation of a fully differentiated, confluent Caco-2 monolayer requires at least 21 days of cell culturing (Liang et al. 2000) as well as intensive cell feeding procedures. To simplify the process of Caco-2 cell culturing, a perfusion system was considered and developed. The functionality of the system was demonstrated in a previously published study (Masungi et al. 2004). Our objective in this previously presented work was to adapt Caco-2 cell culturing to provide a monolayer transport model which is more representative of the *in vivo* situation and to explore its usefulness for metabolism studies.

Environmental conditions for epithelia can be simulated with Minusheet cell and tissue carriers combined with the perfusion system (Minucells and Minutissue) (Minuth et al. 1994; Kloth et al. 1995; Minuth et al. 1997; Sittinger et al. 1997; Minuth et al. 1999; Kloth et al. 2000; Minuth et al. 2000). In this approach, the cell medium is constantly perfused to the apical and basolateral sides of the cultures which results in a continuous supply of nutrients. The perfused culture medium permits efficient nutrient consumption in high-density cell cultures and minimizes the accumulation of toxic metabolites by a constant removal of the waste (Schumacher et al. 2002a). The perfusion system not only optimizes the delivery of culture medium components to the cells but also prevents accumulation of secreted autocrine factors (Risbud and Sittinger 2002). By this design,

the perfusion system allows for the maintenance of cells in a healthy state for an extended period of time. The system also offers other advantages including the ability to select the support filters for optimal cell anchorage.
In the current study, the activity of dipeptidyl peptidases and aminopeptidases were assessed in the apical side of the cell monolayers grown in the perfusion system. In addition, the confocal analysis was applied to assess structural characteristics of Caco-2 cell layers differentiated in the novel system.

2. Investigations and results

2.1. Effect of continuous medium perfusion on the tight junction properties

2.1.1. Transepithelial electrical resistance (TEER)

The epithelial apical junction complex forms a network of transmembrane scaffolds and signalling proteins, and serves as a barrier to control cell polarity, proliferation and differentiation. The integrity of Caco-2 cell monolayers grown in the perfusion system was assessed by measuring the TEER prior to the enzymatic activity assay. When the cells were seeded on polycarbonate or polyethylene terephthalate support filters attached to Minucell (Minusheet) rings and exposed to the perfusion system, they exhibited lower TEER values relative to the traditional Caco-2 cell culture systems. Caco-2 cells seeded on polycarbonate support filters displayed significant lower TEER values ($p < 0.05$) ($171 \pm 52\ \Omega \cdot cm^2$) compared to the cells seeded on polyethylene terephthalate support filters mounted in Minusheets ($251 \pm 62\ \Omega \cdot cm^2$) as illustrated in Fig. 1. To the point above, a TEER value of $644 \pm 119\ \Omega \cdot cm^2$ was obtained for cells differentiated in the traditional Snapwells. The results suggest that the tight junction structure of Caco-2 cell monolayers may be affected by the continuous media perfusion.

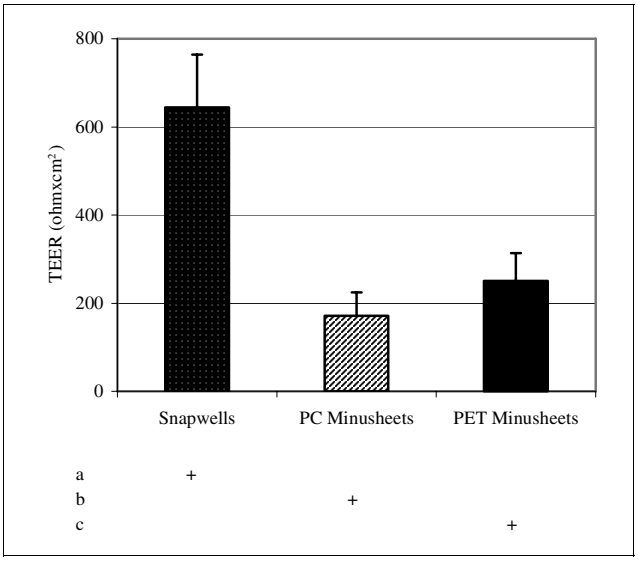


Fig. 1: TEER values of Caco-2 cells seeded on Snapwells or polycarbonate or polyester support filters mounted in Minusheets. The cells were cultivated according to the standard procedures or in the perfusion system as detailed in the Experimental section. Prior to enzymatic activity experiments, the resistance of the cell monolayers was controlled. Each bar represents the mean and standard deviation of 11 individual Snapwell, 31 polycarbonate (PC) and 42 polyethylene terephthalate (PET) inserts. At the bottom of the graph, homogenous groups are identified using the letters a, b, and c. There are no statistically significant differences between data belonging to a homogenous group

2.1.2. Confocal microscope

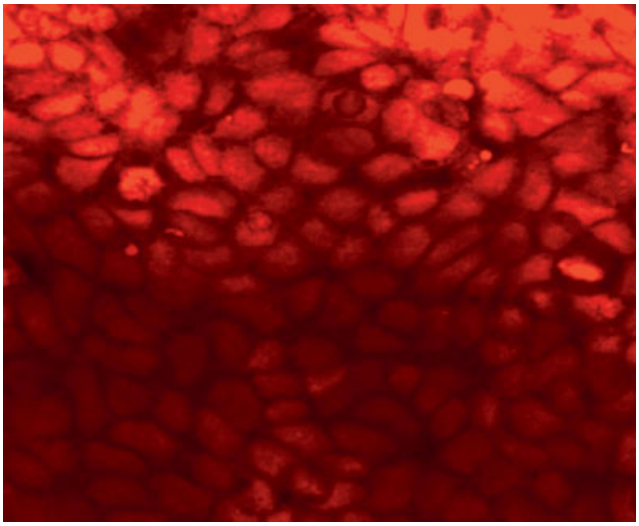
The confluence of Caco-2 cells grown in the perfusion system was further investigated using a confocal microscope. Caco-2 cells reached a monolayer density at day 6 after seeding on polycarbonate or polyethylene terephthalate support filters mounted in Minusheets and coated with collagen IV or Matrigel extracellular proteins (data not shown). That is, while Caco-2 cells, subjected to the perfusion system, displayed lower TEER values, confocal microscopy of the cell nuclei stained by propidium iodide showed that full monolayer structure was obtained and maintained (Fig. 2A). To provide 3-D information of the cell layer, a series of confocal images from different optical planes containing whole image information was further investigated. The thickness of the slices in off/off focus was 18 μm, suggesting that Caco-2 cells seeded on polycarbonate or polyethylene terephthalate support filters attached to Minucell rings formed monolayers (Fig. 2b).

2.2. Functionality of dipeptidases and aminopeptidases

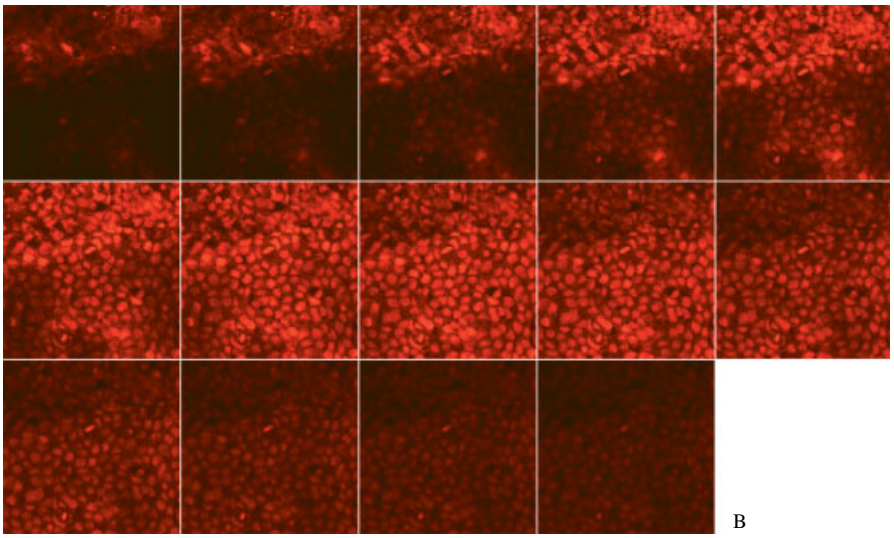
Caco-2 cells were seeded on Snapwells coated with $2.5\ \mu g/cm^2$ of collagen IV for 28 days. The cell monolayers were incubated with glycine-proline-p-nitroanilide or leucine-p-nitroanilide with or without diprotin A. The absorbance of released p-nitroaniline was recorded and the enzymatic activity calculated as indicated in the Experimental section. The enzymatic activity of dipeptidases was demonstrated by the generation of p-nitroaniline (pNA) from gly-pro-p-nitroanilide whereas the generation of p-NA from leu-p-nitroanilide indicated the presence of the active form of aminopeptidases. Dipeptidase activity was higher than the aminopeptidase activity under our experimental conditions. The dipeptidase activity of Caco-2 cells grown in Snapwells was $9.4 \pm 0.5\ nmol \cdot min^{-1} \cdot cm^{-2}$ while the aminopeptidase activity was $1.1 \pm 0.1\ nmol \cdot min^{-1} \cdot cm^{-2}$. The dipeptidase activity significantly decreased to $4.2 \pm 0.3\ nmol \cdot min^{-1} \cdot cm^{-2}$ by the addition of the specific inhibitor Diprotin A while this inhibitor has no effect on aminopeptidase activity as shown in Fig. 3.

2.3. Comparison of dipeptidases and aminopeptidases of Caco-2 cells grown using traditional techniques versus in a perfusion system

Caco-2 cells were seeded on Snapwells or polycarbonate inserts mounted in Minusheets. The inserts were coated with $2.5\ \mu g/cm^2$ of collagen IV. The cells were allowed to grow either in a CO_2 incubator (Snapwells) for 28 days (standard protocol), or for 28 days in a perfusion system. The dipeptidase and aminopeptidase activities of Caco-2 cells were compared. The results showed that the dipeptidase activity of Caco-2 cells grown in a perfusion system was significantly higher ($p < 0.05$) than with the cells differentiated in a CO_2 incubator; 18.20 ± 0.43 and $9.45 \pm 0.5\ nmol \cdot min^{-1} \cdot cm^{-2}$ for Minusheets and Snapwells, respectively (Fig. 4).



A



B

Fig. 2:
A: Confocal microscopy images of Caco-2 cells seeded on polyethylene terephtalate support filter mounted in Minusheets and coated with 20 μg/cm^2 of Matrigel and grown in the perfusion system for 28 days. The cell nuclei were stained with propidium iodide. B: Representative set of 3-D data stacks of Caco-2 cells seeded on polyethylene terephtalate support filters mounted in Minusheets and grown in the perfusion for 28 days. The slices are 18 μm. The object information will vary with the Z position (magnification 40 ×)

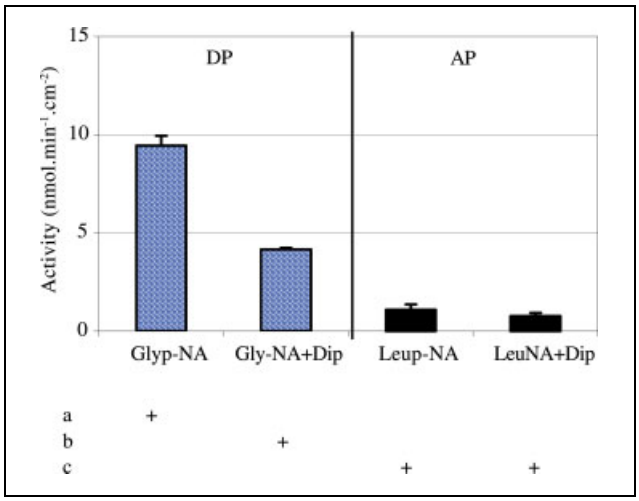


Fig. 3: Dipeptidyl-peptidase (DP, blue bars) and aminopeptidase (AP, black bars) activities of Caco-2 cells grown in Snapwells. Caco-2 cells (passage 63) were seeded on polycarbonate Snapwell inserts coated with 2.5 μg/cm^2 of collagen IV. The cells were maintained in culture for 28 days. The enzymatic activities were performed as described in the Experimental section. The bars represent the means and standard deviations of 3 individual experiments.
Gly-NA = gly-pro-p-nitroanilide, Dip = Diprotin A and Leu-pNA = leu-p-nitroanilide.
At the bottom of the graph, homogenous groups are identified using the letters a, b, and c. There are no statistically significant differences between data which belong to a homogenous group

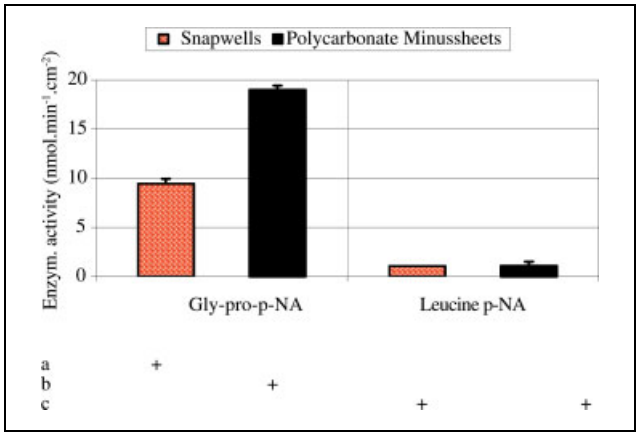


Fig. 4: Dipeptidase (DP) and aminopeptidase (AP) activities of Caco-2 cells seeded on Snapwells or polycarbonate Minucells (Minusheets).
Caco-2 cells (passage 63) were seeded on Snapwells or polycarbonate support filters mounted in Minusheets. The inserts were coated with 2.5 μg/cm^2 of collagen IV. The cells were allowed to grow according the procedure described in the Experimental section and the enzymatic activities were determined. The bars represent the mean and standard deviation of 3 individual experiments. At the bottom of the graph, homogenous groups are identified using the letters a, b, and c. There are no statistically significant differences between data belonging to a homogenous group

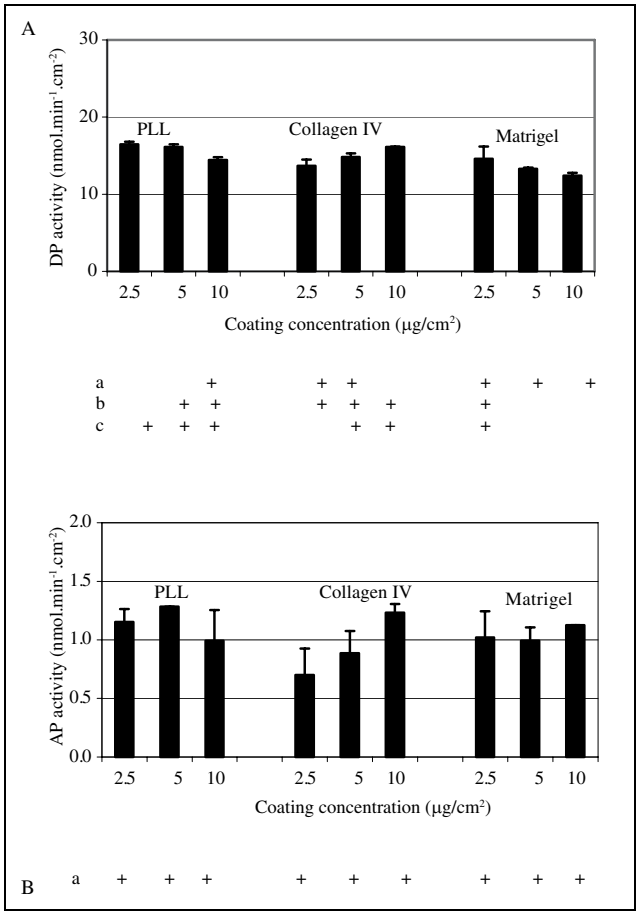


Fig. 5: Dipeptidase (DP) and aminopeptidase (AP) activities of Caco-2 cells grown in the perfusion system. Caco-2 cells (passage 63) were seeded on polyethylene terephtalate support filters mounted in Minusheets. The inserts were coated with different amounts of poly-L-lysine (PLL), collagen IV or Matrigel extracellular matrix proteins. The cells were grown for 28 days in the perfusion system. The dipeptidase (A) and aminopeptidase (B) activities were performed as described in the section Materials and Methods. The bars represent the mean and standard deviation of 3 individual experiments. At the bottom of the graph, homogenous groups are identified using the letters a, b, and c. There are no statistically significant differences between data belonging to a homogenous group

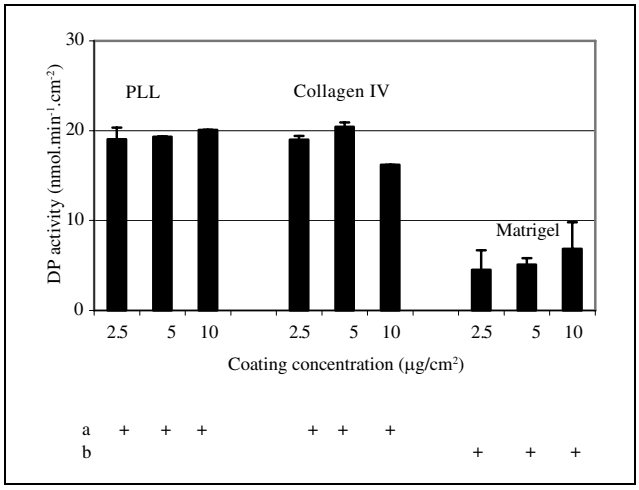


Fig. 6: Effect of coating amount on the dipeptidase (DP) activities. The polycarbonate support filters mounted in Minusheets were coated with increasing concentration of poly-L-lysine, collagen IV or Matrigel. Caco-2 cells were seeded and grown for 28 days in the perfusion system. Data represent the mean ± SD of 3 individual experiments. At the bottom of the graph, homogenous groups are identified using the letters a, and b. There are no statistically significant differences between data belonging to a homogenous group

2.4. Effect of extracellular matrix protein coating on dipeptidase and aminopeptidase activities

In a previous work, we showed that unlike Caco-2 cells grown on non-coated polycarbonate Snapwell inserts, the cells did not grow on non-coated polycarbonate support filters mounted in Minusheets. To verify whether the basement membrane proteins used to improve the cell attachment and growth have an influence on the cell differentiation, polycarbonate as well as polyethylene terephthalate support filters mounted in Minusheets were coated with increasing amounts of poly-L-lysine (PLL), collagen IV or Matrigel. The cells were allowed to grow in the perfusion system. Multiple range tests indicated that the dipeptidase activity was similar when polyethylene terephthalate support filters were coated with PLL or collagen IV whereas the activity decreased upon coating of the support filters with Matrigel compared to poly-L-lysine (Fig. 5A). The analysis of aminopeptidases did not show any difference when the cells were seeded on polyethylene terephthalate coated with increasing amounts of poly-L-lysine, collagen IV or Matrigel (Fig. 5B). While the coating of polycarbonate support filters with Matrigel matrix protein did not affect the growth of the cells or the monolayer formation, it inhibited the activities of dipeptidases and aminopeptidases in Caco-2 cells. As mentioned in Fig. 6, the cells seeded on polycarbonate support filters coated with Matrigel showed significant lower dipeptidase activities (6.5 ± 1.9 nmol $\cdot$ min^{-1} $\cdot$ cm^{-2}) compared to the cells grown on polycarbonate support filters coated with PLL (19.5 ± 0.5 nmol $\cdot$ min^{-1} $\cdot$ cm^{-2}) or collagen IV (17.9 ± 1.3 nmol $\cdot$ min^{-1} $\cdot$ cm^{-2}).

2.5. Effect of passage number on Caco-2 cell differentiation

Caco-2 cells from passage 63 and 73 were seeded on polycarbonate or polyethylene terephthalate support filters coated with extracellular matrix proteins. The cells were grown in the perfusion system under similar conditions and the differentiation parameters were assessed. Figure 7 indicates that Caco-2 cells from passage 63 expressed significant lower dipeptidases and aminopeptidases compared to the activities of cells from passage 73 seeded on polyethylene terephthalate support filters and grown in the perfusion system.

3. Discussion

The use of Caco-2 cells, the most popular *in vitro* cell line for predicting intestinal drug absorption and drug metabolism, is a high cost and time-consuming endeavour. Faced with the increased number of drugs generated by combinatorial chemistry, high-throughput assays are performed in microplates combined with robotic systems to assess the permeability and metabolic characteristics of the drug candidates. Laboratories have also sought alternatives to speed up the procedure to promote the rapid differentiation of Caco-2 cell monolayers. This includes, for example, the three-day system of BD Biosciences (Chong et al. 1997). However, the short life span of the cells obtained by the accelerated protocol restricts their use and suggests that the cells were not fully differentiated (Van de Water-

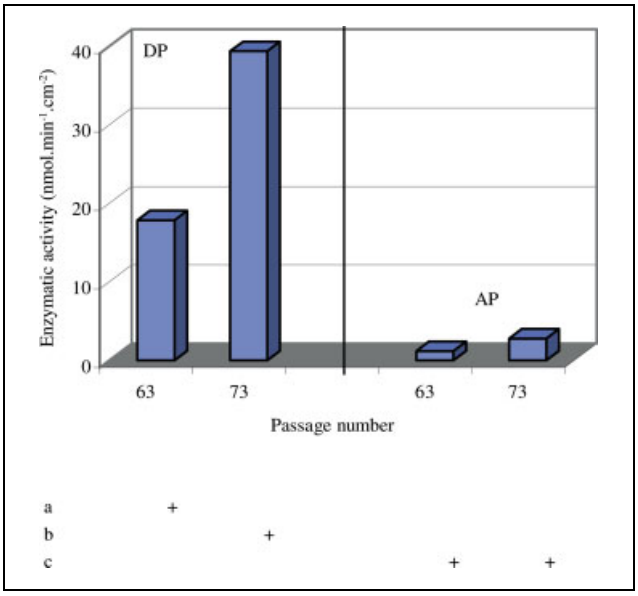


Fig. 7: The effect of passage number on dipeptidase (DP) and aminopeptidase (AP) activities of Caco-2 cells grown in the perfusion system. Caco-2 cells (passage numbers 63 and 73) were grown for 28 days in the perfusion system. The results represent the means of a pool of 9 polycarbonate support filters coated with 2.5, 5 and 10 μg/cm^2 of collagen IV.
At the bottom of the graph, homogenous groups are identified using the letters a, b, and c. There are no statistically significant differences between data belonging to a homogenous group

beemd et al. 2003). In a search for an alternative to the labor-intensive Caco-2 cell culture, a perfusion system for culturing Caco-2 cell monolayers was implemented. The perfusion cell culture is characterized by the continuous supply of fresh medium in the apical and basolateral side of the cell monolayers and the withdrawal of used medium, thus mimicking as closely as possible the *in vivo* situation. The functionality of the novel system has been previously demonstrated (Masungi et al. 2004). The system arguably approximates the *in vivo* situation regarding the functionality of the cells.

The objective of this work was to assess the functional aspect and the differentiation parameters, mainly the dipeptidase and aminopeptidase activities, of Caco-2 cells grown in a perfusion system. Under perfusion conditions, the cells were found to differentiate. Caco-2 cell monolayers cultivated in a perfusion system showed lower TEER compared to the cells grown in the traditional inserts. The lower TEER of Caco-2 cell monolayers suggested that the paracellular pathway of the cell monolayers, generated following the continuous perfusion of the medium, was affected as previously demonstrated by an increase of transport for moderate and low permeable drugs (Masungi et al. 2004). These TEER values are closer to those associated with human intestine.

In contrast to the static, traditional Caco-2 cell culture approach, where long-term culture induces cell dedifferentiation, the perfusion system, while maintaining the monolayer structure, showed a useful differentiation of the cells which can be maintained over a period longer than two months. The differentiation of Caco-2 cells in the perfusion system was accompanied by an increased expression of dipeptidases and aminopeptidases compared to the cells differentiated in the traditional Snapwell system. The majority of epithelia demonstrates barrier functions upon being exposed to different fluids from the luminal and basal sides. In the perfusion system, this natural situation was simulated under *in vitro* conditions and was shown to withstand mechanical and fluid stress over a prolonged period of time (Schumacher et al. 2002b). The highly differentiated Caco-2 cells obtained from the perfusion system may have advantages. Perfusion allows for the delivery of nutrients to high-density cell cultures and minimizes the accumulation of metabolic products. In addition, the system stabilizes the secreted levels of autocrine factors such as morphogenetic signals and does not allow build up of synthesized paracrine factors (von Woedtke et al. 2002). The aminopeptidase activity of Caco-2 cells was lower compared to the dipeptidase activities. Different patterns of peptidases expressed on Caco-2 cell membranes were observed. The percentage of surface staining in intact cultures ranged from 22% for aminopeptidases to nearly 100% for dipeptidases (Howell et al. 1993a). Therefore, the dipeptidase and aminopeptidase activities might reflect their expression on the cell surface of Caco-2 grown in the perfusion system. Caco-2 cells seeded on polycarbonate support filters and grown in the perfusion system showed similar dipeptidase and aminopeptidase activities as the cells seeded on polyethylene terephtalate support filters. However, for polycarbonate support filters, Matrigel coating inhibited dipeptidase and aminopeptidase activities. Inhibition of sucrase and alkaline phosphatase activities has been demonstrated in the presence of collagen and Matrigel (Jumarie and Malo 1991). In our hands, the inhibition of dipeptidase and aminopeptidase activities was induced on support filters coated with Matrigel. We do not yet understand the down regulation of dipeptidases and aminopeptidases induced by Matrigel. It was suggested that inhibition of sucrase and alkaline phosphatase activities in the presence of collagen and Matrigel reflects the absence of specific receptors for those proteins in Caco-2 cells or the expression of other phenotypes of Caco-2 cells.

The influence of culture passage number on the functional and physiological development of Caco-2 cells has been previously demonstrated. It has been reported that growth rate, TEER and sucrase-isomaltase activity increase with the passage number. On the other hand, alkaline phosphatase activity was lower in high passage numbers while sucrase activity was consistently lower in low passage cultures (Briske-Anderson et al. 1997; Yu et al. 1997). In our experimental procedure, dipeptidases and aminopeptidases were up regulated in Caco-2 cells grown in the perfusion system as a function of passage number. In addition to the TEER measurement, confocal microscopy was applied to assess the mono- or multilayer structure. The single plane of Caco-2 cells grown in the perfusion system and three-dimensional analysis allowed us to get multiple slices through different planes. The results of the 3-D data set indicate that the cells seeded either on polycarbonate or polyethylene terephtalate support filters maintained their monolayer structure during growth and differentiation in the perfusion system. This result contrasts previous data showing mixed mono- and multilayers of Caco-2 cells seeded on Cyclopore inserts (Rothen-Rutishauser et al. 2000). Our approach using the continuous supply of the fresh medium and constant removal of the used medium might avoid the possibility of Caco-2 overgrowth giving rise to multilayers.

In conclusion, the perfusion system implemented here, by reducing manual manipulations, provides an experimental tool for highly differentiated Caco-2 cell culture.

4. Experimental

4.1. Chemicals

Glycine-proline-p-nitroanilide, leucine-p-nitroanilide, p-nitroaniline, bestatin and diprotin A were purchased from Sigma (Bornem, Belgium). All supplements and cell culture media were purchased from Invitrogen LTD, Merelbeke, Belgium.

4.2. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC), and were used between passage 63 and 73. The cultures were mycoplasma free (mycoplasma detection kit; Roche Gmbh, Mannheim, Germany). The cells were maintained in 175 cm^2 plastic culture flasks (Becton Dickinson, Erembodegem, Belgium). Caco-2 cells were subcultured with 0.25% trypsin and 0.2% EDTA (15 min) at 37 °C and seeded in new flasks. Culture medium (Dulbecco's Modified Eagle Medium (DMEM)) was supplemented with 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10% fetal bovine serum (FBS). For the experiments using the perfusion system, Caco-2 cells were grown for an initial period of one week in a CO_2 incubator (5% CO_2) at 37 °C prior to being mounted in the perfusion system. The cell monolayers were used at 28 days post initiation of perfusion. For the conventional system, Caco-2 cells were seeded in Snapwell™ polycarbonate inserts (24 mm insert diameter, 0.4 μm pore size) (Corning Costar, Cambridge, U.K.) at a seeding density of 1.4×10^5 cells/cm^2. The culture medium was exchanged every two days and cells were used 28 days post-seeding.

4.3. Matrix proteins

Poly-L-lysine hydrobromide 150,000–300,000 MW (PLL) and collagen IV were supplied by Sigma (Bornem, Belgium) 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. 250 μl 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.

4.4. Perfusion culture system

The 0.45 μm pore size support filters of polycarbonate or a mixture of cellulose acetate and cellulose nitrate were obtained from Millipore (Brussels, Belgium). Polyethylene terephthalate was purchased from Cyclopore, Whatman (Louvain-La-Neuve, Belgium). The support filters were mounted in Minusheets (Minucells and Minutissue Gmbh, Bad Abbach, Germany) and autoclaved. The sterile support filters were placed into 24-well plates (BD Biosciences, Erembodegem, Belgium) and Caco-2 cells were seeded at 5.26×10^5 cells/cm^2 (7×10^5 cells/ml/insert). The effective growth surface of the support filter fixed to Minusheets is 0.5 cm^2. Following an overnight incubation, the support filters were transferred to 6 well plates containing 6 ml of complete DMEM medium (10% FBS). The medium was replaced every other day. After a period of one week in a CO_2 incubator, Caco-2 cells grown on support filters were placed in Minucell rings and transferred into the perfusion culture chambers which were maintained at 37 °C. CO_2 independent medium supplemented with 5% FBS, 100 U/ml penicillin and streptomycin, 2mM glutamine, and 1% non-essential amino acids was used as the perfusion medium. A peristaltic pump was operated to guarantee a constant supply of 1 ml medium/hour. The fresh medium was taken from a cooled reservoir, warmed to 37 °C prior to passing over the cultured cells and spent medium was pumped into a waste bottle. The temperature inside the perfusion culture system was maintained by a heating plate to guarantee a constant temperature of 37 °C. Otherwise, the system was operated on a laboratory bench, at ambient temperature (Masungi et al. 2004).

4.5. Measurement of monolayer integrity

Measurement of transepithelial electrical resistance (TEER) was used to evaluate 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 voltohm 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 $\Omega \cdot cm^2$ were used in these experiments.

4.6. Confocal microscopy

The Minusheet rings containing Caco-2 cells were removed from the incubator and put in an empty 24-well plate. The cells were incubated with 1 ml glutaraldehyde (1%) for 10 min at room temperature, in the dark. Subsequently, they were washed twice with 1 ml of Ca^{2+} and Mg^{2+} free PBS (Invitrogen, Merelbeke, Belgium) and stained with 500 μl of propidium iodide (0.4 μg/ml in PBS) (Sigma, Bornem, Belgium). The cells were incubated for 5 min at room temperature in the dark and washed twice with milliQ water (Millipore, Brussels, Belgium). The support filters were removed from the inserts and put on a microscopic glass slide with a drop of mounting medium and covered. The mounting media is a solution of polyvinyl alcohol (Mw = 10,000) in saline, buffered to pH 7.2 and mixed with glycerol. A Zeiss (Carl Zeiss, Jena, Germany) LSM 410 inverted microscope was used to analyze the slides. Optical sections in off-focus of 18 μm thickness were recorded. The Z scans were performed with fluorescein/rhodamin channel for the nuclei stained with propidium iodide.

4.7. Dipeptidase and aminopeptidase enzymatic activity

Enzymatic activity was assessed under static conditions. The apical side of the cell monolayers was incubated for 5 min with one of the following substrates: gly-pro-p-anilide or leu-p-anilide (100 μM), in the presence or the absence of diprotin A. The activity of the enzymes was measured spectrophotometrically by analysing the UV absorbance of p-nitroaniline (404 nm), a metabolite generated by these enzymes. The UV absorbance values were processed using a spectramax 190 Spectrophotometer (Molecular Devices).

4.8. Calculation

The activity of the enzyme expressed in $nmol \cdot min^{-1} \cdot cm^{-2}$ was calculated as follows:

$$A = \frac{V \times C}{t \times S}$$

where V is the volume of the solution (ml), C, the concentration of p-nitroaniline (μM), t, the time of incubation (min) and S the surface of the monolayer (cm^2).

4.9. Statistical analysis

Statistical analysis was 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.

Part I of this series: Masungi et al. (2004).

Acknowledgements: The authors wish to express their gratitude to Drug Evaluation & CMC Innovation Fund for its support.

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