Differentiation 84 (2012) 294–304

Contents lists available at SciVerse ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Lung endothelial cells strengthen, but brain endothelial cells weaken barrier properties of a human alveolar epithelium cell culture model

Winfried Neuhaus [a,b], Fabian Samwer [a], Steffen Kunzmann [c], Ralf M. Muellenbach [a], Michael Wirth [d], Christian P. Speer [c], Norbert Roewer [a], Carola Y. Förster [a,e,*]

[a] Department of Anaesthesia and Critical Care, University Hospital Wuerzburg, Oberduerrbacherstrasse 6, 97080 Wuerzburg, Germany
[b] Department of Medicinal Chemistry, University of Vienna, Althanstraße 14, 1090 Vienna, Austria
[c] University Children's Hospital Wuerzburg, Josef-Schneider Straße 2, 97080 Wuerzburg, Germany
[d] Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Althanstraße 14, 1090 Vienna, Austria
[e] Comprehensive Heart Failure Center (CHFC) Würzburg, University Hospital Würzburg, Straubmühlweg 2a, 97078 Wuerzburg, Germany

ARTICLE INFO

Article history:
Received 9 January 2012
Received in revised form
21 August 2012
Accepted 24 August 2012
Available online 27 September 2012

Keywords:
Alveolar epithelium *in vitro* model
Claudin-1
Claudin-3
Claudin-4
Claudin-5

ABSTRACT

The blood–air barrier in the lung consists of the alveolar epithelium, the underlying capillary endothelium, their basement membranes and the interstitial space between the cell layers. Little is known about the interactions between the alveolar and the blood compartment. The aim of the present study was to gain first insights into the possible interplay between these two neighbored cell layers. We established an *in vitro* Transwell model of the alveolar epithelium based on human cell line H441 and investigated the influence of conditioned medium obtained from human lung endothelial cell line HPMEC-ST1.6R on the barrier properties of the H441 layers. As control for tissue specificity H441 layers were exposed to conditioned medium from human brain endothelial cell line hCMEC/D3. Addition of dexamethasone was necessary to obtain stable H441 cell layers. Moreover, dexamethasone increased expression of cell type I markers (caveolin-1, RAGE) and cell type II marker SP-B, whereas decreased the transepithelial electrical resistance (TEER) in a concentration dependent manner. Soluble factors obtained from the lung endothelial cell line increased the barrier significantly proven by TEER values and fluorescein permeability on the functional level and by the differential expression of tight junctional proteins on the molecular level. In contrast to this, soluble factors derived from brain endothelial cells weakened the barrier significantly. In conclusion, soluble factors from lung endothelial cells can strengthen the alveolar epithelium barrier *in vitro*, which suggests communication between endothelial and epithelial cells regulating the integrity of the blood–air barrier.

1. Introduction

The blood–air interface in the lung represents one of the vital barriers in the human body. The alveoli of the human lung – with an area of approximately 100–140 m^2 – are functionally the most important element of the lung (Gehr et al., 1978). The alveolar epithelial barrier plays a central role in lung diseases, like acute lung injury (ALI) and its most severe extreme form, the acute respiratory distress syndrome (ARDS) (Maniatis et al., 2008; Matthay, 1994). A key function of the epithelium is the formation of diffusion barriers that allow the generation and maintenance of compartments with different compositions, a fundamental requirement for the physiological organ function like in the lung (Gorin and Stewart, 1979; Balda and Matter, 1998). The lung alveolar epithelium *in vivo* consists of two epithelial cell types, the terminally differentiated squamous alveolar epithelial type I (ATI) cell, which constitutes approximately 93% of the alveolar

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; ATI, alveolar epithelial type I; ATII, alveolar epithelial type II; BBB, blood–brain barrier; SP-B, surfactant protein B; SP-C, surfactant protein C; TEER, transepithelial electrical resistance

* Corresponding author at: Department of Anaesthesia and Critical Care, University Hospital Wuerzburg, Oberduerrbacherstrasse 6, 97080 Wuerzburg, Germany. Tel.: +49 931 201 30065; fax: +49 931 201 30069.
E-mail addresses: winfried.neuhaus@univie.ac.at (W. Neuhaus), fasamwer@hotmail.com (F. Samwer), kunzmann_s@kinderklinik.uni-wuerzburg.de (S. Kunzmann), muellenbach_r@klinik.uni-wuerzburg.de (R.M. Muellenbach), michael.wirth@univie.ac.at (M. Wirth), speer_c@kinderklinik.uni-wuerzburg.de (C.P. Speer), Anaesthesie-Direktion@klinik.uni-wuerzburg.de (N. Roewer), foerster_c@klinik.uni-wuerzburg.de (C.Y. Förster).

0301-4681/$ - see front matter © 2012 International Society of Differentiation. Published by Elsevier B.V. All rights reserved.
Join the International Society for Differentiation (www.isdifferentiation.org)
http://dx.doi.org/10.1016/j.diff.2012.08.006

epithelial surface area, and the surfactant-producing cuboidal alveolar epithelial type II (ATII) cell (Crapo et al., 1982). The major functions of ATII cells are surfactant synthesis, transepithelial ions and water movement as well as regeneration of alveolar epithelium after lung injury (Uhal, 1997). The main functions of ATI cells are the control of peptide growth factors metabolism, transcellular ion transport, alveolar fluid regulation and immune modulation (Williams, 2003). It is believed that ATII cells are the sole progenitor for ATI cells (Fehrenbach, 2001). In ATI cells the caveolin-1 synthesis and the formation of caveolae are discussed as a characteristic feature of an ATI-like cell phenotype in the alveolar epithelium *in vivo* and *in vitro* (Campbell et al., 1999; Kasper et al., 1998; Kunzmann et al., 2011; Matthay, 1994). The caveolae membrane system is of interest because of its important transport function of molecules across the blood–air barrier in the lung (Gumbleton et al., 2000). As a second ATI marker the receptor for advanced glycation end-products (RAGE) was discussed, especially as a marker of ATI cell injury in ALI (Uchida et al., 2006). In comparison, surfactant proteins are restricted to the ATII phenotype; especially surfactant protein B (SP-B) and C (SP-C) are known to be synthesized with a high specificity by ATII cells (Phelps and Floros, 1991a; Weaver et al., 1988). In addition, glucocorticoids enhance fetal lung maturation by increasing production of SP-B in ATII cells (Phelps and Floros, 1991b; Ladenburger et al., 2010). Cell–cell contacts within the alveolar epithelium are sealed by tight junctions. Tight junctions, located at the most apical region of lateral membranes of epithelial cells, create a paracellular barrier in epithelial and endothelial cell layers, which protect the underlying tissue from the external environment (Forster, 2008; Rodriguez-Boulan and Nelson, 1989). Two different classes of integral membrane proteins constitute the tight junction strands in epithelial cells and endothelial cells, occludin and members of the claudin protein family, which are responsible for changes in the electrolyte and solute permeability in cells layers (Forster, 2008). The flux of fluid, ions, macromolecules, and inflammatory cells across airway epithelium depends in part upon the integrity of its apico-lateral tight junctions (Godfrey, 1997).

Because of the complexity of the pulmonary alveolar system, little is known about the molecular interactions between alveolar epithelial and endothelial cells. Up to now only few reports were published supporting the idea of a functional unit at the blood–air barrier consisting of alveolar epithelial and endothelial cells (Hermanns et al., 2004, 2009, 2010). Therefore, the establishment of *in vitro* models of the alveolar epithelial barrier, which could mimic different conditions in the alveolar region, would be useful to analyze the role of lung endothelial cells. Ideally, the *in vitro* model would form a tight barrier and consist of cells expressing ATI and ATII cell markers such as caveolin-1, RAGE and SP-B. NCI H441 cells have emerged as a well-established model system for distal pulmonary epithelial cells (Hermanns et al., 2004; Shlyonsky et al., 2005).

The aim of this study was to establish an alveolar cell culture model based on human cell line H441 and to use this model to study the influence of soluble factors derived from lung endothelial cells (HPMEC-ST1.6R) on the alveolar epithelium barrier. Human cell line HPMEC-ST1.6R was chosen as a model of the lung endothelium since it was reported that only HPMEC-ST1.6R exhibited the major constitutive and inducible endothelial cell characteristics and showed an angiogenic response on Matrigel similar to that of primary HPMEC in contrast to several other endothelial cell lines (Unger et al., 2002). Human brain endothelial cell line hCMEC/D3 represents an established model of the human blood–brain barrier (Weksler et al., 2005) and was used to check if possible effects of the lung endothelial cells on the alveolar epithelium model are tissue-specific.

2. Material and methods

2.1. Cell culture

Human lung epithelial cell line H441 was purchased from ATCC. Cells were maintained in RPMI1640 (R8758, Sigma, Munich, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mol/l Glutamax I, 1 × ITS (100 × ITS, 41400-045) (all from Gibco®, Life Technologies GmbH, Darmstadt, Germany) and 1 mol/l Na-pyruvate (L0473, Biochrom, Berlin, Germany) and were subcultured in a ratio 1:6 on gelatine (0.5%) coated cell culture tissue flasks (GreinerBioone, Frickenhausen, Germany) once a week. Human lung endothelial cell line HPMEC-ST1.6R was a kind gift from Prof. Kirkpatrick and Dr. Unger of the University Hospital Mainz in Germany (Krump-Konvalinkova et al., 2001). They were cultured on gelatine coated flasks in M199 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mol/l Glutamax I (all from Gibco®, Life Technologies GmbH), 25 μg/ml sodium heparin (H3149, Sigma) and 25 μg/ml ECGS (E2759, Sigma) and were trypsinized in a ratio of 1:8 once a week. Human brain endothelial cell line hCMEC/D3 were obtained from Prof. Couraud (Weksler et al., 2005) and were maintained in EBM-2 (C-4147, Lonza, Cologne, Germany) medium supplemented according to the manufacturer's instruction with the components of the EGM®-2 bullet kit (C-3162, Lonza, FBS, VEGF, R^3-IGF-1, hEGF, hbFGF, heparin, ascorbic acid, gentamycine and amphotericin B) without hydrocortisone. hCMEC/D3 cells were splitted in a ratio of 1:3 onto gelatine coated cell culture flasks once a week. Growth media were renewed every 2–3 days. In order to obtain soluble factors secreted from HPMEC-ST1.6R and hCMEC/D3 cells growth medium supernatants were collected from non-confluent cell layers after 3–5 days after seeding on 75 cm^2 tissue flasks and sterile-filtered before usage. The terms ST1, ST1-cond., D3 and D3-cond. are used in the text for fresh or conditioned (cond.) growth medium of the corresponding cells (ST1 = HPMEC-ST1.6R, D3 = hCMEC/D3). H441 cells were used between passages 56 and 76, HPMEC-ST1.6R between passages 30 and 37 and hCMEC/D3 between passages 17 and 34.

2.2. Transwell model experiments

For Transwell insert experiments H441 cells were seeded at a cell density of 23,000 cells/cm^2 onto collagen-I (0.01%, C7661, Sigma) coated 6-well inserts (353090, BD, Heidelberg, Germany, pore size 0.4 μm, PVDF, transparent, apical volume 2 ml, basolateral volume 3 ml). Medium was renewed every 2–3 days. After 5 days of culture it was started to add dexamethasone (10, 100, 300 or 1000 nmol/l) to the apical side with every medium change in a ratio of 1:1000 of the appropriate ethanolic stock solution. In order to assess the development of the barrier properties the transepithelial electrical resistance (TEER) was determined using a WPI device with chopstick electrodes (World Presicion Instruments, Berlin, Germany) as previously published (Neuhaus et al., 2008). In addition to the TEER measurement, transport studies with the paracellular marker fluorescein were undertaken. 10 μmol/l fluorescein was added in the apical compartment to begin the transport studies. 300 μl samples were taken after 15, 45, 105, 165 and 225 min from the basolateral side and were replaced after each sampling step with 300 μl fresh, prewarmed H441 medium to maintain the same hydrostatic pressure conditions during the entire transport experiment. Fluorescence of samples, stock solution and supernatants of the apical compartment after the end of the experiment (90 μl/well in a black 96-well plate from GreinerBioone) were measured with a Tecan GeniosPro (excitation wavelength: 485 nm; emission wavelength: 535 nm). Calculation of the permeability coefficients was accomplished according the clearance principle as previously published (Neuhaus et al., 2008) and described in the supplementary file in detail. In order to ease the