Pharmaceutical Research, Vol. 23, No. 9, September 2006 (© 2006)
DOI: 10.1007/s11095-006-9057-7

Research Paper

Porcine Alveolar Epithelial Cells in Primary Culture: Morphological, Bioelectrical and Immunocytochemical Characterization

Anne Steimer,[1,3] Michael Laue,[2,4] Helmut Franke,[1] Eleonore Haltner-Ukomado,[1] and Claus-Michael Lehr[3,5]

Received December 13, 2005; accepted May 8, 2006; published online August 9, 2006

Purpose. The purpose of this study was to establish a primary culture of porcine lung epithelial cells as an alternative to the currently existing cell cultures from other species, such as e.g., rat or human. Primary porcine lung epithelial cells were isolated, cultivated and analyzed at distinct time points after isolation.

Materials and Methods. The main part of the work focused on the morphology of the cells and the detection of alveolar epithelial cell markers by using electron microscopy, immunofluorescence microscopy and immunoblotting. Regarding a later use for *in vitro* pulmonary drug absorption studies the barrier properties of the cell monolayer were evaluated by monitoring bioelectrical parameters and by marker transport.

Results. Epithelial cells isolated from porcine lung grew to confluent monolayers with typical intercellular junctions within a few days. Maximum transepithelial resistance of about 2,000 Ωcm^2 was achieved and demonstrated the formation of a tight epithelial barrier. Permeability data of sodium fluorescein recommended a minimal transepithelial resistance of 600 Ωcm^2 for transport studies. The cell population changed from a heterogeneous morphology and marker distribution (caveolin-1, pro-SP-C, surface sugars) towards a monolayer consisting of two cell types resembling type I and type II pneumocytes.

Conclusions. The porcine alveolar epithelial primary cell culture holds promise for drug transport studies, because it shares major hallmarks of the mammalian alveolar epithelium and it is easily available and scaled up for drug screening.

KEY WORDS: differentiation; epithelial barrier; pneumocytes; porcine lung; pulmonary cell culture.

INTRODUCTION

Inhalation of systemically acting drug substances into the lung (pulmonary drug delivery), as an alternative to injection, is a frequently addressed topic in modern drug development. In this context transport processes across the pulmonary air-blood barrier came into focus and the demand for *in vitro* models, which imitate the mammalian lung, increased. Since whole organ models are very complex and difficult in handling, *in vitro* reconstructed epithelia are preferred as models. For the bronchiolar level characterized cell lines are available, whereas alveolar epithelial models are prevailed by primary cell culture. Isolation and culture of primary alveolar epithelial cells is possible e.g., in case of rat (1) and man (2,3). However, because of ethical and logistical problems related to human tissue or the small amount of tissue gained from small mammalian species like rat, alternative models should be developed, especially for high-throughput drug screening.

The pig closely resembles man in its anatomy and physiology as well as in histological and biochemical aspects (4). These are the main reasons for considering xenotransplantation from pig to man to overcome organ shortage for transplantation (5). Porcine cells and tissue have been widely used as models in studies investigating oral, ocular, transdermal, intestinal, buccal and nasal drug delivery (6–11). However, porcine alveolar epithelium has not yet been characterized for its suitability in drug absorption studies. One essential advantage of porcine tissue is its availability, because animals intended for slaughter can be used (i.e., reduction of animal use for research purposes). Furthermore the morphology and

[1] Across Barriers GmbH, Department R&D Cell & Tissue based Systems, Science Park Saar, Saarbrücken, Germany.
[2] Center for Electron Microscopy, Department of Anatomy and Cell Biology, Medical Faculty, Saarland University, Homburg, Germany.
[3] Biopharmaceutics and Pharmaceutical Technology, Saarland University, PO Box 151150, 66041, Saarbrücken, Germany.
[4] Present Address: Robert Koch-Institute, Berlin, Germany.
[5] To whom correspondence should be addressed. (e-mail: lehr@mx.uni-saarland.de)

ABBREVIATIONS: (ab), transport direction from apical to basolateral; AP, alkaline phosphatase; ATCC, american type culture collection; Flu-Na, sodium fluorescein; hAEpC, human alveolar epithelial cells; kDa, kilo dalton; MPA, maclura pomifera lectin; pAEpC, porcine alveolar epithelial cells; pAEpC-*n*, pAEpC isolation number *n* (cell batch); P_{app}, apparent permeability coefficient; PD, potential difference; rAEpC, rat alveolar epithelial cells; RCA, ricinus communis lectin; rpm, revolutions per minute; SAGM, small airway growth medium; SDS, sodium dodecyl sufate; TEER, transepithelial electrical resistance; $TEER_{max}$, maximum transepithelial electrical resistance; TRIS, Tris(hydroxymethyl)methylamine (buffer).

physiology of porcine mucosa seems to be comparable to that of man (11). In addition it is generally acknowledged that the enzymatic equipment of cells from these two species is very similar. For instance similarities between man and pig are evident with regard to the overall metabolic status of the two species, the structure and function of the gastrointestinal tract, pharmacokinetics of compounds, and the relative size and properties of mucin glycoproteins from mucus secretions of human and swine trachea epithelium (12,13).

Cell culture based models for drug absorption studies frequently use epithelial cells, which are grown to confluence on permeable cell culture supports. To prove the tightness of the intercellular junctions and to study the permeation characteristics of such an artificial epithelium is an essential pre-requisite for their use in transport studies. Moreover, the epithelium formed *in vitro* should possess all other essential morphological, biochemical and biophysical characteristics of the epithelium *in vivo*. In this study alveolar cells (mainly type II pneumocytes) of porcine lung were isolated and cultivated up to 13 days. Cells were characterized morphologically and by measuring general epithelial and alveolar marker molecules using immunocytochemistry and immunoblotting. In order to identify the barrier properties of the epithelium formed *in vitro*, bioelectrical measurements (i.e., transepithelial resistance and potential difference) were conducted. The functionality of this epithelial barrier depending on the development of cell monolayers was assessed by transport studies with the low permeability marker sodium fluorescein (Flu-Na). The results prove that porcine alveolar epithelial cells in primary culture (pAEpC) develop a tight epithelium, which resembles the alveolar epithelium *in vivo* and other cell culture models in many aspects. Therefore the porcine cell culture provides a good alternative for rat and human cell culture models.

MATERIALS AND METHODS

Cell Isolation and Culture Conditions

Primary Porcine Alveolar Epithelial Cells

Primary porcine alveolar epithelial cells were isolated according to a procedure originally developed for human alveolar epithelial cells (hAEpC) and published by Elbert *et al.* (2) as well as recently by Fuchs *et al.* (14) or Ehrhardt *et al.* (3). This method, originally described by Bingle *et al.* (15), Dobbs (1) and Cunningham *et al.* (16), was slightly modified and adapted to larger amounts of tissue. In this context the use of another species required the adjustment of at least some steps of the original isolation procedure (17).

Porcine lung organs from female, 6 months old pigs were obtained from an abattoir. The recommended starting amount of tissue material for a preparation is one pulmonary lobe of such a slaughtered animal. The fresh organ was transferred into sterile phosphate buffered saline and kept on ice during transport to the laboratory. Time between collecting the tissue and starting the isolation procedure was between 1 and 2 h. The mechanical mincing of tissue pieces from diaphragmal and cranial regions of the pulmonary lobe was followed by washing and the removal of visible bronchioles. Tissue cubes of approx. 5 mm edge length were subjected to a fine manual cutting process to enlarge the surface for enzymatic digestion. Resulting tissue pieces underwent several washing steps and filtration over 100 μm pore-sized nylon gauze. In contrast to the isolation of human alveolar epithelial cells/human protocol (2) there was a pre-incubation at 4°C for 1.5 h before entering the enzymatic digestion by a trypsin-elastase-combination at 37°C for another 30 min. Cell strainers were replaced by nylon filters combined with a glass device (Millipore GmbH, Schwalbach, Germany), which could be connected to a pump and contained greater amounts of tissue. Subsequent to the enzymatic treatment the resulting tissue mass got triturated (mechanically stressed), filtrated via nylon gauze and finally over 40 μm cell strainers. Differential adherence on plastic surfaces served to remove macrophages (incubation at 37°C for 90 min) and dissociation from blood cells and cell debris was done by a discontinuous percoll density gradient (σ = 1.089 g/ml and σ = 1.040 g/ml). Cells collected from the interface were washed and plated at a density of 8×10^5 cells/cm^2. Further removal of macrophages with magnetic beads, as described and published for human alveolar epithelial cells, was omitted in view of high costs and, because it was unclear if this antibody-based technique would work with porcine cells. However, satisfactorily results were obtained without this additional depletion of macrophages. An average of 24.1 (±5.0) g starting tissue mass yielded 9.50 (±6.89) $\times 10^6$ cells/g. Variances in this yield may reflect differences in age, gender or state of health of the respective donor animals which was, however, not further investigated.

Isolated cells were cultured on permeable fibronectin (2 μg/cm^2)/collagen(8 μg/cm^2) coated Transwell Clear filter inserts (12 mm diameter, pore size 0.4 μm, Corning Costar, Bodenheim, Germany). Cells were plated at a density of 8×10^5 cells/cm^2 and grown under liquid covered conditions (i.e., 500 μl apically and 1,500 μl basally) using small airway growth medium, SAGM, (Cell Systems, St. Katharinen, Germany) supplemented with gentamycin, ampicillin (50 μg/ml each) and penicillin G (200 U/ml). Fetal calf serum (1% (v/v), FCS) (Greiner Labortechnik, Frickenhausen, Germany) was added in order to reduce fibroblasts. Cultivation temperature was 37°C in a 5% CO_2 atmosphere and the cell culture fluid was replaced at least every second day. Subsequent isolation batches are referred to as "pAEpC-*n*" (*p*orcine *a*lveolar *ep*ithelial *c*ells) with *n* indicating the batch number.

Other cell culture models, such as Caco-2 and Calu-3, primary porcine brain endothelial cells (PBEC), were cultivated according to established protocols (18–20).

Electron Microscopy

Fixation

Porcine alveolar epithelial cell layers cultured on Transwell filters were quickly washed two times in phosphate buffered saline (PBS) and fixed in a mixture of 4% (w/v) paraformaldehyde (freshly depolymerized) and 1% (v/v) glutaraldehyde in 0.1 M cacodylate buffer. After 5 min at room temperature (RT) under agitation, the fixative was replaced twice by fresh fixative for another 30 min (RT, agitation) and then for about 12–24 h (4°C). Afterwards cells were washed three times using PBS and stored at 4°C.

After the initial fixation cells were postfixed with 2% (w/v) osmium tetroxide in 0.1 M phosphate buffer and dehydrated in a series of acetone (50, 70, 80, 90, 96, 100, 100%; each for 15 min at RT). Cells were then prepared for transmission or scanning electron microscopy according to the following protocols.

Transmission Electron Microscopy of Ultrathin Sections

Cells were infiltrated with an Epon resin (EMbed 812, Science Services International, EMS, Fort Washington, USA) according to Luft *et al.* (21) using acetone/resin mixtures (3:1, 1:1, 1:3) and pure resin. For embedding the Transwell filters were cut into pieces, placed into fresh resin and polymerized at 60°C for at least 2 days. Ultrathin sections were taken perpendicular to the filter surface (60–80 nm thick) and inspected with a transmission electron microscope EM10C (Zeiss, Germany) after staining with uranyl acetate and lead citrate for more contrast. In total, two randomly collected samples were analyzed for each time point.

Scanning electron microscopy of the resin block face was done as described by Laue *et al.* (22).

Scanning Electron Microscopy of Cell Surfaces

After dehydration, cells were dried by critical point drying. Subsequently Transwell filters were mounted on aluminium stubs and sputter coated with platinum. Two samples were inspected and analyzed for each time point/age of cells.

Analysis of the filter area covered by cells was based on two randomized samples. Digital images were taken at a magnification of 800× and overlaid with a lattice-grid (2.5 × 2.5 μm). The number of points lying over free filter surface was determined (point-counting method of basic stereology) and related to the total number of points.

Determination of Bioelectrical Parameters: Transepithelial Electrical Resistance and Potential Difference

Bioelectrical parameters were measured using an electrical voltohmmeter (EVOM, World Precision Instruments (WPI), Berlin, Germany) equipped with STX-2 "chopstick" electrodes. Transepithelial electrical resistance (TEER) was estimated relative to the corresponding surface area (=filter surface) and given in Ωcm^2. The potential difference (PD), defined as an open circuit membrane potential, was measured in millivolt. All measurements were corrected for the value measured with a coated cell-free Transwell filter.

Marker Transport of Sodium Fluorescein (Flu-Na)

In solute transport studies, Flu-Na at a final concentration of 10 μg/ml served as marker for passive paracellular diffusion. Due to the wide use of Flu-Na for this purpose, these data enable to compare different *in vitro* models (e.g., alveolar *versus* bronchial epithelial cells). The culture medium SAGM served as transport buffer in these studies.

Unidirectional fluxes (J) were determined from steady-state appearance rates over 2 h of each compound accumulating in the receiver fluid. The apparent permeability coefficient, P_{app}, is calculated according to the equation $P_{app} = J/(A \times C_i)$, where C_i is the initial concentration of the substance under investigation in the donor fluid and A the nominal surface area of cell monolayers (1.13 cm^2) utilized in this study.

Fluorescence of samples was analyzed in 96-well plates using a fluorescence plate reader (Victor^2, Wallac Perkin Elmer, Rodgau, Germany) at excitation and emission wavelengths of 485 and 553 nm, respectively.

Immunocytochemistry

Antibodies

The following primary antibodies served for qualitative detection of tight junction proteins ZO-1 (zonula occludens protein-1) and occludin: mouse anti ZO-1 antibody with species reactivity human and mouse monoclonal anti occludin antibody (both Becton Dickinson (BD) Transduction Laboratories, Heidelberg, Germany), as well as rat anti ZO-1 including porcine species reactivity (Chemicon, Hofheim, Germany). Localization of the adherent junction protein E-cadherin was examined by means of mouse anti E-cadherin (BD Transduction Laboratories, Heidelberg, Germany). Furthermore, mouse anti-cytoceratin antibody (Transduction Laboratories, Heidelberg, Germany) and mouse monoclonal LRP-56 (lung resistance protein) antibody (Sanbio, Beutelsbach, Germany) were used as primary antibodies. Caveolin-1 expression was analyzed using a rabbit polyclonal cav-1 (BD Transduction Laboratories, Heidelberg, Germany) antibody, which recognizes both the α- and β-isoforms of caveolin-1. Surfactant protein-C (SP-C) was detected by means of a rabbit polyclonal proSP-C anitbody (Chemicon, Hofheim, Germany), which recognizes the SP-C proprotein as well as processing intermediates.

Mouse IgG1κ (Sigma, M 5284, Deisenhofen, Germany), IgG rabbit serum (Dako, X0903, Hamburg, Germany) and rat IgG1, Clone DD9 (Chemicon, CBL 604, Hofheim, Germany) were used for isotypic controls and diluted to the protein content of the primary antibodies listed above. Fluorescence visualization of bound antibodies was done by incubation with secondary FITC-labeled (FITC = fluoresceinisothiocyanate) goat anti-mouse $F(ab')_2$ fragment, goat anti-rabbit $F(ab')_2$ fragment or FITC-conjugated rat-immunoglobulins (Dako, F 0479, F 1262, F 0234, Hamburg, Germany).

Immunofluorescence

Filter-grown pAEpC cell layers were washed three times in PBS, fixed on Transwell filter inserts with 2% (w/v) paraformaldehyde in PBS and blocked for 10 min in 50 mM NH_4Cl, followed by permeabilization for 8 min with 0.1% (w/v) Triton X-100. Subsequent to the fixation cells were incubated with a primary antibody diluted 1:100 in PBS plus 1% (w/v) bovine serum albumin (BSA), pH 7.2, anti-pro-SP-C was diluted 1:40. After a 60-min incubation with 100 μl of the diluted primary antibody, the cell monolayers were washed three times with PBS before incubation with 100 μl of a 1:100 dilution in PBS containing 1% (w/v) BSA of the respective FITC-labeled secondary antibody. Propidium iodide (PI; 1 μg/ml) was then added to counterstain cell nuclei. After 30 min incubation, the specimens were washed

three times with PBS and embedded in FluorSave anti-fade medium (Calbiochem, Bad Soden, Germany).

Lectin Binding

For cytochemical detection of cell-specific lectin binding, Ricinus communis agglutinin (RCA) and Maclura pomifera agglutinin (MPA) served as differentiation markers for type I and type II pneumocytes, respectively, a modified Krebs-Ringer buffer (KRB) (glucose replaced by manitol) was used instead of PBS. After three washing steps in modified KRB cell monolayers were fixed with 2% (w/v) paraformaldehyde and blocked in 50 mM NH_4Cl.

Cell layers were incubated with lectin at a concentration of 50 µg/ml each, for 1 h at 37°C in a water saturated 5% CO_2 atmosphere. The incubation was performed with either vital or fixed cells. In the first case cells were subjected to three washing steps, incubated and mounted in FluorSave or else fixed as described above, followed by incubation and subsequent mounting.

The type I cell-specific lectin Ricinus communis agglutinin (RCA) was used in its rhodamine-labeled, the type II cell-specific Maclura pomifera agglutinin (MPA) in its FITC-labeled form (Vector Laboratories, Burlingame, CA, USA over Boehringer Ingelheim Bioproducts, Ingelheim, Germany). Freshly plated cells (1 day) were air-dried instead of fixation and examined the following day. Cell nuclei were not counterstained. Cells at the age of 4 or 7 days were subjected to both, single- and co-incubation.

Images were obtained with a confocal laser scanning microscope (MRC-1024, Bio-Rad, Hemel, Hempstead, UK) with the instrument settings adjusted so that no positive signal was observed in the channel corresponding to the green fluorescence of the isotypic controls.

Staining for Alkaline Phosphatase

Expression of alkaline phosphatase, as another marker of differentiation, was tested comparing pAEpC immediately after plating, with those cultured for a period of 6 days. A Sigma Diagnostics test kit (Sigma, kit 86R, Deisenhofen, Germany) was used for histochemical detection of phosphatase activity. Cells were plated onto fibronectin/collagen(Fn/Col)-coated glass cover slips, which were placed on the bottom of 12-well plates. Freshly isolated cells (1 day) were air-dried, whereas cultured cells were washed twice with PBS and fixed with methanol (4°C, 30 min). After three more washing steps 400 µl of the staining solution was added into each well and incubated at RT for 10–30 min under protection from light. Staining was performed according to the instructions of the test kit. Stained pAEpC were examined under an inverted light microscope (Leica Microsystems Wetzlar GmbH) connected to a Nikon coolpix990 digital camera for documentation of micrographs.

Western Blot

Detection of proteins by Western blot analysis served to check the specificity of the used antibodies and to compare pAEpC in primary culture with cell lines (Calu-3, Caco-2). Different cell isolations of pAEpC were examined at 0, 2, 4 and 7 days in order to obtain at least semi-quantitative knowledge about changes in protein expression in dependence on cultivation time of cells. Caco-2 and Calu-3 cells were cultured for 1 week before they were analyzed in Western blot studies for comparison with the primary cells.

Detection of individual proteins was performed according to Roche's Manual (23) and current literature as referred in Table I.

Protein Extraction

Cells either cultured on Transwell Clear filter inserts or freshly isolated pAEpC (day 0) were washed twice with PBS (4°C) and lysed in sodium dodecyl sulfate (SDS) sample buffer for approximately 5–10 min while cooled on ice. This buffer contained 50 mM TRIS/HCl, (pH 8), 1% SDS and 50 µM Dithiothreitol (DTT) (Carl Roth GmbH, Karlsruhe, Germany). Lysates were clarified by centrifugation at 16,000 g for 2–5 min, the resulting supernatant was boiled at 95°C for 10 min (denaturation) and stored at −20°C.

Total protein determination of samples was performed by means of MicroBC-Assay protein quantitation kit (Uptima, Montlucon, France) using bovine serum albumin as standard (24).

Gel Electrophoresis

Lysate supernatants were diluted 2:1 (to give a total protein concentration of 1 µg/ml) with 2× electrophoresis sample buffer (1× = 150 mM TRIS/HCl (pH 6.8); 1.2% SDS; 30% glycerol; 15% β-mercaptoethanol and 18 mg/l bromophenol blue); all reagents obtained from Carl Roth GmbH, Karlsruhe, Germany. Aliquots equivalent to 15 µg total protein per sample were loaded and resolved in a 8–15% SDS/polyacrylamide gel (SDS-PAGE) according to Laemmli (25) using a Minigel-Twin (Whatman Biometra, Göttingen, Germany). A full-range rainbow prestained molecular weight marker (Amersham Biosciences, Freiburg, Germany) was concurrently electrophoresed.

Immunoblotting

Gels were electroblotted to Roti-PVDF (polyvinylidene difluoride) Transfer Membrane (Carl Roth GmbH, Karlsruhe, Germany) in a Tank Blot (Whatman Biometra, Göttingen, Germany) filled with Towbin blotting buffer (25 mM TRIS/HCl, 192 mM glycin, 0.1% SDS, 10% methanol). A cooling jacket allowed blotting either at high current (1 A) for 1 h or at low current (100 mA) over night. Non-specific protein binding on the membrane was blocked in 1–2% Slim Fast (Allpharm

Table I. Proteins Analyzed by Immunoblotting

Protein	Molecular Weight	Localization/ Purpose	Method for Western Blot Analysis
Caveolin	22 kDa	Type I pneumocyte	(14,27,40)
pro-SP-C	21 kDa	Type II pneumocyte	(44,45)
Occludin	65 kDa	Tight junction	(46,47)
E-cadherin	120 Da	Adherent junction	(46,48)

Vertriebs GmbH, Messel, Germany) in Tween-buffer (50 mM TRIS (pH 7.5); 150 mM NaCl; 0.1% Tween 20) for 2 h at RT and overnight at 4°C.

After washing in Tween-buffer, membranes were incubated with primary antibody for 1 h at RT, then washed again and probed for 45 min with the secondary HRP (horseradish peroxidase)-conjugated antibody. For quantitative results SDS-PAGE gels were loaded with samples of identical total protein content and membranes were co-incubated with primary antibody plus anti-beta-Actin, which was used as an internal standard and detected in parallel.

Primary antibodies:

Anti-ZO-1, human, (BD Transduction Laboratories 610966, Heidelberg, Germany)

Rat Anti-ZO-1, porcine, (Chemicon, MAB1520, Hofheim, Germany)

Anti-Occludin (BD Transduction Laboratories 611091, Heidelberg, Germany)

Anti-E-Cadherin (BD Transduction Laboratories 610182, Heidelberg, Germany)

Anti-Caveolin (BD Transduction Laboratories 610059, Heidelberg, Germany)

Rabbit Anti-Human proSP-C (Chemicon, AB3428, Hofheim, Germany)

Anti-β-Actin, monoclonal, mouse (Sigma, A5441, Deisenhofen, Germany)

Anti-Actin, rabbit (rabbit Anti-Actin affinity isolated antibody), (Sigma, A2066, Deisenhofen, Germany)

Secondary antibodies:

Goat Anti Rat IgG, HRP conjugated (Chemicon, AP136P, Hofheim, Germany)

Goat Anti Rabbit IgG, HRP conjugated (Chemicon, AP132P, Hofheim, Germany)

Goat Anti Mouse IgG, HRP conjugated (Chemicon, AP124P, Hofheim, Germany)

Bound antibodies were detected by a chemiluminescent signal generated by the use of a Roti–Lumin kit (Carl Roth GmbH, Karlsruhe, Germany), which is based on an enhanced chemiluminescence method (ECL). After incubating the blot with the corresponding substrate, the signal was autoradiographically detected on Kodak BioMax Light-1-Film (Amersham biosciences, Freiburg, Germany). Digital image acquisition and band quantification was performed via BioRad Gel Doc-Documentation System (Scanner) (Bio-Rad, Hemel, Hempstead, UK).

To quantify protein expression in pAEpC at different developmental stages, cells from two different cell isolations were studied and protein expression was determined on days 0, 2, 4 and 7 after isolation. In addition to loading samples of constant total protein content onto the SDS-gels, beta-actin served as internal standard with its molecular weight (42 kDa) sufficiently distinct from that of the studied proteins. Band quantification was performed by densitometry. The ratio between marker protein and beta-actin (internal standard) was compared over time in culture. Background intensity, which was proportional to the protein content, was subtracted to get the real signal for the individual time points. Signals were captured via a rectangle-technique or by means of a "free-hand"-tool and the average mean of both measurements used for further evaluation. In order to exclude systematical errors, samples of both cell isolations were analyzed in parallel.

RESULTS

Morphology of pAEpC

Isolated porcine lung cells were plated on Transwell filters and cultivated for 13 days. The morphology of three cell isolations was analyzed at distinct time points of culture (2, 4, 6, 8, 10, 13 days) using scanning and electron microscopy. The overall morphology and development of cultured cells in three individual preparation batches were the same, starting with single cells or small separated cell groups of a heterogeneous cell morphology at day 2 and ending at day 13 with a flat monolayer of a rather uniform cell population (Fig. 1).

Cell population at day 2 of culture comprised cells of various shape and ultrastructure. The cell shape varied between round and flat spread out onto the substrate. Most cells adhered to the substrate and formed numerous filopodia at their rims, frequently contacting other cells (Fig. 1A). Often cells were observed which settled on other cells or cell groups. These cells appeared either spherical with numerous surface microvilli or more irregular with many filopodia and resembled lymphocytes and macrophages, respectively. Erythrocytes were also present in the culture but disappeared during further cultivation. Ciliated cells were regularly intermingled between other cells and most likely derived from bronchial epithelium. In thin sections the typical ciliary morphology could be identified (not shown). In some flat cells but also occasionally in round cells on top of other cells numerous small holes were detected between the origins of the microvilli (compare Fig. 2C for similar structures in cells at day 13). Thin sectioning revealed the typical omega-shaped morphology of caveolae, which are characteristic for either endothelial cells or type I alveolar epithelial cells (not shown). The internal ultrastructure of the cells was as variable as their shape. In Fig. 3A a cell profile of a round cell is shown. Frequently several vacuole-like inclusions were present which often included membrane lamellae and/or vesicles (compare Fig. 3B which shows similar structures in a cell at day 4).

Cells grew to confluence after 1 week, starting from a surface coverage of 50–60% on day 2, over 80–90% on day 4, 96% for day 6 up to almost 100% at the age of 10 or 13 days (Fig. 1). However, even 13 day-samples revealed very few but clearly visible spots of uncovered filter surface. In parallel to the development of confluence, the average height of the cells was reduced. The height declined from about 5 μm on day 2 down to about 2–2.5 μm from day 4 on, as measured in images taken from the block face of embedded filters after sectioning.

Cells on culture days 10 and 13 formed a monolayer (Fig. 1E, F) with few locally restricted multi-layered spots (Fig. 2A). The monolayer was dominated by flat cells (even in their nuclear region) with broad cytoplasmic extensions. Cells with a protruding nucleus were interspersed in between these flat cells (Fig. 1F). Ciliated cells or small groups of multi-layered cells disrupt either the homogeneous surface appearance or the monolayer organization of the cells

(Fig. 2B). The internal ultrastructure of cells on days 10 and 13 revealed mainly two morphological forms: 1) flat cells with or without vacuole-like structures, which contain fragments of membrane lamellae and/or vesicles (Fig. 3F), and 2) round cells with several multilamellar bodies (Fig. 3E). Fields of caveolae were only occasionally detected in flat cells (Fig. 2C).

The development of cell preparations from day 2 of culture towards days 10 and 13 was not only characterized by the flattening and spreading of most cells. Cellular junctions, i.e., tight and adherent junctions, between cells were already clearly visible in young cultures (Fig. 3D). With ongoing time of culture and confluence the presence of these epithelial cell contacts increased. Erythrocytes and cells with the morphology of lymphocytes, macrophages, endothelial cells or bronchial cells were significantly reduced in frequency or disappeared. Most cells showed vacuoles containing membrane fragments or vesicles in their cytoplasm (Fig. 3B, C). Vacuoles with densely stacked membrane lamellae, like in typical multilamellar bodies observed in cells on days 10 and 13 (Fig. 3E) were rarely detected in cultures up to day 8 of cultivation (Fig. 3C). Some cells revealed few dispersed caveolae at their apical plasma membrane.

Development of Barrier Properties

Bioelectrical Parameters (TEER, PD)

Transepithelial electrical resistance (TEER; unit: Ωcm^2) was used as a standard parameter to quantify the tightness of the epithelial barrier. While the TEER gives information about the integrity of the barrier, the potential difference (PD) describes active ion transport in epithelial cells (unit:

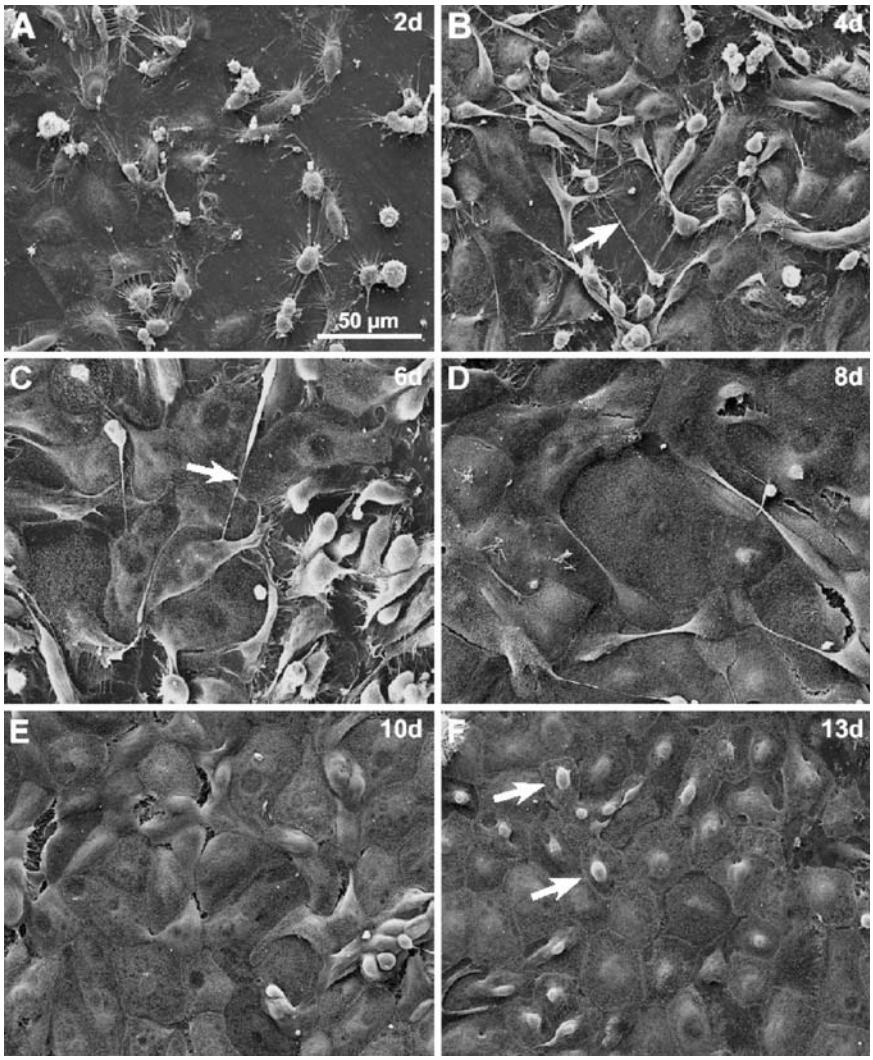

Fig. 1. Scanning electron microscopy of cultivated pAEpC at different days post plating. (A) On day 2 (2 days), cells of a different shape with numerous filopodia are adhered onto the filter substrate. (B, C) With time (4, 6 days), many cells become flat, spread out onto the substrate and thereby covering the filter surface. Fine cytoplasmic bridges connect some cells with each other (*arrows*). (D, E, F) On day 8 (D, 8 days) the coverage of the filter area is almost 100%. The monolayer appears flat with few cytoplasmic bridges between some of the cells, which disappear at later time points. On days 10 (E, 10 days) and 13 (F, 13 days) the monolayer appears quite homogenous. Occasionally single cells with elevated nuclei and small surface area (*arrows* in F) are inserted in between the flat cell monolayer.

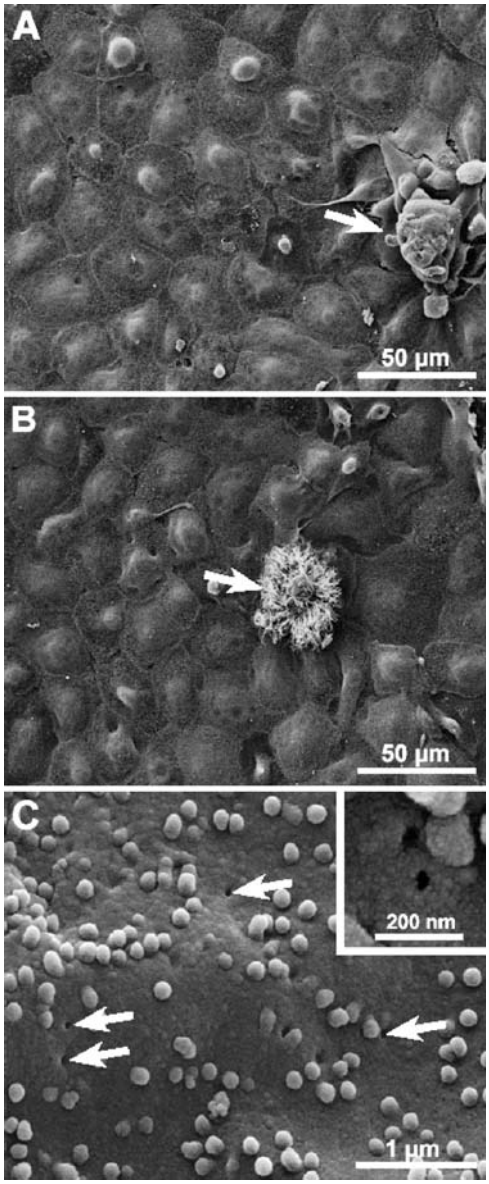

Fig. 2. Scanning electron microscopy of cultivated pAEpC at day 13 post plating. Cells form a flat monolayer. (A) Cluster of multilayered cells (*arrow*) exist at rare sites. (B) At few positions in the monolayer cells with brush-like surface projections are localized (*arrow*). (C) In some flat cells the apical plasma membrane possesses characteristic holes of about 40–60 nm in diameter, which most likely represent the apical openings of caveolae.

mV). Characteristic progressions of these parameters observed for pAEpC during the cultivation period are illustrated in Fig. 4A.

Cell cultures from different isolation batches showed different growth behavior and thus variation in the corresponding TEER and PD curves over time of culture. This seems to be a consequence of varying purity among the different cell isolations as indicated by microscopic observations. In particular, cell preparations differed in parameters, such as maximum transepithelial resistance ($TEER_{max}$), the time when it was reached, as well as duration of the TEER plateau and the time when it started. In most cases cultured pAEpC displayed an initial rise in TEER after 4–5 days, which was followed by a plateau (Fig. 4A, type A) lasting for 3–4 days. More heterogeneous cell preparations usually showed a retarded development of the transepithelial resistance but a similar curve shape (Fig. 4A, type B). Apart from that, some exceptional cell isolations featuring a slower but continuous increase in barrier properties and/or an extended plateau phase were observed in a few cases (data not shown).

The potential difference (PD) was monitored in parallel and gave similar results (data not shown). If referred to a single isolation batch, it consistently followed the development of TEER, reaching a maximum (PD_{max}) of 6–14 mV between culture days 5–10. At the same time pAEpC monolayers achieved their maximum TEER of 1,200–2,400 Ωcm^2.

Integrity of pAEpC Monolayers

In addition to measuring TEER values, the paracellular integrity of pAEpC monolayers was assessed by transport studies using the hydrophilic marker Flu-Na in absorptive (ab) direction. The average permeability coefficient P_{app} for pAEpC was calculated as $3.49 \pm 1.66 \times 10^{-7}$ cm/s ($n = 39$ filters, obtained from 14 independent cell preparations, transport buffer SAGM). As expected, an inverse correlation between permeability coefficient and TEER was observed. Fig. 4B illustrates that P_{app}(Flu-Na) declined with increasing TEER values. According to these data no further significant decline in permeability was detected for cell monolayers with TEER values > 600 Ωcm^2, which therefore was defined as a limit for monolayer integrity in drug absorption experiments.

Due to these results further characterization concentrated on cell monolayers exceeding a minimum TEER of 600 Ωcm^2 and focused on the plateau phase (culture days 6 and 7) as the potential time window for later transport experiments.

Immunocytochemical Characterization of pAEpC

Immunostainings should identify the presence of epithelial proteins and cell markers at the cellular level. Since most of the used antibodies were initially not tested for their species reactivity in swine (apart from ZO-1), their specificity was also investigated by Western blot analysis (compare 3.4).

Junction Proteins

Porcine alveolar epithelial cells developed an increasing transepithelial resistance with ongoing time of cultivation most likely by establishing functional cell-cell contacts. For a further characterization, pAEpC monolayers were analyzed for marker proteins of epithelial cell-cell contacts and for the presence of the epithelial marker cytoceratin.

In young cultures (4 days) ZO-1 immunofluorescence was distributed as small bright spots all over the cytoplasm and lined more or less clearly the cell border (data not shown). In pAEpC monolayers stained after 7 days in culture, the fluorescent signal in the cytoplasm had disappeared and the cells showed a bright and continuous immunofluorescence marking the circumference of the cell (Fig. 5A).

An analogous labeling pattern was obtained for occludin, another tight junction protein (Fig. 5B).

In addition pAEpC (6 days) stained specifically for the adherent junction protein E-cadherin, as well as for the common epithelial cell marker cytoceratin (Fig. 5C, D). In

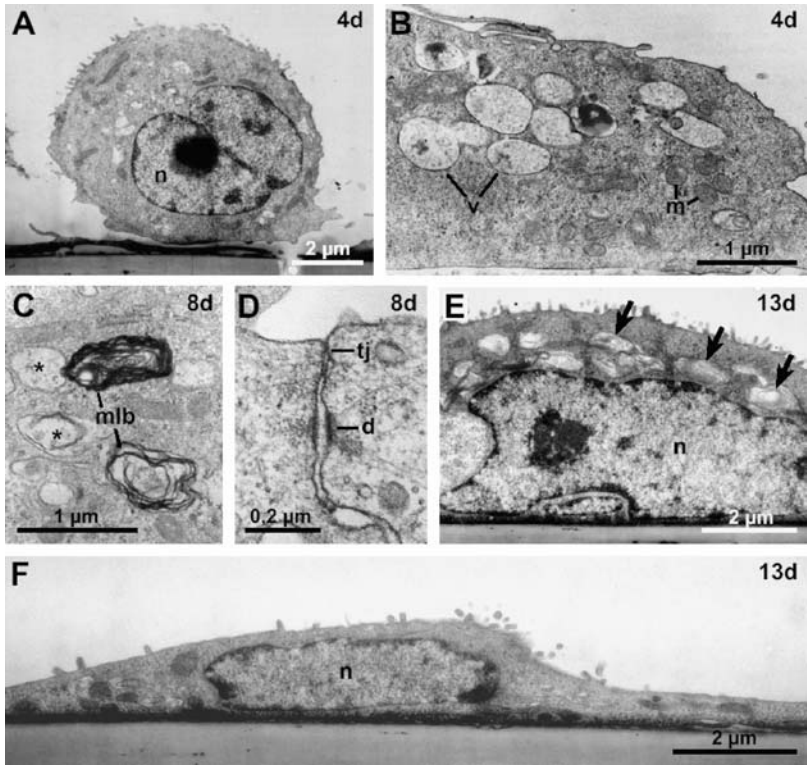

Fig. 3. Transmission electron microscopy of cultivated pAEpC at different time points post plating. (A, B) Typical cell forms at day 4 of culture. A round cell without particular cell organelles is localized on the flat extensions or filipodia of other cells (A). (*n*) nucleus. Many of the flat cells contain vacuoles (*v*) with some vesicles or amorphous electron dense material (B). (*m*) mitochondria. (C, D) On day 8, in few cells multilamellar bodies (*mlb*) can be detected besides vacuoles (*asterisk*) (C). The cell monolayer is already highly confluent and the cells display the typical epithelial cell contacts, i.e., tight junctions (*tj*) and desmosomes (*d*). (E, F) With ongoing cultivation time, two morpho-types develop. At day 13 some cells reveal a more round morphology with several characteristic multilamellar bodies in the apical cytoplasm (*arrows*) (E). The other cells are comparatively flat spread out on to the substrate and reveal no particular ultrastructure (F).

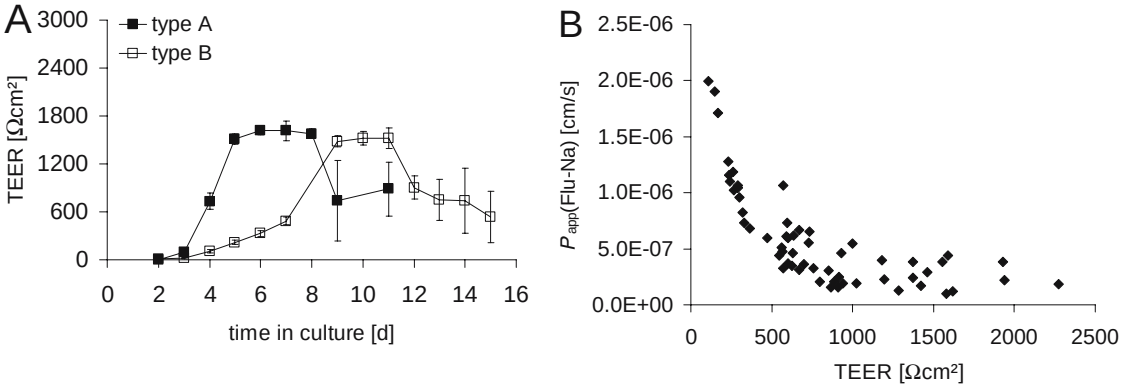

Fig. 4. Bioelectrical parameters monitored during culture of pAEpC monolayers, and their integrity assayed by solute permeability. (A) Typical TEER-time-courses observed in different cell isolations; data are given as mean ± SD; $n = 3-6$. Cells of some cell isolations (*type A*) grow faster, i.e., they feature a steeper increase in TEER and confluence is reached earlier. Whereas other cell preparations (*type B*) comprise a retarded development. (B) Correlation between P_{app} of Flu-Na (10 µg/ml) across pAEpC monolayers, and initial TEER values. Each data point represents the result obtained from a single filter. The observed inverse correlation (rising TEER values paralleled by a decrease in permeability) suggests a threshold of 600 Ωcm^2.

case of E-cadherin the observed fluorescence was distributed within the cytoplasm but especially concentrated close to the cell border. Staining for cytoceratin was detected as diffuse cytoplasmic fluorescence and in some cells as groups of small bright fluorescent dots. Qualitative detection of E-cadherin underlines the formation of functional cell-cell contacts in this primary cell culture and the presence of cytoceratin identifies pAEpC monolayers as epithelial cells.

Alveolar Epithelial Cell Marker

Antibodies against caveolin-1, surfactant proteins and the lung resistance protein (LRP) were used to identify alveolar epithelial cells. Subsequent to proving that the used isolation method results in epithelial cells, it was important to classify different types of alveolar cells; i.e., to differ between type I and type II pneumocytes in particular, by applying cell-type specific cytochemical staining. In addition to characteristic proteins, lectin binding is also proposed to trace a differentiation process (2,26). Typical markers for type II cells, the progenitor of type I cells, are surfactant protein-C, alkaline phosphatase and binding to Maclura pomifera agglutinin (MPA), while caveolin and binding to Ricinus communis agglutinin (RCA) are considered as markers for type I cells. The lung resistance protein (LRP) is characteristic for secreting cells and therefore attributed to type II pneumocytes. Characterization was mainly focused on 6 day-old monolayers because most cell preparations achieved TEER exceeding 600 Ωcm^2 within this cultivation time indicating the development of a tight epithelial barrier.

Since the ultrastructural diagnosis of invaginations as caveaolae is not unequivocal, the biochemical presence of caveolin (27) in pAEpC monolayers was analyzed. The caveolin-1 immunofluorescence was detected at the cell surface and in form of bright fluorescent spots in the cytoplasm of a subpopulation of cells. As illustrated in Fig. 6A not all cells within the population stained positive for caveolin.

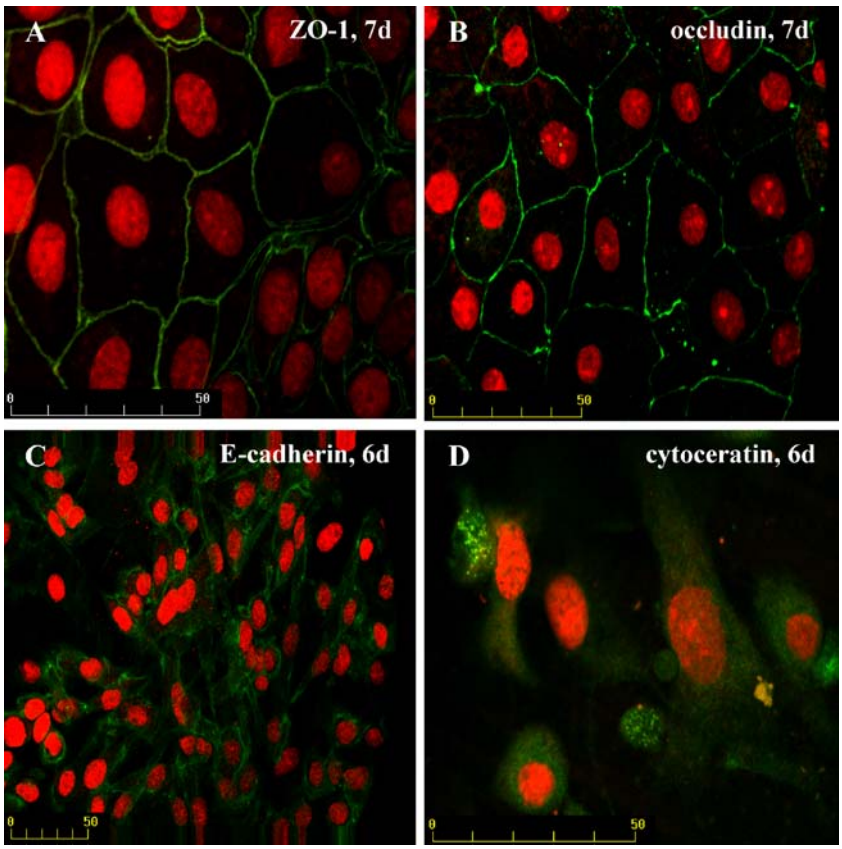

Fig. 5. Confocal laser scanning microscopy of pAEpC monolayers during the plateau phase, immunostained for epithelial cell-cell contact markers: Monolayers after 7 days in culture are analyzed for the tight junction proteins ZO-1 (A) and occludin (B), and on day 6 in culture for the adherent junction protein E-cadherin (C) and epithelial cell marker cytoceratin (D); cell nuclei are counterstained with propidium iodide. *Scale bars* represent micron (μm). (A) At day 7 ZO-1 staining is sharply confined to the cell border, resulting in a circumferential labeling of cells. (B) A similar labeling pattern is obtained for the tight junction protein occludin. Cell cultures on day 7 feature a few occludin-positive cytoplasmic spots besides a more or less continuous cell border staining. (C, D) E-cadherin and cytoceratin are present in pAEpC monolayers on day 6 in culture. The adherent junction protein is overall detected in the cytoplasm as well as at the cell border region, whereas fluorescent signals for the epithelial marker are distributed at the surface and in the cytoplasm of cells, but also display positive cytoplasmic spots.

Surfactant protein-C (SP-C) is an important component of the surfactant film covering the alveolar epithelium and is mainly secreted by type II cells (28). As shown in Fig. 6B, pro-surfactant protein-C (pro-SP-C), a pre-mature form of SP-C, was present in pAEpC monolayers (6 days). However, cells of a flat, type I-like morphology gave a less intensive signal. Isotypic control for these antibodies (both anti-rabbit) was analyzed in parallel and did not show specific fluorescence (not shown).

The lung resistance protein LRP-56 is known to be intensely expressed in epithelial cells with secretory and excretory functions, as well as in cells chronically exposed to xenobiotics, such as bronchial epithelial cells. The protein is found in vesicles and works as major vault transporter protein (vaults = multi-subunit structures) (29). In pAEpC some cells showed intense labeling of the cytoplasm as densely arranged spots indicating a vesicular association (Fig. 6C).

Alkaline phosphatase (AP) is a differentiation marker that is known to be expressed in alveolar type II pneumocytes, but absent in type I cells (30). Isolation of pAEpC is supposed to predominantly gain type II pneumocytes, which are able to undergo differentiation into type I cells with time in culture. Cytochemical staining performed subsequent to isolation of pAEpC could detect the presence of the type II cell specific marker enzyme (AP activity) in freshly isolated (analyzed as soon as possible, 1 day) pAEpC-cultures, whereas pAEpC monolayers at the age of 6 days did not stain (not shown).

As additional cell type marker lectin binding proved to be complementary to the change of expression pattern of cellular proteins in human type I- and type II-pneumocytes (2,26). In tissue sections the Ricinus communis agglutinin (RCA) stains the surface of type I pneumocytes, while the Maclura pomifera agglutinin (MPA) binds to the surface of type II cells. In pAEpC, RCA and MPA stained the surface of cells and in case of MPA their nuclei as well (only surface labeling has a diagnostic relevance). Freshly isolated cells were air-dried, not fixed, because fixation usually removes cells from the preparation.

Co-incubation with both lectins at the same time in 4 day-old pAEpC monolayers showed double labeling with both lectins in a subpopulation of cells, indicating a mixture of type I and type II characteristics in these cells. Besides those double-labeled (MPA + RCA) cells, single-labeled cells, which only bound RCA, were observed (Fig. 7A). A comparison of 1 day (not shown) *versus* 7 day-old pAEpC after single-incubation with either MPA (Fig. 7C) or RCA (Fig. 7D) revealed an identical labeling pattern. The fluorescence was concentrated as dense, fine granulation at the cell surface and along the cell borders. At both time points many cells stained for MPA and only few stained strongly for RCA. Also lectin-incubation of living instead of fixed cells did not change this result. In summary, the lectin binding argues rather for a mixture of cell type specific features in pAEpC at the analyzed time of culture than for a clear segregation into two cell types.

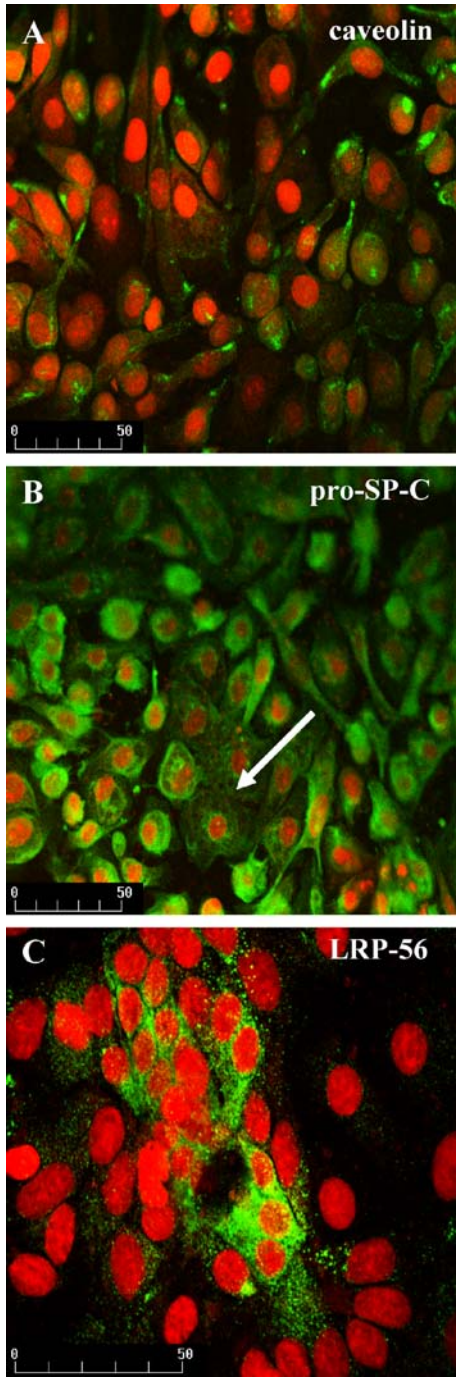

Fig. 6. Immnunostaining for lung epithelial cell markers caveolin, pro-surfactant protein-C (*pro-SP-C*) and bronchial epithelial cell marker lung resistance protein (*LRP*) in pAEpC monolayers after 6 days in culture. Cell nuclei are counterstained with propidium iodide (*red fluorescence*). (A) The caveolin-1 staining (*green fluorescence*) is rather weak and only concentrated to brighter spots in the cytoplasm and to the cell surface in some cells. (B) Staining for pro-SP-C is found in all cells. The distribution is rather diffuse with a slight concentration around the nucleus and the cell borders. Cells, which cover a large area, indicative for a type I-like morphology, show a less intensive staining (*arrow*). (C) The staining with antibodies against LRP shows a gradual intensity among the cells of the monolayer ranging from strong staining to no staining. Staining appears granular, which is in accordance with the vesicular localization of the LRP. *Scale bars* represent micron (μm).

Qualitative and Semi-Quantitative Western Blot Analysis of Cell Marker Proteins in pAEpC

In a qualitative Western blot analysis pAEpC were compared with two tumor-derived human epithelial cell lines, the bronchial Calu-3 and the intestinal Caco-2. Due to their

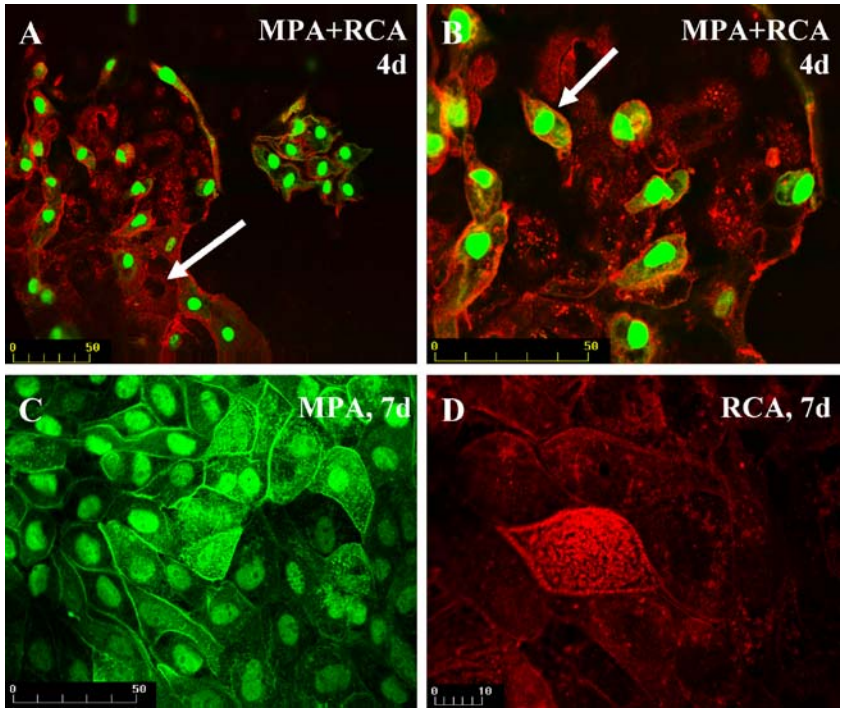

Fig. 7. Confocal laser scanning microscopic detection of lectin binding patterns in pAEpC. Images depict pAEpC after incubation with rhodamine-labeled, type I cell-specific ricinus communis lectin (*RCA*, *red fluorescence signal*) or FITC-labeled, type II cell-identifying maclura pomifera lectin (*MPA*, *green fluorescence signal*) or both at the same time. (A, B) Co-incubated pAEpC monolayers on day 4 in culture show cells binding both lectin markers or cells binding only RCA [*arrow* in panel (A)]. *Arrow* in panel B marks a co-stained cell (*yellow fluorescence*). Note that MPA also labels the nuclei of cells. (C, D) Single-incubation of pAEpC monolayers with either MPA (C) or RCA (D) on day 7 in culture reveals a fine granular staining of the cell surface and borders. Many cells are stained strongly by MPA (C) and only few cells are strongly stained by RCA (D). However, at least a faint staining for both lectins is visible in all cells. *Scale bars* represent micron (μm).

morphological and functional similarity to the human airway or small intestine mucosa, respectively, they are widely used as *in vitro* models of these biological barriers. To model the alveolar epithelium there seems to be no cell line available today, which displays sufficient barrier tightness as required for transport experiments. Similarities of a new primary culture to epithelial models of the same organ (Calu-3) or differences to that of other epithelial barriers of the human body, like the gastro-intestine (Caco-2), should help to evaluate a new approach.

A semi-quantitative analysis served to monitor protein expression in pAEpC at different time points of culture. Analyzed proteins as well as their corresponding molecular weights are listed in Table I. The specificity of the used antibodies was ascertained, because they generated autoradiographic signals at the reported molecular weights. Pro-SP-C could not be transferred on to the blotting membrane and therefore could not be analyzed by immunoblotting. Nevertheless, the immunostaining pattern obtained in case of pro-SP-C was comparable to that observed for a human *in vitro* model (hAEpC) (14) indicating specific labeling. The specificity of the antibody against tight junction protein ZO-1 was already documented by the manufacturer.

Qualitative Detection

Results are summarized in Table II. Caveolin-1 was detected in pAEpC over a period of 8 days, as well as in isolated (0 day) cells. Bronchial Calu-3 cells, which were examined at the same age (8 days), also seemed to express caveolin-1, although showing a less intensive signal. The tight junction protein occludin was present in both epithelial cell lines Caco-2 and Calu-3. In pAEpC occludin could not be detected after the isolation (0 day), whereas a positive signal was obtained for pAEpC at the age of 2, 4 or 8 days (Fig. 8B). A possible explanation for this result is that cell-cell contacts were destroyed during the isolation process (e.g., by the digesting enzymes). Afterwards isolated cells have to recover and to reorganize in an epithelial monolayer. Therefore, a new synthesis of junction proteins would be required as a precondition to re-build cell-cell contacts. Expression of the adherent junction protein E-cadherin was proved in pAEpC at the age of 4 days and in both cell lines, suggesting the development of functional cell-cell contacts.

In summary, proteins which contribute to the tightness of an epithelium (occludin and E-cadherin) were detected in all of the three considered *in vitro* models, whereas

Table II. Qualitative Detection of Caveolin, Occludin and E-cadherin by Immunoblotting, Comparison of Primary pAEpC with Caco-2 and Calu-3 Cell Lines

	pAEpC (0 day)	pAEpC (8 days)	Calu-3 (8 days)	Caco-2 (8 days)
Caveolin	+++	+++	(+)	−−
Occludin	−−	++	++	+++
E-cadherin	+	+++	++	+

"−−": no signal/detection; "+": positive signal; number of symbols indicates signal intensity.

caveolin was only found in pAEpC cultures and to a far less extent in the bronchial cell line.

Semi-Quantitative Detection

To get information about the development of marker protein expression in pAEpC over time of culture, cells from two different cell isolations were investigated. Protein expression was determined on days 0, 2, 4 and 7 after isolation and related to the expression of beta-actin.

The alveolar epithelial cell marker *caveolin-1* was detected in all analyzed cell samples at the same molecular weight as the positive control (Fig. 8A). For some samples (pAEpC-35: 2 days; pAEpC-36: 0, 2 days; positive control) an additional band appeared just beneath the caveolin band with the indicated molecular weight. This band was also recognized in the positive control, but was excluded from quantitative assessment. The time course on caveolin expression was different for the two analyzed cell isolations pAEpC-35 and -36 as shown by the protein density ratios (Fig. 8A). Both passed a minimum on day 4 in culture, followed by an increase, which was steeper in case of isolation batch 35.

Signals of the tight junction protein *occludin* were located between marker bands of 50 and 75 kDa in all samples, except in those of freshly prepared cells (Fig. 8B). The fact that the density of occludin-spots increased continuously with time in culture suggests a predominant expression of the protein in older cells, which is in accordance with the formation of cell-cell contacts. Ratios calculated in relation to beta-actin as an internal standard, which was detected throughout all cell lysates, showed that expression of occludin increased from days 0 to 2, holding a plateau level up to day 4 before increasing again up to day 7 (Fig. 8B). Both cell preparations developed in a similar way.

Detection of *E-cadherin* revealed positive spots localized between marker bands of 105 and 160 kDa, i.e., according to the positive control (Fig. 8C). The internal reference beta-actin was observed in each lane. The examined cell isolations showed some differences in expression of E-cadherin. While in pAEpC-36 the protein was equally expressed at all tested time points, in pAEpC-35 E-cadherin expression started at low expression levels and increased with time of culture (Fig. 8C). Despite these differences, a considerable and constant expression of E-cadherin was detected for cells older than 4 days, which was paralleled by the formation of cell–cell contacts and a corresponding TEER development.

DISCUSSION

The aim of the present study was the characterization of a primary porcine epithelial cell culture, which may serve as a model for the human alveolar epithelium. The results are

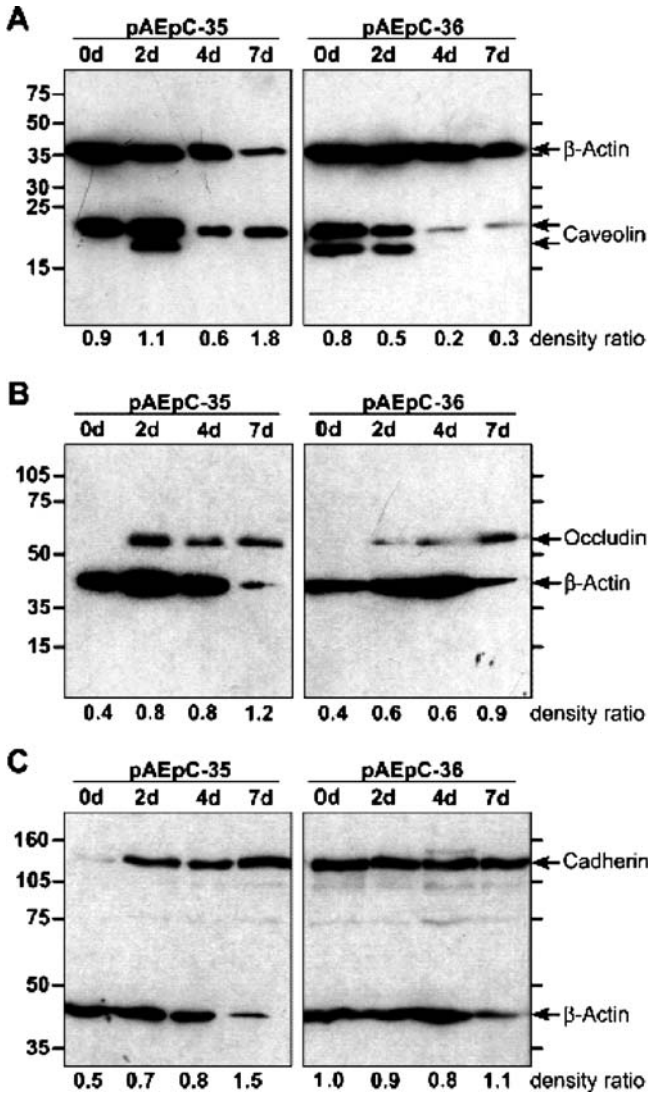

Fig. 8. Quantitative detection of caveolin, occludin and E-cadherin by Western blotting, comparing two different cell preparations of pAEpC at the age of 0, 2, 4 and 7 days; beta-actin serves as internal standard with 42 kDa molecular weight. The development of protein expression is described by density ratios: protein/internal standard and given as numerical values below each lane. Monolayers from 6 filter inserts are pooled for analysis of one time point. (A) Expression of caveolin is detected on all tested time points but differs for the two analyzed cell isolations with more intensive signals observed for pAEpC-35. Nevertheless, in both cases cell lysates of younger cultures (0, 2 days) feature a higher protein content than older ones (4, 7 days). According to densitometry both cell isolations feature a minimum in caevolin expression on day 4, followed by an increase. (B) Occludin is not found in freshly isolated cells (0 day). Expression increases in both cell preparations with time of culture. (C) Expression of E-cadherin is different in the two analyzed cell preparations. While in pAEpC-36 the expression level is almost constant, in pAEpC-35 it increases from low levels to a higher level than in pAEpC-36.

discussed in comparison with data published for similar cell cultures of other mammalian species, including man (31). The human or the porcine alveolar epithelium *in situ* was not analyzed, because the overall hallmarks in both species are the same (4,32,33) and are well documented (11).

Differentiation of Porcine Alveolar Epithelial Primary Cells (pAEpC)

The mammalian alveolar epithelium is a single-layered epithelium basically comprised out of two different cell types, type I and type II pneumocytes, which possess a distinct morphology. Type I pneumocytes are flat cells with a protruding nucleus and cover a comparatively large area. 93% of the surface of the alveolar air space is covered by type I cells, although representing only 8% of the total number of alveolar cells (i.e., mean number of cells $19 \pm 3 \times 10^9$) (33–36). In contrast type II pneumocytes, with their mean volume being half that of type I cells, comprise 16% of the total alveolar cells (number of cells $37 \pm 5 \times 10^9$) but cover only 7% of the alveolar surface (1,34). The apical and the basal plasma membrane are characterized by caveolae, which are usually not formed by type II pneumocytes. The latter are of a cubical shape and bear particular intracellular structures, the multilamellar bodies, which form the surfactant of the lung.

Cultivation of pAEpC starts with a morphologically heterogeneous cell population, including pneumocytes, endothelial cells, erythrocytes and probably macrophages as well as lymphocytes, which then develop a thin monolayer with time of culture. After 10 to 13 days the cells of the monolayer can be assigned to two different morpho-types, which resemble the described type I and type II pneumocytes of the alveolar epithelium *in situ*. Most cells are flat and cover a large surface of the substrate representing type I morphology, while few smaller, but elevated cells are intermingled between these flat cells, like the type II cells *in vivo*. The typical hallmarks of pneumocytes, i.e., multilamellar bodies and caveolae, are also present at least in some of the cultivated cells.

The morphological analysis did not reveal cells with a clear type II morphology on day 2 of culture. In contrast, many cells possess vacuole-like structures in their cytoplasm, which contain vesicles and/or membrane lamellae. These structures might be remnants of intact multilamellar bodies of the isolated type II cells. It has been reported that type II cells lose their multilamellar bodies in the course of the isolation process and by cultivation (26,37,38). During the ongoing development of the culture most of the cells flatten but retain the characteristic vacuole-like compartments. This decrease in the overall thickness of a cell monolayer was confirmed by epithelial height measurements and findings agreed with data reported for *in vitro* models of other species (2,34,37). The other cells do not flatten and probably reconstruct multilamellar bodies from these vacuole-like compartments, which contain membrane structures similar to the lamellar material of typical type II cell multilamellar bodies. At late time points some of the flat cells develop caveolae, indicative for a type I cell, but many cells still show the vacuole-like inclusions. Thus, the overall morphology seems to represent a mixture between type I and type II features. This notion is supported by the analysis of cell type specific marker molecule expression in the cells (i.e., expression of pro-SP-C, caveolin-1, sugars). While the expression of marker molecules showed a tendency towards a particular morphological type, in many cells an overlap of marker molecule expression was detectable. This mixture of cell-type specific features can be explained by assuming that cells pursue an ongoing differentiation process, which starts with the isolated but morphologically impaired type II cells (caused by the isolation process) and proceeds towards the two cell types. The initial phase of the differentiation is characterized by spreading, formation of cell contacts and establishing of cell shape. The later steps end up with the development of the typical hallmarks, which are important for function of the differentiated cells. In this scenario cells with a mixture of cell type specific features would represent intermediate stages which have conserved the type II pattern from their progenitor and not yet fully established type I characteristics. This interpretation is in accordance with the theory that type II cells are the progenitor cells of type I cells (39). Studies on primary cell culture models of the alveolar epithelium of other animals and man showed a comparable differentiation process (2,14,26,30,32,34,37,38,40). However, further studies should investigate if differentiation can be fully completed, i.e., if cells with a complete segregation of type I and type II features develop.

Integrity of Epithelial Monolayers and Barrier Properties of pAEpC

The alveolar epithelium has a barrier and a transport function. Intercellular cell contacts formed by junction proteins are a pre-requisite for establishing an efficient diffusion barrier. In pAEpC the typical epithelial cell contacts (tight junction, adherent junction) could be shown by electron microscopy. In addition, the junction proteins ZO-1, occludin and E-cadherin are expressed in pAEpC. With ongoing cultivation more occludin, a tight junction protein, is expressed. This development is paralleled by an increasing surface coverage of the cells and a rise in TEER. These features are comparable with that of other primary cell culture systems for the alveolar epithelium in other species (14,31,41). Table III shows the bioelectrical parameters of cell culture models derived from three species (rat, swine and man). The values of TEER and PD are about the same, indicating similar barrier properties in these different culture systems.

TEER values can serve as an indicator for the developmental stage of the culture in respect to epithelial integrity. A non-invasive measurement to determine the developmental stage of a culture system is necessary for its use in transport assays. Suited cultures should rest at high TEER levels as a consequence of a functional epithelial barrier

Table III. Comparison of Bioelectrical Parameters

Cell Culture	Origin	$TEER_{max}$ [Ωcm^2]	PD_{max} [mV]	Reference
rAEpC	Rat	>2,000	10	(41)
pAEpC	Porcine	1,200–2,400	6–14	Own data
hAEpC	Human	2,100	13.5	(14)

Overview of maximal TEER and PD in primary alveolar epithelial cell cultures obtained from different species.

during the time required for a transport experiment (usually 1.5–4 h). In most cultures of pAEpC TEER values reach a plateau, lasting 2–3 days, after an initial fast rise, rendering this time span suitable for transport studies. Daily monitoring of the TEER and calculation of the slope (for the respective curve segment) may help to identify the beginning plateau. After an initial increase the slope remains almost constant before it declines indicating the beginning plateau. Cultures, which do not show this behavior, are most likely different in their growth and differentiation.

Apart from bioelectrical measurements the determination of transepithelial permeability for paracellularly leaking solutes is also suited to gain information on the barrier properties of an *in vitro* model, i.e., finally about the functionality of its cell-cell junctions. Results from absorptive transport studies with Flu-Na across pAEpC monolayers prove the functionality of this epithelial diffusion barrier. The further characterization of this primary culture with respect to the transepithelial permeability as well as the presence or absence of active transporter systems or multidrug resistance is another important subject of ongoing investigations.

The formation of the epithelial barrier in most cell isolations was finished around day 6 of culture according to the TEER values and the coverage of the substrate surface by the cells. At this time differentiation of cells is not fully complete because functional important structures, like caveolae and intact multilamellar bodies are not yet formed, although their molecular components are already detectable. Between days 8 and 10 the cells start to develop the typical morphological structures, which proceeds and is not completed until the end of the observation time. In this time span the further development of the TEER varies: some cell isolations show a further increase, others stabilize at a less high and more extended plateau, but usually the TEER values decrease again. Nevertheless, this decrease following the plateau does not mean a sudden change (values move between 1,000 and 600 Ωcm^2 for over 4 days) and may be because of initiated transport processes by the cells. Thus further studies have to find conditions, where fully differentiated cells display suitable epithelial barrier properties for the use as an experimental model.

From the pharmaceutical point of view the described plateau phase is certainly the most interesting aspect. During the development of such an artificial epithelium, the presence of junction proteins as well as of the selected alveolar epithelial cell markers was confirmed by immunostaining. Results of parallel TEER measurements and their correlation with permeability data underline the functionality of these cell-cell contacts. Even if binding of specific lectins did not succeed in a clear distinction between type I and type II pneumocytes, the plateau phase unified the presence of characteristic alveolar cell markers and appropriate barrier properties for drug transport studies.

Comparison to Alveolar and Other Epithelial Cell Cultures from Different Species

Cell lines generated from the airways of the respiratory system, e.g., Calu-3 and 16HBE14o−, are already available and model the airway epithelium to a certain extent (31). In general immortal cell lines call for easy maintenance and avoid several difficulties, including reproducibility or high costs, which are commonly associated with primary culture approaches. In contrast there has been only limited success in generating cell lines representing the alveolar epithelium of the lung, which is the preferred target area for systemically acting drugs. This is why cultured primary pneumocytes still take priority over cell lines. Among the primary cultures of the alveolar epithelium the isolation from the rat has been described first and the model is meanwhile well established (1,37,41,42). Primary culture of human alveolar cells has been described and characterized more recently (2,3,14).

While pig and man are closer related to each other than to rodents, this human cell culture model is certainly the most interesting comparison. Nevertheless, some species differences were observed, especially between the porcine and human alveolar epithelial cells in primary culture: in contrast to the human model lectin binding could not be used to differentiate porcine type II from type I pneumocytes. However, morphological analysis revealed the formation of these characteristic cell types. Species differences also occurred e.g., when a human anti-ZO-1-antibody did not work with porcine monolayers. But an anti-ZO-1-antibody, which was also tested for species reactivity in swine, succeeded in demonstrating the presence of this tight junction protein. This equivalence on the structural protein level is also confirmed by the fact that each of the other tissue specific markers used in this study was detected by the same antibody as used for human cell cultures.

Depending on the respective donor, larger amounts of tissue are available for the preparation of cells: rat < man < pig. In spite of differences in their macro-anatomy, i.e., the number of lobes constituting the lung organ, the same cell types and morphologies are detected on the cellular level. In terms of bioelectrical parameters porcine cells feature less high mean transepithelial resistances (Table III), but single batches of monolayers generate 2,000 Ωcm^2 as well. Similarities are found in the morphological development of isolated primary cells, in a proceeding differentiation and in

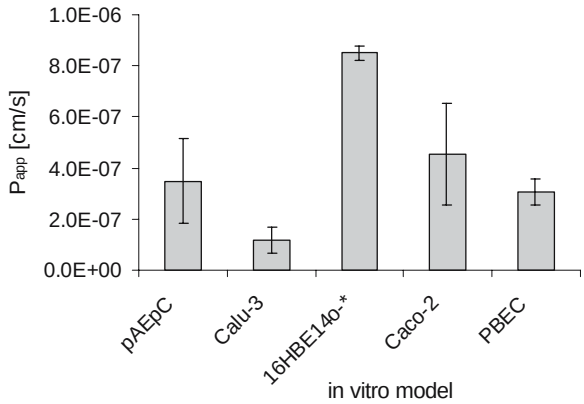

Fig. 9. Comparison of epithelial barrier properties quantified for different *in vitro* models, including cell lines and primary cells. Absorptive permeability coefficients (mean ± SD) were determined for the hydrophilic marker compound Flu-Na. *(*Asterisk*) indicates data from Saarland University, i.e., according to Ehrhardt *et al.* (49) (n = 12 except for pAEpC: n = 39 and Caco-2: n = 57). Despite the differences in cell type and donor species, the passive paracellular diffusion across the monolayers was within the same order of magnitude ($\times 10^{-7}$).

the presence of type II and type I pneumocyte-like cells. All the three cell culture systems reveal their epithelial origin by the formation of cell-cell contacts and functional tight junctions, enabling an imitation of typical barrier properties. According to immunocytochemical studies they commonly display alveolar epithelial cell markers. Electron microscopy detects a good resemblance in the ultrastructure of cells e.g., multilamellar bodies or caveolae.

Finally the integrity of pAEpC monolayers was evaluated against that of other epithelial *in vitro* models. Flu-Na was chosen as marker, because of the vast amount of data available from transport studies using the substance across other *in vitro* cell cultures: an intestinal human cell line (Caco-2), bronchial human cell lines (Calu-3, 16HBE14o−) and primary porcine brain endothelial cells (PBEC). Figure 9 compares permeability coefficients across these barrier-forming models (data either generated at Saarland University (*) or Across Barriers GmbH). Permeability coefficients for Flu-Na in all models ranged between 1.18 and 8.50×10^{-7} cm/s. Considering variations reported in literature for cells cultured in different laboratories, the paracellular leakiness of pAEpC monolayers was similar to the other models, indicating comparable barrier properties.

CONCLUSION

Porcine alveolar epithelial cells in primary culture (pAEpC) differentiate into a tight monolayer formed by two cell types, which resemble morphologically and biochemically type I and type II pneumocytes. Although differentiation of the cells is not completed after 13 days of culture (end point of observation in this study), pAEpC monolayers are similar to primary barrier forming cell cultures from other species, such as e.g., rat or human. The comparability of functional barrier properties (TEER, Flu-Na) with those of other epithelial *in vitro* models is encouraging. Variations between different cell preparations, which generally occur in primary cell cultures, could be kept within acceptable limits. Moreover, the porcine system has relevant advantages: 1) the pig is closer related to man than rodents (43); and 2) porcine tissue is easily available at high amounts from abattoirs without raising ethical concerns. Especially this last point makes the pAEpC attractive as a potential screening tool for drug development.

ACKNOWLEDGMENTS

We would like to thank Dr. Carsten Ehrhardt for helpful discussions and cooperation in immunostaining and confocal laser scanning microscopy, Birgit Leis and Norbert Pütz for technical assistance in electron microscopy and Manuel Birke (Symbiotec) for his kind introduction to the BioRad Scanner.

REFERENCES

1. L. G. Dobbs. Isolation and culture of alveolar type II cells. *Am. J. Physiol.* **258** (4 Pt 1):L134–L147 (1990).
2. K. J. Elbert, U. F. Schäfer, H. J. Schäfers, K. J. Kim, V. H. Lee, and C. M. Lehr. Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies. *Pharm. Res.* **16** (5):601–608 (1999).
3. C. Ehrhardt, K.-J. Kim, and C.-M. Lehr. Isolation and culture of human alveolar epithelial cells. In J. Picot (ed.), *Human Cell Culture Protocols*, Humana, Totowa, NJ, 2005, pp. 207–216.
4. W. G. Pond and K. A. Houpt. The pig as a model in biomedical research. In Cornell University Press Ithaca (ed.), *The Biology of the Pig*, Comstock, London, 1978, pp. 13–64.
5. C. Hammer. Xenotransplantation—will it bring the solution to organ shortage? *Ann. Transplant* **9** (1):7–10 (2004).
6. N. Gardner, W. Haresign, R. Spiller, N. Farraj, J. Wiseman, H. Norbury, and L. Illum. Development and validation of a pig model for colon-specific drug delivery. *J. Pharm. Pharmacol.* **48**:689–693 (1996).
7. O. Camber and P. Edman. Factors influencing the corneal permeability of prostaglandin F2alfa and its isopropyl ester *in vitro*. *Int. J. Pharm.* **37**:27–32 (1987).
8. M. Fujii, S. Yamanouchi, N. Hori, N. Iwanaga, N. Kawaguchi, and M. Matsumoto. Evaluation of yucatan micropig skin for use as an *in vitro* model for skin permeation study. *Biol. Pharm. Bull.* **20** (3):249–254 (1997).
9. M. B. Hansen, J. E. Thorboll, P. Christensen, N. Bindslev, and E. Skadhauge. Serotonin-induced short-circuit current in pig jejunum. *Zentralbl. Veterinarmed. A.* **41** (2):110–120 (1994).
10. A. J. Hoogstraate, J. Coos Verhoef, A. Pijpers, L. A. van Leengoed, J. H. Verheijden, H. E. Junginger, and H. E. Bodde. *In vivo* buccal delivery of the peptide drug buserelin with glycodeoxycholate as an absorption enhancer in pigs. *Pharm. Res.* **13** (8):1233–1237 (1996).
11. C. Wadell, E. Bjork, and O. Camber. Nasal drug delivery—evaluation of an *in vitro* model using porcine nasal mucosa. *Eur. J. Pharm. Sci.* **7** (3):197–206 (1999).
12. S. Sangadala, P. Wallace, and J. Mendicino. Characterization of mucin glycoprotein-specific translation products from swine and human trachea, pancreas and colon. *Mol. Cell. Biochem.* **106** (1):1–14 (1991).
13. M. Larsen and B. Rolin. Use of the Gottingen minipig as a model of diabetes, with special focus on type1 diabetes research. *ILAR J.* **45** (3):303–313 (2004).
14. S. Fuchs, A. J. Hollins, M. Laue, U. F. Schaefer, K. Roemer, M. Gumbleton, and C.-M. Lehr. Differentiation of human alveolar epithelial cells in primary culture: morphological characterization and synthesis of caveolin-1 and surfactant protein-C. *Cell. Tissue Res.* **311**:31–45 (2003).
15. L. Bingle, T. B. Bull, B. Fox, A. Guz, R. J. Richards, and T. D. Tetley. Type II pneumocytes in mixed cell culture of human lung: a light and electron microscopic study. *Environ. Health Perspect.* **85**:71–80 (1990).
16. A. C. Cunningham, D. S. Milne, J. Wilkes, J. H. Dark, T. D. Tetley, and J. A. Kirby. Constitutive expression of MHC and adhesion molecules by alveolar epithelial cells (type II pneumocytes) isolated from human lung and comparison with immunocytochemical findings. *J. Cell. Sci.* **107** (Pt 2):443–449 (1994).
17. C. Gindorf, A. Steimer, C. M. Lehr, U. Bock, S. Schmitz, and E. Haltner. Markertransport über biologische Barrieren *in vitro*: Vergleich von Zellkulturmodellen für die Dünndarmschleimhaut, die Blut-Hirn Schranke und das Alveolarepithel der Lunge. *Altex* **18** (3):155–164 (2001).
18. LGC Promochem Offices (ATCC homepage). Cell lines and Hybridomas, Product Description. http://www.lgcpromochem-atcc.com/common/catalog/CellBiology/CellBiologyIndex.cfm (accessed 12/04/05), part of ATCC homepage. http://www.lgcpromochem-atcc.com (accessed 12/04/05).
19. C. Jumarie and C. Malo. Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation *in vitro*. *J. Cell. Physiol.* **149** (1):24–33 (1991).
20. H. Franke, H.-J. Galla, and C. T. Beuckmann. Primary cultures of brain microvessel endothelial cells: a valid and flexible model to study drug transport through the blood-brain barrier. *Brain Res. Brain Res. Prot.* **5**:248–256 (2000).
21. J. H. Luft. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9** (Feb):409 - 414 (1961).
22. M. Laue, G. Kiefer, B. Leis, N. Pütz, and P. Mestres. Environmental scanning electron microscopy of resin block face. *Eur. Microsc. Anal.* **97**:13–15 (2005).

23. Roche Molecular Biochemicals. Lab FAQS: find a quick solution. Urs W. Hoffmann-Rohrer (DFKZ Heidelberg) (ed.), Mannheim, 2000.
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem. Baltimore* **193**:265–275 (1951).
25. U. K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (259):680–685 (1970).
26. L. G. Dobbs, M. C. Williams, and A. E. Brandt. Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. *Biochim. Biophys. Acta* **846** (1):155–166 (1985).
27. G. R. Newman, L. Campbell, C. Ruhlandvon, B. Jasani, and M. Gumbleton. Caveolin and its cellular and subcellular immunolocalisation in lung alveolar epithelium: implications for alveolar epithelial type I cell function. *Cell. Tissue Res.* **295** (1):111–120 (1999).
28. J. L. Alcorn, M. E. Smith, J. F. Smith, L. R. Margraf, and C. R. Mendelson. Primary cell culture of human type II pneumonocytes: maintenance of a differentiated phenotype and transfection with recombinant adenoviruses. *Am. J. Respir. Cell. Mol. Biol.* **17** (6):672–682 (1997).
29. Loxo Ltd. Product catalogue: LRP antibody. Novocastra Laboratories Ltd: p. 126. (2001).
30. J. D. Edelson, J. M. Shannon, and R. J. Mason. Alkaline phosphatase: a marker of alveolar type II cell differentiation. *Am. Rev. Resp. Dis.* **138** (5):1268–1275 (1988).
31. A. Steimer, E. Haltner, and C.-M. Lehr. Cell culture models of the respiratory tract relevant to pulmonary drug delivery. *J. Aerosol. Med.* **18** (2):137–182 (2005).
32. E. R. Weibel. *Morphometry of the Human Lung*, Academic, New York, 1963.
33. E. R. Weibel, P. Gehr, D. Haies, J. Gil, and M. Bachofen. The cell population of the normal lung. In A. Bouhhuys (ed.), *Lung Cells in Disease*, Amsterdam, North-Holland, 1976, pp. 3–16.
34. J. D. Crapo, B. E. Barry, P. Gehr, M. Bachofen, and E. R. Weibel. Cell number and cell characteristics of the normal human lung. *Am. Rev. Respir. Dis.* **126** (2):332–337 (1982).
35. L. G. Dobbs, M. C. Williams, and R. Gonzalez. Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. *Biochim. Biophys. Acta* **970** (2):146–156 (1988).
36. D. M. Haies, J. Gil, and E. R. Weibel. Morphometric study of rat lung cells. I. Numerical and dimensional characteristics of parenchymal cell population. *Am. Rev. Respir. Dis.* **123** (5):533–541 (1981).
37. J. M. Cheek, M. J. Evans, and E. D. Crandall. Type I cell-like morphology in tight alveolar epithelial monolayers. *Exp. Cell Res.* **184** (2):375–387 (1989).
38. C. A. Diglio and Y. Kikkawa. The type II epithelial cells of the lung. IV. Adaption and behavior of isolated type II cells in culture. *Lab. Invest* **37** (6):622–631 (1977).
39. I. Y. Adamson and D. H. Bowden. The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. *Lab. Invest* **30** (1) :35–42 (1974).
40. L. Campbell, A. J. Hollins, A. Al-Eid, G. R. Newman, C. von Ruhland, and M. Gumbleton. Caveolin-1 expression and caveolae biogenesis during cell transdifferentiation in lung alveolar epithelial primary cultures. *Biochem. Biophys. Res. Commun.* **262** (3):744–751 (1999).
41. Y. Matsukawa, H. Yamahara, F. Yamashita, V. H. Lee, E. D. Crandall, and K. J. Kim. Rates of protein transport across rat alveolar epithelial cell monolayers. *J. Drug Target* **7** (5):335–342 (2000).
42. K. J. Kim, Z. Borok, and E. D. Crandall. A useful *in vitro* model for transport studies of alveolar epithelial barrier. *Pharm. Res.* **18** (3):253–255 (2001).
43. P. Vodicka, K. J. Smetana, B. Dvorankova, T. Emerick, Y. Xu, J. Ourednik, V. Ourednik, and J. Motlik. The miniature pig as an animal model in biomedical research. *Ann. NY Acad. Sci.* **1049**:161–171 (2005).
44. T. F. Allred, R. R. Mercer, R. F. Thomas, H. Deng, and R. L. Auten. Brief 95% O2 exposure effects on surfactant protein and mRNA in rat alveolar and bronchiolar epithelium. *Am. J. Physiol.* **276** (6 Pt 1):L999–L1009 (1999).
45. G. F. Ross, M. Ikegami, W. Steinhilber, and A. H. Jobe. Surfactant protein C in fetal and ventilated preterm rabbit lungs. *Am. J. Physiol.* **277** (6 Pt 1):L1104–L1108 (1999).
46. V. Wong, D. Ching, P. D. McCrea, and G. L. Firestone. Glucocorticoid down-regulation of fascin protein expression is required for the steroid-induced formation of tight junctions and cell-cell interactions in rat mammary epithelial tumor cells. *J. Biol. Chem.* **274** (9):5443–5453 (1999).
47. H. Wan, H. L. Winton, C. Soeller, G. A. Stewart, P. J. Thompson, D. C. Gruenert, M. B. Cannell, D. R. Garrod, and C. Robinson. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o−. *Eur. Respir. J.* **15** (6):1058–1068 (2000).
48. Y. Man, V. J. Hart, C. J. Ring, S. Sanjar, and M. R. West. Loss of epithelial integrity resulting from E-cadherin dysfunction predisposes airway epithelial cells to adenoviral infection. *Am. J. Respir. Cell Mol. Biol.* **23** (5):610–617 (2000).
49. C. Ehrhardt, C. Kneuer, M. Laue, U. F. Schaefer, K. J. Kim, and C. M. Lehr. 16HBE14o− human bronchial epithelial cell layers express P-glycoprotein, lung resistance-related protein, and caveolin-1. *Pharm. Res.* **20** (4):545–551 (2003).