Optimizing culture conditions of a porcine epithelial cell line IPEC-J2 through a histological and physiological characterization

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Abstract The high similarity between pigs and humans makes pigs a good gastrointestinal (GI) model for humans. Recently an epithelial cell line originating from the jejunum of pig (IPEC-J2) became available. Once validated, this model can be used to investigate the complex interactions occurring in the intestine. The advantages of using IPEC-J2 as in vitro model of the GI tract are the high resemblance between humans and pigs, and the ease of extrapolating in vitro to in vivo characteristics. In this study, the IPEC-J2 cells were functionally characterized by measuring the trans-epithelial electrical resistance (TEER), and by histological and ultrastructural studies. IPEC-J2 cells grown on six different permeable support systems, were investigated. The Transwell®-COL collagen-coated membrane (1.12 cm²) showed the best results concerning time efficiency and TEER values. The optimum seeding density of 12×10^5 cells/mL ensured that after 9 days of differentiation a confluent monolayer was formed. The decrease in TEER values after a maximum had been reached, coincided with the ultrastructural development of apical microvilli. We conclude that IPEC-J2 cells grown on collagen-coated membranes represent a valuable in vitro model system

for the small intestinal epithelium which can be of great interest for intestinal research.

Keywords Pig · IPEC-J2 · Boyden chamber · Model system

Introduction

Experimental animals and in vitro models are an ethically justified alternative to study gastrointestinal (GI) problems and disorders such as inflammatory diseases like Crohn's disease and ulcerative colitis that occur in humans. The currently available animal models and in vitro systems have provided significant insights into the basic properties of living cells, relevant to human biology, and numerous experimental models of human diseases have been established (Lloyd et al. 2006; Schmid et al. 2008; Bauer et al. 2009; Wolfe 2009). As in vitro model, Caco-2, a human colon cell line, is frequently used because of its great morphological, ultrastructural, and biochemical similarities with small intestinal epithelial cells (Pinto et al. 1983; Hidalgo et al. 1989; Perlmutter et al. 1989), although questions remain about their functional resemblance to the in vivo situation.

An appropriate in vivo model for the GI tract has preferably a high similarity with the one of humans. Of all non-primates, pigs are most similar to humans in size, weight and diet, and the anatomy and

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physiology of the GI system. Moreover, pigs are less descend from humans in contrast to rodents at the evolutionary level, and as a consequence the pig sequence is much more similar to the human sequence (Wernersson et al. 2005). The pig is as well an obvious choice because they have a subset of biological functions important to humans. Nowadays, a lot of intestinal research is performed on rodents e.g. mice and rats (Choi et al. 2009). However, the validity when extrapolating results to humans can be questioned. It is not possible to determine if differences are due to e.g. the treatment or to changes in the rodent or primate lineage since their divergence [approximately 90 million years ago (Springer et al. 2003)], when comparing results from rodents to humans. Pigs may be a more appropriate model. Altogether, research using pigs can help to elucidate and investigate human diseases especially GI diseases e.g. Crohn's disease and ulcerative colitis.

Recently, a cell line from jejunum epithelium isolated from a neonatal unsuckled piglet, small intestinal porcine epithelial cell line (IPEC-J2; Berschneider 1989) was characterized and used as an in vitro model system for studying porcine intestinal pathogen-host interactions, porcine-specific pathogenesis, and innate immune responses (Schierack et al. 2006; Botic et al. 2007; Brown and Price 2007; Skjolaas et al. 2007; Koh et al. 2008; Arce et al. 2010; Liu et al. 2010). This cell line, a non-transformed, non-tumorigenic small intestinal cell line, can secrete mucin, produce cytokines/chemokines, and express Toll-like receptors similar to those of the original tissue. It conserves its epithelial nature, and can serve as a convenient model to simulate innate immune functions of the intestinal epithelium. Two major points demonstrate that IPEC-J2 represents a better model of normal intestinal epithelial cells than transformed cell lines: (1) they maintain their differentiated characteristics and exhibit strong similarities to primary intestinal epithelial cells (Schierack et al. 2006), and (2) IPEC-J2 cells can be an appropriate model through the advantage of direct comparison with the experimental animal, which in turn might serve as a good model for humans. Therefore, IPEC-J2 was selected for this study.

The system used, namely the Boyden chamber, which consist of a porous membrane, allowed working with separate apical and basolateral compartments promoting cell polarization, approaching

the in vivo differentiated situation. Numerous membranes and systems are commercially available, each with their individual characteristics. However, none of the prior studies examined the actual characteristics of IPEC-J2 cells in response to the membrane being used, and the influence of the membrane on the IPEC-J2 morphology and functionality.

We aimed to determine the time course effect of different types of membranes on the progression in trans-epithelial electrical resistance (TEER) of IPEC-J2 cell monolayer, and the differentiation, including microvilli, in time. Subsequently, culture conditions were optimized in order to realize the most appropriate and optimal in vitro system to be implemented into GI research as a good in vitro alternative for human research.

Materials and methods

Cell culture

The porcine jejunal intestinal cell line IPEC-J2, is a non-transformed cell line originating from a neonatal, unsuckled piglet (Berschneider 1989). IPEC-J2 were cultured in 1:1 DMEM (Dulbecco's modified Eagle medium)/Ham's F-12 mixture (Invitrogen, Belgium) supplemented with 0.12% sodium bicarbonate (Sigma–Aldrich, Belgium), 15 mM HEPES (Invitrogen, Belgium), 0.5 mM sodium pyruvate (Invitrogen, Belgium), 5% heat-inactivated fetal bovine serum (Biocrom AG, Germany) and 1% antibiotic–antimycotic mixture (Sigma–Aldrich, Belgium). The cells were grown at 37 °C in a humidified atmosphere of 5% CO₂. Every other day, culture medium was refreshed.

IPEC-J2 cells between passages 70–90 were seeded onto the different membranes at a high density of 12×10^5 cells/mL to saturate the available area for attachment, hereby avoiding the need for cell division (Cereijido et al. 1978). Every other day, medium was refreshed according to the instruction manual provided by the supplier for each insert system.

Boyden chamber inserts

Six different cell culture Boyden chamber inserts, of which the membranes are imbedded in a solid plastic support, were tested for specific requirements. They

differ in growth surface area, membrane pore size and other characteristics of the permeable membrane (Table 1). The six cell culture systems are: Transwell® polycarbonate membrane (growth surface area 4.67 cm²; membrane pore size 0.4 µm; Corning, Belgium), Transwell® polycarbonate membrane which we treated with collagen (4.67 cm²; 0.4 µm; Corning, Belgium), Anopore™ membrane without coating (1.12 cm²; 0.2 µm; Nunc, Thermo Scientific, Germany), Transwell®-COL collagen-coated membrane (4.67 cm²; 0.4 µm; Corning, Belgium), Transwell®-COL collagen-coated membrane (1.12 cm²; 0.4 µm; Corning, Belgium), and Transwell®-COL collagen-coated membrane (0.33 cm²; 0.4 µm; Corning, Belgium).

The second cell culture inserts (Transwell® polycarbonate membrane treated with collagen), were coated with collagen I from rat tail (Sigma–Aldrich, Belgium). The collagen was first dissolved in 0.1% acetic acid (3 mg/mL; VWR, Belgium). This stock solution was diluted 1:1 with absolute ethanol (VWR, Belgium), 500 µL/insert of the diluted collagen solution was added to the Transwell® polycarbonate membrane inserts (4.67 cm²) and were dried overnight in the incubator (37 °C).

Trans-epithelial electrical resistance measurements

The monolayer formation was determined through trans-epithelial electrical resistance (TEER) measurements which is a convenient, reliable and non-destructive method to monitor for confluence, viability of tissue culture monolayer, and presence of tight junctions. TEER values were measured using an epithelial voltohmmeter (EVOM) with STX2 electrodes (World Precision Instruments, USA), and expressed as kΩ cm².

Validation of monolayer by permeability test

The enzyme horseradish peroxidase (HRP) was used to measure macromolecular permeation through the IPEC-J2 monolayer. In brief, medium of the Transwell®-COL collagen-coated membrane (1.12 cm²) was removed. In the top well it was replaced by 500 µL of a 100 µg/mL HRP solution (Sigma–Aldrich, Belgium) and in the bottom well by 1.5 mL Hank's balanced salt solution (HBSS;

Table 1 Characteristics of six permeable membrane supports studied

	Transwell® polycarbonate membrane	Transwell® polycarbonate membrane treated with collagen	Anopore™ membrane without coating	Transwell®-COL collagen-coated membrane	Transwell®-COL collagen-coated membrane	Transwell®-COL collagen-coated membrane
Growth surface area (cm ²)	4.67	4.67	4.2	4.67	1.12	0.33
Membrane pore size (µm)	0.4	0.4	0.2	0.4	0.4	0.4
Type of membrane	Polycarbonate	Polycarbonate	Rigid inorganic	PTFE	PTFE	PTFE
Surface treatment	Tissue culture-treated	Tissue culture-treated and coated with collagen I from rat tail	Tissue culture-treated	Collagen coated ^a	Collagen coated ^a	Collagen coated ^a
Thickness of membrane (µm)	10	10	45	50	50	50
Nominal pore density	1 × 10 ⁸ pores per cm ²	N.D.	1 × 10 ⁹ pores per cm ²	N.D.	N.D.	N.D.
Cell visibility	Poor	Poor	Good	Good with cell outlines	Good with cell outlines	Good with cell outlines

PTFE polytetrafluoroethylene, N.D. not defined

^a Equimolar mixture of type I and III bovine placental collagens

Invitrogen, Belgium). Plates were further incubated at 37 °C. Every 15 min samples were taken from the bottom well. Subsequently, samples were incubated for 20 min with ABTS substrate (KPL, USA) to detect HRP enzymatically and reaction was stopped by addition of H₂O₂ (Merck, Belgium) before reading at 450 nm. Samples were obtained from controls (insert in culture, for 10 and 21 days, without IPEC-J2 cells), and of an IPEC-J2 monolayer of 10 days and one of 21 days in culture tested.

Histology

Preceding the fixation step, both the top and bottom well were carefully rinsed (2×) with HBSS (Invitrogen, Belgium; 37 °C). The cultured cells were fixed at room temperature during 1 h by buffered formalin (VWR, Belgium) and rinsed with HBSS (Invitrogen, Belgium; 37 °C). Inserts were dehydrated with a graded ethanol series (VWR, Belgium), membranes were embedded into paraffin (VWR, Belgium), 7 µm sections were cut, mounted on glass slides, allowed to dry, and stained with hematoxylin (VWR, Belgium) — eosin (Sigma–Aldrich, USA).

Transmission electron microscopy

The protocol used for transmission electron microscopy (TEM) is supplied by Corning. In brief, monolayers were fixed with 2.5–3.0% glutaraldehyde (Polyscience, USA) in cacodylate buffer (0.1 M), pH 7.2–7.4, for 1 h at room temperature and rinsed (2×) with cacodylate buffer (Agar scientific, UK) adjusted to 300 milli-osmols with 6.84% sucrose (Acros, Belgium). This was followed by postfixation with 1% osmium tetroxide (Polyscience, USA) in cacodylate buffer for 1 h at 4 °C and rinsed (2×) with cacodylate-sucrose buffer. Monolayers were dehydrated in a graded ethanol series and embedded with LR white resin (medium grade; Ted Pella, Inc., USA). Semithin 1 µm sections were stained with methylene blue (UCB, Belgium) and thionin (Sigma–Aldrich, USA) and viewed with an Olympus BX51 microscope. Double-stained 70 nm thin sections, with uranyl acetate (Agar scientific, UK) and lead citrate (Tab laboratories, UK), were examined in a Zeiss EM900 electron microscope.

Microvilli detected with TEM were counted over an epithelial surface of 5 µm. The length and diameter of each microvilli was measured and expressed in µm. For every time point (day 9, 21 and 28) three TEM observations were used to determine the mean average of a single time point for the number, length and diameter of microvilli present, also the average standard deviation (±SD) is given.

Statistical analysis

Resulting data were analyzed with a paired two-sample *t* test.

Results

Monolayer formation of IPEC-J2

The Transwell® polycarbonate membranes with and without coating reached a resistance maximum after 16 (Fig. 1b) or 22 (Fig. 1a) days, respectively. For the membranes coated with collagen the TEER values dropped rapidly after reaching the maximum (Fig. 1b). A disadvantage of the Transwell® polycarbonate membranes was that cells were not visible during monolayer formation (Table 1), due to the material of which the membranes consisted.

The Anopore™ membranes (Fig. 1c) did offer a good clarity for microscopy (Table 1). However, monolayer formation could not be determined by resistance measurements. The Transwell®-COL collagen-coated membranes were tested in three variants of surface area (4.67 cm²; 1.12 and 0.33 cm²), all possessing the same type of membrane. As presented in Fig. 2 the Transwell®-COL collagen-coated inserts with the largest membrane (Fig. 2a) reached a maximum TEER value at day 15. However, just like in Fig. 1b, these Transwell®-COL collagen-coated inserts (4.67 cm²) showed a rapid decline of TEER values after reaching the maximum (Fig. 2a). The Transwell®-COL collagen-coated membrane with the smallest surface area, 0.33 cm², was not appropriate due to the lower resistance maximum after 12 days of culturing (Fig. 2c), and difficulties during maintenance and measurements. The Transwell®-COL collagen-coated membrane with a membrane surface of 1.12 cm² is most applicable when taking TEER

Fig. 1 The progression of TEER values of Transwell® polycarbonates membrane (a) the same membrane coated with collagen I (b) and the Anopore™ membrane inserts without coating (c) were measured (means $\pm$ SD of $n = 5$)

values (Fig. 2b) and visibility into account. The maximum was reached after 9–11 days of culturing. Afterwards a stable value was maintained during the progression of the time interval (Fig. 2b).

The permeability tests on IPEC-J2 grown on the most suitable filter support, Transwell®-COL

Fig. 2 Progression of TEER values of Transwell®-COL collagen-coated membranes [4.67 cm²; 0.4 μm (a) (means $\pm$ SD of $n = 5$), 1.12 cm²; 0.4 μm (b) (means $\pm$ SD of $n = 12$), 0.33 cm²; 0.4 μm (c) (means $\pm$ SD of $n = 11$)]

collagen-coated membrane (1.12 cm²), confirmed the TEER values. After 10 days (and 21 days) of culturing, IPEC-J2 had been differentiated into a tight epithelia impermeable to HRP (Fig. 3), confirming the TEER values (Fig. 2b).

Fig. 3 Macromolecular permeability of IPEC-J2 monolayer on Transwell®-COL collagen-coated membrane (1.12 cm²; 0.4 µm). Basolateral concentration of HRP (mean ± SD of $n = 6$) over 2 h (*closed circle* insert without IPEC-J2 cells, *open triangle* IPEC-J2 monolayer 10 days in culture and *open circle* IPEC-J2 monolayer 21 days in culture)

Histological characterization

Histological slides of the IPEC-J2 cells grown on the Transwell®-COL collagen-coated membrane (1.12 cm²) showed that at day 7 (Fig. 4), the IPEC-J2 cells formed a tight monolayer as indicated by the previous TEER values (Fig. 2b).

The transmission electron study revealed that from day 9 onwards (Fig. 5a), well formed apical microvilli were present on the IPEC-J2 cells. Over time we observed a significant increase in length (day 21 to day 28) and diameter (day 9 to day 28) of the microvilli (Table 2).

Junctional complexes such as tight junctions, desmosomes, zonula adherens and intercellular spaces were observed (Fig. 5). These intercellular

Fig. 4 IPEC-J2 cells on the permeable filter support of Transwell®-COL collagen-coated membranes (1.12 cm²; 0.4 µm). At day 7 there is a well formed monolayer, as indicated previously by the TEER measurements (312×)

Fig. 5 Electron micrographs of IPEC-J2 cells. Cells were grown on a Transwell®-COL collagen-coated membranes for 9 (a), 21 (b), and 28 (c) days. Demonstration of junctional complexes e.g. tight junctions (T.J.); intercellular space (I.S.); zonula adherens; desmosome (D.); microvilli (M.)

Table 2 Summary of three TEM observations

	Number*	Length*	Diameter*
Day 9	21.0 ± 4.95	1.51E-07 ± 4.85E-08 ^a	1.02E-07 ± 1.76E-08 ^a
Day 21	26.0 ± 4.45	1.56E-07 ± 5.24E-08 ^a	1.07E-07 ± 2.81E-08 ^{ab}
Day 28	21.3 ± 1.77	2.14E-07 ± 7.31E-08 ^b	1.16E-07 ± 2.30E-08 ^b

Number, length and diameter of microvilli observed on IPEC-J2 on a surface of 5 µm were determined

* Numbers are an average (±SD) of three TEM observations, expressed in µm for the columns length and diameter. Numbers with the same letter are not significantly different ($p < 0.05$)

spaces displayed cellular protrusions similar to those seen in Schierack et al. (2006) but not observed in the Caco-2 cell line (Costa de Beauregard et al. 1995).

Discussion

To allow investigation of important GI disorders in both humans and pigs, an in vitro model was developed for which the results can easily be verified and extrapolated to in vivo situations. However, porcine in vitro studies have been restricted by the limited availability of intestinal epithelial cell lines [to date only three: IPEC-1, IPEC-J2 (Berschneider 1989) and IPI-2I (Kaeffer et al. 1993)], whereas the use of human and rodent intestinal cell lines have been widely documented. Nevertheless, the use of porcine cell lines should be encouraged because of the similarities with the originated cell tissue and with humans (Schierack et al. 2006; Wernersson et al. 2005).

To our knowledge, there are no previous published reports of IPEC-J2 where the most appropriate culture system was demonstrated by comparing different systems by their specific characteristics. As well the development of microvilli formed by IPEC-J2, mainly for their surface enlargement, have never been studied in time.

During this study six different membrane systems were tested for growing IPEC-J2 cells. It was concluded that the membranes coated with collagen reached a maximal TEER value more rapidly (Figs. 1 and 2) since collagen is a determinant factor in the orientation (Chambard et al. 1981). The membranes with the largest surface (4.67 cm²), namely Transwell[®] polycarbonate membrane with and without coating (Fig. 1a, b) and Transwell[®]-COL collagen-coated membrane (Fig. 2a), all displayed a rapid

decrease in TEER value after a maximum had been reached, irrespective of brand. This can be caused through the detachment of cells at the edges of the support (Pinto et al. 1983). So we deem that the inserts with the largest surface are not suitable when longer culture periods are required. Application of the Anopore[™] membrane resulted in extremely low TEER values without reaching a maximum. The Transwell[®]-COL collagen-coated membrane with a surface of 1.12 cm², however, reached a high TEER maximum in a relative short time and allowed good visualization of the cell monolayer. Therefore it is concluded that Transwell[®]-COL collagen-coated membrane (1.12 cm²) is the most appropriate model system for in vitro intestinal research with IPEC-J2.

The decline of TEER values (Fig. 2b) coincided with a significant increase in length and diameter of the microvilli at the IPEC-J2 monolayer on day 28 in comparison to day 9 (Table 2) as confirmed by the electron micrographs (Fig. 5). This association can be explained by the fact that the presence of microvilli on the apical side of IPEC-J2 cells makes them more electrical negative as compared to the basal side (Mauchamp et al. 1979), thereby causing a decrease in TEER values. The TEER values observed and the permeability tests, indicate the development of a fully differentiated and functional epithelium. The values obtained in this study are higher than those of the small intestine in vivo (approximately 50 Ω cm²), however, still comparable to other intestinal cell lines such as T-84 and DIEC (Terrés et al. 2003; Weng et al. 2005).

Summarizing, IPEC-J2 cells cultured on Transwell[®]-COL collagen-coated membranes differentiated to a single monolayer of polarized enterocyte-like cells with apical microvilli, junctional complexes and intercellular spaces (Fig. 5). This was also supported at the genetical level. In microarray

experimentations, where IPEC-J2 were stimulated with different strains of *Escherichia coli*, data showed gene expression of occludin reported as present call (MAS 5.0 detection calls; Geens and Niewold 2010), which is a specific marker for the presence of tight junctions in differentiated cells.

A number of data demonstrate the functional resemblance of IPEC-J2 to the well defined human colon carcinoma cell line Caco-2. First, both cell lines, at confluency, are covered by typical brush border microvilli, so exhibiting structural and functional differentiation pattern characteristics of mature enterocytes (Pinto et al. 1983). Morphologically, IPEC-J2 are polarized the same as Caco-2. Secondly, the decrease of TEER values after day 20 (Fig. 2b) indicates a limited culture time, which is similar to Caco-2, and most probably caused by deterioration of tight junctions relative to the culture's age and initiated apoptosis (Da Violante et al. 1994).

In conclusion, using the Transwell®-COL collagen-coated membrane with a surface area of 1.12 cm², an IPEC-J2 in vitro model system of porcine origin was realized and positively validated, electrophysiologically as well as morphologically. This system allows easy comparison with the in vivo situation and could be very helpful in intestinal research, for diseases common to both pigs and humans.

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