Svenja Nellinger*, Simon Heine, Lothar Steeb, Petra J. Kluger

Completely defined cell culture medium for advanced alveolar models

https://doi.org/10.1515/cdbme-2024-2110

Abstract: In vitro alveolar models are an important tool study respiratory physiology, investigate lung diseases, develop and test new therapies, and simulate inhalation exposure to environmental pollutants or therapeutic agents. A defined cell culture medium is essential for maintaining consistent and reliable in vitro models. In this study a defined cell culture medium for advanced alveolar models based on epithelial A549 cell line and endothelial EaHy926 cell line is developed. Cellular survival was proven by quantification of LDH release. It was shown that co-culture of the cells enhances cellular survival compared to the endothelial monoculture. The formation of a homogeneous monolayer on laminin coated membranes was confirmed by actin staining. Further, the expression of cell type specific proteins (endothelial: CD31, VE-cadherin; epithelial: ZO1 and E-cadherin) was shown. Overall, we demonstrated a completely defined cell culture medium that can be used for the setup of an advanced in vitro alveolar model for biomedical application like drug development or breath gas analysis.

Keywords: alveolar model, defined medium, A549, EaHy926

1 Introduction

The endothelial/epithelial barrier of the alveoli constitutes a critical interface for gas exchange in the lungs. It comprises a single layer of endothelial cells on one side and a thin layer of epithelial cells on the other side separated by a specialized extracellular matrix – the basal membrane. This barrier is highly specialized to allow for the efficient diffusion of oxygen from the alveolar air into the bloodstream while facilitating the removal of carbon dioxide from the bloodstream into the alveoli [1]. Disruption of the endothelial/epithelial barrier, whether due to inflammation, injury, or disease, can impair gas exchange and lead to respiratory dysfunction.

To resemble the alveolar structures for research in vitro models were developed. Alveolar models are used for various applications in both research and clinical settings [2]. They are utilized to study respiratory physiology, investigate lung diseases, develop and test new therapies, and simulate inhalation exposure to environmental pollutants or therapeutic agents. Furthermore, they play a crucial role in the development and optimization of medical devices such as inhalers, nebulizers, and respiratory support systems. A precisely defined cell culture medium is essential for maintaining consistent and reliable in vitro models, providing the necessary nutrients, growth factors, and hormones for maintaining the characteristic cellular functions. These tailored formulations mimic the physiological environment, ensuring cells behave as they would in vivo, thus enhancing the relevance and translatability of experimental findings. Careful optimization of the medium composition is critical for supporting specific cell types and studying complex biological processes accurately.

In this study we developed a completely defined cell culture medium for an advanced in vitro alveolar model based on a co-culture of the lung epithelial cell line A549 and the endothelial cell line EaHy926. Impact of the defined medium on the cellular survival was investigated in mono- and co-culture. Further, the lung model was optimized by coating of the insert membrane with laminin and the expression of cell type specific proteins was confirmed.

2 Materials and Methods

2.1 Cell culture

For the setup of the alveolar model the epithelial cell line A549 and the endothelial cell line EaHy926 were used. The A549 cell line is a human adenocarcinoma-derived cell line isolated from a lung cancer patient. These cells are particularly useful for investigating cellular processes involved in lung cancer progression, drug resistance mechanisms, and responses to

***Corresponding author: Svenja Nellinger:** Reutlingen Research Institute, Alteburgstr. 150, 72762 Reutlingen, Germany, Svenja.nellinger@reutlingen-university.de
Simon Heine: Reutlingen Research Institute, Alteburgstr. 150, 72762 Reutlingen, Germany
Lothar Steeb: PELObiotech GmbH, Planegg, Germany
Petra J. Kluger: School of Life Science, Reutlingen University, Alteburgstr. 150, 72762 Reutlingen, Germany

therapeutic agents. Moreover, their ability to form polarized monolayers makes them valuable models for studying lung epithelial cell biology and respiratory infections [3]. The EaHy926 cell line is an immortalized hybrid cell line derived from the fusion of human umbilical vein endothelial cells (HUVECs) with the aforementioned A549 cell line. EaHy926 cells retain many characteristics of primary endothelial cells, including the expression of endothelial-specific markers. Cells were purchased from DSMZ – German Collection of Microorganisms and Cell Cultures GmbH.

Expansion of the cells was performed in Dulbecco´s modified Eagles Medium DMEM supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin/streptomycin (DMEM10).

For the co-culture of the cells as an alveolar model, transwell cell culture inserts (Greiner Bio-One International GmbH, Germany) were used. The membrane was coated with human laminin from fibroblasts (Merck KGaA, Darmstadt). EaHy926 were seeded at a density of 150.000 cells/cm^2 on the basolateral side of the membrane and left for adhesion for 3h at 37°C and 5 % CO2. Afterwards, the apical side of the inserts was seeded with A549 at a density of 150.000 cells/cm^2. Cells were cultured in defined medium (DefM) based on the Endothelial Growth Basal Medium from PELOBiotech GmbH (Cat.No. PB-MH-100-2199) Germany or serum containing medium (DMEM10) After 3 days the lung model was changed to air lift culture by removing the medium from the apical side of the insert. At day 7 cells were fixed with RotiHistofix (Carl Roth, Germany) for immunofluorescent staining.

2.2 Lactate dehydrogenase assay

To determine the survival of the cells in the defined cell culture medium, lactate dehydrogenase (LDH) assay was performed. Cells were cultured in monolayer in a 24-well plate and in co-culture in inserts. As a control (dead) cells were treated with 0.5 % triton X. LDH release in the supernatant was determined after 24h according to the manufacturers protocol (Merck KGaA Darmstadt, Germany, 11644793001 Roche).

2.3 Immunofluorescent staining

To investigate the effect of membrane coating on the formation of the cell layer, actin staining was performed using the ActinRed™ 555 ReadyProbes™ and ActinGreen™ 488 ReadyProbes™ staining kits (ThermoFisher Scientific Inc., Darmstadt, Germany). Fixed cells were washed once with PBS. Staining solution for the endothelial cells (PBS with one drop per mL Actin Red) was filled into the well for the staining of the cells on the lower side of the membrane. Staining solution for the epithelial cells (PBS with one drop per mL Actin Green) was filled into the insert for the staining of the cells on the upper side of the membrane. Cells were incubated for 30 min and washed three times with PBS.

For staining of the cell specific proteins cluster of differentiation (CD) 31 (Dako Germany; 1:50), zonula occludes (ZO) 1 (ProteinTech Germany GmbH; 1:300) and cadherins (Cell Signaling Technology Inc.; 1:200), specific antibodies were used. Cells were washed once with PBS and incubated for 10 min with permeabilization solution (0.1 % triton X in PBS). Unspecific binding sites were blocked with blocking solution (3 % bovine serum albumin in PBS) for 30 min. Primary antibody was diluted in blocking solution and incubated for 1h. Cells were washed three times with washing buffer (0.1 % Tween 20 in PBS) and secondary antibody (diluted in blocking buffer) was incubated for 30 min. Counter staining of nuclei was performed with DAPI. After three washing steps images were taken using the Axio Observer from Carl Zeiss, Germany.

2.4 Statistics

All experiments were performed at least three times. Data were analyzed by one-way analysis of variance (ANOVA) with a Tukey posthoc test using Origin 2018b. Statistical significances were stated as $p < 0.05$ (*), very significant as $p < 0.01$ (**), and highly significant as $p < 0.001$ (***).

3 Results and Discussion

In this study a completely defined culture medium based on the Endothelial Growth Medium from PELOBiotech for the setup of a lung model was developed. To determine the survival of the cells in the DefM LDH release in the supernatant was quantified after 24h of culture (Figure 1). LDH serves as an indicator for cell death due to its release into the extracellular environment upon cellular damage or disruption. When cells undergo necrosis or apoptosis, their membranes become compromised, leading to the leakage of LDH into the surrounding medium. The A549 cells exhibited the lowest amount of LDH in both media (DefM: 3.4 (± 0.9) %; DMEM10: 10.8 (± 0.4) %) indicating a survival rate of the cells in DefM comparable to DMEM10. The EaHy926 cells exhibited the highest LDH release in both media (DefM: 50.1(±1.4) %; DMEM10: 27.5(±2.5) %). Interestingly, the negative effect of DefM on EaHy926 is compensated in the co-culture with the lung epithelial cells. LDH release in co-

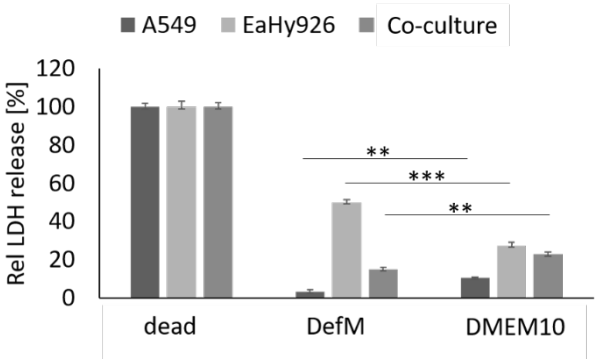

Figure 1: Determination of the released LDH from cells in different culture media. Cells were cultured in monolayer and co-culture in either defined medium (DefM) or serum containing medium (DMEM10) for 24h. As a control (dead) cells were treated with 0.5 % Triton X which was set as 100%. Other values were normalized to the control.

culture in DefM (15.1(±0.8) %) is lower compared to the serum containing DMEM10 (23.1(±2.1) %), which clearly demonstrates the suitability of the DefM medium for serum-free culture of advanced alveolar models.

The positive effect of the co-culture on the survival of the cells, especially in the DefM, can be due to paracrine effects between the two cell types. It is well known that secreted cytokines of one cell type can have a positive impact on the cellular behavior of other cell types [4]. This effect can be utilized in the development of serum-free media.

In order to optimize the lung model (co-culture of the cells in an insert), the laminin coating of the membrane was tested (Figure 2). In the present lung model the membrane of the insert represents the basal membrane between the endothelial and epithelial cell layer. Laminin is a prominent extracellular matrix protein in basal membranes and therefore a suitable protein for the coating for the setup of a lung model [5]. Actin staining of the cells revealed a more homogeneous monolayer on laminin coated membranes. This effect is most visible in DMEM10, which may be due to the various adhesion proteins that are contained in FCS that are able to adsorb more likely to the coated membrane [6]. But also, for the DefM the monolayer appears more homogeneous. As there is no FCS in the DefM this medium is lacking of FCS-derived adhesion proteins that physisob on the culture surface and enhance cell adhesion. Thus, laminin coating may enable the faster and stronger adhesion of the cells on the membrane. For the following experiments the membrane was coated with laminin.

To further characterize the lung model in the DefM expression of specific endothelial and epithelial proteins were proven (Figure 3). CD31, also known as PECAM-1, is a glycoprotein found on endothelial cells, platelets, and leukocytes, facilitating cell adhesion and migration. Its homophilic binding function aids in processes like leukocyte

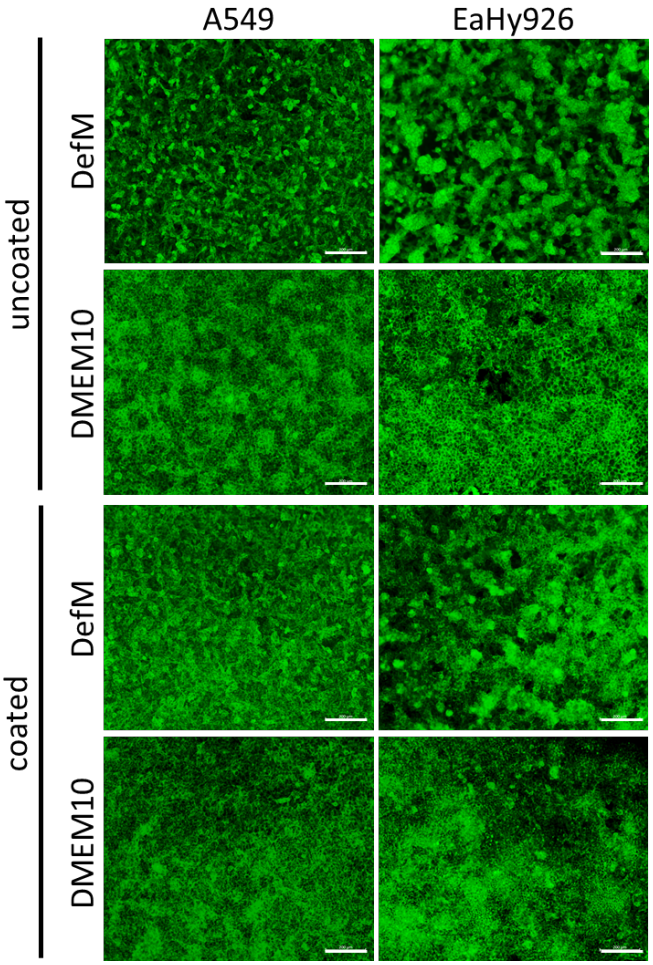

Figure 2: Comparison of laminin coated and uncoated Membranes. Cells were seeded on laminin coated and uncoated membranes at a density of 150.000 cells/cm^2. After 7 days of culture in DefM or DMEM10 actin (green) was stained to determine the development of the monolayer. Scale bar: 200µm

migration across endothelial barriers and angiogenesis. Dysregulation of CD31 has implications in diseases such as inflammation, cancer, and cardiovascular conditions, making it a significant target for research and potential therapeutic interventions [7]. VE-cadherin, short for Vascular Endothelial Cadherin, is a cell adhesion molecule predominantly expressed in endothelial cells lining blood vessels. It plays a crucial role in maintaining the integrity and barrier function of the endothelium by mediating cell-cell adhesion. Dysregulation of VE-cadherin has been implicated in vascular diseases such as inflammation, thrombosis, and vascular leakage [8]. Both proteins are expressed in both culture media. However, the expression pattern is more homogeneous in the DMEM10 compared to the DefM. This indicates the formation of a tighter monolayer in the serum containing medium.

ZO-1, or Zonula Occludens-1, is a cytoplasmic protein found in tight junctions, specialized structures between epithelial and endothelial cells. It serves as a scaffolding protein, linking transmembrane proteins to the actin cytoskeleton, thereby regulating the permeability and integrity

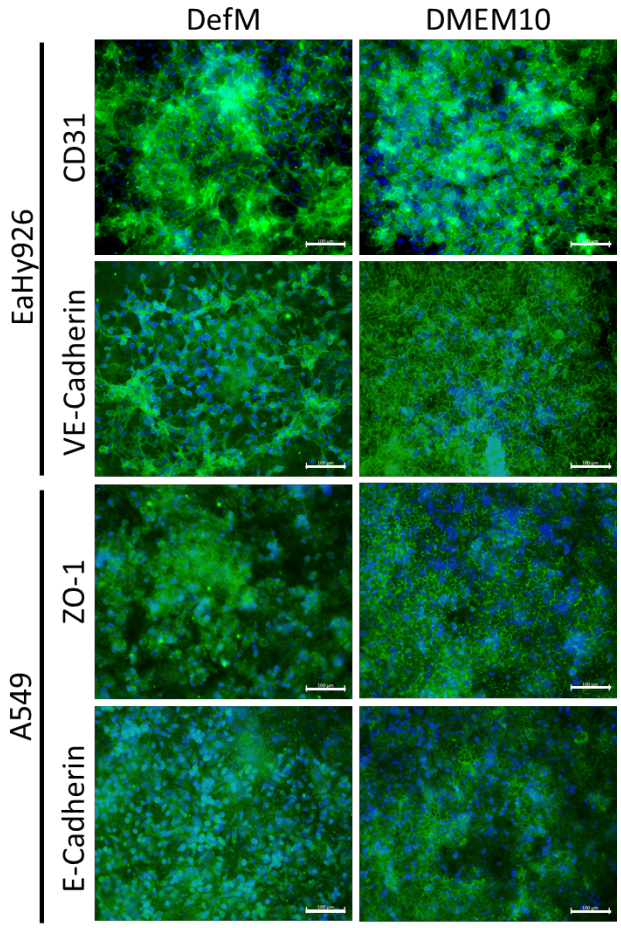

Figure 3: Staining of specific endothelial and epithelial proteins. Cells were seeded at a density of 150.000 cells/cm2. At day 7 of culture in DefM and DMEM10 cells were fixed and specific proteins (CD31, VE-Cadherin, ZO-1, E-Cadherin) were stained in green and nuclei were stained with DAPI (blue). Scale bar: 100µm

of epithelial and endothelial barriers. Dysregulation of ZO-1 has been associated with various diseases, highlighting its importance in maintaining tissue homeostasis [9]. E-cadherin, or Epithelial cadherin, is a calcium-dependent cell adhesion protein primarily found in epithelial tissues. It mediates homophilic cell-cell adhesion, playing a key role in maintaining the structural integrity and polarity of epithelial layers [8]. The expression pattern of these proteins is comparable in the both culture media which demonstrate a comparable integrity of the barrier of the monolayer.

Overall in this study we demonstrated a completely defined cell culture medium that can be used for the setup of an in vitro alveolar model based on the cell lines A549 and EaHy926. The cells built a homogeneous monolayer and express the cell type specific proteins. This defined medium can be used to conduct robust and reproducible experiments for clinical research on lung physiology and pathological lung conditions using corresponding in vitro lung models.

Author Statement

Research funding: The authors thank the Ministry of Science, Research and the Arts (Baden-Württemberg, Germany) for financial support.

Conflict of interest: Dr. Lothar Steeb is CEO and CSO of PELOBiotech GmbH.

References

[1] L. Knudsen, M. Ochs, The micromechanics of lung alveoli: structure and function of surfactant and tissue components, Histochem Cell Biol 150 (2018) 661–676. https://doi.org/10.1007/s00418-018-1747-9.

[2] K.V. Evans, J.-H. Lee, Alveolar wars: The rise of in vitro models to understand human lung alveolar maintenance, regeneration, and disease, Stem Cells Translational Medicine 9 (2020) 867–881. https://doi.org/10.1002/sctm.19-0433.

[3] K.A. Foster, C.G. Oster, M.M. Mayer, M.L. Avery, K.L. Audus, Characterization of the A549 Cell Line as a Type II Pulmonary Epithelial Cell Model for Drug Metabolism, Experimental Cell Research 243 (1998) 359–366. https://doi.org/10.1006/excr.1998.4172.

[4] D.M.L.W. Kruk, M. Wisman, J.A. Noordhoek, M. Nizamoglu, M.R. Jonker, H.G. de Bruin, K. Arevalo Gomez, N.H.T. ten Hacken, S.D. Pouwels, I.H. Heijink, Paracrine Regulation of Alveolar Epithelial Damage and Repair Responses by Human Lung-Resident Mesenchymal Stromal Cells, Cells 10 (2021) 2860. https://doi.org/10.3390/cells10112860.

[5] M. Aumailley, The laminin family, Http://Dx.Doi.Org/10.4161/Cam.22826 7 (2012) 48–55. https://doi.org/10.4161/CAM.22826.

[6] A. Kikuchi, H. Taira, T. Tsuruta, M. Hayashi, K. Kataoka, Adsorbed serum protein mediated adhesion and growth behavior of bovine aortic endothelial cells on polyamine graft copolymer surfaces, Journal of Biomaterials Science, Polymer Edition 8 (1997) 77–90. https://doi.org/10.1163/156856296X00165.

[7] H. Askari, M. Sadeghinejad, I.S. Fancher, Chapter Three - Mechanotransduction and the endothelial glycocalyx: Interactions with membrane and cytoskeletal proteins to transduce force, in: I.S. Fancher, A.Z. Chignalia (Eds.), Current Topics in Membranes, Academic Press, 2023: pp. 43–60. https://doi.org/10.1016/bs.ctm.2023.02.003.

[8] H.M. DeLisser, ADHESION, CELL–CELL | Vascular, in: G.J. Laurent, S.D. Shapiro (Eds.), Encyclopedia of Respiratory Medicine, Academic Press, Oxford, 2006: pp. 29–37. https://doi.org/10.1016/B0-12-370879-6/00008-9.

[9] S. Ni, L. Xu, J. Huang, J. Feng, H. Zhu, G. Wang, X. Wang, Increased ZO-1 expression predicts valuable prognosis in non-small cell lung cancer, Int J Clin Exp Pathol 6 (2013) 2887–2895.