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AN IMPROVED METHOD FOR ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS FROM NORMAL AND INFLAMED HUMAN LUNG

JIN NING LOU,[1] NABIL MILI, CHRISTINE DECRIND, YVES DONATI, SYLVIE KOSSODO, ANASTASE SPILIOPOULOS, BARA RICOU, PETER M. SUTER, DENIS R. MOREL, PHILIPPE MOREL, AND GEORGES E. GRAU

Division of Anesthesiological Investigations (J. N. L., N. M., C. D., Y. D., S. K., B. R., P. M. S., D. R. M., G. E. G.), Division of Surgical Intensive Care, Clinics of Cardiovascular and Thoracic Surgery (A. S.), and Division of Investigative Surgery (J. N. L., P. M.), Department of Surgery, University Hospital and University Medical Center, CH-1211 Geneva 4, Switzerland

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SUMMARY

Microvascular endothelial cells (MVEC), which differ from large vessel endothelial cells, have been isolated successfully from lungs of various species, including man. However, contamination by nonendothelial cells remains a major problem in spite of several technical improvements. In view of the organ specificity of MVEC, endothelial cells should be derived from the tissue involved in the diseases one wishes to study. Therefore, to investigate some of the immunopathological mechanisms leading to acute respiratory distress syndrome (ARDS), we have attempted to isolate lung MVEC from patients undergoing thoracic surgery for lung carcinoma and patients dying of ARDS. The method described here includes four main steps: (1) full digestion of pulmonary tissue with trypsin and collagenase, (2) aggregation of MVEC induced by human plasma, (3) Percoll density centrifugation, and (4) selection and transfer of MVEC after local digestion with trypsin/EDTA under light microscopy. Normal and ARDS-derived lung MVEC purified by this technique presented contact inhibition (i.e., grew in monolayer), and expressed classical endothelial markers, including von Willebrand factor (vWF), platelet endothelial cell adhesion molecule 1(PECAM-1, CD31), and transcripts for the angiotensin converting enzyme (ACE). The cells also formed capillarylike structures, took up high levels of acetylated low-density lipoprotein (Ac-LDL), and exhibited ELAM-1 inducibility in response to TNF. Contaminant cells, such as fibroblasts, smooth muscle cells, or pericytes, were easily recognized on the basis of morphology and were eliminated by selection of plasma-aggregated cells under light microscopy. The technique presented here allows one to study the specific involvement and contribution of pulmonary endothelium in various lung diseases.

Key words: lung microvascular endothelial cell; vWF; CD31; LDL; ACE; ELAM-1.

INTRODUCTION

Since the endothelial cell culture was developed in 1973 (19), the awareness has grown that these cells play active roles in homeostasis and pathology. In particular, microvascular endothelial cells (MVEC) have been shown to have more physiological and pathological significance than large vessel endothelial cells (LVEC) [for review *see* Scott and Bicknell (29)]. In physiological conditions, MVEC are crucial in modulating metabolisms and tissue functions, while in pathological conditions, they are central to the process of inflammation (31). MVEC have been successfully isolated from almost all organs and tissues (29). MVEC differ from LVEC by various morphological and functional variables (14). Furthermore, endothelial cells from arterial origin are different from those of venous origin (1). These data suggest that LVEC may not be adequate for the study of pathological events occurring in microvessels. Moreover, MVEC derived from various organs also differ in some characteristic (2). Indeed, MVEC derived from different areas of the microcirculation exhibit differential adhesive properties for granulocyte (23).

In view of the organ specificity of MVEC, endothelial cells should be derived from the tissue involved in the diseases one wishes to study (29). Therefore, to further investigate the pathophysiology of acute respiratory distress syndrome (ARDS), a clinical condition during which lung MVEC express increased levels of cell adhesion molecules and thereby mediate the sequestration of polymorphonuclear leukocytes in microvessels (35), we attempted to isolate lung MVEC from patients who died of this syndrome.

MVEC have been successfully isolated from bovine (11), sheep (25), rat (33), mouse (2), rabbit (36), and human (16) lungs, but isolation and culture of these MVEC seems more laborious than from other organs. Although more than 25% of MVEC of the body are found in the lung, the presence of about 40 other cell types in this organ renders the isolation of pure MVEC difficult. The contamination by nonendothelial cells, such as fibroblasts, pericytes, and smooth muscle cells remains a major problem in lung MVEC culture. Several techniques have been established to purify MVEC, including the use of selective culture medium (13), treatment with trypsin (27), subcellular cloning (25), weeding of nonendothelial cells by manual manipulation (24), labeling endothelial cells with fluorescent probes and subsequent cell sorting by fluorescence-activated cell sorter (3),

[1]To whom correspondence should be addressed at Division of Investigative Surgery, Department of Surgery, University Hospital, University of Geneva, 24, rue Micheli-du-crest 1211, Geneva 14, Switzerland.

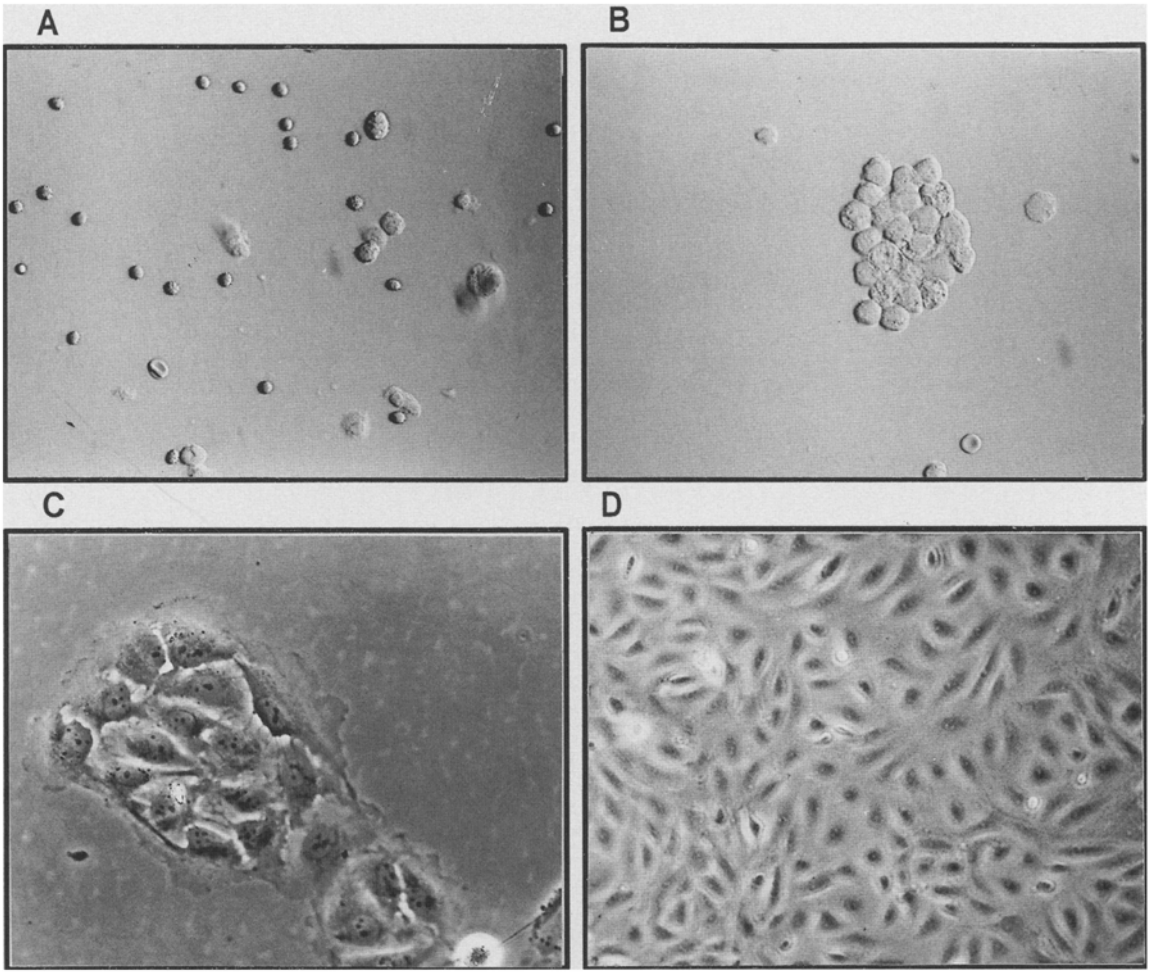

FIG. 1. Four steps of lung microvascular endothelial cell (MVEC) isolation. *A*, Cell suspension after digestion with trypsin and collagenase; *B*, induction of MVEC aggregates by plasma; *C*, formation of MVEC colony after a 3-d culture; *D*, purified lung MVEC grown to confluence.

and sorting using magnetic beads conjugated with *Ulex europaeus* agglutinin-1 (UEA-1) (14). However, it was noted that adherent beads are phagocytosed by cells and thereby interfere with cell functions (8). Moreover, the antibody conjugated on magnetic beads seriously affects the phenotypic analysis, as discussed below.

Here, we describe a method allowing a highly pure MVEC population to be obtained from human lung, either in normal or diseased conditions. These purified lung MVEC were identified by three typical features of endothelial cells: expression of von Willebrand factor (vWF), platelet endothelial cell adhesion molecule 1(PECAM-1, CD31), and transcripts for the angiotensin converting enzyme (ACE). The cells also formed capillarylike structures, took up high levels of acetylated low-density lipoprotein (Ac-LDL), and exhibited endothelial cell leukocyte Adhesion Molecule 1 (ELAM-1) inducibility in response to tumor necrosis factor alpha (TNF).

MATERIALS AND METHODS

Isolation of MVEC From Human Lung

Human lung tissue samples (20–30 g) were collected from ARDS patients who died in the surgical intensive care unit of our hospital. Informed consent for postmortem biopsy of the lung was obtained from the next of kin. Control human lung tissues were obtained from patients undergoing thoracic surgery. Samples were collected from macroscopically and microscopically normal tissue. The study protocol was examined and approved by the institutional Ethical Committee for Research in Humans (15). Peripheral lung parenchyma was aseptically removed and stored in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% ethylene diaminetetraacetic acid (EDTA) at 4° C and kept up to 6 h prior to processing. The tissue was washed with DMEM and the pleuron was carefully dissected from the underlying tissue with scissors to preclude contamination by mesothelial cells. The peripheral lung tissue devoid of large vessels was dissected, finely minced into 3 × 3 mm pieces and washed with DMEM on a sterile 20-μm metal mesh to remove blood cells. The tissue was then digested for 20–30 min at 37° C in 0.1% trypsin (Sigma Chemical Co., St. Louis, MO, USA) containing 0.1% EDTA (1 ml/g tissue) pH 7.2, followed by 0.2% collagenase (Sigma II type) pH 7.4, for another 20 min at 37° C. The digested solution was filtered through 100-μm nylon mesh to discard large fragments of connective tissue. The filtrate was centrifuged at 500 ×g for 5 min at 4° C and the pellet was resuspended in 5 ml DMEM containing 20% human plasma and incubated for 5 min to induce the aggregation of MVEC. The cell suspension was overlaid on 20 ml of 20% Percoll and centrifuged at 1500 ×g for 15 min at 4° C. The pellet was collected and washed twice in DMEM by centrifugation at 500 ×g for 5 min. The cells were resuspended in DMEM containing 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 30% fetal calf serum (FCS),

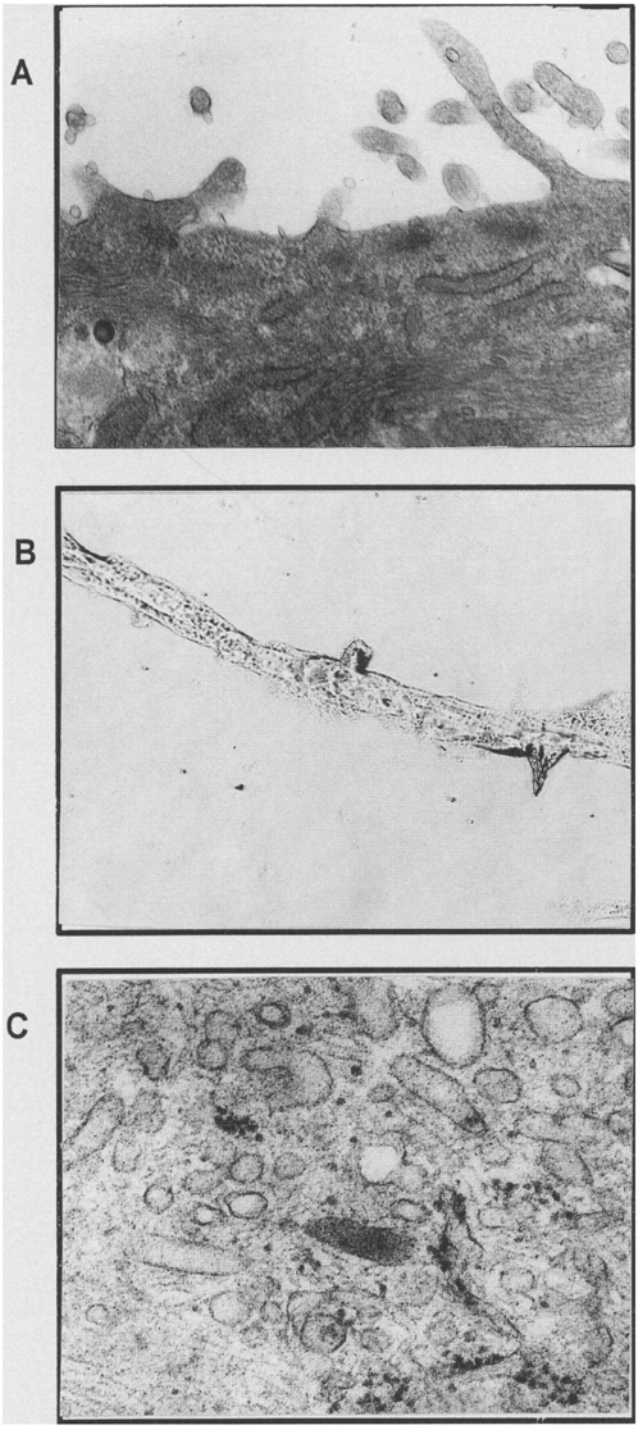

FIG. 2. Morphology of lung MVEC. *A*, Microvilli (×10 000), *B*, capillary-like tube formation (×400); *C*, Weibel-Palade bodies (×28 000).

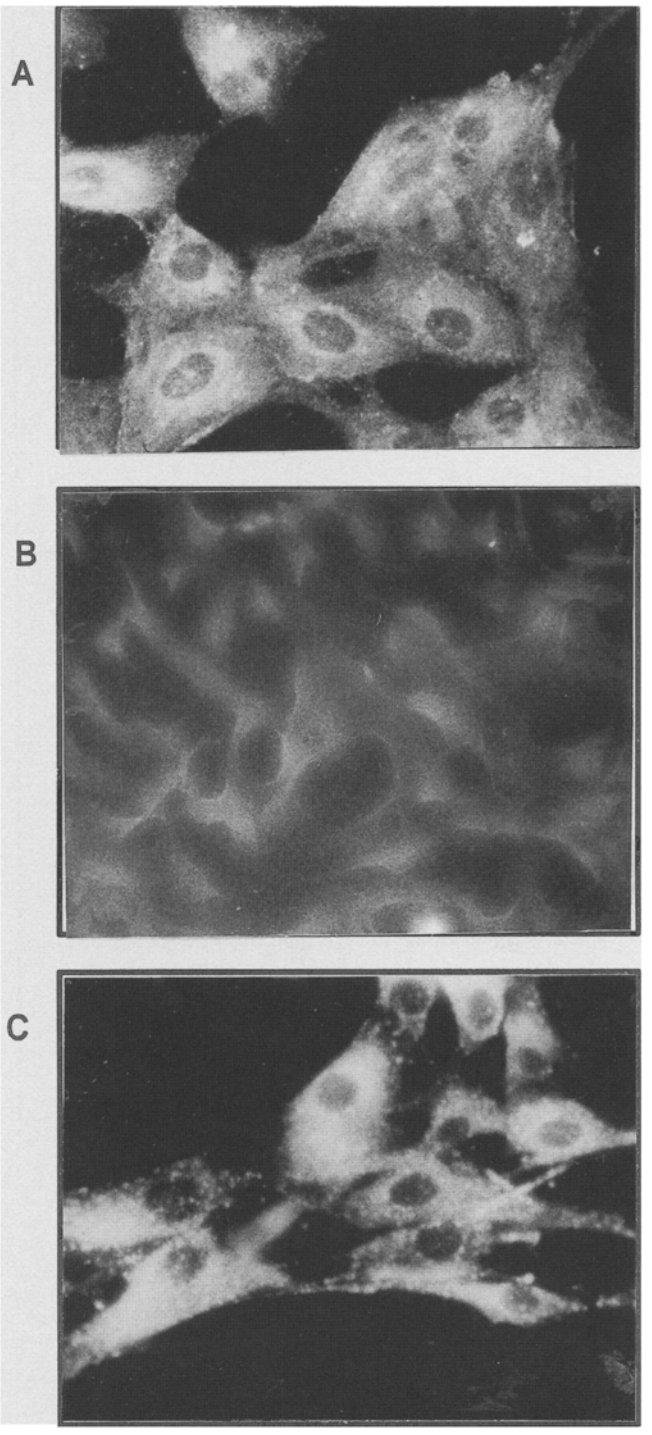

FIG. 3. Immunocytochemical staining for endothelial markers. *A*, von Willebrand factor; *B*, CD31; *C*, acetylated low-density lipoprotein uptake on normal lung microvascular endothelial cells.

40 U/ml heparin, and 100 μg/ml endothelial cell growth supplement (Sigma). The cells were plated onto T25 flasks precoated with 2% gelatin (Sigma) and cultured at 37° C in a 5% CO_2 atmosphere.

Purification of Human Lung MVEC

The cells were cultured for 48 h, washed with DMEM to remove nonadherent cells, and fresh growth medium was added. After 1 wk, MVEC grew out as typical "cobblestone" colonies and exhibited the characteristics of monolayer growth and contact inhibition. These "cobblestone" colonies were marked under an inverted microscope in a laminar flow hood. The nonendothelial cells around the colonies were weeded by manual manipulation, as described (24). The medium was removed, the flask was washed with DMEM,

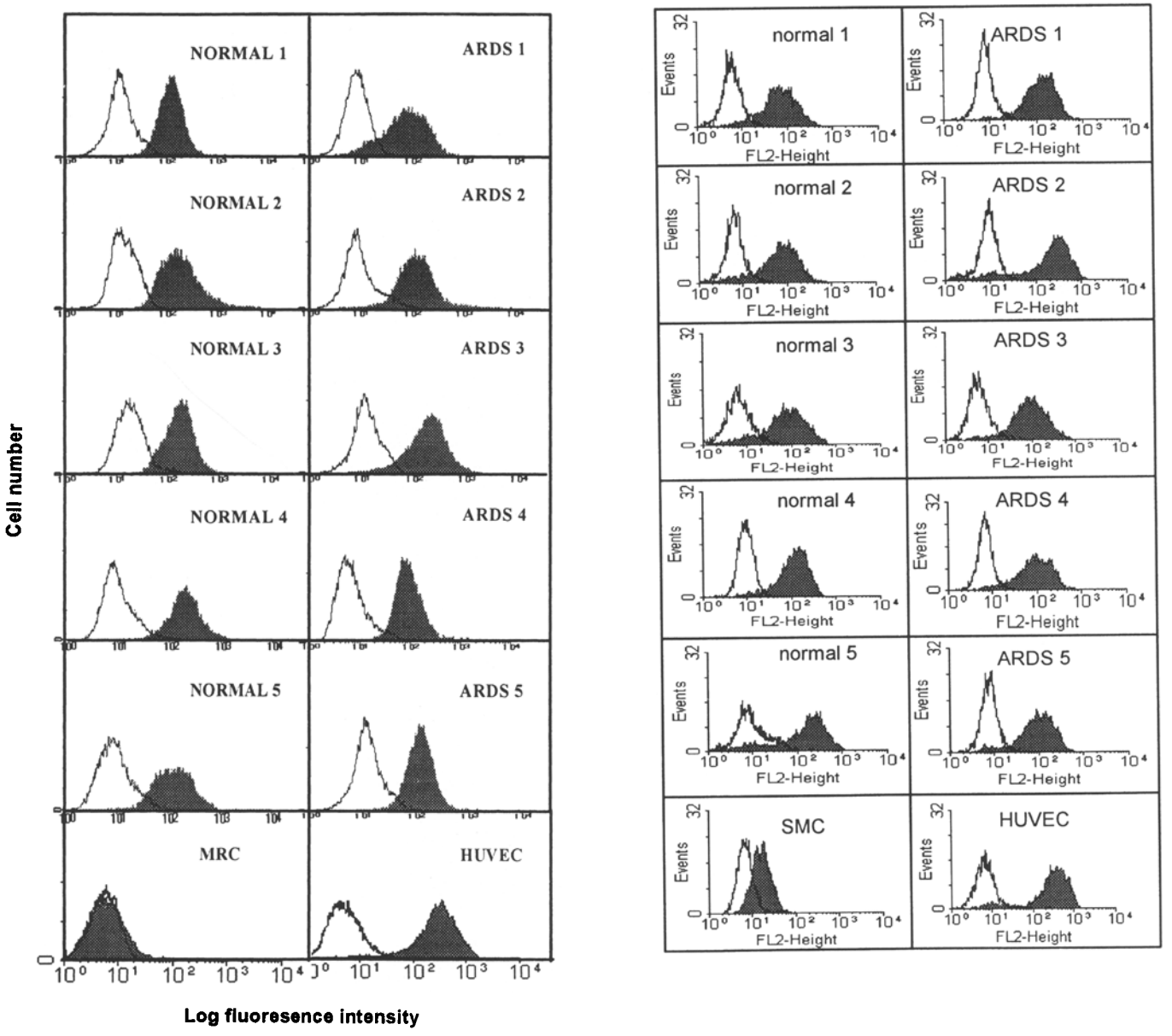

FIG. 4. Profiles of von Willebrand factor (vWF) (*A*) and acetylated low-density lipoprotein (Ac-LDL) (*B*). Flow cytometric analysis of lung microvascular endothelial cells (MVEC) from control patients and patients who died of acute respiratory distress syndrome (ARDS). (*A*). Analysis of vWF expression. The *open histograms* represent the fluorescence with second step only, and the *shaded histograms* indicate the specific staining with anti-human vWF antibody. Human umbilical vein endothelial cells (HUVEC) and human fibroblasts (MRC) used respectively as positive and negative controls. (*B*). Analysis of Ac-LDL uptake. The *open histograms* represent the lung MVEC alone, and the *shaded histograms* indicate uptake of Ac-LDL after 4 h incubation with Ac-LDL. HUVEC and human smooth muscle cells (SMC) used respectively as positive and negative controls.

and a drop of trypsin/EDTA was added onto the marked colonies with a micropipette. Under an inverted microscope, the digested cells were carefully transferred to a new flask with a micropipette. Purified MVEC were cultured until cells reached confluence, then passaged after trypsin/EDTA treatment at a rate dependent on their growth.

Characterization of Human Lung MVEC

1. Morphology. Purified lung MVEC were seeded on a chamber slide (Nunc, Inc., Naperville, IL) and grown to confluence. To check their ability to form capillarylike structures *in vitro*, the cells were cultured in chamber slides coated with 3% gelatin. After 3 d of culture, the formation of capillarylike structures was observed under an inverted phase contrast microscope.

2. Transmission electron microscopy. Cells were seeded in a gelatin-coated cell culture insert with 0.45 μm pore size (Falcon, Meylan Cedex, France) and grown to confluence. The cells were washed with DMEM and then fixed with 2.5% glutaraldehyde for 30 min at 4° C, washed with 0.1 *M* phosphate-buffered saline (PBS) pH 7.2, and postfixed with 1% osmium tetroxide for 20 min. The cells were dehydrated with different concentrations of ethanol and embedded in Epon 812. Selected areas of monolayer cell cultures were stained with uranyl acetate and lead citrate, and examined in a Philips EM 400 electron microscope.

3. Expression of endothelial markers: vWF and PECAM-1. Lung MVEC (5×10^3 cells in 0.5 ml) were plated in a chamber slide and grown to subconfluence. Human umbilical vein endothelial cells (HUVEC), purified as de-

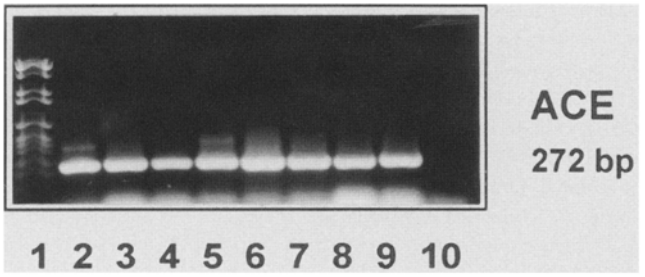

FIG. 5. Expression of angiotensin converting enzyme (ACE) transcripts in normal and acute respiratory distress syndrome (ARDS)-derived lung microvascular endothelial cells (MVEC) Reverse transcription/polymerase chain reaction (RT/PCR) analysis. *Lane 1*: a DNA ladder. *Lanes 2–5*: normal lung MVEC. *Lanes 6–9*: ARDS-derived lung MVEC. *Last lane*: THP-1 cells used as negative control.

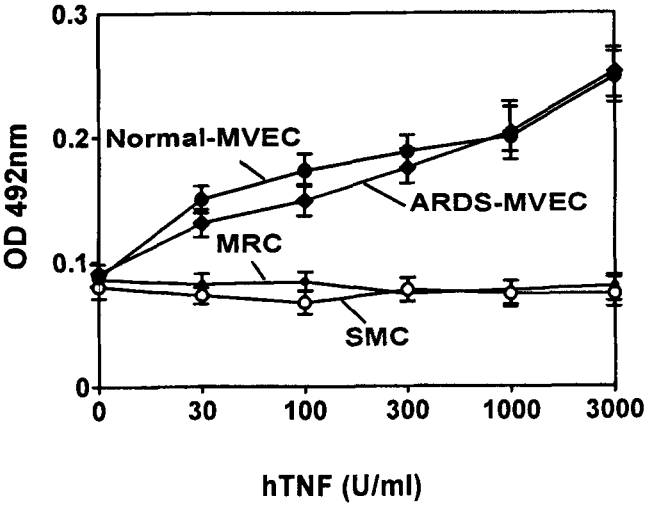

FIG. 6. Inducibility of ELAM-1 on lung microvascular endothelial cells (MVEC). Normal or acute respiratory distress syndrome (ARDS)-derived lung MVEC were stimulated for 4 h with recombinant human TNF (hTNF). Intensity of staining was determined by a cell-based enzyme-linked immunosorbent assay (ELISA). Negative controls consisted of human lung fibroblasts (MRC) and smooth muscle cells (SMC).

scribed (19), and human fibroblasts (MRC, a kind gift from Dr. M. Pepper, University of Geneva Medical School), were used as positive and negative controls, respectively. The cells were fixed with methanol at −20° C for 10 min, washed three times with PBS containing 1% bovine serum albumin (BSA), and preincubated with PBS containing 5% FCS, 0.05% Tween 20 for 20 min at 37° C to block nonspecific binding. The cells were stained with 1/20 diluted rabbit antiserum to human vWF (Sigma) or 1/100 diluted monoclonal antibody to human PECAM-1 (9G11, from British Biotechnology) for 45 min at room temperature. Cells were washed three times with PBS/BSA and incubated with 1/100 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) or 1/100 diluted FITC-conjugated goat anti-mouse IgG antibody (Sigma) for 30 min at room temperature. After washing and mounting, the staining was observed under a fluorescence microscope. Alternatively, for flow cytometry, single-cell suspensions were prepared by trypsinization, fixed 5 min with −20° C methanol, and then incubated with PBS containing 5% FCS and 0.05% Tween 20 for 10 min to block nonspecific binding. Cells were washed, stained as above at room temperature, and analyzed with a FACScan (Becton-Dickinson, San Jose, CA).

4. Uptake of acetylated LDL. Uptake was demonstrated with the fluorescent probe 1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanide perchlorate

TABLE 1

SUMMARY OF DIFFERENCES BETWEEN MICROVASCULAR ENDOTHELIAL CELLS (MVEC), LARGE VESSEL ENDOTHELIAL CELLS (LVEC), AND POSSIBLE CONTAMINANT CELLS, SUCH AS FABROBLASTS AND SMOOTH MUSCLE CELLS (SMC)[a]

	MVEC	LVEC	fibroblasts	SMC
Morphology	cobblestone/ spindle	cobblestone	spindle	spindle
Growth characteristic	monolayer	monolayer	polylayer	polylayer
Capillarylike structures	yes	yes	no	no
Microvilli	+ + +	−	−	−
Weibel-Palade Bodies	+	+ + +	−	−
vWF	+	+ + +	−	−
CD31	+/−	+	−	−
Ac-LDL uptake	high	high	low	low
ACE	+ + +	+	+	−
ELAM-1 inducibility	+	+	−	−

[a] vWF = von Willebrand factor; Ac-LDL = acetylated low-density lipoprotein; ACE = angiotensin converting enzyme; ELAM-1 = endothelial cell leukocyte adhesion molecule 1; + = positive; − = negative.

conjugated to Ac-LDL (Dil-Ac-LDL) (Paesel + Lorei, Frankfurt, Germany). The cells were incubated with 10 μg/ml Dil-Ac-LDL in DMEM containing 10% FCS, for 4 h at 37° C. The medium was removed and cells were incubated for 10 min in fresh medium. For fluorescence microscopy, cells were fixed with 10% buffered formalin, washed, mounted, and then observed under fluorescence microscopy. For flow cytometry, after 4 h of incubation with Ac-LDL, single-cell suspensions were prepared by trypsinization. HUVEC and human smooth muscle cells (SMC, kind gift from Dr. G. Gabbiani, University of Geneva Medical School) were used as positive and negative controls, respectively. The fluorescence intensity was analyzed with a FACScan.

5. Expression of angiotensin converting enzyme. The expression of ACE mRNA was studied by reverse transcription/polymerase chain reaction (RT/PCR) as described (10). Briefly, total RNA was isolated from 1×10^6 cells by the single guanidinium thiocyanate-phenol-chloroform mixture extraction method (5). RNA was incubated for 30 min at 37° C in 40 m*M* Tris-HCl pH 7.5, 10 m*M* NaCl, 6 m*M* $MgCl_2$, and 2.5 units of RQ1 DNAse (Promega Corp., Madison, WI, USA) to remove any contaminating genomic DNA from the preparations. After phenol-chloroform extraction and ethanol precipitation, pellets were resuspended in water. Synthesis of the first strand of cDNA was performed according to the instructions delivered with the cDNA Synthesis Kit (Boehringer Mannheim, Germany), using random primers and AMV reverse transcriptase (10 units/sample) in a final volume of 20 μl. After 1 h incubation at 42° C, samples were heat inactivated and kept frozen (−20° C) until use. Two μl of cDNA were then amplified in a 25 μl reaction mixture containing buffer, deoxynucleotide triphosphates (dNTP), and 2.5 units of ampliTaq (Perkin Elmer Cetus, Emeryville, CA). Samples were overlaid with mineral oil and amplified at 94° C for 5 min, 60° C for 1 min, and 72° C for 30 s followed by 35 cycles at 94° C for 30 s, 60° C for 30 s, and 72° C for 1 min, 30 s. PCR was carried out in an automated DNA Thermal Cycler (Perkin Elmer Cetus), in the presence of 0.2 μ*M* of each primer. The following oligonucleotides were used:

GAPDH 1,5′-TGAAGGTCGGTGTGAACGGATTTGG-3′
GAPDH 2,5′-ACGACATACTCAGCACCAGCATCAC-3′
ACE 1,5′-GATGTGGCCATCACATTCGTCAGA-3′
ACE 2,5′-GCTGCAGAAGAACATGCAAA-3′

One half of the reaction was electrophoresed on 1.5% agarose gels containing ethidium bromide and the appropriate bands were visualized under UV light.

6. Inducibility of ELAM-1. A cell-based enzyme-linked immunosorbent assay (ELISA) was used to detect and quantitate TNF-inducible ELAM-1 expression. Briefly, purified MVEC were seeded in 96-well plates and cultured to confluence. Cells were then stimulated with different doses of recombinant human TNF in DMEM containing 10% FCS for 4 h at 37° C. Cells were washed with DMEM and fixed with −20° C methanol for 10 min. After fixation, PBS containing 5% FCS and 0.05% Tween 20 was added for 20 min to block nonspecific binding. Cells were incubated with monoclonal antibody

to human ELAM-1 (28.109.76 from Janssen Biochemica, Beerse, Belgium), diluted in PBS/BSA (final concentration 10 μg/ml) for 45 min at room temperature, washed with PBS/BSA, and then incubated with 1/1000 diluted peroxidase-conjugated anti-mouse IgG (Sigma) for 30 min at room temperature. Cells were further incubated with diluted substrate, tetramethylbenzidine (TMB, from Medgenix, Fleurus, Belgium) for 30 min, stopped with 2 *N* H_2SO_4, and optical density was read in an automatic ELISA reader at 492 nm.

Results

The four steps of lung MVEC purification are shown in Fig. 1 *A–D*. The cell aggregation induced by human plasma was demonstrated to be vWF positive by immunofluorescence (data not shown). After Percoll centrifugation, the aggregated cells were found in the pellet. When cells adhered to the plastic well, the aggregated MVEC presented a cobblestonelike morphology and exhibited the characteristics of monolayer growth and contact inhibition. Lung MVEC from both types of tissues formed classical capillarylike "tubular" structures when cultured on 3% gelatin-coated slides. Transmission electron microscopy showed the presence of Weibel-Palade bodies in the cytoplasm and abundant microvilli on MVEC plasma membrane (Fig. 2 *A–C*). Although lung MVEC are positive for vWF, the immunofluorescence staining pattern was less granular, because they have fewer Weibel-Palade bodies than HUVEC (9,17,21). Mesothelial cells can also exhibit cell surface microvilli, but we found the microvilli and Weibel-Palade bodies within the same cells. The isolated cells were stained with cytokeratin 8, a marker of mesothelial cells by immunocytochemistry. The results indicated that less than 1% of the cells were positive in both normal and in ARDS lung MVEC. The isolated cells expressed vWF and CD31, as shown by immunofluorescence staining (Fig. 3 *A* and *B*), and took up large amounts of Ac-LDL (Fig. 3 *C*). The staining for vWF and uptake of Ac-LDL were also studied by flow cytometry, to analyze the intensities of fluorescence (Fig. 4 *A* and *B*). By RT/PCR, all purified MVEC from normal and ARDS lung showed the expression of ACE mRNA and its absence in the monocytic THP-1 cell line (a kind gift of Dr. J. Pugin, University of Geneva Medical School), used as negative control (Fig. 5). Moreover, both normal and ARDS-derived lung MVEC exhibited a clear inducibility of ELAM-1 after a 4-h *in vitro* stimulation with recombinant human TNF, in a dose-dependent manner, as shown by cell-based ELISA (Fig. 6). In contrast, both MRC and SMC remained negative for ELAM-1 induction.

Discussion

In the present study, we report a method to isolate and purify human lung MVEC, from either normal or inflamed lung tissue, that leads to cells expressing classical morphological and functional markers of endothelial cells, including monolayer growth, expression of vWF, CD31, ACE, high Ac-LDL uptake, and, most importantly, ELAM-1 inducibility in response to TNF. This method consists of four main steps:

1. Pulmonary tissue was digested fully with trypsin and collagenase to release MVEC from vascular walls. We found that trypsin is capable of digesting perivascular connective tissues while collagenase is beneficial to separate intercellular junctions. Using trypsin/collagenase digestion sequentially can result in higher yield in MVEC from microvessels.

2. Aggregation of MVEC was induced by human plasma. In usual conditions, plasma does not induce MVEC aggregation, but it did after trypsin/collagenase digestion. The aggregated cells were demonstrated to be vWF positive by immunocytochemical staining. The mechanism involved in MVEC aggregation is not known. A probable explanation is that trypsin/collagenase may induce the expression of a glycoprotein IIb/IIIa—like molecule (26) on the surface of MVEC acting as a fibrinogen receptor, or the adherence of blood platelets to isolated MVEC. That MVEC aggregation may be induced by the fibrinogen contained in plasma.

3. The aggregated MVEC were collected after 20% Percoll density centrifugation.

4. Because endothelial colonies were formed quickly after aggregated MVEC adhered to plastic wells, these colonies were easily selected and transferred after local digestion with trypsin/EDTA under light microscopy. Lung MVEC were successfully isolated from normal human lung using magnetic beads conjugated with anti-endothelial antibody. Using anti-CD31-conjugated magnetic beads, we obtained pure MVEC, but the anti-CD31 antibody on the bound beads affected the phenotypic analysis of purified MVEC by flow cytometry. Indeed, FITC-conjugated second antibody bound to both antibodies to CD31 and to other surface molecules, leading to false positive results (data not shown).

The appearance of MVEC as "cobblestone" or "spindle-shaped" may depend on the culture medium and the origin of MVEC (arteriole, capillary, postcapillary venule, or venules). It has been shown that MVEC from arteriolar and capillary origin exhibit typical cobblestonelike morphology, while MVEC from postcapillary venules are spindle-shaped (30). We selected the cell colonies with cobblestonelike morphology because they can be easily recognized from fibroblasts and SMC that present spindle-shaped morphology. MVEC from normal lungs consistently presented the "cobblestone" pattern, while those purified from lungs of ARDS patients often change morphology to "spindle-shaped" after cell transfer. This morphological change may imply that MVEC derived from ARDS patients were activated *in vivo*, because these MVEC exhibited significant phenotypic change (15). Also, it has been shown that upon activation, such as stimulation by TNF, endothelial cells can shift from the cobblestone- to the spindle-shape morphology (28). Contamination by fibroblasts or SMC is frequently seen in lung MVEC cultures. Unlike MVEC, both fibroblasts and SMC exhibit overlay growth (i.e., without contact inhibition), express neither vWF nor CD31, and take up only low levels of Ac-LDL (34). Although a contamination by fibroblasts or SMC is easily recognized on the basis of morphology and other characteristics (summarized in Table 1), their eradication from MVEC cultures is laborious or impossible. Therefore, selection of pure endothelial colonies is critical at the purification step. The induction of aggregation of lung MVEC by human plasma makes this selection easier.

Mesothelial cell contamination can be troublesome when lung MVEC are isolated from small animals such as mice or rats, because in these cases it is difficult to remove the pleura completely. In the case of human lung, nevertheless, this mesothelial cell contamination can be minimized by careful dissection of the visceral pleura prior to proceeding with the isolation procedure. Mesothelial cells present the same morphology as MVEC, by light microscopy, and can take up Dil-Ac-LDL (22,32). Mesothelial cells have also been found to express ACE and vWF (6) but, unlike MVEC, they fail to express CD31, to form capillarylike structures and to express ELAM-1 in response to TNF (20). Staining for cytokeratin 8, a marker of mesothelial cells, indicated that less than 1% of the cells are positive in

MVEC isolated from either normal or ARDS lung (data not shown). Finally, contamination by pericytes can sometimes occur in MVEC cultures. These cells do not express endothelial markers, and are easily detached by strong washings with a Pasteur pipette, as they are less adherent to plastic than MVEC (our unpublished observation).

To rule out a contamination of MVEC cultures by LVEC is not easy because both cell types express the same endothelial markers and morphology. Avoiding contamination of LVEC is also important in isolation of MVEC. Lung MVEC derived from ARDS patients exhibited a marked ICAM-1 upregulation, while the pulmonary vein endothelial cells isolated from the same patients did not (our unpublished data). Several differences exist between MVEC and LVEC, such as density of Weibel-Palade bodies, prostaglandin metabolism, and ACE activity (4,12,18,21), but some specific markers of MVEC would be necessary (Table 1). The abundance of microvilli have been found on brain MVEC (30), but not LVEC such as HUVEC. This surface feature was also demonstrated on lung MVEC, but not on pulmonary artery endothelial cells (7). Moreover, microvilli were also found in microvessels in tissue sections of brain and lung (our unpublished data). These data indicated that cell surface microvilli are a feature of MVEC. Because large vessels are not found in the peripheral lung parenchyma (i.e., under the visceral pleura), we selected these areas for the MVEC isolation in order to avoid contamination by LVEC. In conclusion, by allowing the study of the relevant cell from the relevant tissue, this method may be useful for investigations dedicated to various immunopathological reactions occurring in the lung.

Acknowledgments

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References

1. Beekhuizen, H.; Vanfurth, R. Growth characteristics of cultured human microvascular venous and arterial and microvascular endothelial cells. J. Vasc. Res. 31:230–239; 1994.
2. Belloni, P. N.; Carney, D. H.; Nicolson, G. L. Organ-derived microvessel endothelial cells exhibit differential responsiveness to thrombin and other growth factors. Microvas. Res. 43:20–45; 1992.
3. Carlay, W. C. M.; Niedbala, J.; Gerritsen, M. E. Isolation, cultivation and partial characterization of microvascular endothelium derived from human lung. Am. J. Respir. Cell Mol. Biol. 7:620–630; 1992.
4. Charo, I.; Karasek, S.; Davision, M. A., et al. Prostaglandins I2 is not a major metabolite of arachidonic acid in cultured endothelial cells from human foreskin microvessels. J. Clin. Invest. 74:914–919; 1984.
5. Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159; 1987.
6. Chung-Welch, N.; Patton, W. F.; Ameia Yen-Patton, G. P., et al. Phenotypic comparison between mesothelial and microvascular endothelial cell lineage using conventional endothelial cell markers, cytoskeletal protein markers, and in vitro assays of angiogenic potential. Differentiation 42:44–53; 1989.
7. Chung-Welch, N.; Shepro, D.; Dunham, B., et al. Prostacyclin and prostaglandin E2 secretion by bovine pulmonary microvascular endothelial cells are altered by changes in culture conditions. J. Cell. Physiol. 135:224–234; 1988.
8. Danielsen, H.; Funderud, S.; Nustad, K., et al. The interaction between cell-surface antigens and antibodies bound to monodisperse polymer particles in normal and malignant cells. Scand. J. Immunol. 24:179–187; 1986.
9. Davison, P. M.; Bensch, K.; Karasek, M. A. Isolation and growth of endothelial cells from the microvessels of newborn human foreskin in cell culture. J. Invest. Dermatol. 75:316–321; 1980.
10. de Kossodo, S.; Grau, G. E. Profiles of cytokine production in relation with susceptibility to experimental cerebral malaria. J. Immunol. 151:4811–4820; 1993.
11. Delvecchio, P. J.; Siflingerbirnboim, A.; Belloni, P. N., et al. Culture and characterization of pulmonary microvascular endothelial cells. In Vitro Cell. Dev. Biol. 28A:711–715; 1992.
12. Gerritsen, M. E.; Printz, M. P. Site of prostaglandin synthesis in bovine heart and isolated bovine coronary microvessels. Circ. Res. 5:1152–1163; 1981.
13. Gitlin, J. D.; D'Amore, P. A. Culture of retinal capillary cells using selective growth media. Microvas. Res. 26:74–80; 1983.
14. Grau, G. E.; Lou, J. TNF in vascular pathology: importance of platelet-endothelium interactions. Res. Immunol. 144:355–363; 1993.
15. Grau, G. E.; Mili, N.; Lou, J. N., et al. Phenotypic and functional analysis of pulmonary microvascular endothelial cells from patients with acute respiratory distress syndrome. Lab. Invest. 74:761–770; 1996.
16. Hewett, P. W.; Murray, J. C. Human lung microvessel endothelial cells—isolation, culture, and characterization. Microvas. Res. 46:89–102; 1993.
17. Hewett, P. W.; Murray, J. C.; Price, E. A., et al. Isolation and characterization of microvessel endothelial cells from human mammary adipose tissue. In Vitro Cell. Dev. Biol. 29A:325–331; 1993.
18. Hviid, L.; Elhassan, I. M.; Dodoo, D., et al. Differential t-cell expression of LFA-1 in residents from Africa and Denmark—description of the phenomenon and its possible basis. Immunol. Lett. 39:147–151; 1994.
19. Jaffe, E. A.; Nachman, R. L.; Becker, C. G. Culture of human endothelial cells derived from umbilical veins: identification by morphologic criteria. J. Clin. Invest. 52:2745–2758; 1973.
20. Jonjic, N.; Peri, G.; Bernasconi, S., et al. Expression of adhesion molecules and chemotactic cytokines in cultured human mesothelial cells. J. Exp. Med. 176:1165–1174; 1989.
21. Kern, P. A.; Knedler, A.; Eckel, R. H. Isolation and the culture of the microvascular endothelium from adipose tissue. J. Clin. Invest. 71:1822–1829; 1983.
22. Latron, Y.; Alessi, M. C.; George, F., et al. Characterization of epithelial cells from human omentum: comparison with endothelial cells from umbilical veins. Thromb. Haemostasis 66:361–367; 1991.
23. Ley, K.; Gaehtgens, P.; Spanelborowski, K. Differential adhesion of granulocytes to five distinct phenotypes of cultured microvascular endothelial cells. Microvas. Res. 43:119–133; 1992.
24. Marks, R. M.; Czerniecki, M.; Penny, R. Human dermal microvascular endothelial cells: an improved method for tissue culture and a description of some singular properties in culture. In Vitro Cell. Dev. Biol. 21:627–635; 1985.
25. Meyrick, B.; Hoover, R.; Jones, M. R., et al. In vitro effects of endotoxin on bovine and sheep lung microvascular and pulmonary artery endothelial cells. J. Cell. Physiol. 138:165–174; 1989.
26. Newman, P. J.; Kawai, Y.; Montgomery, R. R., et al. Synthesis by cultured human umbilical vein endothelial cells of two proteins structurally and immunologically related to platelet membrane glycoproteins IIb and IIIa. J. Cell Biol. 103:81–86; 1986.
27. Rupnick, M. A.; Carey, A.; Williams, S. K. Phenotypic diversity in cultured cerebral microvascular endothelial cells. In Vitro Cell. Dev. Biol. 24:435–444; 1988.
28. Sato, N.; Goto, T.; Haranaka, K., et al. Actions of tumor necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition, and cytotoxicity. J. Natl. Cancer Inst. 76:1113–1121; 1986.
29. Scott, P. A. E.; Bicknell, R. The isolation and culture of microvascular endothelium. J. Cell Sci. 105:269–273; 1993.
30. Spanel-Borowski, K.; Van der Bosch, J. Different phenotypes of cultured microvessel endothelial cells obtained from the bovine corpus luteum. Cell Tissue Res. 261:35–47; 1990.

31. Swerlick, R. A.; Lawley, T. J. Role of microvascular endothelial cells in inflammation. J. Invest. Dermatol. 100:S111–S115; 1993.
32. Van Hinsbergh, V. W. M.; Kooistra, T.; Scheffer, M. A., et al. Characterization and fibrinolytic properties of human omental tissue mesothelial cells: comparison with endothelial cells. Blood 75:1490–1497; 1990.
33. Visner, G. A.; Chesrown, S. E.; Monnier, J., et al. Regulation of manganese superoxide dismutase—il-1 and tnf induction in pulmonary artery and microvascular endothelial cells. Biochem. Biophys. Res. Commun. 188:453–462; 1992.
34. Voyta, J. C.; Via, D. P.; Butterfield, C. E., et al. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. J. Cell Biol. 99:2034–2040; 1984.
35. Wautier, J. L.; Setiadi, H.; Vilette, D., et al. Leukocyte adhesion to endothelial cells. Biorheology 27:425–432; 1990.
36. Woodley, N.; Barclay, J. K. Cultured endothelial cells from distinct vascular areas show differential responses to agonists. Can. J. Physiol. Pharmacol. 72:1007–1012; 1994.