Stem Cell Antigen-1 (Sca-1) Expression in the Pulmonary Vascular Endothelium

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  Bone marrow transplant (BMT)
  Side Population (SP)
ABSTRACT

Although the function of the cell surface protein 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 FACS analysis of digested lung tissue; each of these methods showed co-localization with the endothelial marker, PECAM-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.
INTRODUCTION

Sca-1 is a phosphatidylinositol-anchored glycoprotein found on the surface of several murine marrow stem cell subtypes, including hematopoietic stem cells (HSCs) (28;30), mesenchymal stem cells, multipotent adult progenitor cells (14), and Hoechst side-population (SP) cells (9;11). Each of these cell types displays a capacity for multi-lineage differentiation, and self-renewal. Notably, the characterization and purification of bone marrow-derived stem cells is often based on the expression of selective cell surface markers, such as stem cell antigen-1 (Sca-1) (30).

Expression of Sca-1, like other stem cell markers, is not restricted to the bone marrow. Cells expressing this antigen can be found in murine peripheral lymphocyte sub-populations, within the thymic medulla, and in the spleen (21;22;30). Sca-1 expression is also present in the parenchyma of non-hematopoietic tissues such as the tubular epithelium of the kidney, and the vasculature of the brain, heart, and liver (30). Recently, Sca-1+ cells in murine muscle interstitium have been identified; these cells are able to serve as progenitors for muscle and endothelium (29). In breast tissue, epithelial progenitors with a Hoechst negative staining profile express Sca-1 (31). Despite these observations, it remains unclear whether Sca-1 expression in non-hematopoietic tissue connotes cells with stem and progenitor cell capacity, or marks cells of bone marrow origin. Indeed, the function of Sca-1 in stem cells remains uncertain, though it appears to have a role in lymphocyte activation and has, thus, also been referred to as T-cell activating protein (TAP) (20;25).
In the adult murine lung, Sca-1 mRNA and protein can be detected, but whether this expression is restricted to circulating cells present in lung blood vessels, or differentiated parenchymal cells is not currently known (24;30). In this paper, we sought to identify cell types expressing Sca-1 in the adult lung. Our findings indicate that Sca-1 expression is localized to the surface 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 month old C57Bl/6j mice (Jackson Labs, Bar Harbor, ME) that were 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 NIH guidelines for the use and care of experimental animals.

Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% polyacrylamide) was performed, under non-reducing conditions on protein extracts from homogenized murine lungs. Proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore Corporation, Bedford, MA, 300 mA, 4°C, 1 hour). This membrane was blocked with 5% non-fat 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 hour, 22°C). After washing in TBST, monoclonal rat anti-mouse Sca-1 was applied (eBioscience #14-5981, San Diego, CA,
diluted 1:250 in TBST, overnight, 4EC) followed by further washes and incubation with an HRP-conjugated goat anti rat IgG secondary antibody (Santa Cruz #2065, Santa Cruz, CA, diluted 1:4000 in 1% milk TBST, 1 hour, 22°C). Bound antibody was detected with a western blotting chemiluminescent kit (ECL, Amersham, England) according to the manufacturers instructions.

**Reverse transcription-polymerase chain reaction (RT-PCR).** RNA extracts from lung and marrow samples were analyzed by generating cDNA using a reverse transcription kit (Promega, Madison, WI) followed by PCR using primers for Sca-1 (forward primer: CTCTGAGGATGGACACTTCT, reverse primer: GGTCTGCAGGAGGACTGAGC; 94EC 1 minute, 56EC 1 minute, 72EC 1 minute, 35 cycles).

**Fluorescence-activated cell sorting (FACS).** To prepare single cell suspensions of lung tissue euthanized mice underwent perfusion of their lungs via the right ventricle with ice-cold phosphate buffered saline (PBS, pH 7.4). Whole lungs were then dissected free from the thorax, finely minced by razor blade, and enzymatically digested for 50 minutes at 37EC with a solution consisting of 0.1% Collagenase A (Roche Diagnostics, Indianapolis, IN) in 2.4 units/ml of Dispase II (Roche). Lung digests were then filtered (70 μm Falcon Cell Strainer, Becton Dickinson, Franklin Lakes, NJ) and washed twice in HBSS+ (2% fetal bovine serum, 10 mM Hepes in Hank’s buffer) before resuspending at 5x10⁶ cells/ml for antibody staining. Flow cytometric analysis of immuno-labeled cell surface markers was performed by simultaneous staining with three antibodies: phycoerythrin- (PE ), fluorescein isothiocyanate- (FITC ), and
allophycocyanin- (APC-) conjugated monoclonal rat anti-mouse IgG’s against Sca-1, CD-45, and PECAM-1 respectively (BD Pharmingen, San Diego, CA). In addition, cells were exposed to propidium iodide (PI; 1 ug/ml in PBS) to identify dead cells which were excluded from analysis. Only experiments where >85% of cells were alive (PI negative) were included. Non-specific control rat IgG antibodies of identical isotype (IgG2a, 6-PE, IgG2b, 6-FITC, IgG2a, 6-APC, Pharmingen) were included in all experiments, and were used to set 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). Lungs from each adult mouse were analyzed separately, and experiments were repeated on twelve C57/Bl6j mice from six separate litters and three FVB/NJ male mice (Jackson Labs, Bar Harbor, ME). FACS analysis for Sca-1 expression in a pulmonary endothelial cell line was similarly performed using the MFLM-4 cell line (generous gift of Dr. Ann Akeson, Children’s Hospital Medical Center, Cincinnati, OH) which was grown and harvested under established conditions (2;3).

**Sca-1 and PECAM-1 immunohistochemistry of tissue sections.** Formalin-fixed lungs, and kidneys were prepared for frozen or paraffin sectioning through standard methods. 5 um thick paraffin sections were rehydrated by exposure to solvent (Citrisolv, Fisher Scientific, Hanover Park, IL), graded alcohols, and distilled water. Antigen retrieval was performed by heating sections to 90EC in a citric acid buffer (Antigen Retrieval Solution, Vector Laboratories, Burlingame, CA) for 20 minutes and slowly cooling to room temperature. Before staining, sections were treated with hydrogen peroxide in methanol (3%, 15 minutes, 22EC) to quench
endogenous peroxidases. Sections were blocked with 1% goat serum in PBS (60 minutes), and incubated overnight (4EC) with the appropriate antibody: Biotinylated monoclonal rat anti-mouse Sca-1 diluted 1:100 (Pharmingen #553334), Biotinylated rat IgG2a,6 isotype control (Pharmingen), or polyclonal goat anti-mouse PECAM-1 diluted 1:4000 (Santa Cruz #sc-1506). Biotinylated anti-Sca-1 antibody was detected using an ABC kit (Vector Laboratories) followed by tyramide signal amplification (TSA-Biotin System, NEN Life Science Products, 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) prior to tyramide signal amplification. For fluorescent immunostaining, 5 um thick frozen sections were quenched with 1% sodium borohydride for 30 minutes prior to identical immunostaining conditions. 7-amino-4-methylcoumarin-3-acetic acid- (AMCA-) or Texas red-conjugated avidin (5 ug/ml, Vector Laboratories) were substituted for HRP-streptavidin during tyramide 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,6 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 C57/Bl6j 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 approximately 8 kD protein; this is the expected weight of Sca-1 protein when analyzed by SDS-PAGE under non-reducing 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, CD-45. Using this method, we found that, on average, 60% of Sca-1+ lung cells were CD-45 negative (Fig. 1). These results established that Sca-1 is expressed in a non-hematopoietic 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; co-localization of PECAM-1 and Sca-1 staining was confirmed by dual fluorescent staining (Fig. 3). To examine this further, we analyzed single cell suspensions of lung tissue by FACS for Sca-1, CD-45, and PECAM-1 expression. Using 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/CD-45 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). Using 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 auto-immune 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 immuno-staining in capillaries.

**DISCUSSION**
These findings demonstrate that expression of stem cell antigen (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/CD-45 negative) express Sca-1, we can not exclude the possibility that additional rare cells present in the lung express Sca-1. Indeed, of Sca-1 positive/CD-45 negative lung cells, 90% were PECAM-1 positive; on histologic sections non-endothelial lung cell types that stained for Sca-1 were not identified. Specifically, bronchial ciliated and non-ciliated cells, type I and II pneumocytes, and vascular and bronchial smooth muscle cells all lacked Sca-1 immunostaining. Taken 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 non-hematopoietic 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, endothelial progenitor cells and hematopoietic stem cells are believed to arise from a common flk-1+ embryonic ancestor, the hemangioblast (26). Moreover, many marrow stem cell markers are present in both endothelial cells and HSCs in adults, including CD-34, 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+ 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+ 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 non-hematopoietic compartment of adult bone marrow, Sca-1+ multipotent adult progenitor cells (MAPCs) 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. 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 (4). Careful histological analysis of lungs derived from chimeric animals and humans, may provide additional data that supports a role for the 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, 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+ 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+ lung cells. These models will likely require tissue specific injuries in transplant recipients. The use of cell-specific lineage labels rather than ubiquitously expressed GFP 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 based on 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 thus 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 immuno-bead based sorting.

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Reference List


FIGURE LEGEND

**Figure 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. (B) Western blot analysis of lung protein extracts using an anti-Sca-1 antibody. (C) FACS analysis of lung digests using non-specific fluorescence-conjugated control antibodies of identical isotypes to those employed in D. (D) FACS analysis of lung digests using PE-conjugated anti-Sca-1 and FITC-conjugated anti-CD45 antibodies. CD45- and CD45+ populations of Sca-1+ cells are illustrated. The percentage of cells in each quadrant is indicated. FACS images and percentages are representative of experiments repeated individually on twelve C57/Bl6 and three FVB/NJ mice.

**Figure 2**: Sca-1 and 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 endothelium (PA) 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. Black arrows indicate two Sca-1+ 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 RBCs, illustrating Sca-1 immunostaining of the endothelial wall. An adjacent Sca-1 negative type I pneumocyte is shown (black 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. Methyl green nuclear counter-stain. A-D is representative of immunohistochemical analyses from 40 sections taken from three C57/Bl6 mice.

**Figure 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) AMCA (blue) fluorescence labeling using anti-Sca-1 antibody. (D) Merged image of B and C. (E) Enlarged merged image of A,B,C revealing endothelial cells lining the vessel lumen expressing PECAM-1 and Sca-1.

**Figure 4:** (A) FACS analysis of live lung cells in single cell suspension showing PECAM-1, CD-45, and Sca-1 surface staining. (A) Anti-Sca-1-PE antibody vs Anti-PECAM-1-APC antibody staining of lung cells. PECAM-1+ cells are Sca-1+. (B) Anti-PECAM-1-APC antibody vs. CD-45-FITC antibody staining. PECAM-1+/CD45- cells are gated for analysis in C (10% of all cells). (C) Histogram showing Sca-1 surface staining of lung endothelial cells (PECAM-1+/CD45-). 97% of PECAM-1+/CD45- cells are Sca-1+. Shown for comparison is histogram of cells stained with isotype control IgG2a,6-PE antibody. (D) MFLM-4 mouse lung endothelial cell line FACS analysis with anti-Sca-1-PE and isotype control IgG2a,6-PE antibodies. This endothelial cell line is Sca-1+. A-C is representative of FACS data from twelve C57/Bl6 and three FVB/NJ mice.
Figure 5: Sca-1 immunostaining in the adult murine kidney. (A) Low power view indicating Sca-1 immunostaining (brown) in the tubule epithelium. (B) High power view of glomerulus (*) and adjacent distal tubule illustrating Sca-1+ tubule epithelium and Sca-1 negative glomerulus. (C) High power view of Sca-1+ distal tubule epithelium shown in longitudinal section with phase contrast image (D) for comparison. (E) Sca-1+ endothelium (arrow) lining a renal vessel. Adjacent glomerulus (*) shows no Sca-1 labeling in glomerular capillaries. (F) In contrast, PECAM-1 immunostained glomerulus illustrates the typical endothelial marker staining pattern of glomerular capillaries. Methyl green nuclear counter-stain.
Fig 1

A

BM MSC Lung Lung Neg

C

Isotype-PE

Isotype-FITC

B

Lung: 1 2 3

-30kD

-14kD

-6.5kD

D

Sca-1-PE

CD45-FITC

Fig 2

A

PA

BR

C

PA

BR

D

PA

BR
Fig 5