Stem cell antigen-1 expression in the pulmonary vascular endothelium

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Kotton, Darrell N., Ross S. Summer, Xi Sun, Bei Yang Ma, and Alan Fine. Stem cell antigen-1 expression in the pulmonary vascular endothelium. Am J Physiol Lung Cell Mol Physiol 284: L990–L996, 2003. First published February 28, 2003; 10.1152/ajplung.00415.2002.—Although the function of the cell surface protein stem cell antigen-1 (Sca-1) has not been identified, expression of this molecule is a characteristic of bone marrow-derived hematopoietic stem cell populations. Expression of Sca-1, however, is not restricted to hematopoietic tissue. By RT-PCR and Western analysis, we found that Sca-1 is expressed in the adult mouse lung. Sca-1 immunohistochemistry revealed a linear staining pattern on the endothelial surface of large and small pulmonary arteries and veins and alveolar capillaries. Expression of Sca-1 in the pulmonary endothelium was confirmed by dual fluorescent microscopy on lung sections and by fluorescence-activated cell sorting analysis of digested lung tissue; each of these methods showed colocalization with the endothelial marker platelet/endothelial cell adhesion molecule-1. In the kidney, Sca-1 expression was also noted in large vessels, but, in contrast to the lung, was not observed in capillaries. Overall, our data indicate that Sca-1 expression helps define the surface phenotype of endothelial cells throughout the pulmonary vasculature.

MATERIALS AND METHODS

Mice. Protein and RNA extracts, single cell suspensions, and sections for immunohistochemistry were prepared from 2-mo-old C57Bl/6j mice (Jackson Laboratories, Bar Harbor, ME) euthanized by isoflurane anesthesia followed by cervical dislocation and perfusion of lungs with cold saline irrigated through the right ventricle. Animal studies were conducted according to protocols approved by the Boston University Animal Use Committee and adhered strictly to National Institutes of Health guidelines for the use and care of experimental animals.

Western blot analysis. SDS-PAGE (15% polyacrylamide) was performed under nonreducing conditions on protein extracts from homogenized murine lungs. Proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA; 300 mA, 4°C, 1 h). This membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 1 h, 22°C). After being washed in TBST, monoclonal rat anti-mouse Sca-1 was applied (eBioscience no. 14-5981, San Diego, CA; diluted 1:250 in TBST overnight at 4°C) followed by further washes and incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rat IgG secondary antibody (Santa Cruz no. 2065, Santa Cruz, CA; diluted 1:4,000 in 1% milk TBST for 1 h at 22°C). Bound antibody was detected with a Western blotting...
only experiments in which H11022 iodide (PI; 1 U/ml) was used to measure cell death. In addition, cells were exposed to propidium ionization molecule-1 (PECAM-1), respectively (BD Pharmingen, CA). IgGs against Sca-1, CD45, and platelet/endothelial cell adhesion molecule-1 (PECAM-1), respectively (BD Pharmingen, San Diego, CA). In addition, cells were exposed to propidium iodide (PI; 1 μg/ml in PBS) to identify dead cells, which were excluded from analysis. Only experiments in which >85% of cells were alive (PI negative) were included. Nonspecific control rat IgG antibodies of identical isotype (IgG 2a, IgG 2b, IgG 1, FITC, IgG 2a, APC, Pharmingen) were included in experiments and were used to set controls.

**Fluorescence-activated cell sorting.** To prepare single cell suspensions of lung tissue, euthanized mice underwent perfusion of their lungs via the right ventricle with ice-cold PBS (pH 7.4). Whole lungs were then dissected free from the thorax, finely minced by razor blade, and enzymatically digested for 50 min at 37°C with a solution consisting of 0.1% collagenase A (Roche Diagnostics, Indianapolis, IN) in 2.4 U/ml of dispase II (Roche). Lung digests were then filtered (70-μm Falcon cell strainer; Becton Dickinson, Franklin Lakes, NJ) and washed twice in Hanks’ balanced salt solution (2% fetal bovine serum, 10 mM HEPES in Hanks’ buffer) before resuspending at 5 × 10⁶ cells/ml for antibody staining. Flow cytometric analysis of immunolabeled cell surface markers was performed by simultaneous staining with three antibodies: phycoerythrin (PE)-, FITC-, and allophycocyanin (APC)-conjugated monoclonal rat anti-mouse IgGs against Sca-1, CD45, and platelet/endothelial cell adhesion molecule-1 (PECAM-1), respectively (BD Pharmingen, San Diego, CA). In addition, cells were exposed to propidium iodide (PI; 1 μg/ml in PBS) to identify dead cells, which were excluded from analysis. Only experiments in which >85% of cells were alive (PI negative) were included. Nonspecific control rat IgG antibodies of identical isotype (IgG 2a, PE, IgG 2b, K-FITC, IgG 2a, k-APC, Pharmingen) were included in all experiments and were used to set fluorescence-activated cell sorting (FACS) gates for analysis. Fluorescent antibody-exposed live cells were analyzed by flow cytometry (MoFlo; Cytomation, Fort Collins, CO), and data were processed using FlowJo software (Treestar, San Carlos, CA).

**RT-PCR.** RNA extracts from lung and marrow samples were analyzed by cDNA using a reverse transcription kit (Promega, Madison, WI) followed by PCR using primers for Sca-1 (forward primer: CTCTGAGATGGA-CACCTTCT, reverse primer: GGTCTGCAGGAGACTGAGC; 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, 35 cycles).

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**Sca-1 and PECAM-1 immunohistochemistry of tissue sections.** Formalin-fixed lungs and kidneys were prepared for frozen or paraffin sectioning through standard methods. Paraffin sections (5-μm-thick) were rehydrated by exposure to solvent (Citriolv; Fisher Scientific, Hanover Park, IL), graded alcohols, and distilled water. Antigen retrieval was performed by heating sections to 90°C in a citric acid buffer (Antigen Retrieval Solution; Vector Laboratories, Burlingame, CA) for 20 min and slowly cooling to room temperature. Before being stained, sections were treated with hydrogen peroxide in methanol (3%, 15 min, 22°C) to quench endogenous peroxidases. Sections were blocked with 1% goat serum in PBS (60 min) and incubated overnight (4°C) with the appropriate antibody: biotinylated monoclonal rat anti-mouse Sca-1 diluted 1:100 (Pharminogen no. 553334), biotinylated rat IgG₂_k isotype control (Pharminogen), or polyclonal goat anti-mouse PECAM-1 diluted 1:4,000 (Santa Cruz no. sc-1506). Biotinylated anti-Sca-1 antibody was detected using an ABC kit (Vector Laboratories) followed by tyramide signal amplification (TSA-Biotin System; NEN, Boston, MA) according to the manufacturer’s protocol before exposure to diaminobenzadine. Anti-PECAM-1 antibody was detected using an anti-goat secondary antibody kit (Vector Laboratories) before tyramide signal amplification. For fluorescent immunostaining, 5-μm-thick frozen sections were quenched with 1% sodium borohydride for 30 min before identical immunostaining conditions. 7-Amino-4-methylcoumarin-3-acetic acid or Texas red-conjugated avidin (5 μg/ml, Vector Laboratories) were substituted for HRP-streptavidin during tyra-

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**Fig. 1. Stem cell antigen-1 (Sca-1) expression in adult murine lung.** A: RT-PCR showing expression of Sca-1 mRNA in adult murine tissues, including lung extracts. Fresh whole marrow (BM), cultured plastic-adherent marrow (MSC), and lung RNA extracts are shown. Water was used as a negative control (Neg). B: Western blot analysis of lung protein extracts using an anti-Sca-1 antibody. C: fluorescence-activated cell sorting (FACS) analysis of lung digests using nonspecific fluorescence-conjugated control antibodies of identical iso-
mide signal amplification to achieve fluorescent signals. To ensure specificity of immunostaining, for each analysis, adjacent control sections in each experiment underwent identical and simultaneous staining with isotype control antibody (biotinylated rat IgG2a,κ isotype control, Pharmingen) in place of anti-Sca-1, and secondary antibody alone substituted for anti-PECAM-1. Immunohistochemistry was repeated on tissue from three C57Bl/6j mice.

RESULTS

Sca-1 mRNA and protein expression in lung tissue. We found by RT-PCR that Sca-1 mRNA is present in adult lung tissue (Fig. 1). For positive controls, we employed RNA derived from fresh bone marrow cells or cultured marrow-derived mesenchymal stem cells known to express Sca-1. To examine this further, we next performed a Western blot analysis on whole lung extracts for Sca-1 protein expression. In this study, we detected an ~8 kDa protein; this is the expected weight of Sca-1 protein when analyzed by SDS-PAGE under nonreducing conditions (21, 30).

We next sought to determine whether Sca-1 expression in lung was restricted to circulating hematopoietic cells contained within vessels. We prepared single cell suspensions by enzymatically digesting saline-perfused lungs and performed flow cytometry to detect cells expressing Sca-1 and the hematopoietic lineage marker CD45. With this method, we found that, on average, 60% of Sca-1-positive lung cells were CD45 negative (Fig. 1). These results established that Sca-1 is expressed in a nonhematopoietic cell type in the lung.

Localization of Sca-1-expressing cells by immunohistochemistry and FACS. To identify and localize Sca-1-expressing cells, we performed Sca-1 immunohistochemistry on paraffin and frozen sections of adult murine lungs (Fig. 2). We found linear Sca-1 immunostaining in a pattern consistent with expression in endothelial cells of large and small pulmonary arteries, alveolar capillaries, and pulmonary veins. No Sca-1 staining was present in airway epithelium or type I or II alveolar epithelial cells.

We utilized PECAM-1 immunostaining to determine whether the pattern of expression of this endothelial marker was similar to Sca-1. We found that the staining pattern of PECAM-1 and Sca-1 matched; colocalization of PECAM-1 and Sca-1 staining was confirmed.

Fig. 2. Sca-1 and platelet/endothelial cell adhesion molecule-1 (PECAM-1) immunoperoxidase staining of adult lung paraffin sections. A: low-power view of lung tissue showing Sca-1 linear staining (brown) of pulmonary artery (PA) endothelium and alveolar septae of lung parenchyma. Bronchial epithelium (BR) shows no staining. B: high-power view of lung alveoli showing Sca-1 immunostaining in a pattern characteristic of flat alveolar capillary endothelium. Charcoal arrows indicate 2 Sca-1-positive alveolar capillary vessels shown in cross section, one surrounding a single red blood cell. Type II pneumocytes are Sca-1 negative (red arrow). C: high-power view of a small pulmonary vessel, filled with red blood cells, illustrating Sca-1 immunostaining of the endothelial wall. An adjacent Sca-1-negative type I pneumocyte is shown (arrow). Inset: phase-contrast view of boxed region. D: PECAM-1 immunostaining of lung endothelium showing identical pattern to the Sca-1 pattern shown in A. Simultaneous control sections stained with isotype control antibody (substituted for anti-Sca-1) or secondary antibody alone (substituted for anti-PECAM-1) showed no brown labeling. Sections were counterstained with methyl green. A–D is representative of immunohistochemical analyses from 40 sections taken from 3 C57Bl/6j mice.
by dual fluorescent staining (Fig. 3). To examine this further, we analyzed single cell suspensions of lung tissue by FACS for Sca-1, CD45, and PECAM-1 expression. With this method, we found that PECAM-1-positive cells were Sca-1 positive (Fig. 4). To further ensure that all blood cells were excluded from analysis of the Sca-1 status of lung endothelial cells, we analyzed PECAM-1-positive/CD45-negative lung cells; 97% of these cells were Sca-1 positive (Fig. 4).

We also examined Sca-1 expression in the mouse pulmonary fetal endothelial cell line MFLM-4 during in vitro culturing. This cell type expresses features of differentiated endothelial cells (2, 3). RT-PCR demonstrated expression of Sca-1 mRNA in this cell line (data not shown). With the use of FACS, we found that these cells are Sca-1 positive (Fig. 4).

**Localization of Sca-1 in kidney.** The kidney and lung microvasculature share common antigens, as evidenced by autoimmune diseases that preferentially involve the vascular beds of these two organs. We, therefore, examined Sca-1 localization in the kidney. As has been reported (30), we found intense Sca-1 staining in the distal tubule epithelium and in large and small renal vessels (Fig. 5). Unlike the lung, however, Sca-1 expression was not detected by immunostaining in capillaries.

**DISCUSSION**

These findings demonstrate that expression of Sca-1 in the lung is localized to the surface of endothelial cells in large and small pulmonary vessels. Although we found that >97% of lung endothelial cells (PECAM-1 positive/CD45 negative) express Sca-1, we cannot exclude the possibility that additional rare cells present in the lung express Sca-1. Indeed, of Sca-1-positive/CD45-negative lung cells, 90% were PECAM-1 positive; on histological sections, nonendothelial lung cell types that stained for Sca-1 were not identified. Specifically, bronchial ciliated and nonciliated cells, type I and II pneumocytes, and vascular and bronchial smooth muscle cells all lacked Sca-1 immunostaining. Together, these observations add to the growing number of antigens available for immunotyping lung endothelium and raise intriguing questions about the significance of shared gene expression patterns between endothelium and stem or progenitor cells of hematopoietic and nonhematopoietic tissues.

Importantly, a variety of recent studies detail a common embryonic origin of endothelial cells and HSCs and demonstrate the ability of bone marrow-derived cells to participate in angiogenesis and neovascularization during adult life. During fetal development, endo-

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**Fig. 3.** PECAM-1 (red) and Sca-1 (blue) dual fluorescence microscopy of a single lung frozen section. A: high-power view of phase-contrast microscopy of a frozen section showing a single pulmonary vessel wall with adjacent vessel lumen (*). B: Texas red fluorescence labeling of the apical surface membrane of vessel endothelium using anti-PECAM-1 antibody. C: 7-Amino-4-methylcoumarin-3-acetic acid (AMCA, blue) fluorescence labeling using anti-Sca-1 antibody. D: merged image of B and C. E: enlarged merged image of A–C revealing endothelial cells lining the vessel lumen expressing PECAM-1 and Sca-1.
thelial progenitor cells and hematopoietic stem cells are believed to arise from a common flk-1/H11001 embryonic ancestor, the hemangioblast (26). Moreover, many marrow stem cell markers are present in both endothelial cells and HSCs in adults, including CD34, c-kit, MDR-1, and tie-2 (5, 8, 12, 16, 23, 26). Although no single marker has been found that is specific to adult mouse stem cells, the combination of surface markers shared between endothelial cells and HSCs suggests a relationship between these two cell lineages. The finding that Sca-1 is expressed in HSCs and lung endothelial cells further supports such a relationship.

Whether there is any contribution of Sca-1-positive bone marrow-derived circulating cells to the lung endothelium in the adult remains to be established. To date, marrow-derived cells in adults have been demonstrated to contribute to the endothelium in models of cardiac and skeletal muscle injury, wound healing, synthetic graft endothelialization, retinal neovascularization, and tumor angiogenesis (4, 5, 10, 13, 15, 17, 19, 27). Interestingly, Sca-1-positive HSCs purified from marrow by Hoechst side population staining (SP cells) express the endothelial marker, PECAM-1, and can engraft in recipient hearts as endothelial cells and cardiac myocytes (13).

In the nonhematopoietic compartment of adult bone marrow, Sca-1-positive multipotent adult progenitor cells can form differentiated endothelium both in vitro and in vivo during transplantation studies (26). The possibility that lung endothelium may be marrow derived has been proposed by Asahara et al. (4) using a bone marrow transplant model. In that study, RT-PCR of lung RNA showed expression of an endothelial marker that was derived from the donor's marrow. Careful histological analysis of lungs derived from chimeric animals and humans may provide additional data that support a role for bone marrow in pulmonary endothelial reconstitution. Contribution of bone marrow-derived cells to lung endothelium, if definitively proven, could be relevant to pulmonary vascular disease pathogenesis and treatment.

The capacity of endothelial cells to serve as progenitors for differentiated cells of some tissues has been proposed by several studies. For example, during culture of lung endothelial cells, cardiomyocyte markers have been detected (6, 18). Moreover, endothelial cells from the fetal dorsal aorta have been suggested to give rise to blood, cartilage, bone, and smooth, skeletal, and cardiac muscle after injection into embryos (18). Findings from these studies, if confirmed, would suggest
unrecognized plasticity in endothelial cells. Whether Sca-1-positive lung endothelial cells can give rise to other cell types needs further study.

Ultimately, in vivo transplantation studies that employ highly purified lung endothelial cell populations are needed to establish the stem cell potential of Sca-1-positive lung cells. These models will likely require tissue-specific injuries in transplant recipients. The use of cell-specific lineage labels instead of ubiquitously expressed green fluorescent protein or lacZ along with rigorous immunohistochemical and FACS analyses should be used to evaluate engrafted phenotypes. Moreover, single cell transplantation will be necessary to verify pluripotency or clonal expansion of donor cells.

It is noteworthy that endothelial cells display phenotypic heterogeneity on the basis of their organ of residence, developmental stage (embryonic vs. adult), vessel type (arterial, venous, or capillary), and exposure to injury (1). Within the lung, few endothelial surface markers have been extensively characterized (7), and most lung endothelial antigens are not present in both pulmonary arteries, veins, and microvasculature. Despite this phenotypic heterogeneity, some antigens, such as PECAM-1 and Sca-1, appear to be expressed throughout the pulmonary endothelium. As has been reported (30), we also found Sca-1 immunostaining in the vasculature of other organs, such as renal arteries and veins. In contrast to pulmonary alveolar endothelium, the absence of Sca-1 immunostaining in glomerular capillaries may reflect the unique phenotype of the fenestrated filtration bed formed by the glomerular endothelium. We cannot exclude the possibility, however, that glomerular endothelial cells express Sca-1 at low levels below the sensitivity of our staining procedure.

Our data show that Sca-1 expression can be utilized as a reliable marker for the study and analysis of the pulmonary lung endothelium. Finally, our findings suggest novel strategies for the isolation of lung endothelial cells; importantly, we found that Sca-1 is resistant to proteolytic lung digestion and is expressed on the cell surface. These two observations could form the basis for lung endothelial purification protocols that employ anti-Sca-1 antibodies during high-speed flow cytometry or immunobead-based sorting.

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