Molecular phenotype of airway side population cells

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Recent studies indicate that subsets of cells localized within discrete microenvironments throughout the adult conducting airway. Properties of these cells include pollutant resistance, multipotent differentiation, and infrequent proliferation. Goals of the present study were to use Hoechst 33342 efflux, a property of stem cells in other tissues, to purify and further characterize airway stem cells. Hoechst 33342 effluxing lung cells were identified as a verapamil-sensitive side population within flow cytometry. Lung side population cells were further subdivided on the basis of hematopoietic (CD45 positive) or nonhematopoietic (CD45 negative) origin. Nonhematopoietic side population cells were enriched for stem cell antigen-1 reactivity and expression of neuroepithelial body-specific markers specific to both airway and mesenchymal lineages. Analysis of the molecular phenotype of airway-derived side population cells indicates that they are similar to neuroepithelial body-associated variant Clara cells. Taken together, these data suggest that the nonhematopoietic side population isolated from lung is enriched for previously identified airway stem cells.

stem cell; Clara; lung; Hoechst 33342

The recent demonstration in both mammary gland and muscle of nonhematopoietic (CD45-negative) SP cells that maintain both functional and molecular properties of tissue-specific stem cells suggests that the property of rapid Hoechst efflux may be applied to purification of stem cells from other organs.
harbor a rapid Hoechst effluxing population and that these cells would be enriched for markers of lung-specific epithelial stem cells. We demonstrate that isolated lung cells include a verapamil-sensitive SP that can be further fractionated based on the presence or absence of the hematopoietic cell surface marker CD45. CD45-negative SP expressed molecular markers characteristic of mesenchymal and vCE cells.

MATERIALS AND METHODS

Animal housing. FVB/n mice were maintained as an in-house breeding colony under specific pathogen-free conditions, and health status was monitored quarterly via a sentinel-screening program. Animals were maintained on a 12:12-h light-dark cycle and allowed access to food and water ad libitum. All mice used in experiments were 8–12 wk of age.

Lung cell isolation. Animals were killed by intraperitoneal injection of 2.5% avertin to achieve a surgical plane of anesthesia followed by exsanguination. Lungs were perfused with HBSS (Invitrogen, Grand Island, NY), and the lungs were lavaged eight times with 1 ml HBSS/200 μM EGTA (Sigma, St. Louis, MO). Lung cells were isolated by the method of Chichester et al. (6) with minor modifications. Briefly, the entire heart-lung unit was removed and suspended in 2 liters of 37°C saline. One milliliter of elastase (3.5 U/ml in HBSS; Worthington, Lakewood, NJ) was instilled in each lung via a tracheal cannula and incubated for 5 min, and the process was repeated five times. After digestion, lungs were finely minced in <1-mm pieces using a razor blade, strained through a 100-μm nylon membrane (Becton-Dickinson, Franklin Lakes, NJ), and resuspended in 35 ml HBSS containing 0.2 mg/ml DNase. Eight milliliters of sterile FBS (Invitrogen) was underlayered, and cells were pelleted by centrifugation at 300 g for 5 min at 4°C. Cells were washed twice in HBSS containing 2% FBS/10 mM HEPES, pH 7.4 (HBSS+), as described above, and resuspended at 1 × 10⁶ live cells/ml in MEM containing 2% FBS/10 mM HEPES, pH 7.4 (11).

Bone marrow cell isolation. Animals were killed as described above, and the femurs were recovered by blunt dissection. The epiphelial ends were removed, and the lumen of the metaphysis was flushed with 5 ml HBSS. Cell aggregates were dispersed by trituration with 365 nm bandpass filter. Antibodies used for phenotypic analysis of SP cells were directly conjugated with FITC or PE, and emission of these fluorochromes was detected using a 530/40 nm and 575/26 nm bandpass filter.

RNA isolation and RT-PCR. Six-thousand sorted cells belonging to either total, non-SP, or SP categories (see Fig. 4) were directly sorted into cell lysis buffer (Promega, Madison, WI) and frozen before RNA isolation using a glass fiber system (SV Total RNA Isolation Kit; Promega). All RNA samples were eluted in 60 μl of nuclease-free water. First-strand cDNA synthesis was performed on 20 μl RNA using random hexamer primers in the presence (+RT) or absence (−RT) of Superscript II RT (Invitrogen). RT-PCR (40 cycles) was carried out using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 3 μl cDNA template. Primers used in PCR were as indicated in Table 1. PCR reactions for CCSP, CyP450–2F2, platelet-endothelial cell adhesion molecule (PECAM), and vimentin were carried out at an annealing and extension temperature of 60°C, 1 min/cycle. PCR reactions for surfactant protein-C (SP-C) and β-actin were carried out at an annealing temperature of 51°C (30 s/cycle) and an extension temperature of 72°C (1 min/cycle). Products were separated on 3% agarose gels containing ethidium bromide and visualized using an Alpha Innotech video gel documentation system and digital imaging software (Alpha Innotech, San Leandro, CA).

RESULTS

Table 1. Primers used in RT-PCR of lineage-specific gene products

<table>
<thead>
<tr>
<th>Product</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>CCSP</td>
<td>CCAGCTGAAAGAAGACTGTGGAT</td>
<td>TTACAGAAGAGACTGTGTTAGATTTTCT</td>
</tr>
<tr>
<td>2F2</td>
<td>TATGCGACTGCTTGGCTCAAAAA</td>
<td>CATCGACGGAAGTTATTGAA</td>
</tr>
<tr>
<td>SP-C</td>
<td>TTTCCTAGGGCTTGGCTGT</td>
<td>TTTTGGATAGGATGCCC</td>
</tr>
<tr>
<td>PECAM</td>
<td>AACAGAAAAACCTGGAGAGATG</td>
<td>CATCATACAACCTTGGCTTTTGG</td>
</tr>
<tr>
<td>Vimentin</td>
<td>GCACCTGTCAGTCATTCAGA</td>
<td>GATCTGATTCCCGGGCTGAAGC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TTTCCTAGACTGCTTGGCTGT</td>
<td>GGTGATTCCCGGGCTGAAGC</td>
</tr>
</tbody>
</table>

CCSP, Clara cell secretary protein; 2F2, CyP450–2F2; SP-C, surfactant protein-C; PECAM, platelet-endothelial cell adhesion molecule.
shown). Very few basal (cells were of the hematopoietic lineage (Fig. 2 and data not analysis of CD45 expression indicated that 30% of isolated tubulin immunoreactive ciliated cells (Fig. 1). Flow cytometric analysis included stained for airway-specific markers. Controls for antibody specificity included an omission of primary antibody and analysis of Clara cell secretory protein (CCSP) knockout mouse-derived cells. Analysis of 8 cell preps indicated that 32 ± 4% of nucleated cells express the Clara cell marker CCSP (red) and that 20 ± 3% of cells express the ciliated cell marker ATP-binding cassette (ACT; green). The basal cell marker cytokeratin 14 was extremely rare (<0.1% of total cells), and the neuroendocrine cell marker calcitonin gene-related peptide was not detected after immunostaining of the total cell isolate (data not shown). Fluorescence-activated cell sorter analysis (Fig. 2) indicated that ~30% of the total cells expressed the hematopoietic lineage marker CD45 and 20% of all cells lacked both the aforementioned airway markers and CD45. Results are representative of 8 experiments.

32 ± 4% CCSP immunoreactive cells and 20 ± 3% acetylated tubulin immunoreactive ciliated cells (Fig. 1). Flow cytometric analysis of CD45 expression indicated that 30% of isolated cells were of the hematopoietic lineage (Fig. 2 and data not shown). Very few basal (<0.1% of total) and no immunoreactive pulmonary neuroendocrine cells were identified by immunophenotypic analysis of total cells. Thus elastase-isolation of lung cells results in a significant enrichment for cells expressing airway-specific markers. Isolated cells were incubated in the presence of 5 μg/ml Hoechst 33342 for 90 min, washed, and immunostained with mouse anti-CD45 for segregation of hematopoietic (CD45+) and lung (CD45−) cells. Dead cells were identified by addition of 2 μg/ml propidium iodide (PI). For each experiment, a preliminary analysis was used to establish gates based on forward scatter/side scatter that eliminated erythrocytes and debris from subsequent analysis, propidium iodide fluorescence to restrict analysis to living cells, and pulse width to select for single cells. All cells were simultaneously sorted on the basis of these six parameters (Hoechst 33342 red, Hoechst 33342 blue, PI, forward scatter, side scatter, and FITC-CD45 reactivity).

Cells characterized by rapid efflux of Hoechst 33342 were detected in both the CD45+ and CD45− groups (Fig. 2, A and B, respectively). Generation of each of these subpopulations was inhibited by coincubation with the ABC transport inhibitor verapamil, indicating that Hoechst 33342 efflux is dependent on ABC transporter function (Fig. 2, C and D). Identification of verapamil-sensitive CD45+ and CD45− lung SP cells was independent of both gender and genetic background (FVB/n, C57/Bl6, 129/Sv, hybrid strains; data not shown). Within the FVB/n strain, the average representation of CD45+ (hematopoietic) SP cells was 0.09 ± 0.02% of CD45+ cells, whereas the average representation of CD45− (nonhematopoietic) SP cells was 0.87 ± 0.22%. These data demonstrate that cells from elastase-digested lung tissue contain two populations of cells characterized by rapid efflux of Hoechst 33342: the CD45+ and the CD45− side populations. The overall cellular toxicity associated with 5 μg/ml Hoechst 33342 exposure for

Fig. 1. Phenotypic characterization of isolated lung cells. Lung cells were isolated by elastase digestion, fixed, cytospun on glass slides, and immunostained for airway-specific markers. Controls for antibody specificity included an omission of primary antibody and analysis of Clara cell secretory protein (CCSP) knockout mouse-derived cells. Analysis of 8 cell preps indicated that 32 ± 4% of nucleated cells express the Clara cell marker CCSP (red) and that 20 ± 3% of cells express the ciliated cell marker ATP-binding cassette (ACT; green). The basal cell marker cytokeratin 14 was extremely rare (<0.1% of total cells), and the neuroendocrine cell marker calcitonin gene-related peptide was not detected after immunostaining of the total cell isolate (data not shown). Fluorescence-activated cell sorter analysis (Fig. 2) indicated that ~30% of the total cells expressed the hematopoietic lineage marker CD45 and 20% of all cells lacked both the aforementioned airway markers and CD45. Results are representative of 8 experiments.

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90 min was ~60%. Differential susceptibility of the CD45\(^+\) and CD45\(^-\) cell populations to Hoechst 33342 incubation resulted in a shift in the representation of viable cells such that ~60% of cells were CD45\(^+\) and ~40% were CD45\(^-\). This finding was highly consistent and did not appear to influence the representation of hematopoietic or nonhematopoietic SP cells identified in the present study. This lineage-dependent toxicity observed after Hoechst 33342 incubation was not entirely unexpected, as previous studies confirm that there is significant time, species, and cell type-specific dependence associated with Hoechst 33342 exposure (7a, 9a, 16a, 26a).

To address whether the Hoechst 33342-stained, PI-negative, sorted cell population remains viable after the initial FACS analysis, CD45\(^-\) cells were subjected to resorting in the presence of PI 30 min after their initial isolation. Approximately 5% of cells were PI permeable after this time interval, indicating that the vast majority of initially sorted cells retain viability. Similar findings were found evaluating trypan blue dye exclusion up to 90 min after initial sorting (data not shown).

**Stem cell antigen-1 is enriched within lung SP cells.** To further characterize SP cells released by elastase digestion of lung tissue, expression of stem cell antigen-1 (Sca-1), an antigen highly enriched in SP cells of the hematopoietic lineage (11), was assessed. Lung or bone marrow-derived cells were stained with Hoechst 33342, FITC-conjugated mouse anti-CD45, and PE-conjugated mouse anti-Sca-1 antibody. The minimum fluorescence intensity required for classification of cells as Sca-1 positive (Fig. 3, A–C) was established by staining cells with an isotype-matched control antibody (MATERIALS AND METHODS). As has been reported previously, Sca-1-expressing cells were significantly enriched within bone marrow-derived SP (78% positive) and were infrequent within the non-SP fraction (3% positive; Fig. 4A). In contrast, Sca-1 immunoreactivity was not significantly observed among CD45\(^+\) SP or non-SP cells (Fig. 3C). The majority of lung CD45\(^-\) SP cells expressed Sca-1 (80%; Fig. 3B); however, this antigen was also expressed by 30% of cells within the non-SP fraction (Fig. 3B). Although these results demonstrate molecular similarity between SP cells of the bone marrow and CD45\(^-\) SP cells of the lung, expression of Sca-1 by CD45\(^-\) non-SP cells limits the utility of this antigen as a positive selectable marker for putative lung stem cells.

**Nonhematopoietic lung SP cells exhibit a unique molecular phenotype.** To characterize the molecular phenotype of CD45\(^-\) lung SP, RNA was purified from pools of 6,000 unfractionated CD45\(^-\) cells (Fig. 4A), 6,000 CD45\(^-\) non-SP cells (Fig. 4A), and 6,000 CD45\(^-\) SP cells (Fig. 4A), and gene expression was assayed by RT-PCR. Expression of gene products detailed in Table 1 was analyzed. A no-RT negative control reaction was performed for all samples, and total lung RNA served as the positive control.

The cell-specific genes CCSP and CyP450–2F2 (Clara cells), SP-C (type 2 cells), and vimentin (fibroblasts) were expressed in the unfractonated CD45\(^-\) cell population (Fig. 4B). PECAM was not detected in this population, suggesting minimal endothelial cell isolation within the lung cell preparation. All genes expressed within the unfractonated CD45\(^-\) population were also detected in RNA isolated from non-SP cells, indicating that this gate provides an adequate representation of the total CD45\(^-\) population (Fig. 4B). In contrast, only CCSP and vimentin were detected in the CD45\(^-\) SP cells. Based on these findings, it is likely that the CD45\(^-\) lung SP fraction includes at least two populations of cells, one with molecular characteristics of mesenchymal cells (vimentin positive) and the other with characteristics of airway epithelial cells (CCSP positive). The finding that CyP450–2F2 mRNA is undetectable within the CD45\(^-\) SP cells suggests that lung epithelial cells represented within this fraction do not exhibit a molecular phenotype typical of Clara cells. This finding is consistent with our previous identification of an NEB-associated CCSP-expressing cell population that lacks immunoreactive CyP450–2F2 protein (20).

**DISCUSSION**

Results of this study demonstrate the existence of a population of lung cells capable of effluxing the DNA-intercalating...
cell-specific marker CCSP coupled with the absence of CyP450–2F2 further suggests an enrichment of phenotypically variant Clara cells within this SP.

We have previously shown that NEBs serve as a stem cell niche within the bronchiolar epithelium and that a subpopulation of vCE cells within the NEB contains undetectable levels of immunoreactive CyP450–2F2 protein (13, 20, 21). The finding that CCSP-positive, CyP450–2F2-negative cells are enriched within the SP further supports the contention that these cells exhibit properties of stem cell populations previously characterized in vivo. In addition to their localization within NEB and BADJ microenvironments within airways, bronchial and bronchiolar stem cells were stimulated to enter the cell cycle after progenitor cell depletion (13). The relative paucity of nonhematopoietic SP (≤0.9% of viable, CD45-negative cells) is consistent with the low abundance of stem cells throughout the intrapulmonary conducting airways.

Even though stem cells are greatly enriched within the SP of bone marrow, the applicability of this property to enrichment of stem cells from other sources has been an issue of some debate (4, 11, 12, 32). Although numerous studies satisfy this criterion through a comparison of SP phenotype with that of known stem cell populations (e.g., Sca-1, c-kit expression, molecular markers), by far the most common method used to associate SP and stem cells has been a functional assessment of their engraftment potential in vivo (5, 7, 11). This activity has been demonstrated for tissue-derived SP cells from bone marrow, muscle, mammary gland, and liver (2, 11, 14–16, 18, 19, 28, 30). Thus rapid dye efflux is a common property of stem cell populations derived from numerous tissues, although differences in fluorescence intensity and in the shape of the SP profile have been reported (1). In the current study, lung SP cells do exhibit molecular characteristics consistent with their classification as lung stem cells, although future studies are needed to determine the differentiation potential of this population.

It is hypothesized that the genetic integrity of stem cells is critical for maintenance of normal differentiation within a given organ system. Due to their long life and robust differentiation potential, mutations within stem cells could have significant effects on the health status of the entire organism. Although infrequent proliferation and sequestration within a microenvironment are mechanisms for maintenance of this genetic integrity, the expression of ABC transporters (resulting in the SP phenotype) may provide a further level of protection. Targeted deletion of the ABC transporter bcrp1 resulted not only in loss of all SP cells but also increased susceptibility of hematopoietic stem cells to the chemotherapeutic agent mitoxantrone (31). Thus factors directly related to identification of hematopoietic SP cells also serve to protect this cell from toxicant injury. Studies are ongoing to determine the role of ABC transporter activity in maintenance and observed pollutant resistance of lung stem cell populations.

In addition to identification of nonhematopoietic SP, we also confirmed the presence of a subset of CD45+ cells with an SP phenotype in lung. One interpretation of this finding in other organs has been that CD45+ SP cells represent a population of tissue-resident stem cells (16). This hypothesis is contradicted by the recent work of Weissman and colleagues (27, 29) in which parabiotic sex- or CD45 isotype-mismatched mice are
used to identify circulating partner-derived HSC within multiple tissues. This has led to a new theory which states that circulating/transiently resident HSC are common throughout multiple tissues and that these cells are distinct from indigenous tissue-specific stem cells. Further support for this hypothesis comes from a recent study by McKinney-Freeman et al. (19) detailing the differentiation potential of muscle-derived SP cells. In this work, the authors demonstrate that heterogeneity in CD45 expression can be used to segregate cells harboring either hematopoietic or myogenic potential within the muscle-derived SP. Although each of these types of muscle SP show enhanced differentiation potential relative to its corresponding non-SP, it was clear that only CD45+ SP cells were capable of colonizing the hematopoietic lineage in vitro (19). Moreover, studies by Asakura and Rudnicki (1) demonstrate that a large number of tissues contain SP with hematopoietic colony-forming potential. As was the case in muscle, only the CD45+ fraction of these cells demonstrates the ability to undergo any hematopoietic differentiation. Therefore, it is most likely that the CD45+ lung SP cells identified in this study are only capable of hematopoietic differentiation. However, because we are primarily interested in determining the cellular and molecular mechanisms of airway-specific injury and repair, this population has not been extensively characterized in the present study.

A recent paper by Summer et al. (25) also describes the identification of lung-derived SP cells with properties similar to bone marrow SP. SP cells identified in their study are predominately CD45 positive, express high levels of PECAM (CD31), and are uniformly Sca-1 positive. In addition to these features, the SP they identify was significantly less abundant than SP identified in our study (0.05 vs. 0.87% of total cells). This discrepancy likely results from differences in the methods used for lung cell isolation, particularly methods used for enzymatic dissociation of lung tissue. Whereas the SP identified by Summer et al. (25) is predominately positive for PECAM/CD31, endothelial cell-specific mRNA species were not detected in any isolated cell populations (total, non-SP, nor SP) within the current study. These observations support the notion that multiple distinct populations of stem cells likely exist within different lung compartments (3, 8, 10, 13). Future studies are needed to determine whether SP subsets exist within other cellular compartments of the lung that were not adequately sampled with the isolation methods employed in this study.

In summary, studies presented herein demonstrate the existence of rapid Hoechst effluxing cells among isolated lung cell preparations enriched for epithelial cells. Included within the SP identified in elastase digests of lung tissue is a CD45- negative fraction that includes cells expressing marker genes indicative of fibroblast (vimentin) and nonciliated airway epithelial (CCSP) lineages. Expression of CCSP by CD45-negative SP cells was not associated with expression of the mature Clara cell marker CyP450-2F2, a property that is consistent with the molecular phenotype of a subpopulation of cells residing within the NEB, a niche that has been shown to harbor stem cells in vivo (13, 20, 21). Future studies may focus on the establishment of methods for stem/SP cell culture and the identification of unique molecular markers for airway stem cells.

REFERENCES


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