Characterization of CD34+ cells isolated from human fetal lung

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Acarregui, Michael J., Katherine M. England, Joshua T. Richman, and Jennifer L. Littig. Characterization of CD34+ cells isolated from human fetal lung. Am J Physiol Lung Cell Mol Physiol 284: L395–L401, 2003. First published October 11, 2002; 10.1152/ajplung.00202.2002.—The large capillary mass of the newborn lung demands the presence of endothelial cell precursors in lung tissue before development of the pulmonary capillary bed. The objective of this investigation was to isolate and characterize putative endothelial cell precursors from developing human lung. CD34, a cell surface marker for hematopoietic progenitor cells, endothelial precursor cells, and small vessel endothelial cells, was employed as an immunological “handle” for the selection of the desired cells. When CD34+ cells were isolated from midtrimester human fetal lung tissue, then maintained in culture, the isolated cells expressed immunoreactivity for the endothelial cell marker von Willebrand factor and the endothelial cell adhesion molecule CD34. The CD34+ cells endocytosed acetylated low-density lipoprotein and formed capillary-like structures when incubated in a cushion of Matrigel. RT-PCR analysis of mRNA for endothelial cell-related proteins Flt-1, Tie-2, and endothelial nitric oxide synthase demonstrated expression of these mRNAs by the isolated cells for at least 16 cell passages. These observations demonstrated that such cells express the cell surface marker CD34 (11, 16). CD34 is a 105- to 120-kDa transmembrane glycoprotein that is also expressed on stem and progenitor cells of the hematopoietic systems of the human (15, 24) and mouse (25). Therefore, in the present study, our objective was to isolate and characterize CD34+ cells from midtrimester human fetal lung and to determine whether these cells represent capillary endothelial cell precursors. We employed a variety of criteria that are known to be phenotypically associated with endothelial cells. These criteria include the expression of receptors for VEGF, the expression of mRNA for other endothelial cell-specific proteins, the ability to endocytose acetylated low-density lipoproteins, and the ability to form capillary-like tubes in vitro. Fulfillment of these criteria demonstrated that the isolated CD34+ cells possess and maintain an endothelial cell phenotype in culture, suggesting that they are, indeed, pulmonary capillary endothelial precursor cells. This is the first report of the isolation and characterization of such cells from developing human lung. It is speculated that the isolated cells can be employed as a model to investigate factors that regulate existing vessels, and vasculogenesis, defined as the generation of blood vessels from endothelial cell precursors contained within the mesenchyme. Previous studies have demonstrated the role of vasculogenesis in the generation of the pulmonary capillary bed (9). It is during the canalicular phase of human lung development (16–24 wk gestation) that endothelial cell precursors coalesce into capillaries (6, 9) and that the air-blood barrier begins to form (10). Hence, a large pool of endothelial cell precursors must exist within the lung mesenchyme to serve as substrate for the large capillary mass of the maturing lung. The existence of a large population of endothelial cell precursors in the developing lung is further supported by observations of an abundance of the endothelial cell growth factor, vascular endothelial cell growth factor (VEGF), in the epithelium of the developing human lung (1, 20).

Studies of endothelial cell precursors have demonstrated that such cells express the cell surface marker CD34 (11, 16). CD34 is a 105- to 120-kDa transmembrane glycoprotein that is also expressed on stem and progenitor cells of the hematopoietic systems of the human (15, 24) and mouse (25). Therefore, in the present study, our objective was to isolate and characterize CD34+ cells from midtrimester human fetal lung and to determine whether these cells represent capillary endothelial cell precursors. We employed a variety of criteria that are known to be phenotypically associated with endothelial cells. These criteria include the expression of receptors for VEGF, the expression of mRNA for other endothelial cell-specific proteins, the ability to endocytose acetylated low-density lipoproteins, and the ability to form capillary-like tubes in vitro. Fulfillment of these criteria demonstrated that the isolated CD34+ cells possess and maintain an endothelial cell phenotype in culture, suggesting that they are, indeed, pulmonary capillary endothelial precursor cells. This is the first report of the isolation and characterization of such cells from developing human lung. It is speculated that the isolated cells can be employed as a model to investigate factors that regulate

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late, and mechanisms that are involved in, the development of the air-blood barrier in the human.

MATERIALS AND METHODS

Isolation of CD34+ cells from human fetal lung and flow cytometry analysis. Lung tissues were obtained from human abortuses of 16- to 19-wk gestation from a local clinic or from Advanced Biosciences Resources (Alameda, CA) as approved by the University of Iowa Human Subjects Review Committee. Major vessels and airways were removed from midtrimester human fetal lung tissues followed by mincing of the peripheral tissue and digestion with collagenase (0.5%; Sigma, St. Louis, MO) in Eagle's minimal essential medium (EMEM) with gentamicin (50 μg/ml), penicillin (100 U/ml), and amphotericin B (2.5 μg/ml) for 30 min at 37°C. The tissue digest was pipetted through a 10-ml pipette (60 times) and then filtered through a nylon mesh (100 μm). The crude cell filtrate was suspended in EMEM with 10% fetal calf serum and then placed in a 100-mm plastic dish for 60 min at 37°C to allow adhesion of fibroblasts. Nonadherent cells were suspended in EMEM, then incubated with anti-CD34 conjugated magnetic beads (Dynal, Lake Success, NY) for 60 min at 4°C per the manufacturer's protocol followed by a step to remove the beads from the cells. The selected cells were then plated on plastic dishes coated with rat tail collagen (5 μg/cm²; Becton Dickinson, Franklin Lakes, NJ) and maintained in a commercially available endothelial cell growth medium (E-Stim; Becton Dickinson). Aliquots of cells from the crude cell suspension (immediately after filtering through the nylon mesh) and from the final cell selection were plated on glass slides with a cytocentrifuge (Shandon, Pittsburgh, PA) for later immunostaining to verify homogeneity of the isolates. Before plating, the cells were counted on a hemocytometer, and cell viability was evaluated by trypan blue exclusion.

An aliquot (1 × 10⁶ cells) of the freshly isolated CD34+ cells were labeled for fluorescence-activated cell sorting (FACS). After pelleting the cells at 300 g for 10 min at 4°C, we resuspended them in 50 μL of PBS with 1% fetal calf serum containing 10 μL each of monoclonal antibodies against human CD31/platelet endothelial cell adhesion molecule (PECAM) and CD34 (CD34 R-Phycoerythrin conjugated and CD34 FITC conjugated; Caltag Laboratories, Burlington, CA) and incubated for 30 min on ice. After being washed twice with PBS with 1% fetal calf serum, the cells were fixed in 2% paraformaldehyde then washed again two times. The labeled and fixed cells were then separated and analyzed with a Becton Dickinson FACScan.

Immunostaining. Immunoreactivity for endothelial cell markers was performed on cultures of isolated CD34+ cells. Isolated CD34+ cells (10⁵ cells per well) were plated on four-well glass culture slides (Becton Dickinson) coated with collagen (5 μg/ml) and incubated for 48 h. Cytospin preparations and cultures of the incubated cells were then fixed with acetone-MeOH (1:1) for 20 min at −20°C, rinsed 2× with PBS, treated 30 min with 3% H₂O₂, blocked 20 min with 1% bovine serum albumin in PBS, and then incubated for 1 h at room temperature with primary antibody against von Willebrand factor (vWF; 1:50; Labvision, Fremont, CA), Flt-1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or KDR (1:100; Santa Cruz). The remainder of the procedure is as described for the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Negative controls employing nonimmune rabbit serum (for vWF) or antibody preadsorbed with specific blocking peptide (for Flt-1 and KDR; Santa Cruz) in place of primary antibody were performed for each condition. In each case there was no immunoreactivity observed for the negative control (data not shown for Flt-1 or KDR).

For immunostaining performed on midtrimester human fetal lung tissue, paraffin-embedded lung tissue sections were deparaffinized with xylene, then rehydrated through an ethanol-water series. The paraffin sections were then incubated with primary antibody (mouse monoclonal anti-CD34 IgG, 1:200; Vector Laboratories) for 1 h at room temperature. The sections were then incubated with biotinylated secondary antibody (1:50 dilution in PBS), then rinsed two times in PBS, 5 min per rinse. The sections were counterstained with hematoxylin for 30 s, rinsed in PBS for 5 min, rinsed quickly in distilled water, dehydrated, and mounted with glass coverslips. Negative controls were incubated with nonimmune mouse IgG in the place of primary antibody, processed as above with secondary antibody, and were performed for all conditions. The sections were viewed and photographed under a Nikon FX microscope.

Acetylated low-density lipoprotein uptake. Second-passage CD34+ cells were plated on culture slides as above for 24 h and then incubated for 4 h at 37°C in the presence of 10 μg/ml acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiAc-LDL; Biomedical Technologies, Stoughton, MA). The cell cultures were then washed three times with medium, fixed with 4% formaldehyde for 5 min, mounted with coverslips, and then assayed for DiAc-LDL uptake by fluorescent microscopy. Similarly, cultures of human umbilical vein endothelial cells (HUVEC; Clonetics, East Rutherford, NJ) were incubated in the presence of DiAc-LDL for use as positive controls.

Tube formation assays. Second-passage CD34+ cells (50,000 cells/well) were plated on 400-μl cushions of Matrigel basement membrane matrix (Becton Dickinson) and maintained in E-Stim medium. The media were changed daily. After 7 days of incubation at 37°C, the cultures were photographed. Some cultures were fixed with ½ strength Karnovsky's fixative in 0.1 M sodium cacodylate buffer (pH 7.2) with a microwave processor. The gels were postfixed with 1% osmium tetroxide and 1.5% potassium ferricyanide. The fixed gels were then stained en bloc with 2.5% uranyl acetate, dehydrated through an ethanol series, and embedded in Eponate 12 (Ted Pella, Redding, CA). 100-nm-thick sections were examined under a Hitachi H-700 transmission electron microscope.

RT-PCR of RNA derived from isolated CD34+ cells. Isolated CD34+ cells derived from human fetal lung were maintained in E-Stim medium and harvested at various passages. Total cellular RNA was isolated by the method of Chomczynski and Sacchi (7). Qualitative detection of mRNA transcripts for Flt-1, Tie-2, and constitutive endothelial cell nitric oxide synthase (eNOS) was determined by reverse transcriptase and polymerase chain reaction (RT-PCR). Total RNA was incubated with oligo(dT) primer (Promega, Madison, WI) and Moloney murine leukemia virus RT (Invitrogen, Carlsbad, CA) to generate first-strand cDNA for PCR amplification of target cDNA. Reaction mixtures of 50-μl total volume containing aliquots of the RT reaction and Biolase DNA polymerase (Bioline USA, Springfield, NJ) were subjected to
PCR: 94°C for 30 s, 53°C for 30 s, 72°C for 30 s for 35 cycles for Flt-1 and Tie-2 PCR; and 94°C for 30 s, 55°C for 60 s, 72°C for 60 s for 35 cycles for eNOS PCR. The specific primers employed were: Flt-1 forward (Fwd) 5'-GAAGAGGTGAA GGTGTCTATCAC-3', reverse (Rev) 5'-TGCTGATGCTT GAAACACTTTT-3', generating a 372-nt product; Tie-2 Fwd 5'-GCCCTAAGACCAACGACCCAGG-3', Rev 5'-ACCTTCTGGGCTCACATCTCCG-3', generating a 460-nt product; and eNOS Fwd 5'-GACATTTTCGGGCTCACGCTG-3', Rev 5'-TGGGGTAGGCACTTTAGTAGT TCTC-3', generating a 555-nt product. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide staining.

RESULTS

Immunoreactivity for CD34 in midtrimester human fetal lung. Immunoreactivity for CD34 was observed in the distal aspects of the midtrimester human fetal lung in scattered areas of the mesenchyme and in close association to the epithelium of the distal airways (Fig. 1). The CD34 cells were abundant, and the majority of the CD34 cells were not associated with blood vessels, suggesting that such cells may be precursors to the pulmonary capillary bed.

Isolation and characterization of CD34 + cells from midtrimester human fetal lung. Cytospin preparations of crude cell suspensions derived from midtrimester human fetal lung tissue demonstrated a population of cells that were immunoreactive for vWF. After cellular selection with an anti-CD34 monoclonal antibody coupled to magnetic beads, the cell suspension was enriched in vWF (Fig. 2). Approximately 20 x 10⁶ cells were isolated with each procedure. The number of cells isolated was not dependent on the gestational age of the lung tissue. When the CD34 + cells were placed in culture, a relatively homogeneous population of elongated, spindle-shaped cells was observed (Fig. 3). The selected cells proliferated when maintained in a commercially available endothelial cell-supportive medium and became confluent approximately every 4 days when cultures were split 1:4. To determine whether the selected cells are immunoreactive for commonly expressed endothelial cell markers, we assayed cultures of CD34-selected cells for immunoreactivity for vWF and for the VEGF-specific receptors Flt-1 and KDR (also known as VEGF receptor (VEGFR)-1 and VEGFR-2, respectively). The cultured cells were immunoreactive for each of these antigens (Fig. 3) but not to smooth muscle α-actin (data not shown).

Flow cytometry analysis of fluorescently labeled isolated cells confirmed that >97% of the cells were CD34 +. Additional analysis demonstrated that the majority of the CD34 + cells were negative for PECAM, an important component of endothelial intercellular junctions (5) and endothelial cell-cell and cell-matrix interactions (19). In fact, only 3.6% ± 0.6% (means ± SE,
of the isolated cells were positive for PECAM (Fig. 4). Interestingly, we observed that for the CD34+ cells isolated from six different fetal lungs (three lungs from 19-wk gestation and one each from fetuses of 16-, 17-, and 18-wk gestation), two of the three 19-wk lungs yielded ≥5% PECAM-positive cells, whereas all of the other lungs yielded <3% PECAM-positive cells from the CD34+ isolates. This observation of a possible relative increase in PECAM-positive cells at 19-wk gestation compared with earlier gestations is consistent with previous demonstrations that 19 wk is the earliest gestation at which development of the air-blood barrier is observed (10).

Endothelial cells are known to endocytose acetylated low-density lipoproteins (23). Therefore, we incubated second-passage CD34+ cells on glass slides in the presence of DiI-Ac-LDL to determine their ability to take up this fluorescently labeled factor. We observed DiI-Ac-LDL accumulation in all of the cultured CD34+ cells (Fig. 5) in a fashion similar to that for the HUVEC controls (data not shown). These results further support the endothelial cell nature of the isolated human fetal lung cells.

Another important property of endothelial cells is their ability to spontaneously form capillary-like “tubes” when placed on a three-dimensional cushion of Matrigel, a substrate of reconstituted basement membrane proteins and growth factors (4). As demonstrated in Fig. 6, second-passage CD34+ human fetal lung cells formed capillary-like tubes when cultured on Matrigel. The tubes began forming within 24–48 h and became more extensive up to 10–14 days before regressing. Transmission electron microscopy demonstrated that the structures formed were indeed tubes containing lumen that resembled capillaries (Fig. 6C). These findings further support the endothelial cell phenotype of the isolated CD34+ cells, suggesting they may be destined to comprise the capillary bed of the maturing lung.

We have found that the isolated human fetal lung CD34+ cells can be maintained in culture for a number of passages; however, their growth rate diminishes
significantly after 10–12 passages. Therefore, we performed RT-PCR on RNA isolated from CD34+ cells cultured for up to 16 passages to determine whether later passage cells continue to express mRNA for the VEGF receptor Flt-1, the angiopoietin receptor Tie-2, or for eNOS. Surprisingly, we found that mRNA for these endothelial cell-associated proteins is expressed through all 16 passages (Fig. 7). This observation suggests that the observed decrease in rate of growth in later passage cell cultures is not associated with the loss of these endothelial cell-specific markers.

DISCUSSION

In the present study, we isolated a population of cells from midtrimester human fetal lung tissue that expresses an endothelial cell phenotype when maintained in vitro under various conditions. The cells were isolated by an immunomagnetic separation technique dependent on the cellular expression of CD34, a marker for hematopoietic progenitor cells, endothelial cell precursors, and small capillary endothelial cells (16). We demonstrated an endothelial cell phenotype for these CD34+ cells by several accepted techniques. Because the CD34+ cells were derived from distal portions of midtrimester human fetal lung, tissue that is generally void of capillary or vascular endothelium, the isolated cells represent a population of endothelial cell precursors. These cells represent the precursors to the pulmonary capillary bed that will ultimately com...

Fig. 5. Photomicrograph of CD34+ cell cultures after incubation with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL), demonstrating fluorescence in HFL CD34+ cells after the endocytoses of the labeled acetylated low-density lipoprotein. Bar = 10 μm.

Fig. 6. Photomicrographs of "tube formation" by CD34+ cells incubated in Matrigel matrix for 7 days. A: representative patterns of tubes spontaneously formed by CD34+ cells (bar = 25 μm). B: the 3-dimensional nature of the tubes that form within the matrix (bar = 10 μm). C: transmission electron microscopy of the tubes formed in Matrigel demonstrates that the cells form capillary-like tubes (×8,000 magnification).

Fig. 7. Detection of mRNA by RT-PCR for Flt-1, Tie-2, and endothelial nitric oxide synthase (eNOS) in cultures of HFL CD34+ cells from various cell passages. Total RNA was isolated from cells at passages 1, 3, 5, 7, 11, 12, 14, and 16. Analysis of the expression of mRNA for the VEGF receptor Flt-1, the angiopoietin receptor Tie-2, and eNOS was performed by RT-PCR, and the PCR products were separated on agarose as demonstrated. mRNA for Flt-1, Tie-2, and eNOS was detected from cells of all passages assayed up through the 16th passage.
prise the endothelial cell portion of the air-blood barrier.

The isolated CD34\(^+\) cells are relatively homogenous in cell culture, and they express endothelial cell markers including vWF and the VEGF receptors KDR and Flt-1. The human fetal lung-derived cells endocytose acetylated low-density lipoproteins and form tube-like structures when incubated in cushions of Matrigel. We are able to maintain the cells in vitro for a number of cell passages during which time they continue to express mRNA for additional endothelial cell markers including eNOS and Tie-2. These results confirm the endothelial cell nature of the human fetal lung CD34\(^+\) cells and demonstrate our ability to maintain them in culture.

CD34 is frequently employed to select hematopoietic progenitor cells for bone marrow transplant (21). However, CD34 has previously been employed to isolate, via an immunomagnetic method, circulating endothelial cell precursors or angioblasts from human peripheral blood (3). Particularly germane to the present study, the isolated CD34\(^+\) cells exhibit a spindle-shaped morphology in culture, express receptor for vascular endothelial growth factor and mRNA for KDR and eNOS, and form capillary-like tubes when placed in Matrigel (3).

Previous studies have demonstrated that the expression of some commonly employed endothelial cell markers in the lung vary with the developmental stage and type of vessels. For example, vWF is expressed in the endothelium of large pulmonary vessels but not in alveolar capillary endothelial cells (26). However, the majority of CD34\(^+\) endothelial cell precursors and small vessel endothelial cells also label by immunohistochemistry for vWF in the early and midpseudoglandular stage of lung development, but vWF is relatively absent in capillaries in the distal lung by the terminal sac phase of development (17). In contrast, PECAM is expressed in the endothelium of proximal vessels in the developing lung and absent or only weakly expressed by endothelial cell precursors and primitive capillaries.

At later gestation in the fetal rat lung (19–20 days of gestation), PECAM expression extends to the distal vessels and is maintained in all vasculature postnatally (12). These previous findings are consistent with our findings in CD34\(^+\) cells isolated from developing human lung. We found that the majority of isolated CD34\(^+\) cells derived from the distal portions of the midtrimester fetal lung express vWF. Our flow cytometry data demonstrate that relatively few of the isolated CD34\(^+\) cells express PECAM, consistent with the contention that the majority of the CD34\(^+\) cells are precursor cells to the capillary endothelium or are the endothelial cells of primitive capillaries of the developing distal lung. The cells that are both CD34 positive and PECAM positive are likely derived from more mature vessels such as small venules and arterioles or, possibly, such as the endothelium of the nascent air-blood barrier, which is first detectable in the human lung at 19 wk of gestation (10).

Significant endothelial cell heterogeneity is well described and is likely due to endothelial cells being derived from various vascular beds or cells expressing degrees of endothelial cell differentiation (18). For example, HUVEC produce a cobblestone morphology when placed in culture. In contrast, microvascular endothelial cells derived from human placenta are spindle-shaped in culture (14), similar to the cells presented in this paper. Interestingly, there has been one other report regarding the isolation of endothelial cell precursors derived from developing lung. Akeson et al. (2) have isolated and characterized two distinct endothelial cell lines derived from developing mouse lung transformed with SV40 T antigen. These two cell lines designated MFLM-4 [derived from the mesenchyme of embryonic day (E) 14.5-stage mouse lung] and MFLM-91U [derived from the mesenchyme of E19.0-stage developing mouse lung] exhibit different phenotypes when placed in culture. The MFLM-4 line demonstrates a phenotype in culture similar to those in our studies, since it exhibits a spindle-shaped morphology and expresses CD34 and vWF and is able to endocytose acetylated low-density lipoprotein. In contrast, the MFLM-91U cell line demonstrates a cobblestone appearance when placed in culture but also expresses CD34, vWF, and endocytose-acetylated low-density lipoprotein, though more avidly than the MFLM-4 cell line. Interestingly, each of the cell lines forms tube-like structures in Matrigel. However, the time required for each cell line to make tubes and the morphology of the structures are distinctly different, suggesting that even though each line was derived from developing lung mesenchyme, each cell line represents an endothelial cell precursor of different lineage (2).

Histological studies describing the development of the pulmonary vasculature have added to our knowledge of developmental angiogenesis and vasculogenesis in the lung (8, 9, 13). However, there remains a paucity of information regarding mechanisms of regulating development of the pulmonary capillary bed (22). VEGF is produced in abundance by the epithelium of the developing lung (1, 20), suggesting that the developing epithelium is driving development of the pulmonary capillary bed. Results supportive of this hypothesis have been demonstrated by coculturing experiments with epithelium- and Flk-1 (VEGFR-2)-positive cells derived from developing mouse lung (12). These studies demonstrate the dependence of endothelial cell growth on the presence of epithelial cells in situ. A significant amount of further work will be needed to define mechanisms involved in the organization of endothelial cell precursors into a functional pulmonary capillary bed, and further work will be required to define mechanisms regulating endothelial-epithelial cell interactions in the developing lung. These interactions are essential for the development of efficient gas exchange in the newborn lung.

In conclusion, we have demonstrated that CD34\(^+\) capillary endothelial precursor cells can be isolated from developing human lung and that these cells can be maintained in culture through several passages....
while maintaining their endothelial cell phenotype. Our data suggest that the ultimate fate of these CD34+ cells is as the capillary endothelium of the air-blood barrier. Given the relative lack of information regarding mechanisms that control endothelial cell growth, and differentiation and organization in the developing lung, these human fetal lung endothelial precursor cells will provide an important tool for elucidating such mechanisms.

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