The border-crossing behavior of eosinophils and neutrophils in the lung
Zuurbier, A.E.M.

Citation for published version (APA):
CHAPTER VII

Isolation and Cultivation of Human Lung Microvascular Endothelial Cells, and Differences between Human Endothelial Cells from Various Sources

Submitted for publication
Isolation of LMVEC and Differences between Endothelial Cells from Various Sources
Isolation and Cultivation of Human Lung Microvascular Endothelial Cells, and Differences between Human Endothelial Cells from Various Sources

Astrid E.M. Zuurbier, Hans W.M. Niessen*, J. Alain Kummer*, Edward F. Knol† and Dirk Roos

Central Laboratory of the Netherlands Blood Transfusion Service (CLB) and Laboratory for Experimental and Clinical Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

* Department of Pathology, University Hospital Vrije Universiteit, Amsterdam, The Netherlands
† Current address: Department of Dermatology and Allergology, University Medical Center Utrecht, The Netherlands

To study the role of the endothelium in lung inflammation and asthma in vitro, we have tried to isolate human lung microvascular endothelial cells (LMVEC) from lung tissue derived post-operationally from lung cancer patients. We have used several isolation methods based on various specific endothelial features, such as the characteristic cobblestone morphology, the capacity to bind Ulex europaeus agglutinin-1, the cell density, and the expression of PECAM-1. However, the low proliferation rate prevented successful isolation and cultivation of these cells. Recently, human LMVEC derived from human material used for transplantation purposes have become commercially available. Flow-cytometry experiments showed that the expression of adhesion molecules on these LMVEC and on human endothelial cells from umbilical cord veins (HUVEC) differed in that the expression of β1-integrins, PECAM-1 and ICAM-1 was higher on LMVEC than on HUVEC. Furthermore, IL-4 stimulated E-selectin, ICAM-1 and VCAM-1 expression on LMVEC, but not on HUVEC, whereas TNF-α potently stimulated E-selectin expression on HUVEC, but not on LMVEC. Both endothelial cell types showed the highest expression of VCAM-1, ICAM-1 and E-selectin after incubation with the combination of IL-4 and TNF-α. The high expression of VCAM-1 and the high sensitivity to IL-4 support the idea that lung endothelial cells do play a prominent role in allergic inflammation.

Introduction

Endothelial cells play an active regulatory role in the recruitment of leukocytes in inflammatory tissues through various mechanisms, such as vasodilatation, expression of adhesion molecules [e.g. E-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and platelet-endothelial-cell adhesion molecule (PECAM)-1], opening of intercellular junctions, and secretion of chemotactic and activating factors (1;2). The response of the endothelial cells, as to which adhesion molecules are expressed and which cytokines are secreted, depends on the type and site of inflammation, and promotes in this way specific leukocyte transendothelial migration and accumulation in tissues. For instance, allergic asthma is characterized by massive infiltration of eosinophils into the lung (3).

The site of inflammation is a determining factor in the endothelial cell response, because the appearance, organization of intercellular junctions, protein expression (4) and cytokine production (5) of endothelial cells varies from organ to organ, depending on their function (2;6;7). In addition, distinct subsets of endothelial cells with different functions, e.g. microvascular and macrovascular endothelial cells, exist within a single organ. Heterogeneity of endothelial cells develops as a result of variation in exposure to environmental stimuli, such as the microenvironment (8). For instance, murine pulmonary...
post-capillary endothelial cells express a tissue-specific molecule, lung-specific endothelial cell adhesion molecule-1 (Lu-ECAM-1) (9). Cultivation of aortic endothelial cells on extracellular matrix derived from the lung induces expression of Lu-ECAM-1 (9), and these cells develop fenestrac when cultured on matrix derived from kidney-derived MDCK cells (10).

To date, appreciation of endothelial cell function has been largely based on the behavior of cultured endothelial cells from the human umbilical cord veins (HUVEC). To analyze the extravasation process of neutrophilic and eosinophilic granulocytes in the lung as seen in allergic inflammation, we have tried to develop an experimental model in which the interaction of leukocytes with human lung microvascular endothelial cells (LMVEC), in comparison to HUVEC, could be studied. In this short report we discuss the different approaches we have undertaken, and we show that endothelial cells from human umbilical cord and human lung differ in the regulation of the expression of cell surface molecules.

Materials & Methods

Reagents

Interleukin (IL)-4 and tumor-necrosis factor (TNF)-α were purchased from Pepro Tech (Rocky Hill, NJ, USA). Anti-ICAM-1 (15.2) monoclonal antibody (mAb), fibronectin and human serum were obtained from the Central Laboratory of the Netherlands Blood Transfusion Service (CLB) (Amsterdam, The Netherlands). MAb ENA-2 against E-selectin was purchased from Sanbio (San Diego, CA, USA). Anti-VCAM-1 mAb (1G11) was obtained from Immunotech (Marseille, France). MAb 8A2 directed against β1 integrins was a kind gift from Dr. J.M. Harlan (Harbour Medical Center, Seattle, WA, USA). The CD31 mAb HEC170 (11) was isolated from hybridoma supernatant by precipitation with 50% saturated ammonium sulfate and subsequent protein-A affinity chromatography. Fluorescently-labeled secondary antibody to mouse immunoglobulins was from Dako (Glostrup, Denmark). Nu-serum was from Becton Dickinson (Bedford, MA), and basic fibroblast growth factor (bFGF) from Boehringer Mannheim (Mannheim, Germany). Collagenase type II, bovine serum albumin and gelatin were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium was from Gibco Life Technologies (Gaithersburg, MD, USA).

Human lung cell culture

The preparation of a lung cell suspension with single cells was performed as described by Carley et al. (12). In brief, lung tissue was obtained post-operatively from lung cancer patients undergoing lobectomy. The tissue used for endothelial cell isolation was derived from the peripheral lobes with no histological evidence of malignant cells. The outer layer and visible blood vessels were removed before mincing the tissue in culture medium, consisting of RPMI 1640 supplemented with 10% Nu-serum, 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 2 µg/ml heparine, 2 ng/ml bFGF, 100 U/ml penicillin and 100 µg/ml streptomycin. The suspension was centrifuged and the pellet was digested in 0.2% collagenase type II and 0.1% bovine serum albumin in RPMI 1640 for 30 min at 37°C. The tissue was subsequently sheared by pipetting and incubated for another 30 min. The suspension was filtered through a nylon mesh and centrifuged. The pellet was resuspended in pre-warmed culture medium and seeded in T-25 flasks (Nunc, Rochester, NY, USA) pre-coated with 1.5% gelatin in phosphate-buffered saline (PBS). The cells were incubated at 37°C with 5% CO2 and maximal humidity, and the culture medium was refreshed every other day.
**LMVEC culture**

LMVEC cryopreserved as tertiary or quaternary cultures were purchased from BioWhittaker (Walkersville, MD, USA) and cultured in microvascular endothelial growth medium EGM2-MV (BioWhittaker) in fibronectin-coated tissue culture flasks.

**HUVEC culture**

Freshly isolated, primary HUVEC (13) were cultured in HUVEC medium (RPMI 1640 supplemented with 10% (v/v) heat-inactivated human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 1 ng/ml bFGF) in culture flasks coated with 1 mg/ml fibronectin. The 2nd-4th passages of the primary HUVEC were used for sub-culturing in fibronectin-coated tissue culture plates.

**FACS analysis**

The expression of surface antigens on endothelial cells was measured by indirect immunofluorescence with flow cytometry. Cells were detached by means of a 15-min incubation with PBS supplemented with 5 mM EDTA at 37°C. The harvested cells were incubated with mAb (5 μg/ml) directed against β1-integrins (8A2 against CD29 antigen), PECAM-1 (HEC170), E-selectin (ENA-2), ICAM-1 (15.2) or VCAM-1 (1G11) for 30 min at 4°C. After the cells had been washed with ice-cold PBS containing 1% (w/v) bovine serum albumin and 14 μg/ml azide, binding of mAb was detected by incubation with PE-conjugated goat-anti-mouse-lg for 30 min at 4°C. The fluorescence intensity of the cells was measured with a flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA).

**Statistical analysis**

Results were expressed as the mean ± SEM of 3 independent experiments. Results were analyzed with the Student’s paired t-test. Two-tailed P values were calculated, and P values exceeding 0.05 were considered not significant.

**Results**

To purify and culture human lung endothelial cells, we have initially followed a published method to culture lung cells (12), i.e. the lung tissue was minced and digested in collagenase and filtered through a nylon mesh. The filtrate was subsequently centrifuged and the pellet was plated in culture medium. However, the first six attempts failed, because the lung cells with an endothelial morphology, i.e. cobblestone-shaped cells growing in islands, hardly proliferated after isolation. The cells stopped dividing or did not divide at all after a few days, and fibroblasts gradually dominated the cell culture. We hoped that the lung endothelial cells would proliferate when the cells would be cultured in close contact with each other from the beginning. We therefore tried to isolate the endothelial cells from the lung cell preparation by means of a described method (14; 15) based on the specific capacity of endothelial cells to bind *Ulex europaeus* agglutinin-1 (UEA-1). UEA-1 binds specifically to terminal α-L-fucosyl residues of glycoproteins present on the surface of human endothelial cells. Streptavidin-conjugated magnetic beads were coated with biotin-conjugated UEA-1 and incubated with lung cells in suspension. The lung cells were detached from the bottom of the tissue-culture plate by means of incubation in 5 mM EDTA (pH 7.3) in PBS during 30 min at 37°C and in 5% CO2 and maximal humidity. In theory, only endothelial cells are able to bind to these beads, and the cells bound can be isolated by means of magnetic force. Nonetheless, the large majority of the isolated cells did not display a cobblestone morphology. Moreover,
Indirect immunofluorescence analysis showed that the cells did not express the endothelial cell marker PECAM-1 (CD31) either. These results indicate that other lung cells were isolated besides endothelial cells.

Another described method to purify human microvascular endothelial cells is based on differences in specific gravity (density) of the lung cells (16; 17). We have tried to determine the proper centrifugation conditions and Percoll density to purify endothelial cells, but we found that the density of CD31-positive cells varies considerably. CD31-positive cells were generally found in each of the cell populations separated by density centrifugation, i.e. in cell populations with a high density but also in populations with a low density. The large variation in density might be due to differences in the differentiation and activation state of the endothelial cells, and it renders this approach unsuitable for endothelial cell isolation.

As mentioned above, another characteristic feature of endothelial cells is the expression of PECAM-1. We have tried to isolate endothelial cells by selective isolation of the CD31-positive cells. For instance, CD31-positive lung cells were enriched by means of flow cytometric sorting directly after lung digestion. However, the sorted cells did not proliferate either. We presumed that this low proliferation rate was due to the weakened condition of the cells after the collagenase treatment. We therefore continued our research by using a more gentle isolation method, i.e. cell isolation with CD31 mAb-coated magnetic beads (18). To our surprise, the isolated cells remained attached to the bottom of the culture flasks for at least three weeks and finally died. In summary, the major problem that we encountered was that the CD31-positive lung cells, the presumed LMVEC, did not proliferate.

To by-pass the low proliferation rate of the cells, we first immortalized the lung cells with an amphotropic replication-deficient retrovirus containing human papilloma virus 16 E6/E7 DNA (19). The islands of cells with a cobblestone morphology were subsequently trypsinized and the CD31-positive cells were sorted by flow cytometry. Yet, in spite of the immortalization, the CD31-positive cells gradually disappeared and other, CD31-deficient cell types eventually overgrew the cell cultures again. The low proliferation rate of the endothelial cells may also have prohibited successful immortalization, because only dividing cells can be transfected by the retrovirus.

**Figure 1.** Expression of β1 integrins (CD29), CD31 and ICAM-1 on resting HUVEC (open bars) and LMVEC (black bars). Data are mean fluorescence intensity ± SEM of 3 independent experiments. Asterisks indicate that expression of β1 integrins, CD31 and ICAM-1 was significantly higher on LMVEC than on HUVEC (p<0.05).
Flow cytometry experiments with commercially purchased LMVEC showed that the cell surface expression of adhesion molecules on human LMVEC and HUVEC differed in that the constitutive expression of β1-integrins, PECAM-1 and ICAM-1 were significantly higher on LMVEC than on HUVEC (Fig. 1). VCAM-1 and E-selectin were not expressed on resting cells of either cell type (Fig. 2b & 2c). Moreover, on LMVEC, but not on HUVEC, ICAM-1 and VCAM-1 expression was stimulated by IL-4 (Fig. 2a & 2b). The VCAM-1 expression was higher on LMVEC than on HUVEC under each stimulatory condition tested (Fig. 2b), also when the cells were stimulated for 4 hours with IL-1β (data not shown). The highest VCAM-1 and ICAM-1 expression on both LMVEC and HUVEC was reached when the cells were incubated with the combination of IL-4 and TNF-α (Fig. 2a & 2b).

E-selectin expression was differentially regulated on HUVEC and LMVEC, in contrast to the expression of ICAM-1 and VCAM-1. On HUVEC, E-selectin expression was induced by TNF-α alone, and not by IL-4 (Fig. 2c). As a consequence, addition of IL-4 to TNF-α did not yield an additional effect on E-selectin expression by HUVEC (Fig. 2c). On LMVEC, E-selectin expression was induced by IL-4, and not by TNF-α (Fig. 2c). Another difference with VCAM-1 and ICAM-1 expression is that E-selectin expression was higher on HUVEC than on LMVEC under each stimulatory condition except with IL-4 (Fig. 2c).

Figure 2. Expression of antigens on HUVEC (open bars) and LMVEC (black bars) after stimulation for 24 hours with TNF-α (5 ng/ml) and/or IL-4 (100 pM).
(a) Expression of ICAM-1. Data are mean fluorescence intensity ± SEM of three independent experiments.
(b) Expression of VCAM-1. Data are mean fluorescence intensity ± SEM of three independent experiments.
(c) Expression of E-selectin. Data are mean fluorescence intensity ± SEM of three independent experiments. Asterisks indicate that TNF-α stimulates significantly higher E-selectin expression on HUVEC than on LMVEC (p<0.05).
Discussion
On the basis of our experience, it can be concluded that isolation and successful outgrowth of human LMVEC is extremely difficult. Although the lung is potentially a very rich source of microvascular endothelial cells, its structural complexity and the diversity of the cell types in this organ greatly complicate the isolation and culture of pure microvascular endothelial cells. However, recently, human LMVEC have become commercially available (20). These endothelial cells are derived from human material used for transplantation purposes. Entire lungs from transplantation donors, who were in good health at the time of their death, are used for isolation of these LMVEC.

According to the published method to isolate and culture LMVEC (20), the endothelial cells were isolated from a single lung cell suspension, prepared in a similar way as we did, but the exact isolation method used has not been clarified in the publication. We assume that the explanation for our incapability to isolate and culture LMVEC is due to our access to lung tissue only from lung cancer patients. In general, these patients are elderly, smoking persons, and the lungs of these patients are therefore not in a good condition. Simply speaking, the condition of the lung endothelial cells may not have allowed in-vitro survival or proliferation from the very beginning. This assumption is supported by the fact that in nearly each publication reporting successful isolation and cultivation of LMVEC, the lungs were derived from human lung transplant surgery (14) or from animals e.g. from cows (21-23) or from sheep (24).

We intended to compare neutrophil and eosinophil migration across HUVEC versus commercially derived LMVEC monolayers, but we were unfortunately not able to create confluent LMVEC monolayers on Transwell membranes. This incapability also blocked our plans to study migration across bi-layers consisting of LMVEC and primary bronchial epithelial monolayers. However, flow cytometry experiments with LMVEC showed that ICAM-1 and VCAM-1 expression was stimulated by IL-4 on LMVEC, but not on HUVEC. These results are in contrast with those published by Yamamoto et al. (25) and Blease et al. (26), who found hardly any effect of IL-4 on ICAM-1 or VCAM-1 expression on LMVEC. The IL-4 used by these investigators was shown to be biologically active, so the lack of effect was not related to the capacity of the cytokine preparation. In addition, skin microvascular endothelial cells were found not to respond to IL-4 due to undetectable IL-4 receptor levels (27). Moreover, the lack of effect of IL-4 stimulation on VCAM-1 expression by HUVEC is unexpected, because IL-4 has repetitively been reported to induce VCAM-1 expression on HUVEC (27:28:28:29). These conflicting results may partly be due to differences in passage numbers and the condition of the cell cultures, yet the full explanation remains to be elucidated.

The highest VCAM-1 and ICAM-1 expression on both LMVEC and HUVEC and the highest E-selectin expression on LMVEC was reached when the cells were incubated with the combination of IL-4 and TNF-α. This combination has already been reported to be the most potent inducer of VCAM-1 expression on LMVEC (25), but here we show that this also holds for ICAM-1 and E-selectin expression.

The biological impact of the observed differences in regulation of ICAM-1, VCAM-1 and E-selectin expression cannot be fully deduced from our data. However, the moderate E-selectin expression on LMVEC suggests that E-selectin is of less importance for the lung microvascular endothelium. E-selectin is involved in the first step of extravasation, i.e. rolling of leukocytes on the endothelium. This step serves to bring the leukocyte and the endothelium in close contact with each other. Lung venules have small diameters, so leukocytes are already in close contact with the
endothelium when they pass through these venules (30). Moreover, the high expression of VCAM-1 and the high sensitivity to IL-4 support the idea that lung endothelial cells do play a prominent role in allergic inflammation.

Acknowledgements
The authors thank F.P.J. Mul for helpful discussions and technical assistance.

Footnote
This study was financially supported by grant no. 94.47 from the Netherlands Asthma Foundation.

References
Isolation of LMVEC and Differences between Endothelial Cells from Various Sources


